The Epidemiology of Antimicrobial Resistant *Escherichia coli* in Hospitalised Companion Animals

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by

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Abstract

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Antimicrobial resistance (AMR) among *E. coli* is a significant and growing problem in human medicine with particular concern regarding production of extended spectrum beta lactamase (ESBL) and AmpC enzymes which confer resistance to third and fourth generation cephalosporins. AMR among *E. coli* of animal origin is well documented, including ESBL-producing *E. coli* and an increasing number of pets may be at risk of nosocomial colonisation and infection with these organisms.

The mains aims of the work presented in this thesis were to determine the prevalence of antimicrobial resistance *E. coli*, including several important AMR phenotypes and genotypes, from both the faecal microflora of animals hospitalised in referral practices and their practice environment. A further aim was to determine the risk factors for carriage of important resistance phenotypes by faecal commensal *E. coli* in these practices.

Faecal (n = 333) and environmental (n = 257) samples were collected from dogs and cats hospitalised at five referral practices in Northwest England. Microbiological and molecular analyses including sequencing were performed to determine the resistance profile of each *E. coli* isolate and to identify ESBL and AmpC producing *E. coli*. Univariable followed by multivariable analyses were performed to identify risk factors associated with carriage of important resistance outcomes.

The adjusted prevalence of important resistance types among faecal sample isolates were: clavulanic acid potentiated amoxicillin (CAPA) 14% (95% CI 6.7-27); ciprofloxacin 9.2% (95% CI 3.2-23.9); multidrug resistance (MDR) 13.1% (95% CI 6.9-23.6); ESBL-producer 14.0% (95% CI 5.3-35.0) and AmpC-producer 7.7% (95% CI2.5-21.1). There was significant variation by practice suggesting practice factors are potentially important. Among MDR isolates ciprofloxacin and CAPA resistance featured frequently. The $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-158}}$ (inhibitor resistant) gene variants were the two most frequently identified ESBL genes. Among environmental samples similar resistance trends were observed in isolates to those isolated from faecal samples. Resistant isolates were more likely to be found in outside walking areas and ward floors than tables and keyboards. Neurosurgery and soft tissue surgery cases were generally at increased risk of several resistance outcomes compared to medical and orthopaedic cases. Use of fluoroquinolones and CAPA were associated with increased risk of a number of resistance outcomes, including ESBL production, and isolation of environmental AmpC-producers was associated with increased risk of CAPA resistance. Increased hospitalisation time was also identified as a risk factor for some outcomes.

This study shows the presence of high rates of carriage of important AMR types in UK companion animal hospitals. The environment is likely to play an important role in the acquisition and spread of these bacteria within a hospital. Use of antimicrobials, hospitalisation, case type and individual practice were shown to be important risk factors for AMR acquisition.

Chapter 1

General Introduction and Literature Review

Since their discovery in 1928 antimicrobials have been used widely in human and veterinary medicine with great success. The first widespread use of antimicrobials was in the 1940's, resistance in bacteria previously sensitive to antimicrobials in use was reported in the same decade. It is widely acknowledged that the use of antimicrobials exerts a selection pressure on a population of sensitive bacteria which drives the evolution of resistant phenotypes. The link between antimicrobial use and the development of resistance is well established and documented in many studies. In humans a link has been shown between the prescription of antimicrobials in a primary care setting and the subsequent development of resistance to those antimicrobials in the individuals concerned (Costelloe et al., 2010). A link has also been shown between the volume of antimicrobial use and the level of resistance in populations (Austin et al., 1999). The use of certain classes of antimicrobial (tetracyclines, cephalosporins and quinolones) in human hospital patients has also been shown to be independently associated with the development of resistance in *E. coli* (Batard et al., 2013). In animals the use of antimicrobials has also been linked to an increased levels of resistance in bacteria isolated from food producing animals (Mayrhofer et al., 2006, Oliver et al., 2011, Seiffert et al., 2013b, Cabello et al., 2013, Ludwig et al., 2013). Ludwig et al (2013) found an association between the use of beta lactams, tetracyclines and quinolones and the isolation of resistant bacteria from the faeces of pigs (Ludwig et al., 2013).

Though any use of an antimicrobial is likely to contribute to the development of resistance (AMR) inappropriate use, such as treating with a sub optimal dose, use of an inappropriate class of antimicrobial for the pathogen concerned, mis-timing of doses or inadequate length of treatment are likely to have a greater influence due to a reduced efficacy of the drug whilst still exerting a selection pressure on resident bacteria (Holloway, 2011). In humans antimicrobial prescription patterns have been linked to increased colonisation of patients with resistant organisms (Hurford et al., 2012). Inappropriate use of antimicrobials has also been linked to poorer clinical outcomes in many clinical conditions in humans (Kang et al., 2013, Moreira et al., 2013, Vardakas et al., 2013). It is not unreasonable to assume that the drivers and consequences of antimicrobial resistance in animals are similar to those seen in humans.

Bacteria can resist the action of antimicrobial agents by three broad methods (Clarke, 2006). In many cases there are stringent requirements for the interaction of a drug with its target site. Resistance to an antimicrobial agent can be conferred by mutations which alter the structure of these target sites - ribosomal binding sites (e.g. chloramphenicol, tetracyclines), enzymes involved in the synthesis and function of nucleic acids (e.g. quinolones) and enzymes involved in the synthesis of bacterial cell walls (e.g. β lactam antimicrobials). Altering the structure of this site reduces the ability of the antimicrobial to bind and therefore reduces its efficacy.

Another way resistance to antimicrobials can be conferred is by the prevention of the drug from reaching an effective concentration at its site of action either by preventing its transport across the cell membrane or by actively pumping the drug out of the bacterial periplasmic space (Mallea et al., 1998, Clarke, 2006). Reducing the permeability of the cell wall or cell membrane can be achieved by a reduction in the number of transmembrane proteins called porins which allow the movement of water soluble molecules into the cell. The active removal of drugs from the bacterial cell can be achieved by active efflux pumps in the cell membrane. These mechanisms are less specific than the alteration of the structure of a specific target site and are more likely to confer multi-drug resistance (MDR) phenotypes, indeed MDR pumps exist which are active for a number of different substrates with different chemical properties (Clarke, 2006).

The third broad method by which AMR can be achieved is via the active destruction or alteration of the structure of the antimicrobial molecule itself in such a way as to render it ineffective. An example of this is the hydrolysis of the beta-lactam ring of penicillins and cephalosporins by beta lactamase enzymes which can be produced by a variety of pathogenic bacteria (Clarke, 2006).

The most obvious and significant consequence of an increase in antimicrobial resistance for both human and veterinary patients is either the delay of effective treatment or, in the worst case scenario, complete treatment failure. Both of these can be potentially very serious for the patient leading to increased morbidity and mortality (Merz et al., 2010). One estimate places the healthcare costs of antimicrobial resistance in the US to be $20bn and the costs to society in lost productivity to be $35bn (Smith and Coast, 2013). The costs of managing human in-patient infections caused by MDR organisms (including extended spectrum beta lactamase (ESBL)) producing *Enterobactericeae*) have been shown to be significantly higher than the treatment of those caused by non MDR organisms (Tansarli et

al., 2013). Though it is difficult to judge the exact economic impact it is likely to be high. In veterinary patients the treatment costs of MDR infections are likely to be similarly increased. Whether this cost would be met by the owner or practice in the case of nosocomial infection is unclear. There is also potential for significant disruption of income due to a need to close wards and clinics as shown by an outbreak of MDR salmonellosis in a large animal veterinary teaching hospital in the US, where the total loss was estimated at $4.12 million (Dallap Schaer et al., 2010). Added to these potential economic burdens is the problem of public image which is very important in the veterinary industry, given the great publicity given to AMR infections in people the potential for loss of business could be great.

Acquisition and transfer of resistance genes

Antimicrobial resistance arises as a result of random mutations in the genetic code. These mutations can then act in several ways as described above, to reduce the efficacy of an antimicrobial against the organism. Although mutation rates are low the high rate of replication of bacteria means emergence of resistant mutants can occur quickly. The use of antimicrobials favours the emergence and multiplication of resistant mutants by promoting the survival of resistant mutants or inducing the expression of existing resistance genes (Clarke, 2006).

One of the biggest problems with the spread of AMR is the plasmid-mediated horizontal transfer of resistance genes between bacteria of the same generation, in some cases this can occur between different species and genera. This horizontal transfer of genes greatly increases the potential for resistance spread.

The main method of horizontal resistance transfer is via conjugation, where genes on plasmids are transferred from donor to recipient bacteria via an intercellular bridge (Clarke, 2006). It is well established that this can occur between different bacterial species (Rayamajhi et al., 2009, Clarke, 2006, Bourgeois-Nicolaos et al., 2006). Commensal *E. coli* in animals have been shown to possess a number of transferrable elements which can confer AMR (Karczmarczyk et al., 2011, Batchelor et al., 2005a) and it has been shown that gene transfer readily occurs between *E. coli* located in the intestinal tract of humans (Karami et al., 2007), mice and chickens (Hart et al., 2006). In addition plasmid mediated gene transfer has contributed to the dissemination of ESBL's in *Enterobacter* species (Sidjabat et al., 2007) and *E. coli* (Sun et al., 2010) isolated from pets. *E. coli* are good recipients of

mobile genetic elements, combined with its commensal status in many animal species it is regarded as a good indicator of the exposure of an animal to resistant organisms and antimicrobials (Martins et al., 2013). There is also concern that *E. coli* can act as a reservoir of resistance, acting as a source of resistance genes to other bacteria which may be more pathogenic (Hart et al., 2006).

Beta-lactam antimicrobials

The most famous member of the beta-lactam group is penicillin, discovered by Fleming in 1928. Members of the beta-lactam group are characterised by the presence of a beta-lactam ring in the molecular structure. The principle members of the beta-lactam group of antimicrobials are the penicillins, cephalosporins, carbapenams and monobactams (Poole, 2004). Penicillins contain 6-aminopenicillic acid (6APA) and many natural and synthetic penicillins exist with different side chains added conferring different properties. Cephalosporins contain a nucleus of 7-aminocephalosporanic acid (7ACA) which can be modified to give different properties (Kong et al., 2010). They are often grouped into generations. With each generation there is an increase in activity of cephalosporins against Gram negative organisms, this is often at the expense of efficacy against Gram positive organisms, however fourth generation drugs are effective against both and are viewed as broad spectrum agents. Third generation cephalosporins are widely used to treat hospital acquired infections caused by *Enterobacteriaceae* (El Salabi et al., 2013).

Beta-lactam antimicrobials work by inhibition of cell wall formation by entering the periplasmic space via porin channels and targeting enzymes involved in its synthesis called penicillin binding proteins. This results in the cell swelling and lysis (Siu, 2002).

Beta-lactam antimicrobials are commonly used in human and veterinary medicine. In 2010 penicillins were the most commonly used antibiotic in human medicine in the community in European countries (ECDC 2010). In ten of twenty six countries, the penicillins accounted for at least 50% of total antimicrobial consumption in the community and in some cases was a lot higher, the median defined daily dose (DDD) for the penicillins was 8.9 per 1000 individuals. The most commonly used penicillins across Europe were amoxycillin and amoxycillin-enzyme inhibitor combinations. Broad spectrum penicillins were the most commonly used penicillin subgroup in the UK. The use of cephalosporins in human medicine is substantially lower in the 2010 survey with a median DDD (for non-penicillin beta-lactams) of 1.6 per 1000 individuals across Europe. Second generation

cephalosporins were the most commonly used type across Europe though in the UK the most commonly used type was first generation (ECDC, 2010).

The vast majority of veterinary antimicrobials sold in Europe are sold for use in food producing animals (FPA) (including horses) with premixes and oral powders making up the majority of pharmaceutical formulations. Penicillins are commonly used in FPA's making up 19% of sales (these measurements are by ton of active ingredient rather than by animals treated) in the UK (as opposed to 23% across Europe). The use of cephalosporins is much lower making up 0.5% of total sales (0.2% first and second generation and 0.3% third and fourth generation) in the UK with a similar picture across Europe. In the UK veterinary antimicrobials in tablet form (seen as the best estimate for use in companion animals) made up 2.8% of sales. Across Europe there is a similar picture with the maximum proportion of tablets sold being 13.3% (in Finland). Across Europe the most commonly sold antimicrobials in tablet form are penicillins (44% total sales) and first and second generation cephalosporins (30% total sales). The trend in the UK follows these proportions (ESVAC, 2010). From these reports it is clear that penicillins are widely used across Europe in humans, food producing animals and companion animals. This picture suggests a relatively high level (by proportion) of cephalosporin use in companion animals. Beta-lactam antimicrobials are the most commonly prescribed antimicrobial in UK companion animal practice (Radford et al., 2011) which appears to concur with the overall sales figures.

Beta-lactamases

The main resistance mechanism bacteria have developed against beta-lactam antimicrobials is the production of beta-lactamase enzymes. The amide bond in the beta-lactam ring is attacked by a serine residue at the active site of the enzyme. Ultimately the beta-lactam ring is hydrolysed destroying the antimicrobial molecule, the active enzyme is regenerated and is free to attack other beta-lactam molecules (Sykes and Matthew, 1976, Siu, 2002).

To date more than 500 beta lactamases have been reported. The level of activity against different substrates (antimicrobial agents) varies between these types. Beta-lactamases of the enzyme families TEM and SHV have been reported in *Enterobacteriaceae* for a number of decades, these confer resistance to amino-penicillins and first generation cephalosporins. Genes encoding beta lactamases are often found on plasmids and are

therefore readily exchanged between bacteria and as a result are widely disseminated (Pfeifer et al., 2010). These genes can be located on plasmids alongside genes which confer resistance to different antimicrobials (e.g. fluoroquinolones, tetracyclines etc.) thus the transfer of one plasmid between bacteria can potentially confer resistance to a wide range of antimicrobials (not just beta-lactams) (Hawkey and Jones, 2009).

Use of extended spectrum cephalosporins in the 1980's has driven the emergence of more resistance by driving the development of new variants of TEM/SHV beta lactamases which have an extended spectrum of activity (ESBL's) with mutations allowing them to attack the oxymino-cephalosporins and confer greater activity against ceftazidime. Another type of ESBL commonly found in *Enterobacteriaceae* are members of the CTX-M enzyme family. They confer a similar phenotype as TEM and SHV ESBL's, but are likely to have transferred from members of the *Kluyvera* genus on mobile genetic elements probably in response to a selection pressure exerted by the use of antimicrobials. The name CTX-M is a reference to the normally increased activity of these enzymes against cefotaxime compared to ceftazidime (Jacoby and Munoz-Price, 2005, Pitout and Laupland, 2008). There is a lot of heterogenicity in members of the CTX-M family in *Enterbacteriaceae* which probably reflects the fact that gene transfer has occurred in multiple separate events in the past (Bonnet, 2004). In addition the use of carbapenams has led to the emergence of carbapenamases which can confer resistance to all beta-lactams (Pfeifer et al., 2010). Production of these different ESBL's extends the spectrum of resistance of bacteria in a variety of similar phenotypes though they remain susceptible to beta lactamase inhibitors (e.g. clavulanic acid). Table 1 shows summarises the classification scheme for beta-lactamases.

E. coli can also gain AmpC enzyme mediated resistance to cephalosporins. The bla_{AmpC} genes were first reported in 1989 (Bauernfeind et al., 1989), they are present widely in *Enterobacter* species and subject to complex regulation, they are generally not expressed unless derepression occurs. Resistance occurs due to an increased amount of AmpC present, this can happen either by generation of more efficient bla_{AmpC} gene promoters by mutation of the promoter region, or by acquisition of more bla_{AmpC} genes from other bacteria via mobile genetic elements. AmpC production confers broad spectrum resistance to cephalosporins and significantly are unaffected by beta lactamase inhibitors (Pfeifer et al., 2010).

