The Effects of Antimicrobial Therapy on Faecal *Escherichia coli* and Mucosal Staphylococci in Dogs

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

Vanessa Merta Schmidt

March 2014

Acknowledgements

Where do you start after four years of blood sweat and tears; and not all mine. First and foremost I need to thank my ever-suffering husband, Stephen Flynn as without his support I would never of even started let alone finished the PhD - death by starvation at the very least! I love you darling (and I promise to do more housework from now on). I would also like to thank my other two boys (Sebbie & Ziggy) for cuddles, walks and for keeping me company (and sane) whilst writing up.

Very importantly, I would like to thank all of my supervisors Dr Nicola J Williams, Dr Gina Pinchbeck, Dr Neil McEwan, Dr Susan Dawson and Dr Tim Nuttall and my advisor, Dr Eithne Comerford, for their support, advice and mentorship. I would especially like to thank Nicola for all of her very hard work, ideas, positivity, support, reviewing, subbing and especially patience – you're definitely a star!

I would also like to thank Dr Caroline Corless and Erika Tranfield at the Royal Liverpool University Hospital for their amazing and continued help, support, ideas and advice – and especially their valuable time!

Without samples there would be no study – therefore thank you very much to everyone that participated in either sample collection and/or recruitment: Clara MacFarlane, Laura Buckley, Maarja Uri, Ana Martins, Katherine Linney, Helen Yates, Kathleen Gallagher and Atina Unwin. In particular I would like to thank Clara for her co-ordination and organisation in this matter and for accompanying me to dog shows and on home visits when required ☺.

The lab (G10A) has been my second home (sometimes my first) during the last four years. I would like to thank the very many people that work there – THANK YOU ALL for being so nice, understanding and very helpful to this vet!

I would particularly like to thank all the people that have physically helped me at one time or another during the last four years, as without you all I would still be buried under a mountain of plates: Amy Wedley (fountain of all knowledge), Ruth Ryvar, Jenny Fick, Gill Hutchinson, Beatrice Jones, Stevie Snoop, Rebecca Callaby, David Ramsbottom, Mitch Long, Lou Marriage, Chris Ball, Kirsty Kemmett, Nick Harvey, Jackie Lee and IGH ordering team and Karen Ryan (for keeping me in agar and broth). A special thank you to 'Aunty' Ruth for always being there.

Also thank you to Trevor Jones, Dorina Timofte, Andy Watrett, Jan Harris, Tom Maddox, Iuliana Maciuca, Marie McIntyre, Cathy Glover, Cynthia Dare and Anne Forester for your time and kind advice from time to time on all matters microbiological, statistical and grammatical.

Finally thank you to my vet PhD comrades: Dr Camilla Brena and Dr Marco Falchieri, and my friend Dr Steve Shaw. **I still can't believe I have made it!**

Thank you to Zoetis for funding this work.

Abstract

The Effects of Antimicrobial Therapy on Faecal *Escherichia coli* and Mucosal Staphylococci in Dogs

Vanessa Merta Schmidt

Canine infections with antimicrobial resistant (AMR), particularly multi-drug resistant (MDR) bacteria are increasing, severely limiting therapeutic options, and representing an animal health issue. In addition, with potential transfer of AMR bacteria between dogs, their environment, humans and other animals, there may also be a public health risk. Commensal isolates can be a source of clinical infections and studies reporting the prevalence of AMR and risk factors for such isolates are important. Furthermore, one of the most significant impacts upon commensal bacterial populations is antimicrobial therapy that may select for pre-existing AMR organisms or transmission of resistance determinants. The aim of this work was to investigate AMR amongst canine commensal bacterial populations and the effects of five different antimicrobials, authorised to treat dogs in the UK, on these populations both during and after therapy. Three groups of dogs were enrolled: healthy non-antimicrobial treated, non-vet visiting dogs (n = 28), to investigate longitudinal carriage of faecal *E. coli*; healthy non-antimicrobial treated, non-vet visiting, dogs (n = 73) and antimicrobial treated, non-hospitalised dogs (n = 127) to investigate longitudinal carriage of mucosal staphylococci and faecal *E. coli*. Staphylococci and *E. coli* isolated from swabs (nose/perineum) and faecal samples respectively, were tested for phenotypic AMR and carriage of resistance genes by PCR assay. Staphylococci were assigned to species by PCR assay (*nuc* gene), MALDI-TOF-MS and sequencing (*tuf* gene). Healthy dog *E. coli* underwent phylo-typing, and a selection of longitudinal healthy dog *E. coli* isolates were genotyped. Questionnaire data were used to formulate independent variables. Statistical analysis included Pearson's Chi-square, survival analysis and multivariable logistic regression; multilevel for clustered data. The prevalence of meticillin-resistant (MR; 42%) and MDR staphylococci (resistant to ≥ 3 antimicrobial classes; 34%) was high amongst healthy dogs, however MR-coagulase positive staphylococci were not detected. The most common species detected was *S. epidermidis* (52% of dogs), followed by *S. pseudintermedius* (44%). *S. aureus* was only detected in a small number of dogs (8%). Faecal *E. coli* with AMR to at least one tested drug (63%), MDR (30%) and AmpC-production (16%) were prevalent in healthy dogs, however ESBL-producers (1%) were rare. Healthy dogs carried a predominance of phylogenetic group B1; group B2 *E. coli* isolates were less likely to have AMR while group D isolates were more likely. Carriage of *E. coli* with AMR to at least one tested drug was common and persistent, whereas MDR, AmpC- and ESBL-types were intermittent or transient. Genotyping revealed high intra-dog diversity with frequent new genotypes and resistance phenotypes detected over time. AMR staphylococci and *E. coli* were detected in more dogs following antimicrobial treatment than baseline, but generally returned to pre-treatment levels within three months. Eating raw meat/animal stools, living with other dogs/in-contact humans or pets that had been hospitalised/in-contact humans working with farm animals were associated with the detection of AMR canine commensal bacteria. In particular, following treatment with beta-lactams or fluoroquinolones there was a significant increase in the detection of MDR and AmpC-producing *E. coli* or MR- and MDR staphylococci, respectively. However significant differences were not detected at one month after the end of treatment. Antimicrobial therapy is a risk factor for antimicrobial resistant commensal bacteria in dogs and recovery may take up to three months after the end of treatment. This highlights the importance of prudent antimicrobial use and prescribing guidelines. However other factors, such as diet, in-contacts, co-selection and bacterial fitness may be involved in the carriage of resistant bacteria and should be considered.

Abbreviations used

3GCR	Third Generation Cephalosporin Resistance
95% CI	95% confidence interval
Amp	Ampicillin
AMR	Antimicrobial Resistance
BSAC	British Society for Antimicrobial Therapy
CAB	Columbian Blood Agar
CD	Clindamycin
CFX	Cefalexin
Chl	Chloramphenicol
CLSI	Clinical Laboratory Standards Institute
CIP	Ciprofloxacin
CoNS	Coagulase Negative Staphylococci
CoPS	Coagulase Positive Staphylococci
CVN	Cefovecin
ExPEC	Extra-intestinal Pathogenic *E. coli*
ESBL	Extended Spectrum Beta-Lactamases
GNB	Gram Negative Bacteria
M	Mean
MDR	Multidrug resistance
MGEs	Mobile Genetic Elements
MIC	Minimum Inhibitory Concentration
MLST	Multi-Locus-Sequence-Typing
MR	Meticillin resistant
MR-CoNS	Meticillin Resistant Coagulase Negative Staphylococci
MRS	Meticillin Resistant Staphylococci
MRSA	Meticillin Resistant *S. aureus*
MRSS	Meticillin-resistant *S. schleiferi*
MSA	Mannitol Salt Agar
MSSA	Meticillin Susceptible *S. aureus*
MRSP	Meticillin resistant *S. pseudintermedius*
Nal	Nalidixic acid
NOAH	National Office of Animal Health
OR	Odds Ratio
ORSA	Oxacillin Resistance Screening Agar
OX	Oxacillin
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SD	Standard Deviation
SDW	Sterile distilled water
SIG	*Staphylococcus intermedius* Group
Tet	Tetracycline
TM	Trimethoprim
TS	Cotrimazole
VMD	Veterinary Medicines Directorate

Table of Contents

Abbreviations used

3GCR	Third generation cephalosporin resistance
95% CI	95% confidence interval
Amp	Ampicillin
AMR	Antimicrobial resistance
BSAC	British Society for Antimicrobial Therapy
CAB	Columbian blood agar
CD	Clindamycin
CFX	Cefalexin
Chl	Chloramphenicol
CLSI	Clinical Laboratory Standards Institute
CIP	Ciprofloxacin
CoNS	Coagulase negative staphylococci
CoPS	Coagulase positive staphylococci
CVN	Cefovecin
ExPEC	Extra-intestinal pathogenic *E. coli*
ESBL	Extended spectrum beta-lactamases
GNB	Gram-negative bacteria
M	Mean
MDR	Multidrug resistance
MGEs	Mobile genetic elements
MIC	Minimum inhibitory concentration
MLST	Multi-locus-sequence-typing
MR	Meticillin resistant
MR-CoNS	Meticillin resistant coagulase negative staphylococci
MRS	Meticillin resistant staphylococci
MRSA	Meticillin resistant *S. aureus*
MRSS	Meticillin resistant *S. schleiferi*
MSA	Mannitol Salt Agar
MSSA	Meticillin Susceptible *S. aureus*
MRSP	Meticillin resistant *S. pseudintermedius*
Nal	Nalidixic acid
NOAH	National Office of Animal Health
OR	Odds ratio
ORSA	Oxacillin resistance screening agar
OX	Oxacillin
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PFGE	Macro-restriction pulsed field gel electrophoresis
SCC*mec*	Staphylococcal cassette chromosome *mec*
SD	Standard deviation
SDW	Sterile distilled Water
SIG	*Staphylococcus intermedius* group
Tet	Tetracycline
TM	Trimethoprim
TS	Cotrimazole
VMD	Veterinary Medicines Directorate
VPC	Variance partition coefficient

1. General Introduction and Literature Review

1.1 Commensal bacteria

Commensal microorganisms inhabit ecological niches on the body that are exposed to the environment (Berg, 1996), and the bacteria occupying mucous membranes and the gastro-intestinal tract form part of the normal commensal microbiome. Canine commensal bacteria are generally obtained gradually from the dam during the first week of life (Allaker et al., 1992). Commensal organisms benefit by receiving protection and nutrients from the host and other members of the microbiome, but are usually not detrimental to the host (Tenaillon et al., 2010). The population density, phyla and species composition of the commensal microbiome is generally stable over time for a particular habitat and host (Berg, 1996). A stable microbiome is crucial for the health and immune status of the host and helps to provide a colonisation barrier against pathogens (Vollaard and Clasener, 1994). If the host is immune-compromised or the microbiome and/or barrier function is disrupted, commensal bacteria may become opportunistic pathogens (Berg, 1996; von Eiff et al., 2002).

1.2 Intestinal microbiome

The large intestinal microbiota of humans consists of approximately 10^{10} to 10^{11} bacterial cells per gram of large intestinal content with more than 500 species, predominately anaerobes (Backhed et al., 2005; Berg, 1996; Vollaard and Clasener, 1994). Similar findings have been reported in dogs with 10^{8} to 10^{11} intestinal bacteria per gram of dry faeces (Davis et al., 1977; Mentula et al., 2005; Vanhoutte et al., 2005). Bacterial species compete for intestinal niches, but may also be mutually beneficial providing nutrients and optimal growth conditions for each other (Jones et al., 2007). A stable intestinal microbiome has metabolic, trophic and protective functions for the host (Guarner and Malagelada, 2003). Together with anatomical and physiological functions of an intact gut, the microbiome provides colonisation resistance (Vollaard and Clasener, 1994). Mechanisms of this resistance are likely to include production of bacteriocins, competition for attachment sites/nutrients or stimulation of the immune system (Hudault et al., 2001; Tenaillon et al., 2010; Vollaard and Clasener, 1994). One of the most common and significant disturbances of the intestinal microbiome is antimicrobial therapy (Berg, 1996; Vollaard and Clasener, 1994), however fluctuations may also occur with disease e.g. acute enteritis or, to a lesser extent, as a result of dietary modifications (Guarner and Malagelada, 2003).

1.2.1 *Escherichia coli*

The most prevalent Gram-negative aerobic bacteria (GNB) in all hosts are *Escherichia coli* (Backhed et al., 2005; Berg, 1996; Vollaard and Clasener, 1994). The gastro-intestinal tract of warm-blooded animals and reptiles is the main habitat of *E. coli,* but they also frequent secondary environmental habitats such as water and sediments (Berg, 1996; Gordon and Cowling, 2003; Savageau, 1983). The prevalence and density of *E. coli* in the intestine depends on host body size, diet, concurrent microbiota/gut morphology and retention times; human prevalence is > 90% (Penders et al., 2006; Tenaillon et al., 2010). In dogs, *E. coli* was reported to account for 98% of the faecal aerobic coliforms (Mentula et al., 2005). The intestine of all new-born hosts is colonised shortly after birth by *E. coli*, likely from maternal microbiota. The bacteria reside in and obtain nutrition from the mucous layer covering the intestinal epithelium, thereafter they and are shed into the lumen and are excreted in faeces (Poulsen et al., 1994). *E. coli* may contribute to colonisation resistance and inhibit the colonisation of further (exogenous) pathogens (Hudault et al., 2001; Tenaillon et al., 2010; Vollaard and Clasener, 1994).

1.2.1.1 Faecal E. coli: an indicator of intestinal health

E. coli is a well-characterised, widespread gut commensal and potential pathogen that is readily cultured from faeces (Russo and Johnson, 2003; Tenaillon et al., 2010). Faecal culture is an easy, cheap and non-invasive method to study the colonic bacterial microbiota to determine intra or inter-individual differences and investigate antimicrobial selection pressures (Eckburg et al., 2005; Gronvold et al., 2010; Mentula et al., 2005; Wang and Schaffner, 2011). Impairment of the colonisation resistance barrier (mainly composed of anaerobic bacteria) may result in increased detection of antimicrobial resistant *E. coli* (Vollaard and Clasener, 1994).

1.2.1.2. *Faecal E. coli: commensal and pathogen*

E. coli strains have diversified through frequent horizontal gene transfer and recombination events (Rasko et al., 2008; Touchon et al., 2009) and are both gut commensals and adaptable pathogens (Tenaillon et al., 2010). Commensal strains make up the majority of the gut *E. coli* population and occasionally cause extra-intestinal opportunistic infections in compromised patients. However pathogenic strains, intra-intestinal (enteric) and extra-intestinal pathogenic *E. coli* (ExPEC), are more likely to possess virulence genes that facilitate disease (Russo and Johnson, 2000, 2003). Virulence genes encode for factors involved in gut colonisation and

survival within the microbiome and possession of certain genes is likely an adaptive strategy to the strain's environment and only secondarily involved in intra- and extra-intestinal pathogenesis (Tenaillon et al., 2010). Unlike enteric pathogenic strains, ExPEC strains may reside in the microbiome along with commensal *E. coli* and do not cause gastroenteritis. Gut colonisation is a pre-requisite for extra-intestinal infection, which causes a diverse range of clinical syndromes in humans with considerable morbidity, mortality and associated healthcare costs (Russo and Johnson, 2003). ExPEC are also a common cause of urinary tract infections in dogs (Johnson et al., 2009).

1.2.1.3 Classification of E. coli strains by phylogenetic group

Despite frequent recombination, the population structure of *E. coli* is predominantly clonal with division into four major phylogenetic groups (A, B1, B2 and D) (Clermont et al., 2000; Doumith et al., 2012). The original PCR assay to assign isolates to these groups was based on the combination of three marker genes: *chuA* (encoding the outer-membrane hemin receptor gene), *yjaA* (encoding an uncharacterised protein) and TSPE4.C2 (encoding putative lipase esterase) (Clermont et al., 2000). This PCR assay had 80–85% test specificity compared to multi-locus-sequence-typing (MLST) (Gordon et al., 2008). Doumith et al., (2012) recently updated this method with new primers to amplify conserved regions of the same three markers and glutamate decarboxylase-alpha gene (*gadA*), an internal control, with further improved specificity (~ 90%). Clermont et al., (2013) also recently updated their method to improve specificity and identify four additional phylo-groups: C, E, F and *Escherichia* cryptic clade I.

1.2.1.3.1 Phylogenetic groups: characterisation and distribution

Phylo-grouping can help characterise the genetic background, pathogenicity and antimicrobial resistance traits of *E. coli* strains (Sato et al., 2014; Tenaillon et al., 2010). Phylo-groups B2 and D are more likely to carry virulence genes and cause extra-intestinal infections compared to A or B1 (Clermont et al., 2011; Johnson and Stell, 2000; Picard et al., 1999). Phylo-group C is closely related to group B1 (Clermont et al., 2011; Moissenet et al., 2010), while phylo-group E is related to D (Tenaillon et al., 2010) and phylo-group F is related to B2 (Jaureguy et al., 2008). Novel *Escherichia* lineages (cryptic clades) that are genetically distinct, but phenotypically similar to *E. coli* have also been identified (Walk et al., 2009).

The distribution of phylo-groups amongst different hosts is not random, however host-specific strains are uncommon and segregation may depend upon both host and

environmental characteristics such as signalment, body mass, gut morphology, diet, level of hygiene and degree of domestication (Clermont et al., 2011; Escobar-Paramo et al., 2006; Gordon and Cowling, 2003; Gordon et al., 2005; Tenaillon et al., 2010). Generally in humans, phylo-group A predominates followed by groups B2, B1 and D; in animals, group B1 predominates followed by groups A, B2 and D (Tenaillon et al., 2010).

1.2.1.3.2 Phylogenetic groups: antimicrobial resistance

Some genetic backgrounds appear more likely to develop antimicrobial resistance (Tenaillon et al., 2010). This has been reported for the less virulent non-B2 groups in humans, cattle, pigs and dogs (Johnson et al., 2009; Johnson et al., 2003; Moreno et al., 2008). Phylo-group D isolates are more likely to be resistant to fluoroquinolones, third generation cephalosporins (3GCR) or multiple drug classes (MDR) (Deschamps et al., 2009; Platell et al., 2011; Sato et al., 2014; Tamang et al., 2012). Group B2 is more likely to be susceptible (Johnson et al., 2009; Platell et al., 2010; Platell et al., 2011; Sato et al., 2014) than the other groups, however antimicrobial resistance is being reported increasingly amongst B2 (ExPEC) strains (Russo and Johnson, 2003).

1.2.1.4 Gut residents and transient E. coli strains

Intestinal resident *E. coli* strains are adapted to the gut environment and can persist for months or years (Nowrouzian et al., 2001; Nowrouzian et al., 2006; Sears et al., 1950; Sears et al., 1956). Resident strains are defined as being detected from individual hosts on at least two separate occasions, at least three weeks apart (Damborg et al., 2009), or persisting for at least three weeks (Karami et al., 2008). Gut colonisation and persistence may be associated with the carriage of virulence genes encoding adherence factors, for example P fimbriae, and resident strains in humans are more likely to belong to phylo-group B2 than transient strains (Nowrouzian et al., 2006). Clermont et al., (2011b) also identified adhesion factors associated with animal associated non-B2 pathogenic strains. Transient strains may be acquired from the environment, food, water or in-contact humans or animals (Berg, 1996; Johnson et al., 2008; Vollaard and Clasener, 1994; Wellington et al., 2013). In most cases, they do not persist more than a few days or weeks and harmlessly pass through the gut (Sears et al., 1950). However, they may represent a reservoir of antimicrobial resistance determinants for resident bacteria (Wellington et al., 2013), or under antimicrobial selective pressure, they may persist for longer in the host (Dethlefsen et al., 2007; Edlund and Nord, 2000; Vollaard and Clasener, 1994).

1.2.1.3 Diversity of E. coli population structure

The *E. coli* population structure is diverse and dynamic within both the host, the host population, and between host species, for example animals and humans. The aetiology is likely to be multifactorial with host, environmental and bacterial factors all playing a role; possible influences include: host signalment, climate, level of hygiene, diet, health and immune status, bacterial phylogeny, possession of virulence factors, competing bacteria and antimicrobial selection pressure and resistance phenotype (Anderson et al., 2006; Damborg et al., 2009; Schlager et al., 2002; Tenaillon et al., 2010).

1.3 Mucosal microbiome

As with the gastro-intestinal microbiome, the mucosal microbiome consists of a vast number of micro-organisms than inhabit the skin and mucous membranes of humans and other animals; it has been estimated that there are ~ 1 billion bacteria per cm^2 of human skin (Grice et al., 2008). Recent advances in molecular diagnostic methods have used sequencing of bacterial 16S rRNA genes to investigate the microbiome of the mucosa and skin of healthy dogs. This study reported a majority of *Staphlyococcaceae, Oxalobacteriaceae,* and *Enterobacteriaceae* families from the perineum, and *Oxalobacteriaceae, Moraxellaceae* followed by *Staphlyococcaceae* and *Corynebacteriaceae* from the nasal mucosa (Rodrigues Hoffmann et al., 2014). This is in agreement with the majority of previous culture based studies that have identified staphylococci as important commensals of the mucous membranes and skin of humans and other animals (Kloos, 1980; Saijonmaa-Koulumies and Lloyd, 1996). In humans, the diversity of the microbiome is influenced by the characteristics of the ecological niche, for example *Staphylococcus* spp. prefers m oist, humid environments (Grice et al., 2009). Similar findings have been reported for dogs (Saijonmaa-Koulumies and Lloyd, 1996). In humans the most consistent microbiomes are in the ear and nasal cavity (Grice et al., 2009).

The cutaneous barrier is physical, immunological and antimicrobial and together with the microbiome works to prevent adherence, colonisation and infection by pathogens (Elias, 2005; Kong and Segre, 2012; Saijonmaa-Koulumies and Lloyd, 1996). The microbiome may be involved in competitive inhibition for adherence sites and nutrients, production of antimicrobial peptides and priming the local immunity (Kong and Segre, 2012). In addition, competitive interference has been demonstrated between CoNS (e.g. *S. epidermidis*) and CoPS (Park et al., 2011; Saijonmaa-Koulumies and Lloyd, 1995). A compromised cutaneous barrier, microbiome and/or immune system facilitates disease and commensal microbes may

become opportunistic pathogens (Pfaller et al., 2007; von Eiff et al., 2002). Atopic dermatitis in humans and dogs is a common cause of reduced barrier function and sufferers have increased carriage of staphylococci and a predilection for pyoderma (Bibel et al., 1977; Fazakerley et al., 2010; Mason and Lloyd, 1989; Olivry and Hill, 2001).

1.3.1 Coagulase Positive Staphylococci (COPS)

The ability to produce free coagulase and clot rabbit plasma differentiates species of staphylococci. *S. pseudintermedius* is the main commensal coagulase positive staphylococci (CoPS) of dogs, and the main cause of pyoderma (Berg et al., 1984; Ihrke, 1987; Medleau et al., 1986). *S. aureus* is the human counterpart (Mainous et al., 2006). Puppies acquire *S. pseudintermedius* from their mothers during the first week of life (Saijonmaa-Koulumies and Lloyd, 2002) and the main carriage sites appear to be the mucosa (nose, gingiva, oro-pharnyx, perineum or rectum), where they are considered to be residents; licking by the dog is thought to spread bacteria to the skin and hair (Allaker et al., 1992; Devriese and De Pelsmaecker, 1987; Mason et al., 1996). These findings have been corroborated by studies reporting homogeneity of strains from mucosa and skin of an individual dog, but heterogeneity of strains between dogs (Fazakerley et al., 2010; Pinchbeck et al., 2006). In addition, metagenomic analysis identified greater numbers of microbial species and diversity from haired canine skin compared to mucosa (Rodrigues Hoffmann et al., 2014).

The prevalence of mucosal *S. pseudintermedius* carriage in healthy dogs has been reported to be between 37% to 92% (Devriese and De Pelsmaecker, 1987; Fazakerley et al., 2010; Griffeth et al., 2008; Hanselman et al., 2009; Paul et al., 2012; Rubin et al., 2011), with increased carriage for dogs with certain skin diseases such as atopic dermatitis or pyoderma (Fazakerley et al., 2010; Saijonmaa-Koulumies and Lloyd, 1995). Long-term carriage of *S. pseudintermedius* may occur in humans living with dogs (Gomez-Sanz et al., 2013), particularly if owning more than two dogs (Walther et al., 2012).

Fewer healthy dogs carry *S. aureus* (4% to 12% of dogs) (Boost et al., 2007; Fazakerley et al., 2010; Griffeth et al., 2008; Kottler et al., 2010; Loeffler et al., 2005; Wedley et al., 2014), and detected isolates are likely to be of human origin (Weese and van Duijkeren, 2010). The longitudinal carriage of *S. aureus* strains in persistently colonised humans appears to be relatively stable over time compared to dogs with *S. pseudintermedius* carriage, which is more diverse and changing over time (Gomez-Sanz et al., 2013). *S. schleiferi* subsp *coagulans* has also been detected from the skin, mucosa and ears of healthy dogs (Griffeth et

al., 2008; Yamashita et al., 2005). Prior to re-classification of the *S. intermedius* group (SIG) in 2007, *S. pseudintermedius* were referred to as *S. intermedius* (Bannoehr et al., 2007).

1.3.2 Coagulase Negative Staphylococci (CoNS)

Coagulase negative staphylococci (CoNS) are common cutaneous and mucosal residents of humans and dogs (Kloos and Bannerman, 1994; Saijonmaa-Koulumies and Lloyd, 1996). They were historically considered to be apathogenic (Huebner and Goldmann, 1999), however they are now recognised as a significant cause of nosocomial and community-acquired infections in humans (Barbier et al., 2010; Wertheim et al., 2005), and may also cause infections in dogs and other animals (Beck et al., 2012; Hauschild and Wojcik, 2007; Kern and Perreten, 2013). Certain CoNS species (*S. epidermidis*, *S. saprophyticus*, and *S. lugdunensis*), like CoPS, may carry virulence factors that increase their pathogenesis and involvement in severe human infections (Dupont et al., 2010).

There are at least 40 described species of *Staphylococcus* with the majority being CoNS. *S. epidermidis* is the most common CoNS isolated from the mucosa (nares, perineum and inguinal skin), axillae and interdigital skin of humans (Kloos and Bannerman, 1994; Widerstrom et al., 2012). Numerous CoNS have been detected from dogs including S. *schleiferi* subsp *schleiferi, S. epidermidis, S. haemolyticus, S. saprophyticus, S. devriesei, S. warneri, S. simulans, S. xylosus, S. capitis, S. caprae* and *S. sciuri* (Bagcigil et al., 2007; Cox et al., 1988; Fazakerley et al., 2010; Kania et al., 2004; May et al., 2005; Medleau et al., 1986; Wedley et al., 2014). The prevalence of nasal CoNS detection in dogs has been reported in one large UK cross sectional study as 38% (Wedley et al., 2014).

1.4 Antimicrobials

There are a limited number of antimicrobials, mostly broad-spectrum, that are authorised for use in companion animals in the UK. Broad-spectrum antimicrobials are active against both Gram-positive and Gram-negative bacteria, while narrow-spectrum antimicrobials are more specific. Amongst the broad-spectrum antimicrobials, beta-lactam and fluoroquinolone antimicrobials are commonly used and are critically important for the treatment of various bacterial infections in companion animals (Hughes et al., 2012; Li et al., 2007; Mateus et al., 2011). Common use of beta-lactams in dogs has been reported in the UK (Hughes et al., 2012; Mateus et al., 2011) and is mirrored by increased recorded sales (VMD, 2012).

1.4.1 Antimicrobials authorised for dogs in the UK

The majority of antimicrobials are usually administered in an oral form. Cefovecin however, is a long-acting subcutaneous preparation of a semi-synthetic third generation cephalosporin. It is authorised in Europe to use every 14 days, for the treatment of skin and urinary tract infections associated with a number of Gram-positive and negative bacteria (NOAH, 2014). It has reported efficacy in the treatment of canine pyoderma (Summers et al., 2012). The majority of cefovecin is excreted unchanged in the urine; however unchanged drug also occurs in the bile (Stegemann et al., 2006). Oral beta-lactam antimicrobials include cefalexin, a first generation cephalosporin that is authorised to treat susceptible bacteria causing skin and urinary tract infections, and clavulanate-amoxicillin, authorised to treat a broad range of aerobic and anaerobic bacteria associated with skin, soft tissue, dental, urinary or respiratory tract infections or enteritis (NOAH, 2014). Both drugs are commonly used to treat canine pyoderma (Damborg et al., 2011; Summers et al., 2012). Veterinary fluoroquinolones, including oral enrofloxacin and marbofloxacin, are broad-spectrum antimicrobials authorised to treat urinary tract, respiratory and skin infections. Fluoroquinolones have been used frequently in dogs to treat pyoderma (Guardabassi et al., 2004; Ihrke et al., 1999). Clindamycin, an oral lincosamide antimicrobial, is also commonly used to treat bacterial pyoderma and dental disease, and while it is not effective against Gram-negative aerobes it is very effective against anaerobes (NOAH, 2014).

1.4.2 Mechanism of action of antimicrobials

1.4.2.1 Beta-lactams

Beta-lactams include penicillins and cephalosporins. Peptidoglycan is responsible for the structural integrity of bacterial cell walls. Formation of peptidoglycan (transpeptidation) involves enzymatic cross-linking of sugars and amino acids to form a crystal lattice structure. Peptidoglycan makes up ~ 90% of Gram-positive bacterial cell walls, and ~ 10% of Gram-negative bacterial cell walls, which have an additional outer cell membrane. To reach the cell wall of Gram-negative bacteria, antimicrobials need to penetrate the outer cell membrane through porin channels. Beta-lactam antimicrobials inhibit cell wall synthesis by targeting transpeptidation. The structure of beta-lactam antimicrobials consist of a four-member ring (three carbon and one nitrogen atoms) that bind the enzymes responsible for catalysing cross-linking of peptidoglycans. The enzymes are called penicillin-binding proteins (PBP's) (Greene and Watson, 2006; Tipper, 1985).

Amoxicillin is a synthetic derivative of penicillin, with slightly less activity against Gram-positive aerobes and anaerobes, but wider activity against Gram-negative aerobes. The addition of clavulanic acid, a potent inhibitor of beta-lactamase enzymes, improves the effectiveness of amoxicillin. Cephalosporins are divided into four classes based on chemical structure and therapeutic activity with decreasing activity against Gram-positive aerobes and anaerobes and increasing activity against Gram-negative aerobes. Cefalexin is a first generation cephalosporin predominantly active against Gram-positive aerobes and some Gram-negative aerobes including *E. coli*. It is more effective against anaerobes than amoxicillin. Third generation cephalosporins are less effective against Gram-positive aerobes and anaerobes but more effective against Gram-negative aerobes than first generation cephalosporins (Greene and Watson, 2006). Cefovecin is classed as a third generation cephalosporin, but its spectrum of activity is more similar to first- and second-class cephalosporins than third-generation (Hughes et al., 2012).

1.4.2.2 Fluoroquinolones

Fluoroquinolones inhibit DNA synthesis by inhibition of the enzymes DNA topoisomerase II (DNA gyrase) and IV, primarily DNA gyrase in GNB and topoisomerase IV in Gram-positive bacteria. DNA topoisomerases are involved in cutting DNA during replication to remove super-coils and facilitate separation of daughter DNA. The enzymes comprise two pairs of subunits, DNA gyrase consists of GyrA and GyrB and topoisomerase IV consists of ParC and ParE (*E. coli*) or GrlA and GrlB (staphylococci) (Hooper, 2001). Enrofloxacin and marbofloxacin are veterinary third generation fluoroquinolones that are mainly active against Gram-negative aerobes and facultative anaerobes and less so against Gram-positive aerobes. Activity against staphylococci is variable. Metabolism is in the liver and excretion of active drug or metabolites is in urine and bile. Fluoroquinolones may cause arthropathy in juvenile dogs and are contra-indicated during the rapid-growth phase; up to one year old in large breed dogs (Greene and Watson, 2006).

1.4.2.3 Lincosamides

Bacterial ribosomes consist of a 30S and a 50S subunit. This is the location of where mRNA is translated into proteins by tRNA. Lincosamides inhibit protein synthesis by binding the 50S ribosomal subunit and causing dissociation of tRNA from the ribosome (Tenson et al., 2003). Eukaryote ribosomes differ in structure from bacterial ribosomes and therefore are not targeted (Tenover, 2006). Clindamycin is a chloro-substituted lincomycin and is indicated to treat Gram-positive aerobes and obligate anaerobes. Most Gram-negative bacteria are

resistant. The majority of the drug is excreted in bile (Greene and Watson, 2006; Leigh, 1981).

1.4.3 Impact of antimicrobial therapy on the gut microbiome

Antimicrobials may impact the gut microbiome and disrupt colonisation resistance if they are incompletely absorbed and reach the colon in active form, or if they are excreted in saliva, bile or intestinal mucus (Heimdahl et al., 1985). Suppression of the susceptible flora allows pre-existing, possibly undetectable, resistant isolates (either from mutation and/or from acquired resistant determinants) and/or ingested exogenous antimicrobial resistant bacteria to proliferate. In addition, therapy may trigger the bacterial stress response resulting in mobilisation and dissemination of resistance determinants (Donskey, 2006; Edlund and Nord, 2000; Sullivan et al., 2001; Wellington et al., 2013).

Early studies in healthy humans reported disturbances of the gut microflora following administration of amoxicillin, cefotaxime, clindamycin or co-trimoxazole with overgrowth of aerobic bacteria and yeasts and increased concentrations of antimicrobial resistant GNB (Vollaard and Clasener, 1994). Several antimicrobials are more active against anaerobes including clindamycin. Following treatment with this antimicrobial there was reported disturbance of the *Bacteroides* species composition for up to two years (Jernberg et al., 2007; Lofmark et al., 2006) and increased AMR *Enterobacteriaceae* for up to nine months (Nyberg et al., 2007). Aminopenicillins, including clavulanate-amoxicillin, were found to suppress some anaerobic bacteria and aerobic Gram-positive cocci and increase AMR *Enterobacteriaceae,* but with normalisation within a few weeks to months (Jernberg et al., 2010). Both oral cephalosporins and fluoroquinolones strongly suppressed *Enterobacteriaceae* and selected for AMR amongst these bacteria, but had little impact on anaerobes. In addition, cephalosoporins increased aerobic Gram-positive cocci (Edlund and Nord, 2000; Sullivan et al., 2001). Susceptible bacteria may be protected from the antimicrobial in the intestinal crypts or mucous and proliferate again once the antimicrobial pressure is gone (Jernberg et al., 2010).

1.4.4 Impact of antimicrobial therapy on the mucosal microbiome

Similar to the impact on the gut microbiome, the use of antimicrobials may suppress the antimicrobial susceptible mucosal flora leaving a vacant niche that may be filled by pre-existing, possibly undetected, antimicrobial resistant isolates or exogenous strains acquired from the environment or in-contacts. Horizontal transmission of resistant determinants during

therapy may not be as prominent amongst mucosal staphylococci compared with gut GNB. Although staphylococci do commonly possess plasmids, the main mechanism of horizontal gene transfer is by transduction and is species-specific. While SCC*mec* cassettes, associated with meticillin resistance, can transfer between species, they are generally less mobile than other MGEs (Lindsay and Holden, 2006). Furthermore, multiple chromosomal mutations, such as those required for clinical fluoroquinolone resistance, may take time to accumulate (Hacker and Carniel, 2001).

Fluoroquinolone and beta-lactam therapy may select for fluoroquinolone or beta-lactam resistance amongst staphylococci directly or by co-selection (Westh et al., 2004). MRSA, compared to meticillin susceptible *S. aureus* (MSSA), are more prone to develop fluoroquinolone resistance following ciprofloxacin therapy in humans (Isaacs et al., 1988). This may be associated with the up-regulation of adhesion factors, promoting colonisation of resistant strains (Weber et al., 2003). In addition, fluoroquinolone resistant MRSA isolates may demonstrate augmented oxacillin resistance following fluoroquinolone therapy (Venezia et al., 2001). This may be due to a SOS response and increased mutational rates (Mamber et al., 1993), or by repression of *mec*A regulator genes and upregulation of *mec*A gene expression (Venezia et al., 2001). The extent and duration of the effect on the microbiome from antimicrobial therapy depends on the dose, duration, pharmacokinetics and the pharmacodynamics of the antimicrobial, and the level of antimicrobial resistance present before treatment (Edlund and Nord, 2000; Jernberg et al., 2010; Vollaard and Clasener, 1994).

1.5 Antimicrobial resistance

Antimicrobial resistance amongst bacteria is not a new phenonmenom. Even before antimicrobials were used therapeutically, microorganisms had mechanisms to combat naturally produced antimicrobial substances (Piddock, 2006). However in recent years, under intense antimicrobial selection pressure, there has been the development and global dissemination of MDR amongst clinical and commensal bacteria of humans and other animals (Ewers et al., 2012; Gould, 2009; Hunter et al., 2010).

1.5.1 Antimicrobial Resistance (AMR) mechanisms

Intrinsic antimicrobial resistance (AMR) is a functional or structural trait that infers inherent tolerance of an entire bacterial species to one or more antimicrobials, for example *Pseudomonas aeruginosa* is intrinsically resistant to tetracycline due to lack of drug uptake

into the cell. Whereas acquired AMR, that is responsible for the development and spread of resistance amongst bacterial populations, arises from either random gene mutation or horizontal transfer of resistance genes. AMR mechanisms include the prevention of cell entry by porin channel modification, the expulsion of the antimicrobial agents out of the cell via efflux pumps, the inactivation of antimicrobial agents by enzymes and the modification of the antimicrobial target within the bacteria. Multiple mechanisms may occur together and result in MDR (resistance to three or more antimicrobial classes) (Magiorakos et al., 2012) and/or multiple mechanisms may need to occur before there is clinically significant antimicrobial resistance. Efflux pumps in particular are associated with MDR. Some pumps are specific for certain antimicrobials, for example tetracycline in *E. coli*, while other pumps can remove a variety of structurally diverse antimicrobials including antiseptics, for example fluoroquinolone and quaternary ammonium compounds in *S. aureus* (Alekshun and Levy, 2007).

1.5.1.1 Mobile Genetic Elements (MGEs)

Mobile Genetic Elements (MGEs) are segments of DNA that encode factors allowing them to mobilise within or between genomes (Frost et al., 2005). They frequently transfer and often carry AMR and/or virulence genes, and are often responsible for AMR dissemination between bacteria. MGE's include plasmids, transposons, staphylococcal cassette chromosome *mec* (SCC*mec*), integrons and bacteriophages. Plasmids are circular double stranded DNA that can multiply independently of chromosomal DNA and may carry one or more AMR and/or virulence genes. They are ubiquitous amongst bacterial cells, but usually don't carry genes for essential functions, and furthermore multiple plasmids can exist within a single bacterium (Alekshun and Levy, 2007; Frost et al., 2005). Transposons are small fragments of DNA that can change their position in the genome. They are usually integrated into other DNA (chromosomal, other transposons, plasmids or SCC*mec*) and hence may facilitate the incorporation of one or multiple AMR genes (Tenover, 2006). *SCCmec* are large pieces of DNA that frequently carry the *mec*A gene encoding meticillin resistance (Hartman and Tomasz, 1984). They can also carry other resistance genes on transposons or plasmids. SCC*mec* are relatively stable and move infrequently compared to other MGEs. Integrons carry gene cassettes that encode recombination enzymes; they can carry AMR genes and may integrate into transposons. Another method of horizontal gene transfer is via bacteriophages, which are viruses that infect and replicate in bacteria. Bacteriophages may carry genes encoding toxins and virulence factors (Frost et al., 2005).

1.5.1.2 Transformation, conjugation or transduction

Horizontal gene transfer may occur by transformation, conjugation or transduction. Transformation involves the uptake of free DNA or plasmids into the chromosome by some species of bacteria, conjugation involves the synthesis of pilli to allow passage of plasmids or other elements between cells, and transduction involves the packaging of DNA (chromosomal or MGE) into the bacteriophage head and injection into a recipient cell after the donor is lysed. Conjugation is however, the most common mechanism of acquired AMR in GNB such as *E. coli* and transduction in staphylococci (Lindsay and Holden, 2006; Tenover, 2006).

1.5.1.3 Beta-lactam resistance

Resistance to beta-lactam antimicrobials occurs via two resistance mechanisms: inactivation of antimicrobial agents by beta-lactamase enzymes and modification of the antimicrobial target site, with the former important for extended spectrum beta-lactamase resistance (ESBL) in Gram-negative bacteria, and the latter for meticillin resistance in staphylococci (MRS).

1.5.1.3.1 Beta-lactam resistance: Gram-negative bacteria

ESBL- and AmpC-producing GNB, including *E. coli* have emerged (Gould, 2008), and are increasingly detected from clinical and commensal animal samples (Ewers et al., 2012; Li et al., 2007). These beta-lactamase enzymes cleave the beta-lactam ring of penicillins and cephalosporins. ESBL enzymes hydrolyse oxyimino-cephalosporins but can be inhibited by clavulanic acid, whereas AmpC enzymes additionally hydrolyse cephamycins and are not inhibited by clavulanic acid (Bradford, 2001; Thomson, 2010). Genes encoding ESBL- (bla_{SHV}, bla_{TEM}, $bla_{\text{CTX-M}}$ and bla_{OXA}) and AmpC-type (bla_{FOX}, bla_{CIT}, bla_{DHA}, bla_{MOX}, bla_{EBC} and bla_{ACC}) enzymes are carried on mobile genetic elements so can be readily spread between bacteria by horizontal transmission (Li et al., 2007). CTX-M-type ESBL enzymes and CMY-2 (bla_{CIT}) AmpC- beta-lactamases are the most widespread types. They are prevalent amongst *E. coli* isolated from humans and animals, including dogs (Ewers et al., 2012; Jacoby, 2009; Wedley et al., 2011).

AmpC-production may also be chromosomally mediated (Woodford et al., 2007), with the potential for high-level constitutive production in *E. coli* strains. Additionally, mutations encoding the loss of outer membrane porin channels and hence decreased drug uptake, may enhance the resistance phenotype (Jacoby, 2009). ESBL- and plasmid-mediated-AmpC-producers are commonly MDR because of linkage to other antimicrobial resistance determinants on plasmids (Paterson and Bonomo, 2005), including other ESBL- and/or

AmpC-type genes. MDR may result in co-selection of ESBL- or AmpC-producing *E. coli* by non-beta-lactam antimicrobials (Jiang et al., 2008; Livermore and Hawkey, 2005). When carried together with ESBLs, AmpC-type enzymes may mask the production of ESBL-type enzymes complicating detection (Thomson, 2010).

1.5.1.3.2 Beta-lactam resistance: Staphylococci

Staphylococci may also produce beta-lactamase enzyme, encoded by the *bla*Z gene carried on plasmids, resulting in penicillin resistance. However, broad beta-lactam resistance is associated with an altered target site. Meticillin resistant staphylococci (MRS) carry the *mec*A gene, which encodes an altered penicillin binding protein (PBP2a) and confers resistance to all beta-lactam antimicrobials (Hartman and Tomasz, 1984). The *mec*A gene is carried on a large mobile genetic element (MGE), the staphylococcal cassette chromosome *mec* (SCC*mec*) that can be transferred horizontally (mechanism unknown) between staphylococci (Black et al., 2009). CoNS may be the original source of the *mec*A gene (Tsubakishita et al., 2010) and act as reservoirs for CoPS (Barbier et al., 2010; Descloux et al., 2008; Smyth et al., 2011). Integration of SCC*mec* into the host genome is usually stable and transfer occurs less frequently than other MGEs (Lindsay and Holden, 2006). Several SCC*mec* types have been described (Descloux et al., 2008; Perreten et al., 2013). They differ by the combination of resistance (*mec*A) and recombinase (*ccr*) genes that they possess, but smaller classes, for example SCC*mec* type IV, may be more easily transferred between strains and have less fitness cost (Lee et al., 2007). Resistance determinants for other antimicrobials may also be carried on SCC*mec* (Hiramatsu et al., 2001), or other MGEs, and chromosomal mutations may occur also giving rise to resistance. Hence these isolates are commonly MDR, and often fluoroquinolone resistant (Descloux et al., 2008). Co-selection of fluoroquinolone resistant MRS isolates by beta-lactams or fluoroquinolones may lead to the rapid emergence and persistence of these strains (Descloux et al., 2008; Weber et al., 2003; Westh et al., 2004).

1.5.1.4 Fluoroquinolone resistance

Fluoroquinolone resistance is due to altered bacterial target with or without reduced drug uptake. Spontaneous point mutations of DNA gyrase and/or topoisomerase IV genes results in altered antimicrobial target enzymes. Spontaneous mutations conferring various levels of resistance (increased MIC) are thought to occur at a frequency of 10^6 to 10^{10} cell divisions. Step-wise mutations confer higher levels of resistance creating a small number of bacteria within a large population with reduced quinolone susceptibility (Hooper, 2001). Fluoroquinolone selection pressure may lead to proliferation of first-step resistant mutants that may develop secondary mutations, further increasing the MIC. Mutations in the genes

encoding the DNA gyrase are important for *E. coli* while mutations in the genes encoding topoisomerase IV are important for staphylococci. Reduced drug uptake via porin channel modification and up-regulation of efflux pumps are conferred by chromosomal mutations. High-level resistance usually involves multiple mutations in DNA topoisomerase/gyrase genes as well as other mechanisms, such as multidrug efflux pumps (Alekshun and Levy, 2007).

1.5.1.4.1 Fluoroquinolone resistance: Gram-negative bacteria

Fluoroquinolone resistance results from mutations of DNA gyrase and/or topoisomerase IV with or without porin modification and/or upregulation of efflux pumps. Multiple mutations of DNA gyrase and/or topoisomerase IV genes, or other concurrent resistance mechanisms are required for clinical resistance. Plasmid-mediated *qnr* genes encode for a protein that protects DNA gyrase and topoisomerase IV from fluoroquinolones. In addition, plasmid-mediated *qep*A genes encode for a fluoroquinolone-specific efflux pump. Plasmid-mediated resistance mechanisms result in low-level fluoroquinolone resistance but are additive with other mechanisms for clinical resistance. In addition, strains that solely possess plasmid-mediated mechanisms may also have a selective advantage under low fluoroquinolone concentrations (Alekshun and Levy, 2007; Strahilevitz et al., 2009).

1.5.1.4.2 Fluoroquinolone resistance: staphylococci

Fluoroquinolone resistance in staphylococci is associated with mutations of topoisomerase IV and/or DNA gyrase, which may be augmented by the over-expression of multidrug efflux pumps. Single point mutations in *S. aureus* strains have been associated with clinical resistance to ciprofloxacin. For newer fluoroquinolones, particularly dual targeting drugs, additional mutations are generally required (Strahilevitz and Hooper, 2005).

1.5.1.5 Fitness costs

In the absence of antimicrobial exposure, susceptible isolates (endogenous or exogenous) may out-compete resistant isolates due to the fitness cost of resistance (Andersson and Hughes, 2010; Lenski, 1998). However, after a period of adaption, compensatory mechanisms may occur negating such costs (Cottell et al., 2012; Karami et al., 2008; Lofmark et al., 2008). Together with co-selection, this can result in long term carriage of antimicrobial resistant isolates and/or genes in the absence of direct antimicrobial pressure (Jernberg et al., 2010).

1.5.2 AMR in *Escherichia coli*

1.5.2.1 Prevalence of AMR E. coli in dogs

MDR, ESBL- and AmpC-producing *E. coli* have been detected in the rectum or faeces of healthy and hospitalised dogs (Gibson et al., 2011a, b; Guo et al., 2013; Wedley, 2012; Wedley et al., 2011), and have been reported to cause opportunistic infections (O'Keefe et al., 2010; Sanchez et al., 2002; Sidjabat et al., 2006). A small number of studies have reported canine carriage prevalence of AMR *E. coli* in healthy and sick animals. AMR, MDR (resistance to three or more antimicrobial classes), ESBL- or AmpC-producing faecal *E. coli* were detected in 29 - 45%, 15 - 18%, 1 - 4% and 3 – 7% of healthy UK dogs (n = 183 - 581) respectively (Wedley, 2012; Wedley et al., 2011). Similarly, a recent Canadian study, investigating dogs frequenting dog-walking parks, reported AMR, MDR (resistance to two or more antimicrobial classes), ceftiofur resistance and cefoxitin resistance in 18%, 10%, 4% and 5% of dogs (n = 251) respectively (Procter et al., 2013). Ampicillin, tetracycline and sulfamethoxazole/trimethoprim resistance was most frequent while 4 - 5% of dogs carried faecal *E. coli* resistant to clavulanate–amoxicillin and up to 2% of dogs carried fluoroquinolone resistant isolates (Procter et al., 2013; Wedley et al., 2011).

Other smaller prevalence studies include Moreno et al., (2008), that found all isolates from healthy Chilean dogs (n = 15) were resistant to at least one tested antimicrobial, while 3GCR or fluoroquinolone resistance were not detected. Damborg et al., (2011) also did not detect 3GCR isolates in healthy dogs (n = 22) from Denmark. The dogs in both studies had not received antimicrobials within three to six months of sampling, but further potential risk factor information was not provided. Harada et al., (2011) reported a high level of MDR and ESBL-producing faecal *E. coli* (67% and 7% respectively) in pups (< 2 months old; n = 43) from two kennels in Japan. Although the pups had not received antimicrobials, the dams may have received lincomycin post-partum and in addition, extensive sharing of bacterial clones was demonstrated within kennels. This prevalence agrees with the findings of Costa et al., (2004) who reported ESBL-producing *E. coli* in 10% of healthy dogs (n = 39) from Portugal. However, Murphy et al., (2009) reported MDR (resistance to two or more antimicrobial classes) and AmpC-producing *E. coli* in 11% and 1% respectively, of healthy vet-presenting dogs in Canada; fluoroquinolone resistant or ESBL-producing *E coli* were not detected. Finally a study in the Netherlands identified a higher prevalence of either faecal ESBL- or AmpC-producing *Enterobacteriaceae* (45%) in healthy dogs (n = 20) (Hordijk et al., 2013). The treatment history of the dogs in this study was unknown and further detail about the dogs was not given. The reported differences in the prevalence of AMR may be due to different study populations, methodologies, geographical location and local antimicrobial selective pressures.

In sick dogs, including hospitalised dogs and/or dogs under antimicrobial therapy, the reported prevalence of AMR *E. coli* is higher. Hordijk et al., (2013) detected ESBL- or AmpC-producing *Enterobacteriaceae* in 55% of diarrhoeic dogs (n = 20) in the Netherlands and Damborg et al., (2011) detected AmpC-producing *E. coli* in 62% of dogs (n = 13) receiving cephalosporin therapy in Denmark. Guo et al., (2013) detected fluoroquinolone resistant faecal *E. coli* in 19% (n = 123) of hospitalised dogs in an Australian referral hospital and Moreno et al., (2008) detected ESBL-producing *E. coli* in 30% of hospitalised dogs (n = 15) treated with enrofloxacin in Chile.

1.5.2.2 Risk factors for AMR E. coli in dogs

Risk factors for the detection of AMR *E. coli* in dogs have been reported in a small number of studies. Attending a dog day care, where multiple dogs are looked after together, was associated with the carriage of AMR *E. coli*, in particular ampicillin resistance (Procter et al., 2013). This may be due to increased sharing of *E. coli* isolates in multi-dog situations (Johnson et al., 2008). In addition, consumption of commercial dry food and cooked homemade diets were protective against AMR and MDR faecal *E. coli*, possibly due to cooking/processing removing bacteria. Whilst large dogs of a mixed breed were more likely to harbour AMR *E. coli* than smaller mixed or pure breeds, possibly associated with lifestyle differences, and contact with compost was a risk factor for MDR faecal *E. coli* (Procter et al., 2013), possibly due to ingestion of AMR *E. coli* in soil/manure/decomposing foodstuffs. Another study examining dogs, dog-owners and a control population of humans found the receipt of antimicrobials in the previous month to be associated with the detection of MDR (resistance to three or more antimicrobials) faecal *E. coli* in all participants, and drinking from the toilet was a risk factor for ciprofloxacin resistant faecal *E. coli* in dogs (Stenske et al., 2009).

A number of other studies have also found that antimicrobials, in particular beta-lactams or fluoroquinolones, may select for AMR intestinal *E. coli* in dogs (Gibson et al., 2011a, b; Gronvold et al., 2010; Lawrence et al., 2013; Moreno et al., 2008; Trott et al., 2004). Cefalexin was a risk factor for rectal carriage of MDR, predominantly AmpC- producing *E. coli* in hospitalised dogs (Gibson et al., 2011a), and selected for $bla_{\text{CMY-2}}$ (Damborg et al., 2011). Therapy with cefovecin selected for beta-lactam resistance and carriage of $bla_{\text{CMY-2}}$ gene (Lawrence et al., 2013) and fluoroquinolone therapy selected for fluoroquinonlone, ESBL-producing and MDR resistant canine faecal/rectal *E. coli* isolates (Gibson et al., 2011b; Moreno et al., 2008; Ogeer-Gyles et al., 2006). Hospitalisation was associated with increased

risk of acquiring MDR *E. coli* (Gibson et al., 2011a, b; Hamilton et al., 2013; Ogeer-Gyles et al., 2006). Gibson et al., (2011b) reported that cumulative veterinary admission, greater than or equal to four days was associated with the rectal carriage of MDR rectal *E. coli* in dogs.

1.5.2.3 Longitudinal carriage of AMR E. coli in animal faeces

There are very few studies that have examined the longitudinal shedding of AMR faecal *E. coli* in healthy dogs. One study in Denmark, reported the detection of an ESBL-producing *E. coli* on two occasions from one dog; other antimicrobial resistance profiles were not reported. Genotyping was also performed in this study and while one or two resident clones were present in 69% of dogs (n = 13), the overall *E. coli* population was highly diverse with multiple clones being detected in the majority of dogs (Damborg et al., 2009). Anderson et al., (2006) reported similar genotypic diversity in humans, horses and cattle in the USA, and in addition, found that a small number of unique AMR phenotypes persisted in some subjects for up to six months. Likewise Maddox, (2010) reported shedding of faecal *E. coli* with resistance to at least one antimicrobial, in healthy and hospitalised UK horses, for a median of 188 days.

Further studies have examined the longitudinal shedding of AMR faecal *E. coli* following antimicrobial therapy and/or hospitalisation in various animals. Trott et al., (2004), reported shedding of MDR faecal *E. coli* for up to 27 days following 21 days of enrofloxacin in a dog model in Australia; Cavaco et al., (2008) reported the selection of CTX-M-ESBL-producing faecal *E. coli* for up to 22 days following a three-day course of amoxicillin or oxyimino-cephalosporins in pigs in Denmark; Gronvold et al., (2010) reported the selection of AMR faecal *E. coli* for up to two weeks after seven days of amoxicillin in dogs in Norway; Singer et al., (2008) reported the selection of MDR, AmpC-producing faecal *E. coli* for four days following five-days of ceftiofur treatment in dairy cows in the USA; Damborg et al., (2011) demonstrated intermittent faecal shedding of CTX-M-ESBL-producing faecal *E. coli* for up to two weeks following 4 - 7 days of cefquinome in hospitalised horses in Denmark; Boothe and Debavalya, (2011) reported shedding of fluoroquinolone, MDR *E. coli* for at least three weeks following one week of enrofloxacin treatment in laboratory dogs in the USA and Lawrence et al., (2013) reported the selection of beta-lactam resistant, AmpC-producing faecal *E. coli* at 28 days following a single injection of cefovecin in laboratory Beagles in the USA. Two longitudinal studies have been performed in horses under antimicrobial therapy with or without hospitalisation, in UK referral hospitals. Johns et al., (2012) detected AMR faecal *E. coli* in non-hospitalised horses for two weeks and in hospitalised horses for two months following antimicrobial treatment. Similarly, Maddox, (2010) calculated the survival

of MDR and ESBL-producing *E. coli* as a median of 61 and 22 days respectively, in both healthy community and previously hospitalised and antimicrobial-treated horses.

1.5.3 AMR in staphylococci

1.5.3.1 Prevalence of AMR staphylococci in dogs

Meticillin resistant *S. pseudintermedius* (MRSP) has been detected in 0 to 4.5% of healthy dogs (Griffeth et al., 2008; Hanselman et al., 2008; Hanselman et al., 2009; Kania et al., 2004; Murphy et al., 2009; Vengust et al., 2006; Wedley et al., 2014), and from the mucosa and/or skin of between 3.5 - 66% of dogs with skin infections and/or veterinary hospital admission from Germany, USA, or Japan (Beck et al., 2012; Griffeth et al., 2008; Kania et al., 2004; Kawakami et al., 2010; Nienhoff et al., 2011; Onuma et al., 2012; Sasaki et al., 2007). Meticillin resistant *S. aureus* (MRSA) has been reported to colonise up to 4% of healthy community-dogs in the UK, USA, Slovenia and China and up to 9% of hospitalised dogs in the UK, Canada and Denmark (Weese and van Duijkeren, 2010).

Several studies have reported high prevalence of meticillin-resistant CoNS (MR-CoNS) in humans (Diekema et al., 2001), horses (Bagcigil et al., 2007) and livestock (Huber et al., 2011), but there are few canine reports. Bagcigil et al., (2007) and Vengust et al., (2006) detected MR-CoNS in 13% of healthy dogs (n = 100 and 200, respectively) and Malik et al., (2006) isolated MR–*S. haemolyticus* from two healthy dogs out of 252 diseased and healthy dogs.

Meticillin-resistant *S. schleiferi* (MRSS; subsp *coagulans* or *schleiferi*) have been detected from the skin and ears of a small number of healthy dogs, and dogs with pyoderma or otitis (Griffeth et al., 2008; Kawakami et al., 2010; May et al., 2005). However, *S. schleiferi* has not yet been confirmed as a member of canine skin and/or mucosal flora.

1.5.3.2 Risk factors for AMR staphylococci in dogs

The main reported risk factors for the mucosal carriage of, or infection with MRS in dogs are antimicrobial therapy, frequent veterinary premise contact and hospital admission (Eckholm et al., 2013; Faires et al., 2010; Hamilton et al., 2013; Huerta et al., 2011; Lehner et al., 2014; Nienhoff et al., 2011; Soares Magalhaes et al., 2010; Weese et al., 2012; Windahl et al., 2012). Similar risk factors have been reported or proposed for MRSA and MR-CoNS in humans (Barbier et al., 2010; Soares Magalhaes et al., 2010). In particular, risk factors for MRSA infections in dogs (UK, Canada and USA) included the number of previous

antimicrobial courses (greater than three courses in the last six months), treatment with beta-lactams or fluoroquinolones, prolonged hospitalisation, surgical implants, intravenous catheterisation and contact with hospitalised humans (Faires et al., 2010; Soares Magalhaes et al., 2010). Hamilton et al., (2013) reported extended hospitalisation as a risk factor for MRSA carriage. In addition, recent studies have reported surgery, hospitalisation, frequent veterinary premises contact, and antimicrobial therapy and topical ear medication as risk factors for carriage of, or infection with MRSP in dogs from Germany, Canada and Sweden (Bergstrom et al., 2012; Lehner et al., 2014; Nienhoff et al., 2011; Weese et al., 2012; Windahl et al., 2012). Lehner et al., (2014) also reported an association between glucocorticoid therapy and MRSP infection, but this was not reported in a previous study (Weese et al., 2012). Eckholm et al., (2013) reported antimicrobial therapy and hospitalisation within the last 12 months as risk factors for the detection of MRS in dogs with pyoderma from the USA. Similarly, Huerta et al., (2011) reported the detection of MRS/beta-lactam/ fluoroquinolone resistant staphylococci to be associated with recurrent pyoderma in dogs that had received long-term antimicrobial therapy and had frequent veterinary premise contact. Furthermore MRS/MDR staphylococci were more likely detected from urban rather than rural dogs and MRS in male dogs from Spain.

The transfer of MRS isolates has been reported to occur between individuals within households and veterinary clinics, and contaminated environments and clothing may facilitate dissemination (Laarhoven et al., 2011; Paul et al., 2011; Singh et al., 2013; van Duijkeren et al., 2011).

1.5.3.3 Longitudinal carriage of AMR staphylococci in dogs

Longitudinal studies have reported long-term carriage of MRSP in dogs of up to a year following infection (Laarhoven et al., 2011; Windahl et al., 2012) and the duration of detection was extended by three or more weeks of antimicrobial therapy to which the isolates were resistant (Windahl et al., 2012). However, in the absence of ongoing risk factors, MRSA carriage in dogs is likely to be transient (Weese and van Duijkeren, 2010).

1.5.4 Maintenance & spread of AMR by the microbiome

Commensal microbiotas are important in the emergence and dissemination of antimicrobial resistance (Wang and Schaffner, 2011). The gut microbiome in particular, with its dense bacterial populations, large gene pool, potentially high mutation rates (Berg, 1996) and its susceptibility to direct and indirect antimicrobial selection pressures, is an ideal location for

the development of AMR (Jernberg et al., 2010). Thus the intestinal microbiota of humans and other animals is the main reservoir of AMR GNB (Wellington et al., 2013) and potentially pathogenic microorganisms including ExPEC strains (Katouli, 2010; Vollaard and Clasener, 1994).

There is evidence that both intestinal *E. coli* and mucosal staphylococci, including AMR or pathogenic isolates, are shared between in-contact humans and pets in both directions. Transmission is likely to occur within households, but also within veterinary premises (Damborg et al., 2009; Gomez-Sanz et al., 2013; Huebner and Goldmann, 1999; Johnson et al., 2008; Laarhoven et al., 2011; van Duijkeren et al., 2011), where extended veterinary hospitalisation is a risk factor for acquiring MDR *E. coli*, MRSA or MRSP (Hamilton et al., 2013; Nienhoff et al., 2011). Clinical outbreaks of extra-intestinal infections caused by MDR *E. coli* have been reported in veterinary hospitals associated with the carriage of such isolates (Sidjabat et al., 2006). Similarly mucosal microbiomes, particularly after exposure to health-care environments or antimicrobial therapy, may be potential reservoirs of MRS and MDR staphylococci (Eckholm et al., 2013; Guclu et al., 2007; Hamilton et al., 2013; Nienhoff et al., 2011; Salgado et al., 2003) and facilitate the clonal dissemination of strains between patients, staff, the environment and healthy contacts (Eveillard et al., 2004; Huebner and Goldmann, 1999; Miller and Diep, 2008; van Duijkeren et al., 2011).

Very few healthy people carry MRSP (0.4%) (Hanselman et al., 2009). Zoonotic transmission and infection has been reported (Kempker et al., 2009), but isolation of MRSP from humans tends to be transient (Laarhoven et al., 2011). Carriage may be higher among veterinary staff, owners of infected pets and dogs with concurrent skin disease (Griffeth et al., 2008; Ishihara et al., 2010; Kania et al., 2004; Morris et al., 2010; Paul et al., 2011; van Duijkeren et al., 2011); one study reported two veterinarians being MRSP-positive on two occasions, one month apart (Paul et al., 2011). Additionally, while the source of MRSA carriage in dogs is likely to be an in-contact human (Weese and van Duijkeren, 2010), owners and attending veterinarians of MRSA infected pets were more likely to harbour MRSA compared to owners and attending veterinarians of MSSA infected pets (Loeffler et al., 2010).

1.6 Concluding summary and aims

Canine infections with AMR, particularly MDR bacteria are increasing, severely limiting therapeutic options. In addition strain sharing between humans and animals represents a potential public health risk. Antimicrobial therapy is a risk factor for the detection of AMR amongst faecal *E. coli* and mucosal staphylococcal in humans and other animals. It has been

suggested that reducing antimicrobial prescriptions will reduce the emergence and spread of AMR bacteria and antimicrobial prescribing guidelines have proven useful in this respect. However, due to reduced fitness cost and co-selection, AMR strains may continue to persist. It is therefore important to characterise the impact of antimicrobial therapy on canine commensal bacterial populations to be able to make informed decisions on preventative strategies. In addition, microbiomes are diverse in healthy individuals and maybe influenced by factors such as signalment, diet, hospitalisation and contact with human health-care. This diversity and associated factors should be taken into account when investigating the effects of antimicrobial selective pressure on microbiomes.

Firstly this work aimed to investigate the mucosal staphylococci and faecal *E. coli* populations (AMR and community structure), in non-antimicrobial treated and non-vet-visiting healthy dogs. Previous studies have either reported shorter exclusion periods for antimicrobial therapy or not stipulated an exclusion period. Moreover, reports in humans suggest prolonged changes within gut microbiomes following antimicrobial therapy. The second aim of this work was to investigate the longitudinal carriage of faecal *E. coli* in non-antimicrobial, non-vet-visiting healthy dogs. In particular, to investigate the stability, diversity and change within gut *E. coli* populations (AMR phenotype and genotype). One small longitudinal study has reported carriage of resident clones and marked diversity of canine gut *E. coli* populations, but AMR phenotypes were not reported. In addition, both healthy dog studies planned to investigate potential risk factors for the detection of AMR bacteria for consideration in further studies. The final aim of this work was to investigate longitudinal carriage of mucosal staphylococcal and faecal *E. coli* populations in dogs receiving different antimicrobials and to perform risk factor analysis. Antimicrobial therapy has been reported to select for AMR bacteria, but no other canine study has compared the effects of different antimicrobials, or monitored changes in AMR for three months following therapy.

2. General Materials and Methods

2.1 Study populations

2.1.1 Healthy dog cohort study

Labrador retriever dogs were recruited on a convenience basis from two dog shows in the North West UK between November 2010 and June 2011; the aim was to recruit 30 dogs per show. Owners were approached during the shows and 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 veterinary premises 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 questionnaire regarding potential risk factors for the carriage of antimicrobial resistant bacteria. Swabs (one nasal and one perineal) were taken at the time of enrolment and the owners were asked to collect the very next faecal sample. The University of Liverpool, School of Veterinary Science Ethics-Committee approved the study protocol.

2.1.2 Healthy dog longitudinal study

A convenience sample of staff-owned healthy dogs was recruited from the University of Liverpool, Leahurst Campus, between October 2011 and May 2012. Dog owners were made aware of the study by advertisement in a University Bulletin and by staff email. Exclusion criteria included antimicrobial therapy or veterinary admission within three months of enrolment. Dogs were excluded during the study if they became ill, were prescribed systemic antimicrobials or attended veterinary premises. Before enrolment, all owners read the study information sheets and gave written informed consent and completed a questionnaire regarding potential risk factors for the carriage of antimicrobial resistant bacteria. A second questionnaire was completed at the end of the study to detect changes in circumstances during the study. Owners were asked to provide a fresh faecal sample from their dog once daily for seven days (days 0, 1, 2, 3, 4, 5, & 6), once weekly for four weeks (days 13, 20, 27 & 34) and once monthly for two months (days 62 & 90). The University of Liverpool, School of Veterinary Science Ethics-Committee approved the study protocol in October 2011.

2.1.3 Antimicrobial treatment longitudinal study

A convenience sample of dogs attending veterinary consultations at three centres including first opinion and referral practice, in the North West of England between June 2011 and September 2012 were recruited for the study if they met the inclusion and exclusion criteria. Inclusion criteria included diagnosis of a bacterial infection requiring systemic antimicrobial therapy with one of five different antimicrobials authorised for use in dogs in the UK (cefalexin [CFX], clavulanate-amoxicillin [AC], cefovecin [CVN], clindamycin [CD], or a fluoroquinolone [enrofloxacin or marbofloxacin; FL]). Exclusion criteria included antimicrobial therapy or veterinary admission within three months of enrolment and dogs aged less than 12 months old. Dogs were excluded during the study if they were prescribed a further course of systemic antimicrobial. The veterinarian in charge of the case determined if the dog required systemic antimicrobials and approached the owner of the dog regarding the study. Before enrolment, all dog owners read the study outline and gave written informed consent and completed a questionnaire regarding potential risk factors for the carriage of antimicrobial resistant bacteria. In addition vets completed a questionnaire regarding diagnosis and previous therapies and/or supplied the clinical history of each case. Samples were collected before starting treatment (D0), at the end of treatment (End) and at one (M1) and three months (M3) after the end of treatment. Swabs (one nasal and one perineal) were collected at the time of consultations and the owners were asked to collect the very next faecal sample. Questionnaires were completed at each sample point. The University of Liverpool, School of Veterinary Science Ethics-Committee approved the study protocol in June 2011.

2.2 Specimen collection

For all staphylococcal studies, the attending veterinarian or veterinary nurse/technician collected one nasal swab and one perineal swab from each dog (Copan Eswab LQ Amies Minitip Nylon Flocked Applicator, Appleton Woods, Birmingham, UK). The swab was inserted 5 mm into one nostril or rubbed on the skin of the perineum for 3 to 5 seconds, placed in Amies transport media and stored at 4°C. All samples were processed within 36 hours or receipt. For all *E. coli* studies the owner of the dog collected a fresh faecal sample from the ground into a sterile universal faecal pot at the appropriate time points. Sterile disposable gloves were used to collect all samples.

2.3 Processing swab samples

2.3.1 Staphylococcal isolation

On receipt, the swabs and 250 µl of transport media were transferred to 3 ml of nutrient broth with 6.5% sodium chloride and incubated aerobically overnight at 37°C. The broth was vortexed and streaked onto one mannitol salt agar (MSA) plate, one oxacillin resistance screening agar (ORSA) plate, supplemented with 2 µg/ml of oxacillin, and one Columbia 5% horse blood agar (CAB) plate using disposable 5 µl sterile loops; plates were incubated aerobically overnight at 37°C. All media were obtained from LabM Ltd, Bury, UK.

2.3.2 Staphylococcal isolate selection

Where present, isolates typical of staphylococci (small to medium, pink or yellow colonies on MSA, blue colonies on ORSA and white or yellow colonies on CAB) were selected from all plates using a 5 µl sterile loop, sub-cultured onto CAB, and incubated aerobically overnight at 37°C. All fresh cultures on CAB were subject to biochemical tests to identify staphylococci:

1. Gram stain (Sigma-Aldrich Company Ltd., Gillingham, UK). A drop of sterile water was placed onto a clean glass slide. A sterile toothpick was used to touch one colony and emulsify it in the water drop. The slide was air-dried and fixed by passage through the flame of a Bunsen burner. Gram stain was performed according to the standard method. Staphylococci are Gram-positive (dark purple) coccoid bacteria, usually in groups of 2 – 4 cells (X 1000 oil immersion).
   1.1 Slide flooded with crystal violet stain for 30 seconds and then rinsed
   1.2 Slide flooded with Lugol's iodine solution for one minute and then rinsed
   1.3 Slide washed briefly in acetone and rinsed
   1.4 Safranin counter stain flooded over slide for one minute and rinsed
2. Catalase (Sigma-Aldrich Company Ltd., Gillingham, UK) test was performed by placing one to two colonies with a 5 µl loop into a drop of 3% hydrogen peroxide solution in a sterile petri dish. The production of bubbles is a positive reaction and indicates that catalase is causing the formation of hydrogen and oxygen. Staphylococci produce catalase.
3. Free coagulase production (Rabbit plasma, Pro-Lab, Bromborough, UK) was examined by emulsifying one to two colonies with 500 µl of rabbit plasma in a sterile 2 ml eppendorf tube. The tubes were incubated at 37°C and examined once hourly for four hours and again at 24 hours for the presence of a clot. *S. aureus* ATCC®25923 and *S. epidermidis* ATCC® 12228 were used as positive and negative controls, respectively.

2.3.3 Antimicrobial susceptibility testing of staphylococci

Disc diffusion testing was performed on all staphylococcal isolates in accordance with the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2008). Two Mueller Hinton agar plates with 5% defibrinated horse blood were inoculated with each isolate homogenised in saline (0.5 McFarland standards) for semi-confluent growth using a cotton swab and rotary plating device. Ten antimicrobial discs were then applied to the surface: 1 µg oxacillin (OX), 1 µg ciprofloxacin (CIP), 10 µg gentamicin (GM), 10 µg fusidic acid (FA), 30 µg cefalexin (CFX), 30 µg cefovecin (CVN), 25 µg trimethoprim-sulfamethoxazole (TS), 10 µg tetracycline (Tet), 2 µg clindamycin (CD) and 5 µg vancomycin (Va) (Oxoid, Basingstoke, UK). The plates were incubated aerobically at 35°C for 16 to 18 hours for all discs other than oxacillin and vancomycin, which were incubated for 24 hours. The diameter in millimetres of the zone of inhibition for each antimicrobial disc was recorded. Micro-dilution susceptibility testing[1] (Trek Diagnostic Systems, Cleveland, Ohio, USA) was performed on a subset of the CoNS isolates, using the same antimicrobial panel, other than vancomycin (CLSI, 2008).

Interpretation was based on the CLSI guidelines for animal species-specific zone diameter (mm) interpretive standards and minimal inhibitory concentration (MIC; mg/l) breakpoints for veterinary pathogens or human-derived interpretive standards when available. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) zone diameter interpretive standards and MIC breakpoints were used for CIP and FA (EUCAST, 2013). The breakpoints used for interpretation of OX resistance were a zone of inhibition of ≤ 17 mm and MIC ≥ 0.5 mg/l for S. *pseudintermedius* and CoNS, and ≤ 10 mm and MIC ≥ 4 mg/l for *S. aureus* (Bemis et al., 2009; CLSI, 2013). The breakpoints used for interpretation of resistance to CVN were as a zone of inhibition of ≤ 19 mm and MIC ≥ 8 mg/l in accordance with the manufacturer's recommendations. The reference strain *S. aureus* ATCC®25923 (LGC Standards, Teddington, UK) was used for quality control for MIC and zone diameter determinations.

2.3.4 Isolate storage and DNA extraction

Isolates were stored at − 80°C in Microbank vials (Pro-Lab, Bromborough, UK) and recovered by inoculation of one bead onto CAB with overnight aerobic incubation at 37°C. To extract DNA, one fresh staphylococcal colony was homogenised in 90 µl of sterile distilled water (SDW) and 10 µl of lysostaphin (1 mg/ml; Sigma-Aldrich Company Ltd., Gillingham, UK) and vortexed for 5 seconds. The suspensions were then incubated at 37°C

[1] Micro-dilution susceptibility testing was performed by Cindy Lindeman (Zoetis, Kalamazoo, Michigan, USA)

for 10 minutes and heated at 100°C for 10 minutes before adding 400 µl of SDW. DNA extractions were stored at 4°C before use.

2.4 Processing faecal samples

2.4.1 *Escherichia coli* isolation

Faecal samples were mixed with an equal volume of brain heart infusion broth containing 5% glycerol (BHI-G) on receipt. Each faecal homogenate was streaked, using a 5 µl sterile loop, 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) (Sigma-Aldrich Company Ltd, Gillingham, UK) (Liebana et al., 2006), to obtain single colonies. In addition, to detect antimicrobial resistant isolates, one EMBA plate and one MacConkey agar (MAC) plate were inoculated with the faecal homogenate, using a cotton swab and a rotary plating device, for confluent bacterial growth (Bartoloni et al., 2006). Seven antimicrobial discs were applied to the surface: 10 µg ampicillin, 30 µg clavulanate-amoxicillin, 1 µg ciprofloxacin, 30 µg chloramphenicol, 30 µg nalidixic acid, 30 µg tetracycline and 2.5 µg trimethoprim (MAST Group Ltd., Liverpool, UK). A further 500 µl of faecal homogenate was enriched in 4.5 ml of buffered peptone water. All plates and broths were incubated aerobically for 18 to 20 hours at 37°C. If there had been no growth on the EMBA plates impregnated with third generation cephalosporins, the enriched broths were streaked onto EMBA plates impregnated with 1 µg/ml ceftazidime (CZ) and one EMBA plate impregnated with 1 µg/ml cefotaxime (CX) and incubated aerobically for 18 to 20 hours at 37°C.

2.4.2 *Escherichia coli* isolate selection

Random colonies (3 or 10 depending on the study) were selected from the plain EMBA plate if there morphology resembled *E. coli* (medium sized metallic green colonies). If present, colonies growing within the zone of inhibition around each antimicrobial disc on the EMBA and MAC plates (medium sized pink colonies) and from the CX and CZ plates were selected. The selected colonies were streaked, using a 5 µl sterile loop, onto nutrient agar and incubated aerobically for 18 to 20 hours at 37°C. Gram stain and biochemical tests to detect *E. coli* were performed.

1. Gram stain was performed as for staphylococci. *E. coli* are Gram-negative (pink) rod-shaped bacteria (X 1000 oil immersion).
2. Catalase production was tested as for staphylococci. *E. coli* produce catalase.

3. Oxidase activity was tested by streaking colonies, using a 5 µl sterile loop, onto filter paper soaked with oxidase reagent (TestOxidase Reagent; PRO-LAB Diagnostics, Neston, UK). Oxidase production causes a blue colour change within 30 seconds. *E. coli* are negative for oxidase activity. *Pseudomonas aeruginosa* was used as a positive control.
4. Lactose fermentation was investigated by streaking colonies onto a MacConkey agar plate incubated aerobically overnight at 37°C. *E. coli* ferment lactose in the media and produce dark pink colonies.
5. Indole production was investigated by streaking colonies onto a Tryptone Soy Agar (TSA) plate incubated aerobically overnight at 37°C. A small piece of filter paper soaked in Kovac's reagent (bioMerieux, Marcy l'Etoile, France) was added to the colonies. A pink colour change within 30 seconds indicated indole production. *E. coli* produce indole.
6. Citrate use as a carbon source was investigated by streaking colonies onto a Simmon's Citrate Agar (SCA) plate incubated aerobically overnight at 37°C. *E. coli* do not use citrate as a carbon source. They do not grow well and do not change the colour of the media from green to blue.

Isolates identified as *E. coli* by Gram stain and biochemical tests underwent PCR assay for the *uid*A gene for confirmation (McDaniels et al., 1996).

2.4.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility disc diffusion testing was performed on all *E. coli* isolates according to the British Society for Antimicrobial Chemotherapy guidelines (BSAC; Version 11.1 May 2012) (Andrews, 2007). Half a 5 µl sterile loop of fresh isolates, taken from nutrient agar plates, were homogenised in 3 ml of sterile distilled water (SDW) (0.5 McFarland standard) and diluted 1:10 before inoculating one Iso-Sensitest agar plate for semi-confluent bacterial growth with a cotton swab and rotary plate device. Seven antimicrobial discs were applied: 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). After the plates were incubated aerobically at 37°C for 18 to 20 hours, the zone diameters around each disc were measured in millimeters and recorded. Isolates were categorised as susceptible or resistant based on published BSAC guidelines. Isolates with intermediate resistance were classified as susceptible. *E. coli* ATCC® 25922 (LGC Standards, Teddington, UK) cultured overnight on nutrient agar at 37°C was used as a control.

2.4.4 Phenotypic detection of ESBL- and AmpC-producing *E. coli*

Isolates that were resistant to third generation cephalosporins (3GCR) were tested for the production of extended spectrum beta-lactamase enzymes (ESBL). One to two fresh colonies from nutrient agar were emulsified in 3 ml of SDW (0.5 McFarlands) and an Iso-Sensitest agar plate was inoculated for confluent bacterial growth with a cotton swab and rotary plating device. Three pairs of third generation cephalosporin discs (with and without clavulanic acid) were placed on the surface of the agar plate: 30 µg ceftazidime and 30 µg ceftazidime plus 10 µg clavulanic acid; 30 µg cefotaxime and 30 µg cefotaxime plus 10 µg clavulanic acid; and 30 µg cefpodoxime and 30 µg cefpodoxime plus 10 µg clavulanic acid. The plates were incubated aerobically at 37°C for 18 to 20 hours and zone diameters around each disc were measured and recorded. 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, according to the manufacturer's instructions (Extended Spectrum Beta-Lactamase Set D52C, MAST Group Ltd., Liverpool, UK) (M'Zali et al., 2000). *E. coli* isolates that were resistant to 3GCR or clavulanate-amoxicillin were tested for production of AmpC enzyme. An Iso-Sensitest agar plate was inoculated for confluent bacterial growth and three discs applied: 10 µg cefpodoxime plus AmpC inducer (A); 10 µg cefpodoxime, AmpC inducer plus ESBL inhibitor (B); 10 µg cefpodoxime, AmpC inducer, ESBL inhibitor plus AmpC inhibitor (C). The plates were incubated aerobically at 37°C for 18 to 20 hours and zone diameters around each disc were measured and recorded. AmpC production was confirmed when the zone of inhibition around disc C was disc was greater than discs A and B by a minimum of 5 mm, according to the manufacturer's instructions (AmpC detection set D69C, MAST Group Ltd., Liverpool, UK) (Halstead et al., 2012). *E. coli* ATCC® 25922 (LGC Standards, Teddington, UK) cultured overnight on CAB at 37°C was used as a control.

2.4.5 Conjugation experiments

The ability of *E. coli* isolates to transfer antimicrobial resistance determinants was tested as previously described (Karczmarczyk et al., 2011). Rifampin-resistant, lactose-negative strain *E. coli* 26R793 served as a recipient in the assays. Overnight cultures of the donor and recipient strains grown in 5 ml of nutrient broth at 37°C were mixed and re-incubated at 37°C for 18 hours. The transconjugants were selected by streaking broths onto MacConkey agar supplemented with 100 µg/ml rifampin (Sigma-Aldrich) along with either 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, using a 5 µl sterile loop, onto nutrient agar. Plates were incubated at 37°C for 18 hours.

2.4.6 Isolate storage and DNA extraction

All confirmed *E. coli* isolates were stored at – 80°C in Microbank vials (Pro-Lab, Bromborough, UK) and recovered as described for staphylococci, but onto nutrient agar. Cell lysates were prepared by adding three colonies of each isolate into 500 µl of SDW to yield a 0.5 McFarland standard and then vortexed. The suspensions were then heated at 100°C for 10 minutes and the supernatants were stored at 4°C before analysis.

2.5 Polymerase Chain Reaction (PCR) assays

PCR assays were used in this work to investigate the carriage of resistant genes, determine phylogenetic groups or species of bacterial isolates. The exact PCR assays, primers, conditions and references are detailed in individual chapters and appendices.

2.5.1 PCR substrates

All the PCR assays, other than for the *nuc* or *tuf* genes, were performed with 5 µL of bacterial DNA extract, 5 pmol of each primer, 4 µL of 5x FIREPol® Master Mix (12.5 mM MgCl2) (Solis-Biodyne, Tartu, Estonia) and RNase-free water made up to a total reaction volume of 25 µL. For multiplex assays, 0.5 µl of FIREPol® DNA Polymerase 5 U/µl was added per reaction (Solis-Biodyne, Tartu, Estonia). PCR assays for the *nuc* gene were performed in a reaction volume of 25 µl, consisting of 5 µl of bacterial DNA extract, 12.5 µl of master mix (Qiagen® Multiplex PCR Mix; Qiagen, Crawley, UK), 2.5 µl of 10x primer mix (2 µM of each primer) and 5 µl of RNase-free water. Positive control strains were included and molecular grade water (Sigma-Aldrich Company Ltd., Gillingham, UK) was used as the negative control. All primers were synthesised by Eurofins MGW Operon (Ebersberg, Germany). All PCR reactions were performed using ABI 2720 Thermal Cycling Machines (Applied Biosystems, UK).

2.5.2 Visualisation of PCR products

PCR products were analysed by agarose gel (1.5% or 2%) electrophoresis. Agarose gels (35 to 400 ml) were made using Hi-pure EEO agarose (Biogene, Cambridge, UK) in 1 x Tris-acetate EDTA (TAE) buffer (Sigma-Aldrich), with an appropriate volume of peqGREEN (4 – 6 µl per 100 ml of agarose; Peqlab, Fareham, UK). A 100 base pair ladder (10 µl; Solis-

Biodyne, Tartu, Estonia) was loaded into the first well. Products (10 µl) were loaded into the wells in groups of 24 followed by ladder. Larger gels (400 mls) were run in 1 x TAE buffer at a potential difference of 150V and a current of 400mA for 90 minutes; large gels (250 mls) at 120V, 400mA for 60 to 90 minutes; medium gels (150 mls) at 120V, 400mA for 60 to 75 minutes and small gels (35 ml) at 100V, 400mA for 45 to 60 minutes. PCR products were visualised using UviTec Gel Documentation system (UVItec, Cambridge, UK) under ultraviolet (UV) transillumination and images were saved with UVIProMV computer program (UVItec).

2.6 MALDI-TOF-MS identification of staphylococci

The extraction method was performed as previously described (Alatoom et al., 2011). One to three colonies from overnight staphylococcal cultures grown on CAB at 37°C were homogenised in 300 µl of molecular grade water (Sigma-Aldrich Company Ltd., Gillingham, UK) in sterile 1.5 ml eppendorf tubes (Starlab (UK) Ltd., Milton Keynes, UK). The suspensions were mixed with 900 µl of absolute ethanol (Sigma-Aldrich Company Ltd., Gillingham, UK) and then centrifuged at 13,000 rpm for 2 minutes. The supernatant was discarded and the centrifugation repeated. The remaining supernatant was removed, and the pellets dried at room temperature. Depending on the pellet size, 10 to 30 µl of formic acid (Sigma-Aldrich Company Ltd., Gillingham, UK) was added to the pellet. After 2 minutes the same volume of acetonitrile (Sigma-Aldrich Company Ltd., Gillingham, UK) was added gently and the tubes were again centrifuged at 13,000 rpm for 2 minutes. Each target plate (MSP 96 well polished steel; Bruker, Bremen, Germany), was spotted in duplicate with 1 µl of each isolate extract and allowed to air dry before overlaying with 1 µl of HCCA matrix portioned (α-cyano-4-hydroxycinnamic acid; Bruker, Bremen, Germany), according to the manufacturer's instructions. After air-drying, the target plate was inserted into the mass spectrometer for analysis. The bacterial test standard (*E. coli* DH5 alpha, Bruker, Bremen, Germany) was used for calibration before each experiment and included in duplicate on each target plate. Raw spectra were analysed by the MALDI Biotyper 2.0 software programme with default settings (Bruker Daltonics, Bremen, Germany) and the mass peak profiles were matched to the reference database and a score generated based on similarity. A positive identification to species level was made for single result > 2.0 or duplicate results > 1.8 (Carpaij et al., 2011).

2.7 Sequencing for the *tuf* gene

PCR amplification and sequencing of the *tuf* gene is recommended for CoNS assignment to species (Carpaij et al., 2011; Heikens et al., 2005). Initial PCR assays were performed using

HotStarTaq® Master Mix Kit (Qiagen, Crawley, UK) in a 25 µl reaction volume with an initial activation step at 95°C for 15 minutes followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, with a final extension step of 72°C for 5 minutes, according to the manufacturer's protocol. The resulting amplicons were sequenced using BigDye Terminator version 1.1 cycle sequencing (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol on the ABI3131 genetic analyser at the Department of Microbiology, Royal Liverpool University Hospital[2]. The sequences were aligned using the ABI Sequencing analysis software, with contiguous sequences matched to the GenBank database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) and positively identified if there was ≥ 98% sequence similarity with a reference sequence. *S. epidermidis* ATCC® 12228 was used as the control strain.

2.8 Macro-restriction pulsed field gel electrophoresis (PFGE)

Macro-restriction PFGE was performed on a selection of *E. coli* isolates in accordance with standard operating procedure for PULSNET PFGE of *Escherichia coli* O157:H7, *Escherichia coli* Non-O157 (STEC), *Salmonella* serotypes, *Shigella Sonnei* and S*higella Flexneri* (Ribot et al., 2006), with minor changes.

2.8.1 Preparation of agarose plugs

Half a plate of *E. coli* culture, grown overnight on nutrient agar, was emulsified in 2 ml of cell suspension buffer (CSB; 100 mM Tris, 100 mM EDTA [pH 8.0]), and 200 µl of this suspension was added to 180 µl of CSB and vortexed. This 1:10 dilution was used to determine the optical density (OD) at 610 nm using a spectrophotometer (Anthelie Advanced 2; Secomam, Ales, France) and 200 µl of the original suspension was diluted with CSB to OD610 of 1.35, added to 1.5 ml eppendorf tubes containing 10 µl Proteinase K (20 mg/ml), and mixed by pipette. Fresh agarose (1% Bio-Rad PFGE-grade agarose and 1% sodium dodecyl sulfate in Tris EDTA buffer [10 mM Tris, 1 mM EDTA; pH 8.0]) was prepared, added to the eppendorf tube, and gently mixed by pipette before filling duplicate plug molds. The moulds were placed in 4°C for 15 minutes to set and then the plugs were transferred to bijouxs containing 3 ml of cell lysis buffer (CLB; 50 mM Tris, 50 mM EDTA [ph 8.0]; 1% Sarcosyl) with 15 µl of Proteinase K. The bijouxs were incubated at 54°C for 2 hours with shaking (175 rpm). The plugs were then rinsed with sterilised purified water, preheated to 54°C, and re-incubated at 54°C for 15 minutes with shaking (175 rpm). This process was performed three times. After the final incubation, the plugs were rinsed with TE buffer

[2] Sequencing of the *tuf* gene was performed by Caroline Corless (Royal Liverpool University Hospital, UK)

(10 mM Tris, 1 mM EDTA [pH 8.0]), preheated to 54°C, and re-incubated at 54°C for 15 minutes with shaking (175 rpm). This process was repeated four times.

2.8.2 *Xba*I restriction digest

The plugs were transferred to 2 ml eppendorf tubes with 200 µl of 1 x dilution of *Xba*I restriction buffer (Roche, Mannheim, Germany) and incubated at 37°C for 15 minutes. The buffer was removed and replaced with 200 µl of restriction buffer with 50U of *Xba*I before further incubation at 37°C for 2 hours.

2.8.3 Gel electrophoresis

X*ba*I digested genomic DNA was analysed in 1% Bio-rad agarose gel in 0.5 x TBE buffer. Three quarters of the plug was inserted into each well with Lambda DNA marker (New England Biolabs, Ipswich, MA, USA) at the start, middle and end. The plugs were sealed with liquefied 1% agarose before the gel was placed into the CHEF-DRIII PFGE system (Bio-Rad, Hercules, CA, USA) containing 2 litres of 0.5 x TBE buffer and run at 14°C for 20 hours (initial switch 2.2seconds, final switch 54.2 seconds, with a gradient of 6 V/cm^2 and angle of 120°). Gels were stained in 0.5 x TBE containing 200 µl of ethidium bromide for 25 minutes, visualised under UV transillumination as above, and image files were saved. Isolates were considered to be the same genotype if there was less than three bands difference in their banding pattern (Tenover et al., 1995).

3. Manuscript 1

Antimicrobial resistance and characterisation of staphylococci isolated from healthy Labrador retrievers in the United Kingdom

Vanessa Schmidt[1,2], Nicola Williams[2], Gina Pinchbeck[2], Caroline Corless [3], Stephen Shaw[4], Neil McEwan[1], Susan Dawson[2], Tim Nuttall[5]

[1]Department of Infection Biology and [2]Department of Epidemiology and Population Health, The University of Liverpool, Leahurst Campus, Neston, UK, [3]Infection and Immunity, Royal Liverpool University Hospital, Liverpool, UK, [4]UK VetDerm, Coalville, UK and [5]University of Edinburgh, The Royal (Dick) School of Veterinary Studies, Easter Bush Campus, Midlothian, UK.

Manuscript published in BMC Veterinary Research 2014; 10: 17.
DOI: 10.1186/1746-6148-10-17.

Summary

Background: Coagulase-positive (CoPS) and coagulase-negative (CoNS) staphylococci are normal commensals of the skin and mucosa, but are also opportunist pathogens. Meticillin-resistant (MR) and multidrug-resistant (MDR) isolates are increasing in human and veterinary healthcare. Healthy humans and other animals harbour a variety of staphylococci, including MR-CoPS and MR-CoNS.

Objectives: The main aim of the study was to characterise the population and antimicrobial resistance profiles of staphylococci from healthy non-vet visiting and non-antimicrobial treated Labrador retrievers in the UK.

Methods: Nasal and perineal samples were collected from 73 Labrador retrievers. Staphylococci were isolated and identified using phenotypic and biochemical methods. They were also confirmed by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), PCR of the *nuc* gene and PCR and sequencing of the *tuf* gene. Disc diffusion and minimum inhibitory concentration (MIC) susceptibility tests were determined for a range of antimicrobials.

Results: In total, 102 CoPS (*S. pseudintermedius* n = 91; *S. aureus* n = 11) and 334 CoNS isolates were detected from 99% of dogs in this study. In 52% of dogs CoNS only were detected, with both CoNS and CoPS detected in 43% dogs and CoPS only detected in 4% of dogs. Antimicrobial resistance was not common among CoPS, but at least one MDR-CoNS isolate was detected in 34% of dogs. MR-CoNS were detected from 42% of dogs but no MR-CoPS were isolated. *S. epidermidis* (52% of dogs) was the most common CoNS found followed by *S. warneri* (30%) and *S. equorum* (27%), with another 15 CoNS species isolated from ≤ 15% of dogs. *S. pseudintermedius* and *S. aureus* were detected in 44% and 8% of dogs respectively.

Conclusions: MR- and MDR-CoPS were rare. However a high prevalence of MR- and MDR-CoNS was found in these dogs, even though they had no prior antimicrobial treatment or admission to veterinary premises. These findings are of concern due to the potential for opportunistic infections, zoonotic transmission and transmission of antimicrobial resistant determinants from these bacteria to coagulase positive staphylococci.

1. Introduction

Staphylococci are normal commensal bacteria of the skin and mucous membranes of humans and other animals. They can be differentiated by their ability to produce coagulase, with coagulase positive (CoPS) staphylococci regarded as more pathogenic than coagulase negative (CoNS) species (Devriese et al., 2005; Kloos, 1980; Kloos and Bannerman, 1994; Rich, 2005; von Eiff et al., 2002).

Healthy humans and other animals may harbour multiple species and strains of staphylococci. *Staphylococcus aureus* is the main human commensal CoPS species and is carried in the nasal cavity of approximately 30% of healthy humans (Mainous et al., 2006). *S. epidermidis* is the most common CoNS isolated from the nares, perineum, inguinal skin, axillae and interdigital skin of humans (Huebner and Goldmann, 1999; Kloos and Bannerman, 1994). The main commensal CoPS of dogs, *S. pseudintermedius* (Berg et al., 1984), has been isolated from 37% to 92% of healthy dogs (Devriese and De Pelsmaecker, 1987; Fazakerley et al., 2010; Griffeth et al., 2008; Hanselman et al., 2009; Paul et al., 2012; Rubin et al., 2011), while *S. aureus* is carried by 4.3% to 12% of healthy dogs (Boost et al., 2007; Fazakerley et al., 2010; Griffeth et al., 2008; Kottler et al., 2010; Loeffler et al., 2005; Pinchbeck et al., 2006; Sasaki et al., 2007; Wedley et al., 2014). Other species isolated from the mucosa and skin of healthy dogs include the CoPS, *S. schleiferi* subspecies *coagulans* (Griffeth et al., 2008; Yamashita et al., 2005) and numerous CoNS (*S. schleiferi* subspecies *schleiferi*, *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. devriesei*, *S. warneri, S. simulans, S. xylosus, S. capitis, S. caprae,* and *S. sciuri*) (Bagcigil et al., 2007; Cox et al., 1988; Fazakerley et al., 2010; Kania et al., 2004; May et al., 2005; Medleau et al., 1986; Wedley et al., 2014). The carriage rate of CoNS isolated from the nasal mucosae of healthy dogs was reported to be 38% in one large cross-sectional study (Wedley et al., 2014).

Staphylococci are frequent opportunistic pathogens and commensal isolates are the most common source of infection in humans (von Eiff et al., 2002) and dogs (Bannoehr and Guardabassi, 2012; Fazakerley et al., 2010; Pinchbeck et al., 2006). Antimicrobial resistance can increase the morbidity, mortality and treatment cost of staphylococcal infections. Meticillin (oxacillin) resistance associated with carriage of the *mec*A gene confers resistance to all beta-lactam antimicrobials (Hartman and Tomasz, 1984). The *mec*A gene is located on a large mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*), enabling horizontal transmission between staphylococcal isolates (Black et al., 2009). Meticillin resistant staphylococci (MRS) are important pathogens in human and veterinary healthcare and are often multi-drug resistant (MDR; resistant to three or more classes of

antimicrobial) (Diekema et al., 2001; Garza-Gonzalez et al., 2011; Hryniewicz, 1999; Loeffler et al., 2007; Perreten et al., 2010; Weese and van Duijkeren, 2010), extremely limiting therapeutic options. Meticillin resistant *S. pseudintermedius* (MRSP) clones with a broader resistance spectrum than meticillin resistant *S. aureus* (MRSA) or MR-CoNS are increasingly reported in domestic animals throughout Europe, USA and Canada (Loeffler et al., 2007; Perreten et al., 2010). MR-CoNS are associated with infections in humans and animals (Diekema et al., 2001; Hauschild and Wojcik, 2007; Kern and Perreten, 2013). In humans the most prevalent species is MR-*S. epidermidis* (MRSE), which may be a reservoir of *mec*A for *S. aureus* (Barbier et al., 2010; Smyth et al., 2011). In addition, the SCC*mec* cassette of the major European MRSP clone (ST71-J-t02-II–III) (Perreten et al., 2010) consists of a combination of SCC*mec* II from MRSE and SCC*mec* III from MRSA (Descloux et al., 2008).

The prevalence of MRSA and MRSP carriage in healthy humans and dogs in the community is low (Abudu et al., 2001; Boost et al., 2007; Hanselman et al., 2009; Loeffler et al., 2010; Sa-Leao et al., 2001; Shopsin et al., 2000; van Duijkeren et al., 2011; Zanelli et al., 2002). However, human community-based surveys report a wider range of carriage rates for MR-CoNS (11–50%) (Barbier et al., 2010; Lebeaux et al., 2012; Silva et al., 2001). MR-CoNS have also been isolated from the carriage sites of 13% of healthy dogs (Bagcigil et al., 2007; Vengust et al., 2006). The reported prevalence of MRS is higher in animals exposed to veterinary healthcare environments and antimicrobial therapy (Bergstrom et al., 2012; Huerta et al., 2011; Loeffler et al., 2010; Nienhoff et al., 2011) suggesting that these are risk factors for colonisation.

Previous studies looking at the commensal staphylococci in dogs have concentrated on CoPS species, particularly MR-CoPS species, the CoNS group or MR-CoNS species (Bagcigil et al., 2007; Devriese and De Pelsmaecker, 1987; Griffeth et al., 2008; Hanselman et al., 2009; Loeffler et al., 2005; Paul et al., 2012; Rubin et al., 2011; Vengust et al., 2006), but no study has characterised the complete canine commensal staphylococcal population. Moreover, reporting of the antimicrobial treatment history of dogs in these studies has been inconsistent. The aim of this study was to characterise the mucosal staphylococcal population structure and antimicrobial resistance profiles in healthy Labrador retrievers in the UK in the absence of antimicrobial pressure. This will be important in understanding changes in staphylococcal populations and their antimicrobial susceptibility patterns in dogs exposed to antimicrobials and other risk factors.

2. Materials and Methods

2.1 Study Population

Labrador retriever dogs were recruited for the study from dog shows in the North West of England between November 2010 and June 2011. One healthy dog was enrolled from each household if the dog had not received topical or systemic antimicrobial therapy, or had not been admitted to a veterinary clinic within the last 12 months. All dog owners gave written informed consent before enrolment in this study and completed a questionnaire regarding potential risk factors for the carriage of antimicrobial resistant bacteria (Appendix I). The University of Liverpool, School of Veterinary Science ethics committee approved the study protocol.

2.2 Specimen collection and bacterial isolation

One nasal swab and one perineal swab were collected from each dog (Copan Eswab LQ Amies Minitip Nylon Flocked Applicator, Appleton Woods, Birmingham, UK). A sterile swab was either inserted 5mm into one nostril or rubbed on the skin of the perineum for 3–5 seconds before being placed in Amies transport media, stored at 4˚C and processed within 36 hours. Swabs were incubated aerobically overnight at 37˚C in nutrient broth with 6.5% sodium chloride. The broth was streaked onto Mannitol Salt Agar (MSA), Oxacillin Resistance Screening Agar (ORSA) supplemented with 2 μg/ml of oxacillin and Columbia 5% horse Blood Agar (CAB), and incubated aerobically overnight at 37˚C. Where present, isolates typical of staphylococci were selected from all plates, sub-cultured onto CAB and incubated aerobically overnight at 37˚C. Fresh staphylococcal cultures on CAB were subject to Gram stain (Sigma-Aldrich Company Ltd., Gillingham, UK), tested for catalase (Sigma-Aldrich Company Ltd., Gillingham, UK) and free coagulase production (Rabbit plasma, Pro-Lab, Bromborough, UK) according the manufacturer's instructions and stored at -80˚C in Microbank vials (Pro-Lab, Bromborough, UK). All media were obtained from LabM Ltd, Bury, UK.

2.3 Antimicrobial susceptibility testing

Disc diffusion testing was performed on all staphylococcal isolates in accordance with the Clinical and Laboratory Standards Institute (CLSI) and the following panel of ten antimicrobial discs were applied: 1 μg oxacillin (OX), 1 μg ciprofloxacin (CIP), 10 μg gentamicin (GM), 10 μg fusidic acid (FA), 30 μg cefalexin (CFX), 30 μg cefovecin (CVN),

25 μg trimethoprim-sulfamethoxazole (TS), 10 μg tetracycline (Tet), 2 μg clindamycin (CD) and 5 μg vancomycin (Va) (CLSI, 2008). All the discs were purchased from MAST Group Ltd., Liverpool, UK, except for CVN, which were obtained from Oxoid, Basingstoke, UK. Micro-dilution susceptibility testing (Trek Diagnostic Systems, Cleveland, Ohio, USA) was performed on a subset of the CoNS isolates using the same antimicrobial panel, except vancomycin (CLSI, 2008). Interpretation was based on the CLSI guidelines for animal species-specific zone diameter (mm) interpretive standards and Minimal Inhibitory Concentration (MIC; mg/l) breakpoints for veterinary pathogens or human-derived interpretive standards when available. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) zone diameter interpretive standards and MIC breakpoints were used for CIP and FA (EUCAST, 2013). The breakpoints used for interpretation of resistance to OX as a zone of inhibition of ≤ 17 mm and MIC ≥ 0.5 mg/l for S. *pseudintermedius* and CoNS, and ≤ 10 mm and MIC ≥ 4 mg/l for *S. aureus* (Bemis et al., 2009; CLSI, 2013). The breakpoints used for interpretation of resistance to CVN were a zone of inhibition of ≤ 19 mm and MIC ≥ 8 mg/l in accordance with the manufacturer's recommendations. The reference strain *S. aureus* ATCC®25923 (LGC Standards, Teddington, UK) was used for quality control for MIC and zone diameter determinations.

2.4 DNA extraction and characterisation of antimicrobial resistance genes

Three colonies of each staphylococcal isolate were homogenised in 90 μl of sterile distilled water (SDW) and 10 μl of lysostaphin (1 mg/ml; Sigma-Aldrich Company Ltd., Gillingham, UK) and vortexed for 5 seconds. The suspensions were then incubated at 37˚C for 10 minutes and heated at 100˚C for 10 minutes before adding 400 μl of SDW. Samples were stored at 4˚C.

PCR assays were performed to detect the presence of *mec*A gene in staphylococcal isolates that were phenotypically resistant to oxacillin. All the 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) (Solis-Biodyne, Tartu, Estonia) and water to made up to a total reaction volume of 25 μL (Table 3-1, Appendix 1). Molecular grade water (Sigma-Aldrich Company Ltd., Gillingham, UK) was used as the negative control in all PCR assays. PCR products were analysed by agarose gel (1.5%) electrophoresis and the DNA fragments were visualised under UV light after staining with peqGREEN (Peqlab, Fareham, UK).

2.4 Genotypic species identification

PCR assays to detect the presence of the *nuc* genes of *S. pseudintermedius, S. aureus* and *S. schleiferi* were performed on all CoPS isolates using Qiagen® Multiplex PCR Mix (Qiagen, Crawley, UK), according to the manufacturer's instructions with minor modifications. In short, the PCR assays were performed in a reaction volume of 25 µl, consisting of 5 µl of bacterial DNA extract, 12.5 µl of master mix, 2.5 µl of 10x primer mix (2 µM of each primer) and 5 µl of RNase-free water. The cycling conditions consisted of an initial activation step at 95°C for 15 minutes, followed by 30 cycles of 95°C for 30 seconds, 57°C for 90 seconds and 72°C for 60 seconds, and a final extension step at 72°C for 10 minutes (Table 3-1, Appendix 1).

2.5 MALDI-TOF-MS

All isolates were subjected to matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) according to the manufacturer's protocol. Raw spectra were analysed by the MALDI Biotyper 2.0 software programme with default settings (Bruker Daltonics, Bremen, Germany). The extraction method was performed as previously described (Alatoom et al., 2011) on overnight colonies grown on CAB at 37°C and all isolates were tested in duplicate. The bacterial test standard (*E. coli* DH5 alpha, Bruker, Bremen, Germany) was used for calibration before each experiment and included in duplicate on each target plate. The mass peak profiles were matched to the reference database and a score generated based on similarity (Carpaij et al., 2011).

2.6 Sequencing of the *tuf* gene

Two subsets of isolates detected from our group of dogs underwent sequencing following PCR amplification of the *tuf* gene (Carpaij et al., 2011; Heikens et al., 2005); a control group of CoNS isolates (n = 27) identified by MALDI-TOF-MS and a test group of isolates (n = 52) that had not been identified by MALDI-TOF-MS. Initial PCR assays were performed using HotStarTaq® Master Mix Kit (Qiagen, Crawley, UK) in a 25 µl reaction volume with an initial activation step at 95°C for 15 minutes followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, with a final extension step of 72°C for 5 minutes, according to the manufacturer's protocol (Table 3-1, Appendix I). The resulting amplicons were sequenced using BigDye Terminator version 1.1 cycle sequencing (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol on the ABI3131 genetic analyser at the Department of Microbiology, Royal Liverpool University

Hospital. The sequences were aligned using the ABI Sequencing analysis software, with contiguous sequences matched to the GenBank database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) and positively identified if there was ≥ 98% sequence similarity with a reference sequence. *S. epidermidis* ATCC® 12228 was used as the control strain.

2.7 Statistical analysis

Data were analysed using SPSS software package (SPSS 20.0 for Mac, SPSS Inc, Chicago, Illinois). To examine the association between isolation of *S. pseudintermedius* with each of the 16 different CoNS species Pearson's chi-square was calculated ($P < 0.003$; Bonferroni correction). To examine the association between MR and MDR with potential risk factors (previous antimicrobial therapy or hospitalisation within 12 months of enrolment, owner or in-contact pet with health-care association or owner with large animal-association) identified from the questionnaires Pearson's chi-square was calculated ($P < 0.0125$; Bonferroni correction). To examine the agreement between antimicrobial susceptibility tests by disc diffusion and MIC a *kappa* statistic was calculated (Landis and Koch, 1977) and an independent *t*-test was conducted to compare the MIC of oxacillin resistant CoNS isolates that were either positive or negative for the *mec*A gene.

3. Results

3.1 Specimen collection

Seventy-three Labrador retriever dogs were recruited. Twenty-one dogs were aged between 3 to 12 months, 25 dogs were aged between 12 months to 2 years, and 27 dogs were >2 years old, with 35 female dogs and 38 male dogs in total. The demographics of the dogs included three countries of the UK, The Isle of Man, and 23 counties of England (Table 3-2, Appendix I). The majority (40%) were from the north west of England (Figure 3-1, Appendix I).

3.2 Bacterial isolation

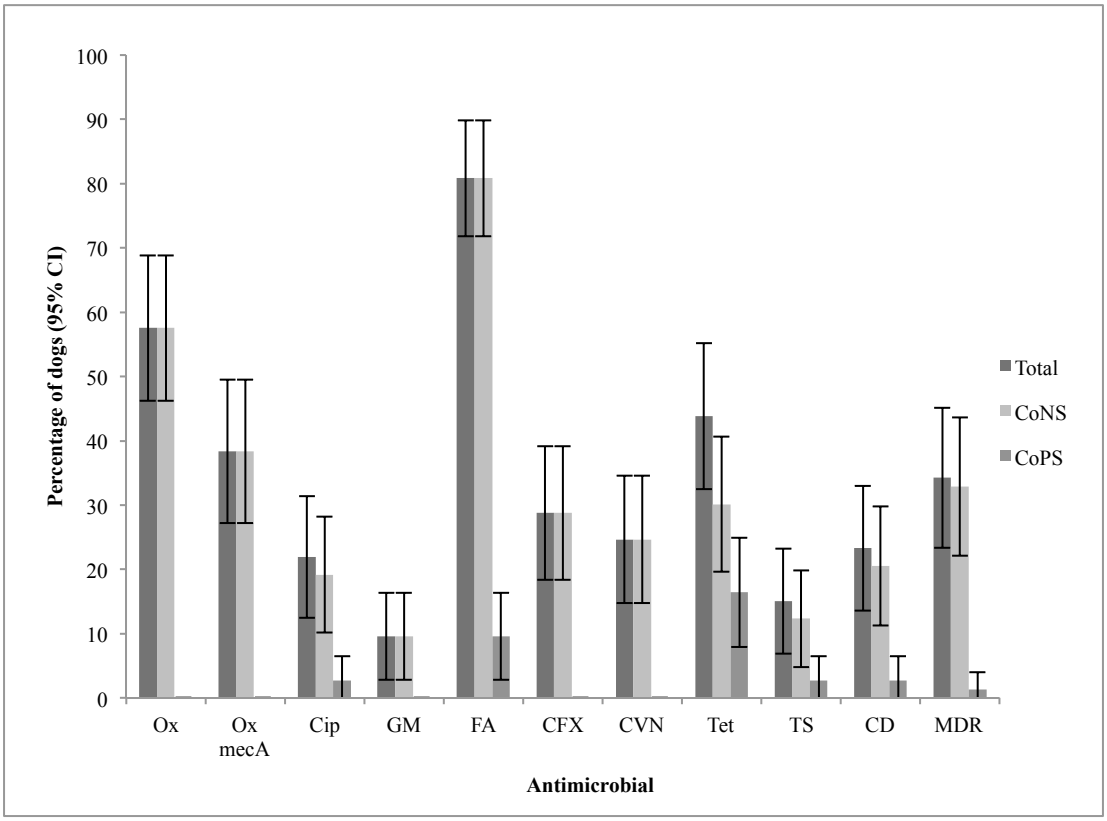
Staphylococci were isolated in 72 out of 73 dogs (99%; 95% CI: 92.6 - 99.8) and from both sample sites in the majority of dogs (78%; 95% CI: 67.3 - 86.0). Isolation of staphylococci from the nasal mucosa (16%; 95% CI: 9.7 - 26.6) or perineum (4%; 95% CI: 1.4 - 11.4) only occurred in a small number of dogs. If only the nasal mucosa had been sampled, CoPS (all *S. pseudintermedius*) would not have been detected in seven dogs (10%; 95% CI: 4.7 - 18.5) and CoNS in six dogs (8%; 95% CI: 3.8 - 16.8). CoNS were detected in the majority of dogs

(95%; 95% CI: 86.7 - 97.8) either alone (52%; 95% CI: 40.8 - 63.1) or with CoPS (43%; 95% CI: 31.8 - 53.9). Detection of CoPS alone was significantly less common (4%; 95% CI: 1.4 - 11.4). In total, there were 436 staphylococcal isolates; 102 of which were CoPS and 334 were CoNS isolates.

3.3 Antimicrobial susceptibility testing by disc diffusion

The overall prevalence of antimicrobial resistance among the isolates detected in this study appeared high, with at least one MDR isolate detected in 34% of dogs. Antimicrobial resistant CoNS isolates were detected in more dogs than antimicrobial resistant CoPS isolates for OX, GM, FA, CFX, CVN and CD and MDR. At least one OX resistant isolate was detected in 58% dogs (n = 126 oxacillin resistant isolates), but resistance to the other tested beta-lactam antimicrobials, CVN (25%) and CFX (29%), was less common. Few CoPS demonstrated antimicrobial resistance; isolates from twelve dogs had Tet resistance, (all *S. pseudintermedius*), seven with FA resistance *(S. pseudintermedius* = 5, *S. aureus* = 3); two with TS resistance (both *S. pseudintermedius*); two with CD resistance (*S. pseudintermedius* = 1*, S. aureus* = 1) and two with CIP resistance (*S. pseudintermedius*). MDR CoPS was detected from only one dog (*S. pseudintermedius* with FA, Tet and CD resistance) (Figure 1).

Figure 1. The proportion of dogs (n = 73) carrying at least one staphylococcal isolate with resistance to each antimicrobial tested in this study by disc diffusion, *mec*A gene positive oxacillin resistance or multidrug resistance (error bars = 95% CI).



95% CI = 95% confidence interval; Total = CoNS and CoPS; Ox = phenotypic oxacillin resistant; Ox mecA = phenotypic oxacillin resistant and *mec*A gene (meticillin resistant); Cip = ciprofloxacin resistant; GM = gentamicin resistant; FA = fusidic acid resistant; CFX = cefalexin resistant; CVN = cefovecin resistant; Tet = tetracycline resistant; TS = Trimethoprim-sulfamethoxazole resistant; CD = Clindamycin resistant; MDR = multidrug resistant (resistant to three or more antimicrobial classes)

3.4 MIC compared to disc diffusion testing for antimicrobial resistance

Micro-dilution susceptibility testing (Trek Diagnostic Systems, Cleveland, Ohio, USA) was performed on 172 CoNS isolates, of which 52 were OX susceptible and 120 were OX resistant by disc diffusion. The OX resistant isolates were further divided into those found to be positive (n = 74) or negative (n = 46) for carriage of the *mec*A gene by PCR. The strength of agreement between antimicrobial resistance detected by MIC and disc diffusion was very good for OX, GM, CVN, Tet and CD resistance, good for CFX and TS resistance and moderate for CIP (*Kappa* = 0.593) and FC resistance (*Kappa* = 0.589). MIC testing identified more isolates as resistant to OX, GM, CFX, CVN and Tet compared to disc diffusion, and disc diffusion identified more isolates as resistant to CIP, FA, TS and CD compared to MIC testing (Table 2).

Table 2. Cross tabulation of the results of 172 staphylococcal isolates classified as resistant or susceptible to the antimicrobials tested in this study by both MIC and disc diffusion testing.

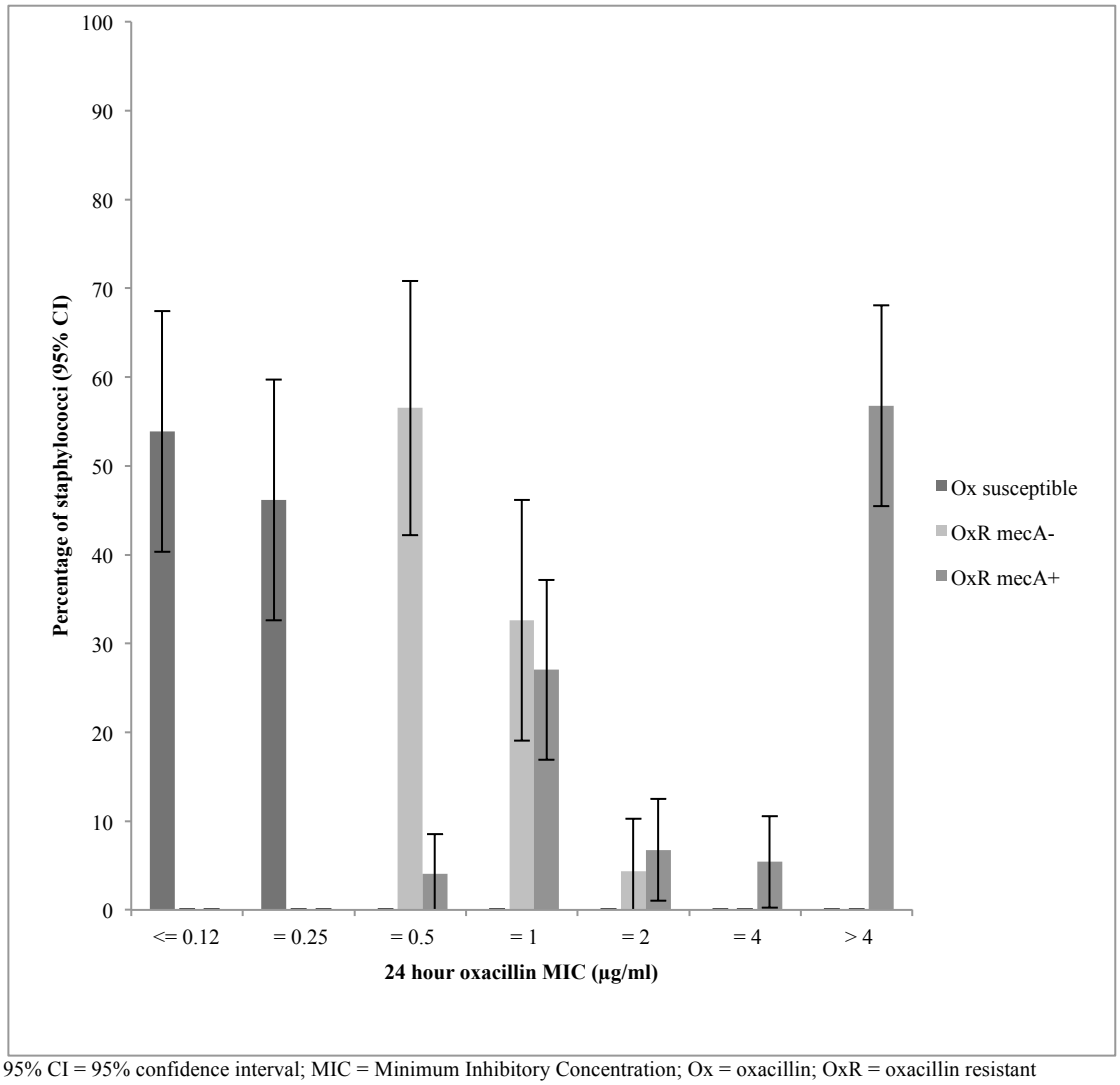
Antimicrobial resistance			**MIC**		
			No	Yes	Total
Oxacillin	**Disc diffusion**	No	50	10	60
		Yes	2	110	112
		Total	52	120	172
		Kappa = 0.842			
			No	Yes	Total
Oxacillin *mec*A positive		No	115	0	115
		Yes	1	56	57
		Total	116	56	172
		Kappa = 0.987			
			No	Yes	Total
Ciprofloxacin		No	146	0	157
		Yes	14	12	15
		Total	160	12	172
		Kappa = 0.593			
			No	Yes	Total
Gentamicin		No	156	1	157
		Yes	0	15	15
		Total	156	16	172
		Kappa = 0.965			
			No	Yes	Total
Fusidic acid		No	36	5	41
		Yes	25	106	131
		Total	61	111	172
		Kappa = 0.589			
			No	Yes	Total
Cefalexin		No	117	15	132
		Yes	0	40	40
		Total	117	55	172
		Kappa = 0.784			
			No	Yes	Total
Cefovecin		No	130	11	141
		Yes	0	31	31
		Total	130	42	172
		Kappa = 0.810			
			No	Yes	Total
Tetracycline		No	148	1	149
		Yes	0	23	23
		Total	148	24	172
		Kappa = 0.975			
			No	Yes	Total
Trimethoprim-sulfamethoxazole		No	156	2	158
		Yes	3	11	14
		Total	159	13	172
		Kappa = 0.799			
			No	Yes	Total
Clindamycin		No	148	2	150
		Yes	4	18	22
		Total	152	20	172
		Kappa = 0.837			

MIC = Minimum Inhibitory Concentration; *Kappa* statistic > 0.8 = very good; 0.61 – 0.8 = good; 0.41 – 0.6 = moderate.

3.5 Characterisation of antimicrobial resistance genes

Of the 126 OX resistant CoNS isolates detected by disc diffusion, 75 isolates (60%; 95% CI: 51 - 68) from 31 dogs (42%; 95% CI: 32 - 54) were positive for the *mec*A gene (Figure 1). Ten additional oxacillin resistant isolates were detected by MIC and two of these were positive for the *mec*A gene, resulting in two additional dogs with MR-CoNS and one additional dog with phenotypic oxacillin resistant CoNS. There was a significant difference between the MIC of *mec*A positive (M = 3.84, SD = 0.18) and *mec*A negative isolates (M = 0.97, SD = 0.12, $P < 0.001$). In addition the epidemiological breakpoint for OX resistant CoNS isolates with *mec*A gene carriage isolated in this study was consistent with the clinical CLSI breakpoint (≥ 0.5 mg/l) (Figure 2).

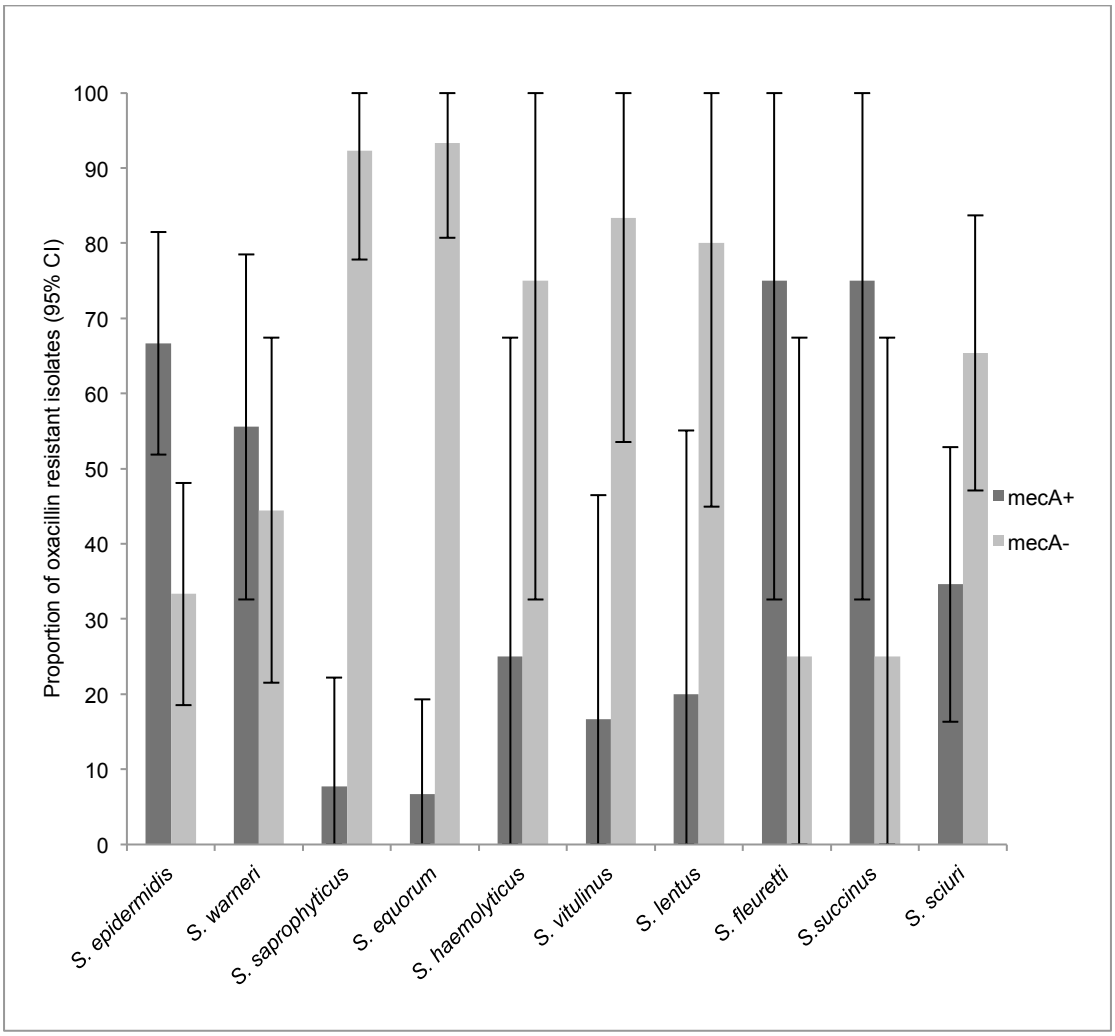
Figure 2. The MIC (µg/ml) data for staphylococcal isolates (n = 172). The isolates consisted of 52 oxacillin susceptible, 46 oxacillin resistant *mec*A negative and 74 oxacillin resistant *mec*A positive (error bars = 95% CI).



95% CI = 95% confidence interval; MIC = Minimum Inhibitory Concentration; Ox = oxacillin; OxR = oxacillin resistant

Eleven different CoNS species (*S. epidermidis, S. warneri, S. sciuri, S. equorum, S. fleurettii, S. vitulinus, S. saprophyticus, S. haemolyticus, S. lentus, S. succinus* and *S. pettenkoferi*) were found to carry the *mec*A gene. Amongst oxacillin resistant CoNS species, *S. epidermidis*, *S. fleuretti* and *S. sciuri* were more likely to carry the *mec*A gene than *S. saprophyticus*, *S. equorum*, *S. vitulinus* and *S. succinus* (Figure 3).

Figure 3. The percentage of each oxacillin-resistant staphylococcal species by disc diffusion and MIC that was either positive (*mec*A+) or negative (*mec*A-) for the *mec*A gene (error bars = 95% CI).



95% CI = 95% confidence interval

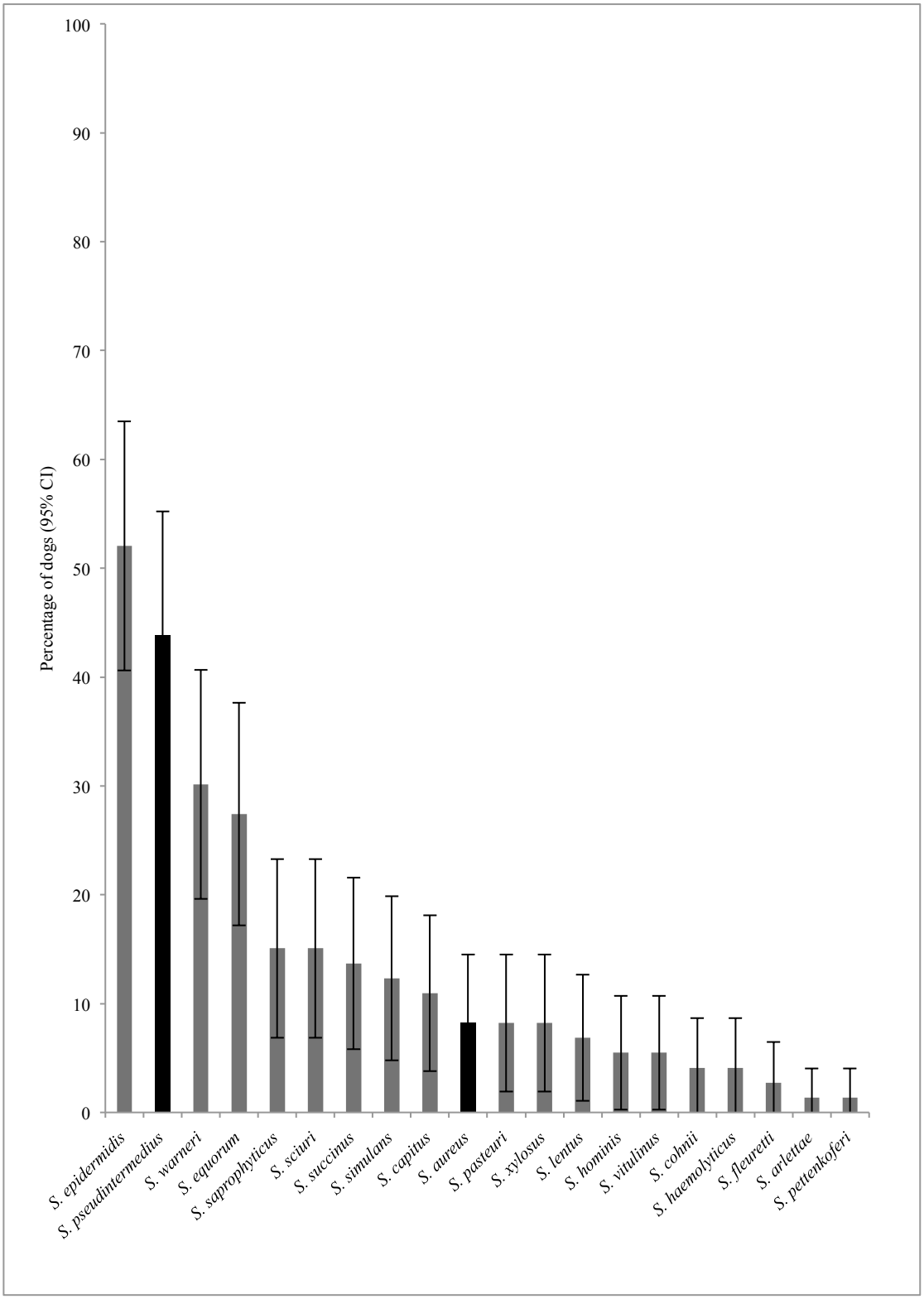
MRSE isolates were detected in 18 dogs (25%, 95% CI: 14.8 - 34.5), meticillin-resistant *S. warneri* were detected in 7 dogs (10%, 95% CI: 2.8 - 16.3) and meticillin-resistant *S. sciuri* were detected in 5 dogs (7%, 95% CI: 1.1 - 12.6). The remaining species were only isolated from one or two dogs. MDR *mec*A positive CoNS were detected in 19 dogs (26%, 95% CI: 17.3 - 37.1). There was no significant association between detection of MR-CoNS or MDR isolates and potential risk factors tested in this study (Pearson's chi-square; $P > 0.0125$).

3.6 Species identification

Phenotypic and biochemical methods identified 436 isolates as *Staphylococcus* species. Using a combination of *nuc* gene PCR, MALDI-TOF-MS and sequencing of the *tuf* gene, 399 isolates (92%, 95% CI: 88.5 - 93.8) were identified to the species level. MALDI-TOF-MS identified 345 isolates to the species level including 264 of 334 CoNS isolates (79%, 95% CI: 74.4 - 83.1). Amplification and sequencing of the *tuf* gene identified 33 out of 51 CoNS isolates (65%, 95% CI: 51 - 76.4) to the species level (n = 11 species; ≥ 98% sequence similarity) and an additional control group (n = 27) of CoNS isolates that had also been identified by MALDI-TOF-MS. There was 100% agreement between the two methods for the identification of the control group. In particular, sequencing of the *tuf* gene identified all of the *S. fleurettii*, *S. arlettae* and *S. pettenkoferi* isolates, 12 isolates closely related to *S. felis* (96% sequence similarity) and an additional 15 isolates to the genus level (*Staphylococcus* spp. ≥ 98% sequence similarity). PCR amplification of the *nuc* gene detected all of the *S. aureus* n = 11 (100%, 95% CI: 74.1 - 100) and *S. pseudintermedius* isolates n = 91 (100%, 95% CI: 96.0 - 100). There was 100% agreement of this assay with MALDI-TOF-MS for the identification of *S. aureus* isolates, however MALDI-TOF-MS only identified 69 out of 91 *S. pseudintermedius* isolates.

Overall from the combined results using PCR amplification of the *nuc* gene, MALDI-TOF-MS and sequencing of the *tuf* gene we detected *S. epidermidis* in 52% (95% CI: 41 - 63) and *S. pseudintermedius* in 44% (95% CI: 33 - 55) of the dogs. *S. warneri* and *S. equorum* were the next most common species, isolated from 30% and 27% of dogs respectively, and the remaining staphylococcal species were carried by no more than 15% of the dogs. *S. aureus* was detected in 6 of the dogs, exclusively from the nasal mucosa, and usually with *S. pseudintermedius* (88%, 95% CI: 52.9 - 97.8). *S. pseudintermedius* was concurrently isolated with 16 different CoNS species, although there was no significant association between the presence of *S. pseudintermedius* and any CoNS species (Pearson's chi-square; P > 0.003) (Figure 4; Table 3).

Figure 4. The proportion of dogs (n = 73) carrying each staphylococcal species identified in this study by MALDI-TOF-MS, PCR of the *nuc* gene and sequencing of the *tuf* gene (error bars = 95% CI).



95% CI = 95% confidence interval; CoNS = grey; CoPS = black

Table 3. The number of staphylococcal isolates identified to species level by MALDI-TOF-MS, *nuc* gene PCR (CoPS), and *tuf* gene sequencing.

Staphylococcal species	Number of isolates	Number (%) of positive dogs	Number (%) identified by MALDI-TOF-MS	Number (%) of CoPS identified by *nuc* PCR	Number (%) identified by *tuf* gene sequencing
S. pseudintermedius	91	32 (44)	70 (77)	91 (100)	0
S. aureus	11	6 (8)	11 (100)	11 (100)	0
S. epidermidis	67	38 (52)	64 (96)	N/A	3 (4)
S. warneri	35	22 (30)	35 (100)	N/A	0
S. equorum	39	20 (27)	36 (92)	N/A	3 (8)
S. saprophyticus	19	11 (15)	15 (79)	N/A	4 (21)
S. sciuri	27	11 (15)	21 (78)	N/A	6 (22)
S. succinus	19	10 (14)	16 (84)	N/A	3 (16)
S. simulans	15	9 (12)	15 (100)	N/A	0
S. capitis	7	8 (11)	7 (100)	N/A	0
S. pasteuri	8	6 (8)	8 (100)	N/A	0
S. xylosus	17	6 (8)	17 (100)	N/A	0
S. lentus	9	5 (7)	4 (44)	N/A	5 (56)
S. hominis	6	4 (5)	6 (100)	N/A	0
S. cohnii	5	3 (4)	3 (60)	N/A	2 (40)
S. vitulinus	14	3 (4)	13 (93)	N/A	1 (7)
S. haemolyticus	4	3 (4)	4 (100)	N/A	0
S. fleurettii	4	2 (3)	0	N/A	4 (100)
S. arlettae	1	1 (1)	0	N/A	1 (100)
S. pettenkoferi	1	1 (1)	0	N/A	1 (100)
Total ID	399[d]	72[e]	345[f]	101[f]	33[f]
Staphylococcus spp.	3	N/A	N/A	N/A	3
Species related to *S. felis*	12	N/A	N/A	N/A	12
No ID	22	N/A	N/A	2	3
Total	436[a]	73[b]	436[c]	102[c]	51[c]

Values in the table are expressed as total numbers and percentage in parenthesis where applicable. [a]Total number of isolates in study; [b]Total number of dogs in study; [c]Total number of isolates tested by each method; [d]Total number of isolates with positive identification (ID); [e]Number of dogs with staphylococcal detection; [f]Number of isolates with positive ID from each method.

4. Discussion

This is the first study incorporating MALDI-TOF-MS to successfully characterise commensal staphylococcal populations in a group of healthy dogs in the absence of antimicrobial pressure. We isolated staphylococci from 99% of our dogs, with 95% carrying CoNS and 47% carrying CoPS. The relative prevalence of the staphylococci concurs with other published studies in humans (Kloos and Bannerman, 1994; von Eiff et al., 2002), horses (Busscher et al., 2006; De Martino et al., 2010; Karakulska et al., 2012; Moodley and Guardabassi, 2009; Yasuda et al., 2000) and dogs (Loeffler et al., 2005; Moon et al., 2012; Wedley et al., 2014), although the overall staphylococcal prevalence was double that reported

for healthy vet visiting dogs (Wedley et al., 2014). This could be related to the study population and techniques, as we sampled both the nose and the perineum to increase detection of CoPS (Fazakerley et al., 2010; Moon et al., 2012; Rubin et al., 2011; Windahl et al., 2012).

We were able to assign 92% of the staphylococcal isolates to 20 different species, including 18 CoNS. This is the first study to demonstrate such diversity in dogs, and carriage of this number of different species has only been previously reported for humans (Bagcigil et al., 2007; Cox et al., 1988; Fazakerley et al., 2010; Griffeth et al., 2008; Kania et al., 2004; Kloos and Bannerman, 1994; May et al., 2005; Medleau et al., 1986; Wedley et al., 2014; Widerstrom et al., 2012; Yamashita et al., 2005). The most common species was *S. epidermidis*, which was detected in 52% of the dogs, mainly from the nasal cavity. This is similar to human reports (Rogers et al., 2009), but apart from one canine study (Bagcigil et al., 2007), *S. epidermidis* has not been commonly reported in different animal species (Garbacz et al., 2013; Huber et al., 2011; Karakulska et al., 2012). *S. pseudintermedius* was the second most common species and the most common CoPS detected, also in agreement with previous reports (Devriese and De Pelsmaecker, 1987; Griffeth et al., 2008; Hanselman et al., 2009; Rubin et al., 2011). Unlike *S. epidermidis*, *S. pseudintermedius* was carried equally in the nose and on the perineum, suggesting that this species may have a wider range of mucosal niches. Very few dogs carried *S. aureus* (8%), which is comparable to other studies that reported carriage rates of ~ 7% from healthy vet visiting dogs (Fazakerley et al., 2010; Wedley et al., 2014). The majority of the CoNS in our study were human-associated and included *S. epidermidis*, *S. hominis, S. haemolyticus, S. capitis, S. saprophyticus, S. warneri, S. cohnii, S. simulans, S. pettenkoferi and S. pasteuri.* Human associated CoNS species have previously been isolated from dogs, horses, cows and pigs (Bagcigil et al., 2007; Gillespie et al., 2009; Huber et al., 2011; Karakulska et al., 2012; Malik et al., 2006; Tulinski et al., 2012). The other CoNS species isolated from our dogs are reported as indigenous to animals (*S. equorum*, *S. vitulinus, S. arlettae S. sciuri*, *S. lentus* and *S. fleurettii*) (Kloos and Bannerman, 1994).

We used several methods to identify staphylococcal isolates to species level. Multiplex PCR for the *nuc* gene is an accurate, rapid and cost efficient method to determine the species of CoPS (Sasaki et al., 2010), which identified 100% of our *S. pseudintermedius* (n = 91) and 100% of our *S. aureus* isolates (n = 11). Recently MALDI-TOF-MS has been reported as a rapid and reliable method to characterise CoNS, *S. aureus* and *S. intermedius* group (SIG) strains (Carpaij et al., 2011; Decristophoris et al., 2011; Dubois et al., 2010; Huber et al., 2011; Szabados et al., 2010). MALDI-TOF-MS identified all of our *S. aureus* isolates, 77%

of our *S. pseudintermedius* isolates and 79% of our CoNS isolates, identified by phenotypic and biochemical characteristics, to the species level. Similar results for the identification of *S. aureus*, *S. pseudintermedius* and CoNS by MALDI-TOF-MS, in comparison to molecular methods, have been reported (Bergeron et al., 2011; Decristophoris et al., 2011; Szabados et al., 2010). The overall performance of MALDI-TOF-MS to speciate the staphylococcal isolates in this study, similar to other reports (Decristophoris et al., 2011), is likely to be directly related to the database, which at the time of analysis consisted mainly of common human-derived species and only one S. *pseudintermedius* strain. However species level identification will improve as more highly characterised reference isolates are added to the database. Amplification and sequencing of the *tuf* gene is regarded as the gold standard to speciate CoNS isolates (Carpaij et al., 2011; Heikens et al., 2005). This method identified 77% of the tested staphylococcal isolates (n = 79) to the species level. The performance of this method in our study may have been affected by the lack of certain-animal derived isolates representing different species in the database. Additionally, we may have improved identification by sequencing a larger region of the *tuf* gene. We sequenced a previously described 412 base pair region of the *tuf* gene that was reported to have successfully identified 88% of human-derived staphylococcal strains (Heikens et al., 2005). However, a more recent publication that sequenced a 660 base pair region of the *tuf* gene, reported 98.9% identification of 186 human and animal-derived staphylococcal strains (Bergeron et al., 2011).

We did not detect any MR-CoPS isolates. Other studies of healthy dogs have similarly reported a low prevalence (Onuma et al., 2012; Vanderhaeghen et al., 2012; Wedley et al., 2014). In contrast, 58% of the dogs in our study carried at least one CoNS isolate with phenotypic oxacillin resistance and 42% carried oxacillin resistant *mec*A positive isolate. Other studies have also reported high levels of meticillin resistance among CoNS isolates from humans (Diekema et al., 2001; Garza-Gonzalez et al., 2011; Hanssen and Ericson Sollid, 2006), horses (Bagcigil et al., 2007; Busscher et al., 2006; De Martino et al., 2010; Moodley and Guardabassi, 2009) and livestock (Huber et al., 2011; Zhang et al., 2009). However, the prevalence of MR-CoNS carriage in our study is markedly higher than the levels reported in other community canine studies (Bagcigil et al., 2007; Malik et al., 2006; Vanderhaeghen et al., 2012; Vengust et al., 2006; Wedley et al., 2014). High community carriage rates of MR-CoNS are of concern for animals and humans, as these organisms may not only be reservoirs of resistance genes for CoPS (Barbier et al., 2010; Smyth et al., 2011; Tsubakishita et al., 2010), but also act as pathogens (Diekema et al., 2001; Duval et al., 2004; Hauschild and Wojcik, 2007; Kern and Perreten, 2013; Moran et al., 2007). Cross-transmission is reported to be an important mechanism for dissemination of MRS (Cimiotti et al., 2004; Silva et al.,

2001), and transmission between dogs and in-contact humans may occur in the community and in veterinary premises (Laarhoven et al., 2011; van Duijkeren et al., 2011).

Nine different CoNS species carried the *mec*A gene in our study with MRSE detected in 25% of our dogs. MRSE is the predominant MR-CoNS species in humans both in hospital and community settings (Barbier et al., 2010; Lebeaux et al., 2012; Silva et al., 2001), and has been reported in one study investigating nasal carriage of MRS in dogs (Bagcigil et al., 2007). Other canine studies have isolated meticillin resistant *S. sciuri* and meticillin resistant *S. warneri* (Bagcigil et al., 2007; Malik et al., 2006). Our research found that the majority of the *S. sciuri* and *S. fleurettii* isolates were *mec*A positive, which is consistent with other studies in humans, livestock and horses (Busscher et al., 2006; De Martino et al., 2010; Garza-Gonzalez et al., 2011; Huber et al., 2011; Karakulska et al., 2012).

MDR CoNS (n = 38) were isolated from 34% of dogs in this study. MDR was generally associated with resistance to beta-lactams, FA and additional antimicrobials. In particular, MDR-MRSE were resistant to at least four antimicrobial classes tested in our study. A similar finding was reported in a study of hospitalised animals, medical equipment and veterinary staff (Moon et al., 2012). MDR among CoNS isolates is widely reported (Garbacz et al., 2013; Huber et al., 2011; Silva et al., 2001; Wedley et al., 2014; Wisplinghoff et al., 2004) and may be associated with the carriage of multiple antimicrobial resistance genes on SCC*mec* cassettes (Smyth et al., 2011). In contrast, the majority of our commensal CoPS isolates were susceptible to a broad range of antimicrobials (apart from Tet), in line with previous reports for clinical isolates (Hoekstra and Paulton, 2002; Kruse et al., 1996; Lloyd et al., 1996) and isolates from healthy vet-visiting dogs (Wedley et al., 2014). There was good to very good agreement between disc and MIC antimicrobial susceptibility testing apart for FC and CIP. These two antimicrobials were the only ones where human breakpoints were applied and emphasises potential species differences in pharmacokinetic and pharmacodynamic data for individual antimicrobials.

The *mec*A gene was not identified in 40% of the phenotypic oxacillin resistant isolates in this study and may include some isolate duplication due to our sampling methods. Other studies have reported phenotypic meticillin resistance with absence of the *mec*A gene in staphylococci (Bignardi et al., 1996; Eckholm et al., 2013; Fessler et al., 2010; Suzuki et al., 1992). Our OX-resistant *mec*A negative isolates may be truly negative for the *mec*A gene as they were less likely to be resistant to the other antimicrobials tested in this study, including CVN and CFX, and had significantly lower MICs compared to the OX resistant *mec*A positive isolates. It is possible that they had low-level resistance associated with other mechanisms such as hyperproduction of beta-lactamases (Rosdahl and Rosendal, 1983), or

production of an oxacillin-specific beta-lactamases (Jones et al., 2007). There are bovine mastitis CoNS isolates with oxacillin MICs of 0.5 – 1 mg/l that lack the *mec*A gene (Fessler et al., 2010), and the CLSI guidelines state that 'oxacillin interpretive criteria may overcall resistance for these CoNS strains' (CLSI, 2013). In addition, many of the published PCR assays to identify and characterise the *mec*A gene have been developed for MRSA (Francois et al., 2003; Kondo et al., 2007; Mehrotra et al., 2000; Zhang et al., 2005) and may therefore lack sensitivity for some CoNS isolates. However, other authors have successfully employed the same methods for *mec*A detection among CoNS isolates as used in our study (Eckholm et al., 2013; Moon et al., 2012; Ruppe et al., 2009). Yet it is possible that additional PCR assay (Murakami et al., 1991), or latex agglutination for PBP2a (Baddour et al., 2007) may have improved the sensitivity of *mec*A detection or detected phenotypic *mec*A-associated resistance in our oxacillin resistant *mec*A negative isolates.

Our study had some limitations, including the small sample size. Nevertheless, these dogs yielded 436 staphylococcal isolates and a high prevalence of resistance was identified among the CoNS isolates even in the absence of antimicrobial exposure. Another weakness was that the study population was limited to one breed (Labrador retrievers) and the dogs were recruited at dog shows. Kennelled dogs have been shown to have higher levels of antimicrobial resistance in faecal *E. coli* than individually owned and non-kennelled dogs (De Graef et al., 2004). Kennelling was transient in our dogs, but this may have affected the results. Many of the dogs came from multi-dog households but only one dog from each household was sampled to avoid cluster effects.

5. Conclusions

This is the first comprehensive study of commensal staphylococcal populations in a group of healthy dogs. Staphylococci, particularly CoNS, form a normal part of the canine commensal population and were detected from almost all the dogs. The most commonly isolated staphylococcal species in this group of dogs was *S. epidermidis*, although a wide variety of other human- and animal-associated CoNS were found. CoPS were less common, and the major species was *S. pseudintermedius*. Antimicrobial resistance amongst the CoPS was uncommon, and no MRSP or MRSA were isolated, however the sample size was small. Antimicrobial resistance (including MDR and meticillin resistance) however was common among the CoNS isolates, even though this was a community population of healthy dogs in the absence of direct-antimicrobial pressure or veterinary contact. The clinical significance of commensal CoNS and MR-CoNS is unclear, but *S. epidermidis* carries a number of virulence factors and is an increasing cause of nosocomial and community-acquired infections in

humans. The possibility of similar infections escalating in companion animals cannot be excluded. In addition, there is potential for cross-species transmission of antimicrobial resistant bacteria and exchange of resistance determinants between bacterial species. In particular, MR- and MDR-CoNS may provide a reservoir of antimicrobial resistance genes that could rapidly spread within bacterial populations under the selection pressure exerted by antimicrobial therapy. Further longitudinal studies in healthy dogs and in dogs receiving antimicrobials are required to assess the population diversity, antimicrobial resistance profiles and persistence of antimicrobial resistant staphylococci in dogs.

4. Manuscript 2

Antimicrobial resistance and characterisation of faecal *Escherichia coli* isolated from healthy Labrador retrievers in the United Kingdom

Vanessa Schmidt[1,2], Tim Nuttall[3], Gina Pinchbeck[2], Neil McEwan[1], Susan Dawson[2], Nicola Williams[2]

[1]Department of Infection Biology and [2]Department of Epidemiology and Population Health, The University of Liverpool, Leahurst Campus, Neston, UK, [3]University of Edinburgh, The Royal (Dick) School of Veterinary Studies, Easter Bush Campus, Midlothian, UK.

Manuscript submitted to Veterinary Microbiology.

Summary

Background: Antimicrobial resistance (AMR), in particular multidrug resistance (MDR; resistance to three or more antimicrobial classes) is increasing amongst canine clinical and commensal isolates. This represents both an animal welfare and public health concern. Risk factors for the detection of AMR faecal *E. coli* include antimicrobial therapy and veterinary admission but few studies have examined commensal isolates in healthy community dogs.

Objectives: The main aim of the study was to characterise the population and AMR profiles of *E. coli* from healthy non-vet visiting and non-antimicrobial treated Labrador retrievers in the UK and to examine potential risk factors for the detection of such bacteria.

Methods: Faecal samples were collected from 73 Labrador retrievers and owners completed questionnaires regarding potential risk factors for AMR *E. coli*. Isolates were identified using phenotypic and biochemical methods and PCR assay for the *uid*A gene. Disc diffusion susceptibility tests were determined for a range of antimicrobials, including combination disc tests for phenotypic ESBL- and AmpC-production. PCR assay was used to detect resistance genes ($bla_{\text{CTX-M}}$ [groups 1, 2 and 9], bla_{SHV}, bla_{TEM}, bla_{OXA}, bla_{CIT}) and phylogenetic groups and conjugation tests were used to detect *in vitro* transfer of resistance determinants. Multivariable logistic regression was used to examine potential risk factors for the outcomes.

Results: AMR-, MDR- and AmpC-producing *E. coli* were detected in 63%, 30% and 16% of dogs, respectively. ESBL-producing *E. coli* ($bla_{\text{CTX-M}}$ group 1) were only detected from one dog. In conjugation experiments, MDR phenotype and $bla_{\text{CTX-M}}$ and bla_{CIT} were transferred from commensal *E. coli* to a recipient *E. coli* strain. The majority of the isolates were phylo-group B1 and group A. Group B2 isolates were more likely to be susceptible ($P < 0.001$) while group D isolates were more likely to be resistant ($P = 0.04$). Eating raw meat was associated with clavulanate-amoxicillin and third generation cephalosporin resistance (3GCR) and phylogenetic groups B1 and D. Multi-animal or multi-dog households were associated with beta-lactam resistance and 3GCR and phylogenetic groups A, E, F or Clades.

Conclusions: AMR, including MDR- and AmpC-producing *E. coli* were prevalent in this group of non-antimicrobial treated and non-vet-visiting dogs; however ESBL-production was rare. The majority of the isolates belonged to phylo-genetic groups B1 and A; potentially gut commensals. The main risk for the detection of AMR was the consumption of raw meat. These findings are of concern due to the potential for opportunistic infections, zoonotic transmission and transmission of antimicrobial resistant determinants from commensal isolates to pathogenic bacteria.

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); this may be disturbed by a number of factors including disease, diet and antimicrobial therapy (Jernberg et al., 2010; Stecher and Hardt, 2008; 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; resistance to ≥ 3 antimicrobial drug classes) (Dahmen et al., 2012; Karczmarczyk et al., 2011; Li et al., 2007; Zhao et al., 2001). AmpC production may also be associated with a chromosomal mutation, but these isolates are less likely to be MDR (Jacoby, 2009; Thomson, 2010). ESBL-, AmpC-producing and MDR *E. coli* have been detected in healthy (Carattoli et al., 2005; Costa et al., 2008; De Graef et al., 2004; Wedley et al., 2011) and sick dogs (Carattoli et al., 2005; Gibson et al., 2011a; Pomba et al., 2009; Steen and Webb, 2007), and increased detection has been associated with exposure to antimicrobials and veterinary healthcare (Damborg et al., 2011; Gibson et al., 2011a, b; Moreno et al., 2008).

E. coli can to some extent be divided into commensal and pathogenic strains. Although commensal strains may cause opportunistic infections in compromised patients, pathogenic strains are more likely to cause disease, either intestinal disease associated with enteric strains (intestinal pathogenic *E. coli*) or extra-intestinal disease associated with ExPEC strains (extra-intestinal pathogenic *E. coli*) (Russo and Johnson, 2000, 2003). 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). Pathogenic intestinal strains usually cause disease in a naïve host upon ingestion, whereas gut colonisation by ExPEC 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., 2009; Johnson et al., 2007; 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), recently updated by Doumith et al., (2012), has been widely used. Additionally a revision of the original method (Clermont et al., 2000) has recently been published (Clermont et al., 2013) and assigns isolates to eight different phylogenetic groups: A, B1, B2, C, D, E, F and *Escherichia* Clade I. Phylo-groups D and particularly B2 are more likely to be involved in extra-intestinal infections compared to A or B1 (Picard et al., 1999). Amongst the newer groups, C isolates are more likely to be commensals (Clermont et al., 2011b; Moissenet et al., 2010), group E and F are more likely to be ExPEC (Jaureguy et al., 2008; Tenaillon et al., 2010) and isolates belonging to *Escherichia* Clades are thought to reside outside of the gut (Walk et al., 2009).

The distribution of these phylo-groups amongst different hosts is not random and may depend on characteristics such as body mass, gut morphology, diet, environment and degree of domestication (Escobar-Paramo et al., 2006; Gordon and Cowling, 2003; Tenaillon et al., 2010). In 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 phylo-group A predominating in carnivores and omnivores, and group B1 in herbivores (Baldy-Chudzik et al., 2008; Carlos et al., 2010; Escobar-Paramo et al., 2006). Moreover domestication is associated with reduced B2 and more phylo-group A strains compared to wild animals (Escobar-Paramo et al., 2006).

Antimicrobial resistance has been linked to the non-B2 phylo-groups in people, cattle, pigs and dogs (Johnson et al., 2009; Johnson et al., 2003; Moreno et al., 2008). In dogs, phylo-group D isolates are more likely to be antimicrobial resistant, including fluoroquinolone, 3GCR and MDR (Platell et al., 2011; Sato et al., 2014; Tamang et al., 2012), and group B2 are more likely to be antimicrobial susceptible (Johnson et al., 2009; Platell et al., 2010; 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 (Johnson et al., 2009; Platell et al., 2010; Russo and Johnson, 2003). This represents both an animal welfare and zoonotic risk.

The majority of human and other animal studies of *E. coli* have concentrated on clinical isolates. However, to further our understanding of the antimicrobial resistance of pathogenic isolates under the influence of antimicrobial pressure and other potential risk factors, it is first necessary to elucidate the characteristics of gastrointestinal *E. coli* populations under natural conditions. The aim of this study was to determine the prevalence of antimicrobial resistance and phylogenetic groups amongst faecal

E. coli from a group of healthy non-vet visiting and non-antimicrobial treated dogs and to investigate the relationship of these findings to potential risk factors for antimicrobial resistance.

2. Materials and Methods

2.1 Study Population

Labrador retriever dogs were recruited on a convenience basis from two dog shows in the North West UK between November 2010 and June 2011. We aimed to recruit 30 dogs per show. 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 questionnaire regarding potential risk factors for the carriage of antimicrobial resistant bacteria. The two-page questionnaire was based on a previous study (Wedley et al., 2014) and consisted of 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 any household member worked with farm animals or in healthcare (Appendix I). The University of Liverpool, School, of Veterinary Science Ethics-Committee approved the study protocol.

2.2 Specimen collection and bacterial isolation

Owners were asked to collect the next fresh faecal sample in a sterile faecal pot (Appendix I). Faecal samples were returned to the University of Liverpool either at the time of enrolment or 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) to obtain single colonies. In addition, to detect antimicrobial resistant isolates, 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 in accordance with the direct plating method (Bartoloni et al., 2006). A further 500 μL of faecal homogenate was enriched in 4.5 ml of buffered

peptone water. All plates and broths were incubated aerobically for 18-20 hours at 37°C. If there had been no growth on the EMBA plates impregnated with third generation cephalosporins, the enriched broths were plated onto the same selective media and incubated aerobically for 18-20 hours at 37°C. Three random colonies, whose morphology resembled *E. coli* were selected from the plain EMBA plate. Where present, one colony growing within the zone of inhibition around each antimicrobial disc on both the EMBA and MAC plates and/or from the CX and/or CZ plates were also selected. These 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 Antimicrobial susceptibility testing

Antimicrobial susceptibility disc diffusion testing was performed on all *E. coli* isolates according to British Society for Antimicrobial Chemotherapy guidelines (BSAC; Version 11.1 May 2012) (Andrews, 2007). Iso-Sensitest agar plates were inoculated with each isolate homogenised in sterile distilled water (0.5 McFarland standards) for semi-confluent bacterial growth and the same panel of seven antimicrobial discs was applied. After the plates were incubated aerobically at 37°C for 18 to 20 hours, the zone diameters around each disc were measured in millimeters and recorded. *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 phenotypic AmpC- and ESBL-producing *E. coli*

Isolates with 3GCR were tested for the production of ESBL enzymes. An Iso-Sensitest agar plate was inoculated for confluent bacterial growth and three pairs of third generation cephalosporin discs (with and without clavulanic acid) were placed on the surface of the agar plate: 30 µg ceftazidime and 30 µg ceftazidime plus 10 µg clavulanic acid; 30 µg cefotaxime and 30 µg cefotaxime plus 10 µg clavulanic acid; and 30 µg cefpodoxime and 30 µg cefpodoxime plus 10 µg clavulanic acid. The plates were incubated aerobically at 37°C for 18 to 20 hours and zone diameters around each disc were measured and recorded. 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, according to the manufacturer's instructions (Extended Spectrum Beta-Lactamase Set D52C, MAST Group Ltd., Liverpool, UK) (M'Zali et al., 2000). *E. coli* isolates with 3GCR or clavulanate-amoxicillin resistance were tested for production of AmpC enzyme. An Iso-Sensitest agar plate was inoculated for confluent bacterial growth and three discs applied: 10 µg cefpodoxime plus AmpC inducer (A); 10 µg cefpodoxime plus AmpC inducer and ESBL inhibitor (B); 10 µg cefpodoxime plus AmpC inducer, ESBL inhibitor and AmpC inhibitors (C). The plates were incubated aerobically at 37°C for 18 to 20 hours and zone diameters around each disc were measured and recorded. AmpC production was confirmed when the zone of inhibition around disc C was disc was greater than that for discs A and B by a minimum of 5 mm, according to the manufacturer's instructions (AmpC detection set D69C, MAST Group Ltd., Liverpool, UK) (Halstead et al., 2012). *E. coli* ATCC® 25922 (LGC Standards, Teddington, UK) cultured overnight on CAB at 37°C was used as a control.

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

Cell lysates were prepared from 188 isolates by adding three colonies of each isolate to 500 µl of SDW to yield a 0.5 McFarland standard solution. The suspensions were then vortexed, heated at 100°C for 10 minutes and centrifuged. The supernatants were stored at 4°C.
PCR assays for the *uid*A gene (McDaniels et al., 1996) were performed to confirm that the isolates were *E. coli* before further characterisation. *E. coli* isolates positive for ESBL production by the double disc method (MAST Group Ltd., Liverpool, UK) and isolates resistant to either cefotaxime and/or ceftazidime and positive for AmpC production (n = 18) 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) genes. If positive for $bla_{\text{CTX-M}}$, isolates (n = 2) were tested for the presence of CTX-M group 1, 2 and 9 genes (Batchelor et al., 2005; Hopkins et al., 2006). Isolates identified as phenotypic AmpC producers by the AmpC detection set (MAST Group Ltd., Liverpool, UK) were tested for the presence of bla_{AmpC} gene carriage (Perez-Perez and Hanson, 2002); absence of this gene was supportive of chromosomal

AmpC. All isolates (n = 188) were tested for the presence of *qnr*A, *qnr*B or *qnr* S genes (Robicsek et al., 2006). All the 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. Positive control strains were included and molecular grade water (Sigma-Aldrich Company Ltd., Gillingham, UK) was used as the negative control (Table 4-1, Appendix II). 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. All phenotypically unique antimicrobial resistant and susceptible confirmed *E. coli,* from each dog were stored at – 80°C in Microbank vials (Pro-Lab, Bromborough, UK) for further characterisation and analysis.

2.6 Phylogenetic groups

E. coli isolates (n = 188) were segregated into phylogenetic groups by PCR assay. In short, a multiplex PCR for the phylo-groups A, B1, B2 and D was performed according to Doumith et al., (2012) and analysed according to Clermont et al., (2000). In addition, a multiplex PCR for amplification of the phylo-groups A, B1, B2, D, C, E, F and Clade I was performed and analysed according to Clermont et al., (2013) (Table 4-1, Appendix II), to compare the results of the two assays .

2.7 Conjugation experiments

The ability to transfer antimicrobial resistance determinants was tested as previously described (Karczmarczyk et al., 2011). The rifampin-resistant, lactose-negative strain *E. coli* 26R793 served as a recipient in the assays. Multidrug resistant *E. coli* isolates or those carrying bla_{AmpC} or $bla_{\text{CTX-M}}$ were tested. 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 (Sigma-Aldrich) 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. These isolates were subjected to antimicrobial susceptibility tests and PCR assay for bla_{AmpC} or $bla_{\text{CTX-M}}$ genes.

2.8 Statistical analysis

All questionnaire derived information and microbiological data were entered into a spreadsheet program (Microsoft Excel for Mac 2008, Microsoft Corporation) and these data were reviewed and

checked for coding of all variables. Independent (risk factor) variables were created from information obtained from the owner questionnaires. Except for the age of the dog, all variables were dichotomous or categorical in nature (Table 4).

Data analysis included descriptive statistics and binary logistic regression. Resistance to each tested antimicrobial, any antimicrobial (AMR) or multidrug resistance (MDR; resistance to greater than or equal to three antimicrobial classes) or the presence of ESBL- or AmpC-producing *E. coli* were expressed as percentages +/- 95% confidence intervals (CI). Each antimicrobial resistance outcome for each phylogenetic group and each phylogenetic group at the isolate level was similarly expressed. The antimicrobial resistance outcomes were AMR, clavulanate-amoxicillin (ACR), ciprofloxacin (CipR), third generation cephalosporin (3GCR; synonymous for phenotypic AmpC- and ESBL-producing isolates) or beta-lactam resistance (BLR; Amp, ACR, 3GCR) or MDR. The phylogenetic group outcomes were group A, group B1, group B2 and group D (Doumith et al., 2012), and groups C, E, F and clades (Clermont et al., 2013). For any pair of variables with a correlation coefficient of ≥ 0.7 only the variable with the smallest *P*-value was considered for further analysis.

Logistic regression was used to examine the association between the test variables and all resistance outcomes, phylogenetic group and antimicrobial resistance outcomes and phylogenetic group and conjugation. Initially all variables were analysed in a univariable multilevel model. All variables that showed some association with the presence of resistant *E. coli* or phylogenetic group on univariable analysis (*P*-value < 0.25) were considered for incorporation into a final multivariable model. The final models were constructed by a manual backwards stepwise procedure where variables with a likelihood ratio *P* value < 0.05 were retained in the model. Tests for correlation (Spearman's rho) and binary logistic regression were performed using SPSS software package (SPSS 20.0 for Mac, SPSS Inc, Chicago, Illinois).

3. Results

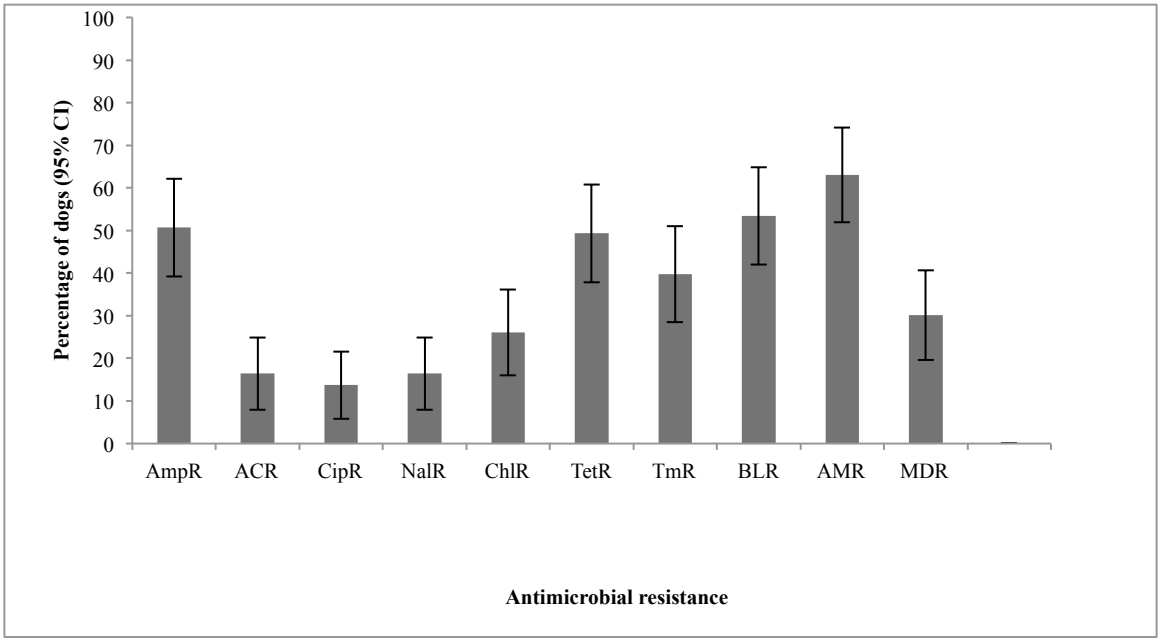
3.1 Study population

In total, 73 Labrador retriever dogs were recruited, with 21 dogs aged between 3 to 12 months, 25 dogs aged 12 months to two years old, and 27 dogs greater than two years old. There were 35 female and 38 male dogs. The demographics of the dogs included three countries of the UK, The Isle of Man, and 23 counties of England (Table 3-2, Appendix I). The majority (40%) were from the north west of the UK (Figure 3-1, Appendix I).

3.2 Antimicrobial resistance

Faecal *E. coli* (n = 188) were isolated from 72 dogs (99%; 95% CI: 92.6-99.8). At least one AMR isolate was detected in 46 dogs (63%; 95% CI: 51.5-73.2) and at least one MDR isolate in 22 dogs (30%; 95% CI: 20.8-41.4) (Figure 1). A high prevalence of resistance to ampicillin and/or tetracycline and/or trimethoprim was observed and this combination was the most common MDR phenotype, detected in 9 dogs (12%; 95% CI: 6.6-21.8) (Table 1). Chloramphenicol was the next most common resistance phenotype, detected in 19 dogs (26%; 95% CI: 17.3-37.1). Fewer dogs had faecal *E. coli* with resistance to ciprofloxacin (10 dogs; 14%) or clavulanate-amoxicillin (12 dogs; 16%). Combined ciprofloxacin and clavulanate-amoxicillin resistance was rare (3 dogs) although the majority of the ciprofloxacin isolates were also detected in association with MDR isolates (8 dogs).

Figure 1. The percentage of dogs (n = 73) with antimicrobial resistant faecal *E. coli* (error bars = 95% CI).



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

Table 1. The different antimicrobial resistance profiles observed amongst the *E. coli* isolates from 73 dogs.

Resistance Profile	Number of dogs carrying profile	Percentage of dogs carrying profile
AMP	36	53
TET	30	44
TM	3	4
NAL	2	3
AMP, TET	16	24
AMP, TM	2	3
TET, TM	7	10
AMP, TET, TM	9	13
CHL, TET	2	3
CHL, TET, TM	1	1
AMP, CHL, TET	7	10
AMP, CHL, TET, TM	5	7
AMP, AC	4	6
AMP, AC, TM	1	1
AMP, AC, TET	2	3
AMP, AC, TET, TM	2	3
AMP, NAL, TET, TM	1	1
AMP, CHL, NAL, TET, TM	1	1
CIP, NAL	1	1
CIP, NAL, TET	1	1
AMP, AC, CHL, TM	1	1
AMP, AC, CHL, TET, TM	1	1
AMP, CIP, CHL, NAL, TET, TM	4	6
AMP, CIP, NAL, TM	1	1
AMP, CIP, NAL, TET, TM	2	3
AMP, AC, CIP, CHL, NAL, TET, TM	2	3

AMP = ampicillin; TET = tetracycline; TM = trimethoprim; NAL = nalidixic acid; CHL = chloramphenicol; AC = clavulanate-amoxicillin; CIP = ciprofloxacin

3.3 ESBL- and AmpC-producing *E. coli*

Only one dog carried ESBL-producing faecal *E. coli* (CTX-M group 1). The isolate was also MDR (AmpR, 3GCR, TetR, TMR) and carried a bla_{TEM} gene. Cefpodoxime and clavulanate-amoxicillin resistant, phenotypic AmpC-producing *E. coli* (n = 16 isolates) were detected in 12 dogs (16%; 95% CI: 9.7-26.6), with five of these isolates from four dogs (6%; 95% CI: 2.2-13.3) MDR. However, only three isolates from three dogs (4%; 95% CI: 1.4-11.4) carried bla_{AmpC} genes (all CIT) and one isolate also carried bla_{TEM}. No isolates carried bla_{SHV}, bla_{OXA} or *qnr* genes. One isolate carrying bla_{AmpC} was also MDR, including ciprofloxacin resistance (Table 2).

Table 2. The resistance profiles of the ESBL- and AmpC-producing *E. coli* isolates (n = 18).

Isolate	Amp	AC	Cip	Chl	Nal	Tet	TM	MDR	AmpC	ESBL	bla_{AmpC}	bla_{CTXM}	bla_{TEM}
1	R	R	S	S	S	R	S	S	Y	N	N	N	N
2	R	R	S	S	S	R	S	S	Y	N	bla_{CITM}	N	Y
3	R	R	S	R	S	R	R	R	Y	N	N	N	N
4	R	R	R	R	R	R	R	R	Y	N	bla_{CITM}	N	N
5	R	R	S	S	S	R	S	S	Y	N	N	N	N
6	R	R	S	S	S	R	S	S	Y	N	N	N	N
7	R	R	S	S	S	R	S	S	Y	N	N	N	N
8	R	R	S	S	S	R	S	S	Y	N	N	N	N
9	R	R	S	S	S	R	S	S	Y	N	N	N	N
10	R	R	S	S	S	S	S	S	Y	N	N	N	N
11	R	R	S	S	S	S	S	S	Y	N	N	N	N
12	R	R	S	S	S	R	R	R	Y	N	N	N	N
13	R	R	R	R	R	R	R	R	Y	N	N	N	N
14	R	R	S	S	S	S	S	S	Y	N	N	N	N
15	R	R	S	S	S	S	S	S	Y	N	bla_{CITM}	N	N
16	R	R	S	S	S	R	R	R	Y	N	N	N	N
17	R	S	S	S	S	R	R	R	N	Y	N	Gp1	Y
18	R	S	S	S	S	R	R	R	N	Y	N	Gp1	Y

Amp = ampicillin resistance; AC = clavulanate-amoxicillin resistance; Cip = ciprofloxacin resistance; Chl = chloramphenicol resistance; Nal = nalidixic acid resistance; Tet = tetracycline resistance; TM = trimethoprim resistance; MDR = multidrug resistance (resistance to ≥ 3 antimicrobial class); ESBL = phenotypic ESBL-producing *E. coli* = ESBL, AmpC = phenotypic AmpC-producing *E. coli*; bla_{AmpC} = carriage of AmpC genes; bla_{CTXM} = carriage of CTX-M genes; bla_{CITM} = carriage of CIT genes; Gp1 = ESBL with CTX-M gene group 1; R = resistant; S = susceptible

3.4 Phylogenetic groups

The first method by Doumith et al., (2012) assigned 58 *E. coli* isolates to phylogenetic group A (31%; 95% CI: 24.7-37.8), 78 to group B1 (42%; 95% CI: 34.4-48.9), 33 to group B2 (18%; 95% CI: 12.8-23.6) and 19 to group D (10%; 95% CI: 6.6-15.2). The second method by Clermont et al., (2013) assigned 15 *E. coli* isolates to phylogenetic group A (8%; 95% CI: 4.9-12.7), 78 to group B1 (42%; 95% CI: 34.4-48.9), 31 to group B2 (17%; 95% CI: 11.9-22.5) and seven to group D (4%; 95% CI: 1.8-7.5). The remaining isolates that were assigned to A group by the first method were reassigned to either group C (n = 39 isolates) or *Escherichia* Clades III-IV (n = 4 isolates). Additionally nine isolates that were assigned to either group B1 (n = 1), B2 (n = 3) or D (n = 5) by the first method were reassigned to group E and three isolates, assigned to group D by the first method, were reassigned to group F. There were also two isolates grouped as B1 by the first method that were classed as unknown by the second method.

The agreement between the two methods was compared using a *kappa* statistic. The agreement was very good for the assignment of phylo-groups B1 (*kappa* = 0.890) and B2 (*kappa* = 0.884), but there was only fair and moderate agreement respectively for phylo-groups A (*kappa* = 0.289) and D (*kappa* = 0.470). However, when the assignment of group A by the first method was compared to the assignment of group A or C, by the second method, the agreement was also very good (*kappa* = 0.924).

3.5 Conjugation studies

All MDR isolates (n = 61), including two ESBL- and three AmpC-producing isolates, were tested for the transfer of antimicrobial resistance genes to a recipient *E. coli* strain. In total, 23 isolates transferred resistance to the recipient strain including ESBL- and AmpC-producing *E. coli*. The isolates that successfully transferred resistance determinants were negatively associated with phylo-group A (OR 0.2; CI: 0.05-0.66; *P* = 0.005) and C (OR 0.12; CI: 0.02-0.57; *P* = 0.002) but positively associated with phylo-group B1 (OR 1.58; CI: 1.55-15.19; *P* = 0.004) (Table 3).

Table 3. Details of the conjugation experiments: multidrug resistance isolates that have transferred resistance profiles/antimicrobial resistance genes onto the recipient strain*.

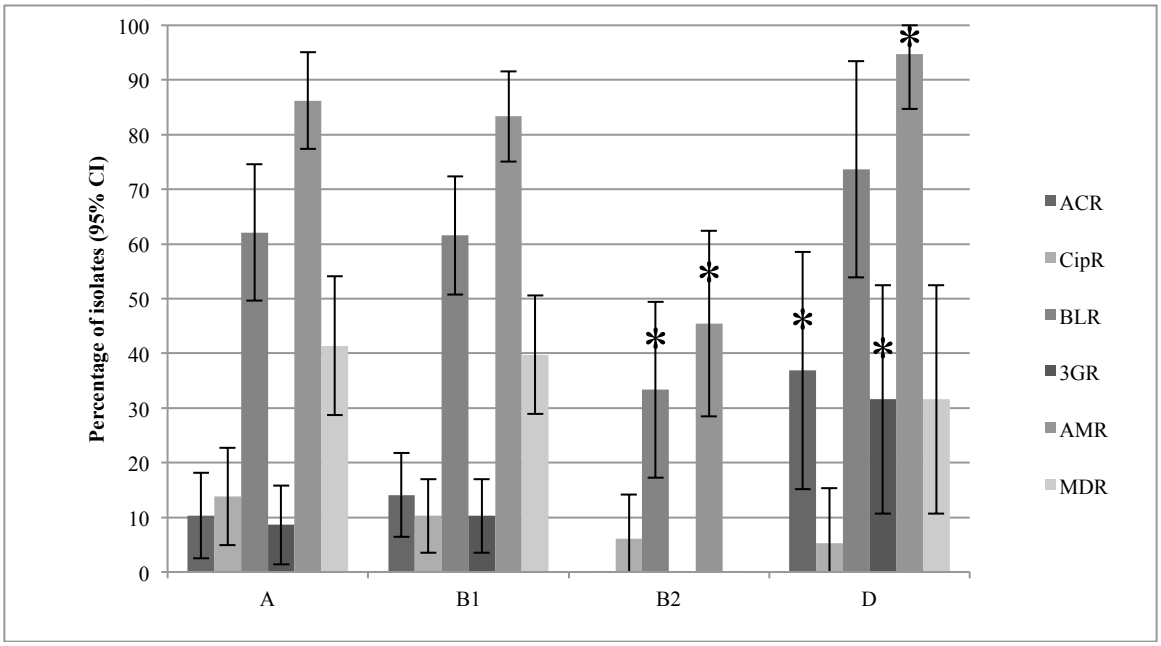
Donor resistance phenotype	Transconjugate resistance phenotype	bla_{CIT} or bla_{CTX-M} gene[1] transconjugate
AMP, TET, TM	AMP, TET, TM	
AMP, CHL, TET, TM	AMP, TET, TM	
AMP, CHL, TET, TM	AMP, CHL, TET, TM	
AMP, TET, TM	TET	
AMP, CHL, TET, TM	AMP, TET	
AMP, TET, TM	AMP, TET, TM	
AMP, TET, TM	AMP, TET, 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 (Karczmarczyk et al., 2011); AMP = ampicillin; AC = clavulanate-amoxicillin; CIP = ciprofloxacin; CHL = chloramphenicol; NAL = nalidixic acid; TET = tetracycline; TM = trimethoprim; [1]present in donor and transconjugate.

3.6 Association between phylo-groups and antimicrobial resistance outcomes

ESBL-producing *E. coli* belonged to phylo-group B2, whereas the 17 AmpC-producing *E. coli* belonged to groups A (n = 5), B1 (n = 8) or D (n = 4). One isolate carrying bla_{AmpC} belonged to group B1 and two isolates to group A (including the ciprofloxacin and MDR isolate). The majority of the isolates in phylo-groups A, B1 and D were resistant to at least one antimicrobial, particularly a beta-lactam, whereas group B2 isolates were unlikely to be antimicrobial resistant ($P < 0.001$) and did not contain isolates with either clavulanate-amoxicillin, 3GCR or MDR. On the other hand, group D isolates were likely to be antimicrobial resistant ($P = 0.04$), in particular to clavulanate-amoxicillin ($P = 0.004$) and 3GCR ($P = 0.005$). Ciprofloxacin resistant isolates were spread between the phylo-groups (Figure 3; Table 4-2, Appendix II).

Figure 3. Percentage of isolates (n = 188) with each antimicrobial resistance outcome (n = 6) and phylo-group (error bars = 95% CI).