Table 1: Classification scheme for beta-lactamases modified from Pfeifer et al, 2010.

Serine beta-lactamases	**β lactamase class**	**β lactamases**	**Examples**	**Resistance phenotype**
	A	Broad spectrum	TEM 1 and 2, SHV 1 and 11	Ampicillin, cephalotin
		ESBL (TEM)	TEM-3 and 52	Penicillins and third gen cephalosporins
		ESBL (SHV)	SHV-5 and 12	
		ESBL (CTX-M)	CTX-M-1, 14 and 15	
		Carbapenemases	KPC GES SME	All beta-lactams
	C	AmpC (chromosomal)	AmpC	Cefamycins and third gen cephalosporins
	D	AmpC (plasmid)	CMY	
		Broad Spectrum Beta Lactamases	OXA-1 and 9	Oxacillin, ampicillin and cephalotin
		ESBL (OXA)	OXA-2 and 10	Pencillins and third gen cephalosporins
		Carbapenemases	OXA-23	All beta lactams
Metallo-beta-lacatmases	B	Carbapenemases	VIM, IMP	

Extended Spectrum Beta-Lactamases in Humans

The first ESBL's to be described were variants of the SHV and TEM beta lactamases which had mutations conferring extended spectrum of activity as described above (Philippon et al., 1989). The CTX-M and AmpC enzymes emerged later in the decade (Bauernfeind et al., 1989, Bauernfeind et al., 1990). The emergence of these ESBL's is likely to have been driven by extensive use of second and third generation cephalosporins in the 1980's.

ESBL-production is now found in bacteria throughout the world though there is significant variation in prevalence in human populations between regions of the world. Prevalence of ESBL production in isolates from human hospital-acquired infections from different areas of the world is shown in table 2.

Table 2: Prevalence of ESBL-producers in *E. coli* and *Klebsiella pneumoniae* isolates from human hospital acquired infections in different areas of the world (Reinert et al., 2007)

	Latin America	Asia/Pacific Rim	Europe	North America
E. coli	13.5%	12%	7.6%	2.2%
K. pneumoniae	44%	22.4%	13.3%	7.5%

Production of CTX-M enzymes is also a significant problem in the community, while TEM and SHV types are more associated with hospital-associated infections than community acquired infections (Pitout and Laupland, 2008), there is potential for crossover with community acquired infections becoming severe enough to warrant hospitalisation. Of particular concern in the community is the presence of CTX-M enzymes in *E. coli* which is common in the gastro-intestinal tract of most humans. The $bla_{CTX\text{-}M}$ genes have been found on plasmids (Accogli et al., 2013) and, given the potential for transfer of AMR between bacteria via plasmids, there is great concern that spread of CTX-M mediated resistance can occur in this way.

Risk factors for community acquired infections by ESBL-producing organisms in humans have been identified as: existing diabetes mellitus; increased age; female gender; recurrent urinary tract infections; residence in a nursing home; renal/liver pathology and recent treatment with beta-lactam or fluoroquinolone antimicrobials (Soraas et al., 2013, Pitout and Laupland, 2008). Risk factors identified in a hospital setting are: length of hospitalisation; severity of illness; urinary catheterisation; length of stay in intensive care unit; ventilation; multiple co-morbid conditions; non-home residence and previous treatment with antimicrobials (Pitout and Laupland, 2008, Hayakawa et al., 2013, Jacoby and Munoz-Price, 2005).

Aside from the obvious impacts on patient morbidity and mortality, healthcare provision and costs the production of ESBL's frequently co-exists with a phenotype of multi-drug resistance (e.g. fluoroquinolones) which bla_{ESBL} genes do not confer (Schultsz and Geerlings, 2012). Surveys from several countries show that co-resistance to non-beta-lactamase antimicrobials among ESBL-producing bacteria in the community is common (Pitout and Laupland, 2008, Pitout et al., 2007). Of particular interest is that ESBL-producing bacteria in a community healthcare setting (as opposed to a hospital) in the UK were identified to be more likely to be multi-drug resistant than those which were not ESBL producers (Woodford et al., 2004).

The $bla_{CTX\text{-}M}$ gene has rapidly become the most widely disseminated and frequently isolated of the beta-lactamase genes. There are many different types of CTX-M enzyme, some of which occur in specific regions but the most important is the CTX-M-15 which is found worldwide (Pitout and Laupland, 2008). Several studies have recently identified $bla_{CTX\text{-}M}$ as the most common ESBL gene. In a case-control study in a large medical centre in the USA 85% of ESBL-producers were $bla_{CTX\text{-}M}$ positive with $bla_{CTX\text{-}M\text{-}15}$ being the most prevalent type.

The presence of a *bla*$_{CTX-M}$ gene was also associated with an increased likelihood of multi-drug resistance (Hayakawa et al., 2013). In Canada, 64% of ESBL-producing *E. coli* isolated from the community were identified as CTX-M producers, here the most prevalent type was CTX-M-14, however this was closely followed by CTX-M-15 and peaks of each types occurred at different time periods within the duration of the study with the peak of CTX-M-15 being towards the end of the study. CTX-M producers were also found to be significantly more resistant to fluoroquinolones (Pitout et al., 2007). In a study of *E. coli* isolated from urinary tract infections (UTI's) over a wide geographical area in Morocco the overall prevalence of ESBL producers was relatively low at 4.1%, the most commonly produced ESBL type was CTX-M though SHV and TEM types were also isolated. Other recent studies in Swiss primary care patients (Nuesch-Inderbinen et al., 2013), hospitalised Indian neonates (Roy et al., 2013), hospitalised children in Gabon (Schaumburg et al., 2013), urinary isolates from the community in Morocco (Barguigua et al., 2013) and even a remote community of Amerindians in French Guiana (Woerther et al., 2013) have also found CTX-M enzymes to be the most commonly produced ESBL. These studies (summarised in table 3 below) show that *bla*$_{CTX-M}$ is the predominant ESBL gene found in many different geographic areas and clinical settings.

Table 3: Summary of selected recent studies in different regions and populations where *bla*$_{CTX-M}$ was detected at a higher prevalence than other ESBL types

Study	Study subjects	CTX-M prevalence	Other ESBL prevalences
(Nuesch-Inderbinen et al., 2013)	Swiss primary care patients	13/15 ESBL producing isolates were positive for *bla*$_{CTX-M}$	1/15 *bla*$_{SHV-12}$ 3/15 *bla*$_{CMY-2}$
(Roy et al., 2013)	Hospitalised Indian neonates	100% of ESBL producers positive for *bla*$_{CTX-M}$	*bla*$_{SHV}$ 5% *bla*$_{TEM}$ 52% *bla*$_{OXA}$-1 81%
(Woerther et al., 2013)	Remote Amerindian community in French Guiana	100% of ESBL producing isolates positive for *bla*$_{CTX-M}$ (in the 2010 isolates)	None (2010 isolates)
(Schaumburg et al., 2013)	Hospitalised children in Gabon	*bla*$_{CTX-M}$ 86.7% (of ESBL producing)	*bla*$_{TEM}$ 56.7% *bla*$_{SHV}$ 6.7%
(Barguigua et al., 2013)	Moroccan community isolates (from UTI's)	Highest CTX-M prevalence was CTX-M-15 at 63% of ESBL producing isolates	Most prevalent non-CTX-M ESBL gene was *bla*$_{SHV-12}$ at 12%

ESBLs in Food Producing Animals

ESBL production has been found in food producing animals in a variety of studies in various regions of the world.

It has been shown that the intestinal tract of the chicken can act as a reservoir for ESBL-producing *E. coli* (Costa et al., 2009). In 2013 a longitudinal study of broilers in Germany, revealed high levels of ESBL and AmpC production in *E. coli* from both the broilers and the environment. Although levels did increase over time there was a high detection rate from both the environment at the onset of the study and 1 day old chicks suggesting that the ESBL-producers found were either brought in with hatched chicks or due to contamination from the environment (Laube et al., 2013). Many other studies have reported the presence of relatively high levels of ESBL-producing bacteria in poultry flocks from Switzerland (Geser et al., 2012), Tunisia (Ben Sallem et al., 2012), Holland (Leverstein-van Hall et al., 2011) and the UK (Horton et al., 2011). The $bla_{\text{CTX-M}}$ and bla_{AmpC} genes have also been found on British turkey farms (Randall et al., 2013) indicating that the problem is not restricted to chickens. A study in 2012 comparing a flock which had feed containing antimicrobials with a flock that did not found no difference in ESBL-producing bacteria levels, indeed in a separate part of the study a flock fed antimicrobials (salinomycin, gentamicin and enramycin) and kept in a controlled environment did not develop ESBL-producer carriage suggesting that environmental contamination plays at least a partial role in the entry of ESBL-producers into a flock (Hiroi et al., 2012). ESBL-producers in poultry are of particular concern from a public health point of view as a number of studies have identified not only a high level of contamination of chicken meat on sale but also genetic similarities between the ESBLs found within poultry flocks, on meat and circulating within the human population (Leverstein-van Hall et al., 2011, Laube et al., 2013, Kola et al., 2012, Stuart et al., 2012, Overdevest et al., 2011). Some of these studies also identified high levels of co-resistance to non-beta-lactam antimicrobials in these ESBL-producers (Overdevest et al., 2011, Stuart et al., 2012). In Spain high levels of CTX-M producing *E. coli* with associated high levels of quinolone resistance have also been found in turkey meat on sale (Egea et al., 2012). These findings are of great concern and indicate that it is highly likely that ESBL producers are circulating between human and poultry populations.

ESBL producing *E. coli* have also been frequently isolated from cattle and pigs. In studies in the UK and Switzerland comparing levels across species cattle and pigs have had a much lower detection rate than that of poultry with pigs having a slightly increased detection rate over cattle. In these studies the most common type of ESBL gene identified was $bla_{\text{CTX-M}}$ (Horton et al., 2011, Geser et al., 2012). In Korea pigs have been found to have very high levels of ESBL-producers compared to very rare isolation rates from cattle (Tamang et al., 2013). In Tunisia a study of healthy food producing animals found high levels of ESBL-

producers in poultry and none in cattle (Ben Sallem et al., 2012). In a recent study of wild hunted deer in Switzerland extremely low levels of ESBL-production were found (Stephan and Hachler, 2012) suggesting that farmed animals are more likely to carry ESBL-producers than wild animals.

Risk factors identified for the occurrence of bla_{ESBL} genes in *E. coli* isolated from food-producing animals (FPAs) are generic antimicrobial use and specific cephalosporin use while international trade is a risk factor for dissemination of these genes. It has been proposed that the use of antimicrobials and particularly cephalosporins in FPA's should be reduced in order to reduce these isolation levels (Liebana et al., 2013). In a study on German dairy and beef farms the use of antimicrobials was identified as a risk factor for the detection of bla_{ESBL}, interestingly most farms in this study did not use beta lactam antimicrobials and the use of non beta-lactam antimicrobials was proposed to be selecting for ESBL-producing bacteria due to co-resistance to different antimicrobial classes (Schmid et al., 2013). In Denmark the levels of extended spectrum cephalosporinase isolation from pigs was significantly reduced after stopping the use of cephalosporins further implicating the use of these antimicrobials in mediating the presence of ESBL producers in food producing animals (Agerso and Aarestrup, 2013).

The detection of high levels of ESBL-producers in FPA's is of particular concern from a public health point of view with evidence for transmission between these animals and humans. In Europe poultry are associated with particularly high levels of ESBL-producer isolation with pigs and cattle having lower isolation rates. Though rates are lower in cattle and pigs they are still cause for concern as high density shedders have been identified (Horton et al., 2011) in these species with potential for the contamination of human food supplies.

Of additional concern is the potential link between FPA's and companion animals where they are fed raw meat diets. Antimicrobial resistance has been demonstrated in bacteria from raw meat fed to dogs (Finley et al., 2008) and it has been shown that dogs fed a raw meat diet are more likely to have *Salmonella* species isolated from their faeces (Leonard et al., 2011). These studies raise the possibility of resistant bacteria from food producing animals colonising dogs which, given closer contact with humans and human living environments may have greater potential to transmit the same bacteria to humans. The isolation of ESBL-producing *E. coli* from raw meat mentioned above suggests that this could

be a route for the colonisation of companion animal gastro-intestinal tracts with ESBL-producing *E. coli*.

ESBLs in Companion animals

ESBL producing *E. coli* are being found frequently in companion animals throughout the world. This is of concern as a significant proportion of pets come into close contact humans whether it be indirect (e.g. via food preparation areas) or direct (Westgarth et al., 2008). This contact with pets is likely to be the closest contact with animals most of the human population will have and the potential for interspecies transmission of both commensal and pathogenic bacteria is a concern. There is also a clear implication for animal welfare if treatment of infections is prolonged or not possible at all.

Antimicrobials are frequently used in companion animal practice – a study of dogs attending a veterinary hospital in the USA found that 56% had received treatment with an antimicrobial in the last 12 months, with 40% being treated with beta-lactam type antimicrobials (Baker et al., 2012). In the UK the prescription of antimicrobials for pets attending veterinary clinics for the investigation of disease is common. A study in the UK found that non routine consultations involved the prescription of a systemic antimicrobial in 35.1% and 48.5% of the time for dogs and cats respectively. The three most frequently prescribed classes being clavulanic acid potentiated amoxicillin (CAPA), amoxicillin and cefovecin. Beta-lactam antimicrobials made up 76% of all the antimicrobials prescribed (Radford et al., 2011). Frequent use of beta-lactams was also shown in a survey of companion animal practitioners in the UK by Hughes and others (2011), where practitioners were presented with four potential clinical scenarios and asked to give information on likely prescriptions. In three out of four scenarios the most commonly prescribed antimicrobial was CAPA and in the remaining scenario it was cefalexin. The study also demonstrated that a proportion of vets are likely to prescribe sub-optimal doses for CAPA (4%), amoxicillin (8%) and cefalexin (7%), in addition to other non-beta-lactam antimicrobials (Hughes et al., 2012). These studies demonstrate the frequent use of beta-lactam antimicrobials in UK veterinary practice and are reinforced by the tablet sales data mentioned previously (ESVAC, 2010). This is likely to be exerting a selection pressure for resistance to these antimicrobials.

In the UK a study of faecal samples from horses across the country in the community showed widespread resistance to antimicrobials. Of the horses samples 69.5% had *E. coli*

resistance to at least one antimicrobial. ESBL-producing *E. coli* were however detected at a much lower rate of 6.3% (Maddox et al., 2012). A recent study investigating the effect of hospitalisation on faecal carriage of antimicrobial resistant *E. coli* found that the levels of resistance to most classes of antimicrobial were significantly increased over the period the horse was hospitalised. At day one of hospitalisation 35% of samples were found to contain MDR *E. coli*, by day 7 this proportion had increased to 80%. Treatment while in hospital, age and breed were not associated with increased risk. The reason for admission was investigated and acute gastrointestinal (medical and surgical) and musculoskeletal cases were associated with increased risk (Williams et al., 2013). Maddox et al (2011) also demonstrated high levels of MDR and ESBL-producing *E. coli* in the faeces of hospitalised horses with an increased risk of isolation during hospitalisation (Maddox et al., 2011). These studies suggest that a widespread low level of AMR exists in horses in the community in the UK. Levels of AMR and ESBL-producer isolation are significantly higher in hospitalised horses with the risk of isolation of these organisms rising with length of hospitalisation.

A cross sectional study of 183 healthy dogs in the community in the UK by Wedley and others (2012) found carriage of AMR in 29% and MDR in 15% of the dogs studied. ESBL production was detected at low levels with one isolate testing positive for ESBL production using phenotypic methods (Wedley et al., 2011). The finding of a relatively high level of resistant and particularly MDR in dogs in the community is cause for concern from both an animal and human health perspective.