95% confidence interval = 95% CI; phylo-groups A, B1, B2 or D (Doumith et al., 2012); ACR = clavulanate-amoxicillin resistance; CipR = ciprofloxacin resistant; BLR = beta-lactam antimicrobial resistant; 3GR = third generation cephalosporin resistant; AMR = any antimicrobial resistance; MDR = multidrug resistance; *significant ($P < 0.05$)

3.7 Logistic regression of antimicrobial resistance, phylo-groups & questionnaire data

The diet of all dogs in this study consisted of dry dog food supplemented either with tinned dog food, home-cooked meats, proprietary dog treats, table scraps or raw meat. In addition, a number of dogs regularly scavenged from the environment. The variables, obtained from questionnaire data, and the investigated outcomes were all categorical other than age (Table 4). Clavulanate-amoxicillin resistance ($P = 0.003$) and 3GCR ($P = 0.002$) were associated with dogs eating raw meat (chicken, red-meat and/or tripe). Multi-animal households were also at increased risk for faecal carriage of *E. coli* with 3GCR ($P = 0.04$) and in particular, multi-dog households were of borderline significance for beta-lactam resistance ($P = 0.08$). Consumption of proprietary dog treats appeared to be protective against ciprofloxacin ($P = 0.003$) and MDR ($P = 0.001$) *E. coli* (Table 5). Univariable models are included in the appendix of this chapter (Table 4-3, Appendix II).

Table 4. The number and percentage of dogs with each categorical variable and outcome for 73 dogs.

Variable	ACR	CipR	BLR	3GCR	AMR	MDR	Phylo-group A	Phylo-group B1	Phylo-group B2	Phylo-group D	Phylo-group C	Phylo-groups E, F, Clades	Total dogs with variable
Dog eats raw meat	6 (50)	4 (40)	10 (26)	6 (55)	11 (24)	7 (32)	7 (30)	9 (30)	1 (8)	5 (38)	7 (41)	3 (25)	15 (21)
Dog eats animal carcass or faeces	9 (75)	5 (50)	22 (56)	7 (64)	25 (54)	12 (55)	15 (65)	13 (43)	7 (58)	8 (62)	12 (71)	2 (17)	42 (58)
Dog eats tinned or cooked meat	1 (8)	2 (20)	7 (18)	3 (27)	10 (22)	4 (18)	1 (4)	6 (20)	2 (17)	2 (15)	1 (6)	1 (8)	16 (22)
Dog fed treats	10 (83)	3 (30)	25 (64)	8 (73)	32 (70)	10 (46)	15 (65)	18 (60)	11 (92)	9 (69)	8 (47)	4 (33)	52 (71)
Dog fed table scraps	7 (58)	6 (60)	19 (49)	6 (55)	21 (46)	10 (46)	12 (52)	13 (43)	3 (25)	6 (46)	7 (41)	1 (8)	37 (51)
Multi-dog household	12 (100)	9 (90)	34 (87)	7 (64)	38 (84)	20 (91)	21 (91)	24 (80)	8 (67)	12 (92)	16 (94)	5 (42)	56 (77)
Multi-animal household	6 (50)	6 (60)	17 (44)	1 (9)	21 (48)	10 (46)	12 (52)	12 (40)	5 (42)	7 (54)	8 (47)	4 (33)	30 (41)
Owner works with farm animals	1 (8)	3 (30)	5 (13)	1 (9)	10 (23)	2 (9)	6 (26)	7 (23)	1 (8)	2 (15)	3 (18)	0	15 (21)
Owner works in healthcare	4 (33)	2 (20)	12 (31)	4 (36)	16 (36)	5 (24)	8 (35)	8 (27)	6 (50)	7 (54)	6 (35)	3 (25)	25 (34)
In-contact been hospitalised[1]	8 (67)	7 (70)	24 (62)	8 (73)	28 (62)	13 (59)	12 (52)	18 (60)	7 (33)	9 (69)	9 (53)	4 (33)	44 (60)
In-contact received antimicrobials[1]	5 (42)	8 (80)	25 (64)	5 (45)	31 (69)	15 (68)	12 (52)	20 (67)	6 (50)	8 (62)	10 (59)	4 (33)	44 (60)
Male	5 (42)	4 (40)	16 (41)	5 (45)	19 (41)	8 (36)	9 (39)	12 (41)	6 (50)	5 (38)	7 (41)	2 (17)	34 (47)
Female	7 (58)	6 (60)	23 (59)	6 (55)	27 (59)	14 (64)	14 (61)	17 (57)	6 (50)	8 (62)	10 (59)	4 (30)	37 (51)
Total dogs with outcome	12 (16)	10 (14)	39 (53)	11 (15)	46 (63)	22 (30)	23 (31)	30 (40)	12 (16)	13 (17)	17 (23)	12 (16)	73 (100)

[1]In-contact person or pet within 12 months before enrolment. ACR = clavulanate-amoxicillin resistance; CipR = ciprolfloxacin resistance; BLR = beta-lactam resistance; 3GCR = third generation cephalosporin resistance; AMR = antimicrobial resistance to at least one disc; MDR = multidrug resistance (resistance to three or more antimicrobial classes); phylo-groups A-D are from (Doumith et al., 2012) and C, E, F and clades from (Clermont et al., 2013).

Table 5. Final multivariable logistic regression models for the six antimicrobial resistance outcomes amongst faecal *E. coli* from 73 dogs.

Resistance outcome and final model variables				
Clavulanate-amoxicillin (ACR)	***β***	***OR***	***95% CI***	***P-value***
Dog eats raw meat[1]	2.25	9.57	2.0-45.68	0.003*
In-contact had antimicrobials[2]	- 1.79	0.19	0.04-0.91	0.028*
Ciprofloxacin resistance (CipR)	***β***	***OR***	***95% CI***	***P-value***
Dog fed treats[1]	- 2.07	0.13	0.03-0.56	0.004*
Beta-lactam resistance (BLR)	***β***	***OR***	***95% CI***	***P-value***
Multi-dog household[1]	1.16	3.19	0.85-11.99	0.08
Owner works with farm animals[1]	- 1.06	0.35	0.1-1.19	0.09
3rd generation cephalosporin resistance (3GCR)	***β***	***OR***	***95% CI***	***P-value***
Dog eats raw meat[1]	2.39	10.86	2.18-54.03	0.002*
In contact had antimicrobials[1]	- 1.55	0.21	0.04-1.07	0.05
Multi-animal household[1]	1.62	5.06	0.97-26.5	0.04
Any antimicrobial resistance (AMR)	***β***	***OR***	***95% CI***	***P-value***
Gender (Male is reference)	0.81	2.25	0.83-6.1	0.11
Multidrug resistance (MDR)	***β***	***OR***	***95% CI***	***P-value***
Dogs fed treats[1]	- 1.9	0.15	0.04-0.49	0.001*
Owner works with farm animals[1]	- 1.5	0.23	0.04-1.3	0.07

[1] Reference category is the absence of the risk factor; [2]Within 12 months of enrolment; β = coefficient; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the likelihood ratio test; AMR = antimicrobial resistance to at least one antimicrobial; MDR = antimicrobial resistance to three or more antimicrobial classes; *significant at $P < 0.05$.

Association with a raw meat diet was also significant for phylo-groups B1 ($P = 0.032$) and D ($P = 0.014$) and of borderline significance ($P = 0.056$) for group B2 isolates. Multi-dog households were associated with phylo-groups A ($P = 0.02$) and C ($P = 0.05$), while having an owner working in healthcare was significant for carriage of group D ($P = 0.015$). Dogs rolling in, or eating animal carcasses or faeces were negatively associated with group B1 ($P = 0.018$) and being fed treats was negatively associated with group C ($P = 0.011$) (Table 6). Univariable models are included in the appendix of this chapter (Table 4-4, Appendix II).

Table 6. Final multivariable logistic regression models for the six-phylogeny outcomes amongst faecal *E. coli* from 73 dogs.

Resistance outcome and final model variables				
Phylogenetic group A	**β**	**OR**	**95% CI**	***P*-value**
Multi-dog household[1]	2.00	7.4	0.89-61.21	0.020*
Phylogenetic group B1	**β**	**OR**	**95% CI**	***P*-value**
Dog eats raw meat[1]	1.38	3.9	1.08-14.64	0.032*
Dog eats animal carcass or faeces[1]	- 1.26	0.28	0.1-0.83	0.018*
Phylogenetic group B2	**β**	**OR**	**95% CI**	***P*-value**
Dog eats raw meat[1]	1.17	3.2	0.94-11.0	0.056
Phylogenetic group D	**β**	**OR**	**95% CI**	***P*-value**
Dog eats raw meat[1]	2.08	8.04	1.37-47.21	0.014*
Owner works in healthcare[1]	1.88	6.57	1.24-34.82	0.015*
Phylogenetic group C	**β**	**OR**	**95% CI**	***P*-value**
Multi-dog household[1]	1.84	6.29	0.68-57.9	0.054
Dog fed treats[1]	- 1.58	0.21	0.06-0.72	0.011*
Age (variable is continuous)	- 0.018	0.98	0.96-1.01	0.075
Phylogenetic groups E, F or Clades	**β**	**OR**	**95% CI**	***P*-value**
Dog eats raw meat	2.85	5.44	1.43-20.72	0.014*

[1] Reference category is the absence of the risk factor; β = coefficient; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the likelihood ratio test; AMR = antimicrobial resistance to at least one antimicrobial; MDR = antimicrobial resistance to three or more antimicrobial classes; phylo-groups A, B1, B2 and D based on (Doumith et al., 2012); C, E, F and Clades based on (Clermont et al., 2013); *significant at $P < 0.05$.

4. Discussion

This study found a high level of antimicrobial and multidrug resistance 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. However, the majority of the isolates were of commensal (B1 and A) phylogenetic backgrounds and detection of ESBL- and plasmid-mediated AmpC-producing *E. coli* was rare.

The level of MDR was high (30% of dogs) compared to that in previous reports of healthy

dogs in the UK of 15% (Wedley et al., 2011). This discrepancy may be due to the five-year difference in sample collection between the studies as the prevalence of AMR may have increased in such populations over time. In addition, the dogs in this study were regularly in close contact, in confined environments, with many other dogs and humans at dog shows around the UK. Sharing of faecal *E. coli* isolates may occur within kennels (Harada et al., 2011), within households between humans and pets (Damborg et al., 2009; Johnson et al., 2008; Stenske et al., 2009) and probable transmission of MDR resistant faecal *E. coli* from humans to dogs (Stenske et al., 2009). Moreover, kenneled dogs can have a higher prevalence of AMR faecal *E. coli* than individually owned dogs (De Graef et al., 2004), possibly associated with antimicrobial selection pressure and/or exposure through diet, the environment or other dogs.

Only one dog had faecal *E. coli* carrying a $bla_{\text{CTX-M}}$ gene and only three dogs carried bla_{AmpC}. A similar prevalence (< 1% of faecal samples) was reported for healthy dogs in semi-rural Cheshire (Wedley et al., 2011). A larger UK wide study reported a slightly higher prevalence of 4% and 7% of ESBL- and AmpC-producing *E. coli* respectively (Wedley, 2012) but these dogs may have received antimicrobials or had other veterinary contact within 12 months of sampling, and may be more representative of the whole UK vet visiting dog population. Antimicrobial therapy with enrofloxacin or cefalexin has been reported to select for faecal *E. coli* carrying $bla_{\text{CTX-M}}$ or bla_{AmpC} respectively (Damborg et al., 2011; Moreno et al., 2008) and the absence of antimicrobial therapy in 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).

The majority of the isolates in this study belonged to phylo-group B1 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 13 healthy dogs in Denmark. However, this predominance of group B1 followed by group A has been reported in farmed and wild herbivorous animals (Carlos et al., 2010; Escobar-Paramo et al., 2006), 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. 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 (O'Brien and Gordon, 2011), although this variable was not examined in the current study. Escobar-Paramo et al., (2006) found increased prevalence of B1 and A and decreased B2 and D from domesticated animals compared to wild animals, which is in agreement with our findings. The authors proposed that this might be associated with high antimicrobial pressure in the farmed animals deselecting antimicrobial susceptible B2 strains, although the dogs in our study had not received antimicrobials. The secondary habitat of *E. coli* is soil, sediment and water (Savageau, 1983) where phylo-group B1 isolates are more likely to persist than the other groups (Walk et al., 2009). Given the propensity for dogs to drink, scavenge and orally explore their environments, these habits may predispose them to a predominance of B1 but many other factors may be involved. French pig-farmers were found to have a faecal *E. coli* phylogeny (Escobar-Paramo et al., 2004) more similar to their livestock than to other human populations (Escobar-Paramo et al., 2006). As dogs live in very close contact with humans it would be interesting to see if dog owners have a divergent phylogeny from non-dog owners and the general human population.

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., 2009; Johnson et al., 2003; Platell et al., 2010; Platell et al., 2011; Sato et al., 2014). Similarly, this study showed that group B2 isolates tended to be more susceptible and in particular, less likely to be clavulanate-amoxicillin, 3GCR or MDR. Phylo-group D was more likely to be antimicrobial resistant; in particular to clavulanate-amoxicillin and 3GCR. Other studies have reported a relationship between group D and fluoroquinolone resistance, 3GCR and MDR (Deschamps et al., 2009; Platell et al., 2011; Sato et al., 2014; Tamang et al., 2012). Phylo-group D was not associated with fluoroquinolone resistance in this study, although the low prevalence of this phylo-group and fluoroquinolone resistance may have precluded accurate statistical analysis.

The analysis of AMR outcomes and phylogenetic groups with potential risk factors found a relationship between dogs fed raw diets (chicken, red meat and/or tripe) with clavulanate amoxicillin and 3GCR, as well as with phylo-groups B1, D and combined E, F and Clades. In addition multi-dog households were associated with 3GCR and phylogenetic group A, and dogs with owners working in either human or veterinary healthcare were more likely to have phylo-group D faecal *E. coli* isolates. In contrast, consumption of proprietary dog treats appeared to be protective against ciprofloxacin resistance and phylogenetic group C; group C was associated ciprofloxacin resistance on univariable analysis. Dogs fed *Salmonella*-contaminated raw-meat diets have been reported to shed *Salmonella* in their faeces (Finley et al., 2007). Food, particularly chicken meat, has been reported as a possible source of antimicrobial resistant bacteria, including ExPEC, for humans and dogs (Johnson et al., 2009;

Johnson et al., 2007; 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 (Cortes et al., 2010; Johnson et al., 2003; Johnson et al., 2007; Unno et al., 2009). 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.

Sharing of faecal *E. coli* between household members correlates with increasing numbers of in-contact humans and pets (Johnson et al., 2008). This may include phylo-group A strains, which appear to be equally present in humans and animals (Tenaillon et al., 2010). Furthermore, people working in healthcare may represent reservoirs of antimicrobial resistant and/or pathogenic bacteria for their home and its occupants. Phylo-group D isolates may potentially cause extra-intestinal infections and, together with probable antimicrobial resistance, may be selected within healthcare environments.

There was very good agreement between the two methods for assigning isolates to group B1 and B2 and differences were mainly associated with the reassignment of the groups. The prevalence of group C in our study was high compared to the prevalence reported for a variety of pathogenic and commensal *E. coli* isolates of human, bird and non-human mammal origin (Clermont et al., 2013) but further studies will be required to confirm this finding in dogs. Phylo-groups E and F are potential ExPEC strains (Jaureguy et al., 2008; Tenaillon et al., 2010) and were re-assigned from group D or B2 with similar risk factors to group D. Four isolates were assigned to *Escherichia* Clades (Clermont et al., 2013). *Escherichia* Clades have been more commonly isolated from non-human mammals and birds than people and are unlikely to be pathogenic (Clermont et al., 2011a). They have not been reported previously in dogs but as they may emanate from an environmental source (Walk et al., 2009) it is not surprising to find them.

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. Another limitation was that the study only included one breed and had limited demographics. It is possible that samples from other breeds and other geographical populations would differ.

5. Conclusions

The overall prevalence of AMR and MDR amongst canine faecal *E. coli* was higher than expected for a group of healthy non-vet-visiting and non- antimicrobial-treated dogs. However the level of ESBL and AmpC-producing *E. coli* was low and had not increased compared to earlier UK studies. The predominant faecal *E. coli* phylogenetic group in this group of dogs was group B1, and group B2 was less likely than the other groups to harbour antimicrobial resistance, in agreement with previous work. Certain risk factors were identified. In particular, raw meat diets were associated with increased detection of beta-lactam resistance and phylo-group D, representing a potential animal welfare and zoonotic risk. Further strain characterisation, including virulence typing, may help to elucidate this further. 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. Transient bacteria may act as a reservoir of antimicrobial resistant determinants for resident bacteria. Additionally, selective pressure through antimicrobial therapy may facilitate establishment as permanent residents of the gut flora. Gut diversity is likely to be dynamic and maybe cyclical. Future research should include longitudinal studies with genotyping to better determine and understand these changes.

5. Manuscript 3

Longitudinal study of antimicrobial resistance and characterisation of faecal *Escherichia coli* isolated from healthy dogs in the United Kingdom

Vanessa Schmidt[1,2], Tim Nuttall[3], Gina Pinchbeck[2], Neil McEwan[1], Susan Dawson[2], Nicola Williams[2]

[1]Department of Infection Biology and [2]Department of Epidemiology and Population Health, The University of Liverpool, Leahurst Campus, Neston, UK, [3]University of Edinburgh, The Royal (Dick) School of Veterinary Studies, Easter Bush Campus, Midlothian, UK.

Summary

Background: The intestinal *E. coli* population structure in healthy humans and other animals is diverse with differing phenotypes and genotypes over time. Risk factors for antimicrobial resistant (AMR) faecal *E. coli* include antimicrobial therapy and veterinary admission but few longitudinal studies have examined commensal isolates in healthy dogs under normal exposures.

Objectives: The main aim of the study was to characterise the population structure and AMR profiles of *E. coli* in healthy non-vet visiting and non-antimicrobial treated dogs over three months and to examine potential risk factors of carriage.

Methods: Faecal samples were collected from 28 dogs; daily for seven days, weekly for four weeks, and monthly for two months. Isolates were identified using phenotypic and biochemical methods and PCR assay for the *uid*A gene. Disc diffusion susceptibility tests were determined for a range of antimicrobials, including combination disc tests for phenotypic ESBL- and AmpC-production. A selection of isolates from three dogs was strain typed using Pulsed Field Gel Electrophoresis (PFGE). Survival analysis was calculated for resistance outcomes and multilevel, multivariable models were used to examine potential risk factors of carriage.

Results: AMR to at least one antimicrobial, MDR, AmpC- and ESBL-producing *E. coli* were detected in 45%, 14%, 20% and 4% of samples (n = 341), respectively. AMR to at least one antimicrobial and AmpC-producing *E. coli* could be persistent, whereas MDR and ESBL-producing isolates were usually intermittent and/or transient. The mean survival of isolates with AMR, MDR, ESBL and AmpC-production was 58, 24, 29 and 18 days, respectively. Marked genetic diversity was demonstrated on an individual animal basis. There was frequent change of resistance phenotype and genotype, however resident clones were detected. Owners working with farm animals were associated with AMR to any antimicrobial, eating raw meat was associated with MDR and ESBL/AmpC-production and the consumption of dog treats was associated with MDR faecal *E. coli*.

Conclusions: AMR was common and persistent. MDR- and AmpC-production was less frequent, transient or intermittent. ESBL-production was rare and transient, suggesting exogenous exposure, and eating raw meat was associated with MDR and ESBL/AmpC production. The *E. coli* population structure was diverse with frequent change of resistance phenotype and genotype over time. These findings highlight the diversity of gut *E. coli* under natural conditions and potential risk factors that should be taken into account when examining the impact of antimicrobial therapy.

3. Introduction

Antimicrobial resistance (AMR), particularly multi-drug resistance (MDR), is increasing amongst *E. coli* from people and other animals (Ewers et al., 2012; Gould, 2009; Hunter et al., 2010). Of major concern is the emergence and dissemination of ESBL-producing *E. coli* that are resistant to a wide range of beta-lactam antimicrobials, including oxyimino-cephalosporins (Gould, 2008). Additionally, AmpC-producing *E. coli* that are common amongst animal isolates, are resistant to cephamycins and not inhibited by clavulanic acid (Jacoby, 2009; Li et al., 2007; Thomson, 2010). Genes encoding these enzymes can be spread between bacteria by horizontal transmission on mobile genetic elements (Li et al., 2007), and due to genetic linkage with other antimicrobial resistance determinants, isolates are commonly MDR (Gould, 2009; Paterson and Bonomo, 2005). MDR, ESBL- and AmpC-producing *E. coli* have been detected in healthy and hospitalised dogs (Gibson et al., 2011b; Guo et al., 2013; Wedley, 2012; Wedley et al., 2011) and in canine clinical samples (O'Keefe et al., 2010; Sanchez et al., 2002; Sidjabat et al., 2006).

Colonisation of the hosts' large intestine is a prerequisite for extra-intestinal infections with *E. coli* (Martindale et al., 2000). Gastrointestinal *E. coli* may include both commensal and ExPEC (extra-intestinal pathogenic *E. coli*) strains. ExPEC strains are more likely to reside within phylogenetic group B2, and to a lesser extent group D (Picard et al., 1999). ExPEC are more likely than commensal strains to carry a range of virulence genes that may be involved in pathogenesis (Russo and Johnson, 2003). In particular, virulence genes may encode colonisation factors, such as P fimbriae that facilitate gut colonisation by resident *E. coli* strains. Resident strains are adapted to the gut environment and can persist for extended periods, and in humans they are often of phylo-group B2 (Nowrouzian et al., 2001; Nowrouzian et al., 2006; Sears et al., 1950; Sears et al., 1956). Transient strains are only present for a few days to weeks but may act as a reservoir of AMR determinants for resident bacteria (Wellington et al., 2013), or antimicrobial selective pressures may lead to longer persistence in the gut (Dethlefsen et al., 2007; Edlund and Nord, 2000).

Longitudinal studies in people, horses, cattle and dogs have reported the intestinal *E. coli* population structure to be diverse and in a state of flux (Anderson et al., 2006; Damborg et al., 2009; Schlager et al., 2002). Schlager et al., (2002) examined faecal *E. coli* from female children, in the absence of antimicrobial pressure, and reported a change of resident clone on a weekly basis in approximately 60% of cases. Anderson et al., (2006) also reported a changing dominant subtype on a monthly basis in horses, cattle and adult humans (Anderson et al., 2006). This latter study also reported greater diversity amongst *E. coli* populations, with

respect to AMR phenotypes and genotypes, in farm animals compared to humans. Damborg et al., (2009) reported either long-term or intermittent detection of one or two resident *E. coli* strains in the majority of people and dogs, however there was high individual diversity with frequent detection of different clones, particularly in dogs. Overall faecal *E. coli* population dynamics can differ between individuals and between host species; possibly associated with particular host factors such as signalment, diet and health status (Anderson et al., 2006).

The intestinal microbiota has a protective barrier function for the host (Vollaard and Clasener, 1994). Disruption of the microbiota and selection of AMR may ensue following exposure to certain factors including disease, diet and medications (Katouli, 2010; Stecher and Hardt, 2008; Vincent et al., 2010). In particular, antimicrobial therapy can disturb the intestinal microbial flora and select for AMR (Edlund and Nord, 2000; Jernberg et al., 2010). Both antimicrobial therapy and veterinary clinic admission are reported risk factors for the detection of MDR, ESBL and AmpC-producing *E. coli* in dogs (Damborg et al., 2011; Gibson et al., 2011a, b; Moreno et al., 2008; Stenske et al., 2009). Additionally, the environment (Wellington et al., 2013), in-contact humans or pets (Damborg et al., 2009; Johnson et al., 2008; Sidjabat et al., 2006) and farm-animal meat (Johnson et al., 2009; Johnson et al., 2007; Vincent et al., 2010) are possible sources of AMR *E. coli* or ExPEC strains.

Shedding of AMR faecal *E. coli* has been reported in dogs and horses receiving antimicrobial therapy (Damborg et al., 2011; Damborg et al., 2012; Gronvold et al., 2010; Johns et al., 2012; Lawrence et al., 2013; Trott et al., 2004). Hospitalisation in addition to antimicrobial therapy increased the duration of shedding in horses in one study (Johns et al., 2012). Faecal shedding of AMR bacteria and/or determinants leads to environmental contamination and potential dissemination to in-contact humans or pets (Johnson et al., 2009; Johnson et al., 2001). However, few studies have examined temporal faecal shedding of antimicrobial resistant *E. coli* in healthy dogs in the community.

E. coli community profiles have been commonly investigated to detect antimicrobial selection pressure on the gastrointestinal flora (Gronvold et al., 2010), however study construction and interpretation should be based on knowledge and understanding of the normal diversity that occurs in healthy individuals over time. This study was performed to characterise the diversity of the canine faecal *E. coli* population structure and AMR shedding patterns in the absence of direct antimicrobial pressure or veterinary premise contact but with normal day-to-day exposures. This study may act as a baseline for further risk factor associated studies in dogs.

2. Methods

2.1 Study population

A convenience sample (n = 28 dogs) of staff-owned healthy dogs was recruited from the University of Liverpool, Leahurst Campus, between October 2011 and May 2012. Exclusion criteria included antimicrobial therapy or veterinary admission within three months of enrolment. Dogs were excluded during the study if they became ill, were prescribed systemic antimicrobials or attended veterinary premises. Before enrolment, all dog owners read the study information sheets and gave written informed consent. Owners were asked to provide a fresh faecal sample from their dog once daily for seven days (days 0, 1, 2, 3, 4, 5, & 6), followed by once weekly for four weeks (days 13, 20, 27 & 34) and then once monthly for two months (days 62 & 90). Labelled sterile faecal pots (n = 13) were provided for the owner of each dog at the time of enrolment. Samples were either delivered in person or by first-class post. Email and phone text reminders were sent before each weekly and monthly sample was due. A two-page questionnaire for potential risk factors for the carriage of AMR bacteria was administered at the start and end of the study. Questionnaires were returned in person, by first-class post or by email and consisted of closed questions with tick box responses and space for additional information (Appendix III). 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, diet, the presence and type of in-contact pets, previous medical history of the household (including antimicrobial therapy or hospital contact of humans and pets) and whether owners worked with farm animals or in healthcare. All questionnaire-derived information was available as potential explanatory variables for inclusion in the multivariable modelling of antimicrobial resistance outcomes. The University of Liverpool, School of Veterinary Science Ethics-Committee approved the study protocol in October 2011.

2.2 Specimen collection and bacterial isolation

Faecal samples were mixed with an equal volume of brain heart infusion broth containing 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), to obtain single colonies. In addition, to detect antimicrobial resistant isolates, one EMBA plate and one MacConkey agar (MAC) plate were inoculated with the faecal homogenate for confluent bacterial growth with seven antimicrobial discs (10 μg ampicillin, 30 μg clavulanate-amoxicillin, 1 μg ciprofloxacin, 30 μg chloramphenicol, 30 μg nalidixic acid, 30 μg tetracycline and 2.5 μg trimethoprim) (Bartoloni et al., 2006; Bartoloni et al., 1998). A further 500 μL of faecal homogenate was enriched in 4.5 ml of buffered peptone water. All plates and broths were incubated aerobically for 18-20 hours at 37°C. If there had been no growth on the EMBA plates impregnated with third generation cephalosporins, the enriched broths were streaked onto the same selective media and incubated aerobically for 18 to 20 hours at 37°C. Three random colonies, whose morphology resembled *E. coli*, were selected from the plain EMBA plate. Where present, one colony growing within the zone of inhibition around each antimicrobial disc on both the EMBA and MAC plates and/or from the CX and/or CZ plates were also selected. These colonies were sub-cultured onto nutrient agar and incubated aerobically for 18 to 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. PCR assays for the *uid*A gene (McDaniels et al., 1996) were performed to confirm that the isolates were *E. coli* before further characterisation. All antimicrobial discs were obtained from MAST Group Ltd., Liverpool, UK, the media from LabM Ltd, Bury, UK, and the CX and CZ powder from Sigma-Aldrich Company Ltd, Gillingham, UK.

2.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility disc diffusion testing was performed on all *E. coli* isolates according to British Society for Antimicrobial Chemotherapy guidelines (BSAC; Version 11.1 May 2012) (Andrews, 2007). Iso-Sensitest agar plates were inoculated, with isolates homogenised in sterile distilled water (1:10 dilution of a 0.5 McFarland standard), for semi-confluent bacterial growth and the same panel of seven antimicrobial discs were applied. After the plates were incubated aerobically at 37°C for 18 to 20 hours, the zone diameters around each disc were measured in millimeters and recorded. *E. coli* ATCC® 25922 (LGC Standards, Teddington, UK), was used as a control.

2.3 Screening for phenotypic AmpC- and ESBL-producing *E. coli*

Isolates that grew on media containing third generation cephalosporins were tested for the production of ESBL enzymes. An Iso-Sensitest agar plate was inoculated for confluent bacterial growth and three pairs of cephalosporin discs (with and without clavulanic acid) were placed on the surface of the agar plate: 30 µg ceftazidime and 30 µg ceftazidime plus 10 µg clavulanic acid; 30 µg cefotaxime and 30 µg cefotaxime plus 10 µg clavulanic acid; and 30 µg cefpodoxime and 30 µg cefpodoxime plus 10 µg clavulanic acid. The plates were incubated aerobically at 37°C for 18 to 20 hours and zone diameters around each disc were measured and recorded. 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 by one or more of the antimicrobial pairs, according to the manufacturer's instructions (Extended Spectrum Beta-Lactamase Set D52C, MAST Group Ltd., Liverpool, UK) (M'Zali et al., 2000). *E. coli* isolates resistant to third generation cephalosporins or clavulanate-amoxicillin were tested for production of AmpC enzyme. An Iso-Sensitest agar plate was inoculated for confluent bacterial growth and three discs applied: 10 µg cefpodoxime plus AmpC inducer (A); 10 µg cefpodoxime plus AmpC inducer and ESBL inhibitor (B); 10 µg cefpodoxime plus AmpC inducer, ESBL inhibitor and AmpC inhibitors (C). The plates were incubated aerobically at 37°C for 18 to 20 hours and zone diameters around each disc were measured and recorded. 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, according to the manufacturer's instructions (AmpC detection set D69C, MAST Group Ltd., Liverpool, UK) (Halstead et al., 2012). *E. coli* ATCC® 25922 (LGC Standards, Teddington, UK) was used as a control.

2.4 Macro-restriction pulsed field gel electrophoresis (PFGE)

PFGE was performed on a selection of isolates from 21 samples (n = 3 dogs) to compare phenotypic resistance outcome with genotype. X*ba*I digested genomic DNA was analysed in 1% agarose gels in 0.5 x Tris-boric acid (TBE) buffer at 14°C in a CHEF-DRIII PFGE system in accordance with (Ribot et al., 2006). Banding patterns were assessed for each dog to examine individual genetic diversity over time.

2.5 Statistical analysis

All questionnaire derived information and microbiological data were entered into a spreadsheet program (Microsoft Excel for Mac 2008, Microsoft Corporation) and the dataset was reviewed and checked for coding of all variables. Independent (risk factor) variables were created from information obtained from the owner questionnaires. Except for the age of the dog, all variables were dichotomous or categorical in nature. The variable body weight was divided into three categories: small (< 11 kg), medium (11 - 20 kg) and large (> 20kg) (Table 1).

Microbiological data were collapsed to the sample level and categorised for time of sample collection. Initial data analysis included calculation of the percentage, with 95% confidence intervals, of samples with antimicrobial resistance to each tested antimicrobial and in addition, antimicrobial resistance to at least one tested antimicrobial (AMR), multidrug resistance (MDR; resistance to three or more antimicrobial classes) and the presence of either extended-spectrum beta-lacatmase (ESBL) or AmpC-producing *E. coli* (AmpC).

Kaplan Meier plots were utilised to calculate the duration of shedding of antimicrobial resistant faecal *E. coli* for the four outcomes: AMR, MDR, ESBL and AmpC. The survival time was the time in days from the appearance of the resistance outcome until the first occurrence of two consecutive negative samples. If the end event (loss of resistance outcome for two consecutive samples) did not occur before the dog left of completed the study it was censored. The first six samples were considered together so that the minimum duration was seven days. Kaplan-Meier plots were created to determine the mean and median duration of each outcome. The Log-rank test was used to detect differences between the resistance outcomes (significance $P < 0.05$).

To examine the effect of independent variables on the presence of resistance, data were analysed using a multilevel logistic regression models with a binomial distribution and logit link. Due to the inclusion of multiple dogs from some owners and repeated samples over time, data were clustered within household (level three) and within dogs (level two units) and this clustering was accounted for by inclusion of second- and third-level random intercept terms in all models. Faecal samples were considered the level one unit of interest. Three binary outcomes were investigated: AMR, MDR and the presence of either ESBL- or AmpC-producing *E. coli* (ESBL/AmpC).

Spearman's rho was used to test for correlation amongst the independent variables. For any pair of variables with a correlation coefficient of ≥ 0.7 only the variable with the smallest *P*-value was considered for further analysis. Initially all variables were analysed in a univariable

multilevel model. All variables that showed some association with the presence of resistant *E. coli* on univariable analysis (*P*-value < 0.25) were considered for incorporation into a final multivariable model for that outcome. The final models were constructed by a manual backwards stepwise procedure where variables with a Wald *P*-value < 0.05 were retained in the model. First order interaction terms were tested for biologically plausible variables remaining in the final models. Multilevel models were analysed using the MLwiN statistical software package (MLwiN Version 2.28 Centre for Multilevel Modelling, University of Bristol). Univariable and multivariable calculations were performed using penalised quasi-likelihood estimates (2nd order PQL for all outcomes). Calculating and graphing the residual +/- 1.96 SD x rank (caterpillar plots) was performed for each household and dog to check for outliers (Figures 5-1 to 5-6, Appendix III). If present, outliers were removed and the models were rerun to assess the effects. Tests for correlation (Spearman's rho) and survival analysis were performed using SPSS software package (SPSS 20.0 for Mac, SPSS Inc, Chicago, Illinois).

3. Results

3.1 Study population

In total, 28 dogs, 18 male and ten female, were recruited from 21 households for this study; the average age was 81 months and ranged from 5 - 146 months. The main diet of all dogs consisted of dry complete dog food and the majority of the dog owners worked in veterinary health care; owners from 10 households worked in veterinary clinical practice. Five dogs did not complete the study; two dogs left the study after seven days, one dog after 14 days, one after 21 days and one after 35 days. One dog left the study due to enteritis, two dogs were prescribed antimicrobials and two owners withdrew for personal reasons. Overall there were 341 faecal samples provided during a 90-day period (Table 1).

Table 1. The variables and outcomes considered for inclusion in the final multivariable model, with the number and percentage (%) of dogs.

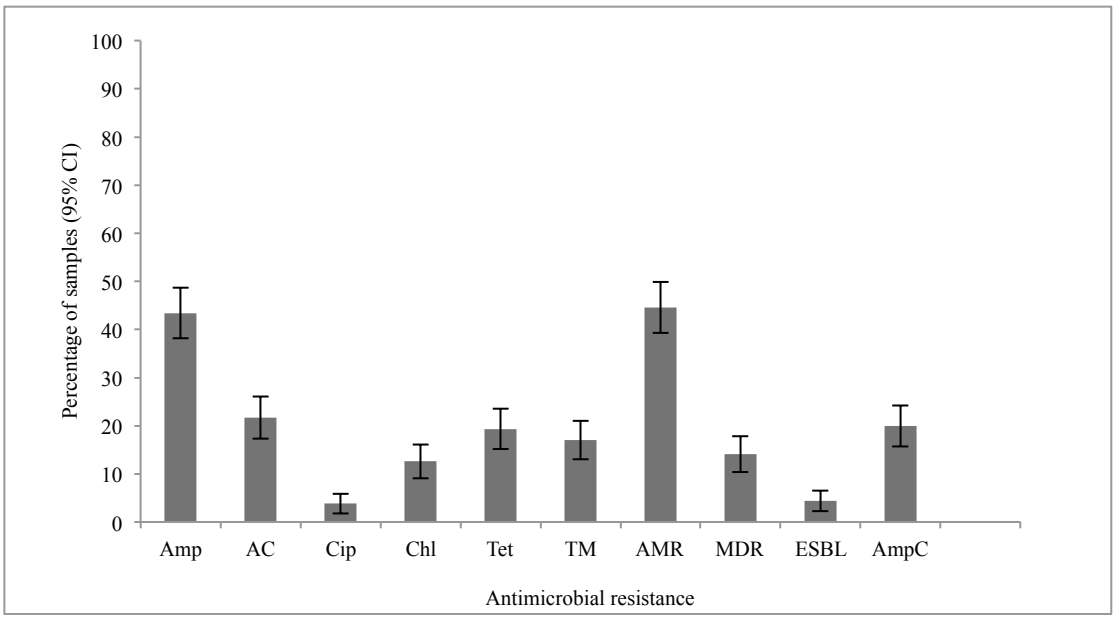
Variable	AMR	MDR	AmpC/ESBL	Total
Age[a] (mean age in months)	83	82	86	81
Purebred REF	19 (68)	14 (50)	13 (46)	21 (75)
Crossbreed	6 (21)	5 (19)	5 (18)	7 (25)
Weight (< 11kg) REF	11 (40)	9 (32)	6 (21)	13 (46)
Weight (11-20kg)	6 (21)	4 (14)	5 (18)	6 (21)
Weight (> 20kg)	8 (29)	6 (21)	7 (25)	9 (32)
Male REF	17 (61)	13 (46)	13 (46)	18 (64)
Female	8 (29)	6 (21)	5 (18)	10 (36)
Eat tinned meat	6 (22)	4 (15)	3 (11)	6 (22)
Eat raw meat	5 (19)	5 (19)	5 (19)	5 (19)
Eat treats	18 (67)	15 (56)	14 (52)	19 (71)
Eat table scraps	19 (70)	15 (56)	14 (52)	20 (74)
Eat or roll carcass	5 (19)	5 (19)	4 (15)	6 (22)
Eat animal faeces	12 (44)	10 (37)	10 (37)	14 (52)
Multi-dog household	17 (63)	14 (52)	15 (56)	18 (67)
Multi-animal household (not dog)	6 (22)	6 (22)	5 (19)	7 (26)
Owner works with farm animals	7 (26)	6 (22)	6 (22)	7 (26)
Owner works in health care	20 (74)	16 (60)	15 (56)	22 (81)
In-contact human or pet received systemic antimicrobials[b]	10 (37)	9 (33)	10 (37)	11 (41)
In-contact human or pet admitted to hospital or veterinary premise[b]	11 (41)	10 (37)	9 (33)	12 (44)

AMR = antimicrobial resistance to at least one tested antimicrobial; MDR = multidrug resistance (resistance to three or more antimicrobial classes); ESBL = extended spectrum beta-lactamase producing *E. coli*; AmpC = AmpC enzyme producing *E. coli*. [a]Age was the only categorical value and is represented by the mean age of dogs in each treatment group rather than the number and percentage of dogs; REF = the reference category for non-dichotomous variables; [b]Within 12-months of enrolment.

3.2 **Prevalence of antimicrobial resistance**

Overall AMR, MDR, AmpC- and ESBL-producing *E. coli* were detected in at least one sample, in 25, 19, 18 and eight dogs respectively. From 341 samples, AMR *E. coli* were detected in 152 samples (45%; 95% CI: 39.4-50), MDR in 48 samples (14%; 95% CI: 10-8-18.2), AmpC-producing *E. coli* in 68 samples (20%; 95% CI: 16-24.5) and ESBL-producing *E. coli* in only 15 samples (4%; 95% CI: 2.7-7) (Figure 1).

Figure 1. Percentage of samples (n = 341) with antimicrobial resistant *E. coli* (error bars = 95% CI)



Amp = ampicillin; AC = clavulanate-amoxicillin; Cip = ciprofloxacin; Chl = chloramphenicol; Tet = tetracycline; TM = trimethoprim; AMR = resistance to at least one tested antimicrobial; MDR = multidrug resistance

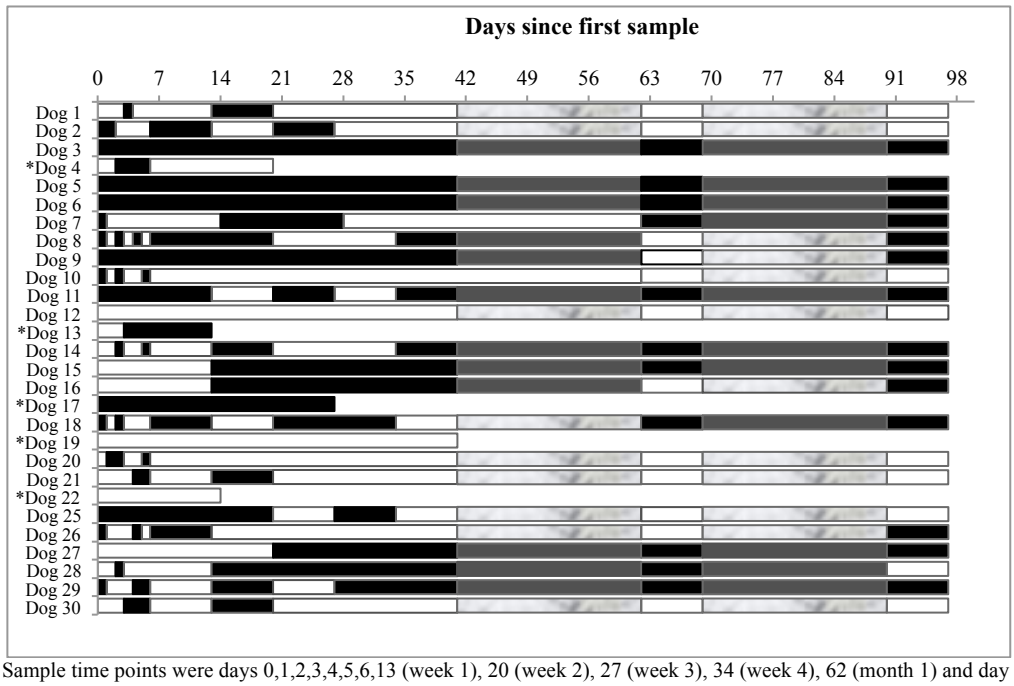
3.3 **Changes in antimicrobial resistance over time**

E. coli isolates with AMR to at least one antimicrobial were not detected in any sample from three dogs, however only one of these dogs completed the study and there was no commonality amongst questionnaire data for these dogs (one Labrador cross, one Pug and one Shih Tzu). *E. coli* isolates with AMR to at least one antimicrobial were detected in every sample submitted from three dogs that completed the study. Ampicillin resistance with or without resistance to other antimicrobials was the most common resistance phenotype. Two dogs that completed the study had stable resistance to ampicillin in every isolate in every sample. Both dogs (one Chihuahua and one Cocker Spaniel) lived in single-animal households, ate only proprietary dog food or cooked meat, rarely ate treats or table scraps and only one dog rarely ate animal faeces. Both dog owners were veterinarians, one small animal and one farm animal.

Other resistance phenotypes, particularly MDR, were generally intermittent and/or transient over time. AmpC-producing *E. coli* however, could be also be detected long term in a small number of dogs whereas ESBL-producing *E. coli* were infrequently isolated and transient (Figure 2).

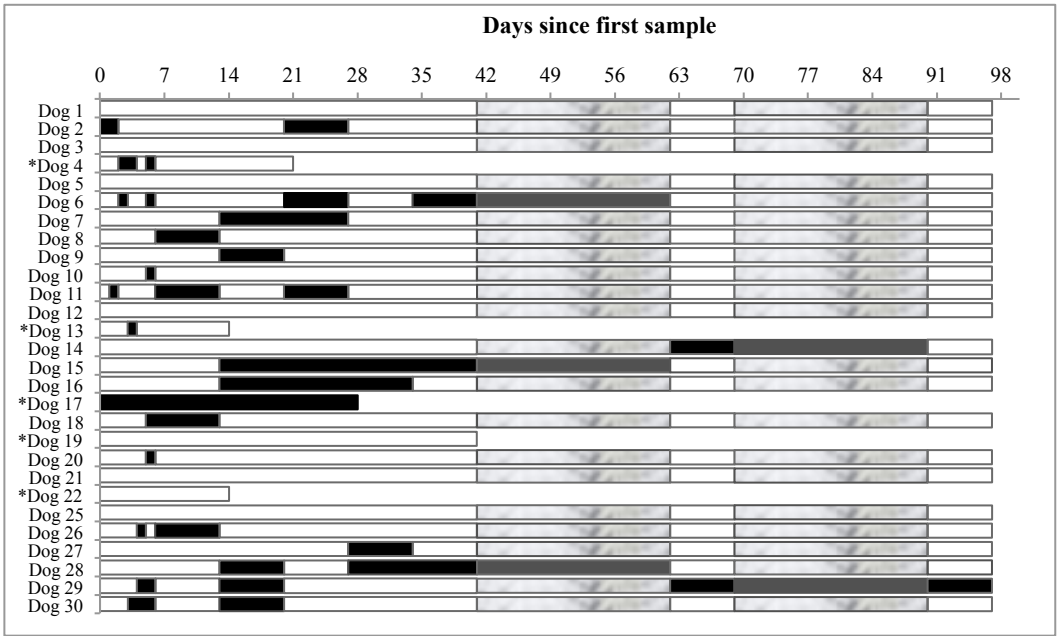
Figure 2. Faecal carriage of a) any resistance (AMR), b) multidrug resistance (MDR), c) ESBL-producing and d) AmpC-producing *E. coli* in 28 healthy community dogs.

a) AMR



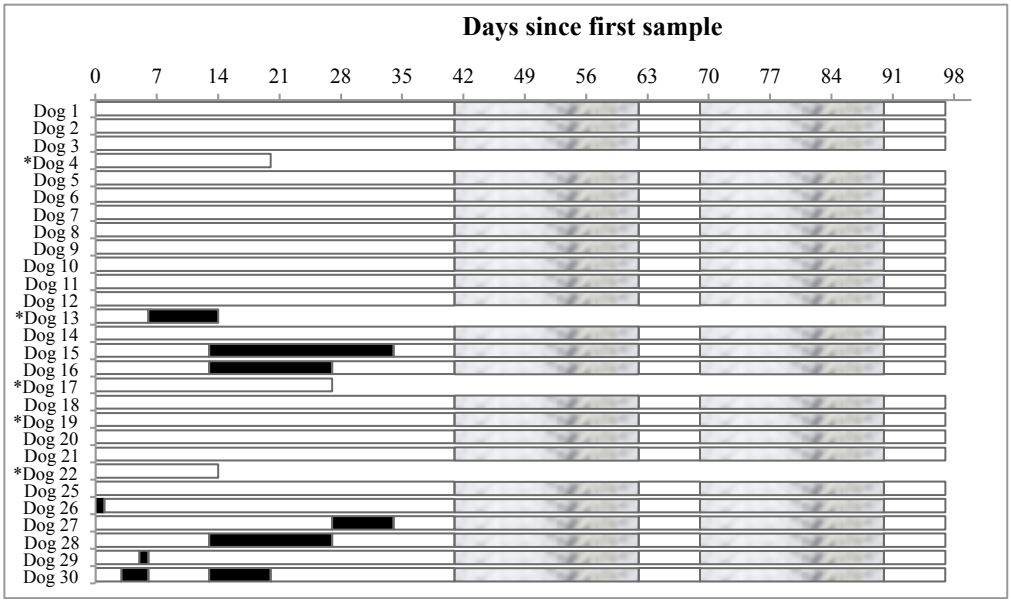
Sample time points were days 0,1,2,3,4,5,6,13 (week 1), 20 (week 2), 27 (week 3), 34 (week 4), 62 (month 1) and day 90 (month 2). After week 4 (34 days) detection is still expressed as seven day intervals: Black bars represent positive detection, white bars represent negative detection, grey solid bars follow positive detection and are expected to be positive but samples were not examined during this period; textured grey bars follow negative detection and are expected to be negative but samples were not examined during this period; * dogs that did not complete the study

b) MDR



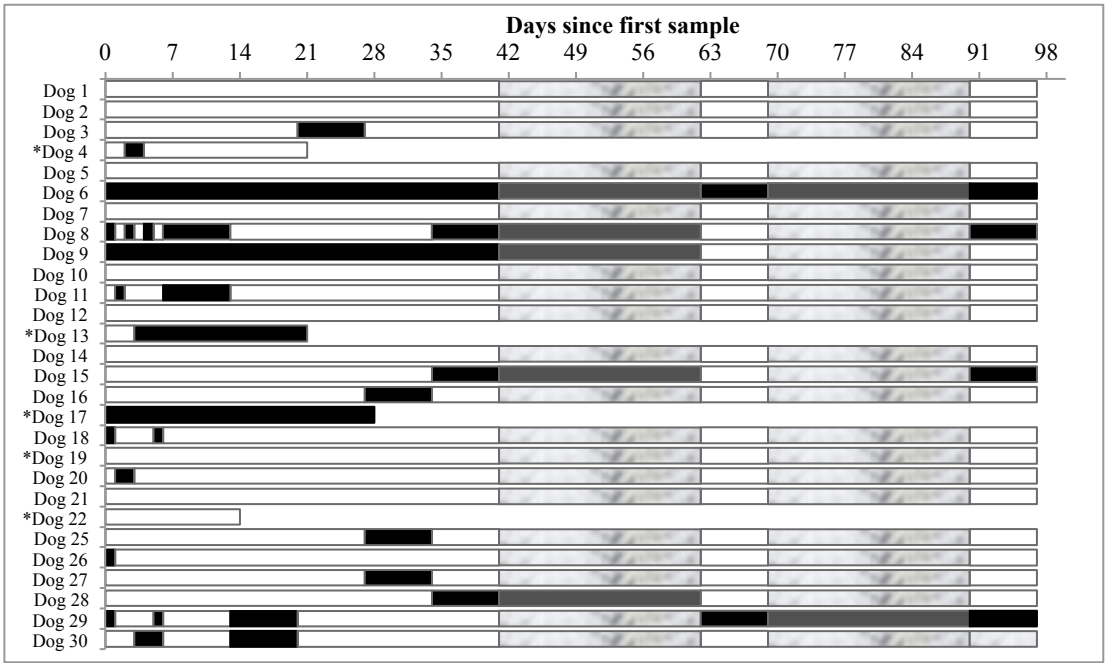
Sample time points were days 0,1,2,3,4,5,6,13 (week 1), 20 (week 2), 27 (week 3), 34 (week 4), 62 (month 1) and day 90 (month 2). After week 4 (34 days) detection is still expressed as seven day intervals: Black bars represent positive detection, white bars represent negative detection, grey solid bars follow positive detection and are expected to be positive but samples were not examined during this period; textured grey bars follow negative detection and are expected to be negative but samples were not examined during this period; * dogs that did not complete the study

c) ESBL-producing *E. coli*



Sample time points were days 0,1,2,3,4,5,6,13 (week 1), 20 (week 2), 27 (week 3), 34 (week 4), 62 (month 1) and day 90 (month 2). After week 4 (34 days) detection is still expressed as seven day intervals: Black bars represent positive detection, white bars represent negative detection, grey solid bars follow positive detection and are expected to be positive but samples were not examined during this period; textured grey bars follow negative detection and are expected to be negative but samples were not examined during this period; * dogs that did not complete the study

d) **AmpC-producing *E. coli***

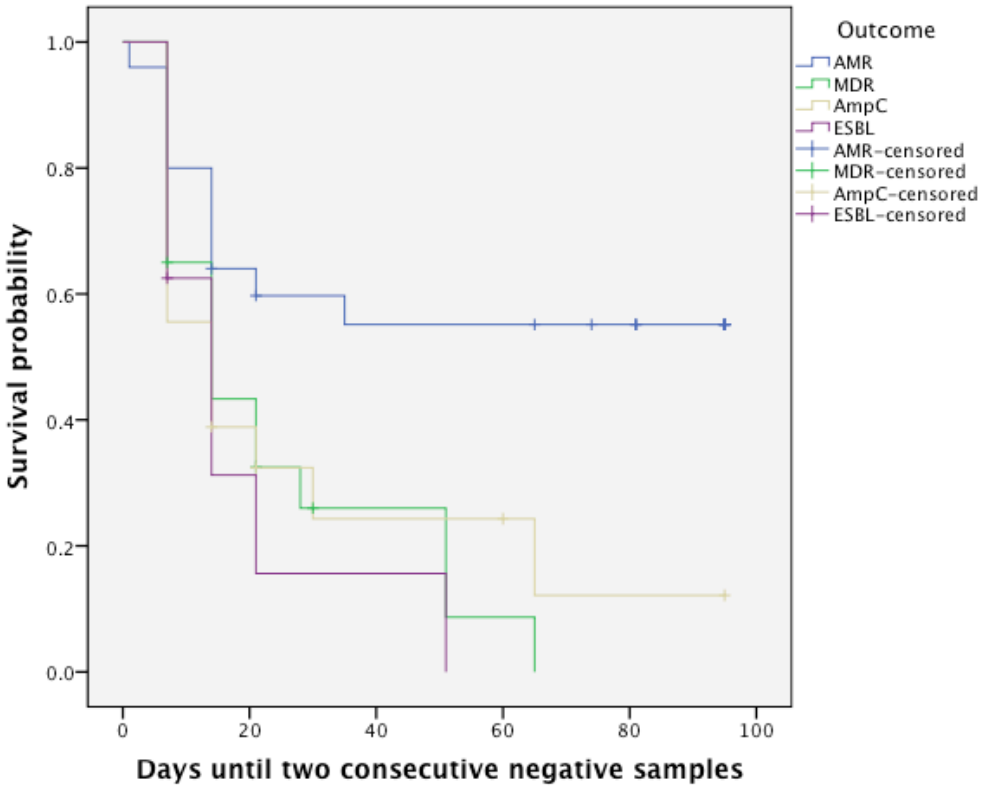


Sample time points were days 0,1,2,3,4,5,6,13 (week 1), 20 (week 2), 27 (week 3), 34 (week 4), 62 (month 1) and day 90 (month 2). After week 4 (34 days) detection is still expressed as seven day intervals: Black bars represent positive detection, white bars represent negative detection, grey solid bars follow positive detection and are expected to be positive but samples were not examined during this period; textured grey bars follow negative detection and are expected to be negative but samples were not examined during this period; * dogs that did not complete the study

3.4 **Survival analysis**

Kaplan-Meier survival analysis was performed for 25 dogs with any AMR resistance; 20 dogs with MDR; 18 dogs carrying AmpC-producing *E. coli*; and eight dogs carrying ESBL-producing *E. coli*. The median survival time for MDR, ESBL and AmpC-producing *E. coli* was 14 days and the mean ranged from 18 – 29 days. Due to the large number of dogs with AMR detected at the end of the study, the median survival time could not be calculated but the mean survival time was 58 days. There was a significant difference between the survival time of AMR and the other three outcomes (Figure 3; Table 2).

Figure 3. Kaplan-Meier plot of survival analysis for AMR, MDR, ESBL and AmpC-producing *E. coli* (n = 28 dogs)



AMR = resistance to at least one tested antimicrobial, MDR = multidrug resistance; ESBL = presence of ESBL-producing E. coli; AmpC = presence of AmpC producing *E. coli;* dogs were censored for an outcome if they did not have two consecutive negative samples before the end of the study or before they left the study

Table 2. Mean and median duration of carriage (days) of AMR, MDR, ESBL and AmpC before two consecutive negative samples (n = 28 dogs)

Outcome	Mean	95% CI	Median	95% CI
AMR*	58	42-75	—	—
MDR	24	15-33	14	7-21
AmpC	29	13-44	14	4.5-23.5
ESBL	18	6-30	14	6-22
Total	38	29-47	14	8-20

95% CI = 95% confidence interval; AMR = any antimicrobial resistance; MDR = multidrug resistance; AmpC = AmpC producing *E. coli*; ESBL = ESBL-producing *E. coli*; '—' = cannot be calculated as > 50% of dogs censored; *significant difference (Log-Rank Test; $P = 0.006$)

3.5 **Genetic diversity over time assessed by PFGE in three dogs**

Marked genetic diversity was demonstrated on an individual animal basis. For example a total of 21 and 12 different strains were detected in only six samples over 90-days from two dogs (dogs 16 and 28 respectively). In addition, 40 genotypes, 10 recurrent, were detected from nine samples during a three-week period from one dog (dog 17). Dog 16 and 28 had five recurrent genotypes each (Table 3). The original antimicrobial susceptible strains were not detected throughout the study however they were replaced by new susceptible strains that were then detected in consecutive samples. There were also susceptible strains that appeared to gain or lose resistance traits, and the same strains had both identical and different antimicrobial resistance phenotypes within and between samples. ESBL-production was detected in dogs 16 and 28. Detection of the identical strain and ESBL-resistance phenotype occurred for both dogs, but only in one consecutive sample. In addition, ESBL production and phenotypic resistance (ampicillin & tetracycline for dog 16 and ampicillin for dog 28), were detected in strains that were previously antimicrobial susceptible.

AmpC-production was detected in all samples from dog 17 (Table 4). The most common resistance phenotype (ampicillin & clavulanate-amoxicillin) and genotype were detected in all but the final sample. The second most common AmpC resistance phenotype (ampicillin, clavulanate-amoxicillin & tetracycline) was detected with at least three different genotypes, but only one genotype was detected in a consecutive sample. One other of the three-*ampC* genotypes was detected again in the last sample but it had a new resistance phenotype (ampicillin & tetracycline). An AmpC-producing isolate with the resistance phenotype ampicillin, clavulanate-amoxicillin & tetracycline was also present in the last sample, but it had a different genotype; a genotype that had been detected in earlier samples with ampicillin, tetracycline and trimethoprim resistance, but without AmpC production. MDR (ampicillin, chloramphenicol, nalidixic acid, tetracycline and trimethoprim/ampicillin, ciprofloxacin,

chlormphenicol, nalidixic acid and trimethoprim) was also detected for two different *ampC*-producing genotypes but from only one sample each.

Table 3. Resistance phenotypes compared to genotypes over different sampling time points for dogs number 16 and 28.

Sample code dog 16	Resistance phenotype	PFGE	ESBL or AmpC	Sample code dog 16	Resistance phenotype	PFGE	ESBL or AmpC
Day 1		A		Week 4	Tet	Q	
Day 1		B		Week 4		H[4]	
Week 1		C[1]		Month 1		T[5]	
Week 1	Amp, Tet	D[2]	ESBL	Month 1		H[4]	
Week 1	Amp, Cip, Chl, Nal, Tet, Tm	E		Sample code dog 28	Resistance phenotype	PFGE	ESBL or AmpC
Week 1	Amp, Chl, Tet	F[3]		Day 1		a	
Week 1	Amp, Tet, Tm	G		Day 1		b	
Week 1	Amp, Chl, Tet, Tm	C[1]	ESBL	Day 4		c	
Week 2		H[4]		Week 1		d[1]	
Week 2	Amp, Nal, Tet, Tm	J		Week 1	Amp, Tet, Tm	e[2]	
Week 2	Amp	—		Week 1	Amp, Tet, Tm	f	
Week 2	Amp, Tm	—		Week 1	Amp, AC	g	ESBL
Week 2	Amp, Nal, Tm	—		Week 1	Amp	h	ESBL
Week 2	Amp, AC, Chl, Nal, Tet, Tm	—		Week 2	Amp	d[1]	ESBL
Week 2	Amp, Chl, Nal, Tet	—		Week 2	Amp	i	
Week 2	Amp, Tet	I		Week 3		J[3]	
Week 2	Amp, Tet	K		Week 3	Amp, Nal, Tet, Tm	e[2]	
Week 2	Amp, Tet	D[2]	ESBL	Week 3	Amp, Tet, Tm	—	
Week 3		L		Week 3	Amp, Nal, Tet	—	
Week 3		P		Week 3	Amp	d[1]	ESBL
Week 3		H[4]		Week 3	Amp	J[3]	ESBL
Week 3	Tet	F[3]		Week 4		j[3]	
Week 3	Nal	N		Week 4	Amp, Tet, Tm	k[4]	
Week 3	Amp, Tet	O		Week 4	Amp, AC, Cip, Nal, Tet, Tm	l[5]	AmpC
Week 3	Amp, Na, Tet, Tm	—		Week 4	Amp, Cip, Nal, Tet, Tm	l[5]	AmpC
Week 3	Chl, Tet	R		Week 4	Amp, AC, Nal, Tet, Tm	k[4]	AmpC
Week 3	Amp, Tm	—		Week 4	Amp, AC, Cip, Chl, Nal, Tet, Tm	—	
Week 3	Amp, Chl, Nal, Tet, Tm	S					
Week 3	Amp, Tet, Tm	M					
Week 3	Amp, Tet, Tm	T[5]					
Week 3	Amp, AC	U	AmpC				
Week 3	Amp, AC, Cip, Na, Tet, Tm	P	AmpC				

Day 1 = baseline; Day 4 = four days; Week 1 = 14 days; Week 2 = 21 days; Week 3 = 28 days; Week 4 = 35 days; Month 1 = 65 days; Amp = ampicillin; AC = clavulanate-amoxicillin; Cip = ciprofloxacin; Chl = chloramphenicol; Nal = nalidixic acid; Tet = tetracycline; Tm = trimethoprim; blank = no resistance detected '— '= PFGE type not identified; [1-5]repeated genotypes; genotypes A - U (dog 16) and a - l (dog 28)

Table 4. Resistance phenotypes compared to genotypes over different sampling time points for dog number 17

Sample code dog 17	Resistance phenotype	PFGE	ESBL or AmpC	Sample code dog 17	Resistance phenotype	PFGE	ESBL or AmpC
Day 1	Tet	1		Day 5	Amp, Chl, Nal, Tet, Tm	20[g]	
Day 1	Amp, AC	2[a]	AmpC	Day 5	Amp, Chl, Nal, Tet, Tm	2[a]	AmpC
Day 1	Amp, Cip, Chl, Nal, Tet, Tm	3[b]		Day 5	Amp, Tet	26	
Day 1	Amp, Chl, Tet	4		Day 5	Amp, Cip, Chl, Nal, Tet, Tm	3[b]	
Day 1	Tm	5		Day 5	Amp, Chl, Tet, Tm	25[j]	
Day 1	Amp	6[c]		Day 5	Tet, Tm	27	
Day 1	Amp, Tet	—		Day 5	Amp, AC, Tet	—	AmpC
Day 1	Amp, Tet, Tm	7		Day 5	Amp, AC	2[a]	AmpC
Day 1	Amp, AC	8	AmpC	Day 6	Tet	—	
Day 1	Amp, Nal, Tet, Tm	—		Day 6	Amp, AC	2[a]	AmpC
Day 2	Tet	9[d]		Day 6	Amp, Chl, Tet	—	
Day 2	Amp, AC	2[a]	AmpC	Day 6	Amp, Tet	28	
Day 2	Amp, Cip, Nal, Tt, Tm	10		Day 6	Amp, Chl, Tm	29	
Day 2	Amp, Chl, Tet	6[c]		Day 6	Amp, AC, Tet	—	AmpC
Day 2	Amp, Nal, Tet, Tm	11		Day 6	Chl, Nal, Tet, Tm	—	
Day 2	Amp, AC, Tet, Tm	12		Day 6	Tet, Tm	30	
Day 2	Chl, Tm	13		Day 7	Amp, AC, Tet	23[h]	AmpC
Day 2	Amp, AC, Tet	14[e]	AmpC	Day 7	Amp, AC	2[a]	AmpC
Day 2	Amp, Cip, Chl, Nal, Tet, Tm	3[b]		Day 7	Cip, Nal, Tet	—	
Day 2	Chl, Tet	15		Day 7	Amp, Chl, Nal, Tet, Tm	20[g]	
Day 2	Amp, Nal, Tet	16		Day 7	Amp, Tet	31	
Day 2	Amp, Tet, Tm	17		Day 7	Tm	32	
Day 3	Amp, Tet, Tm	18[f]		Day 7	Amp, Tet	—	
Day 3	Amp, AC	2[a]	AmpC	Day 7	Amp, Cip, Chl, Nal, Tm	33	AmpC
Day 3	Amp, Chl, Tet	6[c]		Day 7	Chl	34	
Day 3	Cip, Nal	—		Day 7	Amp, Tet, Tm	35	
Day 3	Amp, Nal, Tet	—		Day 7	Amp, AC, Tet	—	AmpC
Day 3	Tet	9[d]		Week 1	Amp, Tet	—	
Day 3	Amp, AC, Tet	—	AmpC	Week 1	Chl, Tet	36	
Day 4	Amp, AC, Chl, Tm	19		Week 1	Chl, Tet, Tm	—	
Day 4	Amp, AC	2[a]	AmpC	Week 1	Amp, Nal, Tet, Tm	37	
Day 4	Amp, Chl, Nal, Tet, Tm	20[g]		Week 1	Tet	38	
Day 4	Amp, Chl, Tet	6[c]		Week 1	Amp, AC, Tet	—	AmpC
Day 4	Cip, Chl, Nal, Tet, Tm	21		Week 1	Amp, AC	2[a]	AmpC
Day 4	Tet	22		Week 2	Amp, Tet, Tm	18[f]	
Day 4	Chl, Tm	—		Week 2	Amp, Tet	14[e]	AmpC
Day 4	Amp, AC, Tet	23[h]	AmpC	Week 2	Amp, AC, Chl, Tet	39	
Day 4	Cip, Nal, Tet	24[i]		Week 2	Chl, Nal, Tet, Tm	—	
Day 4	Nal, Tet	—		Week 2	Amp, Chl, Tet	—	
Day 4	Tet, Tm	25[j]		Week 2	Tet	—	
Day 5	Amp, AC	2[a]	AmpC	Week 2	Amp, Tet, Tm	40	
Day 5	Cip, Nal, Tet	24[i]		Week 2	Amp, AC, Tet	18[f]	AmpC

Day 1 = baseline; Week 1 = 14 days; Week 2 = 21 days; Amp = ampicillin; AC = clavulanate-amoxicillin; Cip = ciprofloxacin; Chl = chloramphenicol; Nal = nalidixic acid; Tet = tetracycline; Tm = trimethoprim; blank = no resistance detected; '—' = PFGE type not identified; [a-j]repeated genotypes; genotypes 1 - 40

3.6 **Univariable multilevel analysis**

Only two variables were statistically significant ($P < 0.05$) in the univariable models: an owner working with farm animals ($P = 0.035$) for AMR and dogs eating raw meat for MDR ($P < 0.001$) and ESBL/AmpC-producing *E. coli* ($P = 0.015$). Variables with $P \leq 0.25$ were considered in multivariable models (Table 5-1, Appendix III).

3.7 **Multivariable multilevel analysis**

Dogs with owners working with farm animals were at increased risk for the detection of AMR faecal *E. coli*. Dogs eating raw meat were at increased risk for the detection of MDR and ESBL/AmpC-producing faecal *E. coli* and of borderline significance for AMR *E. coli*. In addition, dogs being fed treats were at increased risk for the detection of MDR and living in a multi-dog household was of borderline significance for ESBL/AmpC-producing faecal *E. coli*. The variance partition coefficients (VPC) for household were 40% and 49% for outcomes AMR and ESBL/AmpC respectively, and for dog the VPC for ESBL/AmpC was 56%. This suggests that there was substantial clustering within household or dog for these outcomes as expected (Table 5).

Table 5. Multilevel, multivariable final models for outcomes AMR, MDR and ESBL/AmpC-producing *E. coli*

Resistance outcome and final model variables				
Antimicrobial resistance (AMR)	***β***	***OR***	***95% CI***	***P-value***
Dogs fed raw meat[1]	2.6	14.1	0.9-218	0.06
Owner works with farm animals[1]	1.9	6.4	1.7-33.7	0.02*
Household variance (SE); dog variance (SE)		2.192 (0.896); 0.000		
Multidrug resistance (MDR)	***β***	***OR***	***95% CI***	***P-value***
Dogs fed treats[1]	1.4	4.1	1.4-11.9	0.01*
Dogs fed raw meat[1]	4.2	67.5	8.5-537.4	0.000*
Household variance (SE); dog variance (SE)		0.000; 0.000		
ESBL/AmpC-producing *E. coli*	***β***	***OR***	***95% CI***	***P-value***
Dogs fed raw meat[1]	4.5	91.7	2.9-2937.7	0.011*
Multi-dog household[1]	2.6	18.12	1.0-198.4	0.051
Household variance (SE); dog variance (SE)		3.214 (1.94); 4.26 (0.642)		

[1] Reference category is absence of risk factor; β = coefficient; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the Wald chi-squared test; AMR = antimicrobial resistance to at least one tested antimicrobials; MDR = antimicrobial resistance to three or more antimicrobial classes; significant at $P < 0.05$

4. Discussion

This study was performed to examine the natural diversity of faecal *E. coli* populations, with respect to AMR phenotype and genotype, under natural conditions in healthy dogs. In addition, to assess the detection of AMR *E. coli* over time and to investigate the relationship of these findings to potential risk factors for carriage.

AMR to one antimicrobial was very common (45% of samples); three dogs also carried AMR *E. coli* in every sample and only one dog did not have AMR *E. coli* in any sample over the entire study period. The most common type of resistance was to beta-lactam antimicrobials (ampicillin and clavulanate-amoxicillin) that are commonly sold for use in both food and non-food producing animals in the UK (VMD, 2012). These dogs had not recently received antimicrobial therapy, but the majority of owners worked in veterinary healthcare with companion and farm animals. Healthcare and farm animal contact may be risk factors for the carriage of AMR *E. coli* in humans (Ben-Ami et al., 2009; Price et al., 2007) and strains may be shared within households, including pets (Johnson et al., 2008). Hence the frequency of resistance may be lower in the general population of dogs not owned by veterinary healthcare workers.

Sole ampicillin resistance was detected in every isolate in every sample over three months in two unrelated dogs. This resistance phenotype is common amongst canine faecal *E. coli* (Hamilton et al., 2013; Procter et al., 2013; Wedley et al., 2011) and may not exert a fitness cost to such isolates. Long-term gut colonisation with ampicillin resistant faecal *E. coli*, in the absence of direct antimicrobial pressure, has also been reported for Swedish infants (Karami et al., 2008). In addition the stability of the *E. coli* population in these dogs may be associated with a lack of other potential risk factors that could disturb gut flora or select for AMR compared to other dogs in the study.

MDR was less common (14% of samples) and only detected intermittently and/or transiently, and not in any sample from seven of the dogs. These findings may be associated with the absence of antimicrobial selective pressure and the potential fitness cost of carrying multiple resistance determinants on mobile genetic elements, or mutations. Additionally the isolates may lack virulence factors associated with gut adherence. In humans, strains possessing the adhesion factor P fimbriae were more likely to persist in the colon (Nowrouzian et al., 2006). Clavulanate-amoxicillin resistance was common (~ 20% samples) and correlated well with the detection of AmpC-producing *E. coli.* Plasmid-mediated AmpC producing *E. coli* are frequently detected in animal samples and may be selected for by the frequent use of potentiated beta-lactam antimicrobials in animal populations (Li et al., 2007). AmpC producing *E. coli* were found to be either intermittently or transiently shed or could be carried long term in some animals. Whereas ESBL producing *E. coli* were uncommon, and usually only detected short term, suggesting acquisition from an endogenous source. A similar study detected ESBL-producing *E. coli* from only two samples from one dog over six months (Damborg et al., 2009).

The first seven faecal samples were collected on a daily basis. During this period approximately one quarter of dogs had intermittent AMR, one quarter had transient AMR, one quarter had constant AMR and one quarter had no resistance; this trend continued for the remainder of the study. Marked diversity in AMR profiles of faecal *E. coli* have even been detected during a 24-hour period in humans and farm animals (Anderson et al., 2006), highlighting the insensitivity of single point sampling. Also in agreement with our study, high diversity was reported within individual humans, horses and cattle when sampling monthly for six-months; the majority of AMR phenotypes were detected from only one sample (Anderson et al., 2006). AMR faecal *E. coli* were shed for an average of 58 days in the current study, approximately double the duration of the other resistance phenotypes (AmpC = 29 days; MDR = 24 days; ESBL = 18 days). Although AMR (resistance to at least one antimicrobial) included all resistance phenotypes, many AMR *E. coli* isolates were not MDR,

ESBL- or AmpC-producing. Therefore AMR to one or two antimicrobials, in the absence of antimicrobial pressure, may exert less fitness cost than the other phenotypes if adaptation and/or compensatory mechanisms have not occurred (Andersson and Hughes, 2010; Morosini et al., 2000; Schrag et al., 1997). Horses were also found to shed AMR longer than either MDR or ESBL-producing *E. coli*. While ESBL shedding in horses was similar to the dogs in this study, the duration of AMR and MDR shedding was much longer in the horses, half of which had been recently hospitalised and received antimicrobials (Maddox, 2010). Hospitalisation together with antimicrobial therapy may prolong shedding of AMR in horses (Johns et al., 2012), where as the dogs in this study had not received antimicrobials or been admitted to veterinary premises within three months of enrolment.

Genotyping of isolates from three dogs in this study revealed high inter-individual variation between the dogs with new strains detected on a weekly basis. Similar diversity has been reported in female children (Schlager et al., 2002). In two dogs, 12 and 21 distinct *E. coli* strains were detected over three months and in another dog, 40 distinct *E. coli* strains were detected over a three-week period. While high diversity amongst faecal *E. coli* has been previously reported for horses, cattle and dogs when compared to humans (Anderson et al., 2006; Damborg et al., 2009), only a maximum of 10 distinct strains were detected over a six-month sampling period from a group of 13 dogs (Damborg et al., 2009). The difference between the two studies may be associated with different sampling methods and criteria. The previous study only tested one random colony, from non-selective media, per sample, whereas this study tested three random colonies. This increased the sensitivity to detect potentially highly prevalent susceptible clones (Damborg et al., 2009), in our study. The direct plating method (Bartoloni et al., 2006) was employed by both studies to improve detection of less prevalent AMR strains (Damborg et al., 2009), however the previous study only genotyped select isolates according to their study criteria.

Resident clones are defined as strains detected from individual hosts on at least two separate occasions at least three weeks apart (Damborg et al., 2009), or strains persisting for at least three weeks (Karami et al., 2008). Long-term carriage or intermittent shedding of one or two resident *E. coli* clones from 69% of dogs (n = 13) was reported over a six-month period (Damborg et al., 2009). If similar criteria are applied in this study, two resident strains were detected from consecutive samples in one of the two dogs assessed over three months; one strain was a susceptible isolate that was detected on three consecutive weeks and again 30 days later. Potentially this isolate may have possessed virulence factors that facilitated gut adherence, possibly of phylo-group B2; phylo-group B2 isolates are less likely to be AMR than other phylo-groups (Johnson et al., 2009; Nowrouzian et al., 2006; Platell et al., 2010;

Platell et al., 2011; Sato et al., 2014). The third dog was only assessed for three weeks, however an AmpC-producing strain with ampicillin and clavulante-amoxicillin resistance was present at baseline and detected in all consecutive samples apart from the last sample, whereas MDR AmpC-producing *E. coli* were only detected on single occasions. It seems likely that this isolate was a gut resident.

Entirely new genotypes were frequently identified during the study in the three dogs, suggesting endogenous acquisition. Potential sources include the environment (Wellington et al., 2013), farm-animal meat (Johnson et al., 2009; Johnson et al., 2007; Vincent et al., 2010), or in-contact humans or pets (Damborg et al., 2009; Johnson et al., 2008; Stenske et al., 2009). In addition, recurring genotypes gained/changed antimicrobial resistance phenotype during the study, suggesting exchange of antimicrobial resistance determinants between strains by horizontal gene transfer within the gastrointestinal tract. Transfer of antimicrobial resistance genes may occur under natural conditions in the gastrointestinal tract (Lester et al., 2006; Levy et al., 1988). Transmission of plasmid-encoded ampicillin resistance to a co-residing antimicrobial susceptible bowel isolate has been reported in a Swedish infant, in the absence of antimicrobial pressure (Karami et al., 2008). Alternatively, identical genotypes with different AMR phenotypes may have been present concurrently but were undetectable due to small numbers.

A number of variables were found to be associated with AMR, MDR and ESBL or AmpC-producing *E. coli*. Dogs with owners working with farm animals were more likely to have AMR faecal *E. coli,* while eating raw meat was a risk factor for having MDR, ESBL- or AmpC-producing *E. coli*. Contact with farm animals has been recognised as a risk factor for human carriage of AMR bacteria (Price et al., 2007; Silbergeld et al., 2008; Weese et al., 2005). Food, particularly chicken meat, has been reported as a possible source of AMR bacteria (including ExPEC) for humans and dogs (Johnson et al., 2009; Johnson et al., 2007; Vincent et al., 2010). Faecal contamination of carcasses during processing is important for the dissemination of ESBL-producing *E. coli*, that along with AmpC-producing *E. coli*, have been isolated from the faeces of cattle, chickens and pigs in the UK (Horton et al., 2011). Raw meats may therefore be a source of antimicrobial resistant and/or pathogenic organisms that are potential animal and public health risks. In addition, dogs that were regularly fed dog treats were at increased risk for MDR *E. coli,* and multi-dog households for ESBL or AmpC-producing *E. coli.* Details of the type of treats fed to the dogs in this study was not available, however dog treats derived from animal parts, such as pig ears are popular and may be a potential source of AMR bacteria (White et al., 2003). Sharing of *E. coli* between household members correlates with increasing numbers of in-contact humans and pets (Johnson et al.,

2008) and ESBL- and AmpC- enzymes are encoded by genes that are usually plasmid-mediated, facilitating horizontal dissemination amongst bacterial populations (Jacoby, 2009; Thomson, 2010).

Limitations of this study included the small number of observations for some variables and outcomes that reduced the power of the study and resulted in very large confidence intervals. Nevertheless, after accounting for clustering within dog and within household, statistically significant associations were found. Another limitation was the enrolment of a convenience sample of dogs, where the majority of owners worked in veterinary healthcare. This may have increased the detection of AMR and negated the exclusion criteria of veterinary admission, however this information was included in the models. In addition, testing more colonies and genotyping all isolates from all dogs would have allowed better assessment of diversity and stability of canine faecal *E. coli*; individually, within households and overall. Finally, further characterisation of resistance determinants and phylogenetic typing would help to further explain the findings of this study.

5. Conclusions

There was a high prevalence of antimicrobial resistant faecal *E. coli*, including AmpC-producing *E. coli*, detected in this group of non-antimicrobial treated and non vet-visiting dogs over three months. Fewer samples had MDR or ESBL-producing *E. coli.* AmpC producing *E. coli* could be detected long term in some dogs, whereas ESBL-producing isolates tended to be transient. However, from survival analysis, the median duration of carriage for MDR, ESBL- or AmpC-producing *E. coli* was two weeks. This represents a potential source of antimicrobial resistant *E. coli* for contamination of the environment or for in-contact individuals. There was high individual diversity in most cases with changing AMR phenotypes and genotypes on a daily, weekly and monthly basis. However there were also some dogs with recurrent, possibly resident, strains or stable *E. coli* populations over the entire study period. Multi-level, multivariable regression analysis found certain variables to be associated with antimicrobial resistance and it is likely that there are external sources of AMR bacteria for dogs including diet, environment and in-contact humans and animals. *E. coli* colonisation of the canine gut is likely to be multifactorial including AMR phenotype, genotype, fitness and phylo-type. More in-depth characterisation of these isolates with phylogenetic grouping and fitness assays may help to further elucidate these results. Ultimately longitudinal studies investigating the effects of other risk factors, such as antimicrobial therapy, will need to account for the normal diversity detected amongst canine

faecal *E. coli* populations under natural conditions. Allowing for the effects of the risk factors identified in this study will facilitate interpretation of such investigations.

6. Manuscript 4

Antimicrobial resistance amongst canine mucosal staphylococci following antimicrobial therapy

Vanessa Schmidt[1,2], Tim Nuttall[3], Gina Pinchbeck[2], Neil McEwan[1], Susan Dawson[2], Nicola Williams[2]

[1]Department of Infection Biology and [2]Department of Epidemiology and Population Health, The University of Liverpool, Leahurst Campus, Neston, UK, [3]University of Edinburgh, The Royal (Dick) School of Veterinary Studies, Easter Bush Campus, Midlothian, UK.

Manuscript submitted to Veterinary Microbiology.

Summary

Background: Coagulase-positive (CoPS) and coagulase-negative (CoNS) staphylococci are normal commensals of the skin and mucosa, but are also opportunist pathogens. Meticillin-resistant (MR) and multidrug-resistant (MDR) isolates are increasing in veterinary healthcare. Reported risk factors for infections with or carriage of such bacteria include antimicrobial therapy and health care contact.

Objectives: The main aim of the study was to examine staphylococcal populations and antimicrobial resistance profiles following therapy with one of five different antimicrobials (cefalexin, clavulanate-amoxicillin, cefovecin, clindamycin, or a fluoroquinolone), authorised for use in dogs in the UK. Additionally, to investigate potential risk factors for resistance, and the longevity of any change.

Methods: Swab samples (one nasal, one perineal) were collected from 127 dogs attending veterinary consultations in the UK: before treatment (D0), treatment end (End), and one month (M1) and three months (M3) after the end of treatment. Staphylococci were isolated and identified using phenotypic and biochemical methods. Disc diffusion antimicrobial susceptibility tests were determined for a range of antimicrobials and PCR assays detected the *mec*A gene. CoPS were assigned to species by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) and PCR of the *nuc* gene. Questionnaire data and treatment group/sample time were used as independent variables.

Results: The percentage of samples with MRS, MDR and ciprofloxacin resistant staphylococci, increased following beta-lactam (particularly cephalosporin), or fluoroquinolone therapy; this was significant on multilevel, multivariable analysis for fluoroquinolone therapy. Furthermore, the percentage of samples with CoPS decreased and CoNS increased after all treatments. By one month after the end of therapy, there was no significant difference in resistance compared to baseline and by three months the percentage of samples had returned to pre-treatment levels.

Conclusions: Antimicrobial resistant staphylococci were prevalent in this population of dogs. In particular, treatment with a fluoroquinolone was associated with the detection of such bacteria. These findings are of concern due to the potential for resistant opportunistic infection and zoonotic and environmental transmission. In addition, MR-CoNS may be a source of the *mec*A gene for CoPS. Implementation of antimicrobial prescribing guidelines may facilitate prudent use of antimicrobials and reduce the development and spread of resistance.

8. Introduction

Staphylococci are normal mucosal and skin commensals of humans and other animals (Kloos, 1980). The main coagulase positive *Staphylococcus* (CoPS) of humans is *S. aureus* (Mainous et al., 2006) and the main CoPS of dogs is *S. pseudintermedius* (Berg et al., 1984). Prior to re-classification of the *S. intermedius* group (SIG) in 2007, *S. pseudintermedius* were referred to as *S. intermedius* (Bannoehr et al., 2007). The prevalence of mucosal *S. pseudintermedius* carriage in healthy dogs has been reported to be between 37% to 92% in different studies (Devriese and De Pelsmaecker, 1987; Fazakerley et al., 2010; Griffeth et al., 2008; Hanselman et al., 2009; Paul et al., 2012; Rubin et al., 2011; Schmidt et al., 2014), with increased carriage in dogs with atopic dermatitis or pyoderma (Fazakerley et al., 2010; Saijonmaa-Koulumies and Lloyd, 1995). Fewer healthy dogs carry *S. aureus* (4% to 12%) (Boost et al., 2007; Fazakerley et al., 2010; Griffeth et al., 2008; Kottler et al., 2010; Loeffler et al., 2005; Schmidt et al., 2014; Wedley et al., 2014) and isolates are likely to originate from in-contact humans (Weese and van Duijkeren, 2010). In addition, coagulase negative staphylococci (CoNS) are common mucosal commensals of dogs (Schmidt et al., 2014). Commensal staphylococci are also opportunistic pathogens with mucosal isolates often being the source of infection (von Eiff et al., 2002). The most frequent cause of canine pyoderma is *S. pseudintermedius* (Ihrke, 1987; Medleau et al., 1986).

The frequency of antimicrobial resistance (AMR), particularly multi-drug resistance (MDR) in canine staphylococcal infections is increasing, severely limiting therapeutic options. Meticillin resistant staphylococci (MRS) are associated with the carriage of the *mec*A gene, which encodes an altered penicillin binding protein (PBP2a) and confers resistance to all beta-lactam antimicrobials (Hartman and Tomasz, 1984). The *mec*A gene is carried on a large mobile genetic element (MGE), the staphylococcal cassette chromosome *mec* (SCC*mec*) that can be transferred horizontally between staphylococci (Black et al., 2009). CoNS may be the original source of the *mec*A gene (Tsubakishita et al., 2010) and may act as reservoirs of this gene for CoPS (Barbier et al., 2010; Descloux et al., 2008; Smyth et al., 2011). Resistance determinants for other antimicrobials may also be carried on SCC*mec* (Hiramatsu et al., 2001), or other mobile genetic elements, and chromosomal mutations may occur also giving rise to resistance. Hence these isolates are commonly MDR and often, fluoroquinolone resistant (Descloux et al., 2008). Co-selection of fluoroquinolone resistant MRS isolates by beta-lactams or fluoroquinolones may lead to the rapid emergence and persistence of these strains (Descloux et al., 2008; Weber et al., 2003; Westh et al., 2004).

Antimicrobial therapy, frequent veterinary premise contact and hospital admission have been frequently reported as risk factors for the mucosal carriage of or infection with MRS in dogs (Eckholm et al., 2013; Faires et al., 2010; Huerta et al., 2011; Lehner et al., 2014; Nienhoff et al., 2011; Soares Magalhaes et al., 2010; Weese et al., 2012; Windahl et al., 2012). Similar risk factors have been reported or proposed for meticillin resistant *S. aureus* (MRSA) and meticillin resistant coagulase negative staphylococci (MR-CoNS) carriage in humans (Barbier et al., 2010; Soares Magalhaes et al., 2010). Meticillin resistant *S. pseudintermedius* (MRSP) have been detected from 0 to 4.5% of healthy dogs (Griffeth et al., 2008; Hanselman et al., 2008; Hanselman et al., 2009; Kania et al., 2004; Murphy et al., 2009; Schmidt et al., 2014; Vengust et al., 2006; Wedley et al., 2014) and from the mucosa and/or skin of between 3.5 to 66% of dogs with skin infections and/or veterinary hospital admission from Germany, the USA or Japan (Beck et al., 2012; Griffeth et al., 2008; Kania et al., 2004; Kawakami et al., 2010; Nienhoff et al., 2011; Onuma et al., 2012; Sasaki et al., 2007). In addition, transfer of isolates between individuals within households and veterinary clinics, and environmental contamination has been reported (Gomez-Sanz et al., 2013; Laarhoven et al., 2011; Paul et al., 2011; Singh et al., 2013; van Duijkeren et al., 2011).

There are a limited number of antimicrobials authorised for use in companion animals in the UK, mostly broad-spectrum and therefore active against both Gram-positive and Gram-negative bacteria (Hughes et al., 2012; Li et al., 2007; Mateus et al., 2011). The majority of these antimicrobials are usually administered in an oral form, however cefovecin is a long-acting subcutaneous preparation of a semi-synthetic third generation cephalosporin, authorised to use every 14-day (Stegemann et al., 2006). It has reported efficacy in the treatment of canine pyoderma (Summers et al., 2012). Veterinary authorised fluoroquinolones, including enrofloxacin and marbofloxacin, are broad-spectrum antimicrobials that have also been used frequently in dogs to treat pyoderma (Guardabassi et al., 2004; Ihrke et al., 1999).

The majority of the previous studies examining risk factors for MRS in dogs either found no association with antimicrobial therapy, or reported in association with antimicrobial therapy in general, with or without veterinary premise admission, and did not examine the recovery period. The aim of this study was to examine the impact of antimicrobial therapy on the canine mucosal staphylococci, by investigating changes in antimicrobial resistance and community staphylococcal profiles during and after treatment. In addition, to compare therapy with one of five, commonly used and important antimicrobials authorised for the use in dogs in the UK without the confounding factor of veterinary premise admission.

2. Methods

2.1 Study Population

A convenience sample of dogs attending veterinary consultations at three centres including first opinion and referral practice, in the North West of England between June 2011 and September 2012 were recruited for the study if they met the inclusion and exclusion criteria. Inclusion criteria included diagnosis of a bacterial infection (skin, soft tissue, urinary tract, dental, respiratory tract, orthopaedic, gastrointestinal, ocular) requiring systemic antimicrobial therapy with one of five different antimicrobials authorised for use in dogs in the UK (cefalexin [CFX], clavulanate-amoxicillin [AC], cefovecin [CVN], clindamycin [CD], or a fluoroquinolone [enrofloxacin or marbofloxacin; FL]). Exclusion criteria included antimicrobial therapy or veterinary admission within three months of enrolment and dogs aged less than 12 months old. Dogs were excluded during the study if they were prescribed a further course of systemic antimicrobial.

The veterinarian in charge of the case determined if the dog required systemic antimicrobials and decided on and implemented the treatment plan (type of antimicrobial, dose, frequency and duration of therapy). Before enrolment, all dog owners read the study outline and gave written informed consent. Samples were collected before starting treatment (D0), at the end of treatment (End) and at one (M1) and three months (M3) after the end of treatment. Veterinary personnel were responsible for client follow-up and scheduled re-examinations coinciding with the re-sample date. A questionnaire regarding potential risk factors for the carriage of antimicrobial resistant bacteria was administered at the start of the study and at the time of each follow-up sample. The questionnaire was either completed during the consultation or returned by first-class post. The two-page questionnaire used in this study consisted of closed questions with tick box responses and space for additional information (Appendix IV). 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, diet, previous veterinary history (including previous antimicrobial therapy and veterinary admission), the presence, number and type of in-contact pets, previous medical history of the household (including antimicrobial therapy or hospitalisation of humans or other pets) and whether any household member worked with farm animals or in healthcare. The attending veterinary surgeon also completed a one-page questionnaire confirming the reason for the visit, the prescribed antimicrobial and therapeutic regime and any previous antimicrobial therapy the dog had received in the last 12 months. All questionnaire-derived information was available as potential explanatory variables for inclusion in the multivariable

modelling of various antimicrobial resistance outcomes. The University of Liverpool, School of Veterinary Science Ethics-Committee approved the study protocol in June 2011.

2.2 Specimen collection and bacterial isolation

One nasal swab and one perineal swab were collected from each dog (Copan Eswab LQ Amies Minitip Nylon Flocked Applicator, Appleton Woods, Birmingham, UK) at each sample point. A sterile swab was inserted 5mm into one nostril and a further swab rubbed on the skin of the perineum for 3 to 5 seconds before being placed in Amies transport media, stored at 4˚C, and processed within 36 hours of collection. Swabs were incubated aerobically overnight at 37˚C in nutrient broth with 6.5% sodium chloride. The broth was streaked onto mannitol salt agar (MSA), oxacillin resistance screening agar (ORSA) supplemented with 2 μg/ml of oxacillin and Columbia 5% horse blood agar (CAB) to obtain single colonies, and incubated aerobically overnight at 37˚C. Where present, isolates with a staphylococcal phenotype were selected from all plates, sub-cultured onto CAB and incubated aerobically overnight at 37˚C. Fresh staphylococcal cultures on CAB were subject to Gram stain (Sigma-Aldrich Company Ltd., Gillingham, UK), tested for catalase (Sigma-Aldrich Company Ltd., Gillingham, UK) and free coagulase production (Rabbit plasma, Pro-Lab, Bromborough, UK) according the manufacturer's instructions. Testing for free coagulase production was repeated if there was discrepancy between this result and species assignment. Isolates were stored at -80˚C in Microbank vials (Pro-Lab, Bromborough, UK) for further processing. All media were obtained from LabM Ltd, Bury, UK.

2.3 Antimicrobial susceptibility testing

Disc diffusion testing was performed in accordance with accepted standards (CLSI, 2008) as described previously (Schmidt et al., 2014) with the following discs: 1 μg oxacillin (OX), 1 μg ciprofloxacin (CIP), 10 μg gentamicin (GM), 10 μg fusidic acid (FA), 30 μg cefalexin (CFX), 30 μg cefovecin (CVN), 25 μg trimethoprim-sulfamethoxazole (TS), 10 μg tetracycline (Tet), 2 μg clindamycin (CD) and 5 μg vancomycin (Va). All discs were purchased from MAST Group Ltd., Liverpool, UK, except for CVN, which were obtained from Oxoid, Basingstoke, UK. The reference strain *S. aureus* ATCC®25923 (LGC Standards, Teddington, UK) was used for quality control for zone diameter determinations.

2.4 Species identification using MALDI-TOF-MS

All coagulase positive, all CoNS with phenotypic oxacillin resistance, and CoNS from ten randomly selected dogs from each antimicrobial treatment group, were subject to matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) according to the manufacturer's protocol. Raw spectra were analysed by the MALDI Biotyper 2.0 software programme with default settings (Bruker Daltonics, Bremen, Germany). The extraction method was performed as previously described (Alatoom et al., 2011) on overnight colonies grown on CAB at 37°C and all isolates were tested in duplicate. The bacterial test standard (*E. coli* DH5 alpha, Bruker, Bremen, Germany) was used for calibration before each experiment and included in duplicate on each target plate. The mass peak profiles were matched to the reference database and a score generated based on similarity. A positive identification to species level was made for single result > 2.0 or duplicate results > 1.8 (Carpaij et al., 2011). All staphylococcal speciation results are not reported in this thesis.

2.5 Detection of antimicrobial resistance genes and species identification

DNA extraction was performed as previously described (Schmidt et al., 2014). PCR assays were performed to detect the presence of *mec*A gene in staphylococcal isolates that were phenotypically resistant to oxacillin. PCR assays to detect the presence of the *nuc* genes of *S. pseudintermedius, S. aureus* and *S. schleiferi* were performed on CoNS isolates identified as either *S. pseudintermedius* or *S. aureus* by MALDI-TOF-MS using Qiagen® Multiplex PCR Mix (Qiagen, Crawley, UK), according to the manufacturer's instructions with minor modifications (Schmidt et al., 2014). 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 (Table 3-1, Appendix I).

2.6 Statistical analysis

All questionnaire derived information and microbiological data were entered into a spreadsheet program (Microsoft Excel for Mac 2008, Microsoft Corporation) and the dataset was reviewed and checked for coding of all variables. Independent (risk factor) variables were created from information obtained from the owner and veterinary surgeon questionnaires. Except for the age of the dog, all variables were dichotomous or categorical in nature. The variables treatment duration and body weight were divided into three categories: ≤ 1 week, > 1 week and ≤ 3 weeks, and > 3 weeks and small (< 11 kg), medium (11-20 kg) and large (> 20kg) respectively. The variable recruitment site was divided into two categories: first opinion and referral consultations. Microbiological data were collapsed to the sample level and categorised for time of sample collection. The baseline samples were always at day zero (D0) and the end of therapy sample (End) varied depending on the length of the prescription. M1 and M3 time points were always at one month (M1) or three months (M3) after End for individual dogs.

Initial data analysis included Pearson's chi-square tests for the categorical variables and a one-way between-groups analysis of variance (ANOVA) for age to investigate differences between treatment groups at baseline (D0) (Table 2). In addition logistic regression was used to test for differences in antimicrobial resistance outcomes between treatment groups at baseline (D0) after accounting for the other independent variables (Tables 6-7 to 6-9, Appendix IV).

Phenotypic resistance to oxacillin, ciprofloxacin or multidrug resistance (MDR; resistance to three or more antimicrobial classes), the presence of CoNS or CoPS isolates and the presence of phenotypic oxacilllin resistant isolates carrying the *mec*A gene (MRS) were considered as six separate binary outcomes. The percentage of samples, with 95% confidence intervals, were calculated for each treatment group and treatment overall (CFX, AC, CVN, CD, FL, Total) at each time point (D0, E, M1, M3) for each of the six outcome measures. To examine the effect of treatment group and time of sample, and other independent variables on the presence of resistance, data were analysed using a multilevel logistic regression models with a binomial distribution and logit link. Due to repeated samples over time, data were clustered within dogs (level two units) and this clustering was accounted for by inclusion of dog as a random intercept in all models. Swab samples were considered the level one unit of interest. In order to make allowance for all dogs not having received any therapy at baseline (D0), all dogs at baseline were classed as baseline untreated. Ultimately, in addition to no treatment at

baseline (n = 1), there were 15 categories created to account for the different combinations of time (n = 3) and treatment group (n = 5).

Initially all variables were analysed in univariable multilevel models. All variables that showed some association with each outcome on univariable analysis (*P*-value < 0.25) were considered for incorporation into a final multilevel, multivariable model. Treatment group and the time of sample were always retained in the final models. For any pair of variables with a correlation coefficient of ≥ 0.7 only the variable with the smallest *P*-value was considered for further analysis. The final models were constructed by a manual backwards stepwise procedure where variables with a Wald *P*-value < 0.05 were retained in the model. Once a final multivariable model was produced all variables that were significantly ($P < 0.05$) different between treatment groups at day 0 were forced back into the model to assess their effect on the remaining variables, in particular treatment group (Table 1).

Multilevel models were analysed using the MLwiN statistical software package (MLwiN Version 2.28 Centre for Multilevel Modelling, University of Bristol). Univariable and multivariable calculations were performed using penalised quasi-likelihood estimates (2^{nd} order PQL for all outcomes). First order interaction terms were tested for biologically plausible variables remaining in the final models. The residuals +/- 1.96 SD x rank (caterpillar plots) were calculated and graphed for each dog to check for outliers (Figures 6-1 to 6-6, Appendix V). If present, outliers were removed and the models were rerun to assess the effects. Tests for correlation (Spearman's rho), Pearson's chi-square and logistic binary regression were performed using SPSS software package (SPSS 20.0 for Mac, SPSS Inc, Chicago, Illinois).

3. Results

3.1 Study population

One hundred and twenty-seven dogs were enrolled in this study from three centres: 1 (n = 43), 2 (n = 52) and 3 (n = 32) and included first opinion (n = 72) and referral consultations (n = 55). The dogs were treated with one of the following antimicrobials: cefalexin (n = 32), clavulanate-amoxicillin (n =29), cefovecin (n = 26), clindamycin (n = 28), or a fluoroquinolone (n = 13). Treatment was prescribed for ≤ 1 week in 33 dogs, > 1 week and ≤ 3 weeks in 48 dogs, and > 3 weeks in 46 dogs. The dogs were aged from 12 to 204 months (mean = 62 months) with 75 male and 52 female, 21 small, 16 medium and 90 large (> 20kg) dogs enrolled in this study. Owners of 24 dogs worked in health care environments. This

included four owners in veterinary healthcare, 18 owners in human healthcare (hospital, GP surgery, community nursing, paramedical, pharmaceutical, research and nursing home staff), and two owners in undefined healthcare (Table 1).

Table 1. The baseline (D0) variables considered for inclusion in the final multivariable model, with the number and percentage (%) of samples in each treatment group and variable category.

Variable	CFX	AC	CVN	CD	Fl	Total dogs	*P*-value
Mean age[a] (months)	44	50	68	79	83	62	0.016*
Weight							0.003*
Small (< 11kg)	1 (5)	3 (14)	4 (19)	10 (48)	3 (14)	21 (17)	
Medium (11-20kg)	4 (25)	3 (19)	0	7 (44)	2 (13)	16 (13)	
Large (> 20kg) REF	26 (29)	23 (26)	22 (24)	11 (12)	8 (9)	90 (71)	
Gender							0.925
Male REF	18 (24)	17 (23)	14 (19)	17 (23)	9 (12)	75 (59)	
Female	13 (25)	12 (23)	12 (23)	11 (21)	4 (8)	52 (41)	
Treatment duration							0.000*
1 week REF	5 (15)	17 (52)	0	10 (30)	1 (3)	33 (26)	
>1 and < 3 weeks	12 (25)	9 (19)	10 (21)	11 (23)	6 (13)	48 (38)	
> 3 weeks	14 (30)	3 (7)	16 (35)	7 (15)	6 (13)	46 (173)	
Recruitment site							0.000*
First opinion practice REF	23 (32)	28 (39)	4 (6)	17 (24)	0	72 (57)	
Referral consultation	8 (15)	1 (2)	22 (40)	11 (20)	13 (24)	55 (43)	
A diagnosis of pyoderma was made at enrolment	27 (33)	3 (4)	25 (31)	15 (18)	12 (15)	82 (65)	0.000*
Previous systemic antimicrobial treatment[1]	15 (26)	10 (17)	15 (26)	9 (16)	9 (16)	58 (46)	0.031*
Previous beta-lactam antimicrobial treatment[1]	11 (29)	7 (18)	7 (18)	6 (16)	7 (18)	38 (30)	0.111
Previous hospital admission[1]	4 (16)	3 (12)	6 (24)	8 (32)	4 (16)	25 (20)	0.287
In-contact human or pet received antimicrobials[2]	7 (25)	6 (22)	4 (14)	6 (21)	5 (18)	28 (22)	0.720
In-contact human or pet admitted to hospital or veterinary premises[1]	16 (40)	12 (30)	5 (13)	6 (15)	1 (2.5)	40 (31)	0.015*
Owner works in healthcare	5 (21)	2 (8)	5 (21)	9 (38)	3 (13)	24 (19)	0.133
Multi-dog household	17 (30)	15 (27)	12 (21)	8 (14)	4 (7)	56 (44)	0.241
Enrolled dog regularly eats animal stools	7 (18)	5 (13)	8 (21)	10 (26)	8 (21)	38 (30)	0.025*

CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fl = fluoroquinolone. [a]Age was the only continuous variable and is represented by the mean age of dogs in each treatment group; REF = the reference category for non-dichotomous variables; [1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; *significant at $P < 0.05$.

One hundred and twenty-seven dogs provided samples at Day 0 and End, 106 dogs provided samples at M1 and 103 dogs provided samples at M3 with a total of 463 samples (Table 2). Missing samples from M1 (n = 21) were due to prescription of further antimicrobials (n = 6), euthanasia or death due to unrelated reasons (n = 2), or owner non-compliance (n = 13). Missing samples from M3 (n = 24) were due to prescription of further antimicrobial courses (n = 10), euthanasia or death due to unrelated reasons (n = 3), re-homing (n = 3) or owner non-compliance (n = 8).

Table 2. The number of samples provided at each time point in each treatment group.

	Antimicrobial treatment group					
Time point	CFX	AC	CVN	CD	Fl	Total
D0	31	29	26	28	13	127
End	31	29	26	28	13	127
M1	26	27	20	24	9	106
M3	24	25	21	24	9	103
Total	112	110	93	104	44	463

CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fl = fluoroquinolone; D0 = baseline day zero; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy; two swabs per sample.

3.2 Descriptive statistics

3.2.1 Staphylococcal species & antimicrobial resistance during the study period

CoNS isolates were detected in 272 samples (59%; 95% CI: 54 - 63) from 118 dogs (93%; 95% CI: 87-96.2), including five dogs (4%) with *S. schleiferi* subsp *schleiferi*. CoPS isolates were detected in 289 samples (62%; 95% CI: 57.9 – 66.7) from 111 dogs (87%; 95% CI: 80.5-92.2), including *S. aureus,* detected in at least one sample from 27 dogs (21%), and *S. schleiferi* subsp *coagulans*, detected in at least one sample from nine dogs (7%). All *S. schleiferi* isolates were oxacillin susceptible at all time points.

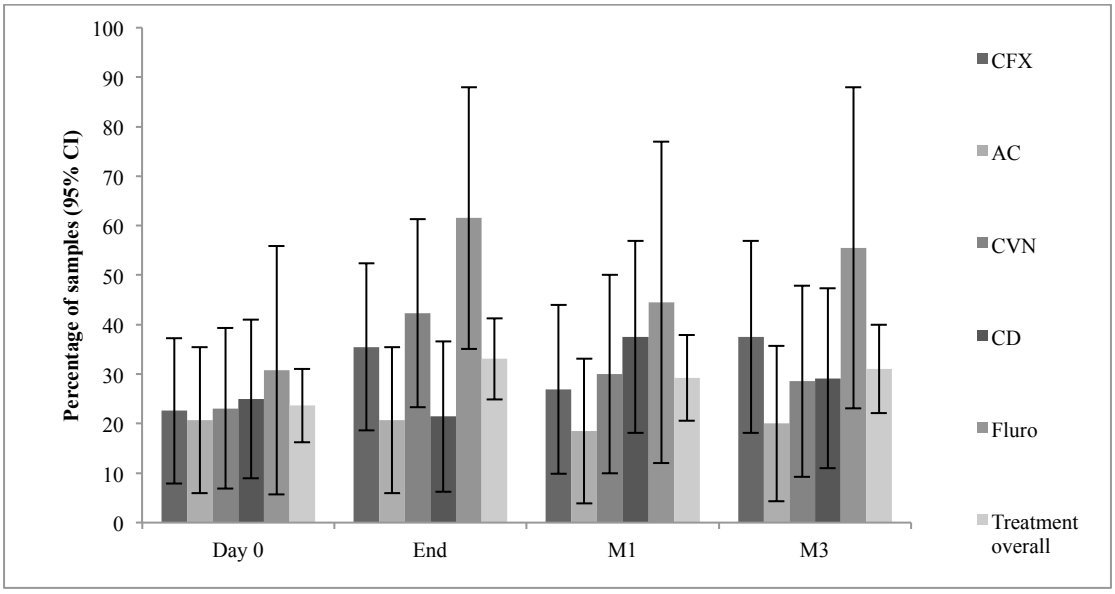
Phenotypic oxacillin resistant staphylococci were detected in 135 samples (29%; 95% CI: 29.2 – 33.5) from 86 dogs (68%; 95% CI: 59.2-75.2), with 27 samples (6%; 95% CI: 4 – 8.4) from 18 dogs (14%; 95% CI: 9-21) having phenotypic oxacillin resistant CoPS. Oxacillin resistant *mec*A positive staphylococci (MRS) were detected in 118 samples (26%; 95% CI: 21.7 - 29.6) from 80 dogs (63%; 95% CI: 54.3 – 70.9). Eighteen of these samples (4%; 95% CI: 2.5-6) from 13 dogs (10%; 95% CI: 6.1 – 16.7) were meticillin resistant coagulase positive staphylococci (MR-CoPS); 11 dogs carried MRSP and two dogs carried MRSA. MRS isolates that were MDR were detected in 80 samples (17%; 95% CI: 14 - 21) from 59 dogs (46.5%; 95% CI: 38-55) and MRS with ciprofloxacin resistance were detected in 49 samples (11%; 95% CI: 8.1 - 13.7) from 44 dogs (35%; 95% CI: 26.9-43.3) at any time point.

Overall MDR staphylococci were detected in 128 samples (28%; 95% CI: 23.8 - 31.9) from 77 dogs (61%; 95% CI: 51.9 – 68.7) and 49 samples (11%; 95% CI: 8.1 – 13.7) from 33 dogs (26%; 95% CI: 19 – 34.2) had MDR CoPS isolates. Ciprofloxacin resistant isolates were detected in 81 samples (18%; 95% CI: 14.3 - 21.2) from 60 dogs (47%; 95% CI: 38.3-55.9) and 25 samples (5%; 95% CI: 3.7 – 7.8) from 17 dogs (13%; 95% CI: 8.5-20.5) had ciprofloxacin resistant CoPS isolates. MDR isolates with ciprofloxacin resistance were detected in 78 samples (17%; 95% CI: 13.7 - 20.5) from 57 dogs (45%; 95% CI: 36.5 – 53.6) at any time point.

3.2.2 Changes in species and antimicrobial resistance following therapy

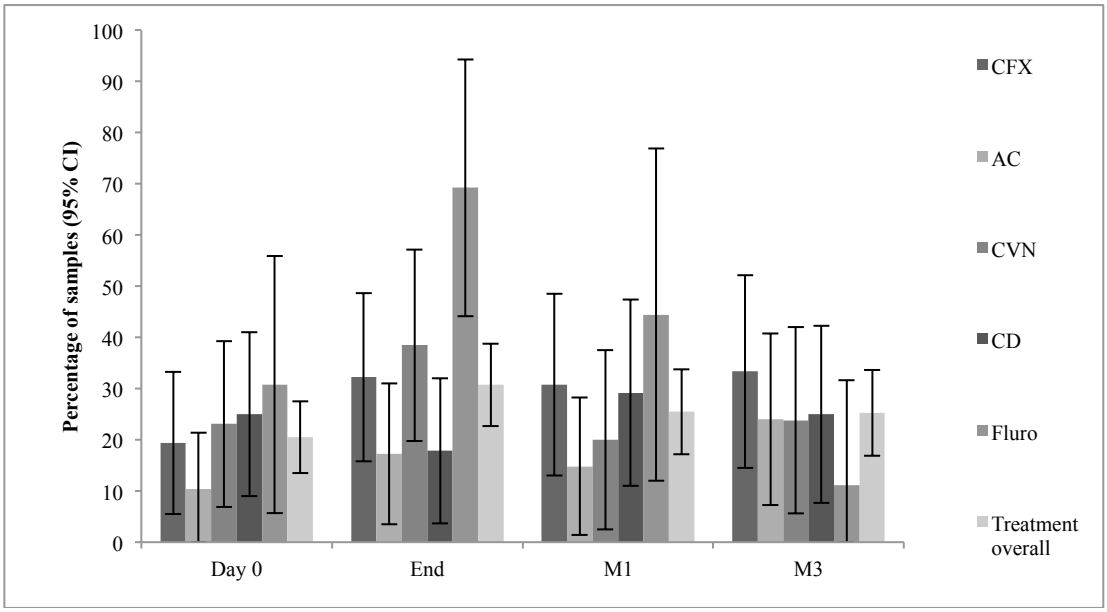
There was an overall trend for the percentage of samples with antimicrobial resistant mucosal staphylococci (phenotypic oxacillin resistance [Figure 1], MRS [Figure 2], ciprofloxacin resistance [Figure 3] or MDR [Figure 4]), to increase following antimicrobial therapy with beta-lactams or fluoroquinolones. In addition, the percentage of samples with CoPS (Figure 5) decreased and CoNS (Figure 6) increased following most therapies. However by M3, the percentage of samples positive for each of these outcomes had returned to pre-treatment levels in the majority of treatment groups and outcomes. A similar tendency for increase following treatment and recovery by M3 was noted for the detection of phenotypic oxacillin resistant CoPS, MR-CoPS, MR-MDR staphylococci and MRS or MDR staphylococci with concurrent ciprofloxacin resistance (Tables 6-1 to 6-6, Appendix IV).

Figure 1. The percentage of samples with phenotypic oxacillin resistant staphylococci at each time point for each treatment group and treatment overall (error bars = 95% CI)



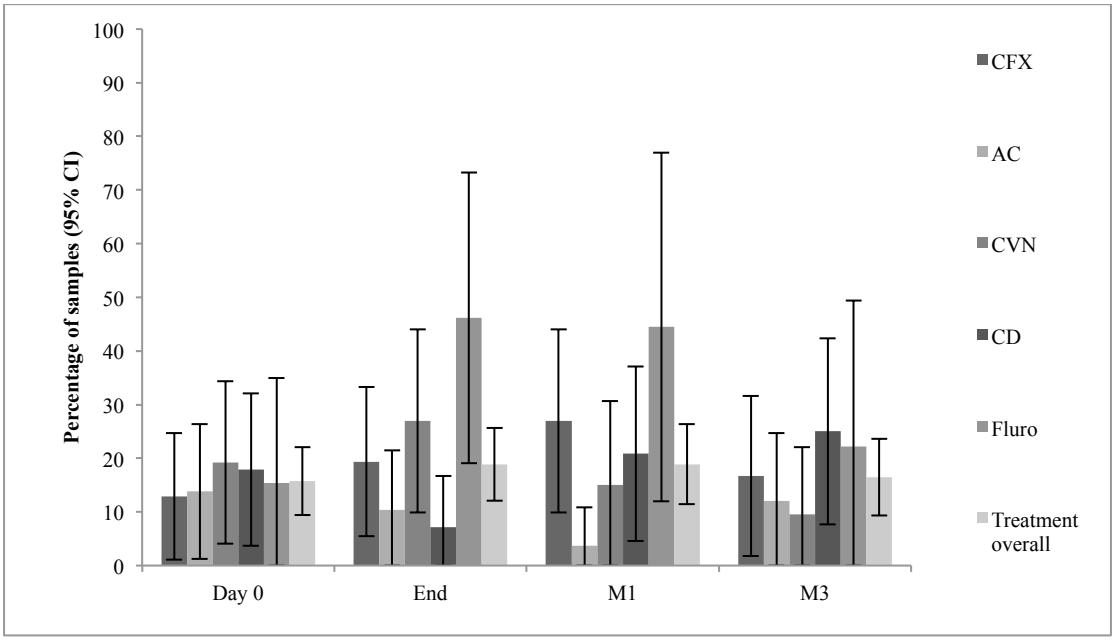
95% CI = 95% confidence interval; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fluro = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy

Figure 2. The percentage of samples with phenotypic oxacillin resistant, *mec*A gene positive, staphylococci (MRS) at each time point in each treatment group (error bars = 95% CI)



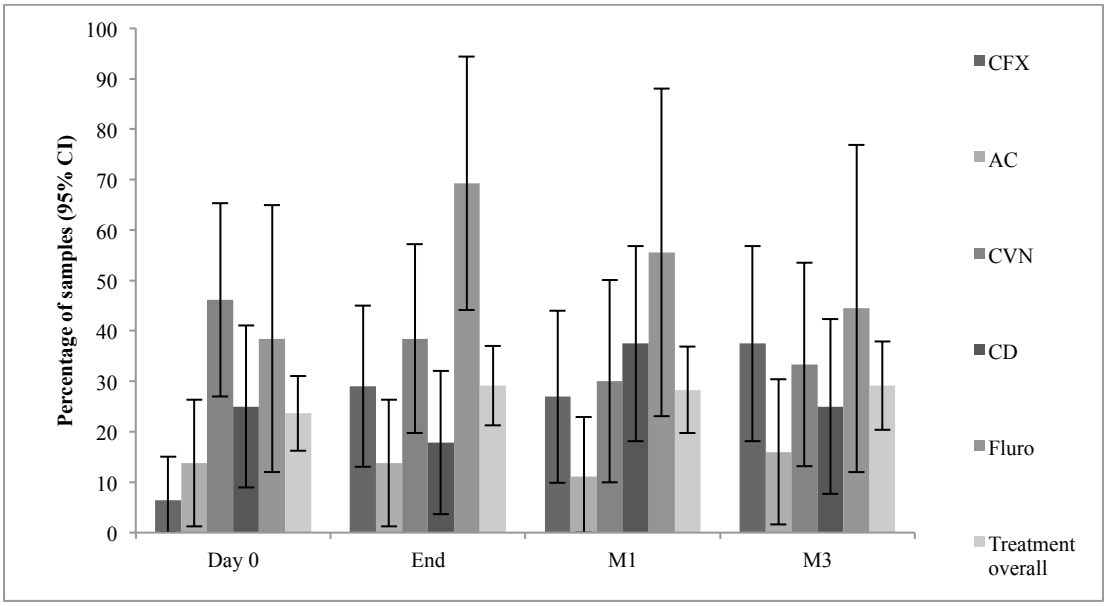
95% CI = 95% confidence interval; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fluro = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy; MRS = meticillin resistant staphylococci

Figure 3. The percentage of samples with ciprofloxacin resistance at each time point in each treatment group (error bars = 95% CI)



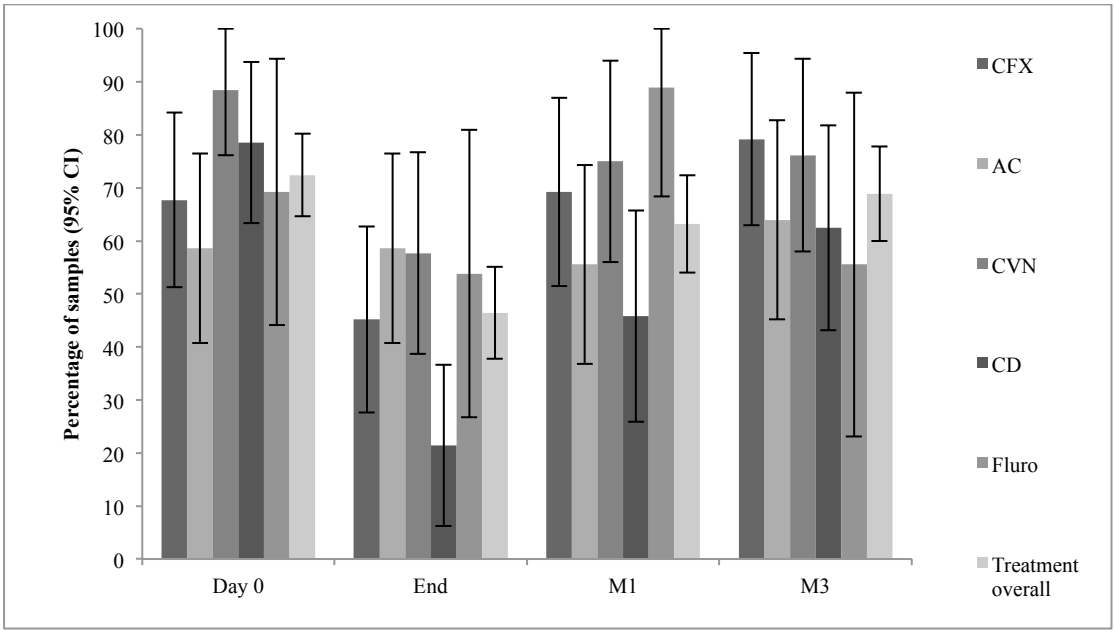
95% CI = 95% confidence interval; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fluro = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy

Figure 4. The percentage of samples with MDR at each time point in each treatment group (error bars = 95% CI)



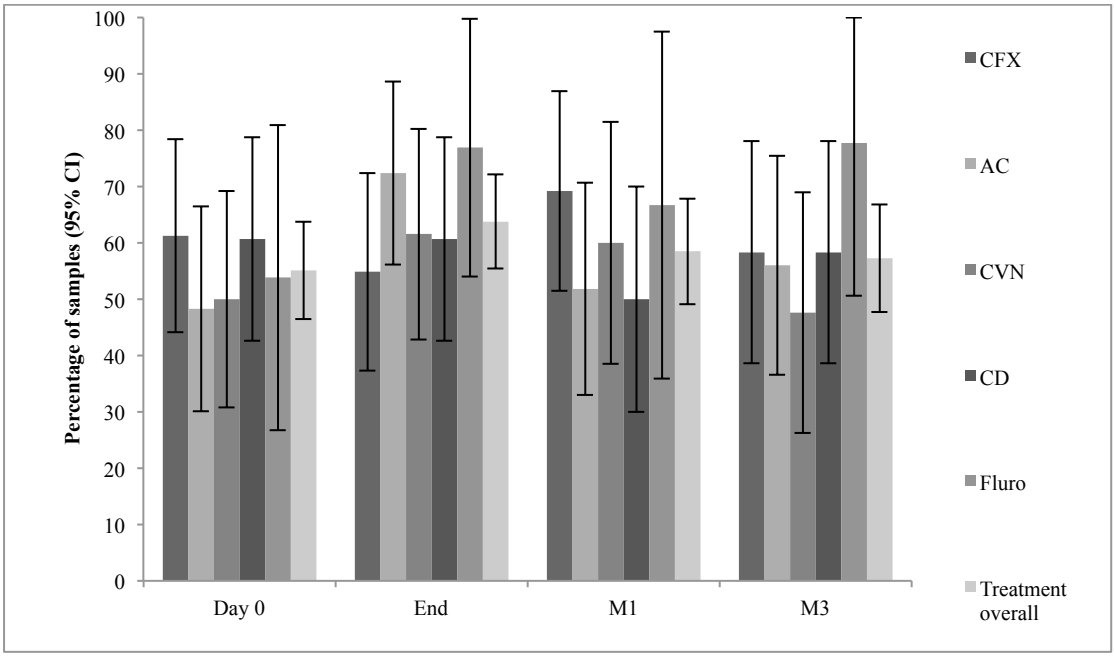
95% CI = 95% confidence interval; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fluro = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy; MDR = multidrug resistance (resistance to ≥ 3 antimicrobial classes)

Figure 5. The percentage of samples with CoPS at each time point in each treatment group (error bars = 95% CI)



95% CI = 95% confidence interval; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fluro = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy; CoPS = coagulase positive staphylococci

Figure 6. The percentage of samples with CoNS at each time point in each treatment group (error bars = 95% CI)



95% CI = 95% confidence interval; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fl = fluoroquinonlone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy; CoNS = coagulase negative staphylococci

3.3 Multilevel, univariable analysis

The overall variable of treatment and time was associated with all outcomes ($P < 0.25$) other than CoNS, and was statistically significant for decreased CoPS detection ($P < 0.001$). In particular, fluoroquinolone therapy at end was significant for all antimicrobial resistance outcomes ($P < 0.01$). In addition univariable analysis found multi-dog household to be significant for increased detection of phenotypic oxacillin resistant, MRS or MDR staphylococci or CoNS ($P < 0.05$). Similarly, recruitment from referral services rather than first opinion clinics increased the risk to detect phenotypic oxacillin resistant ($P = 0.04$) or MDR staphylococci ($P = 0.001$) or CoPS ($P = 0.005$). An initial 'diagnosis of pyoderma' increased the risk to detect phenotypic oxacillin resistant ($P = 0.02$) or MDR staphylococci ($P = 0.001$) and CoPS ($P = 0.002$), while female dogs were more likely than male dogs to harbour MDR staphylococci or CoNS ($P < 0.05$). Increasing age ($P = 0.01$) or smaller breed dogs ($P = 0.006$) were at increased risk for the detection of ciprofloxacin resistant staphylococci, while previous admission to veterinary premises decreased the risk of CoPS detection ($P = 0.01$) and increasing treatment length ($P = 0.02$) was a risk for CoPS detection. Finally MDR was more likely if the owner or in-contact dog had been admitted to hospital ($P = 0.004$), the dog had received beta-lactam antimicrobials prior to enrolment ($P = 0.04$), or if the dog regularly ate animal stools ($P = 0.01$). A number of other variables were associated

($P < 0.25$) with each outcome and were considered for inclusion in the final model (Tables 6-10 and 6-11; Appendix IV).

2.3 Multilevel, multivariable analysis

Treatment and time overall was only significant for the outcome CoPS, however therapy with a fluoroquinolone was a risk for increased detection of phenotypic oxacillin resistant, MRS, ciprofloxacin resistant or MDR staphylococci at the end of therapy compared to all samples at baseline. By M1 there was no significant difference from baseline for the majority of treatment groups and outcomes. There was increased risk for the detection of MDR staphylococci three months after the end of therapy with cefalexin however the aetiology of this finding is unknown. Although cefalexin therapy at the end was a borderline risk for the detection of MDR there was no association at M1. All treatment groups other than clavulanate-amoxicillin were negatively associated with the detection of CoPS staphylococci and this effect continued to M1 for the treatment CD. There was no significant difference at M1 for CFX, AC, CVN or FL treatment groups and M3 for CD treatment group compared to baseline. Other variables were found to be significant for various outcomes including living in a multi-dog household, which was a risk for the detection of phenotypic oxacillin resistant staphylococci or MRS. Small and medium dogs were more likely to harbour ciprofloxacin resistant staphylococci than large dogs. In addition, female dogs, dogs that regularly ate animal stools and dogs that lived with owners or in-contact pets that had been hospitalised were at increased risk for the detection of MDR staphylococci. Previous veterinary admission reduced the risk of detecting CoPS, while conversely living with an owner working in healthcare increased the risk of detecting CoPS and decreased the risk for detecting CoNS. A 'diagnosis of pyoderma' also increased the risk to detect CoPS. The variance partition coefficient (VPC) varied between 8% and 35% for the models, suggesting that there was clustering within dog, particularly for the outcome CoPS (Table 3 and 4).

Table 3. Multilevel multivariable results for outcomes phenotypic oxacillin resistance, oxacillin resistance and carriage of the *mec*A gene (MRS) and ciprofloxacin resistant staphylococci in samples from 127 dogs.

Variables	Phenotypic oxacillin resistance			Oxacillin resistant & carriage of *mec*A gene (MRS)			Ciprofloxacin resistance		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Time D0	REF	—	—	REF	—	—	REF	—	—
Time End and CFX	2.03	0.83-4.98	0.121	2.23	0.86-5.77	0.100	1.56	0.53-4.63	0.421
Time End and AC	0.95	0.33-2.72	0.927	0.98	0.31-3.12	0.977	0.68	0.17-2.70	0.584
Time End and CVN	1.94	0.70-5.34	0.201	2.42	0.84-6.95	0.100	2.36	0.80-6.92	0.118
Time End and CD	0.70	0.23-2.16	0.540	0.63	0.18-2.21	0.466	0.29	0.06-1.46	0.133
Time End and FL	4.56	1.24-16.73	0.022*	9.09	2.22-37.23	0.002*	4.98	1.39-17.80	0.013*
Time M1 and CFX	1.06	0.36-3.07	0.918	1.70	0.59-4.93	0.328	2.43	0.84-7.02	0.102
Time M1 and AC	0.60	0.18-2.02	0.412	0.55	0.14-2.22	0.399	0.23	0.03-2.07	0.190
Time M1 and CVN	1.47	0.45-4.81	0.521	1.27	0.34-4.72	0.717	1.03	0.24-4.34	0.968
Time M1 and CD	1.81	0.65-5.03	0.257	1.39	0.45-4.30	0.571	1.00	0.31-3.27	0.994
Time M1 and FL	2.47	0.56-10.81	0.231	3.22	0.69-15.01	0.137	4.27	0.95-19.23	0.059
Time M3 and CFX	2.03	0.77-5.36	0.155	2.09	0.74-5.91	0.163	1.24	0.35-4.38	0.740
Time M3 and AC	0.69	0.20-2.35	0.556	1.22	0.38-3.94	0.744	0.83	0.21-3.33	0.792
Time M3 and CVN	1.19	0.37-3.84	0.771	1.06	0.29-3.89	0.931	0.58	0.11-3.06	0.519
Time M3 and CD	1.12	0.38-3.34	0.833	1.06	0.32-3.47	0.928	1.30	0.42-4.00	0.644
Time M3 and FL	3.25	0.69-15.18	0.134	0.47	0.04-4.99	0.530	1.60	0.28-9.36	0.599
Time treatment overall	—	—	0.418	—	—	0.207	—	—	0.082
Weight (large)	REF	—	—	REF	—	—	REF	—	—
Weight (small)	—	—	—	—	—	—	3.04	1.45-6.36	0.003*
Weight (medium)	—	—	—	—	—	—	2.48	1.11-5.52	0.026*
Weight overall	—	—	—	—	—	—	—	—	0.005*
Multi-dog household	0.56	0.34-0.93	0.026*	0.56	0.31-0.99	0.045*	—	—	—
Level 2 (dog) Variance [standard error] VPC (%)	0.331 (0.237) 9%	—	—	0.573 (0.299) 15%	—	—	0.349 (0.311) 10%	—	—

OR = odds ratio; 95% CI = 95% confidence interval; VPC = variance partition coefficient; *P* values are from the Wald chi-squared test; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy; significant at $P < 0.05$

Table 4. Multilevel multivariable results for outcomes multidrug resistant staphylococci or the presence of CoPS or CoNS in samples from 127 dogs.

Variables	Multidrug resistance (MDR)			CoPS			CoNS		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Time D0	REF	—	—	REF	—	—	REF	—	—
Time End and CFX	2.41	0.89-6.56	0.085	0.15	0.05-0.46	0.001*	1.05	0.45-2.46	0.911
Time End and AC	0.40	0.08-2.04	0.270	1.22	0.37-4.02	0.745	1.96	0.76-5.06	0.162
Time End and CVN	1.73	0.57-5.19	0.331	0.12	0.03-0.41	0.001*	1.21	0.46-3.21	0.704
Time End and CD	0.63	0.18-2.23	0.478	0.03	0.01-0.10	0.000*	1.54	0.59-4.04	0.375
Time End and FL	7.03	1.76-28.13	0.006*	0.20	0.04-0.98	0.047*	2.94	0.71-12.22	0.137
Time M1 and CFX	1.47	0.48-4.50	0.503	0.54	0.16-1.85	0.327	2.29	0.84-6.21	0.105
Time M1 and AC	0.42	0.08-2.11	0.290	0.67	0.20-2.21	0.513	0.90	0.37-2.20	0.814
Time M1 and CVN	1.56	0.45-5.40	0.480	0.53	0.12-2.38	0.405	1.41	0.46-4.31	0.552
Time M1 and CD	1.60	0.53-4.77	0.403	0.17	0.05-0.59	0.005*	0.79	0.29-2.16	0.648
Time M1 and FL	3.63	0.80-16.53	0.096	1.71	0.11-25.98	0.699	1.95	0.44-8.73	0.381
Time M3 and CFX	2.97	1.06-8.36	0.039*	1.13	0.29-4.45	0.857	1.24	0.49-3.15	0.650
Time M3 and AC	1.06	0.30-3.78	0.927	1.71	0.44-65.59	0.435	1.00	0.38-2.58	0.993
Time M3 and CVN	1.22	0.36-4.19	0.749	0.48	0.11-2.17	0.339	0.77	0.27-2.22	0.627
Time M3 and CD	0.72	0.20-2.59	0.619	0.45	0.13-1.59	0.214	1.22	0.45-3.32	0.703
Time M3 and FL	1.74	0.33-9.07	0.513	0.23	0.03-1.59	0.136	2.89	0.52-16.16	0.226
Time treatment overall	—	—	0.113	—	—	0.000*	—	—	0.814
Pyoderma diagnosis	—	—	—	4.59	1.86-11.33	0.001*	—	—	—
Gender (male REF)	2.01	1.16-3.50	0.013*	—	—	—	—	—	—
Dog eats animal faeces	2.19	1.22-3.92	0.009*	—	—	—	—	—	—
Owner works in healthcare	—	—	—	2.83	1.09-7.38	0.033*	0.55	0.31-0.96	0.036*
In-contact been hospitalised[2]	2.35	1.26-4.35	0.007*	—	—	—	—	—	—
Previous vet admission[1]	—	—	—	0.30	0.13-0.66	0.003*	—	—	—
Level 2 (dog) Variance [standard error] VPC (%)	0.298 (0.263) 8%		—	1.778 (0.491) 35%		—	0.299 (0.198) 8%		—

[1] Within 12 months but more than three months as per enrolment criteria; [2]Within 12 months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; VPC = variance partition coefficient; *P* values are from the Wald chi-squared test; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fl = fluoroquinonlone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy; CoPS = coagulse positive staphylococci; CoNS = coagulase negative staphylococci; significant at $P < 0.05$

3. Discussion

The aim of this study was to examine the effect of routine antimicrobial therapy on canine mucosal staphylococci and to compare five different antimicrobials, authorised for use in dogs in the UK. The percentage of samples with antimicrobial resistant staphylococci increased and the percentage of samples with CoPS decreased at the end of therapy for the majority of the treatment groups. However, for the majority of the examined outcomes and treatment groups, the sample prevalence returned to pre-treatment levels by three months following the end of therapy. In addition, fluoroquinolone therapy was found to be a risk factor for the detection of antimicrobial resistance amongst canine mucosal staphylococci.

CoNS were detected in 93% of dogs in this study in agreement with the prevalence reported for healthy dogs (Schmidt et al., 2014). The CoNS in this study included *S. schleiferi* subsp. *schleiferi*, previously reported from humans and dogs (Kloos and Bannerman, 1994; May et al., 2005). Interestingly coagulase negative *S. aureus,* previously reported from humans and cattle (Malinowski et al., 2009; Obaid et al., 1999), and coagulase negative *S. pseudintermedius* were detected, including MRSP isolates. This finding has not been previously reported for *S. pseudintermedius* and highlights the importance of species determination, particularly of clinical isolates. CoPS were detected in 87% of dogs and *S. pseudintermedius* in 83%, which is double the prevalence reported for healthy non-vet visiting and non-antimicrobial treated dogs using the same methodology (Schmidt et al., 2014). This may be due to consecutive sampling, but 65% of the dogs were diagnosed with pyoderma and skin disease, including atopic dermatitis, which may be associated with increased detection of *S. pseudintermedius* (Fazakerley et al., 2010; Onuma et al., 2012; Saijonmaa-Koulumies and Lloyd, 1995).

The percentage of dogs carrying MRS in this population (~ 60%) was high compared to a group of healthy non-vet visiting and non-antimicrobial treated Labrador retrievers (42%) or healthy vet-visiting dogs (6%) in the UK (Schmidt et al., 2014; Wedley et al., 2014). A higher prevalence of MRS was also reported in dogs with recurrent pyoderma and long-term antimicrobial therapy compared to healthy dogs (Huerta et al., 2011). In addition, MR-CoPS mainly MRSP, were detected in 10% of dogs in this study, whereas no MR-CoPS were detected in previous studies of healthy dogs in the UK (Schmidt et al., 2014; Wedley et al., 2014). A similar prevalence of MRSP (7.4%) was reported in dogs admitted to a small animal hospital in Germany and antimicrobial therapy and hospitalisation were identified as separate risk factors (Nienhoff et al., 2011).

Ciprofloxacin resistant and MDR staphylococci were also prevalent, detected in 47% and 61% of dogs respectively. Similarly, fluoroquinolone resistant staphylococci were more frequently detected in dogs with recurrent pyoderma and long-term antimicrobial therapy than in healthy dogs (Huerta et al., 2011). In addition, ciprofloxacin resistant and MDR CoPS isolates were detected in 13% and 26% of dogs in this study, including MRSP isolates that are often resistant to four or more antimicrobial classes (Descloux et al., 2008; Perreten et al., 2010).

Antimicrobial therapy has been associated with increased infection with or carriage of antimicrobial resistant staphylococci, including MRS and MDR isolates in humans and dogs (Eckholm et al., 2013; Huerta et al., 2011; Jarlov et al., 1996; Nienhoff et al., 2011; Soares Magalhaes et al., 2010; Terpstra et al., 1999; Weese et al., 2012; Westh et al., 2004). Fluoroquinolone therapy was found to be a risk factor for the detection of phenotypic oxacillin resistant staphylococci, MRS, MDR staphylococci and ciprofloxacin resistant staphylococci in this study. Other studies have reported the selection of MRSA or meticillin resistant *S. epidermidis* (MRSE) in humans and MRSA in dogs by fluoroquinolones (Faires et al., 2010; Graffunder and Venezia, 2002; Weber et al., 2003). Phenotypic oxacillin resistant, MRS or MDR-staphylococci were concurrently ciprofloxacin resistant in half of the dogs with each of these resistance outcomes, which may explain selection by fluoroquinolones. Co–selection of MRSA by fluoroquinolones is a significant problem in human hospitals (Westh et al., 2004).

Conversely, it was surprising that beta-lactam therapy was not significantly associated with the investigated resistance outcomes, particularly as the percentage of samples with MRS increased following therapy with cefalexin and cefovecin. Beck et al., (2012) did not find an association between previous beta-lactam therapy and MRSP in dogs however other studies have reported beta-lactam therapy as a risk factor for MRSA infection in humans and dogs (Aldeyab et al., 2008; Faires et al., 2010). In addition, clavulanate-amoxicillin, a broad-spectrum-beta-lactam antimicrobial appeared to have less effect on the detection of MRS than either of the cephalosporins. The majority of the dogs in the clavulanate-amoxicillin group received one week or less of treatment compared to the other two groups, where the majority of dogs received three or more weeks of cephalosporin treatment. Although there was a significant difference for treatment duration between treatment groups at baseline, there was no statistical association between treatment duration and any resistance outcome. Lack of statistical significance may have been associated with the power of the study; however the fluoroquinolone treatment group had the smallest number of observations. Larger studies are required to validate these findings.

Clindamycin therapy did not select for MRS or MDR staphylococci, however it did reduce the detection of CoPS for an extended period compared to the other tested antimicrobials. Prolonged aberrations in gut microbiota have been reported following therapy with clindamycin in humans (Jernberg et al., 2010). Although extended courses of antimicrobials would be expected to prolong selection pressure (Huttner et al., 2013), duration of therapy was not significantly associated with antimicrobial resistance on multivariable analysis in this study. However, due to the small number of observations, it cannot be excluded.

At one and three months following the end of therapy, there was no significant difference between resistance outcomes compared to baseline for the majority of the outcomes and treatment groups. The mechanism of increased antimicrobial resistance following therapy and subsequent recovery was not investigated in this study, but is likely to be multi-factorial. Staphylococci contain a number of mobile genetic elements (MGEs) that may be involved in horizontal transference of antimicrobial resistance determinants between isolates (Forbes and Schaberg, 1983). SCC*mec* cassettes carry the *mec*A gene, and possibly other antimicrobial resistance determinants (Hiramatsu et al., 2001; Holden et al., 2004) but are generally less mobile than other MGEs (Lindsay and Holden, 2006). Furthermore chromosomal mutations, giving rise to clinically relevant fluoroquinolone resistance, may take time to accumulate (Hacker and Carniel, 2001). Therefore selection and co-selection of pre-existing, possibly undetected, antimicrobial resistant strains by fluoroquinolones may have occurred. Additionally, the use of fluoroquinolones, which are broad-spectrum antimicrobials, may have resulted in vacant mucosal niches that could be filled by exogenous strains acquired from the environment or in-contact humans or pets.

However, as co-selection of ciprofloxacin resistant MRS isolates by beta-lactam antimicrobials was not found to be significant, perhaps other mechanisms were involved. *S. aureus* isolates have been shown to up-regulate adhesion factors in response to fluoroquinolones, promoting colonisation by resistant strains (Weber et al 2004). This may occur in other MRS species and further studies are required to investigate this possibility. In addition, fluoroquinolones may affect mutational rates, bacterial SOS response (Mamber et al., 1993) or repress *mec*A regulator genes resulting in up-regulated *mec*A gene expression (Venezia et al., 2001). The recovery phase may represent re-establishment of the original susceptible isolates or newly acquired exogenous antimicrobial susceptible isolates at the expense of the antimicrobial resistant isolates. Antimicrobial resistant isolates may be less fit than antimicrobial susceptible isolates, in the absence of antimicrobial pressure, if compensatory mechanisms have not occurred (Andersson and Hughes, 2010; Lenski, 1998).

In addition to antimicrobial therapy, a number of other factors were found to increase the risk of antimicrobial resistance or change the population structure. These included multi-dog household for phenotypic oxacillin resistance and MRS. Sharing of staphylococci including MRSP, has been reported between in-contact individuals and pets (Gomez-Sanz et al., 2013; Huebner and Goldmann, 1999; Laarhoven et al., 2011) and correlates with the number of in-contacts (Walther et al., 2012). Other findings included females being more likely to carry MDR isolates than males, while other studies have reported male gender more commonly associated with MRS (Huerta et al., 2011; Weese et al., 2012). Additionally, dogs eating animal stools or living with humans or pets with hospital contact increased the risk of detecting MDR staphylococci. Exposure to swine manure was a risk for MRSA infections in humans (Casey et al., 2013), suggesting that animal stools could be source of antimicrobial resistant Gram-positive bacteria for dogs. Hospital contact is a reported risk factor for the detection of MRS in dogs (Eckholm et al., 2013; Hamilton et al., 2013; Nienhoff et al., 2011) and humans (Guclu et al., 2007; Huebner and Goldmann, 1999; Salgado et al., 2003). MRS are often MDR (Diekema et al., 2001; Kern and Perreten, 2013; Perreten et al., 2010) and isolates could be shared with in-contact dogs.

A diagnosis of pyoderma prior to therapy was associated with increased CoPS, mainly *S. pseudintermedius*, which has been isolated more frequently in dogs suffering with pyoderma than from healthy dogs (Beck et al., 2012; Onuma et al., 2012; Saijonmaa-Koulumies and Lloyd, 1995). Additionally, along the same lines, we found that dogs with owners working in health care were more likely to harbour CoPS than CoNS. Dogs in-contact with human health-care workers or facilities were more likely to harbour *S. aureus* or MRSA (Kottler et al., 2010; Lefebvre and Weese, 2009; Soares Magalhaes et al., 2010). The majority of the dog owners working in healthcare in this study worked in the human health care sector, which may account for the predominance of CoPS in this subset of dogs. Approximately half of these dogs carried *S. aureus*, MRSA in one dog.

Limitations of this study included small treatment groups, which reduced the power of the study although the smallest treatment group, fluoroquinolones were found to be a risk factor for a number of outcomes. The recruitment of a clinically-led sample of dogs rather than a fully randomised sample may have introduced sampling bias; however antimicrobial selection and treatment length is based on a number of important individual case factors (diagnosis, culture and susceptibility testing, compliance, cost, contraindications or previous adverse events, drug cascade and UK authorisation) and all antimicrobials under investigation in this study were not appropriate for all cases. In addition, sampling only one nostril per dog may

have reduced the sensitivity to detect staphylococci, however we also sampled the perineum. There were 16 samples missing from M1 and M3 time points due to the exclusion of dogs for receiving further antimicrobial therapy. The majority of these dogs were diagnosed with pyoderma and recurrent infections may occur with ongoing underlying disease. While antimicrobial resistance to the initial prescription was possible, and may be a source of bias, this information was not available. In addition, the non-randomisation to treatment groups led to significant differences of the baseline variables between treatment groups and may have introduced bias. However, the significant variables were all retested in the final models to ensure they did not alter the results. After accounting for the baseline variables there was no significant difference in baseline resistance outcomes across treatment groups. However there was a high level of antimicrobial resistance at baseline. This may have been associated with chronic skin infection, previous veterinary premise contact and antimicrobial treatment; 55 dogs were recruited during referral consultations. High baseline resistance made it harder to identify change following therapy and may have extended the detection of resistance.

3. Conclusions

Overall this study demonstrated changes in antimicrobial resistance profiles and population structure of mucosal staphylococci following routine antimicrobial therapy in dogs. However, the percentage of samples with antimicrobial resistant staphylococci, CoPS and CoNS were similar to pre-treatment levels by three months after the end of therapy. As the infecting isolate often originates from the individuals own mucosal flora, carriage of resistant staphylococci is a risk for resistant infections. This is of particular concern in dogs that are immune suppressed or predisposed to recurrent pyoderma. Additionally, staphylococci can be shared and contaminate environments or clothing, representing potential risks for in-contact individuals. This study provides important information that can be used to design and implement antimicrobial prescribing guidelines and stewardship programs to help limit the development and dissemination of antimicrobial resistance. Larger studies are indicated to validate and build on the findings of this study. Further characterisation and fitness assays of staphylococcal isolates may help to elucidate changes in population structure following therapy and during recovery.

3. Manuscript 5

Antimicrobial resistance amongst canine faecal *Escherichia coli* following antimicrobial therapy

Vanessa Schmidt[1,2], Tim Nuttall[3], Gina Pinchbeck[2], Neil McEwan[1], Susan Dawson[2], Nicola Williams[2]

[1]Department of Infection Biology and [2]Department of Epidemiology and Population Health, The University of Liverpool, Leahurst Campus, Neston, UK, [3]University of Edinburgh, The Royal (Dick) School of Veterinary Studies, Easter Bush Campus, Midlothian, UK.

Manuscript submitted to Journal of Antimicrobial Chemotherapy.

Summary

Background: Antimicrobial resistant, particularly multidrug resistant (MDR) and/or extended spectrum beta-lactam (ESBL) producing *E. coli* are increasingly detected in both healthy and sick dogs. This creates potential animal welfare and public health issues. Reported risk factors include antimicrobial therapy and health care contact.

Objectives: The main aim of the study was to examine the antimicrobial resistance profiles of faecal *E. coli* undergoing treatment with one of five different antimicrobials (cefalexin, clavulanate-amoxicillin, cefovecin, clindamycin, or a fluoroquinolone), authorised to treat dogs in UK. In addition, to investigate potential risk factors for resistance and the longevity of any change.

Methods: Faecal samples were collected from 127 dogs attending veterinary consultations in the UK: before antimicrobial therapy (D0), end of therapy (End), and one month (M1) and three months (M3) after the end of therapy. *E. coli* were isolated and identified using phenotypic, biochemical and genotypic methods. Disc diffusion antimicrobial susceptibility tests were used to determine resistance to a range of antimicrobials and to detect phenotypic ESBL- and AmpC-production. PCR assays investigated the carriage of relevant antimicrobial resistance genes. Questionnaire data and treatment group/time point were independent variables for resistance outcomes.

Results: At the end of therapy**,** multivariable, multilevel modelling found treatment with cephalosporins to be significant for the detection of third generation cephalosporin and ciprofloxacin resistance and phenotypic AmpC-producing *E. coli*; cefovecin and clavulanate-amoxicillin treatment significant for MDR and fluoroquinolones significant for ciprofloxacin resistance, compared to all samples at baseline. Living in a multi-dog household and a 'diagnosis of pyoderma' were associated with the detection of extended spectrum beta lactamase (ESBL)-producing *E. coli*.

Conclusions: Antimicrobial therapy impacts on the gastro-intestinal flora of some dogs. There is a period of at least one month following the end of therapy where treated dogs may have antimicrobial resistant faecal *E. coli*. These findings are of concern due to the potential for resistant opportunistic infections, zoonotic and/or environmental spread and transmission of antimicrobial resistant determinants to pathogens within the gut. Implementation of antimicrobial guidelines may promote rationale antimicrobial use and help limit the development and spread of resistance.

1. Introduction

During the last several years there has been the development and global spread of multidrug resistance and even pan-drug resistance, amongst clinical and commensal bacteria of humans and other animals (Ewers et al., 2012; Gould, 2009; Hunter et al., 2010). Specifically, extended spectrum beta-lactamase (ESBL) and AmpC-producing Gram-negative bacteria, including *Escherichia coli*, have emerged (Gould, 2008) and are increasingly detected from animal samples (Li et al., 2007).

ESBL enzymes hydrolyse oxyimino-cephalosporins but can be inhibited by clavulanic acid whereas AmpC enzymes additionally hydrolyse cephamycins and are not inhibited by clavulanic acid (Bradford, 2001; Li et al., 2007; Thomson, 2010). Genes encoding ESBL and plasmid-mediated AmpC enzymes are carried on mobile genetic elements so can be readily spread between bacteria by horizontal transmission (Li et al., 2007). AmpC-production may also be chromosomally mediated (Woodford et al., 2007). ESBL- and plasmid-mediated AmpC-producers are commonly multidrug resistant (MDR; resistance to three or more antimicrobial classes) because of linkage to other antimicrobial resistance determinants on plasmids (Gould, 2009; Paterson and Bonomo, 2005). This may result in co-selection by therapy with non-oxyimino-cephalosporins (Livermore and Hawkey, 2005) or unrelated antimicrobials such as fluoroquinolones (Jiang et al., 2008).

MDR severely limits therapeutic options for patients resulting in increased morbidity, mortality and costs (Hawkey and Jones, 2009). The gastrointestinal tract of humans and animals is the most important reservoir of antimicrobial resistant (AMR) Gram-negative organisms; the usual source for nosocomial infections (Johnson and Russo, 2002; Livermore and Hawkey, 2005; Wellington et al., 2013). MDR, ESBL- and AmpC-producing *E. coli* have been detected in the rectum or faeces of healthy and hospitalised dogs (Gibson et al., 2011a, b; Guo et al., 2013; Wedley, 2012; Wedley et al., 2011), and have been reported to cause opportunistic infections (O'Keefe et al., 2010; Sanchez et al., 2002; Sidjabat et al., 2006). In addition to the animal welfare concern, due to the close relationship of companion animals with their owners, there is also a potential public health issue (Ewers et al., 2012). Although *E. coli* isolates may be shared within households between humans and pets, the risk appears to be zoonotic and anthroponotic (Damborg et al., 2009; Johnson et al., 2008).

A stable intestinal microflora is required for health with important functions in nutrition, development, metabolism, immune response and pathogen resistance (Dethlefsen et al., 2007). A disruption of this microbiota can occur due to many factors, including diet and

disease, but antimicrobial therapy is the most common and considerable cause (Dethlefsen et al., 2007; Edlund and Nord, 2000; Jernberg et al., 2007). In humans, even short-term antimicrobial therapy may lead to long-term aberration of bacterial populations and prolonged antimicrobial resistance. Ciprofloxacin, cephalosporins and clindamycin have all been associated with the detection of antimicrobial resistant *Enterobacteriaceae* in the intestinal microbiota of humans (Jernberg et al., 2010). The extent and duration of the effect on commensal bacterial populations in humans is reported to depend on the pharmacokinetics, the spectrum of activity, the dose and the duration of the antimicrobial, and the level of antimicrobial resistance present before treatment (Edlund and Nord, 2000; Jernberg et al., 2010; Turnbaugh et al., 2009).

In dogs, treatment with beta-lactam antimicrobials and fluoroquinolones can select for antimicrobial resistant intestinal *E. coli* (Boothe and Debavalya, 2011; Damborg et al., 2011; Gibson et al., 2011a, b; Gronvold et al., 2010; Lawrence et al., 2013; Moreno et al., 2008; Trott et al., 2004). There are a limited number of antimicrobials, mostly broad-spectrum, that are authorised for use in companion animals in the UK. Amongst these, beta-lactam and fluoroquinolone antimicrobials are commonly used and are critically important for the treatment of various bacterial infections in companion animals (Hughes et al., 2012; Li et al., 2007; Mateus et al., 2011; Sato et al., 2013). The majority of these antimicrobials are usually administered in an oral form and active drug that reaches the colon can disturb the microflora (Edlund and Nord, 2000). Cefovecin is a long-acting subcutaneous preparation of semi-synthetic third generation cephalosporin and treatment may be recommended to improve owner compliance (Hughes et al., 2012). Cefovecin is authorised in Europe for administration every 14 days, to treat skin and urinary tract infections, associated with a number of Gram-positive and Gram-negative bacteria in dogs (EMA, 2014). However, its spectrum of activity is narrower than other parenteral third-generation cephalosporins (Hughes et al., 2012). The majority of cefovecin is excreted unchanged in the urine, but unchanged drug also occurs in the bile (Stegemann et al., 2006) and may expose intestinal microflora (Edlund and Nord, 2000). Clindamycin is also commonly used to treat bacterial pyoderma and dental disease, and while it is not effective against Gram-negative aerobes, unlike fluoroquinolones, it has potent activity against anaerobes (Edlund and Nord, 2000; Jernberg et al., 2010; NOAH, 2014).

A number of small case control longitudinal studies, examining the *in-vivo* effects of single antimicrobial agents on the gastrointestinal flora, have been performed in animals. Trott et al., (2004) reported the shedding of MDR faecal *E. coli* for up to 27 days following 21 days of enrofloxacin in a dog model; Cavaco et al., (2008) reported the selection of CTX-M ESBL-

producing faecal *E. coli* for up to 22 days following a three-day course of amoxicillin or oxyimino-cephalosporins in pigs; Gronvold et al., (2010) reported the selection of antimicrobial resistant faecal *E. coli* for up to two weeks after seven days of amoxicillin in dogs; Singer et al., (2008) reported the selection of MDR, AmpC-producing faecal *E. coli* for four days following five-days of ceftiofur treatment in dairy cows; Boothe and Debavalya, (2011) reported the selection of enrofloxacin resistant and MDR faecal *E. coli* for at least 21 days following seven-days of enrofloxacin treatment in dogs and Lawrence et al., (Lawrence et al., 2013) reported the selection of beta-lactam resistant, AmpC-producing faecal *E. coli* at 28 days following a single injection of cefovecin in laboratory Beagles.

In humans, previous therapy with broad-spectrum antimicrobials, particularly oxyimino-cephalosporins, is a risk factor for acquiring infections with antimicrobial resistant bacteria (Wellington et al., 2013), and one of the most useful interventions has been to restrict the use of these antimicrobials (Livermore and Hawkey, 2005). However other authors have questioned whether this therapy is more likely to select for resistance than the use of other broad-spectrum agents and possibly antimicrobial diversification, rather than restriction, may be a more appropriate strategy (Gould, 2008, 2009).

The aim of this study was to ascertain the impact of antimicrobial therapy on the canine gastrointestinal flora by investigating changes in the antimicrobial resistance profiles of faecal *E. coli* over time and to examine the effects of five commonly used and important antimicrobials, authorised for the use in dogs in the UK. This information will help to elucidate the relationship between treatment and the selection and maintenance of antimicrobial resistance amongst canine commensal bacteria, and ultimately aid with the formulation and implementation of strategies to help reduce the burden of antimicrobial resistance in dogs. Considerable inter-individual gastrointestinal flora variability has been reported for both humans and dogs (Dethlefsen et al., 2008; Gronvold et al., 2010; Turnbaugh et al., 2009), which suggests that the impact of antimicrobial therapy is best assessed on an individual basis (Engelbrektson et al., 2006; Jernberg et al., 2010). Therefore the dogs enrolled in this study were used as their own controls.

2. Methods

2.1 Study population

A convenience sample of dogs attending veterinary consultations at three centres including first opinion and referral practice, in the North West UK between June 2011 and September 2012 were recruited for the study. Inclusion criteria included diagnosis of a bacterial infection

(skin, soft tissue, urinary tract, dental, respiratory tract, orthopaedic, gastrointestinal, ocular) requiring systemic antimicrobial therapy with one of five different antimicrobials authorised for use in dogs in the UK (cefalexin [CFX], clavulanate-amoxicillin [AC], cefovecin [CVN], clindamycin [CD], or a fluoroquinolone [enrofloxacin or marbofloxacin; FL]). Exclusion criteria included antimicrobial therapy or veterinary admission within three months of enrolment and dogs aged less than 12 months old. Dogs were excluded during the study if they were prescribed a further course of systemic antimicrobials.

The veterinarian in charge of the case selected and implemented the treatment plan (type of antimicrobial, dose, frequency and duration of therapy). Before enrolment, all dog owners read the study outline and gave written informed consent. Owners were asked to provide a fresh faecal sample from their dog before starting treatment (D0), at the end of treatment (End) and at one (M1) and three months (M3) after the end of treatment. Labelled faecal pots and first-class return-post envelopes were provided at the time of the initial consultation and at each re-examination and/or posted to the client the week before the next sample was due. Veterinary personnel were responsible for client follow-up. This consisted of re-examinations coinciding with the sample due date or by telephone and postal (see above) reminders one week before the sample was due. A questionnaire regarding potential risk factors for the carriage of antimicrobial resistant bacteria was administered at the start of the study and at the time of each faecal sample. The questionnaire was either completed during the consultation or returned with the sample by first-class post.

The two-page questionnaire used in this study consisted of 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, diet and previous veterinary history (including previous antimicrobial therapy and veterinary admission), the presence, number and type of in-contact pets, previous medical history of the household (including antimicrobial therapy or hospitalisation of owners or other pets) and whether any household member worked with farm animals or in healthcare (Appendix V). The attending veterinary surgeon also completed a one-page questionnaire confirming the reason for the visit, the prescribed antimicrobial and therapeutic regime and any previous antimicrobial therapy the dog had received in the last 12 months. All questionnaire-derived information was available as potential explanatory variables for inclusion in the multivariable modelling of various antimicrobial resistance outcomes. The University of Liverpool, School of Veterinary Science Ethics-Committee approved the study protocol in June 2011.

2.2 Specimen collection and bacterial isolation

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, to obtain single colonies, onto one eosin methylene blue agar (EMBA) plate without antimicrobials, and 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, to detect antimicrobial resistant isolates, one EMBA plate and one MacConkey agar (MAC) plate were inoculated with the faecal homogenate for confluent bacterial growth with seven antimicrobial discs: 10 μg ampicillin, 30 μg clavulanate-amoxicillin, 1 μg ciprofloxacin, 30 μg chloramphenicol, 30 μg nalidixic acid, 30 μg tetracycline and 2.5 μg trimethoprim, (Bartoloni et al., 2006). A further 500 μL of faecal homogenate was enriched in 4.5 ml of buffered peptone water. All plates and broths were incubated aerobically for 18 to 20 hours at 37°C. If there was no growth on the EMBA plates impregnated with third generation cephalosporins, the enriched broths were streaked onto the same selective media and incubated aerobically for 18 to 20 hours at 37°C. Ten random colonies, whose morphology resembled *E. coli*, were selected from the plain EMBA plate and where present, one colony growing within the zone of inhibition around each antimicrobial disc on both the EMBA and MAC plates and/or from the CX and/or CZ plates. The selected colonies were sub-cultured onto nutrient agar and incubated aerobically for 18 to 20 hours at 37°C. Gram stain and biochemical tests to confirm *E. coli* were performed (catalase production, lack of oxidase, lactose fermentation, indole production and inability to use citrate as a carbon source) on fresh overnight cultures. All antimicrobial discs were obtained from MAST Group Ltd., Liverpool, UK, and media from LabM Ltd, Bury, UK and the CX and CZ powder from Sigma-Aldrich Company Ltd, Gillingham, UK.

2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility disc diffusion testing was performed on all *E. coli* isolates according to British Society for Antimicrobial Chemotherapy guidelines (BSAC; Version 11.1 May 2012) (Andrews, 2007). Iso-Sensitest agar plates were inoculated, with isolates homogenised in sterile distilled water (0.5 McFarland standard), for semi-confluent bacterial growth and the same panel of seven antimicrobial discs detailed in section 2.3 were applied. After the plates were incubated aerobically at 37°C for 18 to 20 hours, the zone diameters around each disc were measured in millimeters and recorded. *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 phenotypic AmpC- and ESBL-producing *E. coli*

Isolates that were resistant to third generation cephalosporins were tested for the production of extended spectrum beta-lactamase enzymes (ESBL). An Iso-Sensitest agar plate was inoculated for confluent bacterial growth and three pairs of cephalosporin discs (with and without clavulanic acid) were placed on the surface of the agar plate: 30 µg ceftazidime and 30 µg ceftazidime plus 10 µg clavulanic acid; 30 µg cefotaxime and 30 µg cefotaxime plus 10 µg clavulanic acid; and 30 µg cefpodoxime and 30 µg cefpodoxime plus 10 µg clavulanic acid. The plates were incubated aerobically at 37°C for 18 to 20 hours and zone diameters around each disc were measured and recorded. 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 by ≥ 1 of the antimicrobial pairs, according to the manufacturer's instructions (Extended Spectrum Beta-Lactamase Set D52C, MAST Group Ltd., Liverpool, UK) (M'Zali et al., 2000). *E. coli* isolates that were resistant to third generation cephalosporins or clavulanate-amoxicillin were tested for production of AmpC enzyme. An Iso-Sensitest agar plate was inoculated for confluent bacterial growth and three discs applied: 10 µg cefpodoxime plus AmpC inducer (A); 10 µg cefpodoxime, AmpC inducer plus ESBL inhibitor (B); 10 µg cefpodoxime, AmpC inducer, ESBL inhibitor plus AmpC inhibitors (C). The plates were incubated aerobically at 37°C for 18 to 20 hours and zone diameters around each disc were measured and recorded. AmpC production was confirmed when the zone of inhibition around disc C was greater than the zone of inhibition around discs A and B by a minimum of 5 mm, according to the manufacturer's instructions (AmpC detection set D69C, MAST Group Ltd., Liverpool, UK) (Halstead et al., 2012). *E. coli* ATCC® 25922 (LGC Standards, Teddington, UK) cultured overnight on CAB at 37°C was used as a control.

2.5 *E. coli* confirmation and characterisation of antimicrobial resistance genes

Cell lysates were prepared by adding approximately three colonies of each isolate to 500 µl of sterile distilled water (0.5 McFarland standards) and vortexed. The suspensions were then heated at 100°C for 10 minutes and the supernatants were stored at 4°C. PCR assays for the *uid*A gene (McDaniels et al., 1996) were performed to confirm that the isolates were *E. coli* before further characterisation. *E. coli* isolates positive for ESBL production by the double disc method (MAST Group Ltd., Liverpool, UK) and isolates resistant to either CX and/or CZ and positive for 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) genes. If positive for $bla_{\text{CTX-M}}$,

isolates were tested for the presence of CTX-M group 1, 2 and 9 genes (Batchelor et al., 2005; Hopkins et al., 2006). Isolates identified as phenotypic AmpC producers by the AmpC detection set (MAST Group Ltd., Liverpool, UK) were tested for the presence of bla_{AmpC} gene carriage (Perez-Perez and Hanson, 2002). All 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) and water, made up to a total reaction volume of 25 µL. In addition, 0.5 µl of FIREPol® DNA Polymerase 5 U/µl (Solis-Biodyne, Tartu, Estonia) was added per reaction (total volume 25 µL) for multiplex assays. Positive control strains were included and molecular grade water (Sigma-Aldrich Company Ltd., Gillingham, UK) was used as the negative control. 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 (Table 4-1; Appendix II).

2.6 Statistical analysis

All questionnaire derived information and microbiological data were entered into a spreadsheet program (Microsoft Excel for Mac 2008, Microsoft Corporation) and the dataset was reviewed and checked for coding of all variables. Independent (risk factor) variables were created from information obtained from the owner and veterinary surgeon questionnaires. Except for the age of the dog, all variables were dichotomous or categorical in nature. The variables treatment duration and body weight were divided into three categories: ≤ 1 week, > 1 week and ≤ 3 weeks and > 3 weeks and small (< 11 kg), medium (11-20 kg) and large (> 20 kg) respectively. The variable recruitment site was divided into two categories: first opinion and referral consultations (Table 1).

Microbiological data were collapsed to the sample level and categorised for time of sample collection. The baseline samples were always at day zero (D0) and the end of therapy sample (End) varied depending on the length of the prescription. Due to reduced owner compliance towards the end of the study, M1 and M3 time points represent a range of days over which samples were received for processing following the end of therapy; M1 = 21 to 60 days (mean = 35.38, median 33 and mode 28) and M3 = 61 to 150 days (mean = 100.97, median = 97, mode = 84), hence these were categorised as month 1 (M1) and month 3 (M3) samples.

Initial data analysis included Pearson's chi-square tests for the categorical variables and a one-way between-groups analysis of variance (ANOVA) for age to investigate differences between treatment groups at baseline (D0) (Table 2). In addition logistic regression was used to test for differences in antimicrobial resistance outcomes between treatment groups at baseline (D0) after accounting for the other independent variables (Table 7-8 to 7-11, Appendix V). The percentage of samples, with 95% confidence intervals, were calculated for each treatment group and treatment overall (CFX, AC, CVN, CD, FL, Total) at each time point (D0, E, M1, M3) for each of the five outcome measures.

To examine the effect of treatment group and time of sample and other independent variables on the presence of resistance, data were analysed using a multilevel logistic regression models with a binomial distribution and logit link. Due to repeated samples over time, data were clustered within dogs (level two units) and this clustering was accounted for by inclusion of dog as a random intercept in all models. Faecal samples were considered the level one unit of interest. In order to make allowance for all dogs not having received any therapy at baseline (D0), all dogs at baseline were classed as baseline untreated. Ultimately, in addition to no

treatment at baseline (n = 1), there were 15 categories created to account for the different combinations of time (n = 3) and treatment group (n = 5).

Resistance to ciprofloxacin or to third generation cephalosporins (CZ, CPD or CX; including ESBL- and/or AmpC-producing *E. coli*), multidrug resistance (MDR; resistance to ≥ 3 antimicrobial classes) the presence of phenotypic ESBL-producing *E. coli* and the presence of AmpC-producing *E. coli* were considered as five separate binary outcomes.

Initially all variables were analysed in a univariable multilevel model. All variables that showed some association with the presence of resistant *E. coli* on univariable analysis (a *P*-value < 0.25) were considered for incorporation into a final multilevel, multivariable model for that outcome. In addition treatment group and the time of sample were always retained in the final models. For any pair of variables with a correlation coefficient of ≥ 0.7 only the variable with the smallest *P*-value was considered for further analysis. The final models were constructed by a manual backwards stepwise procedure where variables with a Wald *P*-value < 0.05 were retained in the model. Once a final multivariable model was produced all variables that were significantly ($P < 0.05$) different between treatment groups at day 0 were forced back into the model to assess their effect on the remaining variables, in particular treatment group (Table 2).

Multilevel models were analysed using the MLwiN statistical software package (MLwiN Version 2.28 Centre for Multilevel Modelling, University of Bristol). Univariable and multivariable calculations were performed using penalised quasi-likelihood estimates (2^{nd} order PQL for all outcomes other than phenotypic ESBL which was 1^{st} order MQL). First order interaction terms were tested for biologically plausible variables remaining in the final models. The residuals +/- 1.96 SD x rank (caterpillar plots) were calculated and graphed for each dog to check for outliers. Tests for correlation (Spearman's rho), Pearson's chi-square and logistic binary regression were performed using SPSS software package (SPSS 20.0 for Mac, SPSS Inc, Chicago, Illinois).

3. Results

3.1 Study population

One hundred and twenty-seven dogs were enrolled in this study from three centres: practice 1 (n = 44), practice 2 (n = 50) and practice 3 (n = 33) and included first opinion (n = 73) and referral consultations (n = 54). The dogs were treated with the following antimicrobials: cefalexin (n = 32; CFX), clavulanate-amoxicillin (n = 29; AC), cefovecin (n = 27; CVN), clindamycin (n = 29; CD), or a fluoroquinolone (n = 14; FL). Treatment was prescribed for ≤ 1 week in 34 dogs, > 1 week and ≤ 3 weeks in 47 dogs and > 3 weeks in 46 dogs. The dogs were aged between 12 to 204 months (mean = 62 months), there were 75 male and 52 female, and 20 small, 16 medium and 91 large dogs enrolled in this study (Table 1). One hundred and twenty-seven dogs provided samples at Day 0 and End, 105 dogs provided samples at M1 and 98 dogs provided samples at M3. There were a total of 457 samples, 115 samples in the CFX treatment group, 107 samples in AC treatment group, 85 samples in the CVN treatment group, 107 samples in the CD treatment group and 43 samples in the FL treatment group (Table 2). Missing samples from M1 (n = 22) were due to prescription of further antimicrobial therapy (n = 7), euthanasia due to unrelated reasons (n = 3), or owner non-compliance (n = 12). Missing samples from M3 (n = 29) were due to prescription of further antimicrobial therapy (n = 12), euthanasia due to unrelated reasons (n = 4), re-homing (n = 3) or owner non-compliance (n = 10).

Table 1. The baseline (D0) variables considered for inclusion in the final multivariable model, with the number and percentage (%) of samples in each treatment group and variable category.

Variable	CFX	AC	CVN	CD	Fl	Total	*P*-value
Mean age[a] (months)	44	50	68	79	83	62	0.016*
Weight							0.002*
Small (< 11 kg)	1 (5)	2 (10)	3 (15)	10 (50)	4 (20)	20 (16)	
Medium (11-20 kg)	4 (25)	3 (19)	0	7 (44)	2 (13)	16 (13)	
Large (> 20 kg) REF	27 (30)	23 (25)	21 (23)	12 (13)	8 (9)	91 (72)	
Gender							0.784
Male REF	19 (25)	17 (23)	12 (16)	17 (23)	10 (13)	75 (59)	
Female	13 (25)	11 (21)	12 (23)	12 (23)	4 (8)	52 (41)	
Treatment duration							0.000*
1 week REF	6 (18)	16 (47)	0	10 (29)	2 (6)	34 (27)	
> 1 and < 3 weeks	12 (26)	9 (19)	9 (19)	11 (23)	6 (13)	47 (37)	
> 3 weeks	14 (30)	3 (7)	15 (33)	8 (17)	6 (13)	46 (36)	
Recruitment site							0.000*
First opinion practice REF	24 (33)	27 (37)	4 (17)	17 (23)	1 (1)	73 (57)	
Referral consultation	8 (15)	1 (2)	20 (83)	12 (22)	13 (24)	54 (43)	
A diagnosis of pyoderma was made at enrolment	28 (35)	3 (4)	23 (28)	16 (20)	11 (14)	81 (64)	0.000*
Previous systemic antimicrobial treatment[1]	16 (26)	10 (16)	17 (28)	10 (16)	8 (13)	61 (48)	0.048*
Previous beta-lactam antimicrobial treatment[1]	11 (28)	7 (18)	9 (23)	7 (18)	6 (15)	40 (31)	0.408
Previous hospital admission[1]	17 (42)	12 (29)	5 (12)	6 (15)	1 (2)	41 (32)	0.007*
In-contact human or pet received antimicrobials[2]	7 (26)	5 (19)	4 (15)	7 (26)	4 (15)	27 (21)	0.911
In-contact human or pet admitted to hospital or veterinary premises[2]	4 (15)	3 (12)	6 (23)	8 (31)	5 (19)	26 (20)	0.210
Owner works in healthcare	5 (21)	2 (8)	4 (17)	10 (42)	3 (13)	24 (19)	0.078
Multi-dog household	18 (15)	15 (26)	12 (21)	8 (14)	4 (7)	57 (45)	0.123
Enrolled dog regularly eats animal stools	7 (18)	5 (13)	8 (21)	11 (29)	7 (18)	38 (30)	0.083

CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fl = fluoroquinolone. [a]Age was the only categorical value and is represented by the mean age of dogs in each treatment group; REF = the reference category for non-dichotomous variables; [1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; *significant at $P < 0.05$

Table 2. The number of faecal samples provided at each time point in each treatment group.

	Antimicrobial treatment group					
Time point	CFX	AC	CVN	CD	FL	Total
D0	32	28	24	29	14	127
End	32	28	24	29	14	127
M1	27	26	19	26	7	105
M3	24	25	18	23	8	98
Total	115	107	85	107	43	457

CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone; D0 = baseline day zero; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy

3.2 Descriptive statistics

3.2.1 Prevalence of antimicrobial resistance during the full study period

During the full study period, at least one antimicrobial resistant *E. coli* isolate was detected in 287 samples (63%, 95% CI: 58.3 – 67.1) from 115 dogs (91%, 95% CI: 84.2 - 94.5). Only 10 dogs (8%, 95% CI: 4.3 – 13.9) had no antimicrobial resistant faecal *E. coli* detected in any of their samples (four dogs in CD group, three dogs in AC group, two dogs in CFX group and one dog in FL group). There was no commonality between these dogs and and tested

independent variable. MDR *E. coli* were identified in 149 (n = 457) samples (33%; 95% CI: 28.5 - 37.0) from 80 dogs (63%; 95% CI: 54.3 – 70.9) (Tables 7-1 to 7-5; Appendix V). Multiple (greater than one) MDR phenotypes were detected in 48 of the 149 samples (32.2%; 95% CI: 25.2 – 40.1): 32 samples with two phenotypes, 10 samples with three phenotypes, five samples with four phenotypes and one sample with five MDR phenotypes. Resistance to ampicillin, tetracycline and trimethoprim was the most common MDR pattern (Table 3).

Table 3. The ten most frequent multidrug-resistant phenotypes identified among the 149 faecal samples with MDR *E. coli*.

Resistance phenotype	Number of antimicrobial classes	Number of samples	Percentage of samples (95% CI)
AMP, TET, TM	3	47	31.5 (24.6 – 39.4)
AMP, AC, TET, TM	3	21	14.1 (9.4 – 20.6)
AMP, AC, CIP, NAL, TET, TM	4	20	13.4 (8.9 – 19.8)
AMP, CIP, NAL, TET, TM	4	16	10.7 (6.7 – 16.7)
AMP, CHL, TET, TM	4	13	8.7 (5.2 – 14.4)
AMP, NAL, TET, TM	4	11	7.4 (4.2 – 12.7)
AMP, CHL, TET	3	11	7.4 (4.2 – 12.7)
AMP, CHL, TM	3	10	6.7 (3.7 – 11.9)
AMP, AC, CIP, CHL, NA, TET, TM	5	9	6 (3.2 – 11.1)
AMP, CIP, NAL, TET, TM	4	8	5.4 (2.7 – 0.2)

95% CI = 95% confidence intervals; AMP = ampicillin; AC = clavulanate-amoxicillin; CIP = ciprofloxacin; CHL = chloramphenicol, NAL = nalidixic acid, TET = tetracycline; TM = trimethoprim

Overall, 20% (95% CI: 16.3 – 23.6) of samples from 49 dogs (39%; 95% CI: 30.6 – 47.3) and 35% (95% CI: 30.4 – 39) of samples from 79 dogs (62%; 95% CI: 53.5 – 70.2) had at least one ciprofloxacin resistant or 3GCR *E. coli* isolated, respectively. Phenotypic ESBL-producing *E. coli* were detected in 59 samples (11%, 95% CI: 8.2 – 13.9) from 39 dogs (31%, 95% CI: 23.4 – 39.2) and ESBL-producing *E. coli* carrying $bla_{\text{CTX-M}}$ (mainly group 1) in 34 samples (7.4%; 95% CI: 5.4 – 10.2). Additionally, third generation cephalosporin resistant *E. coli* that carried bla_{TEM} and/or bla_{OXA} were detected in 54 samples (12%; 95% CI: 9.2 – 15.1), in conjunction with bla_{CTXM} in 11 samples. Only one sample carried bla_{SHV} along with bla_{TEM} and bla_{OXA}. MDR ESBL-producing *E. coli* were detected in 33 samples (7.2%; 95% CI: 5.2 – 10) and ciprofloxacin resistant ESBL-producing *E. coli* were detected in 21 samples (4.6%; 95% CI: 3.0 – 6.9).

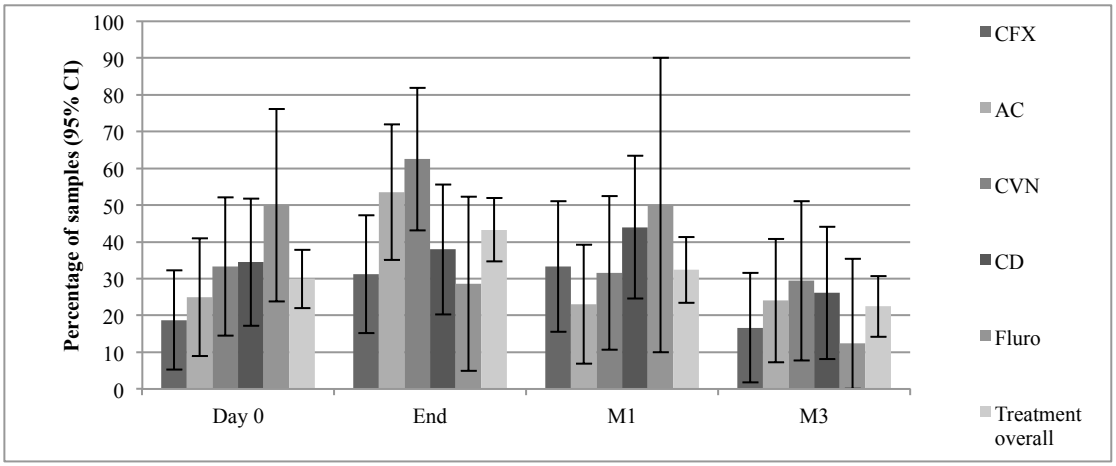
Phenotypic AmpC-producing *E. coli* were detected in 159 samples (35%, 95% CI: 30.6 – 39.3) from 81 dogs (64%, 95% CI: 55.1 – 71.6) and AmpC-producing *E. coli* carrying AmpC genes, mainly bla_{CIT} but also $bla_{\text{DHA-1}}$ and bla_{MOX}, in 133 samples (29.1%; 95% CI: 25.1, 33.4). MDR AmpC-producing *E. coli* were detected in 50 samples (11%; 95% CI: 8.4 – 14.1), while ciprofloxacin resistant AmpC-producing *E. coli* were only detected in 27 samples (5.9%; 95% CI: 4.1 – 8.5).

3.2.2 Changes in antimicrobial resistance following therapy

There was an overall tendency for the percentage of samples with antimicrobial resistant faecal *E. coli,* to increase following antimicrobial therapy and to gradually decrease to pre-treatment levels by M3. In particular, the percentage of samples with MDR, 3GCR or phenotypic ESBL-producing *E. coli* increased following therapy, particularly with beta-lactam antimicrobials, and reduced to pre-treatment levels by M3 for all treatment groups (Figures 1, 2 and 3). MDR ESBL-producing *E. coli* also increased following therapy with beta-lactam or fluoroquinolone antimicrobials but decreased to pre-treatment levels by M1 for the majority of the groups (CFX, CVN and FL). The number of samples with ESBL producing *E. coli* carrying $bla_{\text{CTX-M}}$ increased following therapy in all treatment groups and gradually decreased to or below pre-treatment levels by M3 (Tables 7.1-7.2; Appendix V).

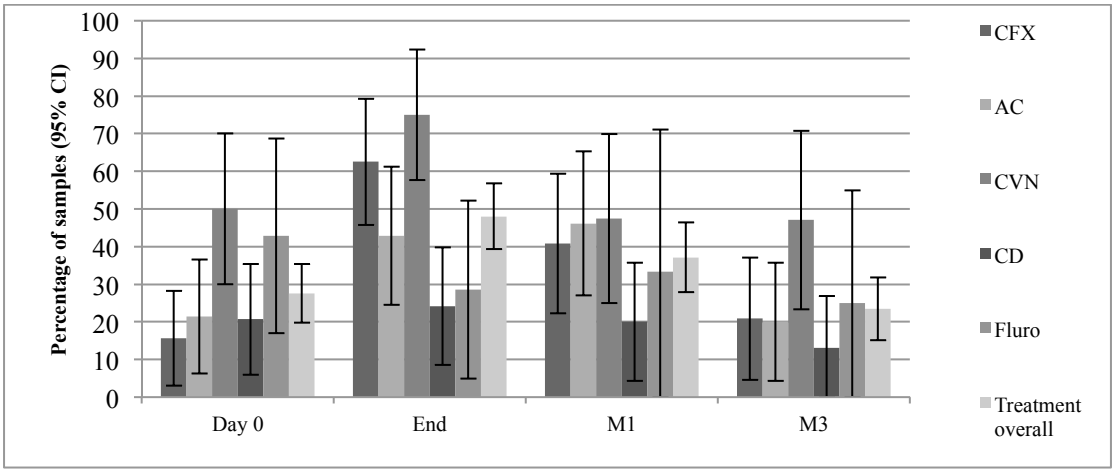
Ciprofloxacin resistance increased after therapy, particularly following CFX, CVN or FL, and then gradually reduced to pre-treatment levels by M3 for all groups (Figure 4). Similar trends occurred for the detection of ciprofloxacin resistant ESBL- or AmpC-producing *E. coli*, however pre-treatment levels were reached by M1. The percentage of samples with phenotypic AmpC-producing *E. coli*, AmpC-producing *E. coli* carrying bla_{AmpC} and MDR AmpC-producing *E. coli* increased following therapy with beta-lactam antimicrobials, but again decreased to pre-treatment levels for all treatment groups by M3 (Figure 5). In fact, pre-treatment levels were reached by M1 for CVN with all AmpC outcomes, for AC with bla_{AmpC} and for CFX with MDR AmpC-producing *E. coli* (Tables 7.1-7.3; Appendix V).