In Tunisia a study of healthy dogs and cats attending a private veterinary clinic for grooming or vaccination collected eighty faecal samples and tested for the presence of AMR *E. coli*. Animals were not hospitalised and were only included if they had received no prior treatment with antimicrobials in the previous three months. Fourteen out of eighty samples contained *E. coli* resistant to cefotaxime, of these thirteen *E. coli* isolates were found to be ESBL producers. All of these ESBL producers were positive for $bla_{\text{CTX-M-1}}$which is the most common ESBL gene found in commensal *E. coli* from FPA's and humans in Tunisia, bla_{TEM} and bla_{CMY} genes were also detected (Ben Sallem et al., 2013). This study demonstrates the presence of ESBL-producers in companion animals which were not hospitalised and which had not been treated with antimicrobials recently, suggesting circulation in a community setting.

A study carried out in China looked at the relative levels of ESBL producing *E. coli* in healthy and unwell pets. Samples (predominantly faecal) were taken from healthy pets from pet shops and visiting veterinary hospitals and from those undergoing treatment. Animals undergoing treatment were more likely to have ESBL producing *E. coli* isolated from their samples than healthy animals with 54.5% of isolates from unwell pets yielding ESBL producers compared to 24.5% from healthy animals. In total the approximate proportion of ESBL-producing isolates was 40% which reflects the situation in the human population of the area of China where the study was carried out. The most common ESBL gene identified was $bla_{\text{CTX-M}}$. The most frequent types being $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-55}}$. It was suggested in the study that the use of amikacin in some of the animals co-selected for ESBL production due to the presence of different resistance genes on the same plasmids and that horizontal spread among the pet population was mediated by these plasmids (Sun et al., 2010). This finding of relatively high levels of ESBL producers in healthy pets not undergoing treatment is a concern and indicates that they are also circulating in healthy pets, rather than just in those undergoing treatment, and shows the great potential for community acquired infections with ESBL-producing *E. coli*. The increased isolation rates from unwell animals undergoing treatment could be a reflection of the use of antimicrobials in these animals or potentially increased exposure to ESBL producing organisms in a hospital environment.

In South Korea a study of *E. coli* isolates from rectal swabs of dogs found high levels of $bla_{\text{CTX-M}}$ and bla_{AmpC}. Of sixty three isolates twenty one were found to be positive for $bla_{\text{CTX-M}}$ fifteen were found to be positive for bla_{AmpC} and twelve were found to be positive for both $bla_{\text{CTX-M}}$ and bla_{AmpC}. All twenty four of these CTX-M/AmpC producing isolates were found to be ciprofloxacin resistant also. Similar mutations were found in *gyrA* and *parC* genes from isolates from dogs as are found in the human population suggesting a similar genetic background. This study also found identical serotypes of *E. coli* in one veterinary hospital suggesting a localised clonal outbreak within that hospital. In most isolates the bla_{ESBL} genes were found to be localised on plasmids lending weight to the theory that spread is greatly facilitated by the horizontal transmission of these mobile genetic elements (So et al., 2012). This study indicates that there may be high levels of ESBL/AmpC producing *E. coli* in Korean veterinary hospitals. The finding that all ESBL/AmpC producers were ciprofloxacin resistant indicates that co-resistance to different antibiotic classes is a very real potential problem for veterinary hospitals.

Also in South Korea a study of *E. coli* isolates from stray dogs isolated ESBL and AmpC producer phenotypes at a relatively low rate – 1.9% and 3.5% respectively. All the isolates with ESBL-producer phenotypes tested positive for $bla_{CTX\text{-}M}$ type ESBL genes with $bla_{CTX\text{-}M\text{-}14}$ being the most common type (Tamang et al., 2012) While the prevalence was low they do indicate the presence of the bla_{ESBL}/bla_{AmpC} genes within the stray dog population in Korea and indicate the potential for community as well as hospital acquired infections. Nam and others (2010) in a different publication from the same study compared the faecal carriage of MDR *E. coli* in dogs from stray animal shelters to that of dogs hospitalised in small animal clinics across the country. Isolation of MDR *E. coli* from the faeces of animals from veterinary clinics was higher than that from dogs housed in shelters (48% of isolates were MDR from clinics as opposed to 32%). Cefotaxime resistance was also higher (2.4% and 3.9% respectively) in isolates from hospitalised animals as opposed to those in shelters. It was also found that more isolates from shelters were sensitive to all antimicrobials tested than those from hospitalised animals. Younger animals were found to have higher levels of resistance among faecal bacteria than older animals (Nam et al., 2010). These studies indicate that ESBL and AmpC-producers are present in the faeces of both hospitalised and non hospitalised dogs in South Korea with a suggestion that hospitalisation is associated with an increased risk of MDR *E. coli* isolation which may reflect environmental contamination or a population of dogs more likely to have been exposed to antimicrobials.

In Europe Franiek and others (2012) in Germany estimated the prevalence of faecal carriage of ESBL-producing *E. coli* in dogs and cats to be 5.3%. Most of the positive samples were from animals housed in shelters. The most commonly isolated bla_{ESBL} type was the $bla_{CTX\text{-}M\text{-}1}$ group with groups 2 and 9 also being detected (Franiek et al., 2012). A recent study in Germany found the most common bla_{ESBL} gene isolated from diseased animals to be $bla_{CTX\text{-}M\text{-}1}$ (Schink et al., 2013). A similar low prevalence (2.5%) of ESBL-producing bacteria in community based animals was detected in a study in Switzerland, prior treatment with antimicrobials was identified as a risk factor for ESBL-producer carriage (Korzeniewska and Harnisz, 2013).

In Holland in 2012, Dierikx and others tested 2700 clinical isolates (mostly from urine samples) of *Enterobacteriaceae* from dogs, cats and horses for resistance to ceftiofur/cefoquinome. Samples were collected from a wide geographical area. Resistance was found in 3%, 4% and 8% of isolates from dogs, cats and horses, respectively. The majority of these isolates (74%) were MDR and in addition to ceftiofur/cefoquinome

resistance most showed resistance to cefotaxime and ceftazidime. Both bla_{ESBL} and bla_{AmpC} genes were identified with $bla_{CTX\text{-}M\text{-}1}$ being the most frequently found. All of the isolates were from animals with different owners apart from two from two horses with the same owner. No relationship was found between any of the serotypes apart from the two from horses with the same owner which were identical suggesting that transmission had occurred between these horses (Dierikx et al., 2012). This study demonstrates the presence of ESBL/AmpC producing *Enterobacteriaceae* in clinical samples and though there is no evidence to suggest that they were pathogenic at the time of sample collection their presence in potentially pathogenic bacteria is cause for concern and indicates the potential for community acquired infections with ESBL/AmpC producing bacteria in companion animals. The most commonly found gene ($bla_{CTX\text{-}M\text{-}1}$) is also the type most commonly isolated from poultry (Dierikx et al., 2010), poultry meat and human patients (Leverstein-van Hall et al., 2011) in Holland indicating that there may be interspecies transmission.

Carattoli and others (2005) tested *E. coli* isolates from healthy and sick animals and from necropsies. In total, 7% of these isolates showed resistance to extended spectrum cephalosporins and within these 76% were found to be CTX-M-1 producers. The $bla_{cmy\text{-}2}$ and $bla_{SHV\text{-}12}$ genes were also detected. Molecular typing of isolates indicated an absence of clonal spread for the most part. However in one kennel isolates from different dogs were similar, suggesting dissemination of the same strain among dogs occupying this kennel (Carattoli et al., 2005).

Huber and others (2013) examined uropathogenic *E. coli* isolates from dogs and cats in Switzerland. Just under 4% of animals in the study had uropathogenic ESBL-producing *E. coli* in their urine. High levels of MDR were associated with ESBL-producing strains compared to non ESBL producers (again including many non beta-lactam agents). The ESBL genes identified in these animals were $bla_{CTX\text{-}M\text{-}15}$ in all four and additionally bla_{TEM} types in three (Huber et al., 2013). In 2010 O'Keefe and others examined 150 isolates from dogs and cats where there was evidence of a UTI. Sixty of these showed evidence of reduced susceptibility to cefpodoxime and ceftazidime. Of these 60, an ESBL-producer phenotype was shown in 6 but the presence of an ESBL gene was demonstrated by sequence analysis in 11 isolates. This discrepancy is likely due to the high prevalence of bla_{AmpC} genes ($bla_{CMY\text{-}2}$) found in this study with 53/60 isolates positive, which can mask ESBL-producer phenotypes. Of the 11 ESBL producers one was identified as an SHV type and 10 were identified as CTX-M types, of these 9 were CTX-M-15 producers and 1 was a CTX-M-14

producer (O'Keefe et al., 2010). This was the first report of SHV and CTX-M type ESBL production in companion animals in the USA. The studies by Huber (2013) and O'Keefe (2010) demonstrate that ESBL-production can occur in pathogenic as well as commensal organisms.

In Portugal Costa and others (2008) examined the prevalence of AMR in from faecal samples of healthy dogs and cats which had not been exposed to antimicrobials in the previous 4 months. The prevalence of resistance was low with most isolates being susceptible to all antimicrobials tested. ESBL production was however detected in two isolates from the same dog. This dog was young and there was no history of exposure to antimicrobial agents (Costa et al., 2008). This was a study of animals with no recent exposure to antimicrobials and therefore a low prevalence is not unexpected. The detection of ESBL production in one animal with no history of AM exposure suggests it was acquired from the environment or another animal or human though there is no evidence for this.

In the USA, Shaheen and others (2011) examined 944 *E. coli* isolates from samples from companion animals with UTI's and other infections. Approximately 6% of these isolates showed reduced susceptibility to ceftazidime or cefotaxime and of these approximately half were shown to be ESBL producers. The prevalence of ESBL-producing *E. coli* causing clinical infections in companion animals in this study was 3%. High levels of resistance to other antimicrobials were found in the ESBL producers with all but one exhibiting a MDR phenotype. The prevalence of resistance to enrofloxacin was particularly high among these isolates at 92%. Among the confirmed ESBL producers all isolates were positive for $bla_{\text{CTX-M}}$ genes and most were positive for bla_{AmpC} genes. The CTX-M-1 group was found in all of these ESBL isolates with CTX-M-15 being found most frequently. *The* bla_{TEM} and bla_{SHV} genes were also identified. Genes were identified on plasmids and the genetic heterogenicity identified in this study suggests that the dissemination of bla_{ESBL} genes in companion animals in the USA is not due to a single clonal outbreak and horizontal transmission of plasmids is highly likely to play an important role (Shaheen et al., 2011). Again this study shows the presence of bla_{ESBL} genes in bacteria causing clinical infections in the USA (though at a low rate). The presence of the pandemic CTX-M-15 ESBL subtype at high prevalence among the ESBL producers is a public health concern, as are the high levels of MDR.

Interestingly in Canada in 2009, Murphy and others found low levels of resistance in commensal *E. coli* in 188 healthy dogs and 39 healthy cats. These animals were presenting at veterinary hospitals over a wide area and mostly were from hospitals/practices with smaller numbers of animals. There was no history of exposure to antimicrobials in any of the animals. Some resistance was found but at low levels, no ESBL producers were detected and bla_{AmpC} genes were detected in two dogs though the absence of plasmids suggested that these were chromosomal in origin (Murphy et al., 2009). This may suggest that levels of resistance are lower in Canada though it is more likely to reflect a reduced risk of antimicrobial resistance without antimicrobial exposure.

A study in Chile by Moreno and others (2008), compared the levels of AMR *E. coli* in commensal *E. coli* from cats and dogs treated with enrofloxacin (n=15) against those which had not (n=15). Compared to animals not treated those which had been treated had broader antimicrobial resistance profiles with high levels of MDR, including resistance to drugs used exclusively in humans. They also had high levels of resistance to cefotaxime, ceftazidime and cefpodoxime. ESBL-production was detected in isolates from five out of fifteen treated animals and no untreated animals (Moreno et al., 2008). This is another study demonstrating the impact of antimicrobial exposure on resistance profiles. The fact that the exposure is to a non-beta-lactam agent lends weight to the theory of co-selection for MDR.

Karczmarczyk and others (2011), carried out a study in an Irish university veterinary hospital of 72 *E. coli* isolates which were resistant to three or more different classes of antimicrobial. bla_{AmpC} genes were detected in an isolate from a dog while $bla_{CTX\text{-}M\text{-}2}$ genes were detected in isolates from horses (Karczmarczyk et al., 2011).

Treatment with antimicrobials was confirmed as a significant risk factor for the recovery of MDR *E. coli* from rectal swabs in a study by Gibson and others (2011) in Australia. A case control study was carried out and treatment with cephalosporins in the 42 days prior to hospitalisation was found to increase the risk of MDR *E. coli* isolation by 5 times and treatment with cephalosporins and metronidazole whilst hospitalised was found to increase the risk by 5 and 7 times respectively. Treatment with other antimicrobial classes was also found to be a risk factor. Hospitalisation for more than six days was also a significant risk factor independent of any treatment with antimicrobials. The strains isolated were in most cases the same (or similar) as those from extra-intestinal clinical infections indicating that pathogenic potential of these commensal organisms (Gibson et

al., 2011). A recent study in Portugal has identified antimicrobial exposure and residence in an animal shelter to be risk factors for the isolation of both ESBL and AmpC-producing *E. coli* from dogs (Belas, 2014).

In summary, ESBL-production has been detected in *E. coli* isolated from companion animals in studies throughout the world (Karczmarczyk et al., 2011, Moreno et al., 2008, Shaheen et al., 2011, Costa et al., 2008, Huber et al., 2013, Carattoli et al., 2005) indicating that, like in humans, it is a global problem. The most common type of bla_{ESBL} identified in these studies is the $bla_{CTX\text{-}M}$ type. This is the case in many different countries indicating that, like in humans (Pitout and Laupland, 2008), the CTX-M ESBLs have a worldwide distribution.

Several studies have suggested a link between antimicrobial exposure and ESBL-producer isolation with higher levels associated with exposure and lower levels associated with an absence of exposure (Gibson et al., 2011, Moreno et al., 2008, Murphy et al., 2009, Costa et al., 2008, Korzeniewska and Harnisz, 2013). Length of hospitalisation has also been implicated as a risk factor for isolation of commensal ESBL-producing *E. coli* (Gibson et al., 2011). ESBL producers have been isolated from both healthy (Murphy et al., 2009, Costa et al., 2008, Ben Sallem et al., 2013) and sick (Carattoli et al., 2005, Sun et al., 2010, Shaheen et al., 2011) populations indicating that, as with humans (Pitout and Laupland, 2008), they are present in the community as well as a hospital setting. Levels of bla_{ESBL} isolation are generally higher in animals described as sick or hospitalised (Sun et al., 2010, Nam et al., 2010) although this may reflect the increased likelihood that a sick or hospitalised animal will have been exposed to antimicrobials or environmental contamination. Multiple studies have demonstrated an association between ESBL production and MDR, including resistance to antimicrobial classes which are unrelated to beta lactam agents (Moreno et al., 2008, Shaheen et al., 2011, Huber et al., 2013, Nam et al., 2010). This is described in human medicine (El Salabi et al., 2013) and lends weight to the theory that co-selection for MDR is occurring in companion animals.

The occurrence of bla_{ESBL} genes on plasmids has been shown (Schink et al., 2013) and it has been demonstrated that transfer between bacteria can occur in companion animals (Karczmarczyk et al., 2011). This has long been acknowledged to be the case in human medicine and it is unsurprising to find the same situation with bacteria from companion animals. As with humans these findings demonstrate the great potential for spread of ESBL-mediated resistance among companion animals.

The finding of ESBL-producing *E. coli* in clinical isolates is relatively rarely reported in animals. To date ESBL-producers have been isolated from urinary tract infections (O'Keefe et al., 2010), preputial infections, wounds (Steen and Webb, 2007) and bile in a cholangiohepatitis case (Timofte et al., 2011). The potential for ESBL production in pathogenic bacteria as opposed to commensal bacterial in companion animals is clearly demonstrated by these studies. In human medicine ESBL production is widely reported in clinical isolates. It is likely that the infrequent reporting in companion animals is a result of a lack of surveillance and the true prevalence is likely to be higher. Indeed it is not unreasonable to assume that any infection caused by (or any other organism capable of ESBL production given the genes) could potentially involve ESBL producers resulting in reduced treatment options and increased morbidity.