Figure 1. The percentage of samples with MDR *E. coli* at each time point for each treatment group and treatment overall (error bars = 95% CI).



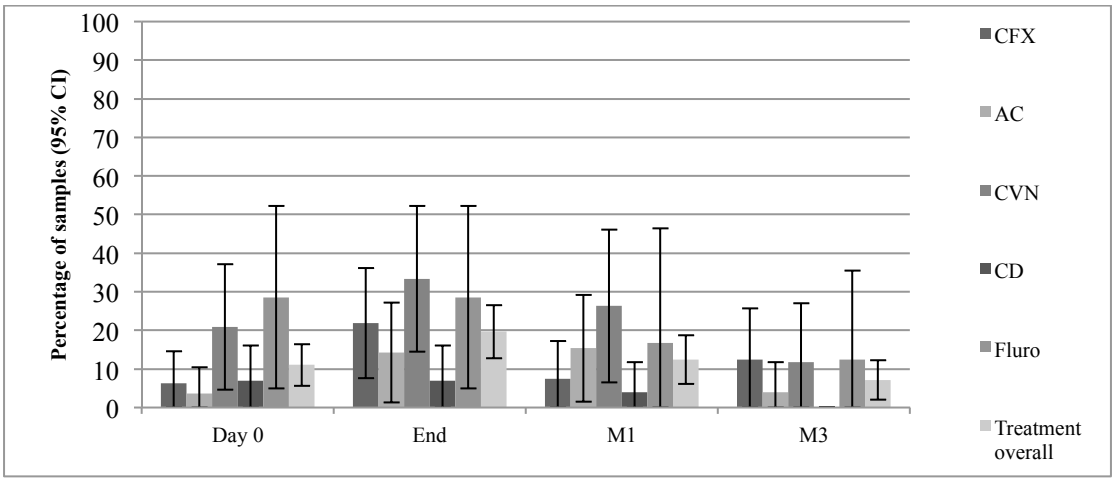
95% CI = 95% confidence interval; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fluro = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy

Figure 2. The percentage of samples with third generation cephalosporin resistant *E. coli* at each time point in each treatment group (error bars = 95% CI).



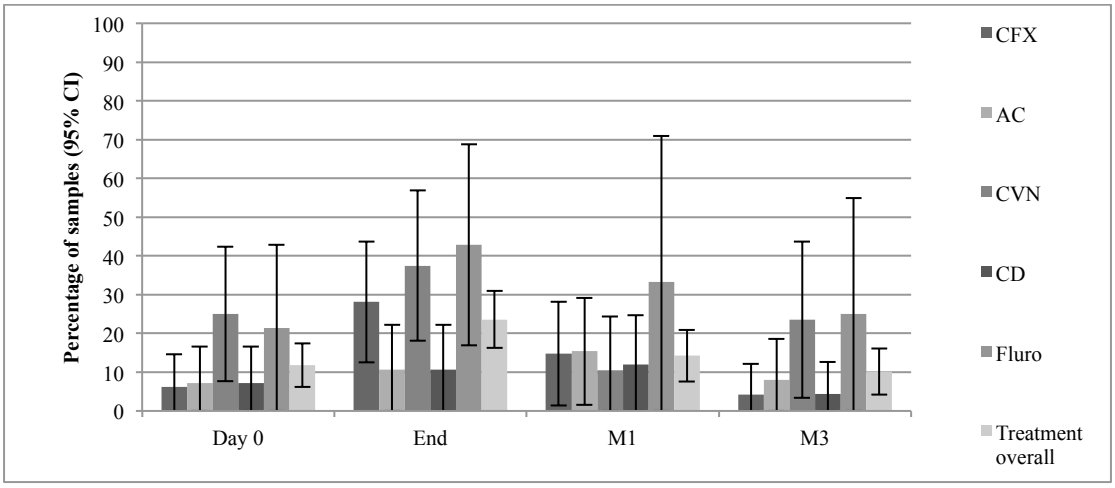
95% CI = 95% confidence interval; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fluro = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy

Figure 3. The percentage of samples with phenotypic ESBL-producing *E. coli* at each time point in each treatment group (error bars = 95% CI).



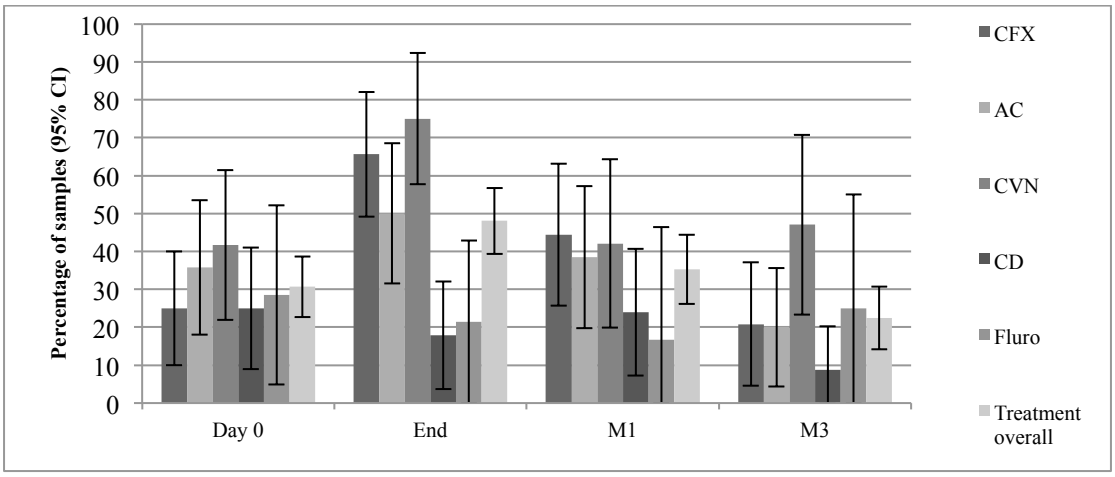
95% CI = 95% confidence interval; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fluro = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy

Figure 4. The percentage of samples with ciprofloxacin resistant *E. coli* at each time point in each treatment group (error bars = 95% CI).



95% CI = 95% confidence interval; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fluro = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy

Figure 5. The percentage of samples with phenotypic AmpC-producing *E. coli* at each time point in each treatment group (error bars = 95% CI).



95% CI = 95% confidence interval; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fluro = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy

3.3 Multilevel, univariable analysis

Univariable analysis found a number of explanatory variables to be either significant for or associated with each of the five outcomes: ciprofloxacin resistance (CipR), third-generation cephalosporin resistance (3GCR), multidrug resistance (MDR), AmpC-producing *E. coli* (AmpC) and ESBL-producing *E. coli* (ESBL). The variables of interest, treatment group (CFX, AC, CVN, CD & FL) at different time points (End, M1 & M3) were compared to the respective baseline outcome detected in all samples from all treatment groups. The overall treatment and time variable was significant for MDR ($P = 0.009$) and 3GCR and AmpC ($P < 0.001$). Living in a multi-dog household was significant for 3GCR ($P = 0.001$), ESBL

($P = 0.004$) and AmpC models ($P = 0.01$). A 'diagnosis of pyoderma' was significant for CipR ($P = 0.012$), 3GCR ($P = 0.008$) and ESBL models ($P = 0.009$). Differences in body weight were significant for 3GCR ($P = 0.002$) and AmpC models ($P < 0.001$) and age (3GCR; $P = 0.004$), duration of treatment (CipR; $P = 0.034$), eating animal stools (CipR; $P = 0.016$), gender (3GCR; $P = 0.004$) and owner working in healthcare (MDR; $P = 0.021$) were significant for one outcome each (outcome and *P*-value in parenthesis). Although recruitment from referral consultations compared to first opinion practice was only close to significance for 3GCR ($P = 0.16$) and AmpC ($P = 0.11$) in the univariable models, this variable was included in the final models for these outcomes (Tables 7-5 and 7-6, Appendix V).

3.4 Multilevel, multivariable analysis

Overall treatment groups and time points were significant for 3GCR, AmpC ($P < 0.001$), and MDR ($P = 0.045$) and borderline significant for CipR ($P < 0.092$). There was significantly increased risk of MDR following treatment with AC or CVN when compared to all baseline samples, but M1 and M3 samples did not have significantly more MDR than the baseline samples. The beta-lactam treatment groups were more likely to be associated with the outcomes 3GCR and AmpC after therapy when compared to baseline, but the risk was greater for treatment with CFX or CVN. In addition there was increased risk for the detection of ciprofloxacin resistant *E. coli* in samples following therapy with CFX, CVN or a fluoroquinolone. Although the odds ratios were still increased at M1 for the majority of outcomes in nearly all treatment groups these were not significantly different to baseline with the exception of AC group and 3GCR.

In addition to the treatment group and time points the only other explanatory variables retained in the final models were living in a multi-dog household (positively associated with 3GCR and ESBL), recruitment from referral consultations compared to first opinion practice (associated with 3GCR and AmpC resistance), eating animal stools (associated with CipR), owner working in healthcare (associated with MDR), a 'diagnosis of pyoderma' (associated with ESBL), and body weight (associated with AmpC); dogs of small or medium weight were less likely than large dogs to harbour AmpC-producing faecal *E. coli* (Table 5 and 6).

All explanatory variables that were significantly different between treatment groups at baseline ($P < 0.05$), and not already present in the final models, were tested in the final models. A 'diagnosis of pyoderma' was significant in the univariable models ($P = 0.008$) for 3GCR. When this variable was forced back into the final model, although it did not reach significance ($P = 0.108$), it narrowed the confidence intervals for cephalosporin therapy,

decreased the variance and resulted in AC treatment group being significant ($P = 0.04$) for this outcome. The variance partition coefficient ranged from 27% to 45% for the different resistance outcomes suggesting that there was substantial clustering within dog. There was no difference between any of the final models compared to models where potential outliers had been excluded (Figures 7-1 to 7-5, Appendix V).

Table 5. Multilevel multivariable results for outcomes ciprofloxacin resistance (CipR) and multidrug resistance (MDR) in 457 faecal samples from 127 dogs.

Variables	CipR			MDR		
	OR	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**
Time D0	REF	—	—	REF	—	—
Time End and CFX	5.14	1.59-16.58	0.006*	1.67	0.55-5.03	0.365
Time End and AC	1.35	0.26-7.07	0.720	4.97	1.54-15.98	0.007*
Time End and CVN	6.97	1.91-25.48	0.003*	8.02	2.10-30.59	0.002*
Time End and CD	0.69	0.10-4.91	0.714	1.59	0.44-5.69	0.479
Time End and FL	5.57	1.21-25.74	0.028*	0.77	0.15-4.00	0.759
Time M1 and CFX	2.02	0.46-8.81	0.352	2.14	0.66-6.94	0.204
Time M1 and AC	2.08	0.46-9.3	0.340	0.58	0.13-2.51	0.465
Time M1 and CVN	0.80	0.11-5.73	0.820	1.59	0.34-7.32	0.553
Time M1 and CD	0.30	0.02-4.12	0.368	1.75	0.47-6.43	0.401
Time M1 and FL	2.70	0.32-22.87	0.363	1.82	0.23-14.09	0.567
Time M3 and CFX	0.44	0.03-5.63	0.528	0.66	0.16-2.80	0.578
Time M3 and AC	1.00	0.15-6.94	0.996	2.02	0.57-7.23	0.278
Time M3 and CVN	2.52	0.51-12.39	0.254	1.24	0.25-6.11	0.792
Time M3 and CD	0.44	0.03-6.02	0.536	0.36	0.07-1.79	0.212
Time M3 and FL	2.88	0.36-22.96	0.317	0.28	0.02-4.53	0.373
Time treatment overall	—	—	0.092	—	—	0.045
Owner works in healthcare	—	—	—	3.61	1.32-9.88	0.012*
Dog eats animal stools	2.89	1.20-7.00	0.018*	—	—	—
Level 2 (dog) Variance [standard error] VPC (%)	1.58 [0.64] 32%	—	—	2.7 [0.66] 45%	—	—

OR = odds ratio; 95% CI = 95% confidence interval; VPC = variance partition coefficient; *P* values are from the Wald chi-squared test; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fl = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months; *significant at $P < 0.05$

Table 6. Multilevel multivariable results for outcomes third generation cephalosporin resistance (3GCR), presence or absence of ESBL- or AmpC-producing *E. coli* in 457 faecal samples from 127 dogs.

Variables	3GCR			ESBL			AmpC		
	OR	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**
Time D0	REF	—	—	REF	—	—	REF	—	—
Time End and CFX	10.04	3.39-29.75	0.000*	1.59	0.52-4.84	0.418	8.84	3.08-25.37	0.000*
Time End and AC	2.86	0.85-9.67	0.091	1.31	0.22-7.59	0.765	2.81	0.95-8.28	0.061
Time End and CVN	11.31	2.89-44.31	0.000*	2.54	0.78-8.26	0.121	9.31	2.72-31.89	0.000*
Time End and CD	0.93	0.25-3.49	0.917	0.42	0.05-3.51	0.426	0.78	0.22-2.73	0.693
Time End and FL	0.58	0.10-3.3	0.537	2.37	0.49-11.53	0.286	0.47	0.09-2.59	0.389
Time M1 and CFX	2.54	0.81-7.97	0.111	0.51	0.11-2.48	0.406	2.76	0.95-8.02	0.063
Time M1 and AC	3.86	1.13-13.14	0.031*	3.01	0.69-13.14	0.143	1.59	0.51-4.92	0.420
Time M1 and CVN	1.50	0.38-5.95	0.561	2.07	0.55-7.72	0.281	1.85	0.56-6.11	0.313
Time M1 and CD	0.64	0.14-2.86	0.562	0.57	0.07-4.59	0.594	1.04	0.29-3.7	0.956
Time M1 and FL	0.69	0.08-5.58	0.724	0.86	0.07-10.42	0.906	0.42	0.03-5.83	0.517
Time M3 and CFX	0.77	0.20-2.98	0.704	0.93	0.23-3.77	0.914	0.62	0.17-2.26	0.471
Time M3 and AC	1.15	0.30-4.41	0.835	0.67	0.07-6.49	0.731	0.42	0.11-1.71	0.227
Time M3 and CVN	3.48	0.86-13.99	0.079	0.76	0.13-4.37	0.759	1.83	0.54-6.24	0.334
Time M3 and CD	0.48	0.09-2.55	0.390	0.65	0.08-5.18	0.686	0.28	0.04-1.85	0.185
Time M3 and FL	0.90	0.12-7.09	0.923	1.13	0.11-11.22	0.916	0.96	0.13-7.21	0.972
Time treatment overall	—	—	0.000*	—	—	0.773	—	—	0.000*
Weight (large)	REF	—	—	REF	—	—	REF	—	—
Weight (small)	—	—	—	—	—	—	0.48	0.17-1.35	0.165
Weight (medium)	—	—	—	—	—	—	0.12	0.03-0.5	0.004*
Weight overall	—	—	—	—	—	—	—	—	0.009*
Diagnosis of pyoderma	—	—	—	3.63	1.18-11.13	0.024*	—	—	
First opinion	REF	—	—	REF	—	—	REF	—	—
Referral consultation	2.61	1.09-6.24	0.031*	—	—	—	2.21	1.06-4.62	0.035*
Multi-dog household	3.78	1.64-8.74	0.002*	2.71	1.24-5.93	0.012*	—	—	—
Level 2 (dog) Variance [standard error] VPC (%)	2.44 [0.6] 43%	—	—	1.190 [0.5] 27%	—	—	1.79 [0.48] 35%	—	—

OR = odds ratio; 95% CI = 95% confidence interval; VPC = variance partition coefficient; *P* values are from the Wald chi-squared test; CFX = cefalexin, AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; Fl = fluoroquinolone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy; *significant at $P < 0.05$

4. Discussion

The aim of this study was to examine the impact of routine antimicrobial therapy on the faecal *E. coli* of dogs attending veterinary outpatient clinics. Treatment with one of five different systemic antimicrobials, authorised to treat dogs in the UK, was investigated. Systemic antimicrobial therapy is known to select for antimicrobial resistant bacteria in the gastrointestinal tract in humans and other animals (Jernberg et al 2010; Singer et al., 2008; Grønvold et al., 2010), and was demonstrated in this study with a significant increase in samples carrying resistant *E. coli* following therapy. For overall antimicrobial treatment, there was approximately a 10 to 20% increase in samples with 3GCR, ciprofloxacin resistance, MDR, phenotypic ESBL-producing *E. coli* and phenotypic and genotypic (bla_{AmpC}) AmpC-producing *E. coli* after therapy. In addition, the percentage of samples with either MDR, ciprofloxacin resistant or CTX-M carrying ESBL-producing *E. coli* doubled immediately following treatment.

Multivariable, multilevel modelling found treatment with beta-lactam antimicrobials, in particular cephalosporins, to be statistically significant for the detection of 3GCR, ciprofloxacin resistance and phenotypic AmpC-producing *E. coli*, and cefovecin and clavulanate-amoxicillin to be significant for MDR, compared to all samples at baseline. AmpC-producing *E. coli* are resistant to oxyimino-cephalosporins (Livermore and Hawkey, 2005) and therefore likely to be selected by therapy with these antimicrobials. Cefalexin was a risk factor for rectal carriage of MDR, predominantly AmpC- producing *E. coli* in hospitalised dogs (Gibson et al., 2011a) and was also reported to select for the carriage of $bla_{\text{CMY-2}}$ (Damborg et al., 2011). In addition, therapy with cefovecin has been reported to select for beta-lactam antimicrobial resistance and carriage of $bla_{\text{CMY-2}}$ gene when compared to untreated dogs (Lawrence et al., 2013). Similar findings have also been reported for humans with increased detection of ampicillin, clavulanate-amoxicillin and second-generation cephalosporin resistant faecal *E. coli* following beta-lactam antimicrobial therapy (Raum et al., 2008). One of the common MDR phenotypes in our study included resistance to clavulanate-amoxicillin, likely accounting for the selection of MDR isolates by clavulanate-amoxicillin or cefovecin therapy. However unexpectedly, clavulanate-amoxicillin treatment was not significantly associated with the detection of AmpC-producing *E. coli* in our study, in agreement with the findings of (Gibson et al., 2011a), where the author proposed further investigation of this result.

Multivariable analysis also found fluoroquinolone therapy to be statistically significant for the detection of ciprofloxacin resistant faecal *E. coli* following therapy. Fluoroquinolone therapy

has been previously reported to select for fluoroquinolone, ESBL-producing and MDR resistant *E. coli* isolates in dogs (Boothe and Debavalya, 2011; Gibson et al., 2011b; Moreno et al., 2008; Ogeer-Gyles et al., 2006), and fluoroquinolone resistant faecal *E. coli* in humans (Raum et al., 2008). *E. coli* strains with high level fluoroquinolone resistance have been reported to be frequently resistant to cephalosporins (Sato et al., 2013). In our study, cephalosporins also selected for fluoroquinolone resistance, suggesting co-selection of multidrug resistant isolates (Livermore and Hawkey, 2005). MDR with clavulanate-amoxicillin and ciprofloxacin resistance were detected in 13% of samples. On the contrary, it was therefore surprising that fluoroquinolone therapy was not significant for MDR or cephalosporin resistance post treatment. It is likely that the full impact of fluoroquinolone therapy on canine faecal *E. coli* in our study may have been limited due to the small sample size of this treatment group.

Multivariable analysis did not find any treatment groups at any time points to be associated with ESBL-producing *E. coli* whereas other studies have reported a link between cephalosporin or fluoroquinolone therapy with ESBL-producing *E. coli* (Cavaco et al., 2008; Moreno et al., 2008). The percentage of dogs that yielded faecal samples positive for ESBL-producing *E. coli* was much lower than the other resistance outcomes examined in the models and therefore associations may have been missed, particularly as cephalosporin therapy was found to be a risk factor for third generation cephalosporin resistance; an outcome that included phenotypic ESBL-producing *E. coli.*

There was an overall trend for the number of positive samples for the majority of resistance outcomes, for the majority of treatment groups to recover to baseline levels by M3; an average of 100 days post-therapy. For some outcomes and treatment groups, including MDR, ciprofloxacin resistant or CTX-M carrying ESBL-producing *E. coli*, recovery to baseline appeared to occur by M1; an average of 35 days. Statistically, there was no difference from baseline in any resistance outcome for any treatment group at one and three months post therapy apart from 3GCR at M1 following clavulanate-amoxicillin therapy. A much shorter recovery period of two weeks was reported by a large prospective cohort study in humans treated with various antimicrobials and a small study of four dogs treated with amoxicillin (Gronvold et al., 2010; Raum et al., 2008). However, the treatment duration in these studies was less than ten days and may have allowed for faster recovery. In addition, the studies only examined one isolate per sample and therefore had lower sensitivity to detect resistance than our study methods. Conversely, our study only sampled four weeks post therapy and may have missed samples with faster recovery time. Other authors have also reported longer recovery times, in agreement with our findings, particularly with longer treatment durations

(Horcajada et al., 2002; Nyberg et al., 2007; Trott et al., 2004). In a canine model, recovery from MDR faecal *E. coli* following oral bacterial inoculation and three weeks of enrofloxacin therapy took one month compared to one week for control animals (Trott et al., 2004), and in humans receiving one month of ciprofloxacin, recovery to baseline took two months (Horcajada et al., 2002). Longer recovery times of up to nine months have been reported for *E. coli* isolates following short-term treatment with clindamycin in humans (Nyberg et al., 2007).

The selection of antimicrobial resistant isolates in the gastrointestinal tract may be a direct effect of treatment suppressing susceptible flora and allowing either pre-existing, possibly undetectable, resistant isolates (either from mutation and/or from acquired resistant determinants) and/or ingested exogenous antimicrobial resistant bacteria to survive and multiply. Also bacterial stress response induced by antimicrobial exposure, may prompt mobilisation and dissemination of resistance determinants amongst the gastrointestinal flora (Donskey, 2006; Edlund and Nord, 2000; Sullivan et al., 2001; Wellington et al., 2013). The exact mechanism of the changes detected in faecal *E. coli* in this study has not yet been elucidated. One prospective study in humans found that isolates detected following therapy were genetically distinct from those detected before or during antimicrobial therapy (Horcajada et al., 2002), suggesting an exogenous source. In the absence of antimicrobial exposure, susceptible isolates (endogenous or exogenous) may out-compete resistant isolates due to the fitness cost of resistance (Andersson and Hughes, 2010; Lenski, 1998). However, compensatory mechanisms have been reported (Cottell et al., 2012; Karami et al., 2008; Lofmark et al., 2008) and together with co-selection, may result in long term gut carriage of antimicrobial resistant isolates and/or genes in the absence of direct antimicrobial pressure (Jernberg et al., 2010).

Faecal *E. coli* populations in humans and other animals are diverse and changeable (Anderson et al., 2006; Damborg et al., 2009). Many factors, including diet and environment (Wang and Schaffner, 2011), in addition to antimicrobial therapy, can be involved in the emergence and dissemination of antimicrobial resistance. A number of factors were found to be associated with certain resistance outcomes. These included: 1) the association of dogs eating animal stools and ciprofloxacin resistance, 2) the owner working in health care and occurrence of MDR and 3) living in a multi-dog household and occurrence of 3GCR and ESBL-producing faecal *E. coli* detection. In addition, large dogs and referral consultations tended to be associated with AmpC-producing *E. coli* and a 'diagnosis of pyoderma' with ESBL-producing *E. coli*. Stenske et al., (2009) reported a link between dogs drinking from toilets and ciprofloxacin resistant faecal *E. coli* and given that antimicrobial resistant faecal *E. coli*

are present in the faeces of wildlife and farmed animals (Guenther et al., 2011; Horton et al., 2011) there is a potential reservoir of resistant bacteria and determinants for ingestion by dogs. Health care contact may be a risk factor for ESBL-producing (Livermore and Hawkey, 2005) and MDR *E. coli* in humans (Stenske et al., 2009) and given the possibility that humans and pets living in the same household can share *E. coli* isolates (Johnson et al., 2008), humans may also be a source of MDR *E. coli* for dogs (Stenske et al., 2009). In addition, Johnson et al., (2008) reported that within-household sharing of *E. coli* was correlated with the number of household members. In particular, living in a multi-dog household was associated with phenotypic ESBL-production, which may be readily transmitted between bacteria by horizontal transmission on mobile genetic elements (Livermore and Hawkey, 2005). One other study has reported large mixed-breed dogs to be at increased risk for AMR (to at least one antimicrobial) and MDR faecal *E. coli* (Procter et al., 2013); perhaps smaller dogs are less likely to scavenge and more likely to undergo regular bathing than larger dogs. A number of other variables including age, duration of therapy and gender appeared significant for various outcomes on univariable analysis but were excluded during the multivariable analysis, in agreement with other studies (Gibson et al., 2011a, b).

Even though the dogs on this study had not received systemic antimicrobials for three months before enrolment, baseline antimicrobial resistance prevalence was high compared to a large cross-sectional study that investigated the prevalence of AMR amongst faecal *E. coli* for vet-visiting dogs in the UK (Wedley, 2012), but similar to AMR prevalence reported for hospitalised dogs, with or without antimicrobials (Guo et al., 2013; Moreno et al., 2008). The high baseline resistance outcomes in our study may be associated with the population demographics and previous health-care contact. Half the dogs had been in contact with veterinary premises, including referral practice, and received systemic antimicrobials during the 12 months prior to the three-month exclusion period for enrolment. Gibson et al., (2011b) reported cumulative veterinary admission of greater than or equal to four days and previous fluoroquinolone therapy as risk factors for rectal carriage of antimicrobial resistant *E. coli* in dogs. This may also account for the finding that dogs with ‘a diagnosis of pyoderma’, often a recurrent condition requiring ongoing veterinary care, or dogs recruited from referral rather than first opinion practice, were more likely to carry AmpC-producing faecal *E. coli*.

There were a number of limitations to this study. The recruitment of a clinically-led sample of dogs rather than a fully randomised sample may have introduced sampling bias; however antimicrobial selection and treatment length is based on a number of important individual case factors (diagnosis, culture and susceptibility testing, compliance, cost, contraindications or previous adverse events, drug cascade and UK authorisation) and all antimicrobials under

investigation in this study were not appropriate for all cases. The small number of dogs enrolled in each treatment group, particularly the fluoroquinolone group, would have reduced the power of the study. Furthermore there was drop out of samples at M1 and M3 time points. This was mainly associated with owner compliance; however 19 samples from 12 dogs were missed due to further prescription of antimicrobials. As these dogs were diagnosed with pyoderma, it is likely that recurrent infection was associated with ongoing underlying disease. Whilst AMR to the initial prescription was possible, and may be a source of bias, this information was not available. In addition the non-randomisation to treatment groups led to significant differences of the baseline variables between treatment groups, which may have also introduced bias. However the significant variables were all retested in the final models to ensure they did not alter the results. After accounting for the baseline variables there was no significant difference in baseline resistance outcomes across treatment groups; however, the high level of AMR at baseline made it more difficult to detect changes following therapy, increased the risk of antimicrobial resistance in the following sample and may have influenced the recovery time. Other limitations included potential regional bias in the sampling of animals and samples delivered by post, possibly with delayed processing in some cases; however Maule (2000) reported *E. coli* could survive in aerated manure for at least a month.

5. Conclusions

Our results suggest that the use of systemic antimicrobials in some dogs will impact the commensal gut flora with a transient shift towards a more resistant bacterial population of *E. coli*. There appears to be at least a month window following the end of therapy where the probability increases for treated dogs to have antimicrobial resistant faecal *E. coli*. This is a potential hazard to the dog, should an infection occur, and for potential transfer to in-contact individuals and the environment. Proactive strategies such as antimicrobial guidelines and stewardship programs to promote judicious and rationale antimicrobial use amongst veterinary health care professionals will help to limit the development and dissemination of antimicrobial resistance. Future research might include the genotyping of *E. coli* isolates to determine the change in populations during and following therapy. In addition, wider spread and longer lasting perturbations may be found with more in depth examination of the canine gut microbiome.

8. Concluding Discussion

8.1 Discussion

The main aim of this work was to examine the effects of routine antimicrobial treatment using five different antibacterial drugs, on canine commensal bacterial populations. Mucosal and intestinal commensal populations were investigated by examining staphylococci and faecal *E. coli* respectively in healthy dogs and those receiving treatment.

Studies of this nature in dogs are rare, particularly longitudinal studies of carriage and AMR. Most studies have only followed a small number of dogs for a limited period after the end of treatment and have not compared different therapies. One longitudinal case-controlled canine study examined the shedding of MDR faecal *E. coli* for three weeks following the end of a three-week course of oral enrofloxacin in dogs (n = 4) (Trott et al., 2004). Additionally, a case-controlled study in laboratory beagles (n = 7) followed AMR faecal *E. coli* for four weeks, after one injection of cefovecin (Lawrence et al., 2013), and Gronvold et al., (2010) followed AMR faecal *E. coli* in healthy community dogs (n = 4), for two weeks, following one-week of amoxicillin. This work encompassed a longitudinal study, comparing the impact of different antimicrobial therapies, on the mucosal staphylococci or faecal *E. coli* of dogs. In addition, recovery was monitored for up to three months following the end of treatment. Studies were not case controlled rather comparison with baseline was used due to expected high inter-individual dog variation of intestinal *E. coli* based on previous studies (Dethlefsen et al., 2008; Engelbrektson et al., 2006; Gronvold et al., 2010; Jernberg et al., 2010; Turnbaugh et al., 2009).

Before performing the main study, it was necessary to investigate the canine commensal bacterial populations in healthy dogs, without the confounding factors of antimicrobial therapy or veterinary premise contact that have been reported as risk factors for AMR bacteria in dogs (Gibson et al., 2011b; Nienhoff et al., 2011). Firstly, a small cross-sectional questionnaire-based study in healthy Labrador retrievers attending dog shows (n = 73) was performed to collect mucosal swab samples and faecal samples. Studies such as these, that report AMR prevalence at the canine level are lacking. Most studies report AMR prevalence at the isolate level, even though multiple colonies per sample are tested. Without genotyping this may lead to an overestimation of resistance prevalence if the results are not aggregated to the canine level. In addition, most studies have concentrated on clinical isolates rather than commensal populations. Commensal bacteria are often the source of infections (von Eiff et al., 2002) and commensal populations may be inadvertently affected by therapy targeting the

infecting pathogen. These populations may then go on to amplify and disseminate AMR. The close relationship between 'man and dog' means that commensal AMR bacteria may be shared between owners and pets in both directions. Furthermore, they may be transferred to the environment and in-contact animals within veterinary premises, representing a possible source for clinical outbreaks. It is therefore just as important, if not more so, to investigate AMR prevalence amongst commensal bacteria.

In addition to reporting AMR prevalence in a cohort of non-antimicrobial treated and non-vet-visiting dogs, the mucosal staphylococci were characterised to the species level using three different methodologies, highlighting the difficulties in performing this accurately. In particular, MALDI-TOF-MS was used for the first time to identify the majority of the canine commensal staphylococcal species in this study. In addition, two different methods to test antimicrobial susceptibility (Disc Diffusion and Minimum inhibitory concentration [MIC]) of oxacillin resistant staphylococci were compared and related to the carriage of antimicrobial resistance genes (*mec*A). Similarly, the healthy dog cohort study reported the prevalence of AMR *E. coli* and examined the population structure by determining phylogenetic groups and investigating the association of these outcomes with potential risk factors. In addition, MDR, ESBL- and AmpC-producing *E. coli* were examined for potential conjugative transfer of antimicrobial resistance determinants.

The healthy cohort study revealed a high level of AMR amongst both mucosal staphylococci and faecal *E. coli* in dogs that had not received antimicrobials or had veterinary premises contact. Amongst mucosal staphylococci, 42% of dogs had MRS and 34% of dogs had MDR isolates. However no MR-CoPS and few MDR-CoPS were identified, in agreement with other studies (Wedley et al., 2014). There was good agreement for the majority of tested antimicrobials for antimicrobial susceptibility testing by Disc Diffusion and MIC methods. In addition, 20 different staphylococcal species were detected from this healthy dog cohort. *S. epidermidis* was the overall predominant species, similar to humans, which is perhaps not surprising given the close association between dogs and their owners, as already mentioned. *S. pseudintermedius*, the main commensal CoPS of dogs, was detected in 44% of dogs, within the reported prevalence range (Devriese and De Pelsmaecker, 1987; Fazakerley et al., 2010; Griffeth et al., 2008; Hanselman et al., 2009; Paul et al., 2012; Rubin et al., 2011). *S. pseudintermedius* were commonly detected along with CoNS species (n = 16) and *S. aureus*, however there was no significant association between *S. pseudintermedius* and any other species, as reported between *S. epidermidis* and *S. aureus* (Park et al., 2011).

While the healthy dog cohort study detected a high prevalence of AMR (63% of dogs), MDR (30% of dogs) and AmpC-producing *E. coli* (mainly bla_{CIT}; 16% of dogs), compared to other cohort studies, few dogs carried ESBL-producing *E. coli* ($bla_{CTX\text{-}M;}$ 1%); resistance to ampicillin, tetracycline and/or trimethoprim was most common (Procter et al., 2013; Wedley et al., 2011). Ampicillin resistant *E. coli* was also commonly detected amongst dogs in the longitudinal healthy study. While the presence of this resistance phenotype may mirror common use of beta-lactam antimicrobials in dogs in the UK, it is also possible that it poses little fitness cost allowing it to persist in intestinal *E. coli* populations, as previously reported in humans (Karami et al., 2008). CTX-M and CIT ($bla_{cmy\text{-}2}$) are the most widespread types of ESBL- and AmpC- beta-lactamases respectively, and are prevalent amongst *E. coli* isolated from humans and animals, including dogs (Ewers et al., 2012; Jacoby, 2009; Wedley et al., 2011), however further characterisation of the ESBL- or AmpC-producing *E. coli* isolates by sequencing was not performed in the current work.

The healthy dog study also found a predominance of phylogenetic group B1, followed by group A *E. coli* isolates in healthy dogs. These phylo-groups are less likely to carry virulence genes and less likely to be associated with extra-intestinal infections compared with the other two phylo-groups (B2 or D) that predominate in humans. Phylo-group B2 isolates were less likely ($P < 0.001$) to be AMR and phylo-group D were more likely ($P = 0.04$) to be AMR compared to the other phylo-groups. This finding concurs with previous work in canine *E. coli* isolates (Platell et al., 2011; Sato et al., 2014). The cohort studies provided a baseline for the antimicrobial treatment study. As the sample population included only one breed type and dogs were recruited at shows, the results may not be fully representative of the general UK dog population. However the prevalence of ESBL-producing *E. coli* was similar to the prevalence reported in a larger UK cohort study in healthy community dogs (Wedley et al., 2011).

In addition, due to the high diversity reported amongst intestinal *E. coli* populations in humans, cattle, horses and dogs (Anderson et al., 2006; Damborg et al., 2009) and the possible influence of external factors such as diet, a longitudinal study was performed to investigate the carriage of faecal *E. coli* in healthy, mainly staff-owned dogs (n = 28), recruited from the University of Liverpool. Only one longitudinal faecal *E. coli* study has been reported in healthy dogs (n = 13) and this study did not report AMR phenotypes, other than the presence of ESBL-producing *E. coli,* and did not assess potential risk factors for carriage.

It was presumed that staphylococcal mucosal populations are more stable over time as has been reported for *S. aureus* in humans (VandenBergh et al., 1999) and *S. pseudintermedius* in dogs (Hartmann et al., 2005). Therefore a similar longitudinal investigation of mucosal staphylococci to the *E. coli* study was not performed in this body of work. However in hindsight, following new information from recent publications following *S. pseudintermedius* strains carried by dogs over time (Gomez-Sanz et al., 2013; Paul et al., 2012), a similar longitudinal investigation of mucosal staphylococci would have added further information to a growing body of evidence and, along with the cohort study, provided a baseline for the antimicrobial study.

The healthy dog longitudinal study found marked inter- and intra-individual diversity of phenotypic antimicrobial resistance traits and genotypes under natural conditions, in agreement with previous work (Anderson et al., 2006; Damborg et al., 2009). This highlights the potential for exogenous *E. coli* isolates to influence the intestinal microbiome. Conjugation experiments revealed the transfer of phenotypic resistance and resistance determinants ($bla_{\text{CTX-M}}$, bla_{CIT}) from commensal faecal *E. coli* isolates, from the healthy dog cohort study, to a recipient strain. Although conjugation experiments and detection of conjugative plasmids was not performed on the isolates from the longitudinal study, transfer of resistance traits between identical genotypes over time is one explanation for the findings in that study. Gene transfer of ampicillin resistance to initially susceptible *E. coli* strains has been reported to occur during intestinal colonisation in human infants (Karami et al., 2008). However intermittent shedding of isolates with identical genotypes and different resistance phenotypes may have also occurred, possibly due to the presence of low numbers.

In the longitudinal healthy dog study, AMR to at least one tested antimicrobial was frequent and prolonged detection occurred in some dogs, whereas MDR, ESBL- or AmpC-producing *E. coli* were less common and detected for shorter periods. Intermittent shedding was common and agrees with previous studies (Anderson et al., 2006), highlighting the lack of sensitivity of cohort analysis to detect AMR prevalence. We cannot however fully exclude non-detection due to the presence of low isolate numbers, rather than intermittent shedding, however the methods used (non-selective and selective media with and without enrichment) were meant to increase the sensitivity to detect both major and minor clones and both susceptible and resistant isolates (Bartoloni et al., 2006). Schlager et al., (2002) calculated that from the random selection of five colonies per plate there was a 99% change of detecting at least one dominant clone. In the longitudinal healthy dog study three random colonies were selected from non-selective media and only one out of three dogs carried resident clone.

Therefore, ten *E. coli* colonies were randomly selected from non-selective media in addition to selective media for the antimicrobial treatment study.

Survival analysis using Kaplan-Meier plots were performed in the longitudinal healthy dog study to calculate the duration of carriage of certain resistance phenotypes. As many dogs continued to carry *E. coli* with resistance to at least one tested antimicrobial when they left or completed the study, the median shedding of this particular outcome was not calculated; however the mean shedding of any AMR was approximately 60 days in agreement with one study following hospitalised/antimicrobial treated horses (Johns et al., 2012). Shedding of MDR, ESBL or AmpC-producing *E. coli* was significantly shorter (median of 14 days), similar to healthy and recently hospitalised/antimicrobial treated horses (Maddox, 2010). The majority of dog owners in the longitudinal healthy dog study worked in veterinary health-care, many in veterinary clinical practice, which may be a source of bias. Therefore the results of this study may not be fully applicable to the general UK dog population.

To determine the effects of anti-bacterials, dogs attending veterinary consultations (n = 127) were recruited that required systemic antimicrobial treatment for a bacterial infection. Following therapy, for the majority of antimicrobials, the prevalence of dogs with AMR bacteria increased and then gradually decreased to baseline by three months after the end of therapy. The use of clinically led samples may be a source of selection bias; however the prescription of antimicrobials could not be assigned randomly. In addition, the interest of this work was to investigate normal dogs in the community. Bias may have also been introduced with non-random allocation to treatment group. There were significant baseline differences for the different independent variables and they were forced back into all final models to ensure that they did not affect the results, particularly treatment and time. In addition, after allowing for independent variables other than treatment, preliminary multivariable regression models were performed for each resistance outcome at baseline to ensure there was no significant difference between treatment groups. Although baseline AMR did not differ across treatment groups, the high prevalence at baseline made it more difficult to detect changes following therapy increased the chance to detect AMR in the following sample and may have influenced the recovery time.

All variables in all studies, other than age, were dichotomous or categorical and all outcomes were dichotomous. Analysis of the cohort studies was performed using Pearson's Chi-square and multivariable regression. However, multilevel multivariable logistic regression was used to investigate the effects of antimicrobials and other independent variables in the antimicrobial treatment study, to allow for potential clustering within dogs due to repeat

sampling over time. This method was also used in the healthy longitudinal study to allow for potential clustering in dogs and in households as more than one dog could be recruited from the same owner in this study. For a number of the resistance outcomes there was still considerable clustering (high variance partition coefficients) that was not accounted for by the tested variables, including antimicrobial therapy. This may be associated with the presence of the resistance outcome in the previous sample for clustering within dogs or sharing of bacteria in multi-dog households, however other unknown variables may also be relevant. Owner-based questionnaires were used as the source of independent test variables in all studies and may be a source of recall bias. In the antimicrobial treatment study this information was supplemented by a veterinarian-based questionnaire and patient clinical history. For all studies the number of observations may have reduced the statistical power, in particular for the fluoroquinolone treatment group in the antimicrobial studies.

In the healthy dog *E. coli* cohort study, the main finding from multivariable logistic regression was that eating raw meats was associated with beta-lactam resistance, including 3GCR, and with phylo-groups B1 and D, which were frequently also AMR. In addition, multi-dog households were associated with 3GCR and phylo-groups A and C (potentially commensal *E. coli* isolates).

Multilevel, multivariable modelling in the longitudinal healthy dog study found that eating dog treats was associated with MDR *E. coli,* whereas the *E. coli* cohort study found them to be protective. The overall effect of eating dog treats may depend on the type of treat, not documented by the questionnaire, which may have differed between the different study populations. Procter et al., (2013) also found that eating cooked meat or proprietary dog food was protective against MDR *E. coli*. However, the low number of observations, particularly in the longitudinal study, may have resulted in this discrepancy between the two studies. In agreement with the cohort study, the healthy longitudinal study also found that eating raw meat was a risk factor for AMR (MDR and ESBL/AmpC-producing faecal *E. coli*) *E. coli*. Farm-animal meat, particularly chicken meat, has been reported as a possible source of AMR *E. coli*, including ExPEC, for people and dogs (Johnson et al., 2009; Johnson et al., 2007; Vincent et al., 2010).

There was a high baseline prevalence of AMR in the dog population participating in the antimicrobial treatment study, most likely due to potential risk factors, including frequent veterinary premises visits, previous antimicrobial therapy and recurrent pyoderma. For instance, in the current work, a diagnosis of pyoderma was associated with increased detection of ESBL-producing *E. coli,* and dogs enrolled via referral clinic rather than first

opinion clinics were more likely to harbour AmpC-producing *E. coli*. There was also an association between beta-lactam therapy and MDR, 3GCR, fluoroquinolone resistance and carriage of AmpC-producing *E. coli.* This highlights the potential for co-selection to drive the selection of AMR isolates. An association was also found between fluoroquinolone therapy and fluoroquinolone resistance in *E. coli*. The antimicrobial study also found an association between dogs eating animal faeces and living in multi-dog households with increased detection of AMR faecal *E. coli*. This last risk factor was also identified in the healthy dog cohort and longitudinal studies.

For staphylococci in the antimicrobial treatment study, multilevel, multivariable modelling found an association between fluoroquinolone treatment with fluoroquinolone resistance, MDR and MRS directly after therapy. However, similar to the findings for faecal *E. coli*, one month after the end of treatment, there was no significant difference from baseline for the majority of treatment groups and outcomes. Surprisingly beta-lactams were not found to influence changes in AMR staphylococci. While this may be associated with insufficient power, the fluoroquinolone group had fewer observations than the other treatment groups and other factors, such as upregulation of adherence factors and/or mutation rate amongst MRS under fluoroquinolone selection pressure, may be involved. Further work is required to investigate these findings. In addition, although the prevalence of MRSP detection (10% of dogs) in this study was higher than reported in healthy dogs, it was still low, preventing assessment of potential risk factors associated with detection.

8.3 Further work

While this body of work was able to provide valuable information of the canine commensal bacterial populations in healthy dogs and during and after antimicrobial therapy the small number of dogs recruited in each study potentially reduced the statistical power. In addition, the study populations were either convenience based or clinically led samples, therefore increasing the likelihood of introducing selection bias. Therefore further similar, ideally randomised, studies with more observations are required to validate the findings of this work. In particular, few dogs carried MRSP (10%; 95% CI: 6 – 17%) in the antimicrobial treatment study. While this is a positive finding, due to the number of dogs enrolled, a larger study is needed to corroborate this prevalence (previous studies report carriage in up to 4.5% and 3.5 - 66% of healthy and sick dogs, respectively) and may help to identify potential risk factors. There are few published studies that have examined the detection of MRSP, and in addition, antimicrobial therapy has not been consistently identified as a risk factor.

In addition, a longitudinal study investigating the carriage of mucosal staphylococci, particularly MRSP, in healthy dogs without antimicrobial pressure or veterinary admission would be beneficial. The fitness of MRSP isolates, compared to meticillin susceptible *S. pseudintermedius*, in the absence of antimicrobial pressure is currently unknown. Longitudinal studies are more likely to detect AMR isolates compared to cohort studies due to transient carriage in some individuals. In addition, speciation and/or genotyping would provide information on the carriage of both CoPS and CoNS species in dogs, the later of which has not been previously reported. Finally, assessment of risk factors for AMR in dogs under natural conditions would provide information that could be used to formulate preventative strategies and used as a baseline for other studies investigating the effects of antimicrobial or veterinary hospital selective pressures.

Further characterisation of the isolates from this work would help to elucidate the findings. For instance, further genotyping, assessment of fitness, phylogenetic grouping and detection of virulence genes with risk factor assessment, particularly in the longitudinal studies, would provide further insight on the carriage of canine gut commensals in healthy dogs versus dogs under antimicrobial therapy. In particular, it would be interesting to investigate the epidemiology of AMR isolates carried by dogs in multi-dog households and in dogs on raw meat diets.

Furthermore, for all *E. coli* studies, characterisation of resistance determinants and plasmids amongst phenotypic ESBL- and AmpC-producing *E. coli* and sequencing of resistance genes and multi-locus sequence typing of (MLST) would provide further information for dogs under different selection pressures e.g. 12% of dogs in the *E. coli* antimicrobial study carried bla_{TEM} and/or $bla_{\text{OXA,}}$ but these isolates were not sequenced to examine variants and determine the significance of such genes for phenotypic resistance. In particular MLST may identify certain dominant ESBL clones and allow comparison with other studies. Moreover, $bla_{\text{DHA-1}}$ and bla_{MOX} were detected in dogs in the antimicrobial resistance study; $bla_{\text{DHA-1}}$ is uncommon and bla_{MOX} has not been previously reported in dogs, so further characterisation would allow comparison with similar isolates detected in humans and other animals. Similarly, for staphylococci, further characterisation of meticillin resistant isolates including determining relative fitness, SCC*mec*, *spa* and strain typing would provide epidemiological information for MRSP isolates carried by dogs in the community in the UK.

Finally, assessment of the microbiomes by metagenomics in healthy dogs and dogs under antimicrobial therapy could investigate the full impact of potential risk factors such as diet, in-contacts and antimicrobial therapy. Examining the impact of different antimicrobials on

anaerobic gut bacteria and the duration of any change is of interest. This is being undertaken as an extension of this work and will be reported elsewhere. Metagenomic determination of the mucosal and skin microbiomes has been recently performed in healthy dogs. Similar longitudinal studies in healthy dogs and dogs under antimicrobial pressure, would give further information on the diversity and stability of such populations.

8.3 Conclusions

The prevalence of antimicrobial resistance was high amongst mucosal staphylococci and faecal *E. coli* in both healthy and sick dogs and the faecal *E. coli* population structure was diverse and dynamic in healthy dogs. In healthy dogs, these findings are likely to be associated with external influences such as diet, environment and in-contact humans and other animals. In particular, dogs that ate raw meat or animal faeces, lived in multi-dog households or had contact with individuals that had been exposed to health-care environments were at increased risk for AMR commensal bacteria. This highlights the potential of bacterial sharing within households and veterinary premises and may represent a human health risk.

Antimicrobial therapy was also associated with the increased risk of antimicrobial resistance amongst these bacterial populations. In particular, beta-lactam treatment was a risk factor for carriage of MDR and AmpC-producing *E. coli,* with fluoroquinolone therapy a risk factor for MDR or MRS staphylococci. The percentage of dogs with each resistance outcome generally returned to baseline within three months of finishing treatment and in the multilevel multivariable models, there was no significant difference for the majority of resistance outcomes and treatment groups between baseline and one month after the end of therapy.

Antimicrobial therapy is a risk factor for the detection of antimicrobial resistant commensal bacteria in dogs and recovery to baseline may take between one to three months after the treatment has finished. This highlights the importance of prudent antimicrobial use, which may be aided by antimicrobial prescribing guidelines. However other factors, such as diet, in-contacts, co-selection and bacterial fitness may be involved in the carriage of resistant bacteria and should be considered

References

Abudu, L., Blair, I., Fraise, A., Cheng, K. K., 2001, Methicillin-resistant *Staphylococcus aureus* (MRSA): a community-based prevalence survey. Epidemiol Infect 126, 351-356.

Alatoom, A.A., Cunningham, S.A., Ihde, S.M., Mandrekar, J., Patel, R., 2011, Comparison of direct colony method versus extraction method for identification of gram-positive cocci by use of Bruker Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 49, 2868-2873.

Aldeyab, M.A., Monnet, D.L., Lopez-Lozano, J.M., Hughes, C.M., Scott, M.G., Kearney, M.P., Magee, F.A., McElnay, J.C., 2008, Modelling the impact of antibiotic use and infection control practices on the incidence of hospital-acquired methicillin-resistant *Staphylococcus aureus*: a time-series analysis. J Antimicrob Chemother 62, 593-600.

Alekshun, M.N., Levy, S.B., 2007, Molecular mechanisms of antibacterial multidrug resistance. Cell 128, 1037-1050.

Allaker, R.P., Jensen, L., Lloyd, D.H., Lamport, A.I., 1992, Colonization of neonatal puppies by staphylococci. Br Vet J 148, 523-528.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990, Basic local alignment search tool. J Mol Biol 215, 403-410.

Anderson, M.A., Whitlock, J.E., Harwood, V.J., 2006, Diversity and distribution of *Escherichia coli* genotypes and antibiotic resistance phenotypes in feces of humans, cattle, and horses. Appl Environ Microbiol 72, 6914-6922.

Andersson, D.I., Hughes, D., 2010, Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol 8, 260-271.

Andrews, J.M., 2007, BSAC standardized disc susceptibility testing method (version 6). J Antimicrob Chemother 60, 20-41.

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., Gordon, J. I., 2005, Host-bacterial mutualism in the human intestine. Science, 307, 1915-1920.

Baddour, M.M., AbuElKheir, M.M., Fatani, A.J., 2007, Comparison of *mec*A polymerase chain reaction with phenotypic methods for the detection of methicillin-resistant Staphylococcus aureus. Curr Microbiol 55, 473-479.

Bagcigil, F.A., Moodley, A., Baptiste, K.E., Jensen, V.F., Guardabassi, L., 2007, Occurrence, species distribution, antimicrobial resistance and clonality of methicillin- and erythromycin-resistant staphylococci in the nasal cavity of domestic animals. Vet Microbiol 121, 307-315.

Baldy-Chudzik, K., Mackiewicz, P., Stosik, M., 2008, Phylogenetic background, virulence gene profiles, and genomic diversity in commensal *Escherichia coli* isolated from ten mammal species living in one zoo. Vet Microbiol, 131, 173-184.

Bannoehr, J., Ben Zakour, N. L., Waller, A. S., Guardabassi, L., Thoday, K. L., van den Broek, A. H., Fitzgerald, J. R., 2007, opulation genetic structure of the *Staphylococcus intermedius* group: insights into agr diversification and the emergence of methicillin-resistant strains. J Bacteriol, 189, 8685-8692.

Bannoehr, J., Guardabassi, L., 2012, *Staphylococcus pseudintermedius* in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. Vet Dermatol,23, 253-66, e51-2.

Barbier, F., Ruppe, E., Hernandez, D., Lebeaux, D., Francois, P., Felix, B., Desprez, A., Maiga, A., Woerther, P.L., Gaillard, K., Jeanrot, C., Wolff, M., Schrenzel, J., Andremont, A., Ruimy, R., 2010, Methicillin-resistant coagulase-negative staphylococci in the community: high homology of SCCmec IVa between *Staphylococcus epidermidis* and major clones of methicillin-resistant *Staphylococcus aureus*. J Infect Dis 202, 270-281.

Bartoloni, A., Benedetti, M., Pallecchi, L., Larsson, M., Mantella, A., Strohmeyer, M., Bartalesi, F., Fernandez, C., Guzman, E., Vallejos, Y., Villagran, A.L., Guerra, H., Gotuzzo, E., Paradisi, F., Falkenberg, T., Rossolini, G.M., Kronvall, G., 2006, Evaluation of a rapid screening method for detection of antimicrobial resistance in the commensal microbiota of the gut. Trans R Soc Trop Med Hyg 100, 119-125.

Batchelor, M., Hopkins, K., Threlfall, E.J., Clifton-Hadley, F.A., Stallwood, A.D., Davies, R.H., Liebana, E., 2005, bla(CTX-M) genes in clinical *Salmonella* isolates recovered from humans in England and Wales from 1992 to 2003. Antimicrob Agents Chemother 49, 1319-1322.

Beck, K.M., Waisglass, S.E., Dick, H.L., Weese, J.S., 2012, Prevalence of meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) from skin and carriage sites of dogs after treatment of their meticillin-resistant or meticillin-sensitive staphylococcal pyoderma. Vet Dermatol 23, 369-375, e366-367.

Bemis, D. A., Jones, R. D., Frank, L. A., Kania, S. A., 2009, Evaluation of susceptibility test breakpoints used to predict mecA-mediated resistance in *Staphylococcus pseudintermedius* isolated from dogs. J Vet Diagn Invest, 21, 53-58.

Ben-Ami, R., Rodriguez-Bano, J., Arslan, H., Pitout, J. D., Quentin, C., Calbo, E. S., Azap, O. K., Arpin, C., Pascual, A., Livermore, D. M., Garau, J., Carmeli, Y., 2009, A multinational survey of risk factors for infection with extended-spectrum beta-lactamase-producing enterobacteriaceae in nonhospitalized patients. Clin Infect Dis, 49, 682-690.

Berg, J.N., Wendell, D.E., Vogelweid, C., Fales, W.H., 1984, Identification of the major coagulase-positive Staphylococcus sp of dogs as *Staphylococcus intermedius*. Am J Vet Res 45, 1307-1309.

Berg, R.D., 1996, The indigenous gastrointestinal microflora. Trends Microbiol 4, 430-435.

Bergeron, M., Dauwalder, O., Gouy, M., Freydiere, A. M., Bes, M., Meugnier, H., Benito, Y., Etienne, J., Lina, G., Vandenesch, F., Boisset, S., 2011, Species identification of staphylococci by amplification and sequencing of the tuf gene compared to the gap gene and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Eur J Clin Microbiol Infect Dis, 30, 343-354.

Bergstrom, A., Gustafsson, C., Leander, M., Fredriksson, M., Gronlund, U., Trowald-Wigh, G., 2012, Occurrence of methicillin-resistant staphylococci in surgically treated dogs and the environment in a Swedish animal hospital. J Small Anim Pract 53, 404-410.

Bibel, D. J., Greenberg, J. H., Cook, J. L., 1977, *Staphylococcus aureus* and the microbial ecology of atopic dermatitis. Can J Microbiol, 23, 1062-1068.

Bignardi, G. E., Woodford, N., Chapman, A., Johnson, A. P., Speller, D. C., 1996, Detection of the mec-A gene and phenotypic detection of resistance in *Staphylococcus aureus* isolates with borderline or low-level methicillinresistance J Antimicrob Chemother, 37, 53-63.

Black, C.C., Solyman, S.M., Eberlein, L.C., Bemis, D.A., Woron, A.M., Kania, S.A., 2009, Identification of a predominant multilocus sequence type, pulsed-field gel electrophoresis cluster, and novel staphylococcal chromosomal cassette in clinical isolates of mecA-containing, methicillin-resistant *Staphylococcus pseudintermedius*. Vet Microbiol 139, 333-338.

Boost, M. V., O'Donoghue, M. M., Siu, K. H., 2007, Characterisation of methicillin-resistant *Staphylococcus aureus* isolates from dogs and their owners. Clin Microbiol Infect, 13, 731-733.

Boothe, D.M., Debavalya, N., 2011, Impact of Routine Antimicrobial Therapy on Canine faecal *Escherichia coli* Antimicrobial Resistance: A Pilot Study. Intern J Appl Res Vet Med 9, 396-406.

Bradford, P.A., 2001, Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev 14, 933-951, table of contents.

Busscher, J. F., van Duijkeren, E., Sloet van Oldruitenborgh-Oosterbaan, M. M., 2006, The prevalence of methicillin-resistant staphylococci in healthy horses in the Netherlands. Vet Microbiol, 113, 131-136.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L., Threlfall, E. J., 2005, Identification of plasmids by PCR-based replicon typing. J Microbiol Methods, 63, 219-228.

Carlos, C., Pires, M. M., Stoppe, N. C., Hachich, E. M., Sato, M. I., Gomes, T. A., Amaral, L. A., Ottoboni, L. M., 2010, *Escherichia coli* phylogenetic group determination and its

application in the identification of the major animal source of fecal contamination. BMC Microbiol, 10, 161.

Carpaij, N., Willems, R.J., Bonten, M.J., Fluit, A.C., 2011, Comparison of the identification of coagulase-negative staphylococci by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and tuf sequencing. Eur J Clin Microbiol Infect Dis 30, 1169-1172.

Casey, J.A., Curriero, F.C., Cosgrove, S.E., Nachman, K.E., Schwartz, B.S., 2013, High-density livestock operations, crop field application of manure, and risk of community-associated methicillin-resistant *Staphylococcus aureus* infection in Pennsylvania. JAMA Intern Med 173, 1980-1990.

Cavaco, L.M., Abatih, E., Aarestrup, F.M., Guardabassi, L., 2008, Selection and persistence of CTX-M-producing *Escherichia coli* in the intestinal flora of pigs treated with amoxicillin, ceftiofur, or cefquinome. Antimicrob Agents Chemother 52, 3612-3616.

Cimiotti, J. P., Wu, F., Della-Latta, P., Nesin, M., Larson, E., 2004, Emergence of resistant staphylococci on the hands of new graduate nurses. Infect Control Hosp Epidemiol, 25, 431-435.

Clermont, O., Bonacorsi, S., Bingen, E., 2000, Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol 66, 4555-4558.

Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013, The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ Microbiol Rep 5, 58-65.

Clermont, O., Gordon, D.M., Brisse, S., Walk, S.T., Denamur, E., 2011a, Characterization of the cryptic *Escherichia* lineages: rapid identification and prevalence. Environ Microbiol 13, 2468-2477.

Clermont, O., Olier, M., Hoede, C., Diancourt, L., Brisse, S., Keroudean, M., Glodt, J., Picard, B., Oswald, E., Denamur, E., 2011b, Animal and human pathogenic *Escherichia coli* strains share common genetic backgrounds. Infect Genet Evol 11, 654-662.

CLSI 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard—Third Edition. CLSI document M31-A3 (Wayne, PA, Clinical and Laboratory Standards Institute).

CLSI 2013. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Approved Standard -- Fourth Edition. CLSI document VET01-A (Wayne, PA, Clinical and Laboratory Standards Institute).

Cortes, P., Blanc, V., Mora, A., Dahbi, G., Blanco, J. E., Blanco, M., Lopez, C., Andreu, A., Navarro, F., Alonso, M. P., Bou, G., Blanco, J., Llagostera, M., 2010, Isolation and

characterization of potentially pathogenic antimicrobial-resistant *Escherichia coli* strains from chicken and pig farms in Spain. Appl Environ Microbiol, 76, 2799-2805.

Costa, D., Poeta, P., Brinas, L., Saenz, Y., Rodrigues, J., Torres, C., 2004, Detection of CTX-M-1 and TEM-52 beta-lactamases in *Escherichia coli* strains from healthy pets in Portugal. J Antimicrob Chemother 54, 960-961.

Cottell, J.L., Webber, M.A., Piddock, L.J., 2012, Persistence of transferable extended-spectrum-beta-lactamase resistance in the absence of antibiotic pressure. Antimicrob Agents Chemother 56, 4703-4706.

Cox, H. U., Hoskins, J. D., Newman, S. S., Foil, C. S., Turnwald, G. H., Roy, A. F., 1988, Temporal study of staphylococcal species on healthy dogs. Am J Vet Res, 49, 747-751.

Dahmen, S., Haenni, M., Madec, J.Y., 2012, IncI1/ST3 plasmids contribute to the dissemination of the blaCTX-M-1 gene in *Escherichia coli* from several animal species in France. J Antimicrob Chemother 67, 3011.

Dallenne, C., Da Costa, A., Decre, D., Favier, C., Arlet, G., 2010, Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. J Antimicrob Chemother 65, 490-495.

Damborg, P., Gaustad, I.B., Olsen, J.E., Guardabassi, L., 2011, Selection of CMY-2 producing *Escherichia coli* in the faecal flora of dogs treated with cephalexin. Vet Microbiol 151, 404-408.

Damborg, P., Nielsen, S.S., Guardabassi, L., 2009, *Escherichia coli* shedding patterns in humans and dogs: insights into within-household transmission of phylotypes associated with urinary tract infections. Epidemiol Infect 137, 1457-1464.

Davis, C. P., Cleven, D., Balish, E., Yale, C. E., 1977, Bacterial association in the gastrointestinal tract of beagle dogs. Appl Environ Microbiol, 34, 194-206.

De Graef, E.M., Decostere, A., Devriese, L.A., Haesebrouck, F., 2004, Antibiotic resistance among fecal indicator bacteria from healthy individually owned and kennel dogs. Microb Drug Resist 10, 65-69.

De Martino, L., Lucido, M., Mallardo, K., Facello, B., Mallardo, M., Iovane, G., Pagnini, U., Tufano, M. A., Catalanotti, P., 2010, Methicillin-resistant staphylococci isolated from healthy horses and horse personnel in Italy. J Vet Diagn Invest, 22, 77-82.

Decristophoris, P., Fasola, A., Benagli, C., Tonolla, M., Petrini, O., 2011, Identification of *Staphylococcus intermedius* Group by MALDI-TOF MS. Syst Appl Microbiol 34, 45-51.

Deschamps, C., Clermont, O., Hipeaux, M. C., Arlet, G., Denamur, E., Branger, C., 2009, Multiple acquisitions of CTX-M plasmids in the rare D2 genotype of *Escherichia coli* provide evidence for convergent evolution. Microbiology, 155, 1656-1668.

Descloux, S., Rossano, A., Perreten, V., 2008, Characterization of new staphylococcal cassette chromosome *mec* (SCC*mec*) and topoisomerase genes in fluoroquinolone- and methicillin-resistant *Staphylococcus pseudintermedius*. J Clin Microbiol 46, 1818-1823.

Dethlefsen, L., Huse, S., Sogin, M.L., Relman, D.A., 2008, The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 6, e280.

Dethlefsen, L., McFall-Ngai, M., Relman, D.A., 2007, An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449, 811-818.

Devriese, L.A., De Pelsmaecker, K., 1987, The anal region as a main carrier site of *Staphylococcus intermedius* and *Streptococcus canis* in dogs. Vet Rec 121, 302-303.

Diekema, D.J., Pfaller, M.A., Schmitz, F.J., Smayevsky, J., Bell, J., Jones, R.N., Beach, M., 2001, Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999. Clin Infect Dis 32 Suppl 2, S114-132.

Donskey, C.J., 2006, Antibiotic regimens and intestinal colonization with antibiotic-resistant gram-negative bacilli. Clin Infect Dis 43 Suppl 2, S62-69.

Doumith, M., Day, M.J., Hope, R., Wain, J., Woodford, N., 2012, Improved multiplex PCR strategy for rapid assignment of the four major *Escherichia coli* phylogenetic groups. J Clin Microbiol 50, 3108-3110.

Dubois, D., Leyssene, D., Chacornac, J. P., Kostrzewa, M., Schmit, P. O., Talon, R., Bonnet, R., Delmas, J., 2010, Identification of a variety of Staphylococcus species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol, 48, 941-945.

Dupont, C., Sivadon-Tardy, V., Bille, E., Dauphin, B., Beretti, J. L., Alvarez, A. S., Degand, N., Ferroni, A., Rottman, M., Herrmann, J. L., Nassif, X., Ronco, E., Carbonnelle, E., 2010, Identification of clinical coagulase-negative staphylococci, isolated in microbiology laboratories, by matrix-assisted laser desorption/ionization-time of flight mass spectrometry and two automated systems. Clin Microbiol Infect, 16, 998-1004.

Duval, X., Selton-Suty, C., Alla, F., Salvador-Mazenq, M., Bernard, Y., Weber, M., Lacassin, F., Nazeyrolas, P., Chidiac, C., Hoen, B., Leport, C., 2004, Endocarditis in patients with a permanent pacemaker: a 1-year epidemiological survey on infective endocarditis due to valvular and/or pacemaker infection. Clin Infect Dis, 39, 68-74.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., Relman, D. A., 2005, Diversity of the human intestinal microbial flora. Science, 308, 1635-1638.

Eckholm, N.G., Outerbridge, C.A., White, S.D., Sykes, J.E., 2013, Prevalence of and risk factors for isolation of meticillin-resistant *Staphylococcus* spp. from dogs with pyoderma in northern California, USA. Vet Dermatol 24, 154-161 e134.

Edlund, C., Nord, C.E., 2000, Ecological Impact of Antimicrobial Agents on Human Intestinal Microflora J Antimicrob Chemother 46, 41-48.

Elias, P. M., 2005, Stratum corneum defensive functions: an integrated view. J Invest Dermatol, 125, 183-200.

EMA 2014. EMA. 2014. European Medicines Agency.

Engelbrektson, A.L., Korzenik, J.R., Sanders, M.E., Clement, B.G., Leyer, G., Klaenhammer, T.R., Kitts, C.L., 2006, Analysis of treatment effects on the microbial ecology of the human intestine. FEMS Microbiol Ecol 57, 239-250.

Escobar-Paramo, P., Grenet, K., Le Menac'h, A., Rode, L., Salgado, E., Amorin, C., Gouriou, S., Picard, B., Rahimy, M.C., Andremont, A., Denamur, E., Ruimy, R., 2004, Large-scale population structure of human commensal *Escherichia coli* isolates. Appl Environ Microbiol 70, 5698-5700.

Escobar-Paramo, P., Le Menac'h, A., Le Gall, T., Amorin, C., Gouriou, S., Picard, B., Skurnik, D., Denamur, E., 2006, Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. Environ Microbiol 8, 1975-1984.

EUCAST 2013. EUCAST. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diamters. Version 3.1, valid from 11.02.2013.

Eveillard, M., Martin, Y., Hidri, N., Boussougant, Y., Joly-Guillou, M. L., 2004, Carriage of methicillin-resistant *Staphylococcus aureus* among hospital employees: prevalence, duration, and transmission to households. Infect Control Hosp Epidemiol, 25, 114-120.

Ewers, C., Bethe, A., Semmler, T., Guenther, S., Wieler, L.H., 2012, Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin Microbiol Infect 18, 646-655.

Faires, M.C., Traverse, M., Tater, K.C., Pearl, D.L., Weese, J.S., 2010, Methicillin-resistant and -susceptible *Staphylococcus aureus* infections in dogs. Emerg Infect Dis 16, 69-75.

Fazakerley, J., Williams, N., Carter, S., McEwan, N., Nuttall, T., 2010, Heterogeneity of *Staphylococcus pseudintermedius* isolates from atopic and healthy dogs. Vet Dermatol 21, 578-585.

Fessler, A. T., Billerbeck, C., Kadlec, K., Schwarz, S., 2010, Identification and characterization of methicillin-resistant coagulase-negative staphylococci from bovine mastitis. J Antimicrob Chemother, 65, 1576-1582.

Finley, R., Ribble, C., Aramini, J., Vandermeer, M., Popa, M., Litman, M., Reid-Smith, R., 2007, The risk of salmonellae shedding by dogs fed *Salmonella*-contaminated commercial raw food diets. Can Vet J 48, 69-75.

Forbes, B.A., Schaberg, D.R., 1983, Transfer of resistance plasmids from Staphylococcus epidermidis to *Staphylococcus aureus*: evidence for conjugative exchange of resistance. J Bacteriol 153, 627-634.

Francois, P., Pittet, D., Bento, M., Pepey, B., Vaudaux, P., Lew, D., Schrenzel, J., 2003, Rapid detection of methicillin-resistant *Staphylococcus aureus* directly from sterile or nonsterile clinical samples by a new molecular assay. J Clin Microbiol, 41, 254-260.

Frost, L.S., Leplae, R., Summers, A.O., Toussaint, A., 2005, Mobile genetic elements: the agents of open source evolution. Nat Rev Microbiol 3, 722-732.

Garbacz, K., Zarnowska, S., Piechowicz, L., Haras, K., 2013, Staphylococci isolated from carriage sites and infected sites of dogs as a reservoir of multidrug resistance and methicillin resistance. Curr Microbiol, 169-173.

Garza-Gonzalez, E., Morfin-Otero, R., Martinez-Vazquez, M. A., Gonzalez-Diaz, E., Gonzalez-Santiago, O., Rodriguez-Noriega, E., 2011, Microbiological and molecular characterization of human clinical isolates of *Staphylococcus cohnii*, *Staphylococcus hominis*, and *Staphylococcus sciuri*. Scand J Infect Dis43, 930-936.

Gibson, J.S., Morton, J.M., Cobbold, R.N., Filippich, L.J., Trott, D.J., 2011a, Risk factors for dogs becoming rectal carriers of multidrug-resistant *Escherichia coli* during hospitalization. Epidemiol Infect 139, 1511-1521.

Gibson, J.S., Morton, J.M., Cobbold, R.N., Filippich, L.J., Trott, D.J., 2011b, Risk factors for multidrug-resistant *Escherichia coli* rectal colonization of dogs on admission to a veterinary hospital. Epidemiol Infect 139, 197-205.

Gillespie, B. E., Headrick, S. I., Boonyayatra, S., Oliver, S. P., 2009, Prevalence and persistence of coagulase-negative *Staphylococcus* species in three dairy research herds. Vet Microbiol, 134, 65-72

Gomez-Sanz, E., Torres, C., Ceballos, S., Lozano, C., Zarazaga, M., 2013, Clonal dynamics of nasal *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in dog-owning household members. Detection of MSSA ST(398). PLoS One 8, e69337.

Gordon, D.M., Clermont, O., Tolley, H., Denamur, E., 2008, Assigning Escherichia coli strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. Environ Microbiol 10, 2484-2496.

Gordon, D. M., Cowling, A., 2003, The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. Microbiology, 149, 3575-86.

Gordon, D. M., Stern, S. E., Collignon, P. J., 2005, Influence of the age and sex of human hosts on the distribution of *Escherichia coli* ECOR groups and virulence traits. Microbiology, 151, 15-23.

Gould, I.M., 2008, The epidemiology of antibiotic resistance. Int J Antimicrob Agents 32 Suppl 1, S2-9.

Gould, I.M., 2009, Antibiotic resistance: the perfect storm. Int J Antimicrob Agents 34 Suppl 3, S2-5.

Graffunder, E.M., Venezia, R.A., 2002, Risk factors associated with nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) infection including previous use of antimicrobials. J Antimicrob Chemother 49, 999-1005.

Greene, C.E., Watson, A.D.J. 2006. Antimicrobial Chemotherapy. In Infectious Diseases of the Dog and Cat, edition 3, Greene, C.E., ed. (St. Louis, Missouri, Elsevier), pp. 274-301.

Grice, E. A., Kong, H. H., Conlan, S., Deming, C. B., Davis, J., Young, A. C., Bouffard, G. G., Blakesley, R. W., Murray, P. R., Green, E. D., Turner, M. L., Segre, J. A., 2009, Topographical and temporal diversity of the human skin microbiome. Science, 324, 1190-1192.

Grice, E.A., Kong, H.H., Renaud, G., Young, A.C., Bouffard, G.G., Blakesley, R.W., Wolfsberg, T.G., Turner, M.L., Segre, J.A., 2008, A diversity profile of the human skin microbiota. Genome Res 18, 1043-1050.

Griffeth, G.C., Morris, D.O., Abraham, J.L., Shofer, F.S., Rankin, S.C., 2008, Screening for skin carriage of methicillin-resistant coagulase-positive staphylococci and *Staphylococcus schleiferi* in dogs with healthy and inflamed skin. Vet Dermatol 19, 142-149.

Gronvold, A.M., L'Abee-Lund, T.M., Sorum, H., Skancke, E., Yannarell, A.C., Mackie, R.I., 2010, Changes in fecal microbiota of healthy dogs administered amoxicillin. FEMS Microbiol Ecol 71, 313-326.

Guardabassi, L., Schwarz, S., Lloyd, D. H., 2004, Pet animals as reservoirs of antimicrobial-resistant bacteria. J Antimicrob Chemother, 54, 321-332.

Guarner, F., Malagelada, J.R., 2003, Gut flora in health and disease. Lancet 361, 512-519.

Guclu, E., Yavuz, T., Tokmak, A., Behcet, M., Karali, E., Ozturk, O., Egeli, E., 2007, Nasal carriage of pathogenic bacteria in medical students: effects of clinic exposure on prevalence and antibiotic susceptibility. Eur Arch Otorhinolaryngol 264, 85-88.

Guenther, S., Ewers, C., Wieler, L.H., 2011, Extended-Spectrum Beta-Lactamases Producing *E. coli* in Wildlife, yet Another Form of Environmental Pollution? Front Microbiol 2, 246.

Guo, S., Brouwers, H.J., Cobbold, R.N., Platell, J.L., Chapman, T.A., Barrs, V.R., Johnson, J.R., Trott, D.J., 2013, Fluoroquinolone-resistant extraintestinal pathogenic *Escherichia coli*, including O25b-ST131, isolated from faeces of hospitalized dogs in an Australian veterinary referral centre. J Antimicrob Chemother 68, 1025-1031.

Hacker, J., Carniel, E., 2001, Ecological fitness, genomic islands and bacterial pathogenicity. A Darwinian view of the evolution of microbes. EMBO Rep 2, 376-381.

Halstead, F.D., Vanstone, G.L., Balakrishnan, I., 2012, An evaluation of the Mast D69C AmpC Detection Disc Set for the detection of inducible and derepressed AmpC beta-lactamases. J Antimicrob Chemother 67, 2303-2304.

Hamilton, E., Kruger, J.M., Schall, W., Beal, M., Manning, S.D., Kaneene, J.B., 2013, Acquisition and persistence of antimicrobial-resistant bacteria isolated from dogs and cats admitted to a veterinary teaching hospital. J Am Vet Med Assoc 243, 990-1000.

Hanselman, B.A., Kruth, S., Weese, J.S., 2008, Methicillin-resistant staphylococcal colonization in dogs entering a veterinary teaching hospital. Vet Microbiol 126, 277-281.

Hanselman, B.A., Kruth, S.A., Rousseau, J., Weese, J.S., 2009, Coagulase positive staphylococcal colonization of humans and their household pets. Can Vet J 50, 954-958.

Hanssen, A. M., Ericson Sollid, J. U., 2006, SCC*mec* in staphylococci: genes on the move. FEMS Immunol Med Microbiol, 46, 8-20.

Harada, K., Morimoto, E., Kataoka, Y., Takahashi, T., 2011, Clonal spread of antimicrobial-resistant *Escherichia coli* isolates among pups in two kennels. Acta Vet Scand 53, 11.

Hartman, B.J., Tomasz, A., 1984, Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. J Bacteriol 158, 513-516.

Hartmann, F.A., White, D.G., West, S.E., Walker, R.D., Deboer, D.J., 2005, Molecular characterization of *Staphylococcus intermedius* carriage by healthy dogs and comparison of antimicrobial susceptibility patterns to isolates from dogs with pyoderma. Vet Microbiol 108, 119-131.

Hauschild, T., Wojcik, A., 2007, Species distribution and properties of staphylococci from canine dermatitis. Res Vet Sci, 82, 1-6.

Hawkey, P.M., Jones, A.M., 2009, The changing epidemiology of resistance. J Antimicrob Chemother 64 Suppl 1, i3-10.

Heikens, E., Fleer, A., Paauw, A., Florijn, A., Fluit, A.C., 2005, Comparison of genotypic and phenotypic methods for species-level identification of clinical isolates of coagulase-negative staphylococci. J Clin Microbiol 43, 2286-2290.

Heimdahl, A., Kager, L., Nord, C.E., 1985, Changes in the oropharyngeal and colon microflora in relation to antimicrobial concentrations in saliva and faeces. Scand J Infect Dis Suppl 44, 52-58.

Hiramatsu, K., Cui, L., Kuroda, M., Ito, T., 2001, The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. Trends Microbiol 9, 486-493.

Hoekstra, K. A., Paulton, R. J., 2002, Clinical prevalence and antimicrobial susceptibility of *Staphylococcus aureus* and *Staph. intermedius* in dogs. J Appl Microbiol, 93, 406-413.

Holden, M.T., Feil, E.J., Lindsay, J.A., Peacock, S.J., Day, N.P., Enright, M.C., Foster, T.J., Moore, C.E., Hurst, L., Atkin, R., Barron, A., Bason, N., Bentley, S.D., Chillingworth, C., Chillingworth, T., Churcher, C., Clark, L., Corton, C., Cronin, A., Doggett, J., Dowd, L., Feltwell, T., Hance, Z., Harris, B., Hauser, H., Holroyd, S., Jagels, K., James, K.D., Lennard, N., Line, A., Mayes, R., Moule, S., Mungall, K., Ormond, D., Quail, M.A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Sharp, S., Simmonds, M., Stevens, K., Whitehead, S., Barrell, B.G., Spratt, B.G., Parkhill, J., 2004, Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc Natl Acad Sci U S A 101, 9786-9791.

Hooper, D.C., 2001, Emerging mechanisms of fluoroquinolone resistance. Emerg Infect Dis 7, 337-341.

Hopkins, K.L., Batchelor, M.J., Liebana, E., Deheer-Graham, A.P., Threlfall, E.J., 2006, Characterisation of CTX-M and AmpC genes in human isolates of *Escherichia coli* identified between 1995 and 2003 in England and Wales. Int J Antimicrob Agents 28, 180-192.

Horcajada, J.P., Vila, J., Moreno-Martinez, A., Ruiz, J., Martinez, J.A., Sanchez, M., Soriano, E., Mensa, J., 2002, Molecular epidemiology and evolution of resistance to quinolones in *Escherichia coli* after prolonged administration of ciprofloxacin in patients with prostatitis. J Antimicrob Chemother 49, 55-59.

Hordijk, J., Schoormans, A., Kwakernaak, M., Duim, B., Broens, E., Dierikx, C., Mevius, D., Wagenaar, J.A., 2013, High prevalence of fecal carriage of extended spectrum beta-lactamase/AmpC-producing Enterobacteriaceae in cats and dogs. Front Microbiol 4, 242.

Horton, R.A., Randall, L.P., Snary, E.L., Cockrem, H., Lotz, S., Wearing, H., Duncan, D., Rabie, A., McLaren, I., Watson, E., La Ragione, R.M., Coldham, N.G., 2011, Fecal carriage and shedding density of CTX-M extended-spectrum {beta}-lactamase-producing *Escherichia coli* in cattle, chickens, and pigs: implications for environmental contamination and food production. Appl Environ Microbiol 77, 3715-3719.

Hryniewicz, W., 1999, Epidemiology of MRSA. Infection, 27, Suppl 2, S13-16.

Huber, H., Ziegler, D., Pfluger, V., Vogel, G., Zweifel, C., Stephan, R., 2011, Prevalence and characteristics of methicillin-resistant coagulase-negative staphylococci from livestock, chicken carcasses, bulk tank milk, minced meat, and contact persons. BMC Vet Res 7, 6.

Hudault, S., Guignot, J., Servin, A. L. 2001, *Escherichia coli* strains colonising the gastrointestinal tract protect germfree mice against *Salmonella typhimurium* infection. Gut, 49, 47-55.

Huebner, J., Goldmann, D.A., 1999, Coagulase-negative staphylococci: role as pathogens. Annu Rev Med 50, 223-236.

Huerta, B., Maldonado, A., Ginel, P.J., Tarradas, C., Gomez-Gascon, L., Astorga, R.J., Luque, I., 2011, Risk factors associated with the antimicrobial resistance of staphylococci in canine pyoderma. Vet Microbiol 150, 302-308.

Hughes, L.A., Williams, N., Clegg, P., Callaby, R., Nuttall, T., Coyne, K., Pinchbeck, G., Dawson, S., 2012, Cross-sectional survey of antimicrobial prescribing patterns in UK small animal veterinary practice. Prev Vet Med 104, 309-316.

Hunter, P.A., Dawson, S., French, G.L., Goossens, H., Hawkey, P.M., Kuijper, E.J., Nathwani, D., Taylor, D.J., Teale, C.J., Warren, R.E., Wilcox, M.H., Woodford, N., Wulf, M.W., Piddock, L.J., 2010, Antimicrobial-resistant pathogens in animals and man: prescribing, practices and policies. J Antimicrob Chemother 65 Suppl 1, i3-17.

Huttner, A., Harbarth, S., Carlet, J., Cosgrove, S., Goossens, H., Holmes, A., Jarlier, V., Voss, A., Pittet, D., 2013, Antimicrobial resistance: a global view from the 2013 World Healthcare-Associated Infections Forum. Antimicrob Resist Infect Control 2, 31.

Ihrke, P. J., 1987, An overview of bacterial skin disease in the dog. Br Vet J, 143, 112-118.

Ihrke, P.J., Papich, M.G., DeManuelle, T.C., 1999, The use of fluoroquinolones in veterinary dermatology. Vet Dermatol, 10, 193-204.

Isaacs, R.D., Kunke, P.J., Cohen, R.L., Smith, J.W., 1988, Ciprofloxacin resistance in epidemic methicillin-resistant *Staphylococcus aureus*. Lancet 2, 843.

Ishihara, K., Shimokubo, N., Sakagami, A., Ueno, H., Muramatsu, Y., Kadosawa, T., Yanagisawa, C., Hanaki, H., Nakajima, C., Suzuki, Y., Tamura, Y., 2010, Occurrence and molecular characteristics of methicillin-resistant Staphylococcus aureus and

methicillin-resistant Staphylococcus *pseudintermedius* in an academic veterinary hospital. Appl Environ Microbiol, 76, 5165-5174.

Jacoby, G.A., 2009, AmpC beta-lactamases. Clin Microbiol Rev 22, 161-182.

Jarlov, J.O., Hojbjerg, T., Busch-Sorensen, C., Scheibel, J., Moller, J.K., Kolmos, H.J., Wandall, D.A., 1996, Coagulase-negative staphylococci in Danish blood cultures: species distribution and antibiotic susceptibility. J Hosp Infect 32, 217-227.

Jaureguy, F., Landraud, L., Passet, V., Diancourt, L., Frapy, E., Guigon, G., Carbonnelle, E., Lortholary, O., Clermont, O., Denamur, E., Picard, B., Nassif, X., Brisse, S., 2008, Phylogenetic and genomic diversity of human bacteremic *Escherichia coli* strains. BMC Genomics 9, 560.

Jernberg, C., Lofmark, S., Edlund, C., Jansson, J. K., 2007, Long-term ecological impacts of antibiotic administration on the human intestinal microbiota, 1, 56-66.

Jernberg, C., Lofmark, S., Edlund, C., Jansson, J.K., 2010, Long-term impacts of antibiotic exposure on the human intestinal microbiota. Microbiology 156, 3216-3223.

Jiang, Y., Zhou, Z., Qian, Y., Wei, Z., Yu, Y., Hu, S., Li, L., 2008, Plasmid-mediated quinolone resistance determinants qnr and aac(6')-Ib-cr in extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in China. J Antimicrob Chemother 61, 1003-1006.

Johns, I., Verheyen, K., Good, L., Rycroft, A., 2012, Antimicrobial resistance in faecal *Escherichia coli* isolates from horses treated with antimicrobials: a longitudinal study in hospitalised and non-hospitalised horses. Vet Microbiol 159, 381-389.

Johnson, J.R., Kuskowski, M.A., Owens, K., Clabots, C., Singer, R.S., 2009, Virulence genotypes and phylogenetic background of fluoroquinolone-resistant and susceptible *Escherichia coli* urine isolates from dogs with urinary tract infection. Vet Microbiol 136, 108-114.

Johnson, J. R., Kuskowski, M. A., Owens, K., Gajewski, A., Winokur, P. L., 2003, Phylogenetic origin and virulence genotype in relation to resistance to fluoroquinolones and/or extended-spectrum cephalosporins and cephamycins among *Escherichia coli* isolates from animals and humans. J Infect Dis, 188, 759-768.

Johnson, J.R., Owens, K., Gajewski, A., Clabots, C., 2008, *Escherichia coli* colonization patterns among human household members and pets, with attention to acute urinary tract infection. J Infect Dis 197, 218-224.

Johnson, J.R., Russo, T.A., 2002, Extraintestinal pathogenic *Escherichia coli*: "the other bad E coli". J Lab Clin Med 139, 155-162.

Johnson, J.R., Sannes, M.R., Croy, C., Johnston, B., Clabots, C., Kuskowski, M.A., Bender, J., Smith, K.E., Winokur, P.L., Belongia, E.A., 2007, Antimicrobial drug-resistant

Escherichia coli from humans and poultry products, Minnesota and Wisconsin, 2002-2004. Emerg Infect Dis 13, 838-846.

Johnson, J. R., Stell, A. L., 2000, Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J Infect Dis, 181, 261-272.

Jones, R.D., Kania, S.A., Rohrbach, B.W., Frank, L.A., Bemis, D.A., 2007, Prevalence of oxacillin- and multidrug-resistant staphylococci in clinical samples from dogs: 1,772 samples (2001-2005). J Am Vet Med Assoc 230, 221-227.

Kania, S.A., Williamson, N.L., Frank, L.A., Wilkes, R.P., Jones, R.D., Bemis, D.A., 2004, Methicillin resistance of staphylococci isolated from the skin of dogs with pyoderma. Am J Vet Res 65, 1265-1268.

Karakulska, J., Fijalkowski, K., Nawrotek, P., Pobucewicz, A., Poszumski, F., Czernomysy-Furowicz, D., 2012, Identification and methicillin resistance of coagulase-negative staphylococci isolated from nasal cavity of healthy horses. J Microbiol, 50, 444-451.

Karami, N., Hannoun, C., Adlerberth, I., Wold, A.E., 2008, Colonization dynamics of ampicillin-resistant *Escherichia coli* in the infantile colonic microbiota. J Antimicrob Chemother 62, 703-708.

Karczmarczyk, M., Abbott, Y., Walsh, C., Leonard, N., Fanning, S., 2011, Characterization of multidrug-resistant *Escherichia coli* isolates from animals presenting at a university veterinary hospital. Appl Environ Microbiol 77, 7104-71012.

Katouli, M., 2010, Population structure of gut *Escherichia coli* and its role in development of extra-intestinal infections. Iran J Microbiol, 2, 59-72.

Kawakami, T., Shibata, S., Murayama, N., Nagata, M., Nishifuji, K., Iwasaki, T., Fukata, T., 2010, Antimicrobial susceptibility and methicillin resistance in *Staphylococcus pseudintermedius* and *Staphylococcus schleiferi* subsp. *coagulans* isolated from dogs with pyoderma in Japan. J Vet Med Sci 72, 1615-1619.

Kempker, R., Mangalat, D., Kongphet-Tran, T., Eaton, M., 2009, Beware of the pet dog: a case of Staphylococcus intermedius infection. Am J Med Sci 338, 425-427.

Kern, A., Perreten, V., 2013, Clinical and molecular features of methicillin-resistant, coagulase-negative staphylococci of pets and horses. J Antimicrob Chemother 68, 1256-1266.

Kloos, W.E., 1980, Natural populations of the genus Staphylococcus. Annu Rev Microbiol 34, 559-592.

Kloos, W.E., Bannerman, T.L., 1994, Update on clinical significance of coagulase-negative staphylococci. Clin Microbiol Rev 7, 117-140.

Kondo, Y., Ito, T., Ma, X. X., Watanabe, S., Kreiswirth, B. N., Etienne, J. Hiramatsu, K., 2007, Combination of multiplex PCRs for staphylococcal cassette chromosome mec

type assignment: rapid identification system for mec, ccr, and major differences in junkyard regions. Antimicrob Agents Chemother, 51, 264-274.

Kong, H.H., Segre, J.A., 2012, Skin microbiome: looking back to move forward. J Invest Dermatol 132, 933-939.

Kottler, S., Middleton, J.R., Perry, J., Weese, J.S., Cohn, L.A., 2010, Prevalence of Staphylococcus aureus and methicillin-resistant *Staphylococcus aureus* carriage in three populations. J Vet Intern Med 24, 132-139.

Kruse, H., Hofshagen, M., Thoresen, S. I., Bredal, W. P., Vollset, I., Soli, N. E., 1996, The antimicrobial susceptibility of Staphylococcus species isolated from canine dermatitis. Vet Res Commun, 20, 205-14.

Laarhoven, L.M., de Heus, P., van Luijn, J., Duim, B., Wagenaar, J.A., van Duijkeren, E., 2011, Longitudinal study on methicillin-resistant *Staphylococcus pseudintermedius* in households. PLoS One 6, e27788.