Transfer of ESBL producing and MDR *E. coli* between companion animals and humans

The potential transfer of ESBL producing *E. coli* from companion animals is of great public health concern given the close relationship between humans and their pets. As veterinary medicine improves and client expectations increase the companion animal population is likely to grow older and the use of antimicrobials in this population more widespread (da Costa et al., 2013). These factors are likely to lead to a higher proportion of the pet community being animals with a history of repeated or long term exposure to antimicrobials. Increased exposure to antimicrobials is likely to lead to increased numbers of resistant bacteria circulating in the population.

Several studies have found similarities between ESBL producers found in companion animals and those circulating within the human population and food producing animals (Dierikx et al., 2012, Wieler et al., 2011, Ewers et al., 2010, So et al., 2012). In 2008 Pomba and others isolated O25-ST131 human virulent *E. coli* producing CTX-M-15 from the bladder of a dog suggesting that transfer between species had occurred (Pomba et al., 2009). However the finding of genetic similarities between isolates does not indicate the direction of transfer (if indeed transfer has taken place), it is entirely possible that transfer is occurring from humans to animals.

In animals there is some variation in the type of $bla_{\text{CTX-M}}$ which is isolated according to geographical area with $bla_{\text{CTX-M-1}}$ being the most widely disseminated type in Europe and $bla_{\text{CTX-M-14}}$ in Asia. In humans it is a different picture, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ are the most widely disseminated and commonly detected regardless of the geographical origin. This

suggests that among humans, person to person transmission is probably the most important route for transmission of AMR (Ewers et al., 2012).

There is a lot of potential for close contact between pets and humans within a household which can aid the transmission of bacteria between humans and dogs (da Costa et al., 2013). Westgarth and others (2008) found a high frequency of behaviours within a community of dogs which have high potential for interspecies bacterial transmission. These include feeding human food from the hand (62%) or directly from the plate (11%) and restriction to the kitchen when alone (24%). Washing hands after touching a dog was only reported by 50% of respondents. Physical interaction with dogs was reported to be frequent in 76% of respondents (Westgarth et al., 2008).

The potential for transmission of *E. coli* clones within a household both between people and a dog has been demonstrated (Johnson and Clabots, 2006). In 2013, Martins and others investigated the resistance profiles of commensal *E. coli* isolates from companion animals, humans and the environment within a household. The highest level of MDR was found in isolates from the dog and a human which had previously been treated with antimicrobials (the dog had a chronic skin condition). However strains from the environment and also from other residents (two humans and a cat) were also found to have high levels of MDR with genetic similarities to those from the dog and other human. These other residents had no history of exposure to antimicrobials suggesting that transfer of resistance had occurred between these two groups although it is unclear by which route this could have occurred (Martins et al., 2013).

In a study of 231 people attending a symposium ESBL-producing *E. coli* were isolated from rectal swabs from 8 attendees. Ownership of domestic animals was identified as a risk factor with an odds ratio of 6.7 though the lower confidence interval extended to one so the significance of this finding is unclear (Meyer et al., 2012).

There is also potential for interspecies transmission of *E. coli* via the environment (rather than directly or within the household). Both antimicrobial agents and resistant bacteria can be excreted into the environment where, if they survive, they could exploit opportunities to colonise or infect new hosts (da Costa et al., 2013). Antimicrobial resistant and ESBL-producing have been isolated from river water in the UK suggesting at least a transient contamination of water supplies is possible (Dhanji et al., 2011). In a recent study in Poland samples were collected from sewage, the air at a waste water treatment plant

and a river receiving effluent from the plant. ESBL-producing *Enterobacteriaceae* were detected in 100%, 23.8% and 33.3% of samples respectively suggesting that sewage is a potent source of environmental contamination and waste water treatment is not effective in removing this (Korzeniewska and Harnisz, 2013). There is no indication that the results in either of these studies was due to animal related contamination of the environment, indeed it is likely to be human sewage that was the source. However it is likely that AMR bacteria excreted by animals have the potential to contaminate the environment in the same way.

Summary and aims

Antimicrobial resistance is a natural phenomenon and purely reflects evolution of bacteria in response to a selection pressure. However the widespread use of antimicrobials has provided a potent selection pressure for the development of AMR throughout the world in both a hospital and a community setting.

ESBL-producing *E.coli* are a major problem in human medicine and are emerging as a serious problem in veterinary medicine. Not only are ESBL-producers resistant to extended spectrum cephalosporins they are often resistant to several different classes of antimicrobial due to co-selection for resistance cause by plasmids expressing multiple AMR genes (including ESBL genes). ESBL-producing bacteria are now commonly found in studies of food producing and companion animals throughout the world including in healthy animals, which is of concern.

Some evidence exists suggesting a link between the epidemiology of ESBL-producers in humans and animals, there are many similarities but also differences so it is difficult to make a definitive judgement at this time. ESBL-producing *E.coli* are present within the companion animal population both as commensals and pathogens and current trends within veterinary practice suggest that levels are likely to increase in the future. There is a high frequency of close contact between pets and their owners, which increases the potential for transmission between these populations. It is likely that transfer is occurring between the populations and is probably dynamic and occurring in both directions.

The challenges facing the veterinary profession regarding ESBL-production by bacteria are similar to those facing the medical profession. However, far less research has been done on the prevalence and risk factors for colonisation or infection with ESBL-producing *E. coli*

in companion animals. Increased collection of data in these areas will help to further our understanding of this area and help combat this very real threat.

The primary aim of this thesis was to determine the prevalence of antimicrobial resistant *E. coli*, including several important AMR phenotypes and genotypes, from both the faecal microflora of animals hospitalised in referral practices and their practice environment. A further aim was to determine the risk factors for carriage of important resistance phenotypes by faecal commensal *E. coli* in these animals .

Chapter two describes the design of the study and the results present the prevalence of important AMR phenotypes and genotypes across the hospitals. This chapter also describes molecular methods and results characterising the genes associated with ESBL-producing and AmpC producing *E. coli*

Chapter 3 describes data collection and multilevel, multivariable analysis to determine risk factors for carriage of specific resistance phenotypes and genotypes by faecal commensal *E. coli*.

Chapter 2

Longitudinal study of antimicrobial resistant *Escherichia coli* in hospitalised companion animals and their hospital environment.

INTRODUCTION

Bacterial resistance to antimicrobials is a global issue in both human and veterinary medicine with serious consequences. The increased financial burden of managing multi-drug resistant (resistant to 3 or more classes of antimicrobial, MDR) infections has been shown to be significant in both human (Smith and Coast, 2013, Tansarli et al., 2013) and veterinary (Dallap Schaer et al., 2010) situations. Furthermore increased patient morbidity and mortality associated with treatment failure, public image problems and the potential public health impact together make antimicrobial resistance one of the most important problems faced by human and veterinary medicine today.

There are a number of different mechanisms by which bacteria can gain resistance to antimicrobials. Among *Escherichia coli* one of the most important mechanisms of resistance is the production of enzymes called beta-lactamases which hydrolyse the beta lactam ring and confer resistance to beta lactam antimicrobial agents (Sykes and Matthew, 1976). This is a mechanism of resistance which has been long established, the early beta-lactamases conferred resistance to only a limited range of beta lactam antimicrobials (principally amino-penicillins and first generation cephalosporins. However use of extended spectrum (third and fourth generation) cephalosporins has driven the evolution of some beta-lactamase enzymes to extend their spectrum of activity to later generation cephalosporins and confer resistance to these agents, although they remain sensitive to beta-lactamase inhibitors, such as clavulanic acid. In human medicine the last few decades has seen this resistance to extended spectrum cephalosporins emerge as an important element of many nosocomial infections. In *E. coli* some extended spectrum beta-lactamases (ESBLs) emerged due to mutations in existing beta-lactamases which extended the spectrum of activity (TEM and SHV type), in other cases the emergence of a new type of ESBL in *E. coli* occurred due to transfer of mobile genetic elements from *Klyuvera* species (CTX-M types)(Bonnet, 2004). This ability of bacteria to exchange resistance genes via

horizontal transfer of plasmids is extremely important to the spread of resistance among bacterial populations (Pfeifer et al., 2010).

E. coli can also gain resistance to beta-lactam agents by the production of the cephamycinase AmpC enzymes. It is important to note that bacteria with this mechanism of resistance are also resistant to beta lactamase inhibitors, which distinguishes them from ESBL-producing bacteria which are sensitive. The production of ESBL's has been shown to be associated with the occurrence of MDR due to the co-existence of different resistance genes on the same plasmids as the bla_{ESBL} genes which can then be shared within bacterial populations (Schultsz and Geerlings, 2012, Pitout et al., 2007, Pitout and Laupland, 2008, Woodford et al., 2004). This adds to the resistance burden within populations with a wider range of resistance further limiting treatment options, for example ESBL production has been shown to be associated with fluoroquinolone resistance in a study of companion animals (Moreno et al., 2008). This is an important phenomenon as it means that the use of one antimicrobial class could select for resistance to multiple classes of antimicrobial by favouring the spread of plasmids conferring MDR.

ESBL-producing bacteria are now being reported commonly in companion animals. They have been reported from healthy dogs in the community in: Tunisia (Ben Sallem et al., 2013); China (Sun et al., 2010); South Korea(Tamang et al., 2012); Switzerland (Gandolfi-Decristophoris et al., 2013); Italy (Carattoli et al., 2005); Germany (Franiek et al., 2012); Portugal (Belas, 2014) and the UK where a high level of MDR *E. coli* was also detected (Wedley et al., 2011). A study in South Korea found a significant ESBL producer burden in commensal *E. coli* from hospitalised animals (So et al., 2012) and another study compared the isolation rates from community animals with hospitalised animals and found a higher rate of isolation from the animals which were hospitalised (Nam et al., 2010). A study in China detected increased levels of ESBL producing bacteria in animals undergoing treatment compared to healthy animals (Sun et al., 2010). ESBL production has been reported in bacteria associated with urinary tract infections in Holland (Dierikx et al., 2012), Switzerland (Huber et al., 2013) and the USA (O'Keefe et al., 2010, Shaheen et al., 2011) and additionally wound infections (Steen and Webb, 2007) and a case of cholangiohepatitis in the UK (Steen and Webb, 2007, Timofte et al., 2011). These studies show that ESBL producing bacteria are circulating within community based healthy animals and hospitalised animals in many parts of the world. ESBL production can occur in isolates

which can cause clinical infections which will lead to limited treatment options with these infections.

The aim of this study was to determine the prevalence of important antimicrobial resistance (AMR) phenotypes from both the faecal microflora of animals hospitalised in referral practices and their practice environment.

MATERIALS AND METHODS

Practice selection and sample collection

Five referral hospitals in Northwest England, that were willing to participate, were selected on a convenience basis to take part in the study. The main criterion for selection was that the hospital must see secondary referral patients with a substantial referral caseload including cases likely to be hospitalised. The limitation to the Northwest was in order to facilitate sample collection and minimise time between collection and processing.

Sample size estimates indicated that with an expected prevalence of ESBL-producing *E. coli* of 10%, a sample size of 385 faecal samples would be required to determine the prevalence with a precision of 3% and 95% confidence. Hence the aim was to collect 385 animal faecal samples in addition to environmental samples.

Once hospitals agreed to participate they were provided with an information sheet and sampling guidelines. Written informed consent was obtained, by the veterinary surgeon, from all owners whose animals took part in the study. Ethical approval for the study was granted by the University of Liverpool's Research Ethics Committee.

In order to avoid clustering of samples from one time of year at one hospital sampling was rotated and performed in three blocks of two weeks (six weeks sampling in total) at each of the practices. Some blocks overlapped such that sampling from 2 hospitals took place at the same time. One practice also included a pilot week (seven weeks sampling in total).

Animals defined as eligible for the study were all dogs and cats hospitalised overnight in the practice whose owners consented to take part. Day cases were excluded as were animals receiving chemotherapy or radiotherapy and those in isolation. The target for sample collection was one faecal sample per hospitalised animal per day. Samples were collected by practice staff and labelled (with name, ID number, date of collection and where the animal was hospitalised at the time of sampling), stored in cool-boxes at the practice and collected at regular intervals (2-3 days) for return to the University of Liverpool for

processing. If staff collected more than one faecal sample for an animal in the same day the first one to be removed from the box was processed and any others were discarded.

Environmental samples were collected once in each week of sampling from each practice. There was some variation in exact sites sampled in each practice necessitated by the different layouts of each practice. However areas sampled from all practices were: ward floors; computer keyboards in kennel rooms and treatment areas; examination tables in treatment areas (not in consulting rooms) and the outside dog walking areas. In addition floor samples were taken from a central treatment area in three practices and a lift used for patient transport in one practice. In the case of multiple keyboards/examination tables in the same area one was selected on the first week of sampling and this was used for all subsequent sample collection. Samples from floors were collected using disposable absorbent overshoes (bootsocks) dampened with 2-3ml saline. These were worn and walked around rooms in the same pattern each week. Samples were taken from keyboards and tables using sterile cloths moistened with sterile saline.

Isolation of resistant bacteria from samples

Samples were processed immediately after collection and transported to the University of Liverpool. Two grams of faeces were placed in a 5ml container and 2ml of brain heart infusion broth (LabM, UK) was added. The mixture was vortexed to create a homogenate which was inoculated onto eosin methylene blue agar (EMBA, LabM, UK). Discs (Mast Ltd, UK) impregnated with ampicillin (10µg), augmentin (30µg), ciprofloxacin (1µg) and trimethoprim (2.5µg) were added to the plate and it was incubated overnight at 37^{o}C (Bartoloni et al., 2006).

Bootsocks were soaked in buffered peptone water (LabM, UK) for two minutes and 10ml of the supernatant incubated overnight. Swabs from surfaces were incubated in buffered peptone water overnight. After incubating overnight environmental samples were processed as described below.

In order to isolate *E. coli* resistant to cephalosporins 0.5ml of the faecal homogenate was added to 4.5ml buffered peptone water (LabM, UK) and incubated overnight at 37^{o}C. Five microlitres of this mixture was then streaked onto one EMBA plate containing 1µg/ml cefotaxime and another EMBA plate containing 1µg/ml ceftazidime. Both plates were incubated overnight at 37^{o}C. If present, for each sample one colony resembling *E. coli* was removed from each of the antimicrobial inhibition zones and from the cephalosporin

containing EMBA plates and inoculated onto nutrient agar (LabM, UK) and incubated overnight at 37^{o}C in order to obtain pure cultures.

In all a maximum of six AMR isolates could be obtained from each faecal sample one isolate each from the four inhibition zones on the plate containing antimicrobial discs, one from the plate containing EMBA with ceftazidime and one from the plate containing EMBA with cefotaxime. Environmental samples were only tested for cephalosporin resistance initially so one environmental sample would yield a maximum of two isolates.

Antimicrobial susceptibility and ESBL phenotypic testing

Full susceptibility testing was performed on all isolates. Colonies from nutrient agar plates were suspended in sterile water to make a solution equivalent to 0.5 McFarlands Turbidity Standard (0.5MTS).

For general antimicrobial susceptibility testing 0.5ml of the above 0.5MTS solution was pipetted into 4.5ml sterile water and this solution was inoculated onto iso-sensitive agar (LabM, UK). Discs impregnated with 10µg ampicillin, 30µg augmentin, 30µg chloramphenicol, 30µg nalidixic acid, 1µg ciprofloxacin, 2.5µg trimethoprim and 30µg tetracycline were placed onto the agar and it was incubated overnight at 37^{o}C. Sensitivity or resistance was interpreted according to BSAC guidelines (BSAC, 2013).