Landis, J.R., Koch, G.G., 1977, Measurement of observer agreement for categorical data. Biometrics 33, 159-174.

Lawrence, M., Kukanich, K., Kukanich, B., Heinrich, E., Coetzee, J.F., Grauer, G., Narayanan, S., 2013, Effect of cefovecin on the fecal flora of healthy dogs. Vet J 198, 259-266.

Lebeaux, D., Barbier, F., Angebault, C., Benmahdi, L., Ruppe, E., Felix, B., Gaillard, K., Djossou, F., Epelboin, L., Dupont, C., Renard, M., Peroz, G., Vandenesch, F., Wolff, M., Andremont, A., Ruimy, R., 2012, Evolution of nasal carriage of methicillin-resistant coagulase-negative staphylococci in a remote population. Antimicrob Agents Chemother, 56, 315-323.

Lee, S.M., Ender, M., Adhikari, R., Smith, J.M., Berger-Bachi, B., Cook, G.M., 2007, Fitness cost of staphylococcal cassette chromosome mec in methicillin-resistant *Staphylococcus aureus* by way of continuous culture. Antimicrob Agents Chemother 51, 1497-1499.

Lefebvre, S.L., Weese, J.S., 2009, Contamination of pet therapy dogs with MRSA and *Clostridium difficile*. J Hosp Infect 72, 268-269.

Lehner, G., Linek, M., Bond, R., Lloyd, D.H., Prenger-Berninghoff, E., Thom, N., Straube, I., Verheyen, K., Loeffler, A., 2014, Case-control risk factor study of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) infection in dogs and cats in Germany. Vet Microbiol 168, 154-160.

Leigh, D. A., 1981, Antibacterial activity and pharmacokinetics of clindamycin. J Antimicrob Chemother, 7, Suppl A, 3-9.

Lenski, R.E., 1998, Bacterial evolution and the cost of antibiotic resistance. Int Microbiol 1, 265-270.

Lester, C. H., Frimodt-Moller, N., Sorensen, T. L., Monnet, D. L., Hammerum, A. M., 2006, In vivo transfer of the *van*A resistance gene from an *Enterococcus faecium* isolate of animal origin to an *E. faecium* isolate of human origin in the intestines of human volunteers. Antimicrob Agents Chemother. 50, 596-599.

Levy, S. B., Marshall, B., Schluederberg, S., Rowse, D., Davis, J., 1988, High frequency of antimicrobial resistance in human fecal flora. Antimicrob Agents Chemother, 32, 1801-1806.

Li, X.Z., Mehrotra, M., Ghimire, S., Adewoye, L., 2007, beta-Lactam resistance and beta-lactamases in bacteria of animal origin. Vet Microbiol 121, 197-214.

Liebana, E., Batchelor, M., Hopkins, K. L., Clifton-Hadley, F. A., Teale, C. J., Foster, A., Barker, L., Threlfall, E. J., Davies, R. H., 2006, Longitudinal farm study of extended-spectrum beta-lactamase-mediated resistance. J Clin Microbiol, 44, 1630-1634.

Lindsay, J.A., Holden, M.T., 2006, Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. Funct Integr Genomics 6, 186-201.

Livermore, D.M., Hawkey, P.M., 2005, CTX-M: changing the face of ESBLs in the UK. J Antimicrob Chemother 56, 451-454.

Lloyd, D.H., Lamport, A.I., Feeney, C., 1996, Sensitivity to antibiotics amongst cutaneous and mucosal isolates of canine pathogenic staphylococci in the UK, 1980–96. Vet Dermatol, 7, 171-175.

Loeffler, A., Boag, A. K., Sung, J., Lindsay, J. A., Guardabassi, L., Dalsgaard, A., Smith, H., Stevens, K. B., Lloyd, D. H., 2005, Prevalence of methicillin-resistant *Staphylococcus aureus* among staff and pets in a small animal referral hospital in the UK. J Antimicrob Chemother, 56, 692-697.

Loeffler, A., Linek, M., Moodley, A., Guardabassi, L., Sung, J. M., Winkler, M., Weiss, R., Lloyd, D. H., 2007, First report of multiresistant, mecA-positive *Staphylococcus intermedius* in Europe: 12 cases from a veterinary dermatology referral clinic in Germany. Vet Dermatol, 18, 412-421.

Loeffler, A., Pfeiffer, D. U., Lloyd, D. H., Smith, H., Soares-Magalhaes, R., Lindsay, J. A., 2010, Meticillin-resistant *Staphylococcus aureus* carriage in UK veterinary staff and owners of infected pets: new risk groups. J Hosp Infect, 74, 282-288.

Lofmark, S., Jernberg, C., Billstrom, H., Andersson, D.I., Edlund, C., 2008, Restored fitness leads to long-term persistence of resistant *Bacteroides* strains in the human intestine. Anaerobe 14, 157-160.

Lofmark, S., Jernberg, C., Jansson, J. K., Edlund, C., 2006, Clindamycin-induced enrichment and long-term persistence of resistant *Bacteroides* spp. and resistance genes. J Antimicrob Chemother, 58, 1160-1167.

M'Zali, F.H., Chanawong, A., Kerr, K.G., Birkenhead, D., Hawkey, P.M., 2000, Detection of extended-spectrum beta-lactamases in members of the family enterobacteriaceae: comparison of the MAST DD test, the double disc and the Etest ESBL. J Antimicrob Chemother 45, 881-885.

Maddox, T.W., 2010. Longitudinal Study of Shedding of Antimicrobial Resistant *Escherichia coli* in Horses in the Community and Recently Discharged from Hospital. University of Liverpool, Liverpool.

Magiorakos, A.P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T., Monnet, D. L., 2012. Multidrug resistant, extensively drug resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 18, 268-281.

Mainous, A.G., 3rd, Hueston, W.J., Everett, C.J., Diaz, V.A., 2006, Nasal carriage of *Staphylococcus aureus* and methicillin-resistant *S. aureus* in the United States, 2001-2002. Ann Fam Med 4, 132-137.

Malik, S., Coombs, G.W., O'Brien, F.G., Peng, H., Barton, M.D., 2006, Molecular typing of methicillin-resistant staphylococci isolated from cats and dogs. J Antimicrob Chemother 58, 428-431.

Malinowski, E., Lassa, H., Klossowska, A., Smulski, S., Kaczmarowski, M., 2009, Atypical *Staphylococcus aureus* as an Aetiological Agent of Mastitis in Cows. Bull Vet Inst Pulawy 53, 383-387.

Mamber, S.W., Kolek, B., Brookshire, K.W., Bonner, D.P., Fung-Tomc, J., 1993, Activity of quinolones in the Ames Salmonella TA102 mutagenicity test and other bacterial genotoxicity assays. Antimicrob Agents Chemother 37, 213-217.

Martindale, J., Stroud, D., Moxon, E.R., Tang, C.M., 2000, Genetic analysis of *Escherichia coli* K1 gastrointestinal colonization. Mol Microbiol 37, 1293-1305.

Mason, I.S., Lloyd, D.H., 1989, The role of allergy in the development of canine pyoderma. J Small Anim Pract, 30, 216-218.

Mason, I.S., Mason, K.V., Lloyd, D.H., 1996, A review of the biology of canine skin with respect to the commensals *Staphylococcus intermedius*, *Demodex canis* and *Malassezia pachydermatis.* Vet Dermatol, 7, 119-132.

Mateus, A., Brodbelt, D. C., Barber, N., Stark, K. D., 2011, Antimicrobial usage in dogs and cats in first opinion veterinary practices in the UK. J Small Anim Pract, 52, 515-521.

Maule, A., 2000, Survival of verocytotoxigenic *Escherichia coli* O157 in soil, water and on surfaces. Symp Ser Soc Appl Microbiol, 71S-78S.

May, E.R., Hnilica, K.A., Frank, L.A., Jones, R.D., Bemis, D.A., 2005, Isolation of *Staphylococcus schleiferi* from healthy dogs and dogs with otitis, pyoderma, or both. J Am Vet Med Assoc 227, 928-931.

McDaniels, A.E., Rice, E.W., Reyes, A.L., Johnson, C.H., Haugland, R.A., Stelma, G.N., Jr., 1996, Confirmational identification of *Escherichia coli*, a comparison of genotypic and phenotypic assays for glutamate decarboxylase and beta-D-glucuronidase. Appl Environ Microbiol 62, 3350-3354.

Medleau, L., Long, R. E., Brown, J., Miller, W. H., 1986, Frequency and antimicrobial susceptibility of *Staphylococcus* species isolated from canine pyodermas. Am J Vet Res, 47, 229-231.

Mehrotra, M., Wang, G., Johnson, W. M., 2000, Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. J Clin Microbiol, 38, 1032-1035.

Mentula, S., Harmoinen, J., Heikkila, M., Westermarck, E., Rautio, M., Huovinen, P., Kononen, E., 2005, Comparison between cultured small-intestinal and fecal microbiotas in beagle dogs. Appl Environ Microbiol 71, 4169-4175.

Miller, L. G., Diep, B. A., 2008, Clinical practice: colonization, fomites, and virulence: rethinking the pathogenesis of community-associated methicillin-resistant *Staphylococcus aureus* infection. Clin Infect Dis, 46, 752-760.

Moissenet, D., Salauze, B., Clermont, O., Bingen, E., Arlet, G., Denamur, E., Merens, A., Mitanchez, D., Vu-Thien, H., 2010, Meningitis caused by *Escherichia coli* producing TEM-52 extended-spectrum beta-lactamase within an extensive outbreak in a neonatal ward: epidemiological investigation and characterization of the strain. J Clin Microbiol, 48, 2459-2463.

Moodley, A., Guardabassi, L., 2009, Clonal spread of methicillin-resistant coagulase-negative staphylococci among horses, personnel and environmental sites at equine facilities. Vet Microbiol, 137, 397-401.

Moon, B. Y., Youn, J. H., Shin, S., Hwang, S. Y., Park, Y. H., 2012, Genetic and phenotypic characterization of methicillin-resistant staphylococci isolated from veterinary hospitals in South Korea. J Vet Diagn Invest, 24, 489-498.

Moran, E., Masters, S., Berendt, A. R., McLardy-Smith, P., Byren, I., Atkins, B. L., 2007, Guiding empirical antibiotic therapy in orthopaedics: The microbiology of prosthetic joint infection managed by debridement, irrigation and prosthesis retention, 55, 1-7.

Moreno, A., Bello, H., Guggiana, D., Dominguez, M., Gonzalez, G., 2008, Extended-spectrum beta-lactamases belonging to CTX-M group produced by *Escherichia coli* strains isolated from companion animals treated with enrofloxacin. Vet Microbiol 129, 203-208.

Morosini, M. I., Ayala, J. A., Baquero, F., Martinez, J. L., Blazquez, J., 2000, Biological cost of AmpC production for Salmonella enterica serotype Typhimurium. Antimicrob Agents Chemother, 44, 3137-3143.

Morris, D. O., Boston, R. C., O'Shea, K., Rankin, S. C., 2010, The prevalence of carriage of meticillin-resistant staphylococci by veterinary dermatology practice staff and their respective pets. Vet Dermatol, 21, 400-407.

Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H., Watanabe, S., 1991, [Detection of methicillin-resistant *Staphylococcus aureus* by polymerase chain reaction]. Rinsho Byori 39, 1325-1330.

Murphy, C., Reid-Smith, R.J., Prescott, J.F., Bonnett, B.N., Poppe, C., Boerlin, P., Weese, J.S., Janecko, N., McEwen, S.A., 2009, Occurrence of antimicrobial resistant bacteria in healthy dogs and cats presented to private veterinary hospitals in southern Ontario: A preliminary study. Can Vet J 50, 1047-1053.

Nienhoff, U., Kadlec, K., Chaberny, I.F., Verspohl, J., Gerlach, G.F., Kreienbrock, L., Schwarz, S., Simon, D., Nolte, I., 2011, Methicillin-resistant *Staphylococcus pseudintermedius* among dogs admitted to a small animal hospital. Vet Microbiol 150, 191-197.

NOAH 2014. NOAH. 2014. National Office of Animal Health Compendium of Data Sheets for Animal Medicines.

Nowrouzian, F., Adlerberth, I., Wold, A. E., 2001, P fimbriae, capsule and aerobactin characterize colonic resident *Escherichia coli.* Epidemiol Infect, 126, 11-18.

Nowrouzian, F.L., Adlerberth, I., Wold, A.E., 2006, Enhanced persistence in the colonic microbiota of *Escherichia coli* strains belonging to phylogenetic group B2: role of virulence factors and adherence to colonic cells. Microbes Infect 8, 834-840.

Nyberg, S.D., Osterblad, M., Hakanen, A.J., Lofmark, S., Edlund, C., Huovinen, P., Jalava, J., 2007, Long-term antimicrobial resistance in *Escherichia coli* from human intestinal microbiota after administration of clindamycin. Scand J Infect Dis 39, 514-520.

O'Brien, C.L., Gordon, D.M., 2011, Effect of diet and gut dynamics on the establishment and persistence of *Escherichia coli*. Microbiology 157, 1375-1384.

O'Keefe, A., Hutton, T.A., Schifferli, D.M., Rankin, S.C., 2010, First detection of CTX-M and SHV extended-spectrum beta-lactamases in *Escherichia coli* urinary tract isolates from dogs and cats in the United States. Antimicrob Agents Chemother 54, 3489-3492.

Obaid, I.A., Udo, E.E., Jacob, L.E., Jonny, M., 1999, Isolation and Characterisation of Coagulase-negative Meticillin-resistant *Staphylococcus aureus* from Patients in an Intensive Care Unit. Med Principles Pract 8, 230-236.

Ogeer-Gyles, J., Mathews, K.A., Sears, W., Prescott, J.F., Weese, J.S., Boerlin, P., 2006, Development of antimicrobial drug resistance in rectal *Escherichia coli* isolates from dogs hospitalized in an intensive care unit. J Am Vet Med Assoc 229, 694-699.

Olivry, T., Hill, P. B., 2001, The ACVD task force on canine atopic dermatitis (VIII): is the epidermal lipid barrier defective? Vet Immunol Immunopathol, 81, 215-218.

Onuma, K., Tanabe, T., Sato, H., 2012, Antimicrobial resistance of *Staphylococcus pseudintermedius* isolates from healthy dogs and dogs affected with pyoderma in Japan. Vet Dermatol 23, 17-22, e15.

Park, B., Iwase, T., Liu, G.Y., 2011, Intranasal application of *S. epidermidis* prevents colonization by methicillin-resistant *Staphylococcus aureus* in mice. PLoS One 6, e25880.

Paterson, D.L., Bonomo, R.A., 2005, Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev 18, 657-686.

Paul, N.C., Bargman, S.C., Moodley, A., Nielsen, S.S., Guardabassi, L., 2012, *Staphylococcus pseudintermedius* colonisation patterns and strain diversity in healthy dogs: a cross-sectional and longitudinal study. Vet Microbiol 160, 420-427.

Paul, N.C., Moodley, A., Ghibaudo, G., Guardabassi, L., 2011, Carriage of methicillin-resistant *Staphylococcus pseudintermedius* in small animal veterinarians: indirect evidence of zoonotic transmission. Zoonoses Public Health 58, 533-539.

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., van den Brandt, P. A., Stobberingh, E. E., 2006, Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics, 118, 511-521.

Perez-Perez, F.J., Hanson, N.D., 2002, Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol 40, 2153-2162.

Perreten, V., Chanchaithong, P., Prapasarakul, N., Rossano, A., Blum, S.E., Elad, D., Schwendener, S., 2013, Novel pseudo-staphylococcal cassette chromosome mec element (psiSCCmec57395) in methicillin-resistant *Staphylococcus pseudintermedius* CC45. Antimicrob Agents Chemother 57, 5509-5515.

Perreten, V., Kadlec, K., Schwarz, S., Gronlund Andersson, U., Finn, M., Greko, C., Moodley, A., Kania, S.A., Frank, L.A., Bemis, D.A., Franco, A., Iurescia, M., Battisti, A., Duim, B., Wagenaar, J.A., van Duijkeren, E., Weese, J.S., Fitzgerald, J.R., Rossano, A., Guardabassi, L., 2010, Clonal spread of methicillin-resistant *Staphylococcus pseudintermedius* in Europe and North America: an international multicentre study. J Antimicrob Chemother 65, 1145-1154.

Pfaller, M. A., Sader, H. S., Jones, R. N., 2007, Evaluation of the in vitro activity of daptomycin against 19615 clinical isolates of Gram-positive cocci collected in North American hospitals (2002-2005). Diagn Microbiol Infect Dis, 57, 459-465.

Picard, B., Garcia, J.S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., Elion, J., Denamur, E., 1999, The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. Infect Immun 67, 546-553.

Piddock, L.J., 2006, Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol 4, 629-636.

Pinchbeck, L. R., Cole, L. K., Hillier, A., Kowalski, J. J., Rajala-Schultz, P. J., Bannerman, T. L., York, S., 2006, Genotypic relatedness of staphylococcal strains isolated from pustules and carriage sites in dogs with superficial bacterial folliculitis. Am J Vet Res, 67, 1337-1346.

Platell, J. L., Cobbold, R. N., Johnson, J. R., Trott, D. J., 2010, Clonal group distribution of fluoroquinolone-resistant *Escherichia coli* among humans and companion animals in Australia. J Antimicrob Chemother, 65, 1936-1938.

Platell, J.L., Trott, D.J., Wetzstein, H.G., Leitner, M., Cobbold, R.N., 2011, Phylogenetic Grouping, Antibiotic Resistance Profile, Fluoroquinolone Susceptibility and ST131 Status of Canine Extra intestinal *Escherichia Coli* Isolated from Submissions to a Veterinary Diagnostic Laboratory 2005-08. J Veterinar Sci Technol S6, 1-8.

Pomba, C., da Fonseca, J. D., Baptista, B. C., Correia, J. D., Martinez-Martinez, L., 2009, Detection of the pandemic O25-ST131 human virulent *Escherichia coli* CTX-M-15-producing clone harboring the qnrB2 and aac(6')-Ib-cr genes in a dog. Antimicrob Agents Chemother, 53, 327-328.

Poulsen, L.K., Lan, F., Kristensen, C.S., Hobolth, P., Molin, S., Krogfelt, K.A., 1994, Spatial distribution of *Escherichia coli* in the mouse large intestine inferred from rRNA in situ hybridization. Infect Immun 62, 5191-5194.

Price, L. B., Graham, J. P., Lackey, L. G., Roess, A., Vailes, R., Silbergeld, E., 2007, Elevated risk of carrying gentamicin-resistant *Escherichia coli* among U.S. poultry workers. Environ Health Perspect, 115-1738-1742.

Procter, T.D., Pearl, D.L., Finley, R.L., Leonard, E.K., Janecko, N., Reid-Smith, R.J., Weese, J.S., Peregrine, A.S., Sargeant, J.M., 2013, A Cross-Sectional Study Examining the Prevalence and Risk Factors for Anti-Microbial-Resistant Generic *Escherichia coli* in Domestic Dogs that Frequent Dog Parks in Three Cities in South-Western Ontario, Canada. Zoonoses Public Health.

Rasko, D. A., Rosovitz, M. J., Myers, G. S., Mongodin, E. F., Fricke, W. F., Gajer, P., Crabtree, J., Sebaihia, M.,Thomson, N. R., Chaudhuri, R., Henderson, I. R., Sperandio, V., Ravel, J., 2008, The pangenome structure of *Escherichia coli*: comparative genomic analysis of *E. coli* commensal and pathogenic isolates. J Bacteriol, 190, 6881-6893.

Raum, E., Lietzau, S., von Baum, H., Marre, R., Brenner, H., 2008, Changes in *Escherichia coli* resistance patterns during and after antibiotic therapy: a longitudinal study among outpatients in Germany. Clin Microbiol Infect 14, 41-48.

Ribot, E.M., Fair, M.A., Gautom, R., Cameron, D.N., Hunter, S.B., Swaminathan, B., Barrett, T.J., 2006, Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog Dis 3, 59-67.

Rich, M., 2005, Staphylococci in animals: prevalence, identification and antimicrobial susceptibility, with an emphasis on methicillin-resistant *Staphylococcus aureus*. Br J Biomed Sci, 62, 98-105.

Rodrigues Hoffmann, A., Patterson, A. P., Diesel, A., Lawhon, S. D., Ly, H. J., Elkins Stephenson, C., Mansell, J., Steiner, J. M., Dowd, S. E., Olivry, T., Suchodolski, J. S., 2014, The skin microbiome in healthy and allergic dogs. PLoS One, 9, e83197.

Rogers, K.L., Fey, P.D., Rupp, M.E., 2009, Coagulase-negative staphylococcal infections. Infect Dis Clin North Am 23, 73-98.

Rosdahl, V.T., Rosendal, K., 1983, Correlation of penicillinase production with phage type and susceptibility to antibiotics and heavy metals in *Staphylococcus aureus*. J Med Microbiol 16, 391-399.

Rubin, J.E., Ball, K.R., Chirino-Trejo, M., 2011, Antimicrobial susceptibility of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* isolated from various animals. Can Vet J 52, 153-157.

Ruppe, E., Barbier, F., Mesli, Y., Maiga, A., Cojocaru, R., Benkhalfat, M., Benchouk, S., Hassaine, H., Maiga, I., Diallo, A., Koumare, A. K., Ouattara, K., Soumare, S., Dufourcq, J. B., Nareth, C., Sarthou, J. L., Andremont, A., Ruimy, R., 2009, Diversity of staphylococcal cassette chromosome *mec* structures in methicillin-resistant *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* strains among outpatients from four countries. Antimicrob Agents Chemother, 53, 442-449.

Russo, T. A., Johnson, J. R., 2000, Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. J Infect Dis, 181, 1753-1754.

Russo, T.A., Johnson, J.R., 2003, Medical and economic impact of extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. Microbes Infect 5, 449-456.

Sa-Leao, R., Sanches, I. S., Couto, I., Alves, C. R., de Lencastre, H., 2001, Low prevalence of methicillin-resistant strains among *Staphylococcus aureus* colonizing young and healthy members of the community in Portugal. Microb Drug Resist, 7, 237-245.

Saijonmaa-Koulumies, L.E., Lloyd, D.H., 1995, Carriage of Bacteria Antagonistic Towards *Staphylococcus intermedius* on Canine Skin and Mucosal Surfaces. Vet Dermatol 6, 187-194.

Saijonmaa-Koulumies, L.E., Lloyd, D.H., 1996, Colonization of the canine skin with bacteria. Vet Dermatol 7, 153-162.

Saijonmaa-Koulumies, L.E., Lloyd, D.H., 2002, Colonization of neonatal puppies by *Staphylococcus intermedius*. Vet Dermatol 13, 123-130.

Salgado, C.D., Farr, B.M., Calfee, D.P., 2003, Community-acquired methicillin-resistant *Staphylococcus aureus*: a meta-analysis of prevalence and risk factors. Clin Infect Dis 36, 131-139.

Sanchez, S., McCrackin Stevenson, M.A., Hudson, C.R., Maier, M., Buffington, T., Dam, Q., Maurer, J.J., 2002, Characterization of multidrug-resistant *Escherichia coli* isolates associated with nosocomial infections in dogs. J Clin Microbiol 40, 3586-3595.

Sasaki, T., Kikuchi, K., Tanaka, Y., Takahashi, N., Kamata, S., Hiramatsu, K., 2007, Reclassification of phenotypically identified *Staphylococcus intermedius* strains. J Clin Microbiol 45, 2770-2778.

Sasaki, T., Tsubakishita, S., Tanaka, Y., Sakusabe, A., Ohtsuka, M., Hirotaki, S., Kawakami, T., Fukata, T., Hiramatsu, K., 2010, Multiplex-PCR method for species identification of coagulase-positive staphylococci. J Clin Microbiol, 48, 765-769.

Sato, T., Yokota, S., Okubo, T., Ishihara, K., Ueno, H., Muramatsu, Y., Fujii, N., Tamura, Y., 2013, Contribution of the AcrAB-TolC efflux pump to high-level fluoroquinolone resistance in *Escherichia coli* isolated from dogs and humans. J Vet Med Sci 75, 407-414.

Sato, T., Yokota, S., Okubo, T., Usui, M., Fujii, N., Tamura, Y., 2014, Phylogenetic association of fluoroquinolone and cephalosporin resistance of D-O1-ST648 *Escherichia coli* carrying *bla*CMY-2 from faecal samples of dogs in Japan. J Med Microbiol 63, 263-270.

Savageau, M.A., 1983, *Escherichia coli* Habitats, Cell Types, and Molecular Mechanisms of Gene Control. Am Nat 122, 732-744.

Schlager, T.A., Hendley, J.O., Bell, A.L., Whittam, T.S., 2002, Clonal diversity of *Escherichia coli* colonizing stools and urinary tracts of young girls. Infect Immun 70, 1225-1229.

Schmidt, V.M., Williams, N.J., Pinchbeck, G., Corless, C.E., Shaw, S., McEwan, N., Dawson, S., Nuttall, T., 2014, Antimicrobial resistance and characterisation of staphylococci isolated from healthy Labrador retrievers in the United Kingdom. BMC Vet Res 10, 17.

Schrag, S. J., Perrot, V., Levin, B. R., 1997, Adaptation to the fitness costs of antibiotic resistance in *Escherichia coli*. Proc Biol Sci, 264, 1287-1291.

Sears, H.J., Brownlee, I., Uchiyama, J.K., 1950, Persistence of individual strains of *Escherichia coli* in the intestinal tract of man. J Bacteriol 59, 293-301.

Sears, H. J., Janes, H., Saloum, R., Brownlee, I., Lamoreaux, L. F., 1956, Persistence of individual strains of *Escherichia coli* in man and dog under varying conditions. J Bacteriol, 71, 370-372.

Shopsin, B., Mathema, B., Martinez, J., Ha, E., Campo, M. L., Fierman, A., Krasinski, K., Kornblum, J., Alcabes, P., Waddington, M., Riehman, M., Kreiswirth, B. N., 2000, Prevalence of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* in the community. J Infect Dis, 182, 359-162.

Sidjabat, H.E., Townsend, K.M., Hanson, N.D., Bell, J.M., Stokes, H.W., Gobius, K.S., Moss, S.M., Trott, D.J., 2006, Identification of bla(CMY-7) and associated plasmid-mediated resistance genes in multidrug-resistant *Escherichia coli* isolated from dogs at a veterinary teaching hospital in Australia. J Antimicrob Chemother 57, 840-848.

Silbergeld, E. K., Graham, J., Price, L. B., 2008, Industrial food animal production, antimicrobial resistance, and human health. Annu Rev Public Health, 29, 151-169.

Silva, F. R., Mattos, E. M., Coimbra, M. V., Ferreira-Carvalho, B. T., Figueiredo, A. M., 2001, Isolation and molecular characterization of methicillin-resistant coagulase-negative staphylococci from nasal flora of healthy humans at three community institutions in Rio de Janeiro City. Epidemiol Infect, 127, 57-62.

Singer, R.S., Patterson, S.K., Wallace, R.L., 2008, Effects of therapeutic ceftiofur administration to dairy cattle on *Escherichia coli* dynamics in the intestinal tract. Appl Environ Microbiol 74, 6956-6962.

Singh, A., Walker, M., Rousseau, J., Monteith, G.J., Weese, J.S., 2013, Methicillin-resistant staphylococcal contamination of clothing worn by personnel in a veterinary teaching hospital. Vet Surg 42, 643-648.

Skurnik, D., Ruimy, R., Andremont, A., Amorin, C., Rouquet, P., Picard, B., Denamur, E., 2006. Effect of human vicinity on antimicrobial resistance and integrons in animal faecal *Escherichia coli*. J Antimicrob Chemother 57, 1215-1219.

Smyth, D.S., Wong, A., Robinson, D.A., 2011, Cross-species spread of SCC*mec* IV subtypes in staphylococci. Infect Genet Evol 11, 446-453.

Soares Magalhaes, R.J., Loeffler, A., Lindsay, J., Rich, M., Roberts, L., Smith, H., Lloyd, D.H., Pfeiffer, D.U., 2010, Risk factors for methicillin-resistant *Staphylococcus aureus* (MRSA) infection in dogs and cats: a case-control study. Vet Res 41, 55.

Stecher, B., Hardt, W.D., 2008, The role of microbiota in infectious disease. Trends Microbiol 16, 107-114.

Steen, S. I., Webb, P. J., 2007, Extended-spectrum beta-lactamase-producing bacteria isolated from companion animals. Vet Rec, 161, 703.

Stegemann, M.R., Sherington, J., Blanchflower, S., 2006, Pharmacokinetics and pharmacodynamics of cefovecin in dogs. J Vet Pharmacol Ther 29, 501-511.

Stenske, K.A., Bemis, D.A., Gillespie, B.E., D'Souza, D.H., Oliver, S.P., Draughon, F.A., Matteson, K.J., Bartges, J.W., 2009, Comparison of clonal relatedness and antimicrobial susceptibility of fecal *Escherichia coli* from healthy dogs and their owners. Am J Vet Res 70, 1108-1116.

Strahilevitz, J., Hooper, D.C., 2005, Dual targeting of topoisomerase IV and gyrase to reduce mutant selection: direct testing of the paradigm by using WCK-1734, a new fluoroquinolone, and ciprofloxacin. Antimicrob Agents Chemother 49, 1949-1956.

Strahilevitz, J., Jacoby, G. A., Hooper, D. C., Robicsek, A., 2009, Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev, 22, 664-689.

Sullivan, A., Edlund, C., Nord, C.E., 2001, Effect of antimicrobial agents on the ecological balance of human microflora. Lancet Infect Dis 1, 101-114.

Summers, J.F., Brodbelt, D.C., Forsythe, P.J., Loeffler, A., Hendricks, A., 2012, The effectiveness of systemic antimicrobial treatment in canine superficial and deep pyoderma: a systematic review. Vet Dermatol 23, 305-329, e361.

Suzuki, E., Hiramatsu, K., Yokota, T., 1992, Survey of methicillin-resistant clinical strains of coagulase-negative staphylococci for *mec*A gene distribution. Antimicrob Agents Chemother, 36, 429-434.

Szabados, F., Woloszyn, J., Richter, C., Kaase, M., Gatermann, S., 2010, Identification of molecularly defined *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization time of flight mass spectrometry and the Biotyper 2.0 database. J Med Microbiol, 59, 787-90.

Tamang, M. D., Nam, H. M., Jang, G. C., Kim, S. R., Chae, M. H., Jung, S. C., Byun, J. W., Park, Y. H., Lim, S. K., 2012, Molecular characterization of extended-spectrum-beta-lactamase-producing and plasmid-mediated AmpC beta-lactamase-producing *Escherichia coli* isolated from stray dogs in South Korea. Antimicrob Agents Chemother, 56, 2705-2712.

Tenaillon, O., Skurnik, D., Picard, B., Denamur, E., 2010, The population genetics of commensal *Escherichia coli*. Nat Rev Microbiol 8, 207-217.

Tenover, F.C., 2006, Mechanisms of antimicrobial resistance in bacteria. Am J Med 119, S3-10; discussion S62-70.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H., Swaminathan, B., 1995, Interpreting chromosomal DNA restriction patterns produced

by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33, 2233-2239.

Tenson, T., Lovmar, M., Ehrenberg, M., 2003, The mechanism of action of macrolides, lincosamides and streptogramin B reveals the nascent peptide exit path in the ribosome. J Mol Biol 330, 1005-1014.

Terpstra, S., Noordhoek, G.T., Voesten, H.G., Hendriks, B., Degener, J.E., 1999, Rapid emergence of resistant coagulase-negative staphylococci on the skin after antibiotic prophylaxis. J Hosp Infect 43, 195-202.

Thomson, K.S., 2010, Extended-spectrum-beta-lactamase, AmpC, and Carbapenemase issues. J Clin Microbiol 48, 1019-1025.

Tipper, D. J., 1985, Mode of action of beta-lactam antibiotics. Pharmacol Ther, 27, 1-35.

Touchon, M., Hoede, C., Tenaillon, O., Barbe, V., Baeriswyl, S., Bidet, P., Bingen, E., Bonacorsi, S., Bouchier, C., Bouvet, O., Calteau, A., Chiapello, H., Clermont, O., Cruveiller, S., Danchin, A., Diard, M., Dossat, C., Karoui, M. E., Frapy, E., Garry, L., Ghigo, J. M., Gilles, A. M., Johnson, J., Le Bouguenec, C., Lescat, M., Mangenot, S., Martinez-Jehanne, V., Matic, I., Nassif, X., Oztas, S., Petit, M. A., Pichon, C., Ruf, C. S., Schneider, D., Tourret, J., Vacherie, B., Vallenet, D., Medigue, C., Rocha, E. P., Denamur, E., 2009, Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. PLoS Genet, 5, e1000344.

Trott, D.J., Filippich, L.J., Bensink, J.C., Downs, M.T., McKenzie, S.E., Townsend, K.M., Moss, S.M., Chin, J.J., 2004, Canine model for investigating the impact of oral enrofloxacin on commensal coliforms and colonization with multidrug-resistant *Escherichia coli*. J Med Microbiol 53, 439-443.

Tsubakishita, S., Kuwahara-Arai, K., Sasaki, T., Hiramatsu, K., 2010, Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. Antimicrob Agents Chemother 54, 4352-4359.

Tulinski, P., Fluit, A. C., Wagenaar, J. A., Mevius, D., van de Vijver, L., Duim, B., 2012, Methicillin-resistant coagulase-negative staphylococci on pig farms as a reservoir of heterogeneous staphylococcal cassette chromosome mec elements. Appl Environ Microbiol, 78, 299-304.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., Egholm, M., Henrissat, B., Heath, A.C., Knight, R., Gordon, J.I., 2009, A core gut microbiome in obese and lean twins. Nature 457, 480-484.

Unno, T., Han, D., Jang, J., Lee, S. N., Ko, G., Choi, H. Y., Kim, J. H., Sadowsky, M. J., Hur, H. G., 2009, Absence of *Escherichia coli* phylogenetic group B2 strains in humans

and domesticated animals from Jeonnam Province, Republic of Korea. Appl Environ Microbiol, 75, 5659-5666.

van Duijkeren, E., Kamphuis, M., van der Mije, I.C., Laarhoven, L.M., Duim, B., Wagenaar, J.A., Houwers, D.J., 2011, Transmission of methicillin-resistant *Staphylococcus pseudintermedius* between infected dogs and cats and contact pets, humans and the environment in households and veterinary clinics. Vet Microbiol 150, 338-343.

VandenBergh, M.F., Yzerman, E.P., van Belkum, A., Boelens, H.A., Sijmons, M., Verbrugh, H.A., 1999, Follow-up of *Staphylococcus aureus* nasal carriage after 8 years: redefining the persistent carrier state. J Clin Microbiol 37, 3133-3140.

Vanderhaeghen, W., Van de Velde, E., Crombe, F., Polis, I., Hermans, K., Haesebrouck, F., Butaye, P., 2012, Screening for methicillin-resistant staphylococci in dogs admitted to a veterinary teaching hospital. Res Vet Sci, 93, 133-136.

Vanhoutte, T., Huys, G., De Brandt, E., Fahey, G. C., Jr., Swings, J., 2005, Molecular monitoring and characterization of the faecal microbiota of healthy dogs during fructan supplementation. FEMS Microbiol Lett, 249, 65-71.

Venezia, R.A., Domaracki, B.E., Evans, A.M., Preston, K.E., Graffunder, E.M., 2001, Selection of high-level oxacillin resistance in heteroresistant *Staphylococcus aureus* by fluoroquinolone exposure. J Antimicrob Chemother 48, 375-381.

Vengust, M., Anderson, M.E., Rousseau, J., Weese, J.S., 2006, Methicillin-resistant staphylococcal colonization in clinically normal dogs and horses in the community. Lett Appl Microbiol 43, 602-606.

Vincent, C., Boerlin, P., Daignault, D., Dozois, C.M., Dutil, L., Galanakis, C., Reid-Smith, R.J., Tellier, P.P., Tellis, P.A., Ziebell, K., Manges, A.R., 2010, Food reservoir for *Escherichia coli* causing urinary tract infections. Emerg Infect Dis 16, 88-95.

VMD 2012. Veterinary Medicines Directorate. Sales of antimicrobial products used as veterinary medicines, growth promoters and coccidostats in the UK from 2006-2011 (Defra).

Vollaard, E.J., Clasener, H.A., 1994, Colonization resistance. Antimicrob Agents Chemother 38, 409-414.

von Eiff, C., Peters, G., Heilmann, C., 2002, Pathogenesis of infections due to coagulase-negative staphylococci. Lancet Infect Dis 2, 677-685.

Walk, S. T., Alm, E. W., Calhoun, L. M., Mladonicky, J. M., Whittam, T. S., 2007, Genetic diversity and population structure of *Escherichia coli* isolated from freshwater beaches. Environ Microbiol, 9, 2274-2288.

Walk, S. T., Alm, E. W., Gordon, D. M., Ram, J. L., Toranzos, G. A., Tiedje, J. M., Whittam, T. S., 2009, Cryptic lineages of the genus *Escherichia.* Appl Environ Microbiol, 75, 6534-6544.

Walther, B., Hermes, J., Cuny, C., Wieler, L.H., Vincze, S., Abou Elnaga, Y., Stamm, I., Kopp, P.A., Kohn, B., Witte, W., Jansen, A., Conraths, F.J., Semmler, T., Eckmanns, T., Lubke-Becker, A., 2012, Sharing more than friendship--nasal colonization with coagulase-positive staphylococci (CPS) and co-habitation aspects of dogs and their owners. PLoS One 7, e35197.

Wang, H.H., Schaffner, D.W., 2011, Antibiotic resistance: how much do we know and where do we go from here? Appl Environ Microbiol 77, 7093-7095.

Weber, S.G., Gold, H.S., Hooper, D.C., Karchmer, A.W., Carmeli, Y., 2003, Fluoroquinolones and the risk for methicillin-resistant *Staphylococcus aureus* in hospitalized patients. Emerg Infect Dis 9, 1415-1422.

Wedley, A.L., 2012. Prevalence and Risk Factors for the Carriage of Antimicrobial Resistant *Escherichia coli* in Dogs. University of Liverpool, Liverpool.

Wedley, A.L., Dawson, S., Maddox, T.W., Coyne, K.P., Pinchbeck, G.L., Clegg, P., Jamrozy, D., Fielder, M.D., Donovan, D., Nuttall, T., Williams, N.J., 2014, Carriage of *Staphylococcus* species in the veterinary visiting dog population in mainland UK: Molecular characterisation of resistance and virulence. Vet Microbiol 170, 81-88.

Wedley, A.L., Maddox, T.W., Westgarth, C., Coyne, K.P., Pinchbeck, G.L., Williams, N.J., Dawson, S., 2011, Prevalence of antimicrobial-resistant *Escherichia coli* in dogs in a cross-sectional, community-based study. Vet Rec 168, 354.

Weese, J.S., Faires, M.C., Frank, L.A., Reynolds, L.M., Battisti, A., 2012, Factors associated with methicillin-resistant versus methicillin-susceptible *Staphylococcus pseudintermedius* infection in dogs. J Am Vet Med Assoc 240, 1450-1455.

Weese, J. S., Rousseau, J., Traub-Dargatz, J. L., Willey, B. M., McGeer, A. J., Low, D. E., Community-associated methicillin-resistant *Staphylococcus aureus* in horses and humans who work with horses. J Am Vet Med Assoc, 226, 580-583.

Weese, J.S., van Duijkeren, E., 2010, Methicillin-resistant Staphylococcus aureus and *Staphylococcus pseudintermedius* in veterinary medicine. Vet Microbiol 140, 418-429.

Wellington, E.M., Boxall, A.B., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., Johnson-Rollings, A.S., Jones, D.L., Lee, N.M., Otten, W., Thomas, C.M., Williams, A.P., 2013, The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. Lancet Infect Dis 13, 155-165.

Wertheim, H. F., Melles, D. C., Vos, M. C., van Leeuwen, W., van Belkum, A., Verbrugh, H. A., Nouwen, J. L., 2005, The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect Dis, 5, 751-762.

Westh, H., Zinn, C.S., Rosdahl, V.T., 2004, An international multicenter study of antimicrobial consumption and resistance in *Staphylococcus aureus* isolates from 15 hospitals in 14 countries. Microb Drug Resist 10, 169-176.

White, D. G., Datta, A., McDermott, P., Friedman, S., Qaiyumi, S., Ayers, S., English, L., McDermott, S.,, Wagner, D. D., Zhao, S., 2003, Antimicrobial susceptibility and genetic relatedness of *Salmonella* serovars isolated from animal-derived dog treats in the USA. J Antimicrob Chemother, 52, 860-863.

Widerstrom, M., Wistrom, J., Sjostedt, A., Monsen, T., 2012, Coagulase-negative staphylococci: update on the molecular epidemiology and clinical presentation, with a focus on *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*. Eur J Clin Microbiol Infect Dis, 31, 7-20.

Windahl, U., Reimegard, E., Holst, B.S., Egenvall, A., Fernstrom, L., Fredriksson, M., Trowald-Wigh, G., Andersson, U.G., 2012, Carriage of methicillin-resistant *Staphylococcus pseudintermedius* in dogs--a longitudinal study. BMC Vet Res 8, 34.

Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P., Edmond, M. B., 2004, Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis, 39, 309-317.

Woodford, N., Reddy, S., Fagan, E.J., Hill, R.L., Hopkins, K.L., Kaufmann, M.E., Kistler, J., Palepou, M.F., Pike, R., Ward, M.E., Cheesbrough, J., Livermore, D.M., 2007, Wide geographic spread of diverse acquired AmpC beta-lactamases among *Escherichia coli* and *Klebsiella* spp. in the UK and Ireland. J Antimicrob Chemother 59, 102-105.

Yamashita, K., Shimizu, A., Kawano, J., Uchida, E., Haruna, A., Igimi, S., 2005, Isolation and characterization of staphylococci from external auditory meatus of dogs with or without otitis externa with special reference to *Staphylococcus schleiferi* subsp. *coagulans* isolates. J Vet Med Sci, 67, 263-268.

Yasuda, R., Kawano, J., Onda, H., Takagi, M., Shimizu, A., Anzai, T., 2000, Methicillin-resistant coagulase-negative staphylococci isolated from healthy horses in Japan. Am J Vet Res, 61, 1451-1455.

Zanelli, G., Sansoni, A., Zanchi, A., Cresti, S., Pollini, S., Rossolini, G. M., Cellesi, C., 2002, *Staphylococcus aureus* nasal carriage in the community: a survey from central Italy. Epidemiol Infect, 129, 417-420.

Zhang, K., McClure, J. A., Elsayed, S., Louie, T., Conly, J. M., 2005, Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol, 43, 5026-33.

Zhang, Y., Agidi, S., LeJeune, J. T., 2009, Diversity of staphylococcal cassette chromosome in coagulase-negative staphylococci from animal sources. J Appl Microbiol, 109, 1375-1383.

Zhao, S., White, D. G., McDermott, P. F., Friedman, S., English, L., Ayers, S., Meng, J., Maurer, J. J., Holland, R., Walker, R. D., 2001, Identification and expression of cephamycinase bla(CMY) genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrob Agents Chemother 45, 3647-3650.

APPENDIX I
(Pertaining to chapter 3)

Detection of antibiotic resistant bacteria in canine faecal samples and mucosal swabs in healthy dogs

Why are we doing this study? Antibiotic resistant normal (commensal) bacteria that live on the mucous membranes of the nose and bottom and in the faeces may cause opportunistic bacterial infections in animals or people or they may pass the resistance onto pathogenic (more invasive) bacteria. Antibiotic resistance is a growing problem in human and canine medicine.
The study will find out whether commensal bacteria of healthy dogs carry antibiotic resistant genes. A questionnaire will be used to try to determine if the findings are related to external influences. By determining the factors that promote antibiotic resistance in bacteria our aim is to develop guidelines that will minimise this occurrence.

To be included in the study your dog must be:

- Healthy without any gastrointestinal (vomiting or diarrhoea) or skin disease e.g. allergy.
- Only one dog from each household can be included in the study.

You dog cannot be included in the study if: :

- It has received antibiotics or been hospitalised at a veterinary surgery in the last 12 months
- Has had diarrhoea in the last 4 weeks.

What does the study involve? Your participation is voluntary and you can decline involvement at any time. Firstly it involves answering a questionnaire. Secondly we will collect the samples (see below). This will take approximately 10 minutes.

Mucosal samples: We will take one surface swab from each of your dog's nostrils and one swab from the rear end. This procedure is non-painful and well tolerated, but if your dog objects at any stage we will not continue.

Faecal sample: We will supply rubber gloves and a faecal pot. Please collect a small amount of faeces and return to us today.

What next? If you are happy to participate in this study please read, initial and sign the consent form.

Thank you for your time.

Vanessa Schmidt BVSc CertVD DipECVD MRCVS
University of Liverpool
Leahurst Campus
Chester Road
Neston
CH64 7TE
vetderm@liv.ac.uk
0151 7956100

If there are any problems, please let us know by contacting Vanessa Schmidt on 0151 7956100, and we will try to help. If you are unhappy or have a complaint which you feel you cannot come to us with then you should contact the Research Governance Officer on 0151 794 8290 (ethics@liv.ac.uk). When contacting the Research Governance Officer, please provide details of the name or description of the study (so that it can be identified), the researcher(s) involved, and the details of the complaint you wish to make.

Version # 3; Created on 01/09/10 at 18.30 pm.

Detection of antibiotic resistant bacteria in canine faecal samples and mucosal swabs in healthy dogs: Questionnaire

Case number:

Please answer all questions to the best of your knowledge.

Name .....

Dogs Name .....

About your dog.

1) Age Years Months Weeks ☐ Don't Know ☐
   a) Is this age Exact ☐ Estimate ☐

2) Breed Pedigree *(please specify)* ☐ .....
   Cross *(please specify)* ☐ .....

3) Sex Male ☐ Female ☐ Neutered

4) How long have you owned him/her?

About your dog's diet.

5) What is s/he fed? (*Tick all that apply)*
   Tinned meat ☐ Dry mixer ☐
   Dry Complete ☐ Raw chicken ☐
   Cooked chicken ☐ Raw red meat ☐
   Cook red meat ☐ Don't Know ☐
   Other .....

6) Is s/he fed commercial dog treats? Never ☐ Rarely ☐ Sometimes ☐
   Often ☐ Don't know ☐

7) Is s/he fed human titbits/ scraps? Never ☐ Rarely ☐ Sometimes ☐
   Often ☐ Don't know ☐

8) Does your dog ever eat stools (faeces)? Never ☐ Rarely ☐ Sometimes ☐
   Often ☐ Don't know ☐
   If so what types of stools? *(Please tick all that apply)*
   Rabbit ☐ Cat ☐ Dog ☐ Horse ☐ Cow ☐
   Sheep ☐ Badger ☐ Other .....

About your household.

9) Are there any other dogs in the household?
   Yes ☐ No ☐ Don't Know ☐
   If yes, how many? 1 ☐ 2 ☐ 3 or more ☐

10) Do you own any other animals (other than dogs)?
   Yes ☐ No ☐ Don't Know ☐
   If yes, what animals? *(Please tick all that apply)*
   Cat ☐ Bird ☐ Rabbit ☐

Rodent *(e.g. hamster)* ☐ Reptile *(e.g. snake)* ☐
Don't know ☐ Other .....

11) Does anyone in your household work with farm animals?
Yes ☐ No ☐ Don't know ☐
If yes, please state which species are worked with .....
.....
.....

12) Has anyone in your family (including other pets) to your knowledge in the last month taken antibiotics?
Yes ☐ No ☐ Don't know ☐

a) If yes, was this a Family Member ☐ Pet ☐

b) Which antibiotic was prescribed *(if known)* .....
.....
..

13) Does anyone in your household work in medical or veterinary healthcare?
Yes No ☐ Don't Know ☐

a) If yes, in what setting? Hospital ☐ Community Nursing ☐
GP surgery ☐ Nursing Home ☐
Dentist ☐ Veterinary practice ☐
Don't Know ☐ Other .....

14) Has anyone in your household attended hospital in the last month?
Yes ☐ No ☐ Don't Know ☐

a) If yes, why? Admission to hospital ☐ Visit ☐
Outpatient appointment ☐ Don't Know ☐
Other .....

Thank you for your time.

Version # 3; Created on 01/09/10 at 19.00 pm.

Table 3-1. Details of PCR assays for *nuc*, *tuf* and *mec*A gene identification.

Primer	Sequence (5'-3')	Amplicon size (bp)	Annealing Temperature (°C)	Control Strain	Reference
au-F3 au-nucR[1]	TCGCTTGCTATGATTGTGG GCCAATGTTCTACCATAGC	359	57	*S. aureus* ATCC®25923 (LGC Standards, Teddington, UK)	Sasaki et al., 2010
pse-F2 pse-R5[1]	TRGGCAGTAGGATTCGTTAA CTTTTGTGCTYCMTTTTGG	926	57	*S. pseudintermedius* 412 (clinical isolate)	Sasaki et al., 2010
SSnucF SSnucR[1]	AATGGCTACAATGATAATCAC TAA CATATCTGTCTTTCGGCGCG	526	57	*S. schleiferi* subspecies *coagulans* ATCC®49545	Sasaki et al., 2010
tuf-F tuf-R	GCCAGTTGAGGACGTATTCT CCATTTCAGTACCTTCTGGTAA	412	55	*S. epidermidis* ATCC®12228	Carpaij et al., 2011
mecAF mecAR mA1 mA2	TGGCTATCGTGTCACAATCG CTGGAACTTGTTGAGCAGAG TGCTATCCACCCTCAAACAGG AACGTTGTAACCACCCCAAGA	310 286	55 57	MRSA (clinical isolate)	Francois et al., 2003; Kondo et al., 2007

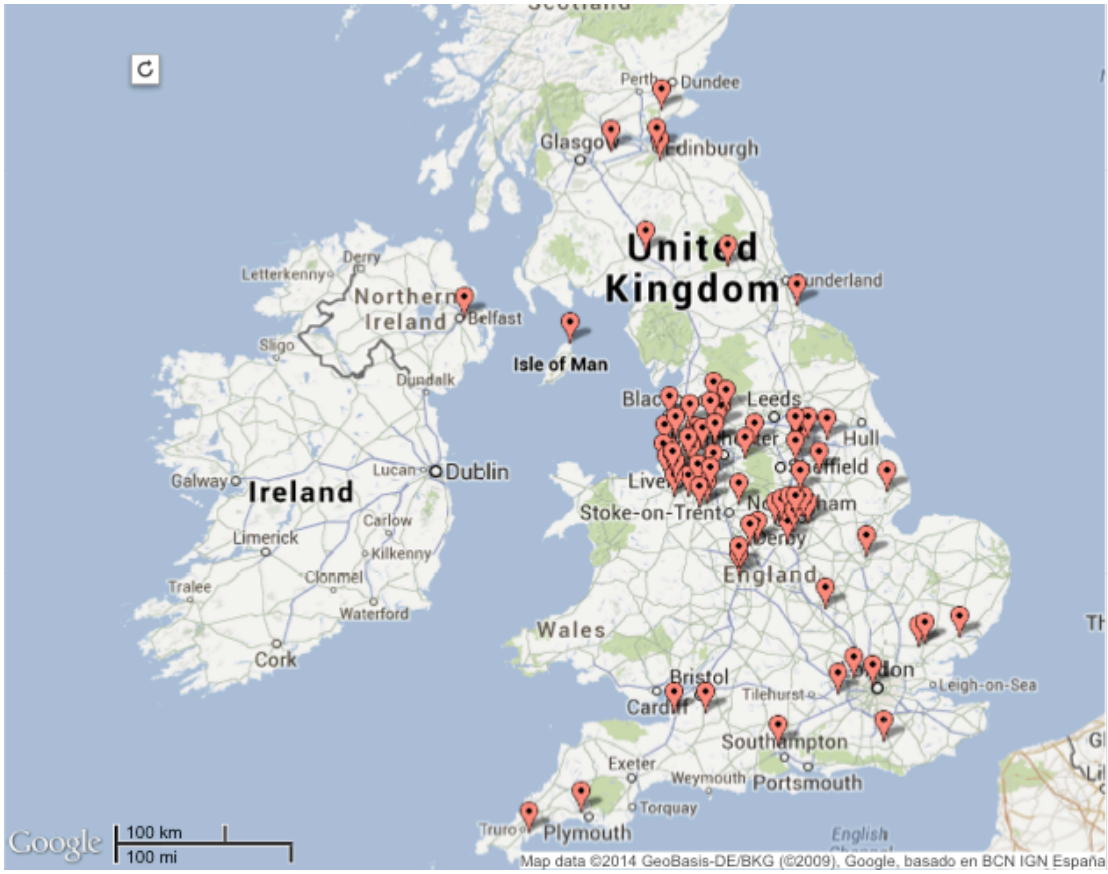
[1]multiplex assay

Table 3-2 The number and percentage of dogs enrolled from different areas of the UK

Location in the UK	Number of dogs	Percentage of dogs
Berkshire[1]	1	1
Cambridgeshire[1]	1	1
Cheshire[1]	8	11
Cleveland[1]	1	1
Cornwall[1]	2	3
Derbyshire[1]	4	5
Essex[1]	3	4
Hampshire[1]	1	1
Isle of Man[2]	1	1
Lancashire[1]	13	18
Leicestershire[1]	3	4
Lincolnshire[1]	2	3
London[1]	1	1
Merseyside[1]	6	8
Middlesex[1]	1	1
Nottinghamshire[1]	3	4
Northamptonshire[1]	2	3
Northern Ireland*	1	1
Northumbria[1]	1	1
Scotland*	5	7
Staffordshire[1]	3	4
Somerset[1]	2	3
West Bromwich[1]	1	1
West Midlands[1]	1	1
West Sussex[1]	1	1
Yorkshire[1]	6	8

[1]English counties; *Countries of the UK, [2]Country associated with the UK

Figure 3-1 Map of the UK (England, Northern Ireland, Scotland and Wales) with markers indicating the location of each dog recruited for this study



APPENDIX II
(Pertaining to chapter 4)

Table 4-1: Details of the primer sequences used in PCR assays in this study

Primer	Sequence (5'-3')	Amplicon Size (bp)	Annealing (°C)	[Primer] /reaction	Reference
uidAF uidAR	CCAAAAGCCAGACAGAGT GCACAGCACATCAAAGAG	623	58	0.25 µM	McDaniels et al., 1996
CTXMU1 CTXMU2	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	593	58	0.25 µM	Bachelor et al., 2005
CTXM1F CTXM1R	ATGGTTAAAAAATCACTGCG TTACAAACCGTCGGTGAC	876	58	0.25 µM	Bachelor et al., 2005
CTXM2F CTXM2R	ATGATGACTCAGAGCATTCGC TCAGAAACCGTGGGTTACGAT	892	58	0.25 µM	Hopkins et al., 2006
CTXM9F CTXM9R	ATGGTGACAAAGAGAGTGCAAC TTACAGCCCTTCGGCGATG	876	58	0.25 µM	Hopkins et al., 2006
SHVF[1] SHVR	AGCCGCTTGAGCAAATTAAAC ATCCCGCAGATAAATCACCAC	713	60	0.25 µM	Dallenne et al., 2010
TEMF[1] TEMR	CATTTCCGTGTCGCCCTTATTC CGTTCATCCATAGTTGCCTGAC	800	60	0.25 µM	Dallenne et al 2010
OXA-1F[1] OXA-1R	GGCACCAGATTCAACTTTCAAG GACCCCAAGTTTCCTGTAAGTG	564	60	0.25 µM	Dallenne et al., 2010
CITMF[2] CITMF	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462	64	0.25 µM	Perez-Perez & Hanson 2002
MOXMF[2] MOXMR	GCTGCTCAAGGAGCACAGGAT CACATTGACATAGGTGTGGTGC	520	64	0.25 µM	Perez-Perez & Hanson 2002
DHAMF[2] DHAMR	AACTTTCACAGGTGTGCTGGGT CCGTACGCATACTGGCTTTGC	405	64	0.25 µM	Perez-Perez & Hanson 2002
ACCMF[2] ACCMR	AACAGCCTCAGCAGCCGGTTA TTCGCCGCAATCATCCCTAGC	346	64	0.25 µM	Perez-Perez & Hanson 2002
EBCMF[2] EBCMR	TCGGTAAAGCCGATGTTGCGG CTTCCACTGCGGCTGCCAGTT	302	64	0.25 µM	Perez-Perez & Hanson 2002
FOXMF[2] FOXMR	AACATGGGGTATCAGGGAGATG CAAAGCGCGTAACCGGATTGG	190	64	0.25 µM	Perez-Perez & Hanson 2002
gadAF[3] gadAR	GATGAAATGGCGTTGGCGCAAG GGCGGAAGTCCCAGACGATATCC	373	65	1 µM	Doumith et al., 2012
chuAF[3] chuAR	ATGATCATCGCGGCGTGCTG AAACGCGCTCGCGCCTAAT	281	65	1 µM	Doumith et al., 2012
yjaAF[3] yjaAR	TGTTCGCGATCTTGAAAGCAAACGT ACCTGTGACAAACCGCCCTCA	216	65	1 µM	Doumith et al 2012
TSPE4.C2F[3] TSPE4.C2R	GCGGGTGAGACAGAAACGCG TTGTCGTGAGTTGCGAACCCG	152	65	1 µM	Doumith et al., 2012
chuA.1b[4] chuA.2	ATGGTACCGGACGAACCAAC TGCCGCCAGTACCAAAGACA	288	59	1 µM	Clermont et al 2013
yjaA.1b[4] yjaA.2b	CAAACGTGAAGTGTCAGGAG AATGCGTTCCTCAACCTGTG	211	59	1 µM	Clermont et al., 2013
TspE4C2.1b[4] TspE4C2.2b	CACTATTCGTAAGGTCATCC AGTTTATCGCTGCGGGTCGC-	152	59	1 µM	Clermont et al 2013
AceK.f[4] ArpA1.r	AACGCTATTCGCCAGCTTGC TCTCCCCATACCGTACGCTA	400	59	2 µM	Clermont et al., 2013
ArpAgpE.f ArpAgpE.r	GATTCCATCTTGTCAAAATATGCC GAAAAGAAAAAGAATTCCCAAGAG	301	57	1 µM	Clermont et al., 2013
trpAgpC.1 trpAgpC.2	AGTTTTATGCCCAGTGCGAG- TCTGCGCCGGTCACGCCC	219	59	1 µM	Clermont et al., 2013
trpBA.f trpBA.r	CGGCGATAAAGACATCTTCAC GCAACGCGGCCTGGCGGAAG	489	57 or 59	0.6 µM	Clermont et al., 2013
qnrAf[5] qnrAr	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	516	53	0.25 µM	Robiseck et al., 2006
qnrBf[5] qnrBr	GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC	468	53	0.25 µM	Robiseck et al., 2006
qnrSf[5] qnrSr	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	417	53	0.25 µM	Robiseck et al., 2006

[1-5] denotes multiplex assays; [Primer] = primer concentration; bp = base pairs

Table 4-2: Association of phylogenetic groups and the presence or absence of one of six antimicrobial resistance outcomes for of 188 faecal *E. coli* isolates

Phylo-group	Number (%) of ACR isolates	Number (%) of PAS isolates	*P* value	Number (%) of CipR isolates	Number (%) of CipS isolates	*P* value
A	6 (25)	52 (32)	0.5	8 (42)	50 (30)	0.27
B1	11 (46)	67 (41)	0.65	8 (42)	70 (41)	0.95
B2	0	33 (20)	*0.002	2 (11)	31 (18)	0.37
D	7 (29)	12 (7)	*0.004	1 (5)	18 (11)	0.43
C	5 (21)	34 (21)	0.99	6 (32)	33 (20)	0.24
E, F, Clades	2 (8)	14 (9)	0.97	0	16 (10)	0.06
Total	**24 (100)**	**164 (100)**		**19 (100)**	**169 (100)**	
Phylo-group	**Number (%) of BLR isolates**	**Number (%) of BLS isolates**	***P* value**	**Number (%) of 3GR isolates**	**Number (%) of 3GS isolates**	***P* value**
A	36 (33)	22 (28)	0.45	5 (26)	53 (31)	0.65
B1	48 (44)	30 (38)	0.4	8 (42)	70 (41)	0.95
B2	11 (10)	22 (29)	*0.002	0	33 (20)	*0.005
D	14 (13)	5 (6)	0.13	6 (32)	13 (8)	*0.005
C	26 (24)	13 (17)	0.21	4 (21)	35 (21)	0.97
E, F, Clades	9 (8)	7 (9)	0.88	1 (5)	15 (9)	0.59
Total	**109 (100)**	**79 (100)**		**19 (100)**	**169 (100)**	
Phylo-group	**Number (%) of AMR isolates**	**Number (%) susceptible isolates**	***P* value**	**Number (%) of MDR isolates**	**Number (%) not MDR isolates**	***P* value**
A	50 (34)	8 (20)	0.08	24 (39)	34 (27)	0.08
B1	65 (44)	13 (33)	0.19	31 (51)	47 (37)	0.07
B2	15 (10)	18 (45)	*0.00	0	33 (26)	*0.00
D	18 (12)	1 (3)	*0.04	6 (10)	13 (10)	0.93
C	35 (24)	4 (10)	*0.044	19 (31)	20 (16)	*0.02
E, F, Clades	11 (7)	5 (13)	0.33	4 (7)	12 (9)	0.5
Total	**148 (100)**	**40 (100)**		**61 (100)**	**127 (100)**	

P value is from the Likelihood ratio test and univariable logistic regression (reference category is absence of phylo-group); ACR = potentiated amoxicillin resistance; PAS = potentiated amoxicillin susceptible; CipR = ciprofloxacin resistant; CipS = ciprofloxacin susceptible; BLR = beta-lactam resistant; BLR = beta-lactam susceptible; 3GR = third generation cephalosporin resistant; 3GS = third generation cephalosporin susceptible; AMR = any antimicrobial resistance; MDR = multidrug resistant; phylo-groups A, B1, B2 and D based on Doumith et al (2012); C, E, F and Clades based on (Clermont et al (2013) *significant $P < 0.05$

Table 4-3. Univariable analysis results for the association of the antimicrobial resistance outcomes with covariates amongst faecal *E. coli* from 73 dogs.

	Potentiated amoxycillin resistance			Ciprofloxacin resistance			Beta-lactam resistance		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Dog eats raw meat[1]	6.67	1.67-26.57	0.007*	2.97	0.72-12.34	0.146*	1.93	0.58-6.38	0.273
Dog eats animal carcass or faeces[1]	2.46	0.6-9.98	0.209*	0.68	0.18-2.58	0.567	0.84	0.33-2.16	0.719
Dog eats tinned or cooked meat[1]	0.29	0.03-2.43	0.189*	0.8	0.15-4.23	0.793	0.55	0.18-1.71	0.300
Dog fed treats[1]	2.14	0.43-10.78	0.326	0.11	0.03-0.5	0.002*	0.39	0.13-1.2	0.09*
Dog fed table scraps[1]	1.4	0.4-4.91	0.597	1.5	0.39-5.84	0.556	0.79	0.31-2.00	0.622
Multi-dog household[1]	3.25	0.38-27.58	0.219*	2.3	0.27-19.95	0.410	3.48	0.95-12.69	0.050*
Multi-animal household[1]	1.65	0.45-6.04	0.448	2.13	0.54-8.35	0.275	1.18	0.45-3.08	0.74
Owner works with farm animals[1]	0.31	0.04-2.62	0.220*	1.64	0.37-7.32	0.525	0.33	0.1-1.1	0.063*
Owner works in healthcare[1]	0.86	0.23-3.2	0.817	0.39	0.08-2.01	0.230*	0.7	0.26-1.88	0.480
In-contact been hospitalised[1,2]	1.22	0.33-4.54	0.764	1.45	0.34-6.18	0.609	1.03	0.39-2.72	0.955
In-contact had antimicrobials[1,2]	0.35	0.1-1.24	0.101*	2.67	0.52-13.66	0.207*	1.32	0.5-3.48	0.580
Age	1.01	0.99-1.02	0.766	0.99	0.98-1.02	0.748	1.01	0.99-1.02	0.324
Gender (Reference is males)	1.35	0.39-4.75	0.635	1.45	0.37-5.66	0.590	1.85	0.72-4.76	0.200*
	Antimicrobial resistance (AMR)			**Multidrug resistance (MDR)**			**3rd generation cephalosporin^R**		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Dog eats raw meat[1]	1.7	0.48-6.03	0.400	2.33	0.72-7.56	0.161*	6.67	1.67-26.57	0.007*
Eat animal carcass or faeces[1]	0.63	0.23-1.7	0.360	0.8	0.29-2.2	0.666	1.3	0.34-4.91	0.697
Dog eats tinned or cooked meat[1]	0.86	0.27-2.74	0.795	0.65	0.18-2.3	0.493	0.29	0.03-2.43	0.189*
Dog fed treats[1]	0.69	0.23-2.08	0.499	6.3	2.04-19.49	0.001*	1.03	0.24-4.35	0.968
Dog fed table scraps[1]	0.53	0.2-1.4	0.194*	0.71	0.26-1.9	0.504	1.16	0.32-4.21	0.820
Multi-dog household[1]	1.52	0.55-4.21	0.415	3.06	0.62-15.18	0.136*	2.61	0.3-22.43	0.333
Multi-animal household[1]	1.81	0.53-6.17	0.347	1.08	0.39-3.01	0.88	2.59	0.68-9.86	0.155*
Owner works with farm animals[1]	0.9	0.27-3.01	0.857	0.25	0.05-1.24	0.058*	0.31	0.04-2.62	0.220*
Owner works in healthcare[1]	1.02	0.37-2.82	0.980	0.44	0.14-1.39	0.147*	1.01	0.26-3.85	0.992
In-contact been hospitalised[1,2]	0.93	0.34-2.56	0.883	0.79	0.28-2.23	0.660	1.7	0.41-7.1	0.452
In-contact had antimicrobials[1,2]	2.04	0.75-5.6	0.163*	1.4	0.48-4.08	0.530	0.427	0.12-1.57	0.200*
Age	1.01	0.99-1.02	0.355	1.01	0.99-1.02	0.732	1.00	0.99-1.02	0.716
Gender (Reference is males)	2.13	0.79-5.75	0.131*	1.98	0.7-5.56	0.190*	1.12	0.31-4.08	0.860

[2]In contact person or pet within 12 months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the likelihood ratio test; AMR = antimicrobial resistance to at least one of the seven antimicrobials tested; MDR = antimicrobial resistance to three or more of the seven antimicrobials tested; *included for analysis in final model $P < 0.25$.

Table 4-4. Univariable analysis results for the association of phylogenetic groups with covariates amongst faecal *E. coli* from 73 dogs.

	Phylo-group A			Phylo-group B1			Phylo-group B2		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Dog eats raw meat[1]	2.13	0.66-6.87	0.207*	2.84	0.88-9.18	0.077*	0.32	0.04-2.74	0.240*
Eat animal carcass or faeces[1]	1.53	0.55-4.26	0.414	0.39	0.15-1.04	0.056*	1.00	0.29-3.52	1.000
Dog eats tinned or cooked meat[1]	0.38	0.1-1.5	0.143*	0.85	0.29-2.67	0.774	0.7	0.14-3.62	0.661
Dog fed treats[1]	1.64	0.56-4.83	0.369	2.31	0.81-6.6	0.116*	5.1	0.61-42.39	0.070*
Dog fed table scraps[1]	0.96	0.35-2.57	0.927	1.56	0.6-4.01	0.360	0.26	0.06-1.04	0.042*
Multi-dog household[1]	7.2	0.87-59.42	0.022*	1.2	0.35-4.13	0.772	0.56	0.13-2.47	0.453
Multi-animal household[1]	0.54	0.19-1.5	0.232*	1.09	0.41-2.89	0.861	1.32	0.34-5.06	0.686
Owner works with farm animals[1]	0.65	0.2-2.13	0.479	0.75	0.24-2.38	0.626	0.35	0.04-3.0	0.284
Owner works in healthcare[1]	1.12	0.39-3.24	0.832	0.52	0.18-1.44	0.200*	2.46	0.67-9.11	0.176*
In-contact been hospitalised[1,2]	0.6	0.21-1.68	0.333	0.94	0.35-2.52	0.909	1.04	0.27-4.0	0.953
In-contact had antimicrobials[1,2]	0.6	0.21-1.68	0.333	1.57	0.58-4.29	0.371	0.66	0.18-2.44	0.538
Age	0.99	0.98-1.01	0.472	0.99	1.01-1.02	0.306	0.99	0.98-1.02	0.910
Gender (Reference is males)	1.69	0.62-4.65	0.305	1.56	0.6-4.05	0.361	0.9	0.26-3.13	0.872
	Phylo-group D			**Phylo-group C**			**Phylo-groups E,F,Clade**		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Dog eats raw meat[1]	2.94	0.79-10.88	0.116*	3.94	1.16-13.39	0.030*	5.44	1.4-20.72	0.014*
Eat animal carcass or faeces[1]	1.18	0.34-4.03	0.795	2.00	0.62-6.45	0.234*	0.44	0.13-1.57	0.203*
Dog eats tinned or cooked meat[1]	0.55	0.11-2.77	0.443	0.15	0.02-1.27	0.031*	1.13	0.26-4.79	0.871
Dog fed treats[1]	0.84	0.23-3.1	0.792	0.22	0.07-0.71	0.011*	0.73	0.19-2.75	0.643
Dog fed table scraps[1]	0.17	0.77-0.23	0.677	0.58	0.9-1.76	0.334	1.4	0.4-4.9	0.597
Multi-dog household[1]	3.27	0.39-27.75	0.215*	4.8	0.58-40.02	0.084*	2.9	0.34-25.03	0.269
Multi-animal household[1]	1.62	0.48-5.47	0.434	1.17	0.39-3.53	0.778	0.89	0.25-3.13	0.850
Owner works with farm animals[1]	0.59	0.12-3.0	0.505	0.7	0.17-2.84	0.606	1.22	0.29-5.23	0.789
Owner works in healthcare[1]	2.46	0.72-8.39	0.149*	0.95	0.3-2.97	0.926	1.32	0.37-4.71	0.669
In-contact been hospitalised[1,2]	1.41	0.39-5.15	0.594	0.58	0.19-1.75	0.335	0.53	0.15-1.85	0.318
In-contact had antimicrobials[1,2]	0.93	0.27-3.23	0.913	0.8	0.26-2.44	0.694	0.795	0.22-2.82	0.723
Age	0.99	0.97-1.02	0.470	0.99	0.97-1.01	0.243*	0.99	0.97-1.02	0.475
Gender (Reference is males)	1.6	0.47-5.48	0.450	1.43	0.47-4.3	0.524	2.07	0.56-7.62	0.264

[1] Reference category is absence of risk factor; [2]In contact person or pet within 12 months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the likelihood ratio test; AMR = antimicrobial resistance to at least one of the seven antimicrobials tested; MDR = antimicrobial resistance to three or more of the seven antimicrobials tested; * included for analysis in final model $P < 0.25$

APPENDIX III
(Pertaining to chapter 5)

Owner information sheet: A longitudinal study to determine if the faecal bacteria of healthy dogs is stable over time

Why are we doing this study? This study forms part of a larger study into the way bacteria develop resistance to antibiotics. In this study we are looking at the bacteria in the gut of healthy dogs and seeing if the types of bacteria change over time.

To be included in the study we need the following (Inclusion Criteria):

- Healthy dogs without a history of chronic gastrointestinal (vomiting or diarrhoea) or skin disease e.g. allergy.
- Only one dog from each household.
- Stable health, environment and diet during the study period.

Your dog cannot be entered into the study if any of the following apply (Exclusion Criteria):

- Received antibiotics or been hospitalised at a veterinary surgery in the last 12 months
- Had diarrhoea in the last 4 weeks.
- Dogs that experience illness, visit and/or are hospitalised at a veterinary surgery/hospital and/or receive medication during the study period will not be eligible to continue in the study.

What does the study involve? Collecting a stool sample once daily for 7 days, then once weekly for 4 weeks and then once two months later. We will supply gloves and faecal (stool) pots. If you would like, we can arrange reminders by phone, text or email to collect the faeces (stools) (please see consent form). Your participation is voluntary and you can decline involvement at any time.

Personal information. All information collected during the course of the study will be anonymous and stored on a secure database. You have the right to see any information that you provide during the course of the study. See also the consent form that we ask you to sign.

Withdrawing from the study. You can withdraw from the study at any time and if you wish all information will be destroyed. See also the consent form, which we ask you to sign.

Complaints. We are happy to discuss any complaints that you may have or if you feel that you cannot contact us then you should contact the Research Governance Officer. Contact details are provided at the end of this information sheet.

Risks. The sample collection we ask you to perform is no more than you would carry out normally during the course of cleaning up after your dog and carries no more additional risk.

What next? If you are happy to participate in this study please read, initial and sign the consent form.

Thank you for your time.

Vanessa Schmidt BVSc CertVD DipECVD MRCVS
University of Liverpool, Leahurst Campus, Chester Road, Neston CH64 7TE
vetderm@liv.ac.uk
0151 7956100

If there are any problems, please let us know by contacting Vanessa Schmidt on 0151 7956100, and we will try to help. If you are unhappy or have a complaint which you feel you cannot come to us with then you should contact the Research Governance Officer on 0151 794 8290 (ethics@liv.ac.uk). When contacting the Research Governance Officer, please provide details of the name or description of the study (so that it can be identified), the researcher(s) involved, and the details of the complaint you wish to make.

Version # 3; Created on 24/11/10 at 08.14

Detection of antibiotic resistant bacteria in dogs longitudinal: Questionnaire

Case number:

Please answer all questions to the best of your knowledge.

Name .....

Dogs Name .....

About your dog.

15) Age Years Months Weeks ☐ Don't Know ☐
a) Is this age Exact ☐ Estimate ☐

16) Breed Pedigree *(please specify)* ☐ .....
Cross *(please specify)* ☐ .....

17) Sex Male ☐ Female ☐ Neutered

18) How long have you owned him/her?

About your dog's diet.

19) What is s/he fed? (*Tick all that apply)*
Tinned meat ☐ Dry mixer ☐
Dry Complete ☐ Raw chicken ☐
Cooked chicken ☐ Raw red meat ☐
Cook red meat ☐ Don't Know ☐
Other .....

20) Is s/he fed commercial dog treats? Never ☐ Rarely ☐ Sometimes ☐
Often ☐ Don't know ☐

21) Is s/he fed human titbits/ scraps? Never ☐ Rarely ☐ Sometimes ☐
Often ☐ Don't know ☐

22) Does your dog ever eat stools (faeces)? Never ☐ Rarely ☐ Sometimes ☐
Often ☐ Don't know ☐
If so what types of stools? *(Please tick all that apply)*
☐ Cat ☐ Dog ☐ Horse ☐ Cow ☐ Sheep ☐ Rabbit
Badger ☐ Other .....

About your household.

23) Are there any other dogs in the household?
Yes ☐ No ☐ Don't Know ☐
If yes, how many? 1 ☐ 2 ☐ 3 or more ☐

24) Do you own any other animals (other than dogs)?
Yes ☐ No ☐ Don't Know ☐
If yes, what animals? *(Please tick all that apply)*
Cat ☐ Bird ☐ Rabbit ☐
Rodent *(e.g. hamster)* ☐ Reptile *(e.g. snake)* ☐
Don't know ☐ Other .....

25) Does anyone in your household work with farm animals?
Yes ☐ No ☐ Don't know ☐
If yes, please state which species are worked with .....
.....

26) Has anyone in your family (including other pets) to your knowledge in the last month taken antibiotics?
Yes ☐ No ☐ Don't know ☐

a) If yes, was this a Family Member ☐ Pet ☐

b) Which antibiotic was prescribed *(if known)* .....
.....

27) Does anyone in your household work in medical or veterinary healthcare?
Yes No ☐ Don't Know ☐

a) If yes, in what setting? Hospital ☐ Community Nursing ☐
GP surgery ☐ Nursing Home ☐
Dentist ☐ Veterinary practice ☐
Don't Know ☐ Other .....

28) Has anyone in your household attended hospital in the last month?
Yes ☐ No ☐ Don't Know ☐

a) If yes, why? Admission to hospital ☐ Visit ☐
Outpatient appointment ☐ Don't Know ☐
Other .....

Thank you for your time.

Version # 3; Created on 24/11/10 at 09.00

Case number:

Detection of antibiotic resistant bacteria in dogs longitudinal follow-up: Questionnaire
Please answer all questions to the best of your knowledge. If you are uncertain of any of the answers please indicate. You may decline to answer any question.

Dog's Name: .....

Owner's name: .....

1) Has your dog visited your own veterinary surgeon since the first faecal sample?
Yes ☐ No ☐ don't know ☐

a. What was the date of the visit (s) .....
.....
.....
.....

b. What was the problem/diagnosis? .....
.....
.....
.....

c. Was an antibiotic prescribed? Yes ☐ No ☐ Don't know ☐

i. If yes, what was prescribed? *(if known, please provide details of drug name and dose)* .....
.....
.....

ii. For how long was the medication prescribed?

One off injection ☐ Up to 5 days ☐ Up to 10 days ☐

Up to 2 weeks ☐ Up to 3 weeks ☐ Over 3 weeks ☐

Don't know ☐ Other .....

d. Was your dog left at the vet premises? Yes ☐ No ☐ Don't know ☐

i. If yes, for how long was s/he hospitalised? ..... Don't know ☐

2) Has your dog's diet changed or has she / he eaten anything out of the ordinary since the first sample was taken?
Yes ☐ No ☐ don't know ☐

Please give details with dates: .....
.....
.....
.....
.....

3) Have any member of your family or other pets visited hospital since the last questionnaire?
Yes ☐ No ☐ don't know ☐

Please give details with dates: .....
.....
.....
.....

4) Have any member of your family or other pets received antibiotics since the last questionnaire?
Yes ☐ No ☐ don't know ☐

Please give details with dates:

Thank you for your time.

Version # 3; Created on 24/11/10 at 9.30

Table 5-1. Multilevel, univariable models for outcomes AMR, MDR and ESBL/AmpC-producing *E. coli*

	AMR			MDR			ESBL/AmpC		
Variable	**OR**	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**
Time	1.01	1-1.02	*0.113	0.99	0.98-1.01	0.309	0.99	0.91.01	0.383
Gender (male REF)	1.02	0.5-2.2	0.965	0.95	0.37-2.5	0.920	0.64	0.14-2.96	0.564
Breed (purebreed REF)	1.57	0.6-4.13	0.362	1.54	0.52-4.6	0.437	1.57	0.51-4.79	0.431
Weight small	REF	—	—	REF	—	—	REF	—	—
Weight medium	0.41	0.1-1.7	0.213	0.70	0.16-2.99	0.632	0.57	0.07-4.69	0.605
Weight large	2.34	0.45-11.97	0.308	1.87	0.38-9.3	0.444	6.49	0.44-95.6	0.173
Weight overall	—	—	*0.172	—	—	0.580	—	—	0.263
Age	0.99	0.98-1.01	0.384	1.00	0.98-1.01	0.963	0.98	0.97-1	0.119
Dog eats tinned food	3.45	0.5-22.5	*0.195	1.88	0.35-10.13	0.464	1.91	0.09-40.95	0.680
Dog eats treats	2.57	0.43-15.2	0.298	5.46	0.85-34.98	*0.073	5.98	0.21-166.27	0.292
Dog fed table scraps	6.66	0.95-46.6	*0.056	2.66	0.34-20.8	0.351	2.73	0.47-15.8	0.264
Dog eats raw meat	11.23	0.8-149.4	*0.067	91.92	8.52-991.9	*0.000	70.10	2.31-2126.9	*0.015
Dog eats carcass	0.75	0.26-2.2	0.603	1.30	0.42-4.05	0.653	2.76	0.77-9.9	*0.120
Dog eats faeces	1.42	0.5-3.8	0.481	2.29	0.68-7.7	*0.180	2.30	0.49-10.8	0.290
Multi-dog household	1.60	0.3-8.3	0.577	1.22	0.24-6.2	0.809	11.58	0.53-251.3	*0.119
Multi-animal household (not dog)	1.60	0.3-8.3	0.577	1.22	0.2-7.4	0.827	0.68	0.02-20	0.824
Owner works in health care	0.30	0.05-1.96	*0.207	2.84	0.4-19.86	0.294	1.00	0.04-27.2	1.000
In contact hospital admission[1]	0.30	0.05-1.96	*0.207	4.04	1-16.28	*0.050	4.79	0.31-74.54	0.263
In contact had antimicrobials[1]	1.24	0.22-6.8	0.809	2.28	0.49-10.49	0.291	5.50	0.31-97.1	*0.244
Owner works with farm animals	7.79	1.15-52.7	*0.035	2.26	0.41-12.4	0.347	3.19	0.11-94.2	0.502

[1]Within 12 months or during enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the Wald chi-squared test; REF = reference category; *$P < 0.25$ included in multivariable model

Figure 5-1. Residuals plot for AMR (antimicrobial resistance to at least one isolate) multilevel, multivariable model (n = 21 households)

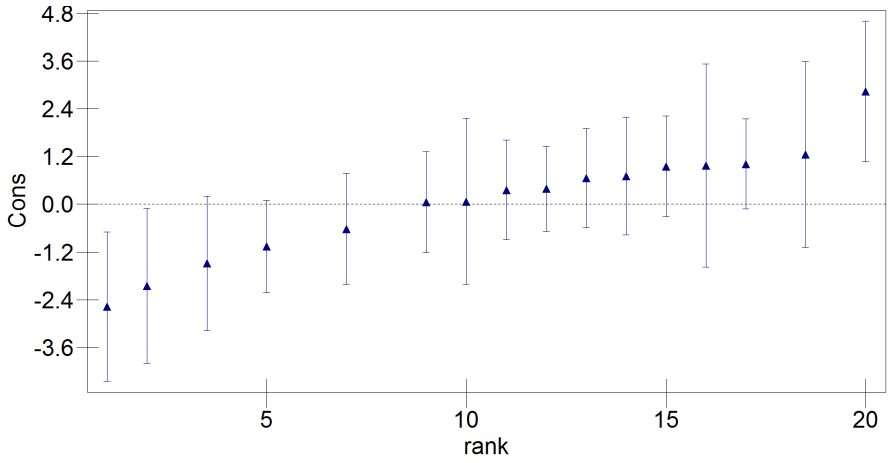


Figure 5-2. Residuals plot for AMR multilevel, multivariable model (n = 28 dogs)

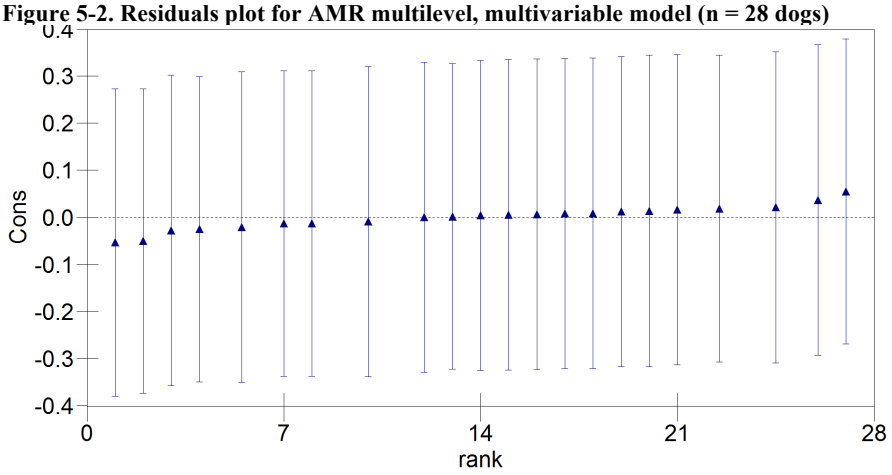


Figure 5-3. Residuals plot for MDR (multidrug resistance to ≥ 3 antimicrobial classes) multilevel, multivariable model (n = 21 households)

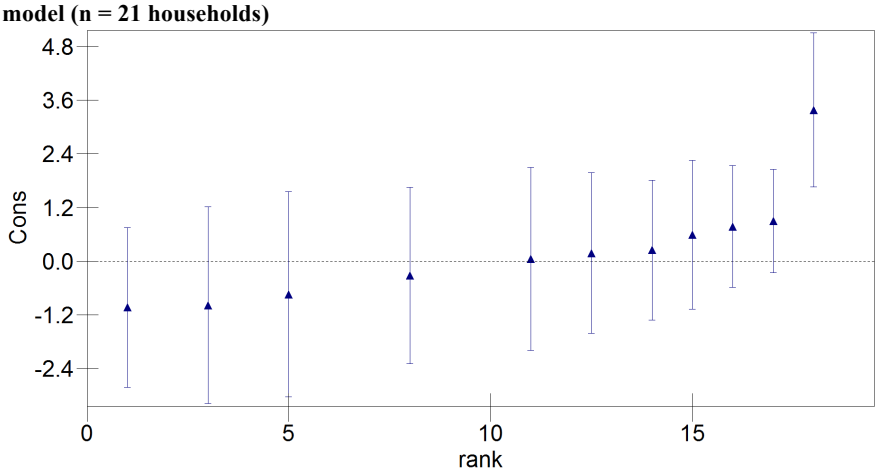


Figure 5-4. Residuals plot for MDR multilevel, multivariable model (n = 28 dogs)

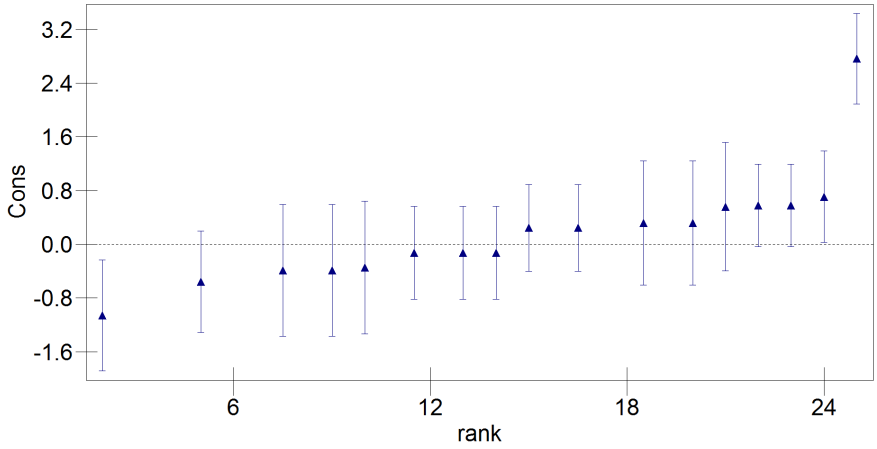


Figure 5-5. Residuals plot for ESBL/AmpC-producing *E. coli* multilevel, multivariable model (n = 21 households)

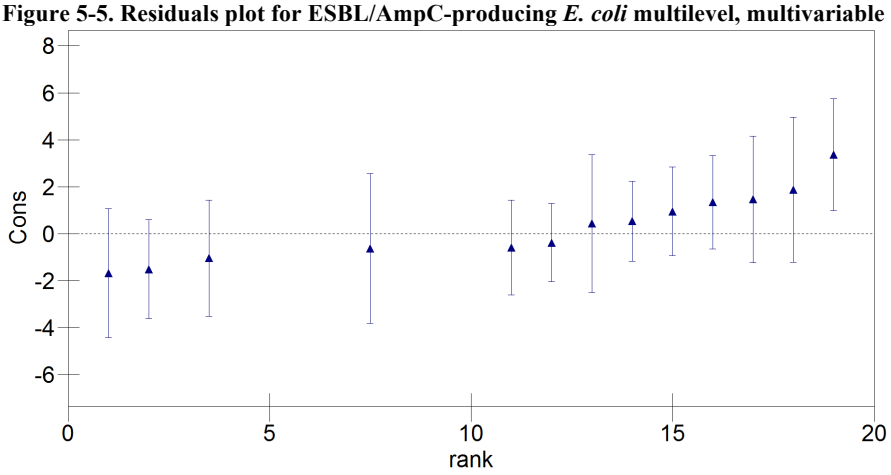
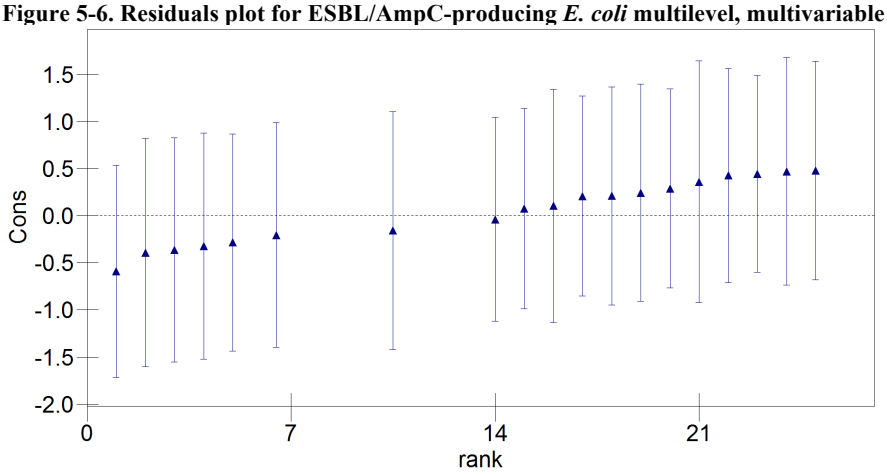


Figure 5-6. Residuals plot for ESBL/AmpC-producing *E. coli* multilevel, multivariable model (n = 28 dogs)



APPENDIX IV
(Pertaining to chapter 6)

Detection of antibiotic resistant bacteria in canine faecal samples and mucosal swabs before and after treatment with antibiotics

Dear Client: Your dog has been chosen as an eligible candidate for our study as she/he has been diagnosed with an infection ..... that requires treatment with systemic (oral or injectable) antibiotics. We would be very grateful if you could fill in a short questionnaire detailing your dogs' environment, diet and previous veterinary treatments along with any antibiotic treatment of in-contact animals and people in the household that could influence the findings in your dog. We ask for your contact details so that we can keep in contact with you during the study and these will remain **confidential**. The information that you give in the questionnaire will be **anonymous**. Please read below for further information on this study and please feel free to ask us for further information or you can contact me at the contact details below.