Isolates demonstrating resistance to third generation cepahalosporins were tested for ESBL-producer phenotypes. The 0.5MTS solution was inoculated directly onto iso-sensitive agar. Discs impregnated with 30µg ceftazidime, 30µg ceftazidime and 10µg clavulanic acid, 30µg cefpodoxime, 30µg cefpodoxime and 10µg clavulanic acid, 30µg ceftazidime and 30µg ceftazidime and 10µg clavulanic acid (ESBL identification set, Mast Ltd, UK) were added and the plates were incubated overnight at 37^{o}C. The diameter of the inhibition zone was measured for each disc and ESBL production was confirmed if there was more than 5mm increase in zone diameter with the disc with clavulanic acid compared to the one without according to the manufacturers (MAST group) instructions (M'Zali et al., 2000).

Cell lysates were prepared by adding 2-3 colonies from the pure culture to 0.5ml sterile water and heating at 100^{o}C for 20 minutes, they were then refrigerated for subsequent DNA analysis.

Genotypic analysis

All isolates consistent with *E. coli* were confirmed using primers uidAF and uidAR targeting the *uidA* gene as described previously (McDaniels et al., 1996). A master mixture of 1.8ml 1.1xReddymix (Abgene) and 20µl each of primers (100pmol/ml) uidAF and uidAR was prepared and 24µl of this was added to each PCR tube. To this was added 1µl of DNA lysate to make a total reaction mixture volume of 25µl. Samples were placed at 94°C for 4 minutes, then subjected to twenty five cycles of: 94°C for 20 seconds; 58°C for 30 seconds and 72°C for 1 minute. After this the mixture was held at 72°C for 7 minutes then at 4°C until analysis.

All isolates demonstrating resistance to cefotaxime and ceftazidime on sensitivity testing were tested for the presence of $bla_{CTX\text{-}M}$ genes using universal $bla_{CTX\text{-}M}$ primers CTXMU1 and CTXMU2 (Batchelor et al., 2005b). A master mixture comprising 1.8ml 1.1xReddymix (Abgene) and 20µl of each primer was made and 24µl of this was pipetted into each PCR tube. Each 25µl reaction contained: 0.625 units Taq DNA polymerase; 75mM Tris-HCl; 20mM $(NH_4)_2SO_4$; 1.5mM $MgCl_2$ and 0.2mM each of dATP, dCTP, dGTP and dTTP. To this was added 1µl of sample DNA.

All isolates positive for the presence of $bla_{CTX\text{-}M}$ genes were then tested to specify the CTX-M group. All CTX-M universal positive isolates were tested using primers specific to $bla_{CTX\text{-}M}$ groups 1 (Carattoli et al., 2005), 2 (Hopkins et al., 2006) and 9 (Batchelor et al., 2005b) using the same thermocycler program as used for the $bla_{CTX\text{-}M}$ universal PCR.

All isolates demonstrating ceftazidime resistance on sensitivity testing were tested for the presence of bla_{TEM}, bla_{SHV} and bla_{OXA} genes using a multiplex PCR assay (Dallenne et al., 2010). Sample DNA (5µl) was added to 4µl 5 x Mastermix (Solis Biodyne) (0.4M Tris-HCl, 0.1M$(NH_4)_2SO_4$, 7.5mM $MgCl_2$, 1mM dNTP's of each) 0.5µl of each primer (10pmol/µl) and 13µl water. Reaction mixtures were subjected to an initial denaturation stage of 94°C for 10 minutes then thirty cycles of 94°C for forty seconds, 60°C for forty seconds and 72°C for sixty seconds. A final elongation step of 72°C for seven minutes was used.

All isolates demonstrating clavulanic acid potentiated amoxicillin (CAPA) resistance on sensitivity testing were tested for the presence of bla_{CITM} using CITM primers (Perez-Perez and Hanson, 2002). A master mix containing 4µl 5 x Mastermix (Solis Biodyne), 0.5µl of CITMF and CITMR primers, 15µl water for each sample was made and pipette to each reaction tube and 5µl sample DNA added. Mixtures were then placed in a thermocycler

and subjected to: 94°C for 3 minutes; twenty five cycles of 94°C for 30 seconds, 64°C for 30 seconds and 72°C for 60 seconds. After this the reaction was held at 72°C for 7 minutes and then at 4°C until analysis.

All isolates identified as producing CTX-M-1 group ESBLs were tested to determine if they belonged to serogroup O25 (Clermont et al., 2008), 20μl of each primer (100pmol/μl) was added to 1.8ml Reddymix (Abgene) and 24μl of this mixture was added to 1μl sample DNA. This reaction mixture was subjected to an initial denaturation step of 94°C for three minutes followed by thirty cycles of: 94°C for thirty seconds; 60°C for thirty seconds and 72°C for sixty seconds. This was followed by a final elongation step of 72°C for five minutes.

Isolates belonging to serogroup O25 were further tested for markers for sequence type (ST) 131 (Clermont et al., 2009). 5μl sample DNA was added to 4μl 5x Mastermix (Solis Biodyne), 0.5μl each primer and 14μl water. The reaction mixture was subjected to an initial denaturation step of 94°C for four minutes then thirty cycles of 94°C for five seconds and 65°C for ten seconds. This was followed by a final elongation step of 72°C for five minutes.

All isolates demonstrating resistance to nalidixic acid were tested for the presence of *qnrA*, *qnrB* and *qnrS* genes using a multiplex PCR assay (Robicsek et al., 2006). 5μl sample DNA was combined with 0.5μl each primer, 4μl 5 x mastermix (Solis Biodyne) and 13μl water. Reaction mixtures were subjected to 94°C for 3 minutes, then 32 cycles of 94°C for 45seconds, 53°C for 45 seconds and 72°C for 60 seconds. A final elongation step of 72°C for 5 minutes was used. Table 1 shows all the primers used in this project along with annealing temperature and expected amplicon size.

Sequencing of resistant isolates

Following successful amplication, products were purified and both forward and reverse strands were amplified using a sequencing PCR. The subsequent products were further purified and plates were sent for reading at the Zoology Sequencing Facility, Oxford. All sequencing reactions and clean ups were carried out according to an in-house protocol (Appendix I). Each sequence was examined and primer sequences were removed using ChromasPRO v1.7.3 (http://technelysium.com.au), as well as the presence of ambiguous background signals. Samples with poor quality sequence data were excluded from further analysis. Where possible, consensus sequences were derived from both the forward and

reverse reads. BLAST searches were conducted on either consensus or single read sequences for each isolate to confirm the *bla*$_{CTX-M}$/*bla*$_{CITM}$/*bla*$_{SHV}$/*bla*$_{TEM}$ related identity. All sequencing was performed by Chris Ball due to time constraints.

Table 1: PCR primers used in this project detailing nucleotide sequences, annealing temperatures and expected amplicon sizes.

Target	Primer name	Sequence (5'-3')	Annealing temperature	Amplicon size
uidA gene (*E. coli* confirmation)	uidAF uidAR	CCAAAAGCCAGACAGAGT GCACAGCACATCAAAGAG	58°C	623bp
bla$_{CTX-M}$ universal (all CTX-M groups)	CTXMU1 CTXMU2	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	58°C	593bp
bla$_{CTX-M}$ group 1	CTXMgp1F CTXMgp1R	CCCATGGTTAAAAAATCACTGC CAGCGCTTTTGCCGTCTAAG	55°C	876bp
bla$_{CTX-M}$ group 2	CTXMgp2F CTXMgp2R	ATGATGACTCAGAGCATTCGC TCAGAAACCGTGGGTTACGAT	55°C	893bp
bla$_{CTX-M}$ group 9	CTXMgp9F CTXMgp9R	ATGGTGACAAAGAGAGTGCAAC TTACAGCCCTTCGGCGATG	55°C	876bp
bla$_{TEM}$, SHV and OXA	TSO-TF TSO-TR TSO-SF TSO-SR TSO-OF TSO-OR	CATTTCCGTGTCGCCCTTATTC CGTTCATCCATAGTTGCCTGAC AGCCGCTTGAGCAAATTAAAC ATCCCGCAGATAAATCACCAC GGCACCAGATTCAACTTTCAAG GACCCCAAGTTTCCTGTAAGTG	60°C (multiplex)	800bp 713bp 564bp
bla$_{CITM}$	CITMF CITMR	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	64°C	462bp
qnr	qnrAF qnrAR qnrBF qnrBR qnrSF qnrSR	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	53°C (multiplex)	520bp 469bp 417bp
serogroup O25	rfb.1bis rfbO25b.r	ATACCGACGACGCCGATCTG TGCTATTCATTATGCGCAGC	60°C	300bp
O125 ST131	O25pabBspe.F rfbO25b.r trpA.F trpA2.R	TCCAGCAGGTGCTGGATCGT GCGAAATTTTTCGCCGTACTGT GCTACGAATCTCTGTTTGCC GCAACGCGGCCTGGCGGAAG	65°C (multiplex)	347bp 427bp

Statistical analysis

All data were entered into a spreadsheet program (Microsoft Excel 2007, Microsoft Corporation) and the dataset was reviewed and checked for coding of all variables. Descriptive statistics and 95% confidence intervals for proportions were calculated for

prevalence data for each practice and overall. Resistance to each of the seven antimicrobials was considered as a separate outcome. Additionally, the presence in a sample of an *E. coli* with multidrug resistance (to three or more antimicrobial classes) or with resistance to third generation cephalosporins were considered. ESBL and AmpC production (both phenotype and genotype demonstrated by PCR) were also considered.

Due to the nature of sampling the data were clustered within veterinary practices and within dog so we also estimated the prevalence of antimicrobial-resistant *E. coli* after allowing for the clustering using separate multilevel models with a binomial distribution and logit link function. Three-level multilevel models were constructed for each outcome, with practice and dog clustering accounted for by incorporation of second- and third-level random intercept terms. Calculations were performed using penalised quasi-likelihood estimates (2nd order or 1st order PQL). The true prevalence (P_T) was estimated using the formula below, by incorporating the constant parameter estimate (β_0) derived from the random intercept-only three-level models constructed for each of the outcomes considered:

$$P_T = \frac{e^{\beta 0}}{1 + e^{\beta o}}$$

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

RESULTS

Samples collected

A total of 341 faecal samples were collected from 214 animals over a total of 31 sampling weeks between 13th May and 21st October 2013 (table 2). Eight samples were discarded from analysis due to being collected from the same animal on the same date. From these 333 faecal samples 363 isolates demonstrating resistance to one or more of the antimicrobial classes tested were obtained corresponding to 167 (50.1%) faecal samples containing *E. coli* resistant to at least one antimicrobial.

A total of 257 environmental samples were collected from areas within the five practices during the same 31 sampling weeks. From these samples 86 isolates demonstrating

resistance were obtained corresponding to 47 (18.3%) of environmental samples having *E. coli* isolates with antimicrobial resistance. The number and sampling location of environmental samples along with the number of faecal samples from each practice are shown in table 2. For some faecal samples (n=13) it was not possible to retrieve clinical records so species was not specified.

Table 2: Number of faecal samples obtained from different species and environmental samples from different areas from each practice and in total.

	Faecal samples				Environmental samples				
Practice	Dogs	Cats	Unspecified	Total	Inside floors	Examination tables	Keyboards	Outdoors walking area	Total
1	122	14	0	136	28	12	18	7	65
2	32	0	5	37	30	6	6	6	48
3	63	10	0	73	24	6	12	6	48
4	34	0	2	36	24	6	12	6	48
5	45	0	6	51	30	6	6	6	48
Total	296	24	13	333	136	36	54	31	257

Resistance phenotypes

The overall prevalence of faecal and environmental samples with at least one isolate resistant to ampicillin, CAPA, chloramphenicol, nalidixic acid, ciprofloxacin, trimethoprim and tetracycline is shown in tables 3 and 4 respectively. The overall prevalence of resistance was higher in faecal samples than environmental samples. Resistance to at least one antimicrobial was detected in isolates from 167/333 (50.1%) faecal samples and 47/257 (18.3%) environmental samples. Overall tetracycline resistance was the least common resistance type in faecal samples and chloramphenicol resistance was the least common type in environmental samples. Resistance to ampicillin was the most common resistance type in both faecal and environmental samples.

There was significant variation by practice within results. This was particularly the case for ciprofloxacin resistance with the highest prevalence in faecal samples of 44% in practice one compared to 7.8% in practice five. This level of variation between practice was not seen for every resistance phenotype, for example the ampicillin resistance prevalence ranged from 38% to 58% between practices, with an overall prevalence when adjusted for

clustering, of 45% indicating that levels of certain resistance phenotype are less affected by practice.

Appendix II shows the percentage of samples from each practice (and in total) which yielded an isolate demonstrating resistance phenotypes deemed to be clinically important. These phenotypes were: CAPA resistance, ciprofloxacin resistance, ESBL-producer phenotype, AmpC-producer phenotype, resistance to third generation cephalosporin and MDR. Practice one demonstrated the highest prevalence of CAPA resistance, ciprofloxacin resistance and AmpC-producer phenotype in both faecal and environmental samples. Practice three demonstrated the highest prevalence of ESBL-producer phenotype and MDR. Practices two and five generally demonstrated low prevalence of resistance.

Isolates displaying MDR varied with regard to which classes of antimicrobial they were resistant to. Table 5 summarises the different MDR phenotypes, the number of each obtained and the proportion of MDR positive samples with isolates of this phenotype for both faecal and environmental samples. In total 18 different MDR phenotypes were detected in this study, 17 of these were in faecal samples and 10 in environmental samples with substantial overlap between the two. Many of the different MDR phenotypes contained ciprofloxacin and CAPA resistance. Figure 1 shows MDR types by practice.

Origin and timing of environmental isolates

Environmental samples commonly associated with important resistance phenotypes (MDR, ESBL-producer and AmpC-producer) were most likely to be isolated from bootsocks from either the outside walking areas or internal floors of the ward communal areas. There was variation in the rate of isolation of resistant bacteria between practices and between areas within practices. The frequency of isolation of different resistance phenotypes from different areas within each practice is shown in figure 2.

The isolation of *E. coli* with important resistance phenotypes varied over the sampling period for each practice with different patterns seen in different practices. Practices two and five had relatively low rates of isolation throughout the study, practices three and four generally had low rates of isolation with marked increases in one sampling week. Practice one had a consistently moderate level of contamination (appendix III).

Table 3: Sample level prevalence of resistance to each antimicrobial, MDR and ESBL and AmpC producer prevalence in faecal samples (n=333) from each practice with 95% confidence intervals and adjustment for clustering within practice and animal. TGCR = 3rd generation cephalosporin resistance, Cx = cefotaxime, Cp = cefpodoxime, Cz = ceftazidime.