Why are we doing this study? Dogs have bacterial flora on their mucous membranes (mouth, nose, rear end) and in their gut (faeces/stool). We know that the bacterial flora carried by one member of a household may be shared with the other members i.e. between dogs and people and vice-versa. We also know that the use of antibiotics may increase the spread of antibiotic resistance. We are monitoring the bacteria carried by dogs for the presence of antibiotic resistance and for the changes that occur in these bacterial populations in response to routine antibiotic therapy. We are also very interested to see what happens 60 days after the antibiotics have finished i.e. does the bacterial flora go back to normal? In summary we are interested in the safe and appropriate use of antibiotics to limit the spread of antibiotic resistance in the community.

What does the study involve? Your participation is voluntary and you can decline involvement at any time. Firstly it involves answering the questionnaire which will take ~ 10 minutes. We would like to take samples (see below for details) from your dog to collect bacteria before and after the antibiotic course that she/he is due to receive. That means we would like to sample your dog **today** and again when you bring she/he back for re-examination in ..... **weeks**. We would also like to repeat the sampling one more time in **two months (60 days)** after the antibiotic course has finished. This is important to see if the bacteria are recovering. There will be three options for the final sampling (see below).

Antibiotic treatment: If you agree to participate in the study we will randomly assign your dog to receive one of five different UK licensed antibiotics, which are appropriate treatment for your dog's infection. The antibiotic course will be **free of charge**. We would ask you to strictly follow the directions given for the antibiotics. Some dogs may develop gastro-intestinal upset (vomiting, diarrhoea, inappetance) when receiving any antibiotic. Please contact us if this occurs so that we can offer advice. One of the antibiotics is an injection that is given every two weeks. If your dog is assigned to this particular antibiotic and the required length of the antibiotic course is longer than two weeks we would ask that you visit your normal veterinary surgery for repeat injections every two weeks. We will contact your veterinary surgeon with this information.

What next? If you are happy to participate in this study please read and sign the consent form. We will then ask you to fill in the questionnaire while we are taking the mucosal swab samples from your dog (this should only take 5 minutes). We will then dispense and explain the antibiotic course.

Mucosal samples: We will take a swab from each of your dog's nostrils (5 seconds each) and from the rear end (5 seconds). This will be repeated at the next re-examination (at the end of the antibiotic course) and again in 60 days after finishing the antibiotics.

Faecal sample: After leaving us, we would ask that you collect the **very next stool sample** i.e. before the antibiotic tablets are started. Please collect the sample into the faecal collection pot and place in the postage paid/addressed envelope to be posted back to us as soon as possible. We will also supply non-powdered, non-sterile rubber gloves. We will supply extra collection pots, gloves and packaging so that you may collect a sample on the **final day of antibiotic therapy** i.e. the day of your next re-examination appointment and **60 days after the antibiotics have finished**. Please bring the stool sample with you to the next appointment. For the third sample please bring the stool sample with you on your appointment date or alternatively please post it to us as soon as possible (see below).

Options for the final sampling (60 days after the antibiotic course is finished):

1. If your dog has a re-examination appointment with us then we can take the mucosal samples while you wait. Again we would ask if you could bring a fresh faecal (stool) sample in for this appointment.
2. If your dog does not need to be re-examined on the date of the 3rd collection then you can either:
    - Make an appointment with Vanessa or the dermatology nurse to come into the hospital free of charge for mucosal sample collection. Please bring the stool sample will you.
    - Visit your own veterinary surgery to have the mucosal samples taken. We would ask if you could post both the mucosal swabs and stool sample into us in the packing that we provide. If you choose this option we will contact your veterinary surgery to arrange this for you.
    - Take the mucosal samples yourself (as demonstrated in the first and second consultations) and collect the faecal sample and post all the samples to us in the packing that we provide. If you choose this option we will also supply you with a demonstration card on how to take the mucosal samples. Please contact us at any stage if you have any questions.

As a gesture of our appreciation for participating in our study we offer discounted flea prevention for your dog. We will dispense or post this product to you once we have received our final samples.

Thank you for your time and co-operation.

Vanessa Schmidt BVSc CertVD DipECVD MRCVS
University of Liverpool
Leahurst Campus
Chester Road
Neston
CH64 7TE
vetderm@liv.ac.uk
0151 7956100

If there are any problems, please let us know by contacting Vanessa Schmidt on 0151 7956100, and we will try to help. If you remain unhappy or have a complaint which you feel cannot come to us with then you should contact the Research Governance Officer on 0151 794 8290 (ethics@liv.ac.uk). When contacting the Research Governance Officer, please provide details of the name or description of the study (so that it can be identified), the researcher(s) involved, and the details of the complaint you wish to make.

Version # 1; Created on 30/03/2014 11:28

Detection of antibiotic resistant bacteria in dogs with bacterial infections before and after treatment with antibiotics: Questionnaire

Case number:

Please answer all questions to the best of your knowledge.

About you (owner or carer)

Could you please provide us with your contact details?
(This information will be held in the strictest of confidence)

Name .....

Address .....

Post Code .....

Contact Telephone Number .....

Contact Email .....

Dogs Name .....

Date of visit .....

About your dog.

1) Age Years ☐ Months ☐ Weeks ☐ Don't Know ☐
   a. Is this age Exact ☐ Estimate ☐

2) Breed Pedigree *(please specify)* ☐
   Cross *(please specify)* ☐ .....

3) Sex Male ☐ Female ☐ Neutered ☐

4) How long have you owned him/her? .....

About your dog's diet.

5) What is s/he fed? (*Tick all that apply)*
   Tinned meat ☐ Dry mixer ☐
   Dry Complete ☐ Raw chicken ☐
   Cooked chicken ☐ Raw red meat ☐
   Cook red meat ☐ Don't Know ☐
   Other .....

6) Is s/he fed commercial dog treats? Never ☐ Rarely ☐ Sometimes ☐
   Often ☐ Don't know ☐

7) Is s/he fed human titbits/ scraps? Never ☐ Rarely ☐ Sometimes ☐
   Often ☐ Don't know ☐

8) Does your dog ever eat stools (faeces)? Never ☐ Rarely ☐ Sometimes ☐
   Often ☐ Don't know ☐

If so what types of stools? *(Please tick all that apply)*

Rabbit ☐ Cat ☐ Dog ☐

Horse ☐ Cow ☐ Sheep ☐ Badger ☐ Other

About your dog's health.

9) Has your dog received any veterinary treatment (other than today) in the last 12 months

Yes ☐ No ☐ don't know ☐

a. Please provide further details

---

b. Was any medication prescribed? Yes ☐ No ☐ Don't know ☐

c. Was it an antibiotic? Yes ☐ No ☐ Don't know ☐

i. If yes, what was prescribed? *(if known, please provide details of drug name and dose)*

---

ii. For how long was the medication prescribed?

One off injection ☐ Up to 5 days ☐ Up to 10 days ☐

Up to 2 weeks ☐ Up to 3 weeks ☐ Over 3 weeks ☐

Don't know ☐ Other

d. Was your dog left at the vet premises? Yes ☐ No ☐ Don't know ☐

i. If yes, for how long was s/he hospitalised? ☐ Don't know ☐

About your household.

10) Are there any other dogs in the household?

Yes ☐ No ☐ Don't Know ☐

If yes, how many? 1 ☐ 2 ☐ 3 or more ☐

11) Do you own any other animals (other than dogs)?

Yes ☐ No ☐ Don't Know ☐

If yes, what animals? *(Please tick all that apply)*

Cat ☐ Bird ☐ Rabbit ☐

Rodent *(e.g. hamster)* ☐ Reptile *(e.g. snake)* ☐

Don't know ☐ Other

12) Does anyone in your household work with farm animals?

Yes ☐ No ☐ Don't know ☐

If yes, please state which species are worked with

---

13) Has anyone in your family (including other pets) to your knowledge in the last month taken antibiotics?

Yes ☐ No ☐ Don't know ☐

a. If yes, was this a Family Member ☐ Pet ☐

b. Which antibiotic was prescribed *(if known)* .....

.....

.....

13) Does anyone in your household work in medical or veterinary healthcare?

Yes ☐ No ☐ Don't Know ☐

a. If yes, in what setting? Hospital ☐ Community Nursing ☐
GP surgery ☐ Nursing Home ☐
Dentist ☐ Veterinary practice ☐
Don't Know ☐ Other .....

14) Has anyone in your household attended hospital in the last month?

Yes ☐ No ☐ Don't Know ☐

a. If yes, why? Admission to hospital ☐ Visit ☐
Outpatient appointment ☐ Don't Know ☐
Other .....

Thank you for your time.

Version # 1: Created on 13th March 2011 at 19.30

Detection of antibiotic resistant bacteria in dogs with bacterial infections before and after treatment with antibiotics: Follow-up sample questionnaire

Case number:

Please answer all questions to the best of your knowledge. If you are uncertain of any of the answers please indicate. You may decline to answer any question.

Date swab sample was obtained: .....

Date faecal sample was obtained: .....

Dog's Name: .....

Owner's name: .....

5) Has your dog visited your own veterinary surgeon since the last samples (swabs and/or faeces) were taken?

Yes ☐ No ☐ don't know ☐

a. What was the problem / diagnosis? .....

.....

.....

.....

b. Was an antibiotic prescribed? Yes ☐ No ☐

Don't know ☐

i. If yes, what was prescribed? *(if known, please provide details of drug name and dose)* .....

.....

.....

ii. For how long was the medication prescribed?

One off injection ☐ Up to 5 days ☐ Up to 10 days ☐

Up to 2 weeks ☐ Up to 3 weeks ☐ Over 3 weeks ☐

Don't know ☐ Other .....

c. Was your dog left at the vet premises? Yes ☐ No ☐

Don't know ☐

i. If yes, for how long was s/he hospitalised? .....

Don't know ☐

6) Has your dog's diet changed or has she / he eaten anything out of the ordinary since the last samples (swabs and/or faeces) were taken?

Yes ☐ No ☐ don't know ☐

Please give details: .....

.....

.....

7) Have any member of your family or other pets visited hospital since the last samples (swabs and/or faeces) were taken?

Yes ☐ No ☐ don't know ☐

Please give details: .....

.....

.....

8) Have any member of your family or other pets received antibiotics since the last samples (swabs and/or faeces) were taken?

Yes ☐ No ☐ don't know ☐

Please give details: .....

.....

.....

Thank you for your time.

Version # 1: Created on 13th March 2011 at 20.00

Table 6-1. Number and percentage (%) of samples with oxacillin resistant staphylococci or oxacillin resistant CoPS at each time point for each treatment group and for treatment overall (95% CI).

		Oxacillin resistant staphylococci		Oxacillin resistant CoPS	
Antimicrobial treatment group	Time Point (Total samples)	Number (%) positive samples	95% CI	Number (%) positive samples	95% CI
CFX	D0 (n = 31)	7 (23)	7.9-37.3	0	—
	E (n = 31)	11 (35)	18.64-52.33	3 (10)	0-20
	M1 (n = 26)	7 (27)	9.87-43.97	1 (4)	0-11.24
	M3 (n = 24)	9 (38)	18.13-56.87	1 (4)	0-12.16
AC	D0 (n = 29)	6 (21)	5.95-35.43	2 (7)	0-16.12
	E (n = 29)	5 (17)	7.60-34.5	2 (7)	0-16.12
	M1 (n = 27)	5 (19)	3.87-22.17	0	—
	M3 (n = 25)	5 (20)	4.32-35.68	0	—
CVN	D0 (n = 26)	6 (23)	6.88-39.27	1 (4)	0-11.24
	E (n = 26)	11 (42)	23.32-61.3	4 (15)	1.5-29.25
	M1 (n = 20)	6 (30)	9.92-50.08	1 (5)	0-14.55
	M3 (n = 21)	6 (29)	9.25-47.9	2 (10)	0. -22.1
CD	D0 (n = 28)	7 (25)	8.96-41.04	0	—
	E (n = 28)	6 (21)	6.23-36.63	1 (4)	0-10.45
	M1 (n = 24)	9 (28)	18.13-56.87	2 (8)	0 -19.39
	M3 (n = 24)	7 (29)	10.98-47.35	1 (4)	0-12.16
FL	D0 (n = 13)	4 (31)	5.68-55.86	2 (15)	0-35
	E (n = 13)	8 (62)	35.09-87.99	1 (8)	0-22.18
	M1 (n = 9)	4 (44)	11.98-76.91	1 (11)	0-31.64
	M3 (n = 9)	5 (56)	23.09-88.02	1 (11)	0-31.64
Total	D0 (n = 127)	30 (23)	16.23-31	5 (4)	0.55-7.3
	E (n = 127)	42 (33)	24.89-41.25	11 (9)	3.77-13.55
	M1 (n = 106)	31 (29)	20.59-37.91	5 (5)	0.68-8.75
	M3 (n = 103)	32 (31)	22.13-40	5 (5)	0.70-9

95% CI = 95% confidence intervals; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone; Total = treatment overall; D0 = baseline day zero; E = end of therapy; M1 = one month after therapy; M3 = three months after therapy; CoPS = coagulase positive staphylococcci

Table 6-2. Number and percentage (%) of samples with *mec*A positive staphylococci or *mec*A positive CoPS at each time point for each treatment group and for treatment overall (95% CI).

		***mec*A positive staphylococci**		***mec*A positive CoPS**	
Antimicrobial treatment group	Time Point (Total samples)	Number (%) positive samples	95% CI	Number (%) positive samples	95% CI
CFX	D0 (n = 31)	6 (19)	5.45-33.26	0	—
	E (n = 31)	10 (32)	15.80-48.7	0	—
	M1 (n = 26)	8 (31)	13.03-48.5	2 (8)	0-17.94
	M3 (n = 24)	8 (33)	14.47-52.19	2 (8)	0-19.39
AC	D0 (n = 29)	3 (10)	0.00-21.43	1 (3)	0-10.09
	E (n = 29)	5 (17)	3.49-30.99	1 (3)	0-10.09
	M1 (n = 27)	4 (15)	1.41-29.2	0	—
	M3 (n = 25)	6 (24)	7.26-40.74	0	—
CVN	D0 (n = 26)	6 (23)	6.88-39.27	1 (4)	0-11.24
	E (n = 26)	10 (38)	19.76-57.16	3 (12)	0-23.8
	M1 (n = 20)	4 (20)	2.47-37.53	1 (5)	0-14.55
	M3 (n = 21)	5 (24)	5.59-42.03	2 (10)	0-22.08
CD	D0 (n = 28)	7 (25)	8.96-41.04	0	—
	E (n = 28)	5 (18)	3.67-32.04	0	—
	M1 (n = 24)	7 (29)	10.98-47.35	1 (4)	0-12.16
	M3 (n = 24)	6 (25)	7.68-42.32	1 (4)	12.16
FL	D0 (n = 13)	4 (31)	5.68-55.86	1 (8)	0-22.18
	E (n = 13)	9 (69)	44.14-94.32	1 (8)	0-22.18
	M1 (n = 9)	4 (44)	11.98-76.9	1 (11)	0-31.64
	M3 (n = 9)	1 (11)	0.00-31.64	0	—
Total	D0 (n = 127)	26 (20)	13.45-27.49	3 (2)	0
	E (n = 127)	39 (31)	22.69-38.73	5 (4)	0.55-7.32
	M1 (n = 106)	27 (25)	17.18-33.77	5 (5)	0.68-8.75
	M3 (n = 103)	26 (25)	16.85-33.63	5 (5)	0.70-9

95% CI = 95% confidence intervals; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone; Total = treatment overall; D0 = baseline day zero; E = end of therapy; M1 = one month after therapy; M3 = three months after therapy; CoPS = coagulase positive staphylococci; *mec*A = oxacillin resistant staphylococci carrying the *mec*A gene

Table 6-3. Number and percentage (%) of samples with MDR staphylococci or MDR CoPS at each time point for each treatment group and for treatment overall (95% CI).

		MDR staphylococci		*mec*A positive MDR staphylococci		CipR & MDR staphylococci	
Antimicrobial treatment group	Time Point (Total samples)	Positive number of samples	Lower 95% CI	Positive number of samples	Lower 95% CI	Positive number of samples	Lower 95% CI
CFX	D0 (n = 31)	2 (6)	0.00-15	2 (6)	0.00-15	2 (6)	0-15
	E (n = 31)	9 (29)	13-45	7 (23)	7.86-37.3	8 (26)	10.4-41.2
	M1 (n = 26)	7 (27)	9.87-43.97	7 (27)	9.87-43.97	7 (27)	9.87-43.97
	M3 (n = 24)	9 (38)	18.13-56.87	6 (25)	7.68-42.32	5 (21)	4.59-37.08
AC	D0 (n = 29)	4 (14)	1.24-26.34	2 (7)	0.00-16.2	2 (7)	0-16.12
	E (n = 29)	4 (14)	1.24-26.34	3 (10)	0.00-21.43	2 (7)	0-16.12
	M1 (n = 27)	3 (11)	0-22.97	1 (4)	0.00-10.83	2 (7)	0-17.3
	M3 (n = 25)	4 (16)	1.63-30.37	4 (16)	1.63-/37	4 (16)	0.00-30.4
CVN	D0 (n = 26)	12 (46)	27-65.3	4 (15)	1.5-29.25	4 (15)	1.52-29.25
	E (n = 26)	10 (38)	19.76-57.16	6 (23)	6.88-39.27	5 (19)	4.08-34.38
	M1 (n = 20)	6 (30)	9.92-50	3 (15)	0.00-30.65	3 (15)	0-30.65
	M3 (n = 21)	7 (33)	13.17-53.5	3 (14)	0.00-29.25	2 (10)	0-22.08
CD	D0 (n = 28)	7 (25)	8.96-41	4 (14)	1.32-27.25	5 (18)	3.67-32
	E (n = 28)	5 (28)	3.67-32	2 (7)	0.00-16.68	2 (7)	0-16.68
	M1 (n = 24)	9 (38)	18.13-56.87	6 (25)	7.68-42.3	6 (25)	7.68-42.32
	M3 (n = 24)	6 (25)	7.68-42.3	4 (17)	1.76-31.58	4 (17)	1.76-31.58
FL	D0 (n = 13)	5 (38)	12.01-64.9	3 (23)	0.17-45.98	3 (23)	0-45.98
	E (n = 13)	9 (69)	44.14-94.3	8 (62)	35.09-88	8 (62)	35.09-87.99
	M1 (n = 9)	5 (56)	23.09-88	4 (44)	11.98-76.9	4 (44)	11.98-76.9
	M3 (n = 9)	4 (44)	11.98-76.9	0	—	0	—
Total	D0 (n = 127)	30 (24)	16.23-31	15 (12)	6.20-17.42	16 (13)	6.83-18.4
	E (n = 127)	37 (29)	21.23-37	26 (20)	13.45-27.49	25 (20)	12.77-26.6
	M1 (n = 106)	30 (28)	19.73-36.9	21 (20)	12.22-27.4	22 (21)	13.03-28.48
	M3 (n = 103)	30 (29)	20.35-37.9	17 (17)	9.34-23.67	15 (15)	7.75-21.38

95% CI = 95% confidence intervals; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone; Total = treatment overall; D0 = baseline day zero; E = end of therapy; M1 = one month after therapy; M3 = three months after therapy; CoPS = coagulase positive staphylococci; MDR = multi-drug resistant (≥ 3 drug classes); CipR = ciprofloxacin resistant; *mec*A = oxacillin resistant staphylococci carrying the *mec*A gene

Table 6-4. Number and percentage (%) of samples with ciprofloxacin resistant staphylococci or *mec*A positive ciprofloxacin resistant staphylococci at each time point for each treatment group and for treatment overall (95% CI).

		CipR		CipR & *mec*A	
Antimicrobial treatment group	Time Point (Total samples)	Positive number of samples	Lower 95% CI	Positive number of samples	Lower 95% CI
CFX	D0 (n = 31)	4 (13)	1-24.7	2 (6)	0-15
	E (n = 31)	6 (19)	5.45-33.26	5 (16)	3.18
	M1 (n = 26)	7 (27)	9.87-43.97	4 (15)	1.5
	M3 (n = 24)	4 (17)	1.76-31.58	3 (13)	0-25.7
AC	D0 (n = 29)	4 (14)	1.24-26.34	1 (3)	0-10
	E (n = 29)	3 (10)	0-21.34	2 (7)	0-16.12
	M1 (n = 27)	1 (4)	0-10.83	1 (4)	0-10.8
	M3 (n = 25)	3 (12)	0-24.74	3 (12)	0-24.7
CVN	D0 (n = 26)	5 (19)	4-34.38	2 (8)	0-17.9
	E (n = 26)	7 (17)	9.87-43.97	4 (15)	1.5-29.25
	M1 (n = 20)	3 (15)	0-30.65	0	—
	M3 (n = 21)	2 (10)	0-22	0	—
CD	D0 (n = 28)	5 (18)	3.67-32	4 (14)	1.3-27.25
	E (n = 28)	2 (7)	0-16.68	0	—
	M1 (n = 24)	5 (21)	4.59-37	4 (17)	1.76-31.6
	M3 (n = 24)	6 (25)	7.68-42.3	4 (17)	1.76-31.6
FL	D0 (n = 13)	2 (15)	0-35	2 (15)	0-35
	E (n = 13)	6 (46)	19-73.25	5 (38)	12-65
	M1 (n = 9)	4 (44)	11.98-76.9	3 (33)	2.5-64
	M3 (n = 9)	2 (22)	0-49.4	0	—
Total	D0 (n = 127)	20 (16)	9.4-22	11 (9)	3.8-13.55
	E (n = 127)	24 (19)	12-25.7	15 (12)	6.2-17.4
	M1 (n = 106)	20 (19)	11.42-26.3	12 (11)	5.3-17.35
	M3 (n = 103)	17 (17)	9.34-23.7	10 (10)	4-15.4

95% CI = 95% confidence intervals; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone; Total = treatment overall; D0 = baseline day zero; E = end of therapy; M1 = one month after therapy; M3 = three months after therapy; CoPS = coagulase positive staphylococci; CipR = ciprofloxacin resistant; *mec*A = oxacillin resistant staphylococci carrying the *mec*A gene

Table 6-5. Number and percentage (%) of CoPS or CoNS at each time point for each treatment group and for treatment overall (95% CI).

		CoPS		CoNS	
Antimicrobial treatment group	Time Point (Total samples)	Number (%) positive samples	95% CI	Number (%) positive samples	95% CI
CFX	D0 (n = 31)	21 (68)	51.29-84.20	19 (62)	44.14-78.44
	E (n = 31)	14 (45)	27.60-62.68	17 (55)	37.32-72.36
	M1 (n = 26)	18 (69)	51.49-86.97	18 (69)	51.49-86.97
	M3 (n = 24)	19 (79)	62.90-95.41	14 (58)	38.61-78.06
AC	D0 (n = 29)	17 (59)	40.70-76.55	14 (48)	30.09-66.46
	E (n = 29)	17 (59)	40.70-76.55	21 (72)	56.15-88.68
	M1 (n = 27)	15 (56)	36.81-74.30	14 (52)	33.00-70.70
	M3 (n = 25)	16 (64)	45.18-82.82	14 (56)	36.54-75.46
CVN	D0 (n = 26)	23 (88)	76.18-100.0	13 (50)	30.78-69.22
	E (n = 26)	15 (58)	38.70-76.68	16 (62)	42.84-80.24
	M1 (n = 20)	15 (75)	56.00-93.98	12 (60)	38.53-81.47
	M3 (n = 21)	16 (76)	57.97-94.41	10 (48)	26.26-68.98
CD	D0 (n = 28)	22 (79)	63.37-93.77	17 (61)	42.62-78.80
	E (n = 28)	6 (21)	6.23-36.63	17 (61)	42.62-78.80
	M1 (n = 24)	11 (46)	25.90-65.77	12 (50)	30.00-70.00
	M3 (n = 24)	15 (63)	43.13-81.87	14 (58)	38.61-78.06
FL	D0 (n = 13)	9 (69)	44.14-94.32	7 (54)	26.75-80.95
	E (n = 13)	7 (54)	26.75-80.95	10 (77)	54.02-99.83
	M1 (n = 9)	8 (89)	68.36-100.0	6 (67)	35.87-97.47
	M3 (n = 9)	5 (56)	23.00-88.02	7 (78)	50.62-104.9
Total	D0 (n = 127)	92 (72)	64.67-80.21	70 (55)	46.47-63.77
	E (n = 127)	59 (46)	37.78-55.13	81 (65)	55.42-72.14
	M1 (n = 106)	67 (63)	54.00-72.39	62 (58)	49.11-67.87
	M3 (n = 103)	71 (69)	59.99-77.87	59 (57)	47.73-66.83

95% CI = 95% confidence intervals; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone; Total = treatment overall; D0 = baseline day zero; E = end of therapy; M1 = one month after therapy; M3 = three months after therapy; CoPS = coagulase positive staphylococci; CoNS = coagulase negative staphylococci

Table 6-6. Number and percentage (%) of CipR CoPS or MDR CoPS at each time point for each treatment group and for treatment overall (95% CI).

		CipR CoPS		MDR CoPS	
Antimicrobial treatment group	Time Point (Total samples)	Number (%) positive samples	95% CI	Number (%) positive samples	95% CI
CFX	D0 (n = 31)	1 (3)	0.00-9.45	0	—
	E (n = 31)	0	—	0	—
	M1 (n = 26)	3 (12)	0.00-23.82	4 (15)	1.52-29.25
	M3 (n = 24)	1 (4)	0.00-12.16	2 (8)	0.00-19.39
AC	D0 (n = 29)	2 (7)	0.00-16.12	3 (10)	0.00-21.43
	E (n = 29)	2 (7)	0.00-16.12	3 (10)	0.00-21.43
	M1 (n = 27)	0	—	1 (4)	0.00-10.83
	M3 (n = 25)	0	—	0	—
CVN	D0 (n = 26)	3 (12)	0.00-23.82	8 (31)	13.03-48.51
	E (n = 26)	4 (15)	1.52-29.25	7 (27)	9.87-43.97
	M1 (n = 20)	2 (10)	0.00-23.15	3 (15)	0.00-30.65
	M3 (n = 21)	2 (10)	0.00-22.08	3 (14)	0.00-29.25
CD	D0 (n = 28)	1 (4)	0.00-10.45	2 (7)	0.00-16.68
	E (n = 28)	0	—	1 (4)	0.00-10.45
	M1 (n = 24)	1 (4)	0.00-12.16	2 (8)	0.00-19.35
	M3 (n = 24)	1 (4)	0.00-12.16	1 (4)	0.00-12.16
FL	D0 (n = 13)	1 (8)	0.00-22.18	2 (15)	0.00-35.00
	E (n = 13)	1 (8)	0.00-22.18	3 (23)	0.17-45.98
	M1 (n = 9)	0	—	2 (22)	0.00-49.38
	M3 (n = 9)	0	—	2 (22)	0.00-49.38
Total	D0 (n = 127)	8 (6)	2.07-10.52	15 (12)	6.20-17.42
	E (n = 127)	7 (6)	1.54-9.48	14 (11)	5.58-16.47
	M1 (n = 106)	6 (6)	1.26-10.06	12 (11)	5.29-17.35
	M3 (n = 103)	4 (4)	0.15-7.61	8 (8)	2.60-12.94

95% CI = 95% confidence intervals; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone; Total = treatment overall; D0 = baseline day zero; E = end of therapy; M1 = one month after therapy; M3 = three months after therapy; CoPS = coagulase positive staphylococci; CipR = ciprofloxacin resistance: MDR = multi-drug resistance (≥ 3 drug classes)

Table 6-7. Multivariable logistic regression results for all baseline outcomes with baseline variables.

Variables	Oxacillin resistance			mecA			CipR		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Age	1.00	0.99-1.02	0.78	1.00	0.99-1.02	0.9	1.00	0.98-1.02	0.9
Weight (large)	REF	—	—	REF	—	—	REF	—	—
Weight (small)	4.3	0.53-35.11	—	1.5	0.18-13.19	—	1.77	0.16-19.26	—
Weight (medium)	1.26	0.22-7.4	—	—	—	—	0.1	0.03-1.4	—
Weight overall	—	—	0.27	—	—	0.044	—	—	0.06
Pyoderma diagnosis	1.3	0.26-6.36	0.76	2.5	0.36-17.24	0.35	0.9	0.08-9.00	0.9
Gender (male REF)	2.3	0.69-7.8	0.17	3.9	0.95-16.8	0.049	7.3	1.4-37.63	0.01
First opinion	REF	—	—	REF	—	—	REF	—	—
Referral consultation	0.98	0.21-4.6	0.98	1.14	0.2-6.57	0.89	3.18	0.3-33.65	0.32
Previous antimicrobial[1]	1.11	0.16-7.72	0.9	3.36	0.4-27.62	0.26	1.2	0.12-12.77	0.87
Previous beta-lactam[1]	1.45	0.2-10.54	0.7	0.86	0.11-6.8	0.88	0.54	0.05-6.09	0.62
Dog eats animal faeces	2.56	0.64-10.24	0.18	3.07	0.59-16.07	0.18	5.14	0.74-35.77	0.085
Owner works in healthcare	0.39	0.09-1.66	0.18	0.63	0.13-3.11	0.57	0.22	0.03-1.67	0.12
In-contact been hospitalised[2]	0.63	0.14-2.9	0.54	0.9	0.16-5.00	0.9	2.00	0.28-14.23	0.49
In-contact had antimicrobials[2]	0.37	0.09-1.6	0.17	0.39	0.06-2.5	0.3	0.4	0.05-3.14	0.37
Previous vet admission[1]	0.24	0.04-1.4	0.096	0.37	0.05-2.77	0.32	1.5	0.11-11.9	0.9
Multi-dog household	0.49	0.13-1.93	0.3	0.43	0.08-2.22	0.3	0.4	0.06-2.9	0.37
	MDR			CoPS			CoNS		
Age	1.00	0.98-1.02	0.9	1.01	0.99-1.02	0.5	1.00	0.99-1.01	0.96
Weight (large)	REF	—	—	REF	—	—	REF	—	—
Weight (small)	0.66	0.06-1.04	—	0.12	0.01-2.05	—	4.9	0.69-35.57	—
Weight (medium)	0.1	0.01-0.8	—	0.35	0.03-4.58	—	2.2	0.46-10.43	—
Weight overall	—	—	0.027	—	—	0.29	—	—	0.26
Pyoderma diagnosis	1.45	0.17-12.45	0.74	1.19	0.18-7.78	0.86	1.2	0.29-4.96	0.79
Gender (male REF)	6.34	1.4-28.27	0.009	1.33	0.32-5.5	0.69	2.8	0.95-8.49	0.05
First opinion	REF	—	—	REF	—	—	REF	—	—
Referral consultation	5.9	0.8-42.86	0.06	2.5	0.33-18.98	0.38	0.97	0.24-3.92	0.97
Previous antimicrobial[1]	3.8	0.4-32.97	0.23	3.38	0.27-42.36	0.3	0.27	0.05-1.55	0.13
Previous beta-lactam[1]	0.3	0.04-2.65	0.28	0.65	0.05-8.83	0.74	5.8	0.89-37.62	0.056
Dog eats animal faeces	1.8	0.34-9.78	0.49	0.78	0.14-4.5	0.78	1.7	0.5-6.1	0.37
Owner works in healthcare	0.63	0.12-3.4	0.58	—	—	0.003	0.64	0.2-2.2	0.47
In-contact been hospitalised[2]	1.69	0.29-10.00	0.57	0.6	0.09-3.9	0.59	0.44	0.11-1.8	0.25
In-contact had antimicrobials[2]	0.5	0.08-3.27	0.47	0.54	0.11-2.7	0.46	1.4	0.39-5.2	0.6
Previous vet admission[1]	0.4	0.05-3.6	0.42	0.43	0.06-3.12	0.4	1.3	0.29-5.67	0.75
Multi-dog household	1.08	0.2-5.5	0.93p	1.17	0.2-6.3	0.86	0.67	0.19-2.39	0.53

[1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values Likelihood ratio test; *mec*A = oxacillin resistant staphylococci with *mec*A gene; CipR = ciprofloxacin resistant; MDR = multi-drug resistant (≥ 3 drug classes); CoPS = coagulase positive staphylococci; CoNS = coagulase negative

Table 6-8. Multivariable logistic regression results for baseline outcomes with baseline variables and treatment.

Variables	Oxacillin resistance			*mec*A			CipR		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Treatment CD	REF	—	—	REF	—	—	REF	—	—
Treatment CFX	0.7	0.11-4.5	—	1.4	0.17-11.9	—			—
Treatment AC	1.18	0.15-9.2	—	0.	0.01-2.6	—			—
Treatment CVN	1.1	0.13-9.2	—	3.7	0.3-44.15	—			—
Treatment Fl	0.57	0.05-5.5	—	2.03	0.13-30.6	—			—
Treatment overall	—	—	0.95	—	—	0.35	—	—	
Age	1.0	0.99-1.02	0.8	1.00	0.98-1.01	0.73	1.00	0.98-1.03	0.8
Weight (large)	REF	—	—	REF	—	—	REF	—	—
Weight (small)	5.3	0.58-48.27	—	2.00	0.2-19.68	—	2.36	0.07-82.19	—
Weight (medium)	1.34	0.19-9.6	—	0.24	0.03-2.08	—	0.09	0.01-1.46	—
Weight overall	—	—	0.22	—	—	0.087	—	—	0.024
Pyoderma diagnosis	1.67	0.22-12.49	0.62	0.9	0.09-9.07	0.9	1.8	0.09-34.5	0.7
Gender (male REF)	2.3	0.67-7.9	0.18	5.00	1.07-23.27	0.03	9.1	66.99	0.015
First opinion	REF	—	—	REF	—	—	REF	—	—
Referral consultation	0.9	0.16-5.6	0.95	0.7	0.1-5.37	0.75	4.23	0.26-69.97	0.3
Previous antimicrobial[1]	0.93	0.11-7.7	0.95	3.4	0.33-35.27	0.3	0.38	0.01-13.12	0.6
Previous beta-lactam[1]	1.8	0.2-16.87	0.6	1.44	0.12-17.00	0.77	0.84	0.02-29.74	0.9
Dog eats animal faeces	2.9	0.62-13.3	0.17	3.4	0.46-25.00	0.22	20.9	1.2-367.9	0.02
Owner works in healthcare	0.38	0.08-1.75	0.2	0.44	0.08-2.48	0.34	0.24	0.02-3.65	0.27
In-contact been hospitalised[2]	0.62	0.13-2.85	0.53	0.94	0.14-6.3	0.95	2.5	0.27-23.6	0.4
In-contact had antimicrobials[2]	0.39	0.09-1.7	0.2	0.22	0.02-1.94	0.14	0.58	0.05-5.33	0.57
Previous vet admission[1]	0.23	0.04-1.3	0.08	0.36	0.04-3.1	0.34	1.15	0.08-16.42	0.9
Multi-dog household	0.5	0.11-2.22	0.4	0.2	0.03-1.45	0.097	0.13	0.01-1.38	0.06

[1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the Likelihood ratio test; *mec*A = oxacillin resistant staphylococci carrying the *mec*A gene; CipR = ciprofloxacin resistant

Table 6-9. Multivariable logistic regression results for baseline outcomes third generation cephalosporin resistance (3GR) and phenotypic ESBL- and AmpC-producing *E. coli* with baseline covariates and treatment.

Variables	MDR			CoPS			CoNS		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Treatment CD	REF	—	—	REF	—	—	REF	—	—
Treatment CFX	1.6	0.12-22.37	—	38.53	0.55-2693.29	—	0.49	0.09-2.84	—
Treatment AC	9.4	0.467-187.78	—	35.75	0.76-1679.84	—	0.39	0.06-2.66	—
Treatment CVN	22.34	0.88-566.74	—	11.76	0.29-479.11	—	0.52	0.07-3.88	—
Treatment Fl	10.08	0.58-174.83	—	0.71	0.03-17.17	—	0.8	0.07-9.15	—
Treatment overall	—	—	0.107	—	—	0.115	—	—	0.88
Age	1.00	0.98-1.02	0.97	1.00	0.99-1.03	0.44	1.00	0.99-1.01	0.89
Weight (large)	REF	—	—	REF	—	—	REF	—	—
Weight (small)	0.8	0.04-17.19	—	0.03	0-1.59	—	5.9	0.7-49.54	—
Weight (medium)	0.03	0.01-0.69	—	0.02	0-1.65	—	3.5	0.56-21.86	—
Weight overall	—	—	0.007	—	—	0.078	—	—	0.22
Pyoderma diagnosis	1.5	0.09-24.5	0.79	0.77	0.02-36.09	0.89	1.1	0.18-6.6	0.93
Gender (male REF)	7.96	1.26-50.27	0.013	1.09	0.2-5.6	0.92	3.2	1.02-9.9	0.038
First opinion	REF	—	—	REF	—	—	REF	—	—
Referral consultation	3.87	0.3-49.3	0.29	15.78	0.25-985.23	0.12	0.9	0.19-4.4	0.9
Previous antimicrobial[1]	1.9	0.09-38.2	0.69	1.57	0.1-25.84	0.75	0.3	0.05-1.96	0.21
Previous beta-lactam[1]	0.66	0.03-15.06	0.79	1.45	0.08-27.42	0.8	5.2	0.7-38.83	0.098
Dog eats animal faeces	3.58	0.35-36.84	0.27	1.98	0.2-20.00	0.55	1.5	0.4-5.67	0.53
Owner works in healthcare	0.8	0.1-6.27	0.84	—	—	0.000	0.55	0.15-1.98	0.36
In-contact been hospitalised[2]	1.88	0.24-14.77	0.55	0.68	0.07-6.5	0.74	0.34	0.09-1.7	0.2
In-contact had antimicrobials[2]	0.33	0.04-3.1	0.3	0.55	0.1-2.97	0.49	1.5	0.39-5.89	0.55
Previous vet admission[1]	0.39	0.04-4.13	0.4	0.52	0.07-3.9	0.52	1.4	0.29-6.6	0.68
Multi-dog household	0.64	0.1-4.28	0.64	0.87	0.13-5.8	0.89	0.63	0.16-2.5	0.5

[1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the Likelihood ratio test; MDR = multi-drug resistant (≥ 3 drug classes); CoPS = coagulase positive staphylococci; CoNS = coagulase negative staphylococci

Table 6-10. Multilevel univariable results for outcomes third generation cephalosporin resistance (3GR), phenotypic ESBL- and AmpC-producing *E. coli* in 457 faecal samples from 127 dogs.

Variables	Oxacillin resistance			*mec*A			CipR		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Time D0 and	REF	—	—	REF	—	—	REF	—	—
Time End and CFX	1.78	0.76-4.19	0.185	1.98	0.79-4.97	0.148	1.35	0.45-4.07	0.598
Time End and AC	0.85	0.31-2.3	0.749	0.85	0.27-2.62	0.771	0.61	0.15-2.56	0.500
Time End and CVN	2.37	0.97-5.81	0.058	2.63	1.01-6.83	0.047	2.07	0.70-6.14	0.189
Time End and CD	0.88	0.32-2.4	0.801	0.81	0.26-2.54	0.723	0.38	0.07-2.08	0.267
Time End and FL	5.08	1.51-17.13	0.009	10.85	2.81-41.89	0.001	5.67	1.54-20.83	0.009
Time M1 and CFX	1.20	0.45-3.17	0.711	1.86	0.69-5.06	0.223	2.19	0.74-6.43	0.155
Time M1 and AC	0.75	0.26-2.16	0.589	0.70	0.20-2.4	0.571	0.20	0.02-2.06	0.178
Time M1 and CVN	1.41	0.49-4.04	0.527	0.98	0.28-3.45	0.970	0.90	0.21-3.90	0.885
Time M1 and CD	1.93	0.75-4.93	0.172	1.63	0.57-4.63	0.360	1.46	0.44-4.84	0.539
Time M1 and FL	2.49	0.61-10.25	0.205	3.31	0.74-14.74	0.116	4.96	1.07-23.01	0.041
Time M3 and CFX	1.95	0.76-4.98	0.164	2.07	0.75-5.7	0.160	1.13	0.31-4.11	0.854
Time M3 and AC	0.81	0.28-2.38	0.705	1.31	0.44-3.86	0.627	0.75	0.18-3.16	0.694
Time M3 and CVN	1.27	0.44-3.64	0.657	1.21	0.38-3.9	0.748	0.49	0.09-2.72	0.415
Time M3 and CD	1.33	0.50-3.57	0.569	1.31	0.44-3.9	0.624	1.90	0.61-5.93	0.267
Time M3 and FL	4.01	0.98-16.44	0.053	0.46	0.05-4.62	0.506	1.78	0.29-10.81	0.532
Time treatment overall	—	—	0.264	—	—	0.110	—	—	0.166
Duration ≤ 1 week	REF	—	—	REF	—	—	REF	—	—
Duration > 1 or ≤ 3 weeks	1.52	0.83-2.8	0.177	1.45	0.75-2.83	0.273	1.27	0.57-2.81	0.561
Duration > 3 weeks	1.63	0.88-3.01	0.119	1.79	0.92-3.48	0.085	1.37	0.61-3.03	0.444
Duration overall	—	—	0.264	—	—	0.226	—	—	0.739
Age	1.00	1.00-1.01	0.356	1.00	1.00	0.282	1.01	1.00-1.01	0.013
Weight (large)	REF	—	—	REF	—	—	REF	—	—
Weight (small)	1.37	0.74-2.54	0.323	1.57	0.81-3.05	0.179	2.71	1.35-5.45	0.005
Weight (medium)	1.29	0.65-2.56	0.465	1.54	0.74-3.2	0.243	2.4	1.10-5.20	0.027
Weight overall	—	—	0.526	—	—	0.267	—	—	0.006
Pyoderma diagnosis	1.8	1.09-2.95	0.021	1.7	0.99-2.93	0.054	1.78	0.92-3.44	0.086
Gender (male REF)	1.47	0.92-2.35	0.103	1.55	0.94-2.56	0.089	1.58	0.87-2.88	0.137
First opinion	REF	—	—	REF	—	—	REF	—	—
Referral consultation	1.61	1.01-2.56	0.044	1.55	0.93-2.57	0.09	1.58	0.86-2.88	0.139

Previous antimicrobial[1]	1.47	0.89-2.43	0.132	1.51	0.88-2.62	0.137	0.83	0.44-1.56	0.563
Previous beta-lactam[1]	1.7	0.99-2.91	0.056	1.54	0.85-2.81	0.156	1.03	0.54-1.98	0.926
Dog eats animal faeces	1.41	0.83-2.41	0.203	1.28	0.7-2.31	0.421	0.87	0.45-1.67	0.679
Owner works in healthcare	0.76	0.4-1.44	0.392	0.56	0.26-1.18	0.127	1.05	0.48-2.28	0.905
In-contact been hospitalised[2]	2	1.11-3.61	0.021	1.59	0.83-3.07	0.163	1.42	0.67-3.00	0.355
In-contact had antimicrobials[2]	1.43	0.79-2.59	0.234	1.54	0.8-2.94	0.193	1.78	0.89-3.59	0.106
Previous vet admission[1]	0.8	0.46-1.37	0.413	1.01	0.56-1.81	0.972	0.73	0.38-1.40	0.341
Multi-dog household	0.55	0.33-1.91	0.019	0.57	0.33-0.99	0.047	0.72	0.39-1.33	0.290

[1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the Wald chi-squared test; **P* < 0.25 included in multivariable model with treatment and time; *mec*A = oxacillin resistant staphylococci carrying the *mec*A gene; CipR = ciprofloxacin resistant

Table 6-11. Multilevel univariable results for outcomes third generation cephalosporin resistance (3GR), phenotypic ESBL- and AmpC-producing *E. coli* in 457 faecal samples from 127 dogs.

Variables	MDR			CoPS			CoNS		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Time D0 and	REF	—	—	REF	—	—	REF	—	—
Time End and CFX	1.56	0.60-4.08	0.362	0.20	0.07-0.57	0.003	0.95	0.41-2.16	0.895
Time End and AC	0.52	0.15-1.84	0.313	0.53	0.18-1.58	0.257	2.33	0.92-5.93	0.076
Time End and CVN	1.91	0.72-5.1	0.194	0.28	0.09-0.84	0.023	1.34	0.54-3.33	0.522
Time End and CD	0.68	0.21-2.15	0.510	0.04	0.01-0.13	0.000	1.36	0.55-3.35	0.501
Time End and FL	9.13	2.31-36.12	0.002	0.33	0.07-1.46	0.143	2.95	0.71-12.23	0.136
Time M1 and CFX	1.35	0.47-3.87	0.577	0.87	0.26-2.88	0.817	1.92	0.74-4.97	0.182
Time M1 and AC	0.42	0.10-1.7	0.223	0.40	0.13-1.22	0.108	0.87	0.37-2.09	0.761
Time M1 and CVN	1.21	0.38-3.84	0.741	0.88	0.22-3.50	0.861	1.16	0.42-3.18	0.773
Time M1 and CD	2.12	0.78-5.77	0.140	0.15	0.05-0.49	0.002	0.83	0.33-2.11	0.695
Time M1 and FL	4.49	1.00-20.24	0.050	2.94	0.20-43.01	0.430	1.85	0.41-8.41	0.424
Time M3 and CFX	2.36	0.86-6.48	0.096	1.59	0.41-6.24	0.506	1.12	0.44-2.85	0.809
Time M3 and AC	0.87	0.27-2.79	0.811	0.67	0.20-2.23	0.517	1.03	0.42-2.55	0.948
Time M3 and CVN	1.35	0.45-4.06	0.592	0.86	0.22-3.37	0.826	0.71	0.27-1.87	0.486
Time M3 and CD	1.08	0.36-3.25	0.893	0.46	0.14-1.49	0.197	1.21	0.47-3.15	0.692
Time M3 and FL	2.79	0.62-12.6	0.182	0.27	0.04-1.66	0.158	3.30	0.59-18.32	0.173
Time treatment overall	—	—	0.095	—	—	0.000	—	—	0.756
Duration ≤ 1 week	REF	—	—	REF	—	—	REF	—	—
Duration > 1 or ≤ 3 weeks	1.67	0.79-3.52	0.179	1.55	0.71-3.39	0.274	0.83	0.48-1.44	0.502
Duration > 3 weeks	2.27	1.08-4.76	0.031	3.17	1.41-7.11	0.005	1.14	0.65-2.00	0.648
Duration overall	—	—	0.097	—	—	0.017	—	—	0.466
Age	1.00	1.00-1.01	0.149	1.00	0.99-1.01	0.814	1.00	1.00-1.01	0.430
Weight (large)	REF	—	—	REF	—	—	REF	—	—
Weight (small)	1.64	0.78-3.43	0.192	0.77	0.24-2.46	0.659	1.62	0.71-3.70	0.254
Weight (medium)	1.87	0.83-4.2	0.131	1.43	0.61-3.32	0.412	0.82	0.45-1.48	0.507
Weight overall	—	—	0.189	—	—	0.379	—	—	0.139
Pyoderma diagnosis	2.77	1.52-5.05	0.001	2.78	1.46-5.29	0.002	0.96	0.61-1.52	0.877
Gender (male REF)	1.77	1.01-3.11	0.045	0.77	0.40-1.46	0.418	1.63	1.04-2.54	0.031
First opinion	REF	—	—	REF	—	—	REF	—	—
Referral consultation	2.63	1.53-4.53	0.000	2.48	1.31-4.71	0.005	1.11	0.71-1.74	0.641

Previous antimicrobial[1]	1.71	0.96-3.05	0.068	1.54	0.79-2.98	0.201	0.89	0.57-1.40	0.622
Previous beta-lactam[1]	1.97	1.03-3.77	0.041	1.35	0.66-2.75	0.411	1.13	0.70-1.84	0.610
Dog eats animal faeces	2.08	1.16-3.73	0.014	1.47	0.74-2.93	0.271	1.16	0.72-1.88	0.542
Owner works in healthcare	0.95	0.46-1.97	0.898	2.24	1.00-5.02	0.051	0.55	0.32-0.93	0.026
In-contact been hospitalised[2]	2.61	1.36-5.00	0.004	1.94	0.90-4.20	0.091	1.06	0.63-1.79	0.828
In-contact had antimicrobials[2]	1.47	0.75-2.88	0.261	1.00	0.48-2.08	0.991	1.64	0.97-2.75	0.062
Previous vet admission[1]	0.64	0.34-1.20	0.165	0.43	0.23-0.83	0.011	1.23	0.76-1.98	0.403
Multi-dog household	0.55	0.31-0.98	0.043	0.53	0.28-1.01	0.055	0.64	0.41-0.99	0.046

[1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the Wald chi-squared test; **P* < 0.25 included in multivariable model with treatment and time; MDR = multi-drug resistant (≥ 3 drug classes); CoPS = coagulase positive staphylococci; CoNS = coagulase negative staphylococci

Figure 6-1. Residuals plot for oxacillin resistance multilevel, multivariable model (n = 127 dogs)

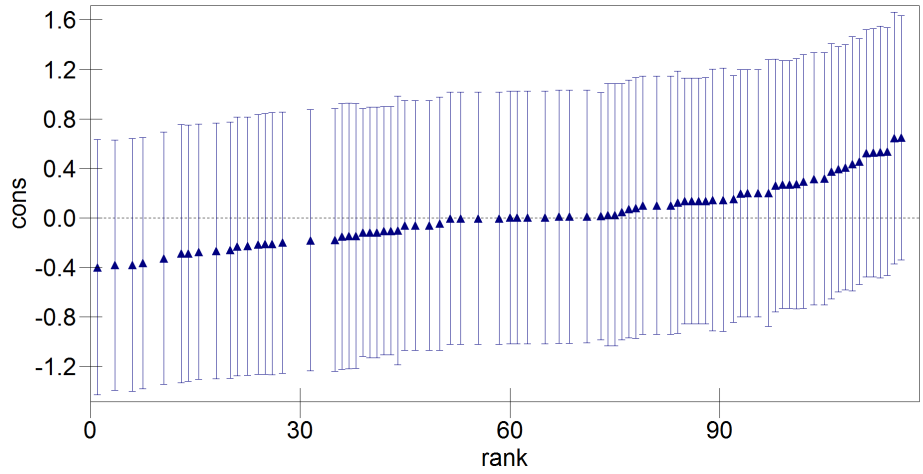


Figure 6-2. Residuals plot for oxacillin resistance and *mec*A carriage multilevel, multivariable model (n = 127 dogs)

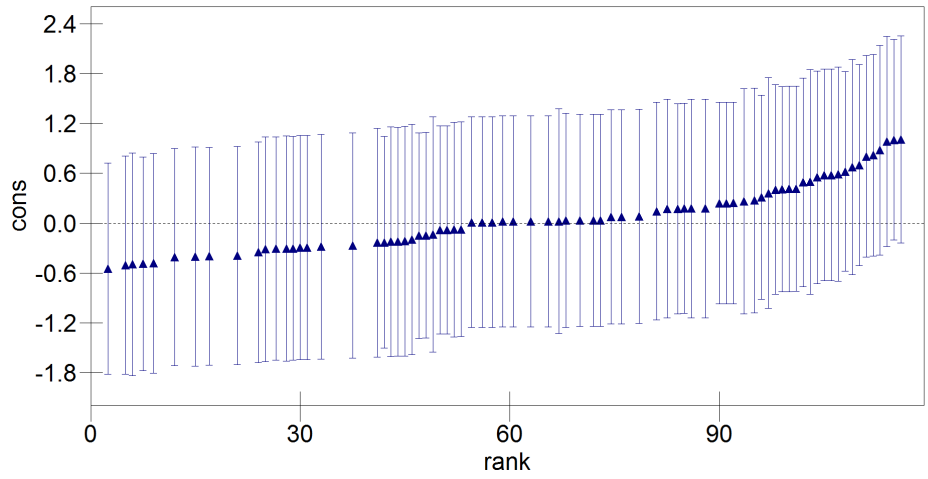


Figure 6-3. Residuals plot for ciprofloxacin resistance multilevel, multivariable model (n = 127 dogs)

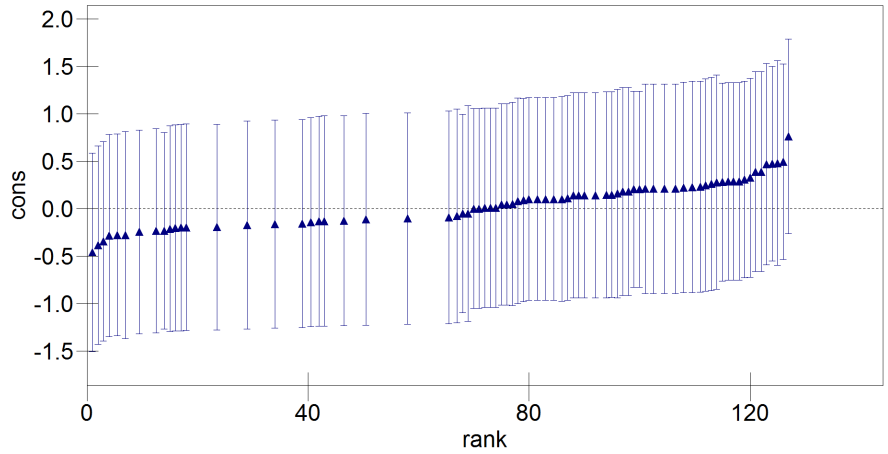


Figure 6-4. Residuals plot for multidrug resistance multilevel, multivariable model (n = 127 dogs)

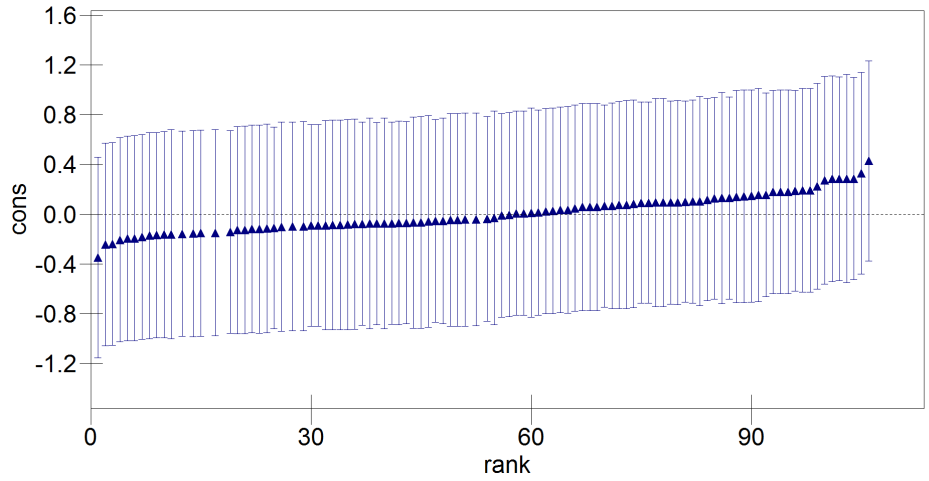


Figure 6-5. Residuals plot for CoPS multilevel, multivariable model (n = 127 dogs)

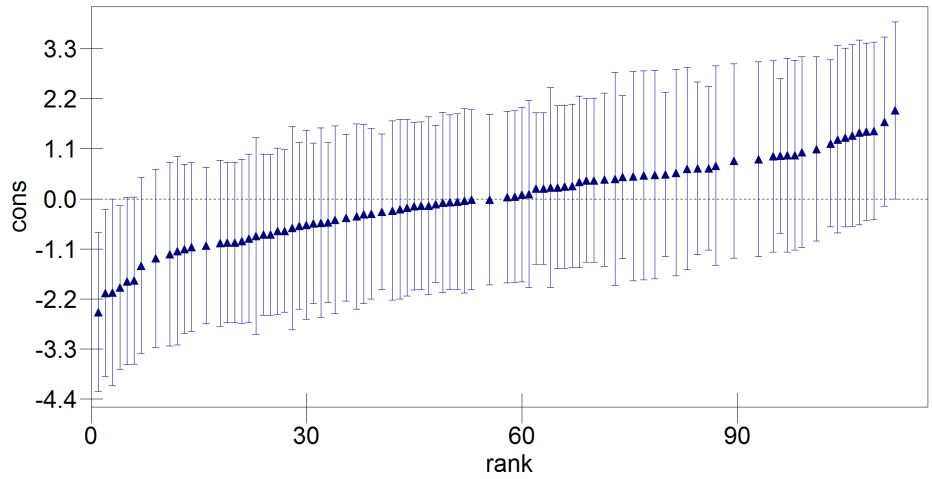
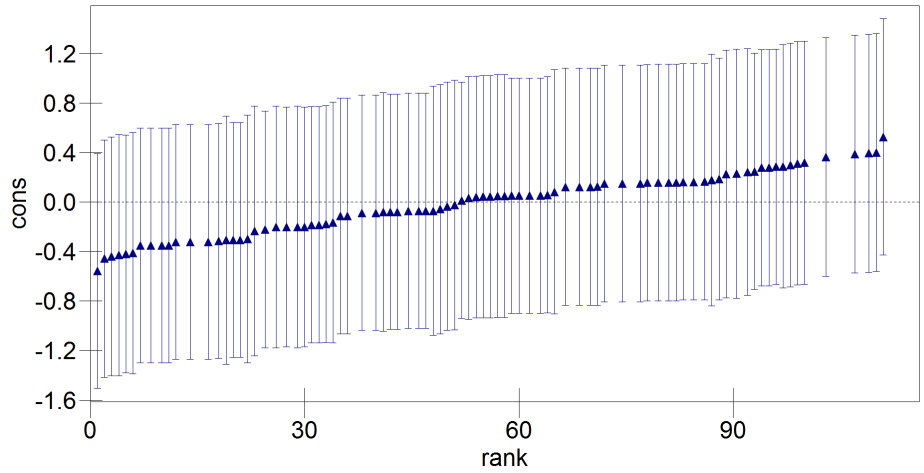


Figure 6-6. Residuals plot for CoNS multilevel, multivariable model (n = 127 dogs)



APPENDIX V
(Pertaining to chapter 7)

Table 7-1: Total number and percentage (%) of samples with each outcome at each time point for each treatment group.

Outcome	Time	CFX	AC	CVN	CD	FL	Total
AMR	D0	14 (44)	13 (46)	17 (71)	15 (54)	8 (57)	69 (54)
	End	24 (75)	21 (75)	23 (96)	20 (71)	5 (43)	94 (74)
	M1	20 (74)	15 (58)	11 (58)	20 (80)	4 (67)	70 (67)
	M3	11 (46)	12 (48)	13 (76)	15 (65)	5 (63)	54 (55)
MDR	D0	6 (19)	7 (25)	8 (33)	10 (34)	7 (50)	38 (30)
	End	10 (31)	15 (54)	15 (63)	11 (38)	4 (29)	55 (43)
	M1	9 (33)	6 (23)	6 (32)	10 (44)	3 (50)	34 (32)
	M3	4 (17)	6 (24)	5 (29)	6 (26)	1 (13)	22 (22)
CipR	D0	2 (6)	2 (7)	6 (25)	3 (7)	3 (21)	15 (12)
	End	9 (28)	3 (11)	9 (38)	3 (11)	6 (43)	30 (24)
	M1	4 (15)	4 (15)	2 (11)	3 (12)	2 (33)	15 (14)
	M3	1 (4)	2 (8)	4 (24)	1 (4)	2 (25)	10 (10)
ESBL	D0	2 (6)	1 (4)	5 (20)	2 (7)	4 (29)	14 (11)
	End	7 (22)	4 (14)	8 (32)	2 (7)	4 (29)	15 (12)
	M1	2 (7)	4 (15)	5 (26)	1 (4)	1 (17)	13 (12)
	M3	3 (13)	1 (4)	2 (12)	0	1 (4)	7 (7)
bla_{CTX-M}	D0	1 (3)	0	5 (21)	0	2 (14)	8 (6)
	End	5 (16)	1 (4)	7 (29)	1 (4)	3 (21)	17 (13)
	M1	1 (4)	1 (4)	5 (26)	1 (4)	1 (17)	9 (9)
	M3	0	1 (4)	2 (12)	0	1 (13)	4 (4)
3GCR	D0	5 (16)	6 (21)	12 (50)	6 (21)	6 (43)	35 (28)
	End	20 (63)	12 (43)	18 (75)	7 (24)	4 (29)	61 (48)
	M1	11 (41)	12 (46)	9 (47)	5 (20)	2 (38)	39 (37)
	M3	5 (21)	5 (20)	8 (47)	3 (13)	2 (25)	23 (23)
AmpC	D0	8 (27)	10 (36)	10 (42)	7 (25)	4 (29)	39 (31)
	End	21 (65)	14 (50)	18 (75)	5 (18)	3 (21)	61 (48)
	M1	12 (44)	10 (38)	8 (42)	6 (24)	1 (17)	37 (35)
	M3	5 (21)	5 (20)	8 (47)	2 (9)	2 (25)	22 (22)
bla_{AmpC}	D0	8 (25)	8 (29)	10 (42)	5 (17)	4 (29)	35 (28)
	End	19 (59)	10 (36)	16 (67)	3 (10)	3 (21)	51 (40)
	M1	11 (41)	7 (27)	6 (32)	3 (12)	1 (17)	28 (27)
	M3	4 (17)	5 (20)	7 (41)	1 (4)	2 (25)	19 (19)

CFX = cefalexin, AC = clavulanate-amoxycillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinonlone; Day 0 = baseline; End = end of therapy; M1 = one month after therapy; M3 = three months after therapy

Table 7-2. Number and percentage (%) of samples with *E. coli* carrying either bla_{CTX} or bla_{AmpC} genes at each time point for each treatment group and for treatment overall (95% CI).

		bla_{CTX}		bla_{AmpC}	
Antimicrobial treatment group	Time Point (Total samples)	Number (%) of positive samples	95% CI	Number (%) of positive samples	95% CI
CFX	D0 (n = 32)	1 (3)	0.00 – 9.15	8 (25)	10.00 – 40.00
	E (n = 32)	4 (13)	1.04 – 23.96	19 (60)	42.36 – 76. 39
	M1 (n = 27)	2 (7)	0.00 – 17.29	11 (41)	22.21 – 59.27
	M3 (n = 24)	0	—	4 (17)	1.76 – 31.58
AC	D0 (n = 28)	0	—	8 (29)	11.84 – 45.30
	E (n = 28)	1 (4)	0.00 – 10.45	10 (36)	17.97 – 53.46
	M1 (n = 26)	1 (4)	0.00 – 11.24	7 (27)	9.87 – 43.97
	M3 (n = 25)	1 (4)	0.00 – 11.68	5 (20)	4.32 – 35.68
CVN	D0 (n = 24)	5 (21)	4.59 – 37.08	10 (42)	21.94 – 61.39
	E (n = 24)	6 (25)	7.68 – 42.32	16 (67)	47.81 – 85.53
	M1 (n = 19)	5 (26)	6.52 – 46.12	6 (32)	10.68 – 52.48
	M3 (n = 18)	1 (6)	0.10 – 25.80	7 (39)	20.30 – 61.40
CD	D0 (n = 29)	0	—	5 (17)	3.49 – 30.99
	E (n = 29)	1 (3)	0.00 – 10.09	3 (10)	0.00 – 21.43
	M1 (n = 25)	1 (4)	0.00 – 11.68	3 (12)	0.00 – 24.74
	M3 (n = 23)	0	—	1 (4)	0.00 – 12.68
FL	D0 (n = 14)	2 (14)	0.00 – 32.62	4 (29)	4.91 – 52.24
	E (n = 14)	3 (21)	0.00 – 42.92	3 (21)	0.00 – 42.92
	M1 (n = 7)	1 (14)	2.60 – 51.30	1 (14)	2.60 – 51.30
	M3 (n = 8)	1 (13)	0.00 – 35.42	2 (25)	0.00 – 55.01
Total	D0 (n = 127)	8 (6)	2.07 – 10.52	35 (28)	19.79 – 35.33
	E (n = 127)	14 (11)	5.58 – 16.47	51 (40)	31.63 – 48.68
	M1 (n = 105)	9 (9)	3.22 – 13.93	28 (27)	18.21 – 35.13
	M3 (n = 98)	3 (3)	0.00 -6.47	19 (19)	11.56 – 27.21

95% CI = 95% confidence intervals; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone; Total = treatment overall; D0 = baseline day zero; E = end of therapy; M1 = one month after therapy; M3 = three months after therapy

Table 7-3. Number and percentage (%) of samples with multidrug resistant and ciprofloxacin resistant ESBL-and AmpC-producing *E.coli* at each time point for each antimicrobial treatment group and treatment overall (95% CI).

		MDR ESBL-producing *E. coli*		MDR AmpC-producing *E. coli*		CipR ESBL-producing *E. coli*		CipR AmpC-producing *E. coli*	
Antimicrobial treatment group	Time Point (Total samples)	Number (%) of positive samples	95% CI	Number (%) of positive samples	95% CI	Number of positive samples	95% CI	Number of positive samples	95% CI
CFX	D0 (n = 32)	0	—	4 (13)	1.04 – 23.96	0	—	2 (6)	0.00 – 14.64
	E (n = 32)	4 (13)	1.04 – 23.96	6 (19)	5.23 – 32.27	3 (9)	0.00 – 19.47	4 (13)	1.04 – 23.96
	M1 (n = 27)	0	—	2 (7)	0.00 – 17.29	1 (4)	0.00 – 10.83	2 (7)	0.00 – 17.29
	M3 (n = 24)	0	—	0	—	0	—	0	0.00
AC	D0 (n = 28)	1 (4)	—	2 (7)	—	0	—	1 (4)	0.00 – 10.45
	E (n = 28)	3 (11)	—	4 (14)	1.32 – 27.25	0	—	1 (4)	0.00 – 10.45
	M1 (n = 26)	2 (8)	—	3 (12)	—	1 (4)	0.00 – 11.24	1 (4)	0.00 – 11.24
	M3 (n = 25)	0	—	1 (4)	—	0	0.00	0	0.00
CVN	D0 (n = 24)	4 (17)	1.76 – 31.58	4 (17)	1.76 – 31.58	2 (8)	0.00 – 19.39	3 (13)	0.00 – 25.73
	E (n = 24)	7 (29)	10.98 – 47.35	8 (33)	14.47 – 52.19	3 (13)	0.00 – 25.73	4 (17)	1.76 – 31.58
	M1 (n = 19)	2 (11)	0.00 – 24.33	2 (11)	0.00 – 24.33	1 (5)	0.00 – 15.39	0	0.00
	M3 (n = 18)	2 (11)	3.10 – 32.80	2 (11)	3.10 – 32.80	1 (6)	0.10 – 25.80	2 (11)	3.10 – 32.80
CD	D0 (n = 29)	1 (3)	0.00 – 10.09	3 (10)	0.00 – 21.43	1 (3)	0.00 – 10.09	1 (3)	0.00 – 10.09
	E (n = 29)	1 (3)	0.00 – 10.09	1 (3)	0.00 – 10.09	0	—	0	0.00
	M1 (n = 25)	1 (4)	0.00 – 11.68	3 (12)	0.00 – 23.82	0	—	1 (4)	0.00 – 11.24
	M3 (n = 23)	0	—	0	—	0	—	0	0.00
FL	D0 (n = 14)	1 (7)	0.00 – 20.63	2 (14)	0.00 – 32.62	2 (14)	0.00 – 32.62	1 (7)	0.00 – 20.63
	E (n = 14)	3 (21)	0.00 – 42.92	2 (14)	0.00 – 32.62	4 (29)	4.91 – 52.24	3 (21)	0.00 – 42.92
	M1 (n = 7)	1 (14)	2.60 – 51.30	0	—	1 (14)	2.60 – 51.30	0	0.00
	M3 (n = 8)	1 (13)	0.00 – 35.42	19 (13)	0.00 – 35.42	1 (13)	0.00 – 35.42	1 (13)	0.00 – 35.42
Total	D0 (n = 127)	7 (6)	1.54 – 9.48	15 (12)	6.20 – 17.42	5 (4)	0.55 – 7.32	8 (6)	2.07 – 10.52
	E (n = 127)	17 (13)	7.46 – 19.31	21 (17)	10.07 – 23.00	10 (8)	3.19 – 12.56	12 (9)	4.36 – 14.54
	M1 (n = 105)	6 (6)	1.27 – 10.15	10 (10)	3.91 – 15.14	4 (4)	0.15 – 7.47	4 (4)	0.15 – 7.47
	M3 (n = 98)	3 (3)	0.00 – 6.47	4 (4)	0.16 – 8.00	2 (2)	0.00 – 4.84	3 (3)	0.00 – 6.47

95% CI = 95% confidence intervals; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone; Total = treatment overall; MDR = multidrug resistance; CipR = ciprofloxacin resistance; D0 = baseline; E = end of therapy; M1 = one month after the end of therapy; M3 = three months after the end of therapy

Table 7-4. Number and percentage (%) of samples with ciprofloxacin resistant (CipR), third generation cephalosporin resistant (3GCR) and multidrug resistant (MDR) *E.coli* at each time point for each antimicrobial treatment group and treatment overall (95% CI).

		CipR		3GCR		MDR	
Antimicrobial treatment group	Time Point (Total samples)	Number (%) of positive samples	95% CI	Number (%) of positive samples	95% CI	Number (%) of positive samples	95% CI
CFX	D0 (n = 32)	2 (6)	0.00-14.64	5 (16)	3.04-28.21	6 (19)	5.23 -32.37
	E (n = 32)	9 (28)	12.55-43.7	20 (63)	45.73-79.27	10 (31)	15.19-47.31
	M1 (n = 27)	4 (15)	1.41-28.21	11 (41)	22.21-59.27	9 (33)	15.55-51.11
	M3 (n = 24)	1 (4)	0.00-12.16	5 (21)	4.59-37.08	4 (17)	1.76-31.58
AC	D0 (n = 28)	2 (7)	0.00-16.68	6 (21)	6.2-36.63	7 (25)	8.96-41.04
	E (n = 28)	3 (11)	0.00-22.17	12 (43)	24.53-61.9	15 (54)	35.10-72.04
	M1 (n = 26)	4 (15)	1.52-29.25	12 (46)	26.99-65.32	6 (23)	6.88-39.27
	M3 (n = 25)	2 (8)	0.00-18.63	5 (20)	4.32-35.68	6 (24)	7.26-40.74
CVN	D0 (n = 24)	6 (25)	7.68-42.32	12 (50)	30.00-70.00	8 (33)	14.47-52.19
	E (n = 24)	9 (38)	18.13-56.87	18 (75)	57.68-92.32	15 (63)	43.13-81.87
	M1 (n = 19)	2 (11)	0.00-24.33	9 (47)	24.92-69.82	6 (32)	10.68-52.48
	M3 (n = 18)	4 (22)	9.00-45.20	8 (44)	24.60-66.30	5 (28)	12.50-50.90
CD	D0 (n = 29)	2 (7)	0.00-16.68	6 (21)	5.95-35.43	10 (34)	17.18-51.78
	E (n = 29)	3 (11)	0.00-22.17	7 (24)	8.56-39.71	11 (38)	20.27-55.59
	M1 (n = 25)	3 (12)	0.00-24.74	5 (20)	4.32-35.68	11 (44)	24.54-63.46
	M3 (n = 23)	1 (4)	0.00-12.68	3 (13)	0.00-26.81	6 (26)	8.14-44.03
FL	D0 (n = 14)	3 (21)	0.00-42.92	6 (43)	16.93-68.78	7 (50)	23.81-76.19
	E (n = 14)	6 (43)	16.93-68.78	4 (29)	4.91-52.24	4 (29)	4.91-52.24
	M1 (n = 7)	2 (29)	8.20-64.10	2 (29)	8.20-64.10	3 (43)	15.80-75.00
	M3 (n = 8)	2 (25)	0.00-55.01	2 (25)	0.00-55.01	1 (13)	0.00-35.42
Total	D0 (n = 127)	15 (12)	6.20-17.42	35 (28)	19.79-35.33	38 (30)	21.96-37.89
	E (n = 127)	30 (24)	16.23-31.01	61 (48)	39.34-56.72	55 (43)	34.69-51.92
	M1 (n = 105)	15 (14)	7.59-20.98	39 (37)	27.90-46.39	34 (32)	23.43-41.33
	M3 (n = 98)	10 (10)	4.21-16.2	23 (23)	15.08-31.86	22 (22)	14.19-30.71

95% CI = 95% confidence intervals; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone; Total = treatment overall; D0 = baseline; E = end of therapy; M1 = one month after the end of therapy; M3 = three months after the end of therapy

Table 7-5. Number and percentage (%) of samples with phenotypic ESBL- and AmpC-producing *E. coli* at each time point for each treatment group (95% CI).

		ESBL-producing *E. coli*		AmpC-producing *E. coli*	
Antimicrobial treatment group	Time Point (Total samples)	Number (%) of positive samples	95% CI	Number (%) of positive samples	95% CI
CFX	D0 (n = 32)	2 (6)	0.00-14.64	8 (25)	10.00-40.00
	E (n = 32)	7 (22)	7.55-36.20	21 (66)	49.17-82.08
	M1 (n = 27)	2 (7)	0.00-17.29	12 (44)	25.70-63.19
	M3 (n = 24)	3 (13)	0.00-25.73	5 (21)	4.59-37.08
AC	D0 (n = 28)	1 (4)	0.00-10.45	10 (36)	17.97-53.46
	E (n = 28)	4 (14)	1.32-27.25	14 (50)	31.48-68.52
	M1 (n = 26)	4 (15)	1.52-29.25	10 (38)	19.76-57.16
	M3 (n = 25)	1 (4)	0.00-11.68	5 (20)	4.32-35.68
CVN	D0 (n = 24)	5 (21)	4.59-37.08	10 (42)	21.94-61.39
	E (n = 24)	8 (33)	14.47-52.19	18 (75)	57.68-92.32
	M1 (n = 19)	5 (26)	6.52-46.12	8 (42)	19.90-64.31
	M3 (n = 18)	2 (11)	3.10 – 32.80	8 (44)	24.60-66.30
CD	D0 (n = 29)	2 (7)	0.00-16.12	7 (25)	8.96-41.04
	E (n = 29)	2 (7)	0.00-16.12	5 (18)	3.67-32.04
	M1 (n = 25)	1 (4)	0.00-11.68	6 (24)	7.26-40.74
	M3 (n = 23)	0	—	2 (9)	0.00-20.21
FL	D0 (n = 14)	4 (29)	4.91-52.24	4 (29)	4.91-52.24
	E (n = 14)	4 (29)	4.91-52.24	3 (21)	0.00-42.92
	M1 (n = 7)	1 (14)	2.60 – 51.30	1 (14)	2.60 – 51.30
	M3 (n = 8)	1 (13)	0.00-35.42	2 (25)	0.00-55.01
Total	D0 (n = 127)	14 (11)	5.58-16.47	39 (31)	22.69-38.73
	E (n = 127)	25 (20)	12.77-26.6	61 (48)	39.34-56.72
	M1 (n = 105)	13 (12)	6.08-18.68	37 (35)	26.10-44.38
	M3 (n = 98)	7 (7)	2.04-12.24	22 (22)	14.19-30.71

95% CI = 95% confidence intervals; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone; Total = treatment overall; D0 = baseline day zero; E = end of therapy; M1 = one month after therapy; M3 = three months after therapy

Table 7-6. Multilevel univariable analysis for the outcomes ciprofloxacin resistance (CipR) and multidrug resistance (MDR) in 457 faecal samples from 127 dogs.

Variables	CipR			MDR		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Time D0	REF	—	—	REF	—	—
Time End and CFX	4.35	1.36-13.85	0.013	1.45	0.52-4.09	0.479
Time End and AC	1.03	0.19-5.5	0.975	4.70	1.63-13.59	0.004
Time End and CVN	5.67	1.57-20.45	0.008	7.50	2.24-25.15	0.001
Time End and CD	0.95	0.18-5.12	0.950	1.72	0.56-5.25	0.343
Time End and FL	8.32	1.81-38.31	0.007	0.65	0.14-3.02	0.581
Time M1 and CFX	1.72	0.39-7.59	0.477	1.86	0.62-5.59	0.267
Time M1 and AC	1.64	0.36-7.49	0.523	0.53	0.14-1.95	0.336
Time M1 and CVN	0.68	0.09-4.89	0.699	1.08	0.27-4.39	0.910
Time M1 and CD	1.15	0.21-6.25	0.869	2.26	0.71-7.14	0.166
Time M1 and FL	3.39	0.38-29.88	0.272	1.68	0.24-11.93	0.602
Time M3 and CFX	0.38	0.03-5.18	0.472	0.58	0.14-2.37	0.449
Time M3 and AC	0.78	0.11-5.62	0.806	1.09	0.33-3.58	0.884
Time M3 and CVN	2.10	0.43-10.13	0.357	0.85	0.19-3.78	0.835
Time M3 and CD	0.38	0.03-5.47	0.476	0.51	0.13-2.04	0.344
Time M3 and FL	4.28	0.50-36.98	0.186	0.26	0.02-3.88	0.327
Time treatment overall	—	—	*0.111	—	—	*0.009
Duration ≤ 1 week	REF	—	—	REF	—	—
Duration > 1or ≤ 3 weeks	1.69	0.52-5.47	0.381	1.48	0.62-3.53	0.379
Duration > 3 weeks	3.89	1.28-11.84	0.017	1.66	0.69-3.96	0.256
Duration overall	—	—	*0.034	—	—	0.509
Age	1.00	0.99-1.01	0.631	1.00	0.99-1.01	0.714
Weight (large)	REF	—	—	REF	—	—
Weight (small)	0.40	0.10-1.57	0.188	1.09	0.43-2.73	0.857
Weight (medium)	0.28	0.06-1.38	0.117	0.40	0.13-1.23	0.109
Weight overall	—	—	*0.150	0.72	0.34-1.54	0.397
Pyoderma diagnosis	3.47	1.32-9.15	*0.012	1.39	0.68-2.82	0.363
Gender (male REF)	0.95	0.39-2.32	0.910	0.76	0.38-1.51	0.429
First opinion	REF	—	—	REF	—	—
Referral consultation	1.95	0.82-4.67	*0.133	1.12	0.56-2.23	0.745
Previous antimicrobials[1]	1.68	0.69-4.07	*0.252	1.05	0.54-2.04	0.889
Previous beta-lactam[1]	1.50	0.61-3.66	0.378	0.91	0.44-1.89	0.796
Dog eats animal stools	2.85	1.22-6.70	*0.016	1.38	0.64-2.97	0.415
Owner works in health care	1.33	0.43-4.11	0.620	2.84	1.17-6.90	*0.021
In-contact hospital admission[2]	0.89	0.28-2.82	0.845	0.84	0.32-2.18	0.724
In-contact had antimicrobials[2]	1.34	0.42-4.31	0.625	0.77	0.30-1.95	0.579
Previous veterinary admission[1]	1.54	0.65-3.66	0.331	1.28	0.60-2.74	0.530
Multi-dog household	2.21	0.88-5.55	*0.092	1.57	0.76-3.24	*0.227

[1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the Wald chi-squared test; **P* < 0.25 included in multivariable model with treatment and time; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone

Table 7-7. Multilevel univariable results for outcomes third generation cephalosporin resistance (3GCR), phenotypic ESBL- and AmpC-producing *E. coli* in 457 faecal samples from 127 dogs.

Variables	3GCR			ESBL			AmpC		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Time D0 and	REF	—	—	REF	—	—	REF	—	—
Time End and CFX	10.14	3.58-28.7	0.000	3.68	0.91-14.84	0.068	8.65	3.09-24.17	0.000
Time End and AC	3.04	1.00-9.22	0.049	1.97	0.36-10.82	0.434	2.43	0.84-6.98	0.100
Time End and CVN	12.98	3.49-48.25	0.000	5.75	1.38-23.97	0.016	12.37	3.51=43.57	0.000
Time End and CD	0.92	0.28-3.07	0.895	0.61	0.06-6.01	0.669	0.62	0.18-2.09	0.440
Time End and FL	0.80	0.17-3.83	0.781	3.34	0.51-21.8	0.208	0.57	0.11-3.06	0.509
Time M1 and CFX	2.90	0.98-8.63	0.055	0.69	0.08-5.7	0.729	2.75	0.96-7.88	0.060
Time M1 and AC	3.62	1.15-11.38	0.028	2.35	0.42-13.01	0.329	1.43	0.47-4.38	0.529
Time M1 and CVN	2.43	0.70-8.51	0.164	3.33	0.66-16.67	0.144	2.32	0.68-7.84	0.177
Time M1 and CD	0.65	0.17-2.49	0.530	0.33	0.01-7.09	0.475	0.78	0.23-2.66	0.690
Time M1 and FL	0.90	0.11-7.22	0.920	0.76	0.04-14.46	0.856	0.47	0.03-6.53	0.572
Time M3 and CFX	0.82	0.22-3.10	0.769	1.40	0.22-8.87	0.720	0.65	0.18-2.35	0.507
Time M3 and AC	0.80	0.22-2.86	0.731	0.34	0.02-5.99	0.463	0.36	0.09-1.44	0.148
Time M3 and CVN	2.23	0.62-8.01	0.220	0.79	0.10-6.2	0.824	2.29	0.65-8.00	0.196
Time M3 and CD	0.33	0.07-1.68	0.183	0.39	0.02-8.33	0.547	0.21	0.03-1.34	0.099
Time M3 and FL	0.97	0.13-7.44	0.973	1.33	0.07-24.72	0.846	1.06	0.14-8.00	0.952
Time treatment overall	—	—	*0.000	—	—	*0.440	—	—	*0.000
Duration ≤ 1 week	REF	—	—	REF	—	—	REF	—	—
Duration > 1 or ≤ 3 weeks	1.81	0.74-5.49	0.190	1.34	0.53-3.36	0.538	1.23	0.53-2.89	0.630
Duration > 3 weeks	2.33	0.96-5.64	0.060	2.19	0.90-5.31	0.084	1.65	0.70-3.85	0.250
Duration overall	—	—	*0.168	—	—	*0.180	—	—	0.500
Age	0.99	0.99-1.00	*0.030	1.00	0.99-1.01	0.849	0.99	0.99-1.00	*0.130
Weight (large)	REF	—	—	REF	—	—	REF	—	—
Weight (small)	0.32	0.12-0.85	0.023	0.61	0.22-1.66	0.330	0.37	0.15-0.90	0.030
Weight (medium)	0.16	0.05-0.52	0.002	0.28	0.07-1.09	0.066	0.10	0.03-0.35	0.000
Weight overall	—	—	*0.002	—	—	*0.140	—	—	*0.000
Pyoderma diagnosis	2.63	1.28-5.39	*0.008	2.91	1.31-6.48	*0.009	1.70	0.85-3.41	*0.130
Gender (male REF)	1.37	0.69-2.73	0.367	0.88	0.25-3.04	0.835	1.01	0.51-1.99	0.980
First opinion	REF	—	—	REF	—	—	REF	—	—

Referral consultation	1.65	0.82-3.29	*0.158	1.71	0.51-5.70	0.382	1.74	0.89-3.42	*0.110
Previous antimicrobial[1]	1.39	0.69-2.81	0.359	1.26	0.29-5.38	0.758	1.24	0.63-2.46	0.530
Previous beta-lactam[1]	1.30	0.63-2.66	0.477	1.12	0.30-4.16	0.864	1.12	0.57-2.21	0.740
Dog eats animal faeces	1.23	0.58-2.62	0.595	1.67	0.78-3.59	*0.185	1.05	0.51-2.20	0.890
Owner works in healthcare	1.61	0.64-4.01	0.309	2.00	0.85-4.71	*0.113	1.11	0.47-2.66	0.810
In-contact hospital admission[1]	0.96	0.38-2.43	0.925	0.41	0.15-1.16	*0.093	0.41	0.15-1.16	*0.093
In-contact had antimicrobials[1]	1.10	0.44-2.73	0.838	0.61	0.23-1.62	0.324	0.61	0.23-1.62	0.324
Previous vet admission[1]	1.29	0.60-2.79	0.511	1.08	0.51-2.30	0.838	1.08	0.51-2.30	0.838
Multi-dog household	3.39	1.67-6.89	*0.001	3.12	1.44-6.75	*0.004	3.12	1.44-6.75	*0.004

[1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the Wald chi-squared test; *$P < 0.25$ included in multivariable model with treatment and time; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone

Table 7-8. Multivariable logistic regression results for baseline outcomes ciprofloxacin resistance (CipR) and multidrug resistance (MDR) with baseline covariates.

Variables	CipR			MDR		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Age	1	0.95-0.99	0.946	1	0.99-1.00	0.701
Weight (large)	REF	—	—	REF	—	—
Weight (small)	0.6	0.02-16.6	—	1.2	0.24-5.7	—
Weight (medium)	1.7	0.12-24.49	—	0.64	0.12-3.5	—
Weight overall	—	—	0.873	—	—	0.822
Pyoderma diagnosis	0.8	0.08-8.5	0.862	2.4	0.61-9.8	0.202
Gender (male REF)	1.3	0.24-7	0.731	0.64	0.22-1.9	0.416
First opinion	REF	—	—	REF	—	—
Referral consultation	10.5	0.93-117.5	0.031	1.4	0.38-5.07	0.628
Previous antimicrobials[1]	1.02	0.6-16.49	0.991	0.42	0.06-2.83	0.354
Previous beta-lactam[1]	0.74	0.05-10.51	0.825	1.9	0.25-14.06	0.527
Dog eats animal stools	0.74	0.11-4.85	0.753	0.72	0.19-2.62	0.617
Owner works in health care	0.9	0.11-7.5	0.918	2.29	0.7-7.3	0.163
In-contact hospital admission[2]	0.63	0.05-7.77	0.717	1.1	0.3-4.04	0.888
In-contact had antimicrobials[2]	1.7	0.22-13.19	0.605	1.04	0.3-3.6	0.954
Previous veterinary admission[1]	1.6	0.2-12.86	0.660	0.43	0.09-2.02	0.274
Multi-dog household	0.64	0.02-17.77	0.796	1.2	0.14-11.14	0.844

[1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the Likelihood ratio test

Table 7-9. Multivariable logistic regression results for baseline outcomes third generation cephalosporin resistance (3GCR), phenotypic ESBL- and AmpC-producing *E. coli* with baseline covariates.

Variables	3GCR			ESBL			AmpC		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Age	1	0.99-1.02	0.622	1	0.98-1.02	0.779	1	0.99-1.02	0.397
Weight (large)	REF	—	—	REF	—	—	REF	—	—
Weight (small)	0.67	0.13-3.5	—	0.49	0.03-7.16	—	1.1	0.11-10.53	—
Weight (medium)	0.56	0.09-3.4	—	—	—	—	0.6	0.08-4.3	—
Weight overall	—	—	0.768	—	—	0.365	—	—	0.824
Pyoderma diagnosis	1.6	0.43-5.87	0.477	1.2	0.14-10.32	0.866	1.6	0.26-9.3	0.623
Gender (male REF)	1.19	0.43-3.31	0.740	0.76	0.15-3.8	0. 739	0.4	0.13-1.26	0.110
First opinion	REF	—	—	REF	—	—	REF	—	—
Referral consultation	1.36	0.37-4.95	0.639	6.46	0.88-47.46	0.048	1.04	0.21-5.2	0.960
Previous antimicrobial[1]	0.6	0.11-3.3	0.551	0.48	0.04-6.22	0.560	0.74	0.12-4.53	0.742
Previous beta-lactam[1]	0.8	0.14-4.5	0.802	1.06	0.08-14.95	0.964	1.48	0.22-9.99	0.683
Dog eats animal faeces	1.5	0.48-4.95	0.475	0.74	0.13-4.12	0.729	1.16	0.29-4.63	0.831
Owner works in healthcare	1.48	0.46-4.72	0.511	5.9	1.11-31.28	0.029	1.5	0.38-6.03	0.560
In-contact hospital admission[2]	1.61	0.43-6.03	0.481	0.43	0.04-4.24	0.447	1.15	0.31-4.28	0.837
In-contact had antimicrobials[2]	0.99	0.29-3.41	0.987	0.81	0.12-5.7	0.835	2	0.57-7	0.278
Previous vet admission[1]	0.52	0.13-2.1	0.358	1.28	0.18-8.92	0.803	0.74	0.18-3.11	0.679
Multi-dog household	1.07	0.13-8.85	0.947	0.62	0.04-10.69	0.741	10.5	0.8-137.24	0.073

[1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the Likelihood ratio test

Table 7-10. Multivariable logistic regression results for baseline outcomes ciprofloxacin resistance (CipR) and multidrug resistance (MDR) with baseline covariates and treatment.

Variables	CipR			MDR		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Treatment CD	REF	—	—	REF	—	—
Treatment CFX	—	—	—	0.35	0.05-2.3	—
Treatment AC	—	—	—	0.38	0.04-3.6	—
Treatment CVN	—	—	—	1.2	0.12-9.6	—
Treatment FL	—	—	—	1.6	0.17-15.8	—
Treatment overall	—	—	0.322	—	—	0.596
Age	1	0.97-1.02	1.000	0.99	0.98-1.01	0.906
Weight (large)	REF	—	—	REF	—	—
Weight (small)	0.7	0.01-43.5	—	0.7	0.1-4.9	—
Weight (medium)	5.6	0.2-142.11	—	0.58	0.09-3.8	—
Weight overall	—	—	0.603	—	—	0.836
Pyoderma diagnosis	1.4	0.07	0.826	2.4	0.42-14.1	0.312
Gender (male REF)	1.13	0.18-7.11	0.900	0.6	0.19-1.9	0.372
First opinion	REF	—	—	REF	—	—
Referral consultation	7.6	0.26-213	0.206	0.74	0.15-3.75	0.722
Previous antimicrobials[1]	0.48	0.02-11.32	0.642	0.37	0.05-3.08	0.339
Previous beta-lactam[1]	1.33	0.07-27.46	0.851	0.48	0.25-19.18	0.464
Dog eats animal stools	1.11	0.12-10.84	0.925	0.55	0.12-2.59	0.438
Owner works in health care	1.48	0.13-17.33	0.757	1.79	0.5-6.42	0.376
In-contact been hospitalised[2]	0.4	0.03-6.5	0.529	0.88	0.22-3.5	0.852
In-contact had antimicrobials[2]	2.06	0.23-18.26	0.521	1.19	0.32-4.42	0.799
Previous veterinary admission[1]	2.25	0.24-21.36	0.479	0.49	0.1-2.4	0.371
Multi-dog household	0.5	0.01-28.16	0.733	0.05	0.1-9.2	0.778

[1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the Likelihood ratio test; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone

Table 7-11. Multivariable logistic regression results for baseline outcomes third generation cephalosporin resistance (3GCR) and phenotypic ESBL- and AmpC-producing *E. coli* with baseline covariates and treatment.

Variables	3GCR			ESBL			AmpC		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Treatment CD	REF	—	—	REF	—	—	REF	—	—
Treatment CFX	0.66	0.09-4.52	—	1.57	0.07-33.52	—	1.66	0.26-10.85	—
Treatment AC	0.91	0.1-8.6	—	0.76	0.025-23.1	—	4.55	0.49-42.38	—
Treatment CVN	4.4	0.51-36.94	—	10.9	0.48-246.73	—	2.42	0.3-19.69	—
Treatment FL	0.54	0.05-5.4	—	3.02	0.09-98.11	—	0.75	0.06-8.84	—
Treatment overall	—	—	0.265	—	—	0.434	—	—	0.612
Age	1	0.99-1.01	0.870	1.0	0.98-1.02	0.959	1.01	0.99-1.02	0.310
Weight (large)	REF	—	—	REF	—	—	REF	—	—
Weight (small)	0.54	0.07-4.02	—	0.39	0.02-9.14	—	0.85	0.08-9.15	—
Weight (medium)	0.86	0.11-6.66	—	—	—	—	0.58	0.07-4.77	—
Weight overall	—	—	0.827	—	—	0.326	—	—	0.874
Pyoderma diagnosis	1.35	0.23-7.8	0.737	0.34	0.03-4.44	0.407	1.56	0.26-9.34	0.465
Gender (male REF)	0.87	0.29-2.66	0.808	0.51	0.09-2.99	0.451	0.41	0.13-1.26	0.068
First opinion	REF	—	—	REF	—	—	REF	—	—
Referral consultation	0.87	0.15-5.1	0.878	3.76	0.32-44.9	0.288	1.04	0.21-5.2	0.223
Previous antimicrobial[1]	0.24	0.03-1.98	0.159	0.14	0.01-3.2	0.178	0.74	0.12-4.53	0.499
Previous beta-lactam[1]	1.9	0.24-15.02	0.538	3.44	0.15-78.08	0.421	0.69	0.22-9.99	0.296
Dog eats animal faeces	2.44	0.63-9.42	0.194	1.7	0.25-11.79	0.587	1.16	0.29-4.63	0.666
Owner works in healthcare	1.57	0.43-5.69	0.496	4.97	0.84-29.39	0.066	1.51	0.38-6.03	0.274
In-contact hospital admission[2]	1.4	0.35-5.54	0.638	0.4	0.04-4.27	0.429	1.15	0.31-4.28	0.998
In-contact had antimicrobials[2]	1.13	0.31-4.11	0.859	0.63	0.09-4.55	0.637	2.0	0.57-7.0	0.258
Previous vet admission[1]	0.65	0.16-2.72	0.551	1.53	0.2-11.79	0.684	0.74	0.18-3.11	0.548
Multi-dog household	0.5	0.05-5.18	0.166	1.01	0.15-7.79	0.944	0.1	0.01-1.55	0.091

[1]Within 12-months but more than three months as per enrolment criteria; [2]Within 12-months of enrolment; OR = odds ratio; 95% CI = 95% confidence interval; *P* values are from the Likelihood ratio test; CFX = cefalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FL = fluoroquinolone

Figure 7-1. Residuals plot for multidrug resistance multilevel, multivariable model (n = 127 dogs)

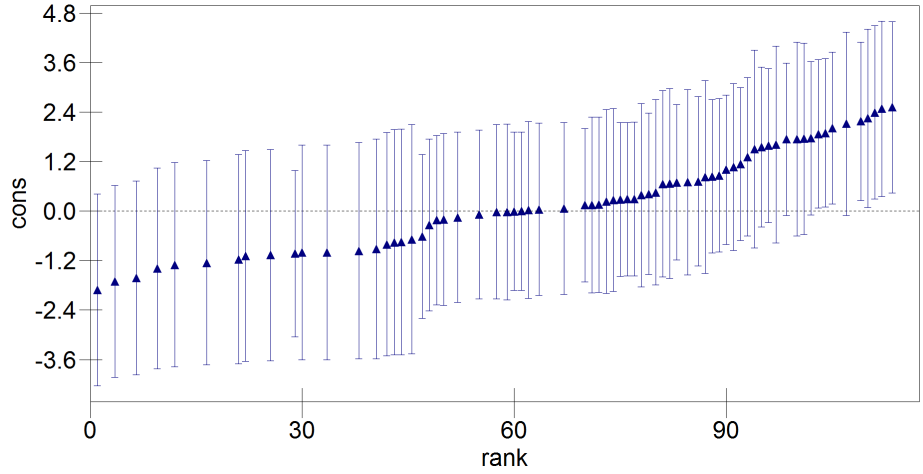


Figure 7-2. Residuals plot for ciprofloxacin resistance multilevel, multivariable model (n = 127 dogs)

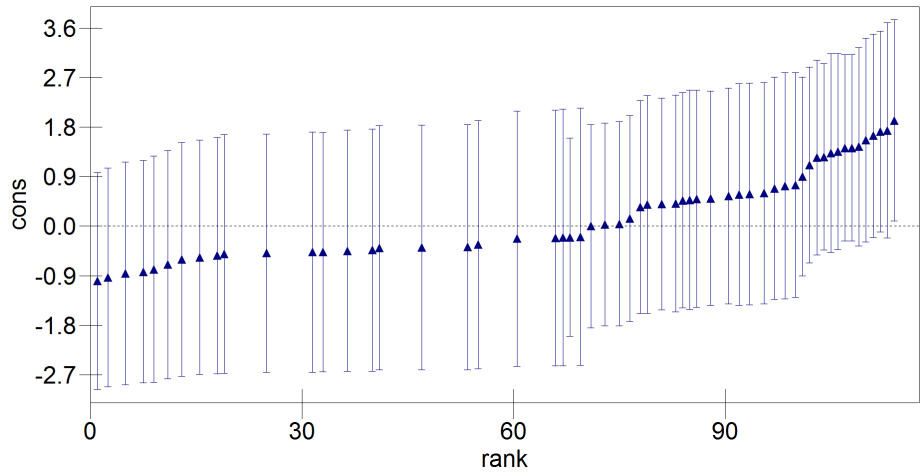


Figure 7-3. Residuals plot for third generation cephalosporin resistance multilevel, multivariable model (n = 127 dogs)

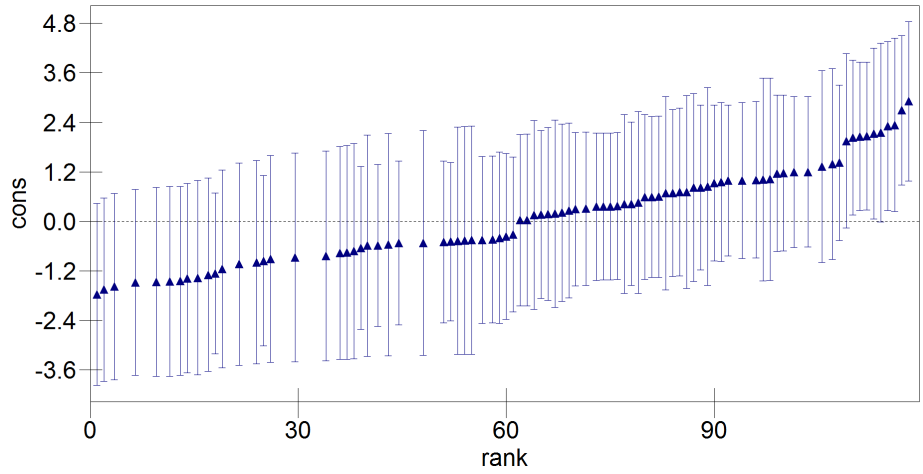


Figure 7-4. Residuals plot for phenotypic AmpC-producing *E. coli* multilevel, multivariable model (n = 127 dogs)

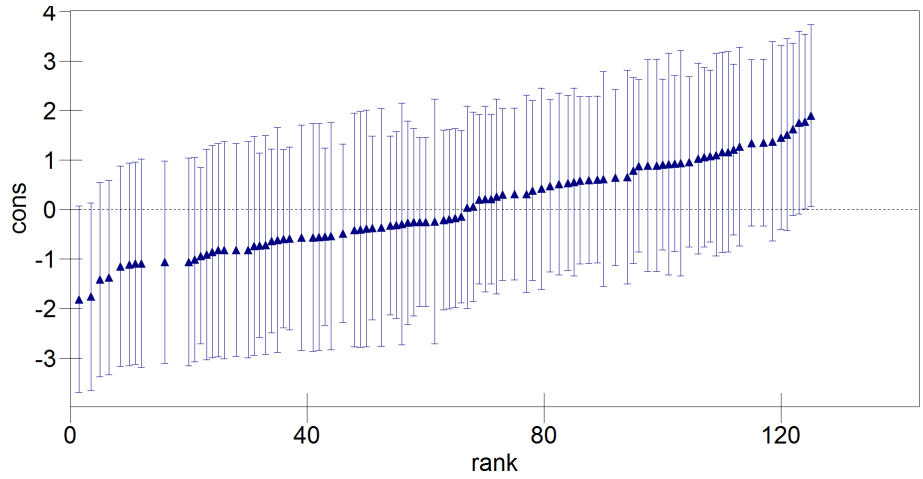
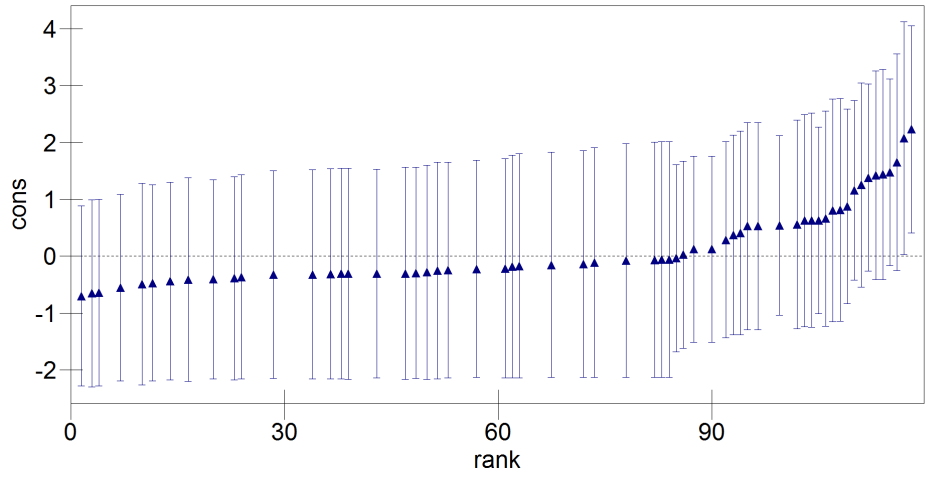


Figure 7-5. Residuals plot for phenotypic ESBL-producing *E. coli* multilevel, multivariable model (n = 127 dogs)



APPENDIX VI

Published paper:

Antimicrobial resistance and characterisation of staphylococci isolated from healthy Labrador retrievers in the United Kingdom

Vanessa Schmidt[1,2], Nicola Williams[2], Gina Pinchbeck[2], Caroline Corless [3], Stephen Shaw[4], Neil McEwan[1], Susan Dawson[2], Tim Nuttall[5]

[1]Department of Infection Biology and [2]Department of Epidemiology and Population Health, The University of Liverpool, Leahurst Campus, Neston, UK, [3]Infection and Immunity, Royal Liverpool University Hospital, Liverpool, UK, [4]UK VetDerm, Coalville, UK and [5]University of Edinburgh, The Royal (Dick) School of Veterinary Studies, Easter Bush Campus, Midlothian, UK.

Manuscript published in BMC Veterinary Research 2014; 10: 17.
DOI: 10.1186/1746-6148-10-17.

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Antimicrobial resistance and characterisation of staphylococci isolated from healthy Labrador retrievers in the United Kingdom

Vanessa M Schmidt[1,6*†], Nicola J Williams[2†], Gina Pinchbeck[2†], Caroline E Corless[3†], Stephen Shaw[4†], Neil McEwan[1,6†], Susan Dawson[2†] and Tim Nuttall[5†]

Abstract

Background: Coagulase-positive (CoPS) and coagulase-negative (CoNS) staphylococci are normal commensals of the skin and mucosa, but are also opportunist pathogens. Meticillin-resistant (MR) and multidrug-resistant (MDR) isolates are increasing in human and veterinary healthcare. Healthy humans and other animals harbour a variety of staphylococci, including MR-CoPS and MR-CoNS. The main aims of the study were to characterise the population and antimicrobial resistance profiles of staphylococci from healthy non-vet visiting and non-antimicrobial treated Labrador retrievers in the UK.

Results: Nasal and perineal samples were collected from 73 Labrador retrievers; staphylococci isolated and identified using phenotypic and biochemical methods. They were also confirmed by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), PCR of the *nuc* gene and PCR and sequencing of the *tuf* gene. Disc diffusion and minimum inhibitory concentration (MIC) susceptibility tests were determined for a range of antimicrobials. In total, 102 CoPS (*S. pseudintermedius* n = 91, *S. aureus* n = 11) and 334 CoNS isolates were detected from 99% of dogs in this study. In 52% of dogs CoNS only were detected, with both CoNS and CoPS detected in 43% dogs and CoPS only detected in 4% of dogs. Antimicrobial resistance was not common among CoPS, but at least one MDR-CoNS isolate was detected in 34% of dogs. MR-CoNS were detected from 42% of dogs but no MR-CoPS were isolated. *S. epidermidis* (52% of dogs) was the most common CoNS found followed by *S. warneri* (30%) and *S. equorum* (27%), with another 15 CoNS species isolated from ≤ 15% of dogs. *S. pseudintermedius* and *S. aureus* were detected in 44% and 8% of dogs respectively.

Conclusions: MR- and MDR-CoPS were rare. However a high prevalence of MR- and MDR-CoNS were found in these dogs, even though they had no prior antimicrobial treatment or admission to veterinary premises. These findings are of concern due to the potential for opportunistic infections, zoonotic transmission and transmission of antimicrobial resistant determinants from these bacteria to coagulase positive staphylococci.

Keywords: Coagulase-postive staphylococci, Coagulase-negative staphylococci, Meticillin-resistant, Dogs, MALDI-TOF-MS, *Tuf* gene, *Nuc* gene, Antimicrobial-susceptibility

* Correspondence: v.schmidt@liv.ac.uk
†Equal contributors
[1]Department of Infection Biology, The University of Liverpool, Leahurst Campus, Neston, UK
[6]The University of Liverpool School of Veterinary Science, Leahurst Campus, Chester High Road, Neston, Wirral CH64 7TE, UK
Full list of author information is available at the end of the article

Background

Staphylococci are normal commensal bacteria of the skin and mucous membranes of humans and other animals. They can be differentiated by their ability to produce coagulase, with coagulase positive (CoPS) staphylococci regarded as more pathogenic than coagulase negative (CoNS) species [1-5].

Healthy humans and other animals may harbour multiple species and strains of staphylococci. *Staphylococcus aureus* is the main human commensal CoPS species and is carried in the nasal cavity of approximately 30% of healthy people [6]. *S. epidermidis* is the most common CoNS isolated from the nares, perineum, inguinal skin, axillae and interdigital skin in man [2,7]. The main commensal CoPS of dogs, *S. pseudintermedius* [8], has been isolated from 37% to 92% of healthy dogs [9-14], while *S. aureus* is carried by 4.3% to 12% of healthy dogs [10,12,15-20]. Other species isolated from the mucosa and skin of healthy dogs include the CoPS *S. schleiferi* subspecies *coagulans* [10,21] and numerous CoNS (*S. schleiferi* subspecies *schleiferi*, *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. devriesei*, *S. warneri, S. simulans, S. xylosus, S.capitus, S. caprae,* and *S. sciuri*) [12,15,22-26]. The carriage rate of CoNS isolated from the nasal mucosae of healthy dogs was reported to be 38% in one large cross-sectional study [15].

Staphylococci are frequent opportunistic pathogens and commensal isolates are the most common source of infection in humans [3] and dogs [12,16,27]. Antimicrobial resistance can increase the morbidity, mortality and treatment cost of staphylococcal infections. Meticillin (oxacillin) resistance associated with carriage of the *mec*A gene confers resistance to all β-lactam antimicrobials [28]. The *mec*A gene is located on a large mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*), enabling horizontal transmission between staphylococcal isolates [29]. Meticillin resistant staphylococci (MRS) are important pathogens in human and veterinary healthcare and are often multi-drug resistant (MDR; resistant to three or more classes of antimicrobial) [30-35], extremely limiting therapeutic options. MRSP clones with a broader resistance spectrum than MRSA or MR-CoNS are increasingly reported in domestic animals throughout Europe, USA and Canada [32,34]. MR-CoNS are associated with infections in humans and animals [31,36-38]. In humans the most prevalent species is MR *S. epidermidis* (MRSE), which may be a reservoir of MR for *S. aureus* [39,40]. In addition, the SCC*mec* cassette of the major European MRSP clone (ST71-J-t02-II–III) [34] consists of a combination of SCC*mec* II from MRSE and SCC*mec* III from MRSA [41].

The prevalence of MRSA and MRSP carriage in healthy humans and dogs in the community is low [11,18,36,42-47]. However, human community-based surveys report a wider range of carriage rates for MR-CoNS (11–50%) [39,48,49]. MR-CoNS have also been isolated from the carriage sites of 13% of healthy dogs [23,50]. The reported prevalence of MRS is higher in animals exposed to veterinary healthcare environments and antimicrobial therapy [47,51-53] suggesting that these are risk factors for colonisation.

Previous studies looking at the commensal staphylococci in dogs have concentrated on CoPS species, particularly MR-CoPS species, the CoNS group or MR-CoNS species [9-11,13,14,17,23,50], but no study has characterised the complete canine commensal staphylococcal population. Moreover, reporting of the antimicrobial treatment history of dogs in these studies have been inconsistent. The aim of this study was to characterise the mucosal staphylococcal population structure and antimicrobial resistance profiles in healthy Labrador retrievers in the UK in the absence of antimicrobial pressure. This will be important in understanding changes in staphylococcal populations and their antimicrobial susceptibility patterns in dogs exposed to antimicrobials and other risk factors.

Methods

Study population

Labrador retriever dogs were recruited for the study from dog shows in the UK between November 2010 and June 2011. One healthy dog was enrolled from each household if the dog had not received topical or systemic antimicrobial therapy, or had not been admitted to a veterinary clinic within the last 12 months. All dog owners gave written informed consent before enrolment in this study and completed a questionnaire regarding potential risk factors for the carriage of antimicrobial resistant bacteria. The University of Liverpool School of Veterinary Science ethics committee approved the study protocol.

Staphylococci

Specimen collection and bacterial isolation

One nasal swab and one perineal swab were collected from each dog (Copan Eswab LQ Amies Minitip Nylon Flocked Applicator, Appleton Woods, Birmingham, UK). A sterile swab was either inserted 5 mm into one nostril or rubbed on the skin of the perineum for 3–5 seconds before being placed in Amies transport media, stored at 4°C and processed within 36 hours. Swabs were incubated aerobically overnight at 37°C in nutrient broth with 6.5% sodium chloride. The broth was streaked onto mannitol salt agar (MSA), oxacillin resistance screening agar (ORSA) supplemented with 2 μg/ml of oxacillin and Columbia 5% horse blood agar (CAB), and incubated aerobically overnight at 37°C. Where present, isolates typical of staphylococci were selected from all plates, subcultured onto CAB and incubated aerobically overnight at 37°C. Fresh staphylococcal cultures on CAB were subject

to Gram stain (Sigma-Aldrich Company Ltd., Gillingham, UK), tested for catalase (Sigma-Aldrich Company Ltd., Gillingham, UK) and free coagulase production (Rabbit plasma, Pro-Lab, Bromborough, UK) according the manufacturer's instructions and stored at – 80°C in Microbank vials (Pro-Lab, Bromborough, UK). All media were obtained from LabM Ltd, Bury, UK.

Antimicrobial susceptibility testing

Disc diffusion testing was performed on all staphylococcal isolates in accordance with the Clinical and Laboratory Standards Institute (CLSI) and the following panel of ten antimicrobial discs were applied: 1 μg oxacillin (OX), 1 μg ciprofloxacin (CIP), 10 μg gentamicin (GM), 10 μg fusidic acid (FA), 30 μg cefalexin (CFX), 30 μg cefovecin (CVN), 25 μg trimethoprim-sulfamethoxazole (TS), 10 μg tetracycline (Tet), 2 μg clindamycin (CD) and 5 μg vancomycin (Va) [54]. All the discs were purchased from MAST Group Ltd., Liverpool, UK, except for CVN, which were obtained from Oxoid, Basingstoke, UK. Micro-dilution susceptibility testing (Trek Diagnostic Systems, Cleveland, Ohio, USA) was performed on a subset of the CoNS isolates using the same antimicrobial panel, except vancomycin [54]. Interpretation was based on the CLSI guidelines for animal species-specific zone diameter (mm) interpretive standards and minimal inhibitory concentration (MIC; mg/l) breakpoints for veterinary pathogens or human-derived interpretive standards when available. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) zone diameter interpretive standards and MIC breakpoints were used for CIP and FA [55]. The breakpoints used for interpretation of OX resistance were a zone of inhibition of ≤ 17 mm and MIC ≥ 0.5 mg/l for S. *pseudintermedius* and CoNS, and ≤ 10 mm and MIC ≥ 4 mg/l for *S. aureus* [56,57]. The breakpoints used for interpretation of resistance to CVN as a zone of inhibition of ≤ 19 mm and MIC ≥ 8 mg/l in accordance with the manufacturer's recommendations. The reference strain *S. aureus* ATCC®25923 (LGC Standards, Teddington, UK) was used for quality control for MIC and zone diameter determinations.

DNA extraction

Three colonies of each staphylococcal isolate were homogenised in 90 μl of sterile distilled water (SDW) and 10 μl of lysostaphin (1 mg/ml; Sigma-Aldrich Company Ltd., Gillingham, UK) and vortexed for 5 seconds. The suspensions were then incubated at 37°C for 10 minutes and heated at 100°C for 10 minutes before adding 400 μl of SDW. Samples were stored at 4°C.

Characterisation of antimicrobial resistance genes

PCR assays were performed to detect the presence of *mec*A gene (Table 1) in staphylococcal isolates that were phenotypically resistant to oxacillin. All the PCR assays were performed with 0.5 μl of each primer (10 pmol/μl), 1 μl of DNA and 1.1x PCR master mix (ReddyMix™, Thermo Fisher Scientific Inc., Surrey, UK) made up to a total reaction volume of 25 μl. Molecular grade water (Sigma-Aldrich Company Ltd., Gillingham, UK) was used as the negative control in all PCR assays. PCR products were analysed by agarose gel (1.5%) electrophoresis and the DNA fragments were visualised under UV light after ethidium bromide staining.

Species identification

Genotypic species identification

PCR assays to detect the presence of the *nuc* genes of *S. pseudintermedius, S. aureus* and *S. schleiferi* were performed on all CoPS isolates using Qiagen® Multiplex PCR Mix (Qiagen, Crawley, UK), according to the manufacturer's instructions with minor modifications. In short, the PCR assays were performed in a reaction volume of 25 μl, consisting of 5 μl of bacterial DNA extract, 12.5 μl of master mix, 2.5 μl of 10x primer mix (2 μM of each primer) and 5 μl of RNase-free water. The cycling conditions consisted of an initial activation step at 95°C for 15 minutes, followed by 30 cycles of 95°C for 30 seconds, 57°C for 90 seconds and 72°C for 60 seconds, and a final extension step at 72°C for 10 minutes (Table 1).

MALDI-TOF-MS

All isolates were subjected to matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) according to the manufacturer's protocol. Raw spectra were analysed by the MALDI Biotyper 2.0 software programme with default settings (Bruker Daltonics, Bremen, Germany). The extraction method was performed as previously described [58] on overnight colonies grown on CAB at 37°C and all isolates were tested in duplicate. The bacterial test standard (*E. coli* DH5 alpha, Bruker, Bremen, Germany) was used for calibration before each experiment and included in duplicate on each target plate. The mass peak profiles were matched to the reference database and a score generated based on similarity [59].

Sequencing

Two subsets of isolates detected from our group of dogs underwent sequencing following PCR amplification of the *tuf* gene [59,60]; a control group of CoNS isolates (n = 27) identified by MALDI-TOF-MS and a test group of isolates (n = 52) that had not been identified by MALDI-TOF-MS. Initial PCR assays were performed using HotStarTaq® Master Mix Kit (Qiagen, Crawley, UK) in a 25 μl reaction volume with an initial activation step at 95°C for 15 minutes followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, with a final

Table 1 Details of PCR assays used in this study for *nuc*, *tuf* and *mec*A gene identification

Primer	Sequence (5′-3′)	Amplicon size (bp)	Annealing Temperature (°C)	Control strain	Reference
au-F3	TCGCTTGCTATGATTGTGG	359	57	*S. aureus* ATCC®25923 (LGC Standards, Teddington, UK)	[77]
au-nucR*	GCCAATGTTCTACCATAGC				
pse-F2	TRGGCAGTAGGATTCGTTAA	926	57	*S. pseudintermedius* (clinical isolate)	
pse-R5*	CTTTTGTGCTYCMTTTTGG				
SSnucF	AATGGCTACAATGATAATCACTAA	526	57	*S. schleiferi* subspecies *coagulans* ATCC®49545	
SS*nuc*R*	CATATCTGTCTTTCGGCGCG				
tuf-F	GCCAGTTGAGGACGTATTCT	412	55	*S. epidermidis* ATCC®12228	[105]
tuf-R	CCATTTCAGTACCTTCTGGTAA				
*mec*AF	TGGCTATCGTGTCACAATCG	310	55	MRSA (clinical isolate)	[103,104]
*mec*AR	CTGGAACTTGTTGAGCAGAG				
mA1	TGCTATCCACCCTCAAACAGG	286	57		
mA2	AACGTTGTAACCACCCCAAGA				

(*multiplex assay).

extension step of 72°C for 5 minutes according to the manufacturer's protocol. The resulting amplicons were sequenced using BigDye Terminator version 1.1 cycle sequencing (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol on the ABI3131 genetic analyser at the Department of Microbiology, Royal Liverpool University Hospital. The sequences were aligned using the ABI Sequencing analysis software, with contiguous sequences matched to the GenBank database using the Basic Local Alignment Search Tool (BLAST) [61] and positively identified if there was ≥ 98% sequence similarity with a reference sequence. *S. epidermidis* ATCC®12228 was used as the control strain.

Statistical analysis

Data were analysed using SPSS software package (SPSS 20.0 for Mac, SPSS Inc, Chicago, Illinois).

To examine the association between isolation of *S. pseudintermedius* with each of the 16 different CoNS species Pearson's chi-square was calculated ($P < 0.003$; Bonferroni correction). To examine the association between MR and MDR with potential risk factors (previous antimicrobial therapy or hospitalisation within 12 months of enrolment, health-care or large animal-association by in-contact people or pets) identified from the questionnaires Pearson's chi-square was calculated ($P < 0.0125$; Bonferroni correction). To examine the agreement between antimicrobial susceptibility tests by disc diffusion and MIC a *kappa* statistic was calculated [62] and an independent *t*-test was conducted to compare the MIC of oxacillin resistant CoNS isolates that were either positive or negative for the *mec*A gene.

Results

Staphylococci

Specimen collection

Seventy-three Labrador retriever dogs were recruited. Twenty-one dogs were aged between 3 to 12 months, 25 dogs were aged between 12 months to 2 years, and 27 dogs were > 2 years old, with 35 female dogs and 38 male dogs in total.

Bacterial isolation

Staphylococci were isolated from in 72 out of 73 dogs (99%; 95% confidence interval (CI): 99.6-95.8) and from both sample sites in the majority of dogs (78%; 95% CI: 67.3, 86.0). Isolation of staphylococci from the nasal mucosae (16%, 95% CI 9.7, 26.6) or perineum (4%, 95% CI 1.4, 11.4) only occurred in a small number of dogs. If only the nasal mucosae had been sampled, CoPS (all *S. pseudintermedius*) would not have been detected in seven dogs (10%, 95% CI 4.7, 18.5) and CoNS in six dogs (8%, 95% CI 3.8, 16.8). CoNS were detected in the majority of dogs (95%, 95% CI 86.7, 97.8) either alone (52%, 95% CI 40.8, 63.1) or with CoPS (43%, 95% CI 31.8, 53.9). Detection of CoPS alone was significantly less common (4%, 95% CI 1.4, 11.4). In total, there were 436 staphylococcal isolates; 102 of which were CoPS and 334 were CoNS isolates.

Antimicrobial susceptibility testing by disc diffusion

The overall prevalence of antimicrobial resistance among the isolates detected in this study appeared high, with at least one MDR isolate detected in 34% of dogs. Antimicrobial resistant CoNS isolates were detected in more dogs than antimicrobial resistant CoPS isolates for OX,

GM, FA, CFX, CVN and CD and MDR (Figure 1). At least one OX resistant isolate was detected in 58% dogs (n = 126 oxacillin resistant isolates), but resistance to the other tested β-lactam antimicrobials, CVN (25%) and CFX (29%), was less common. Few CoPS demonstrated antimicrobial resistance; isolates from twelve dogs had Tet resistance (all *S. pseudintermedius*), seven with FA resistance *(S. pseudintermedius* = 5, *S. aureus* = 3); two with TS resistance (both *S. pseudintermedius*); two with CD resistance (*S. pseudintermedius* = 1, *S. aureus* = 1) and two with CIP resistance (both *S. pseudintermedius*). MDR CoPS was detected from only one dog (*S. pseudintermedius* with FA, Tet and CD resistance) (Figure 1).

MIC compared to disc diffusion testing for antimicrobial resistance

Micro-dilution susceptibility testing (Trek Diagnostic Systems, Cleveland, Ohio, USA) was performed on 172 CoNS isolates, of which 52 were OX susceptible and 120 were OX resistant by disc diffusion. The OX resistant isolates were further divided into those found to be positive (n = 74) or negative (n = 46) for carriage of the *mec*A gene by PCR. The strength of agreement between antimicrobial resistance detected by MIC and disc diffusion was very good for OX, GM, CVN, Tet and CD resistance, good for CFX and TS resistance and moderate for CIP (*Kappa* = 0.593) and FC resistance (*Kappa* = 0.589). MIC testing identified more isolates as resistant to OX, GM, CFX, CVN and Tet compared to disc diffusion, and disc diffusion identified more isolates as resistant to CIP, FA, TS and CD compared to MIC testing (Table 2).

Characterisation of antimicrobial resistance genes

Of the 126 OX resistant CoNS isolates detected by disc diffusion, 75 isolates (60%, 95% CI 51, 68) from 31 dogs (42%, 95% CI 32, 54) were positive for the *mec*A gene (Figure 1). Nine additional oxacillin resistant isolates were detected by MIC and two of these were positive for the *mec*A gene, resulting in two additional dogs with MR-CoNS and one additional dog with phenotypic oxacillin resistant CoNS. There was a significant difference between the MIC of *mec*A positive (M = 3.84, SD = 0.18) and *mec*A negative isolates (M = 0.97, SD = 0.12, $P < 0.001$). In addition the epidemiological breakpoint for OX resistant CoNS isolates with *mec*A gene carriage isolated in this study was consistent with the clinical CLSI breakpoint (≥ 0.5 mg/l) (Figure 2). Eleven different CoNS species (*S. epidermidis, S. warneri, S. sciuri, S. equorum, S. fleurettii,*

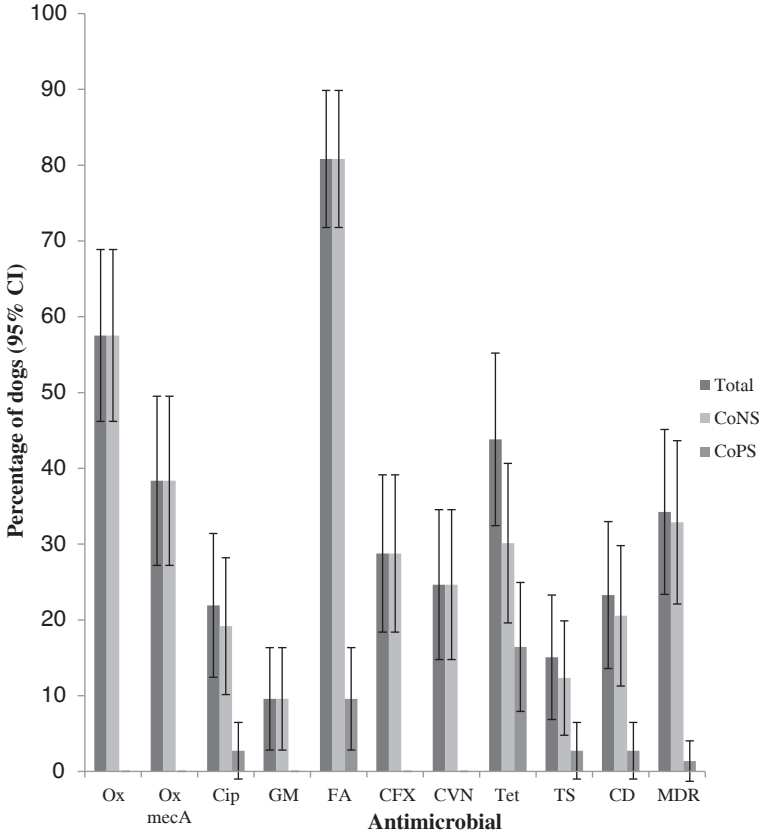


Figure 1 The proportion of dogs (n = 73) carrying at least one staphylococcal isolate with resistance to each antimicrobial, MDR and *mec*A gene positive oxacillin resistance by the disc diffusion method. Total = CoNS and CoPS.

Table 2 Cross tabulation of the results of 172 staphylococcal isolates classified as resistant or susceptible to the antimicrobials tested in this study by both MIC and disc diffusion testing

Antimicrobial resistance			MIC		
			No	Yes	Total
Oxacillin (OX)	**Disc diffusion**	No	50	10	60
		Yes	2	110	112
		Total	52	120	172
		Kappa = 0.842			
			No	Yes	Total
Oxacillin *mec*A positive		No	115	0	115
		Yes	1	56	57
		Total	116	56	172
		Kappa = 0.987			
			No	Yes	Total
Ciprofloxacin (CIP)		No	146	0	157
		Yes	14	12	15
		Total	160	12	172
		Kappa = 0.593			
			No	Yes	Total
Gentamicin (GM)		No	156	1	157
		Yes	0	15	15
		Total	156	16	172
		Kappa = 0.965			
			No	Yes	Total
Fusidic acid (FA)		No	36	5	41
		Yes	25	106	131
		Total	61	111	172
		Kappa = 0.589			
			No	Yes	Total
Cefalexin (CFX)		No	117	15	132
		Yes	0	40	40
		Total	117	55	172
		Kappa = 0.784			
			No	Yes	Total
Cefovecin (CVN)		No	130	11	141
		Yes	0	31	31
		Total	130	42	172
		Kappa = 0.810			
			No	Yes	Total
Tetracycline (T10)		No	148	1	149
		Yes	0	23	23
		Total	148	24	172
		Kappa = 0.975			
			No	Yes	Total
Trimethoprim-sulfamethoxazole (TS)		No	156	2	158
		Yes	3	11	14
		Total	159	13	172
		Kappa = 0.799			
			No	Yes	Total
Clindamycin (CD)		No	148	2	150
		Yes	4	18	22
		Total	152	20	172
		Kappa = 0.837			

S. vitulinus, S. saprophyticus, S. haemolyticus, S. lentus, S. succinus and *S. pettenkoferi*) were found to carry the *mec*A gene. Among oxacillin resistant CoNS species, *S epidermidis* and *S. sciuri* were more likely to carry the *mec*A gene than *S. saprophyticus*, *S. equorum*, *S. vitulinus* and *S. succinus* (Figure 3). MRSE isolates were detected in 18 dogs (25%, 95% CI 14.8, 34.5), meticillin-resistant *S. warneri* were detected in 7 dogs (10%, 95% CI 2.8, 16.3) and meticillin-resistant *S. sciuri* were detected in 5 dogs (7%, 95% CI 1.1, 12.6). The remaining species were only isolated from one or two dogs. MDR *mec*A positive CoNS were detected in 19 dogs (26%, 95% CI 17.3, 37.1). There was no significant association between detection of MR-CoNS or MDR isolates and potential risk factors tested in this study (Pearson's chi-square; $P < 0.0125$).

Species identification

Phenotypic and biochemical methods identified 436 isolates as *Staphylococcus* species. Using a combination of *nuc* gene PCR, MALDI-TOF-MS and sequencing of the *tuf* gene, 399 isolates (92%, 95% CI 88.5, 93.8) were identified to the species level. MALDI-TOF-MS identified 345 isolates to the species level including 264 of 334 CoNS isolates (79%, 95% CI 74.4, 83.1). Amplification and sequencing of the *tuf* gene identified 33 out of 51 CoNS isolates (65%, 95% CI 51, 76.4) to the species level (n = 11 species; ≥ 98% sequence similarity) and an additional control group (n = 27) of CoNS isolates that had also been identified by MALDI-TOF-MS. There was 100% agreement between the two methods for the identification of the control group. In particular, sequencing of the *tuf* gene identified all of the *S. fleurettii*, *S. arlettae* and *S. pettenkoferi* isolates, 12 isolates closely related to

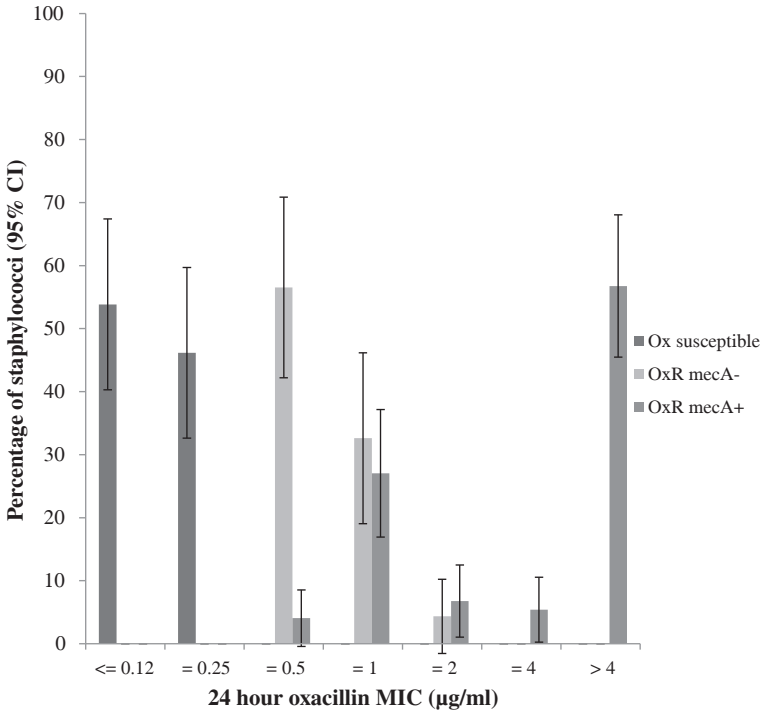


Figure 2 The MIC (μg/ml) data for staphylococcal isolates (n = 172). The isolates consisted of 52 oxacillin susceptible isolates, 46 oxacillin resistant *mec*A negative isolates and 74 oxacillin resistant *mec*A positive isolates.

S. felis (96% sequence similarity) and an additional 15 isolates to the genus level (*Staphylococcus* spp. ≥ 98% sequence similarity). PCR amplification of the *nuc* gene detected all of the *S. aureus* n = 11 (100%, 95% CI 74.1, 100) and *S. pseudintermedius* isolates n = 91 (100%, 95% CI 96.0, 100). There was 100% agreement of this assay with MALDI-TOF-MS for the identification of *S. aureus* isolates, however MALDI-TOF-MS only identified 69 out of 91 *S. pseudintermedius* isolates.

Overall from the combined results using PCR amplification of the *nuc* gene, MALDI-TOF-MS and sequencing of the *tuf* gene we detected *S. epidermidis* in 52% (95% CI 41, 63) and *S. pseudintermedius* in 44% (95% CI 33, 55) of the dogs. *S. warneri* and *S. equorum* were the

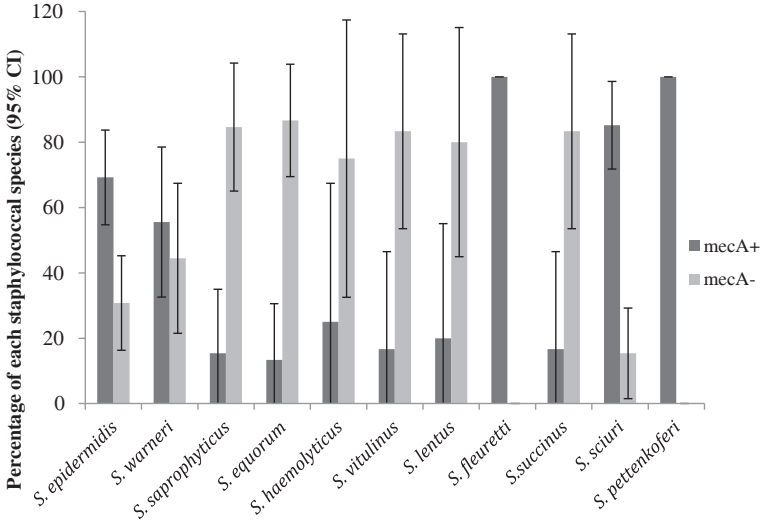


Figure 3 The percentage of each oxacillin-resistant staphylococcal species by disc diffusion and MIC that was either positive (*mec*A+) or negative (*mec*A-) for the *mec*A gene.

next most common species, isolated from 30% and 27% of dogs respectively, and the remaining staphylococcal species were carried by no more than 15% of the dogs. *S. aureus* was detected in 6 of the dogs, exclusively from the nasal mucosae, and usually with *S. pseudintermedius* (88%, 95% CI 52.9, 97.8). *S. pseudintermedius* was concurrently isolated with 16 different CoNS species, although there was no significant association between the presence of *S. pseudintermedius* and any CoNS species (Pearson's chi-square; $P < 0.003$) (Figure 4 and Table 3).

Discussion

This is the first study incorporating MALDI-TOF-MS to successfully characterise commensal staphylococcal populations in a group of healthy dogs in the absence of antimicrobial pressure. We isolated staphylococci from 99% of our dogs, with 95% carrying CoNS and 47% carrying CoPS. The relative prevalence of the staphylococci concurs with other published studies in humans [2,3], horses [63-67] and dogs [15,17,68], although the overall staphylococcal prevalence was double that reported for healthy vet visiting dogs [15]. This could be related to the study population and techniques, as we sampled both the nose and the perineum to increase detection of CoPS [12,13,68,69].

We were able to assign 92% of the staphylococcal isolates to 20 different species, including 18 CoNS. This is the first study to demonstrate such diversity in dogs, and carriage of this number of different species has only been previously reported for humans [2,10,12,15,21-26,70]. The

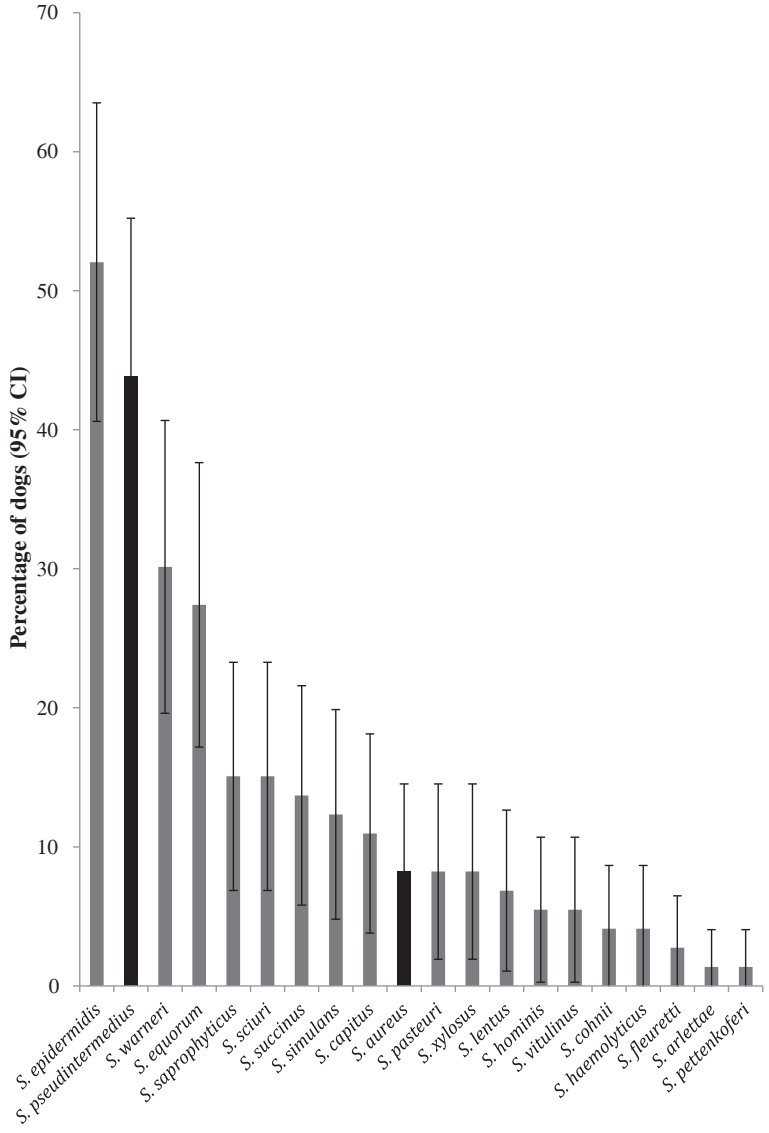


Figure 4 The percentage of dogs (n = 73) carrying each staphylococcal species identified in this study by MALDI-TOF-MS, PCR of the *nuc* gene and sequencing of the *tuf* gene (CoNS grey and CoPS black).

Table 3 The number of staphylococcal isolates identified to species level by MALDI-TOF-MS, *nuc* gene PCR (CoPS), and *tuf* gene sequencing

Staphylococcal species	Number of isolates	Number (%) of positive dogs	Number (%) identified by MALDI-TOF-MS	Number (%) of CoPS identified by *nuc* PCR	Number (%) identified by *tuf* gene sequencing
S. pseudintermedius	91	32 (44)	70 (77)	91 (100)	0
S. aureus	11	6 (8)	11 (100)	11 (100)	0
S. epidermidis	67	38 (52)	64 (96)	N/A	3 (4)
S. warneri	35	22 (30)	35 (100)	N/A	0
S. equorum	39	20 (27)	36 (92)	N/A	3 (8)
S. saprophyticus	19	11 (15)	15 (79)	N/A	4 (21)
S. sciuri	27	11 (15)	21 (78)	N/A	6 (22)
S. succinus	19	10 (14)	16 (84)	N/A	3 (16)
S. simulans	15	9 (12)	15 (100)	N/A	0
S. capitus	7	8 (11)	7 (100)	N/A	0
S. pasteuri	8	6 (8)	8 (100)	N/A	0
S. xylosus	17	6 (8)	17 (100)	N/A	0
S. lentus	9	5 (7)	4 (44)	N/A	5 (56)
S. hominis	6	4 (5)	6 (100)	N/A	0
S. cohnii	5	3 (4)	3 (60)	N/A	2 (40)
S. vitulinus	14	3 (4)	13 (93)	N/A	1 (7)
S. haemolyticus	4	3 (4)	4 (100)	N/A	0
S. fleurettii	*4*	2 (3)	0	N/A	4 (100)
S. arlettae	1	1 (1)	0	N/A	1 (100)
S. pettenkoferi	1	1 (1)	0	N/A	1 (100)
Total ID	399[d]	72[e]	345[f]	101[f]	33[f]
Staphylococcus spp.	3	N/A	N/A	N/A	3
Species related to *S. felis*	12	N/A	N/A	N/A	12
No ID	22	N/A	N/A	2	3
Total	436[a]	73[b]	436[c]	102[c]	51[c]

Values in the table are expressed as total numbers and percentage in parenthesis where applicable. [a]Total number of isolates in study, [b]total number of dogs in study, [c]total number of isolates tested by each method, [d]total number of isolates with positive identification (ID), [e]number of dogs with staphylococcal detection, [f]number of isolates with positive ID from each method.

most common species was *S. epidermidis*, which was detected in 52% of the dogs, mainly from the nasal cavity. This is similar to human reports [71], but apart from one canine study [23], *S. epidermidis* has not been commonly reported in different animal species [67,72,73]. *S. pseudintermedius* was the second most common species and the most common CoPS detected, also in agreement with previous reports [9-11,13]. Unlike *S. epidermidis*, *S. pseudintermedius* was carried equally in the nose and on the perineum, suggesting that this species may have a wider range of mucosal niches. Very few dogs carried *S. aureus* (8%), which is comparable to other studies that reported carriage rates of approximately 7% from healthy vet visiting dogs [12,15]. The majority of the CoNS in our study were human-associated and included *S. epidermidis*, *S. hominis, S. haemolyticus, S. capitus, S. saprophyticus, S. warneri, S. cohnii, S. simulans, S. pettenkoferi and S. pasteuri.* Human associated CoNS species have previously been isolated from dogs, horses, cows and pigs [23,67,72,74-76]. The other CoNS species isolated from our dogs are reported as indigenous to animals (*S. equorum*, *S. vitulinus, S. arlettae S. sciuri*, *S. lentus* and *S. fleurettii*) [2].

We used several methods to identify staphylococcal isolates to species level. Multiplex PCR for the *nuc* gene is an accurate, rapid and cost efficient method to speciate CoPS [77], which identified 100% of our *S. pseudintermedius* (n = 91) and 100% of our *S. aureus* isolates (n = 11). Recently MALDI-TOF-MS has been reported as a rapid and reliable method to characterise CoNS, *S. aureus* and *S. intermedius* group (SIG) strains [59,72,78-80]. MALDI-TOF-MS identified all of our *S. aureus* isolates, 77% of our *S. pseudintermedius* isolates and 79% of our CoNS isolates, identified by phenotypic and biochemical characteristics, to the species level. Similar results for the identification of *S. aureus*, *S. pseudintermedius* and CoNS by

MALDI-TOF-MS, in comparison to molecular methods, have been reported [79-81]. The overall performance of MALDI-TOF-MS to speciate the staphylococcal isolates in this study, similar to other reports [80], is likely to be directly related to the database, which at the time of analysis consisted mainly of common human-derived species and only one *S. pseudintermedius* strain. However species level identification will improve as more highly characterised reference isolates are added to the database. Amplification and sequencing of the *tuf* gene is regarded as the gold standard to speciate CoNS isolates [59,60]. This method identified 77% of the tested staphylococcal isolates (n = 79) to the species level. The performance of this method in our study may have been affected by the lack of certain-animal derived isolates representing different species in the database. Additionally, we may have improved identification by sequencing a larger region of the *tuf* gene. We sequenced a previously described 412 base pair region of the *tuf* gene that was reported to have successfully identified 88% of human-derived staphylococcal strains [60]. However, a more recent publication that sequenced a 660 bp region of the *tuf* gene, reported 98.9% identification of 186 human and animal-derived staphylococcal strains.

We did not detect any MR-CoPS isolates. Other studies of healthy dogs have similarly reported a low prevalence [15,82,83]. In contrast, 58% of the dogs in our study carried at least one CoNS isolate with phenotypic meticillin resistance and 42% carried a meticillin resistant *mec*A positive isolate. Other studies have also reported high levels of meticillin resistance among CoNS isolates from humans [31,35,84], horses [23,64-66] and livestock [72,85]. However, the prevalence of MR-CoNS carriage in our study is markedly higher than the levels reported in other community canine studies [15,23,50,74,83]. High community carriage rates of MR-CoNS are of concern for animals and humans, as these organisms may not only be reservoirs of resistance genes for CoPS [39,40,86], but also act as pathogens [31,36-38,87-89]. Cross-transmission is reported to be an important mechanism for dissemination of MRS [49,90], and transmission between dogs and in-contact humans may occur in the community and in veterinary premises [36,83].

Nine different CoNS species carried the *mec*A gene in our study with MRSE detected in 25% of our dogs. MRSE is the predominant MR-CoNS species in humans both in hospital and community settings [39,48,49], and has been reported in one study investigating nasal carriage of MRS in dogs [23]. Other canine studies have isolated meticillin resistant *S. sciuri* and meticillin resistant *S. warneri* [23,74]. Our research found that the majority of the *S. sciuri* and *S. fleurettii* isolates were *mec*A positive, which is consistent with other studies in humans, livestock and horses [35,64,66,67,72].

MDR CoNS (n = 38) were isolated from 34% of dogs in this study. MDR was generally associated with resistance to β-lactams, FA and additional antimicrobials. In particular MDR-MRSE were resistant to at least four antimicrobial classes tested in our study. A similar finding was reported in a study of hospitalised animals, medical equipment and veterinary staff [68]. MDR among CoNS isolates is widely reported [15,49,72,73,91] and may be associated with the carriage of multiple antimicrobial resistance genes on SCC*mec* cassettes [40]. In contrast, the majority of our commensal CoPS isolates were susceptible to a broad range of antimicrobials (apart from Tet), in line with previous reports for clinical isolates [92-94] and isolates from healthy vet-visiting dogs [15]. There was good to very good agreement between disc and MIC antimicrobial susceptibility testing apart for FC and CIP. These two antimicrobials were the only ones where human breakpoints were applied and emphasises potential species differences in pharmacokinetic and pharmacodynamic data for individual antimicrobials.

The *mec*A gene was not identified in 40% of the phenotypic oxacillin resistant isolates in this study and may include some isolate duplication due to our sampling methods. Other studies have reported phenotypic meticillin resistance with absence of the *mec*A gene in staphylococci [95-98]. Our OX-resistant *mec*A negative isolates may be truly negative for the *mec*A gene as they were less likely to be resistant to the other antimicrobials tested in this study, including CVN and CFX, and had significantly lower MICs compared to the OX resistant *mec*A positive isolates. It is possible that they had low-level resistance associated with other mechanisms such as hyperproduction of β-lactamases [99], or production of an oxacillin-specific β-lactamases [100]. There are bovine mastitis CoNS isolates with oxacillin MICs of 0.5 – 1 mg/l that lack the *mec*A gene [97], and the CLSI guidelines state that 'oxacillin interpretive criteria may overcall resistance for these CoNS strains' [57]. In addition, many of the published PCR assays to identify and characterise the *mec*A gene have been developed for MRSA [101-104] and may therefore lack sensitivity for some CoNS isolates. However, other authors have successfully employed the same methods for *mec*A detection among CoNS isolates as used in our study [68,98,105]. Nevertheless it is possible that additional PCR assay [106], or latex agglutination for PBP2a [107] may have improved the sensitivity of *mec*A detection or detected phenotypic *mec*A-associated resistance in our oxacillin resistant *mec*A negative isolates.

Our study had some limitations, including the small sample size. Still, these dogs yielded 436 staphylococcal isolates and a high prevalence of resistance was identified among the CoNS isolates even in the absence of antimicrobial exposure. Another weakness was that the study population was limited to one breed (Labrador

retrievers) and the dogs were recruited at dog shows. Kennelled dogs have been shown to have higher levels of antimicrobial resistance in faecal *E. coli* than individually owned and non-kennelled dogs [108]. Kennelling was transient in our dogs, but this may have affected the results. Many of the dogs came from multi-dog households but only one dog from each household was sampled to avoid cluster effects.

Conclusions

This is the first comprehensive study of commensal staphylococcal populations in a group of healthy dogs. Staphylococci, particularly CoNS, form a normal part of the canine commensal population and were detected from almost all the dogs. The most commonly isolated staphylococcal species in this group of dogs was *S. epidermidis*, although a wide variety of other human- and animal-associated CoNS were found. CoPS were less common, and the major species was *S. pseudintermedius*. Antimicrobial resistance among the CoPS was uncommon, and no MRSP or MRSA were isolated, however the sample size was small. Antimicrobial resistance (including MDR and meticillin resistance) was common among the CoNS isolates, even though this was a community population of healthy dogs in the absence of direct-antimicrobial pressure or veterinary contact. The clinical significance of commensal CoNS and MR-CoNS is unclear, but *S. epidermidis* carries a number of virulence factors and is an increasing cause of nosocomial and community-acquired infections in humans. The possibility of similar infections escalating in companion animals cannot be excluded. In addition, there is potential for cross-species transmission of antimicrobial resistant bacteria and exchange of resistance determinants between bacterial species. In particular, MR- and MDR-CoNS may provide a reservoir of antimicrobial resistance genes that could rapidly spread within bacterial populations under the selection pressure exerted by antimicrobial therapy. Further longitudinal studies in healthy dogs and in dogs receiving antimicrobials are required to assess the population diversity, antimicrobial resistance profiles and persistence of antimicrobial resistant staphylococci in dogs.

Abbreviations

CoPS: Coagulase positive staphylococci; CoNS: Coagulase negative staphylococci; MDR: Multidrug resistance; MR: Meticillin resistance; MIC: Minimum inhibitory concentration; MRSA: Meticillin resistant *Staphylococcus aureus*; MRSP: Meticillin resistant *staphylococcus pseudintermedius*; MRSE: Meticillin resistant *staphylococcus epidermidis*; OX: Oxacillin; CIP: Ciprofloxacin; CFX: Cefalexin; CVN: Cefovecin; TS: Cotrimazole; Tet: Tetracycline; CD: Clindamycin.

Competing interests

Vanessa Schmidt, Neil McEwan, Stephen Shaw 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.

Authors' contributions

VS was responsible for sample collection and processing, data analysis and writing the manuscript. NJW was responsible for advising on the microbiology methodology used in the study and interpretation of results and contributed to the writing of the manuscript. GP advised on statistical analysis. NM advised on ethical permission and sample collection and contributed to the writing of the manuscript. SS assisted sample collection and contributed to the writing of the manuscript. CEC was responsible for advising on and performing sequencing. SD advised on interpretation of results and contributed to the writing of the manuscript. TN advised on sample collection, interpretation of results and contributed to the writing of the manuscript. NW, GP, NM, SD and TN supervised VS during this project. All authors were involved in the design of this project and reviewed and approved the final manuscript.

Acknowledgements

The authors would like to thank Zoetis (previously Pfizer Animal Health) for funding this project, and Maureen D'Arcy and the North West and Midland Counties Labrador Retriever Clubs for allowing us to obtain samples. We would like to thank Erica Tranfield from Bruker (Liverpool, UK) for training and access to MALDI-TOF-MS Biotyper. We would also like to thank Ruth Ryvar and Gill Hutchinson for their technical support, Dorina Timofte and Andy Wattret for their technical advice, and Clara MacFarlane, Atina Unwin and Camilla Brena for their help in sample collection.

Author details

[1]Department of Infection Biology, The University of Liverpool, Leahurst Campus, Neston, UK. [2]Department of Epidemiology and Population Health, The University of Liverpool, Leahurst Campus, Neston, UK. [3]Infection and Immunity, Royal Liverpool University Hospital, Liverpool, UK. [4]UK VetDerm, Coalville, UK. [5]The Royal (Dick) School of Veterinary Studies, Easter Bush Campus, University of Edinburgh, Midlothian, UK. [6]The University of Liverpool School of Veterinary Science, Leahurst Campus, Chester High Road, Neston, Wirral CH64 7TE, UK.

Received: 19 April 2013 Accepted: 6 January 2014
Published: 14 January 2014

References

1. Kloos WE: **Natural populations of the genus *Staphylococcus*.** *Annu Rev Microbiol* 1980, **34:**559–592.
2. Kloos WE, Bannerman TL: **Update on clinical significance of coagulase-negative staphylococci.** *Clin Microbiol Rev* 1994, **7**(1):117–140.
3. von Eiff C, Peters G, Heilmann C: **Pathogenesis of infections due to coagulase-negative staphylococci.** *Lancet Infect Dis* 2002, **2**(11):677–685.
4. Rich M: **Staphylococci in animals: prevalence, identification and antimicrobial susceptibility, with an emphasis on methicillin-resistant *Staphylococcus aureus*.** *Br J Biomed Sci* 2005, **62**(2):98–105.
5. Devriese LA, Vancanneyt M, Baele M, Vaneechoutte M, De Graef E, Snauwaert C, Cleenwerck I, Dawyndt P, Swings J, Decostere A, *et al*: ***Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals.** *Int J Syst Evol Microbiol* 2005, **55**(Pt 4):1569–1573.
6. Mainous AG 3rd, Hueston WJ, Everett CJ, Diaz VA: **Nasal carriage of *Staphylococcus aureus* and methicillin-resistant *S aureus* in the United States, 2001–2002.** *Ann Fam Med* 2006, **4**(2):132–137.
7. Huebner J, Goldmann DA: **Coagulase-negative staphylococci: role as pathogens.** *Annu Rev Med* 1999, **50:**223–236.
8. Mason IS, Mason KV, Lloyd DH: **A review of the biology of canine skin with respect to the commensals *Staphylococcus intermedius, Demodex canis* and *Malassezia pachydermatis*.** *Vet Dermatol* 1996, **7**(3):119–132.
9. Devriese LA, De Pelsmaecker K: **The anal region as a main carrier site of *Staphylococcus intermedius* and *Streptococcus canis* in dogs.** *Vet Rec* 1987, **121**(13):302–303.
10. Griffeth GC, Morris DO, Abraham JL, Shofer FS, Rankin SC: **Screening for skin carriage of methicillin-resistant coagulase-positive staphylococci and *Staphylococcus schleiferi* in dogs with healthy and inflamed skin.** *Vet Dermatol* 2008, **19**(3):142–149.
11. Hanselman BA, Kruth SA, Rousseau J, Weese JS: **Coagulase positive staphylococcal colonization of humans and their household pets.** *Can Vet J* 2009, **50**(9):954–958.

12. Fazakerley J, Williams N, Carter S, McEwan N, Nuttall T: **Heterogeneity of *Staphylococcus pseudintermedius* isolates from atopic and healthy dogs.** *Vet Dermatol* 2010, **21**(6):578–585.
13. Rubin JE, Ball KR, Chirino-Trejo M: **Antimicrobial susceptibility of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* isolated from various animals.** *Can Vet J* 2011, **52**(2):153–157.
14. Paul NC, Bargman SC, Moodley A, Nielsen SS, Guardabassi L: ***Staphylococcus pseudintermedius* colonisation patterns and strain diversity in healthy dogs: a cross-sectional and longitudinal study.** *Vet Microbiol* 2012, **160**(3–4):420–427.
15. Wedley AL: *Prevalence of Staphyococcus spp. carriage in dogs.* Department of Epidemiology and Population Health, Institute of Infection and Global Health, University of Liverpool; 2012.
16. Pinchbeck LR, Cole LK, Hillier A, Kowalski JJ, Rajala-Schultz PJ, Bannerman TL, York S: **Genotypic relatedness of staphylococcal strains isolated from pustules and carriage sites in dogs with superficial bacterial folliculitis.** *Am J Vet Res* 2006, **67**(8):1337–1346.
17. Loeffler A, Boag AK, Sung J, Lindsay JA, Guardabassi L, Dalsgaard A, Smith H, Stevens KB, Lloyd DH: **Prevalence of methicillin-resistant *Staphylococcus aureus* among staff and pets in a small animal referral hospital in the UK.** *J Antimicrob Chemother* 2005, **56**(4):692–697.
18. Boost MV, O'Donoghue MM, Siu KH: **Characterisation of methicillin-resistant *Staphylococcus aureus* isolates from dogs and their owners.** *Clin Microbiol Infect* 2007, **13**(7):731–733.
19. Sasaki T, Kikuchi K, Tanaka Y, Takahashi N, Kamata S, Hiramatsu K: **Reclassification of phenotypically identified *Staphylococcus intermedius* strains.** *J Clin Microbiol* 2007, **45**(9):2770–2778.
20. Kottler S, Middleton JR, Perry J, Weese JS, Cohn LA: **Prevalence of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* carriage in three populations.** *J Vet Intern Med* 2010, **24**(1):132–139.
21. Yamashita K, Shimizu A, Kawano J, Uchida E, Haruna A, Igimi S: **Isolation and characterization of staphylococci from external auditory meatus of dogs with or without otitis externa with special reference to Staphylococcus schleiferi subsp. coagulans isolates.** *J Vet Med Sci* 2005, **67**(3):263–268.
22. May ER, Hnilica KA, Frank LA, Jones RD, Bemis DA: **Isolation of *Staphylococcus schleiferi* from healthy dogs and dogs with otitis, pyoderma, or both.** *J Am Vet Med Assoc* 2005, **227**(6):928–931.
23. Bagcigil FA, Moodley A, Baptiste KE, Jensen VF, Guardabassi L: **Occurrence, species distribution, antimicrobial resistance and clonality of methicillin- and erythromycin-resistant staphylococci in the nasal cavity of domestic animals.** *Vet Microbiol* 2007, **121**(3–4):307–315.
24. Kania SA, Williamson NL, Frank LA, Wilkes RP, Jones RD, Bemis DA: **Methicillin resistance of staphylococci isolated from the skin of dogs with pyoderma.** *Am J Vet Res* 2004, **65**(9):1265–1268.
25. Medleau L, Long RE, Brown J, Miller WH: **Frequency and antimicrobial susceptibility of *Staphylococcus species* isolated from canine pyodermas.** *Am J Vet Res* 1986, **47**(2):229–231.
26. Cox HU, Hoskins JD, Newman SS, Foil CS, Turnwald GH, Roy AF: **Temporal study of staphylococcal species on healthy dogs.** *Am J Vet Res* 1988, **49**(6):747–751.
27. Bannoehr J, Guardabassi L: ***Staphylococcus pseudintermedius* in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity.** *Vet Dermatol* 2012, **23**(4):253–266. e251-252.
28. Hartman BJ, Tomasz A: **Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*.** *J Bacteriol* 1984, **158**(2):513–516.
29. Black CC, Solyman SM, Eberlein LC, Bemis DA, Woron AM, Kania SA: **Identification of a predominant multilocus sequence type, pulsed-field gel electrophoresis cluster, and novel staphylococcal chromosomal cassette in clinical isolates of *mec*A-containing, methicillin-resistant *Staphylococcus pseudintermedius*.** *Vet Microbiol* 2009, **139**(3–4):333–338.
30. Hryniewicz W: **Epidemiology of MRSA.** *Infection* 1999, **27**(Suppl 2):S13–S16.
31. Diekema DJ, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones RN, Beach M: **Survey of infections due to *Staphylococcus species*: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999.** *Clin Infect Dis* 2001, **32**(Suppl 2):S114–S132.
32. Loeffler A, Linek M, Moodley A, Guardabassi L, Sung JM, Winkler M, Weiss R, Lloyd DH: **First report of multiresistant, *mec*A-positive *Staphylococcus intermedius* in Europe: 12 cases from a veterinary dermatology referral clinic in Germany.** *Vet Dermatol* 2007, **18**(6):412–421.
33. Weese JS, van Duijkeren E: **Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine.** *Vet Microbiol* 2010, **140**(3–4):418–429.
34. Perreten V, Kadlec K, Schwarz S, Gronlund Andersson U, Finn M, Greko C, Moodley A, Kania SA, Frank LA, Bemis DA, *et al*: **Clonal spread of methicillin-resistant *Staphylococcus pseudintermedius* in Europe and North America: an international multicentre study.** *J Antimicrob Chemother* 2010, **65**(6):1145–1154.
35. Garza-Gonzalez E, Morfin-Otero R, Martinez-Vazquez MA, Gonzalez-Diaz E, Gonzalez-Santiago O, Rodriguez-Noriega E: **Microbiological and molecular characterization of human clinical isolates of *Staphylococcus cohnii*, *Staphylococcus hominis*, and *Staphylococcus sciuri*.** *Scand J Infect Dis* 2011, **43**(11–12):930–936.
36. van Duijkeren E, Kamphuis M, van der Mije IC, Laarhoven LM, Duim B, Wagenaar JA, Houwers DJ: **Transmission of methicillin-resistant *Staphylococcus pseudintermedius* between infected dogs and cats and contact pets, humans and the environment in households and veterinary clinics.** *Vet Microbiol* 2011, **150**(3–4):338–343.
37. Hauschild T, Wojcik A: **Species distribution and properties of staphylococci from canine dermatitis.** *Res Vet Sci* 2007, **82**(1):1–6.
38. Kern A, Perreten V: **Clinical and molecular features of methicillin-resistant, coagulase-negative staphylococci of pets and horses.** *J Antimicrob Chemother* 2013, **68**(6):1256–1266.
39. Barbier F, Ruppe E, Hernandez D, Lebeaux D, Francois P, Felix B, Desprez A, Maiga A, Woerther PL, Gaillard K, *et al*: **Methicillin-resistant coagulase-negative staphylococci in the community: high homology of SCC*mec* IVa between *Staphylococcus epidermidis* and major clones of methicillin-resistant *Staphylococcus aureus*.** *J Infect Dis* 2010, **202**(2):270–281.
40. Smyth DS, Wong A, Robinson DA: **Cross-species spread of SCC*mec* IV subtypes in staphylococci.** *Infect Genet Evol* 2011, **11**(2):446–453.
41. Descloux S, Rossano A, Perreten V: **Characterization of new staphylococcal cassette chromosome *mec* (SCC*mec*) and topoisomerase genes in fluoroquinolone- and methicillin-resistant *Staphylococcus pseudintermedius*.** *J Clin Microbiol* 2008, **46**(5):1818–1823.
42. Abudu L, Blair I, Fraise A, Cheng KK: **Methicillin-resistant *Staphylococcus aureus* (MRSA): a community-based prevalence survey.** *Epidemiol Infect* 2001, **126**(3):351–356.
43. Shopsin B, Mathema B, Martinez J, Ha E, Campo ML, Fierman A, Krasinski K, Kornblum J, Alcabes P, Waddington M, *et al*: **Prevalence of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* in the community.** *J Infect Dis* 2000, **182**(1):359–362.
44. Maudsley J, Stone SP, Kibbler CC, Iliffe SR, Conaty SJ, Cookson BD, Duckworth GJ, Johnson A, Wallace PG: **The community prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in older people living in their own homes: implications for treatment, screening and surveillance in the UK.** *J Hosp Infect* 2004, **57**(3):258–262.
45. Sa-Leao R, Sanches IS, Couto I, Alves CR, de Lencastre H: **Low prevalence of methicillin-resistant strains among *Staphylococcus aureus* colonizing young and healthy members of the community in Portugal.** *Microb Drug Resist* 2001, **7**(3):237–245.
46. Zanelli G, Sansoni A, Zanchi A, Cresti S, Pollini S, Rossolini GM, Cellesi C: ***Staphylococcus aureus* nasal carriage in the community: a survey from central Italy.** *Epidemiol Infect* 2002, **129**(2):417–420.
47. Loeffler A, Pfeiffer DU, Lindsay JA, Magalhaes RJ, Lloyd DH: **Prevalence of and risk factors for MRSA carriage in companion animals: a survey of dogs, cats and horses.** *Epidemiol Infect* 2010:1–10.
48. Lebeaux D, Barbier F, Angebault C, Benmahdi L, Ruppe E, Felix B, Gaillard K, Djossou F, Epelboin L, Dupont C, *et al*: **Evolution of nasal carriage of methicillin-resistant coagulase-negative staphylococci in a remote population.** *Antimicrob Agents Chemother* 2012, **56**(1):315–323.
49. Silva FR, Mattos EM, Coimbra MV, Ferreira-Carvalho BT, Figueiredo AM: **Isolation and molecular characterization of methicillin-resistant coagulase-negative staphylococci from nasal flora of healthy humans at three community institutions in Rio de Janeiro City.** *Epidemiol Infect* 2001, **127**(1):57–62.
50. Vengust M, Anderson ME, Rousseau J, Weese JS: **Methicillin-resistant staphylococcal colonization in clinically normal dogs and horses in the community.** *Lett Appl Microbiol* 2006, **43**(6):602–606.
51. Nienhoff U, Kadlec K, Chaberny IF, Verspohl J, Gerlach GF, Kreienbrock L, Schwarz S, Simon D, Nolte I: **Methicillin-resistant *Staphylococcus***

pseudintermedius among dogs admitted to a small animal hospital. *Vet Microbiol* 2011, **150**(1–2):191–197.
52. Bergstrom A, Gustafsson C, Leander M, Fredriksson M, Gronlund U, Trowald-Wigh G: **Occurrence of methicillin-resistant staphylococci in surgically treated dogs and the environment in a Swedish animal hospital.** *J Small Anim Pract* 2012, **53**(7):404–410.
53. Huerta B, Maldonado A, Ginel PJ, Tarradas C, Gomez-Gascon L, Astorga RJ, Luque I: **Risk factors associated with the antimicrobial resistance of staphylococci in canine pyoderma.** *Vet Microbiol* 2011, **150**(3–4):302–308.
54. CLSI (Ed): *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard—Third Edition. CLSI document M31-A3.* Wayne, PA: Clinical and Laboratory Standards Institute; 2008.
55. EUCAST: **Breakpoint tables for interpretation of MICs and zone diameters. Version 3.1, 2013.** http://www.eucast.org.
56. Bemis DA, Jones RD, Frank LA, Kania SA: **Evaluation of susceptibility test breakpoints used to predict mecA-mediated resistance in *Staphylococcus pseudintermedius* isolated from dogs.** *J Vet Diagn Invest* 2009, **21**(1):53–58.
57. CLSI (Ed): *Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Approved Standard – Fourth Edition. CLSI document VET01-A.* Wayne, PA: Clinical and Laboratory Standards Institute; 2013.
58. Alatoom AA, Cunningham SA, Ihde SM, Mandrekar J, Patel R: **Comparison of direct colony method versus extraction method for identification of gram-positive cocci by use of Bruker Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry.** *J Clin Microbiol* 2011, **49**(8):2868–2873.
59. Carpaij N, Willems RJ, Bonten MJ, Fluit AC: **Comparison of the identification of coagulase-negative staphylococci by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and tuf sequencing.** *Eur J Clin Microbiol Infect Dis* 2011, **30**(10):1169–1172.
60. Heikens E, Fleer A, Paauw A, Florijn A, Fluit AC: **Comparison of genotypic and phenotypic methods for species-level identification of clinical isolates of coagulase-negative staphylococci.** *J Clin Microbiol* 2005, **43**(5):2286–2290.
61. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *J Mol Biol* 1990, **215**(3):403–410.
62. Landis JR, Koch GG: **Measurement of observer agreement for categorical data.** *Biometrics* 1977, **33**(1):159–174.
63. Yasuda R, Kawano J, Onda H, Takagi M, Shimizu A, Anzai T: **Methicillin-resistant coagulase-negative staphylococci isolated from healthy horses in Japan.** *Am J Vet Res* 2000, **61**(11):1451–1455.
64. Busscher JF, van Duijkeren E, Sloet van Oldruitenborgh-Oosterbaan MM: **The prevalence of methicillin-resistant staphylococci in healthy horses in the Netherlands.** *Vet Microbiol* 2006, **113**(1–2):131–136.
65. Moodley A, Guardabassi L: **Clonal spread of methicillin-resistant coagulase-negative staphylococci among horses, personnel and environmental sites at equine facilities.** *Vet Microbiol* 2009, **137**(3–4):397–401.
66. De Martino L, Lucido M, Mallardo K, Facello B, Mallardo M, Iovane G, Pagnini U, Tufano MA, Catalanotti P: **Methicillin-resistant staphylococci isolated from healthy horses and horse personnel in Italy.** *J Vet Diagn Invest* 2010, **22**(1):77–82.
67. Karakulska J, Fijalkowski K, Nawrotek P, Pobucewicz A, Poszumski F, Czernomysy-Furowicz D: **Identification and methicillin resistance of coagulase-negative staphylococci isolated from nasal cavity of healthy horses.** *J Microbiol* 2012, **50**(3):444–451.
68. Moon BY, Youn JH, Shin S, Hwang SY, Park YH: **Genetic and phenotypic characterization of methicillin-resistant staphylococci isolated from veterinary hospitals in South Korea.** *J Vet Diagn Invest* 2012, **24**(3):489–498.
69. Windahl U, Reimegard E, Holst BS, Egenvall A, Fernstrom L, Fredriksson M, Trowald-Wigh G, Andersson UG: **Carriage of methicillin-resistant *Staphylococcus pseudintermedius* in dogs–a longitudinal study.** *BMC Vet Res* 2012, **8**:34.
70. Widerstrom M, Wistrom J, Sjostedt A, Monsen T: **Coagulase-negative staphylococci: update on the molecular epidemiology and clinical presentation, with a focus on *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*.** *Eur J Clin Microbiol Infect Dis* 2012, **31**(1):7–20.
71. Rogers KL, Fey PD, Rupp ME: **Coagulase-negative staphylococcal infections.** *Infect Dis Clin North Am* 2009, **23**(1):73–98.
72. Huber H, Ziegler D, Pfluger V, Vogel G, Zweifel C, Stephan R: **Prevalence and characteristics of methicillin-resistant coagulase-negative staphylococci from livestock, chicken carcasses, bulk tank milk, minced meat, and contact persons.** *BMC Vet Res* 2011, **7**:6.
73. Garbacz K, Zarnowska S, Piechowicz L, Haras K: **Staphylococci isolated from carriage sites and infected sites of dogs as a reservoir of multidrug resistance and methicillin resistance.** *Curr Microbiol* 2013, **66**(2):169–173.
74. Malik S, Coombs GW, O'Brien FG, Peng H, Barton MD: **Molecular typing of methicillin-resistant staphylococci isolated from cats and dogs.** *J Antimicrob Chemother* 2006, **58**(2):428–431.
75. Gillespie BE, Headrick SI, Boonyayatra S, Oliver SP: **Prevalence and persistence of coagulase-negative *Staphylococcus* species in three dairy research herds.** *Vet Microbiol* 2009, **134**(1–2):65–72.
76. Tulinski P, Fluit AC, Wagenaar JA, Mevius D, van de Vijver L, Duim B: **Methicillin-resistant coagulase-negative staphylococci on pig farms as a reservoir of heterogeneous staphylococcal cassette chromosome *mec* elements.** *Appl Environ Microbiol* 2012, **78**(2):299–304.
77. Sasaki T, Tsubakishita S, Tanaka Y, Sakusabe A, Ohtsuka M, Hirotaki S, Kawakami T, Fukata T, Hiramatsu K: **Multiplex-PCR method for species identification of coagulase-positive staphylococci.** *J Clin Microbiol* 2010, **48**(3):765–769.
78. Dubois D, Leyssene D, Chacornac JP, Kostrzewa M, Schmit PO, Talon R, Bonnet R, Delmas J: **Identification of a variety of *Staphylococcus* species by matrix-assisted laser desorption ionization-time of flight mass spectrometry.** *J Clin Microbiol* 2010, **48**(3):941–945.
79. Szabados F, Woloszyn J, Richter C, Kaase M, Gatermann S: **Identification of molecularly defined *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization time of flight mass spectrometry and the Biotyper 2.0 database.** *J Med Microbiol* 2010, **59**(Pt 7):787–790.
80. Decristophoris P, Fasola A, Benagli C, Tonolla M, Petrini O: **Identification of *Staphylococcus intermedius* Group by MALDI-TOF MS.** *Syst Appl Microbiol* 2011, **34**(1):45–51.
81. Bergeron M, Dauwalder O, Gouy M, Freydiere AM, Bes M, Meugnier H, Benito Y, Etienne J, Lina G, Vandenesch F, *et al*: **Species identification of staphylococci by amplification and sequencing of the tuf gene compared to the gap gene and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.** *Eur J Clin Microbiol Infect Dis* 2011, **30**(3):343–354.
82. Onuma K, Tanabe T, Sato H: **Antimicrobial resistance of *Staphylococcus pseudintermedius* isolates from healthy dogs and dogs affected with pyoderma in Japan.** *Vet Dermatol* 2012, **23**(1):17–22. e15.
83. Vanderhaeghen W, Van de Velde E, Crombe F, Polis I, Hermans K, Haesebrouck F, Butaye P: **Screening for methicillin-resistant staphylococci in dogs admitted to a veterinary teaching hospital.** *Res Vet Sci* 2012, **93**(1):133–136.
84. Hanssen AM, Ericson Sollid JU: **SCC*mec* in staphylococci: genes on the move.** *FEMS Immunol Med Microbiol* 2006, **46**(1):8–20.
85. Zhang Y, Agidi S, LeJeune JT: **Diversity of staphylococcal cassette chromosome in coagulase-negative staphylococci from animal sources.** *J Appl Microbiol* 2009, **107**(4):1375–1383.
86. Tsubakishita S, Kuwahara-Arai K, Sasaki T, Hiramatsu K: **Origin and molecular evolution of the determinant of methicillin resistance in staphylococci.** *Antimicrob Agents Chemother* 2010, **54**(10):4352–4359.
87. Duval X, Selton-Suty C, Alla F, Salvador-Mazenq M, Bernard Y, Weber M, Lacassin F, Nazeyrolas P, Chidiac C, Hoen B, *et al*: **Endocarditis in patients with a permanent pacemaker: a 1-year epidemiological survey on infective endocarditis due to valvular and/or pacemaker infection.** *Clin Infect Dis* 2004, **39**(1):68–74.
88. Moran E, Masters S, Berendt AR, McLardy-Smith P, Byren I, Atkins BL: **Guiding empirical antibiotic therapy in orthopaedics: the microbiology of prosthetic joint infection managed by debridement, irrigation and prosthesis retention.** *J Infect* 2007, **55**(1):1–7.
89. Piette A, Verschraegen G: **Role of coagulase-negative staphylococci in human disease.** *Vet Microbiol* 2009, **134**(1–2):45–54.
90. Cimiotti JP, Wu F, Della-Latta P, Nesin M, Larson E: **Emergence of resistant staphylococci on the hands of new graduate nurses.** *Infect Control Hosp Epidemiol* 2004, **25**(5):431–435.
91. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB: **Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study.** *Clin Infect Dis* 2004, **39**(3):309–317.
92. Lloyd DH, Lamport AI, Feeney C: **Sensitivity to antibiotics amongst cutaneous and mucosal isolates of canine pathogenic staphylococci in the UK, 1980–96.** *Vet Dermatol* 1996, **7**:171–175.

93. Kruse H, Hofshagen M, Thoresen SI, Bredal WP, Vollset I, Soli NE: **The antimicrobial susceptibility of *Staphylococcus* species isolated from canine dermatitis.** *Vet Res Commun* 1996, **20**(3):205–214.
94. Hoekstra KA, Paulton RJ: **Clinical prevalence and antimicrobial susceptibility of *Staphylococcus aureus* and *Staphylococcus intermedius* in dogs.** *J Appl Microbiol* 2002, **93**(3):406–413.
95. Suzuki E, Hiramatsu K, Yokota T: **Survey of methicillin-resistant clinical strains of coagulase-negative staphylococci for *mec*A gene distribution.** *Antimicrob Agents Chemother* 1992, **36**(2):429–434.
96. Bignardi GE, Woodford N, Chapman A, Johnson AP, Speller DC: **Detection of the *mec*-A gene and phenotypic detection of resistance in *Staphylococcus aureus* isolates with borderline or low-level methicillinresistance.** *J Antimicrob Chemother* 1996, **37**(1):53–63.
97. Fessler AT, Billerbeck C, Kadlec K, Schwarz S: **Identification and characterization of methicillin-resistant coagulase-negative staphylococci from bovine mastitis.** *J Antimicrob Chemother* 2010, **65**(8):1576–1582.
98. Eckholm NG, Outerbridge CA, White SD, Sykes JE: **Prevalence of and risk factors for isolation of meticillin-resistant *Staphylococcus* spp. from dogs with pyoderma in northern California, USA.** *Vet Dermatol* 2013, **24**(1):154–161. e134.
99. Rosdahl VT, Rosendal K: **Correlation of penicillinase production with phage type and susceptibility to antibiotics and heavy metals in *Staphylococcus aureus*.** *J Med Microbiol* 1983, **16**(4):391–399.
100. Jones RD, Kania SA, Rohrbach BW, Frank LA, Bemis DA: **Prevalence of oxacillin- and multidrug-resistant staphylococci in clinical samples from dogs: 1,772 samples (2001–2005).** *J Am Vet Med Assoc* 2007, **230**(2):221–227.
101. Mehrotra M, Wang G, Johnson WM: **Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance.** *J Clin Microbiol* 2000, **38**(3):1032–1035.
102. Zhang K, McClure JA, Elsayed S, Louie T, Conly JM: **Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant Staphylococcus aureus.** *J Clin Microbiol* 2005, **43**(10):5026–5033.
103. Kondo Y, Ito T, Ma XX, Watanabe S, Kreiswirth BN, Etienne J, Hiramatsu K: **Combination of multiplex PCRs for staphylococcal cassette chromosome *mec* type assignment: rapid identification system for *mec*, *ccr*, and major differences in junkyard regions.** *Antimicrob Agents Chemother* 2007, **51**(1):264–274.
104. Francois P, Pittet D, Bento M, Pepey B, Vaudaux P, Lew D, Schrenzel J: **Rapid detection of methicillin-resistant *Staphylococcus aureus* directly from sterile or nonsterile clinical samples by a new molecular assay.** *J Clin Microbiol* 2003, **41**(1):254–260.
105. Ruppe E, Barbier F, Mesli Y, Maiga A, Cojocaru R, Benkhalfat M, Benchouk S, Hassaine H, Maiga I, Diallo A, *et al*: **Diversity of staphylococcal cassette chromosome mec structures in methicillin-resistant *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* strains among outpatients from four countries.** *Antimicrob Agents Chemother* 2009, **53**(2):442–449.
106. Murakami K, Minamide W, Wada K, Nakamura E, Teraoka H, Watanabe S: **Detection of methicillin-resistant *Staphylococcus aureus* by polymerase chain reaction.** *Rinsho Byori* 1991, **39**(12):1325–1330.
107. Baddour MM, AbuElKheir MM, Fatani AJ: **Comparison of *mec*A polymerase chain reaction with phenotypic methods for the detection of methicillin-resistant *Staphylococcus aureus*.** *Curr Microbiol* 2007, **55**(6):473–479.
108. De Graef EM, Decostere A, Devriese LA, Haesebrouck F: **Antibiotic resistance among fecal indicator bacteria from healthy individually owned and kennel dogs.** *Microb Drug Resist* 2004, **10**(1):65–69.

doi:10.1186/1746-6148-10-17
Cite this article as: Schmidt *et al.*: Antimicrobial resistance and characterisation of staphylococci isolated from healthy Labrador retrievers in the United Kingdom. *BMC Veterinary Research* 2014 **10**:17.