	Practice 1	Practice 2	Practice 3	Practice 4	Practice 5	All practices (N)	All Practices	All practices adjusted for clustering[#]
Ampicillin resistant	58.1 (49.8-66.4)	37.8(22.2-53.5)	49.3 (37.8-60.8)	41.7 (25.6-57.8)	39.2 (25.8-52.6)	164	49.2 (43.9-54.6)	45.4 (36.6-54.6)
CAPA resistant	42.6 (34.3-51)	24.3 (10.5-38.1)	20.5 (11.3-29.8)	19.4 (6.5-32.4)	15.7 (5.7-25.7)	97	29.1 (24.2-34)	14.0 (6.7-27.0)
Chloramphenicol res.	19.9 (13.1-26.6)	24.3 (10.5-38.1)	24.7 (14.8-24.5)	2.8 (0-8.1)	5.9 (0-12.3)	58	17.4 (13.3-21.5)	13.5 (8.7, 20.4)
Tetracycline res.	12.5 (6.9-18.1)	5.4 (0-12.7)	15.1 (6.9-23.3)	11.1 (0.8-21.4)	19.6 (8.7-30.5)	44	13.2 (9.6-16.9)	12.8 (9.5, 17.0)
Trimethoprim res.	32.4 (24.5-40.2)	24.3 (10.5-38.1)	30.1 (19.6-40.7)	13.9 (2.6-25.2)	13.7 (4.3-23.2)	87	26.1 (21.4-30.8)	12.7 (6.8, 22.4)
Nalidixic acid res.	44.1 (35.8-52.5)	10.8 (0.8-20.8)	17.8 (9-26.6)	22.2 (8.6-35.8)	9.8 (1.6-18)	90	27.0 (22.3-31.8)	13.1 (5.5, 28.1)
Ciprofloxacin res.	44.1 (35.8-52.5)	8.1 (0-16.9)	11.0 (3.8-18.1)	13.9 (2.6-25.2)	7.8 (0.5-15.2)	80	24.0 (19.4-28.6)	9.2(3.2, 23.9)
MDR	31.6 (23.8-39.4)	24.3 (10.5-38.1)	34.2 (23.4-45.1)	22.2 (8.6-35.8)	9.8 (1.6-18.0)	115	27.0 (22.3-31.8)	13.1 (6.9-23.6)
TGCR	50.0 (41.6-58.4)	24.3 (10.5-38.1)	41.1 (29.8-52.4)	36.1 (20.4-51.8)	11.8 (2.9-20.6)	126	37.8 (32.6-43)	27.2 (14.9-44.3)
CxR	39.7 (31.5-47.9)	8.1 (0-16.9)	16.4 (7.9-24.9)	16.7 (8.6-35.8)	2.0 (0-5.8)	76	22.8 (18.3-27.3)	9.9 (3.5-25.4)
CpR	50.0 (41.6 -58.4)	24.3 (10.5-38.1)	41.1 (29.8-52.4)	36.1 (20.4-51.8)	11.8 (2.9-20.6)	126	37.8 (32.6-43)	27.1 (14.8-44.3)
CzR	37.5 (29.4-45.6)	13.5 (2.5-24.5)	13.7 (5.8-21.6)	22.2 (8.6-35.8)	3.9 (0-9.2)	76	22.8 (18.3-27.3)	9.9 (3.8-23.4)
ESBL producer phenotype	14.0 (8.1-19.8)	8.1 (0-16.9)	30.1 (19.6-40.7)	25.0 (10.9-39.1)	0.0	53	15.9 (12-19.8)	9.7 (3.4-24.6)
AmpC producer phenotype	33.1 (25.2-41)	0.0	1.4 (0-4)	5.6 (0-13)	2.0 (0-5.8)	49	14.7 (10.9-18.5)	4.3 (1.1-15.6)

*Estimates from 1st order PQL # Estimates from intercept-only multilevel models.

Table 4: Sample level prevalence of resistance to each antimicrobial class, MDR and ESBL and AmpC producer prevalence in environmental samples (n=257) from each practice with 95% confidence intervals. CxR = cefotaxime resistant, CpR = cefpodoxime resistant, CzR = Ceftazidime resistant, 3GCR = any third gen. cephalosporin resistance.

	Practice 1	Practice 2	Practice 3	Practice 4	Practice 5	All practices (N)	Total
Ampicillin res.	33.8 (22.3-45.3)	6.3 (0-13.1)	25.0 (12.8-37.3)	12.5 (3.1-21.9)	6.3 (0-13.1)	46	17.9 (13.2-22.6)
CAPA res.	29.2 (18.2-40.3)	6.3 (0-13.1)	8.3 (0.5-16.2)	4.2 (0-9.8)	2.1 (0-6.1)	29	11.3 (7.4-15.2)
Chloraphenicol res.	6.2 (0.3-12)	0.0	14.6 (4.6-24.6)	4.2 (0-9.8)	0.0	13	5.1 (2.4-7.7)
Tetracycline res.	6.2 (0.3-12)	6.3 (0-13.1)	6.3 (0-13.1)	4.2 (0-9.8)	4.2 (0-9.8)	14	5.4 (2.7-8.2)
Trimethoprim res.	12.3 (4.3-20.3)	4.2 (0-9.8)	18.8 (7.7-29.8)	2.1 (0-6.1)	2.1 (0-6.1)	23	8.9 (5.5-12.4)
Nalidixic acid res.	29.2 (18.2-40.3)	2.1 (0-6.1)	6.3 (0-13.1)	2.1 (0-6.1)	2.1 (0-6.1)	29	11.3 (7.4-15.2)
Ciprofloxacin res.	29.2 (18.2-40.3)	2.1 (0-6.1)	6.3 (0-13.1)	4.2 (0-9.8)	2.1 (0-6.1)	27	10.5 (6.8-14.3)
MDR	15.4 (6.6-24.2)	4.2 (0-9.8)	14.6 (4.6-24.6)	4.2 (0-9.8)	4.2 (0-9.8)	23	8.9 (5.5-12.4)
3GCR	33.8 (22.3-45.3)	2.1 (0-6.1)	22.9 (11-34.8)	8.3 (0.5-16.2)	6.3 (0-13.1)	41	16.0 (11.5-20.4)
CxR	29.2 (18.2-40.3)	0.0	14.6 (4.6-24.6)	2.1 (0-6.1)	0.0	27	10.5 (6.8-14.3)
CpR	33.8 (22.3-45.3)	2.1 (0-6.1)	22.9 (11-34.8)	8.3 (0.5-16.2)	6.3 (0-13.1)	41	16.0 (11.5-20.4)
CzR	26.2 (15.5-36.8)	0.0	12.5 (3.1-21.9)	4.2 (0-9.8)	0.0	25	9.7 (6.1-13.4)
ESBL-producer phenotype	3.1 (0-7.3)	0.0	16.7 (6.1-27.2)	8.3 (0.5-16.2)	0.0	14	5.4 (2.7-8.2)
AmpC producer phenotype	16.9 (7.8-26)	0.0	2.1 (0-6.1)	0.0	0.0	12	4.7(2.1-7.2)

Table 5: The proportion of different MDR phenotypes in both faecal and environmental samples. amp = ampicillin resistant, aug = CAPA resistant, chl = chloramphenicol resistant, tet = tetracycline resistant, trim = trimethoprim resistant, nal = nalidixic acid resistant and cip =ciprofloxacin resistant.

MDR phenotype	**Number of antimicrobial classes resistant to**	**FAECAL SAMPLES**		**ENVIRONMENTAL SAMPLES**	
		Number of samples	**% (95% CI)**	**Number of samples**	**% (95% CI)**
amp aug chl trim nal cip	4	20	23.3 (14.3-13.2)	2	8.7 (0-20.2)
amp chl trim	3	13	15.1 (7.5-22.7)	4	17.4 (1.9-32.9)
amp aug chl tet trim nal cip	5	8	9.3 (3.2-15.4)	4	10.5 (0.8 – 20.3)
amp aug trim nal cip	3	7	8.1 (2.4-13.9)	3	13 (0-26.8)
amp aug chl trim	3	6	7.0 (1.6-12.4)	1	4.3 (0-12.7)
amp tet trim nal cip	4	5	5.8 (0.9-10.8)	2	8.7 (0-20.2)
amp chl tet trim nal cip	5	5	5.8 (0.9-10.8)		
amp tet nal	3	4	4.7 (0.2-9.1)		
amp aug tet trim nal cip	4	4	4.7 (0.2-9.1)	1	4.3 (0-12.7)
amp tet trim	3	3	3.5 (0-7.4)		
amp aug tet trim	3	3	3.5 (0-7.4)	2	8.7 (0-20.2)
amp chl trim nal cip	4	2	2.3 (0-5.5)		
amp trim nal cip	3	1	1.2 (0-3.4)		
amp tet nal cip	3	1	1.2 (0-3.4)	2	8.7 (0-20.2)
amp chl tet trim nal	5	1	1.2 (0-3.4)		
amp aug chl tet trim	4	1	1.2 (0-3.4)		
amp aug chl nal cip	3	1	1.2 (0-3.4)		
amp chl tet trim	4			2	8.7 (0-20.2)

Figure 1: Breakdown of MDR types by practice. Showing the relative frequency of isolation of each type in each practice (practice 1 = dark blue, practice 2 = red, practice 3 = green, practice 4 = purple and practice 5 = light blue).

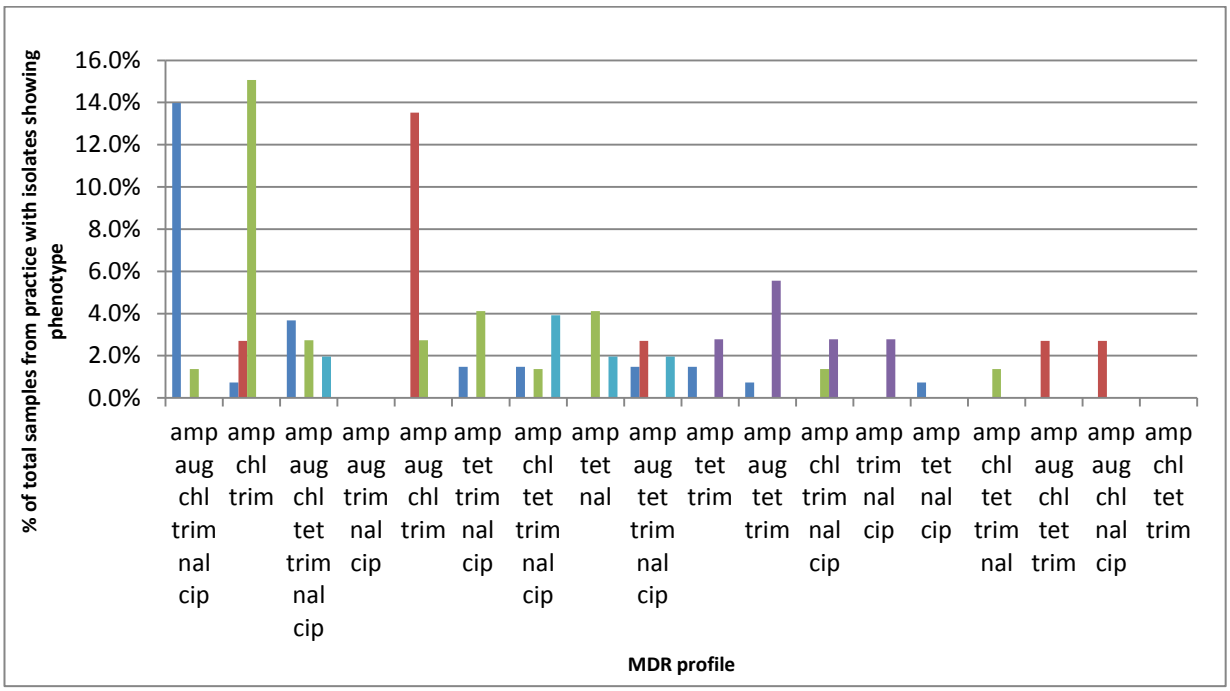
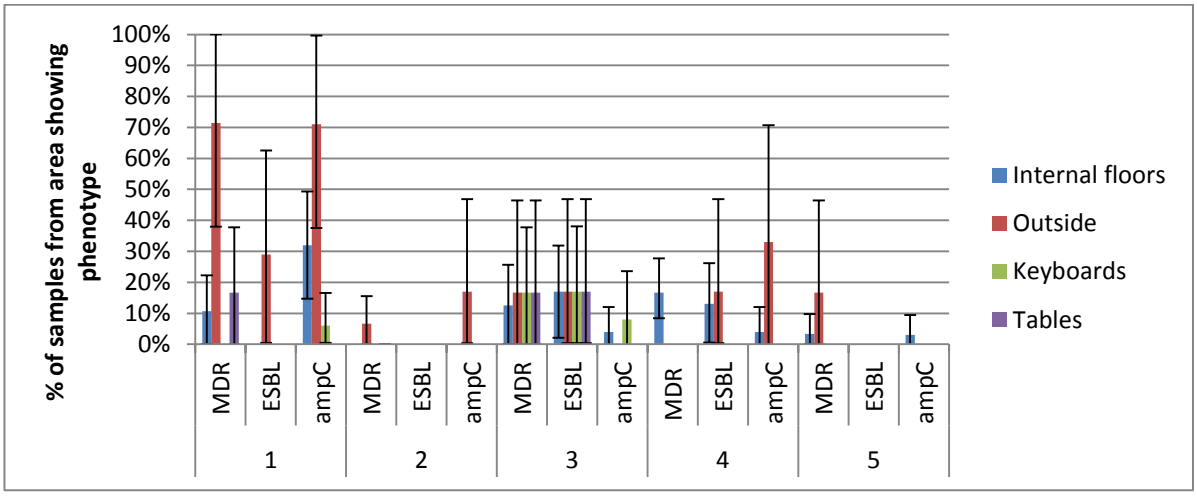


Figure 2: The percentage of samples from each area (represented by different coloured bars) within each practice yielding an isolate positive for MDR, ESBL- or AmpC-producer phenotypes. The Y axis represents the percentage of all samples collected from that area in the whole sampling period that showed the resistance phenotype. The X axis represents the three important resistance types in each practice (one to five).



Molecular characterisation of resistance genes

In total 348/363 faecal sample isolates and 77/86 environmental sample isolates tested positive using the *uidA* PCR test confirming the identity of these isolates.

Of all isolates (environmental and faecal) 216 were resistant to either cefotaxime or ceftazidime. PCR detected $bla_{CTX\text{-}M}$ in 78 (26 environmental isolates and 52 faecal isolates) isolates. Further PCR analysis detected $bla_{CTX\text{-}M}$ genes belonging to group one in 32 isolates from 22/333 (6.6%) faecal samples and 15 isolates from 12/257 (4.7%) environmental samples. The presence of group one $bla_{CTX\text{-}M}$ was detected in isolates from all practices except practice five and in environmental samples from practices one, three and four. Group nine $bla_{CTX\text{-}M}$ production was detected in 5 faecal sample isolates from 3/333 (0.9%) samples and 3 environmental isolates from 2/256 (0.8%) environmental samples, all of which were from practice one. No isolates were positive for group two or found to belong to O25/ST131. Of the 78 isolates positive on the universal $bla_{CTX\text{-}M}$ PCR the group was not identified in 5 isolates. The inhibitor resistant TEM-158 was detected in one environmental sample and in *E. coli* isolated from ten faecal samples from practice one and was not detected in any other practices. A sample was taken to be a confirmed ESBL producer if it either had an ESBL producer phenotype, was positive on the universal $bla_{CTX\text{-}M}$ PCR or, in the case of TEM and SHV producer types, returned a sequencing result corresponding to an ESBL. The prevalence of confirmed ESBL-producers and AmpC producers (bla_{CITM} positive) in faecal and environmental samples is shown in table 6.

The bla_{CITM} gene was detected in isolates from 81% and 69% of faecal and environmental samples which had isolates demonstrating CAPA resistance. All bla_{CITM} positive isolates which returned a result on sequence analysis corresponded to $bla_{cmy\text{-}2}$. There was significant masking of ESBL producer phenotype by the production of AmpC with the effect being particularly marked in practice one where there was a high level of AmpC-producers. Twenty (41%) faecal samples and two (17%) environmental samples with an AmpC producer phenotype were also positive for ESBL production.

Table 6: Prevalence of confirmed ESBL producer (either phenotype or genotype) and *bla*$_{CITM}$ positive samples in faecal and environmental samples by practice and in total. For faecal samples overall prevalence with adjustment for clustering within animal and practice is also demonstrated.

	Practice 1	Practice 2	Practice 3	Practice 4	Practice 5	All practices (N)	All practices (%)	All practices adjusted for clustering (%)
ESBL-production confirmed* (faecal)	26.5 (19.1-33.9)	8.1 (0-16.9)	34.2 (23.4-45.1)	22.2 (8.6-35.8)	2.0 (0-5.8)	73	21.9 (17.5-26.4)	14.0 (5.3,35.0)
bla$_{CITM}$ positive PCR (faecal)	38.2 (30.1-46.8)	13.5 (2.5-24.5)	11.0 (3.8,18.1)	13.9 (2.6,25.2)	3.9 (0-9.2)	72	21.6 (17.2-26)	7.7 (2.5,21.1)
ESBL production confirmed* (environmental)	13.8 (5.4-22.2)	0.0	16.7 (6.1-27.2)	10.4 (1.8-19.1)	0.0	22	8.6 (5.1-12)	NA
bla$_{CITM}$ positive PCR (environmental)	23.1 (12.8-33.3)	2.1 (0-6.1)	4.2 (0-9.8)	6.3 (0-13.1)	2.1 (0-6.1)	22	8.6 (5.1-12)	NA

*Confirmed by PCR, phenotype or sequencing

Not all isolates which were positive on PCR successfully returned sequence analysis results. Sequencing results are summarised in table 7. Group one and nine *bla*$_{CTX-M}$ were detected in isolates from four and one sample respectively by PCR but sequence analysis could not confirm the individual gene they carried belonging to these groups. The remaining isolates categorised as ESBL producers were phenotypically ESBL producers but did not amplify using PCR. All TEM-158 and SHV-12 ESBL producers were from the same practice (practice one).

Table 7: Number and percentage of faecal (n = 333) and environmental (n=257) samples with at least one *E. coli* isolate having a resistance gene identified by sequence analysis.

Gene	**Faecal samples**		**Environmental samples**	
	N	**% (95% CI)**	**N**	**% (95% CI)**
bla$_{CTX-M-15}$	20	6 (3.5-8.6)	8	3 (1-5.2)
bla$_{CTX-M-1}$	1	0.3 (0-0.9)	1	0.4 (0-1.2)
bla$_{CTX-M-9}$	1	0.3 (0-0.9)	0	
bla$_{CTX-M-82}$	1	0.3 (0-0.9)	0	
bla$_{TEM-158}$	10	3 (1.2-4.8)	1	0.4 (0-1.2)
bla$_{SHV-12}$	0		2	0.8 (0-1.9)
bla$_{CMY-2}$	72	22 (17.2-26.0)	22	9 (5.1-12.0)

A low prevalence of *qnr* genes was found with just 3 (1%) faecal samples having at least one *E. coli* isolate which tested positive for the presence of *qnr* genes on PCR. Two were positive for *qnrS* and one was positive for *qnrB.* Among environmental samples only *qnrB* was detected, in isolates from four (1.5%) samples.

DISCUSSION

The aim of this study was to determine carriage rates and the level of hospital environment contamination with commensal gastro-intestinal bacteria with important antimicrobial resistance (AMR) phenotypes and genotypes in small animal referral hospitals. Overall, in isolates from both faecal and environmental samples there were relatively high levels of resistance to a number of important antimicrobials including CAPA, fluoroquinolones and third generation cephalosporins. Antimicrobial resistance has important implications for veterinary practice from both a clinical and public health perspective. An initial step towards mitigating the problem is first understanding the patterns of prevalence in both patients and the practice environment.

CAPA resistance among samples from all practices was high, 14% overall with some variation between practices. CAPA is an antimicrobial used commonly in companion animal practice in the UK (Radford et al., 2011, Mateus et al., 2011) and the frequent carriage of resistance is likely to be related to frequent use in veterinary practice and is of concern. In human medicine the incidence of ESBL-producers in hospital has been linked to use of CAPA in the community highlighting the importance of interaction between community antimicrobial use and the development of AMR in hospitals (Aldeyab et al., 2012). Resistance to CAPA has been found in other studies albeit at lower levels: 6.3% in hospitalised dogs in Korea (Nam et al., 2010); 3.8% in clinical isolates from pets in Denmark (Pedersen et al., 2007); 7% in community dogs in the UK (Wedley et al., 2011) and 8.3% in animals about to be hospitalised in the USA (Hamilton et al., 2013). The high levels in this study are likely a reflection of the hospitalised status of animals given that the study of dogs in the UK community in a similar geographical area showed a relatively low level of CAPA resistance. CAPA resistance was lower in Korean veterinary hospitals which could reflect lower use of CAPA in Korea. Although there are no published reports of frequency of use in Korea in a study of hospitalised dogs in China amoxicillin had only been given to

3% of dogs (Lei et al., 2010) which could reflect different prescribing patterns in the region compared to the UK.

The majority of *E. coli* with CAPA resistance also tested positive for bla_{AmpC} genes making this the most likely mechanism responsible. Previous studies have identified bla_{AmpC} genes at low levels in healthy dogs in the community in the UK (Wedley et al., 2011), Tunisia (Ben Sallem et al., 2013), Korea (Tamang et al., 2012), Portugal (Belas, 2014) and Canada (Murphy et al., 2009). They have also been found in clinical *E. coli* isolates from animals in the USA at low levels (Shaheen et al., 2011) and in Holland at high levels (Dierikx et al., 2012). Significantly, studies of hospitalised dogs in Korea (So et al., 2012) and Australia (Sidjabat et al., 2006) found the prevalence of AmpC-production in *E. coli* of 23.8% and 16.5% respectively. This study concurs with this pattern, with an overall prevalence of 7.7% although there was significant variation between practices with practice 1 having a significantly higher prevalence. This suggests that hospitalised animals are more at risk of carriage of AmpC-producing *E. coli*, possibly as a result of infection from the environment or other hospitalised animals, increased exposure to antimicrobials or increased levels of morbidity among these animals. The differences between practices suggest that practice level factors can have an important influence. High levels of AmpC have also been found in a human healthcare setting where this was putatively linked with a high use of CAPA in the same facility (Seiffert et al., 2013a).

All practices in this study had environmental samples which were positive for *E. coli* with bla_{AmpC} production though again there was significant variation between practices which followed a similar pattern to that seen in the faecal samples, suggesting a link between commensal faecal and environmental AmpC-producing *E. coli.* Colonisation of dogs and humans and contamination of the veterinary environment by the same AmpC-producing *E. coli* strains has been reported previously (Sidjabat et al., 2006) and the environmental prevalence (4.1%) reported is similar to levels found in most of the practices in this study. This study shows that AmpC producing bacteria can contaminate the hospital environment and are a potential source of colonisation or infection of patients, though it is likely to be a complex picture with exchange between animals and the environment occurring in both directions. ESBL-production in *E. coli* has been shown to confer better environmental survival compared to AmpC production in a human healthcare setting (Starlander et al., 2014). The latter is of interest given the high level of AmpC producing *E. coli* compared to

ESBL producing *E. coli* in environmental samples from practice one, it is possible this high level of AmpC production will reduce over time in the favour of ESBL production.

The prevalence of resistance to ciprofloxacin was high but variable between practices ranging from 8 to 44%. Recent studies have found ciprofloxacin resistance at 2.2% in dogs in the UK community (Wedley et al., 2011), 2.9% in clinical samples from pets in Denmark (Pedersen et al., 2007); 1.3% in healthy dogs from the community in Portugal (Costa et al., 2008), 16.1% in stray dogs in Korea (Nam et al., 2010) and 48.2% in hospitalised dogs and cats in China (Lei et al., 2010). This study demonstrates a substantially higher prevalence of ciprofloxacin resistance in hospitalised companion animals compared to those in the UK community (Wedley et al., 2011). Of the 80 faecal samples positive for ciprofloxacin resistant *E. coli* 58 (73%) were MDR, 53 (66%) were AmpC producers and 39 (49%) were ESBL producers. Fluoroquinolones are an important class of antimicrobial for treatment of important infections in both human and veterinary medicine and developing resistance to these drugs is a serious concern for animal and public health. The *qnr* genes were detected at low rates among both faecal and environmental samples and are therefore unlikely to be a significant contributor to the levels of fluoroquinolone resistance seen in this study. The mechanisms for quinolone resistance in this study were not studied but are most likely to be due to chromosomal mutations in the gyrase genes.

The prevalence of MDR *E. coli* in faecal samples from this study was 27%, again there was a large amount of variation between individual practices. The similarity of resistance profiles between MDR faecal and environmental samples is suggestive of cross contamination between animals and their environment. Particularly of concern is the relatively frequent isolation of bacteria with resistance to all seven antimicrobials tested. Other studies of companion animals have found MDR *E. coli* at rates of: 48% in hospitalised dogs and 32% of stray dogs from Korea (Nam et al., 2010); 15% of community based dogs in the UK (Wedley et al., 2011) and 9% of animals due to be hospitalised in the USA (Hamilton et al., 2013). The level of MDR found by Wedley et al (2011) in community dogs in the UK using similar methods is approximately half that found in this study. Levels of resistance would be expected to be higher in a hospitalised setting than a community setting. It has been shown in several studies that MDR organisms are likely to be isolated at a higher rate from hospitalised or sick animals compared to non-hospitalised or healthy animals (Nam et al., 2010, Gibson et al., 2011, Sun et al., 2010) and humans (Cardoso et al., 2012). It is also worth noting that the majority of MDR isolates identified by Wedley et al (2011) were

resistant to ampicillin, tetracycline and potentiated sulphonamides, with resistance to CAPA occurring infrequently. MDR isolates in this study frequently included resistance to CAPA and fluoroquinolones.

Resistance to extended spectrum cephaloporins (ESC's) was detected at high rates in this study but were again variable by practice. Studies of companion animals have found resistance to ESC's at: 60.5% and 30.2% in hospitalised pets treated and not treated with antimicrobials respectively in China (Lei et al., 2010); 13% in dogs and cats from the community and nursing homes in Switzerland (Gandolfi-Decristophoris et al., 2013) and 2.4% and 3.9% from stray and hospitalised dogs respectively in Korea (Nam et al., 2010).

The confirmed prevalence of ESBL-producers in this study was 22%. ESBL production has been detected at prevalences ranging from 54.5% and 24.5% in sick and healthy animals respectively in veterinary hospitals in China (Sun et al., 2010) and 33.3% in Korean veterinary hospitals (So et al., 2012) to 5% of faecal samples from cats and dogs in shelters in Germany (Franiek et al., 2012); 13.2% healthy dogs in Portugal (Belas, 2014) and 16% of faecal samples from healthy cats and dogs in Tunisia (Ben Sallem et al., 2013). ESBL-producers have also been isolated at lower levels from clinical urinary isolates in the USA (Shaheen et al., 2011, O'Keefe et al., 2010) and Switzerland (Huber et al., 2013). There is clearly variation in ESBL-producer prevalence by location and setting, with hospitalised animals being associated with increased isolation rates, though this study demonstrates that some hospitals have low levels compared to others and there are likely to be hospital level factors which have an important influence. The prevalence of ESBL production by phenotypic findings only led to an underestimation in several practices in this study due to masking by AmpC producer phenotypes. This was particularly the case in the practice which had high levels of AmpC production demonstrating the importance of production of AmpC not only in the resistance it confers but also in its ability to make the detection of ESBL production more difficult.

The most commonly identified bla_{ESBL} in this study was $bla_{CTX\text{-}M\text{-}15}$. While other studies have identified $bla_{CTX\text{-}M\text{-}15}$ in companion animals at low levels (Dierikx et al., 2012, Huber et al., 2013, So et al., 2012, Sun et al., 2010) it has been more common for CTX-M-1 production to be detected (Costa et al., 2008, Dierikx et al., 2012). However a recent study in healthy dogs found $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}1}$ to be the second and third most prevalent types with $bla_{CTX\text{-}M\text{-}32}$ being the most commonly reported (Belas, 2014). High levels of $bla_{CTX\text{-}M\text{-}15}$ are more associated with studies of humans however in Europe $bla_{CTX\text{-}M\text{-}15}$ is the second most

commonly isolated ESBL gene from companion animals (Ewers et al., 2012), this is not surprising given the close contact between humans and companion animals (Westgarth et al., 2008) and may reflect transmission into the companion animal population. It is worth remembering that a number of bla_{ESBL} positive samples in this study did not return a sequencing result and therefore the true prevalence of CTX-M-1 production (and other CTX-M type ESBLs) may be higher.

TEM-158 was the second most prevalent ESBL produced in isolates in this study, it was first detected from a faecal sample from an intensive care patient in France and demonstrates both ESBL and inhibitor resistant TEM (IRT) characteristics (Robin et al., 2007) and due to this is often referred to as a complex mutant TEM (CMT). Other studies have identified TEM-158 production in urinary *E. coli* isolates in the human community in Morocco (Barguigua et al., 2013) and clinical isolates from human patients in Kenya (Kiiru et al., 2012). Evolution of TEM-158 has been shown to occur as a result of antimicrobial therapy in humans (Jacquier et al., 2013). Occurrence of this beta lactamase at relatively high levels in one hospital is of concern given the resistance it confers and the fact that its inhibitor resistant characteristics can make identification difficult. We are not aware of any other studies in companion animals which have identified the presence of $bla_{TEM-158}$ and as the only studies reporting this previously were from humans it may be that inter-species transmission has occurred.

Contamination of the human hospital environment with ESBL-producing *Enterobacteriaceae* originating from patients has been reported, though *Klebsiella* species were reported to be more prolific contaminants compared to *E. coli* (Guet-Revillet et al., 2012). Furthermore in a companion animal veterinary hospital in Canada environmental contamination with *E. coli* was detected in 92% and CMY-2 producing *E. coli* in 9% of the hospitals sampled (Murphy et al., 2010). This study concurs with these results and confirms that *E. coli* with important resistance phenotypes are present in the practice environment and may act as a source of infection and reservoir of resistance determinants. To our knowledge there have been no other reports of *E. coli* contamination of the veterinary hospital environment in the UK.

For most practices the outside walking area where dogs were taken to urinate and defecate (with faeces being picked up and disposed of by practice staff) was most associated with isolation of resistant bacteria. A high level of contamination of this area with *E. coli* may be expected due to a combination of a large number of dogs from different wards mixing on

the area and the obvious difficulty with disinfection of the surface. Practices 1, 2 and 5 all had grassy walking areas and practice 4 had a wood chipping surface. Only practice 3 had a concrete surface which would be easily disinfected, and interestingly the isolation rate from the outside in this practice was similar to other areas within the practice. Internal floors were the area associated with the second highest isolation of important resistance phenotypes, tables and keyboards were associated with a low isolation rate in most practices which may be due to the ease of disinfection of tables and the lack of direct animal contact with keyboards.

The fact that data were collected in a different six week period for each practice means that practice environments cannot be compared temporally, however the changing magnitude over time gives a crude picture of the situation in each practice. It should be remembered that practices were not sampled in consecutive six week blocks and the gaps between sampling periods varied. The picture is suggestive of a continually low level of contamination in practices two and five, a low level of contamination in practices three and four with spikes in the isolation rate suggesting short term contamination of the environment and a moderate persistent contamination of the environment in practice one. Longer term sampling of the hospital environment may reveal differing patterns.

The vast majority of isolates were confirmed as *E. coli.* There was very little change in the results for important resistance phenotypes once non *E. coli* confirmed isolates were excluded. The non *E. coli* confirmed isolates were included in overall analysis as their numbers are small and they may be clinically relevant.

Due to some practices contributing more samples than others and the repeated sampling within dogs, prevalence estimates adjusted for this clustering were calculated. These showed quite different estimates for some outcomes suggesting substantial clustering within dogs, as well as practice.

One problem encountered in this study was the collection of sufficient numbers of samples from practices. Some weeks some practices returned fewer samples than others due to low throughput of animals and a lack of hospitalised patients. In addition faecal samples collected were those naturally voided by the animal, due to ethical implications, however as many animals (particularly non-ambulatory animals) will not defecate when hospitalised over short periods they may be under-represented. Several samples were also lost due to inadequate labelling. Ideally faecal sample collection would be carried out consistently by

the same member of staff with a minimal amount of time before processing. In practice this was not possible and there was therefore some variation in time elapsed between sample collection and processing which was never more than three days. Bags were used to collect faeces, therefore faecal consistency may have had an impact upon sample collection with diarrhoeic dogs underrepresented. We included cats in this study as previous estimates of resistance in cats is lacking, however sample numbers were low due to lower hospitalisation rates for cats.

Environmental sample collection was designed to fit around practice routine in order to minimise any disruption to practices, in some cases cleaning may have taken place a short time before sample collection which would be expected to lower the chances of *E. coli* isolation. Isolation rate from inhibiton zones was low compared to the isolation rates from agar containing cefotaxime/ceftazidime, though this is not directly comparable it demonstrates the value of an enrichment step when bacteria are present in low numbers in projects like this.

In summary, this project demonstrates the presence of important AMR phenotypes and genes, including the detection of production of the IRT TEM-158 in both commensal and environmental *E. coli* in the practice environment. Given the demonstration of the presence of these phenotypes and genes in pathogenic isolates (Huber et al., 2013, O'Keefe et al., 2010, Steen and Webb, 2007, Timofte et al., 2011), there is clearly potential for these to limit treatment options for important infections in companion animals. The close contact between pets and their owners (Westgarth et al., 2008) indicates a potential public health issue with the zoonotic transmission of resistant organisms, or resistance determinants a real concern. The contamination of the practice environment with these bacteria has also been shown in this and other studies, which is of concern as these environmental bacteria may act as a source of infection for new animals in the environment and a source of resistance genes for exchange with previously susceptible *E. coli* with public health implications for staff and clients in the practice environment. More work is required to look at the transfer of resistant bacteria between companion animals and their practice environment and how this contributes to clinical infections with resistant organisms and to determine the risk factors associated with both contamination of the practice environment and the carriage of resistant commensal *E. coli.*

Chapter 3

Risk factors for carriage of antimicrobial resistant *Escherichia Coli* in hospitalised companion animals.

INTRODUCTION

Antimicrobial resistance is an important problem facing human and veterinary medicine. It leads to increased morbidity and mortality among patients by limiting treatment options, increased cost and length of treatment and is a potential threat to public health. Resistance among *E. coli* is of particular interest given the ubiquitous nature of these bacteria and their potential to act as a reservoir for resistance genes. Among animals and humans *E. coli* are a common commensal organism in the gastro-intestinal tract (GIT) and they are able to readily accept plasmids, thus horizontal gene exchange is common meaning that spread of resistance genes can occur rapidly and widely (Hart et al., 2006). In addition to this *E. coli* can be opportunistic pathogens themselves and have been isolated from a variety of infections in companion animals including urinary tract infections (Huber et al., 2013, O'Keefe et al., 2010); wounds (Steen and Webb, 2007) and bile in a case of cholangiohepatitis (Timofte et al., 2011).

Beta-lactam antimicrobials are commonly used in UK companion animal practice (Radford et al., 2011, Mateus et al., 2011) and resistance to this class of drug is principally conferred by production of beta-lactamase enzymes which hydrolyse the beta-lactam ring and confer resistance to the penicillins and first generation cephalosporins. Over the last few decades the use of third and fourth generation cephalosporins to treat resistant infections has driven the evolution and emergence of extended spectrum beta lactamases (ESBL's) which confer resistance to third and fourth generation cephalosporins. Resistance to later generation cephalosporins can also be conferred by the production of the cephamycinase AmpC, which unlike ESBL's is resistant to beta lactamase inhibitors (Pfeifer et al., 2010).

ESBL-producing bacteria are widely reported in humans in both community and hospital acquired infections involving *E. coli* (Reinert et al., 2007, Pitout and Laupland, 2008) and they constitute a major problem in human medicine. Risk factors for human carriage of ESBL-producing bacteria have been identified as: length of hospitalisation; severity of illness; urinary catheterisation; length of stay in intensive care unit; mechanical ventilation;

multiple co-morbid conditions; non-home residence and previous treatment with antibiotics (Pitout and Laupland, 2008, Hayakawa et al., 2013, Jacoby and Munoz-Price, 2005). A number of these risk factors are potentially applicable to hospitalised companion animals and some have been shown to be risk factors in hospitalised horses (Maddox et al., 2011).

Multi-drug resistant (MDR), ESBL-producing and AmpC-producing *E. coli* have been isolated from companion animals in the UK (Wedley et al., 2011), Tunisia (Ben Sallem et al., 2013), South Korea (Nam et al., 2010), China (Sun et al., 2010), the USA (O'Keefe et al., 2010), Switzerland (Huber et al., 2013) and Germany (Franiek et al., 2012). Thus indicating that they are widely found in companion animal populations. Prior exposure to antimicrobials, hospitalisation and lower age have all been implicated as risk factors in the isolation of MDR, ESBL-producing and AmpC-producing *E. coli* from companion animals (Hernandez et al., 2014, Sun et al., 2010, Nam et al., 2010, Moreno et al., 2008, Gibson et al., 2011, Belas, 2014). However, there have been no detailed studies examining carriage of resistance in companion animal referral hospitals in the UK, where improvements in pet care and longevity, as well as in the treatment options available, result in an increasing number of pets that may be at risk of nosocomial colonisation and infection with these organisms. The aim of this study was to determine the risk factors for carriage of important resistance phenotypes by faecal commensal *E. coli* in veterinary referral practices.

MATERIALS AND METHODS

Data and sample collection

Faecal and environmental samples were collected from five referral practices in Northwest England and processed as described previously (chapter 2). Practices 1 and 4 were referral only and 2, 3 and 5 saw first opinion cases in addition to referrals. In brief all dogs and cats hospitalised overnight in the practices were sampled daily from admission until discharge. Samples were collected and labelled (with name, ID number, date of collection and where the animal was hospitalised at the time of sampling). Standard bacteriological techniques were used to determine resistance to important antimicrobials and ESBL/AmpC production. The presence of genes encoding for ESBL or AmpC production was confirmed using PCR and sequencing (see methods in chapter two).

Data for each animal providing a faecal sample were collected from practices. Four out of five practices elected for data to be collected manually from the practice computer system. The author collected all data from these practices. One practice (practice two) elected to provide data themselves using a set group of questions in order to standardise the data collected. Data collected from practices were: species; date of hospitalisation; age; gender and neutered status; breed (cats were included with small breed dogs); whether the animal had been hospitalised in the last three months, procedures carried out in current hospital stay (for example radiography, ultrasound, MRI or CT scan) and antimicrobials used both in the three months prior to hospitalisation and whilst being hospitalised up to the date of the sample. Antimicrobial use was assessed as use in the previous 24 hours, 48 hours, 7 days and 3 months. Other data considered included the presence of resistance in environmental samples taken from the practices at the same time as the animal samples. Data were inputted into a database (Microsoft Excel) along with bacteriology and PCR results for each sample.

Statistical methods

The binary outcomes of interest for each sample were the presence or absence of an *E. coli* isolate with; clavulanic acid potentiated amoxycillin (CAPA) resistance, ciprofloxacin resistance, third generation cephalosporin resistance (TGCR), multidrug resistance to 3 or more drug classes (MDR), bla_{AmpC} (CITM) detection and bla_{ESBL} (CTX-M, TEM or SHV) detection.

Due to repeated measures, data were clustered within dogs and therefore factors affecting the occurrence of antimicrobial resistant *E. coli* were examined in multilevel logistic regression models. Within animal clustering was accounted for by inclusion of animal as a random intercept in all models. Initial univariable screening was performed and all variables with p value of <0.25 were considered in a multivariable model.

The correlation of all the exposures was assessed using correlations coefficients and for any correlated variables (correlation coefficient >0.8) the one selected to be included in the model was the one with the lowest p value. The days hospitalised and the age of the animal were the only continuous variables; the functional form of these variables with respect to each outcome was assessed using generalised additive models (GAM). The GAM models were fitted using cubic spline smoothers in the S-Plus software package (S-plus

2000, Mathsoft Inc). The functional forms of the relationships were then used to inform the polynomial fits in the multivariable logistic regression models, which were then tested for significance (see figures 1a-f and 2a-f, appendix V).

The final multivariable models for each outcome of interest were constructed using a manual backward stepwise procedure where variables with a Wald P-value <0.05 were retained in the model. Confounding was considered if elimination of any one variable effected a change of more than 25% in the coefficient of another variable. First order interaction terms were tested for biologically plausible variables remaining in the final models. Finally all variables with P<0.25 on univariable analysis and anything considered a priori to be of importance, were checked in the final model for significance

Data were analysed using the MLwiN statistical software package (MLwiN Version 2.3 Centre for Multilevel Modelling, University of Bristol). Univariable and multivariable calculations were performed using penalised quasi-likelihood estimates (2^{nd} order PQL except in the case of AmpC outcome where it was necessary to use 1^{st} order).

RESULTS

Samples

Of the 333 samples in chapter two 13 were discarded from risk factor analysis due to inadequate labelling or data collection. In total 320 samples from 200 animals remained for risk factor analysis. Cats provided 24 (7.5%) samples and dogs provided 296 (92.5%) samples. Table 1 shows the number and prevalence of resistance for each of the outcomes considered.

The average age of animals in the study was 6.1 years ranging from three months to eighteen years. In 111/320 (34.7%) samples the animal providing the sample had been hospitalised in the last three months (not including the current period of hospitalisation). The average number of days an animal had been hospitalised for when samples were collected was 3.5 days, ranging from 0 (collected on day of admittance) to 20 days. Table 2 shows the numbers of samples in each group for categorical variables and previous exposures to different antimicrobials are shown in table 3.

Table 1: Number and sample level prevalence (with 95% confidence intervals) from 320 samples from 200 animals used in analysis. CAPA= clavulanic acid potentiated amoxycillin, MDR = multidrug resistance to three or more classes in *E. coli*.

Resistance outcomes considered	**Numbers of resistant samples (n=320)**	**Prevalence**	**Lower and Upper 95% CI**
CAPA resistance	95	29.7%	24.7, 34.7
Ciprofloxacin resistance	79	24.7%	20.0, 29.4
MDR	86	26.9%	22.0, 31.7
Resistance to one or more TGC's	122	38.1%	32.8, 43.4
ESBL producer*	73	22.8%	18.2, 27.4
AmpC (CITM confirmed on PCR)	71	22.2%	17.6, 26.7

***** Confirmed by PCR, phenotype or sequencing

Table 2: Categorical variables with number and percentage of samples exposed in faecal samples (n=320) collected from 200 animals. Where the variable status could not be accurately determined it was recorded as unknown.

Exposure variable	Number	%
Breed		
Small breed <10kg (including cats)	94	29.4%
Medium breed dog 10-20kg	113	35.3%
Large breed dog >20kg	96	30.0%
Unknown	17	5.3%
Gender		
Male	113	35.3%
Male neutered	85	26.6%
Female	39	12.2%
Female neutered	80	25.0%
Case type		
Neurosurgery	56	17.5%
Orthopaedic	95	30.0%
Soft tissue surgery	52	16.2%
Medicine	85	26.6%
Other (unidentified)	32	10.0%
X-ray	70	21.9%
Ultrasound scan	39	12.2%
MRI or CT scan	78	24.4%
Environmental MDR *E. coli* isolated in same week	134	41.9%
Environmental ESBL-producing *E. coli* isolated in same week	134	41.9%
Environmental AmpC-producing *E. coli* isolated in same week	164	51.3%

NB some samples had missing data for some variables hence they do not always add to 320 (or 100%)

Table 3: Prevalence of exposures to different antimicrobial agents at any point in the three months prior to sample collection in faecal samples (n=320) collected from 200 animals. It should be noted that these have not been adjusted for clustering within animal and simply reflect sample level prevalence.

Antimicrobial	**Number**	**% (+/- 95% CI)**
Amoxycillin	13	4.1 (1.9-6.2)
CAPA	120	37.5 (32.2-42.8)
Cephalexin	27	8.4 (5.4-11.5)
Cefuroxime	52	16.3 (12.2-20.3)
Metronidazole	37	11.6 (8.1-15.1)
Fluoroquinolones	18	5.6 (3.1-8.1)
Clindamycin	13	4.1 (1.9-6.2)
Any antimicrobial	185	57.8 (52.4-63.2)

Univariable Analysis

Univariable analysis showed multiple significant ($p<0.05$) associations. A summary of these is shown in table 4. Further details are shown in appendix IV tables 1 to 6. For all outcomes increasing number of days hospitalised was significantly associated with increasing risk and antimicrobial use was also significant although which antimicrobials varied with the outcome considered. The type of case and the practice were was also significant as was resistance identified in environmental samples.

The GAM's (appendix V) showed that the days hospitalised demonstrated a significantly non-linear relationship ($p>0.05$) with many of the resistance outcomes being considered. The risk appeared to increase up until approximately 10 days and then there was either no further increase or a decrease in risk, however data points after 10 days were sparse. In the final model this variable was explored as linear, as a quadratic polynomial and as a piecewise fit allowing risk to increase up to 10 days with no further change in risk after this time. Age had mostly a linear relationship except for CAPA and AmpC resistance and quadratic terms were also tested for these outcomes

Significant correlation was found between some variables. This was primarily between variables where the same antimicrobial had been given in different time frames (e.g. receiving metronidazole in the last 7 days was strongly correlated with receiving

metronidazole in the last 48 hours). Kennelling area was correlated with species, both of which are to be expected.

Table 4: Summary of significant associations with the 6 outcomes (P<0.05) on multilevel univariable analysis of 320 faecal samples from 200 dogs and cats in 5 referral practices.

Outcome	**Significant associations on univariable analysis:**
CAPA Resistance	Practice, Hospitalisation length, Environment, Antimicrobial use (various), Case type and X-ray
Ciprofloxacin Resistance	Practice, Hospitalisation length, Environment, Antimicrobial use (various) and Case type
MDR	Practice, Hospitalisation length, Environment, Antimicrobial use (various), Breed and Case type
TGCR	Practice, Hospitalisation length, Environment, Antimicrobial use (various) and Case type
ESBL producer	Practice, Breed, Gender, Antimicrobial use (various)
AmpC (confirmed bla_{CITM})	Practice, Hospitalisation length, Environment, Antimicrobial use (various), Case type and Xray

Multivariable results

The final multivariable results for each of the six outcomes are shown in tables 5 and 6. Practice was a significant risk factor for all outcomes apart from CAPA resistance with practice five having generally lower levels of risk compared to other practices. Practices one, two and three were associated with higher risk for third generation cephalosporin and ciprofloxacin resistance, with practice 1 and 3 having increased risk of ESBL-producing *E. coli* and practices 1 and 2 with AmpC-producing *E. coli*. Case type was significant for some resistance outcomes with neurosurgery cases being associated with increased risk for CAPA, ciprofloxacin and AmpC resistance outcomes and soft tissue surgery cases associated with increased risk for CAPA and ciprofloxacin resistance. Duration of hospitalisation was associated with increased risk for some outcomes. The best fit was provided by inclusion as a piecewise fit allowing risk to increase up to 10 days. Breed was also a significant risk factor for several outcomes with a consistent pattern of small breeds (which included cats) being associated with lower risk than medium and large breeds. Receiving an X-ray was found to be associated with reduced risk of AmpC *E. coli* isolation and receiving an MRI or CT scan was shown to be associated with a higher risk of isolating MDR organisms. Every resistance outcome was positively associated with the use of at least one antimicrobial.

Fluoroquinolone use was strongly associated with the outcomes of ciprofloxacin resistance, 3GCR and ESBL-producing *E. coli*. Use of CAPA was associated with CAPA resistance, 3GCR and the presence of ESBL and AmpC producers. Cephalexin was associated with CAPA resistance and clindamycin with CAPA resistance, 3GCR and AmpC producers. Metronidazole exposure in the last 3 months appeared to be associated with MDR *E. coli* faecal isolation.

Isolation of resistant *E. coli* in the environment in the same week as sampling was assessed for association with the outcomes. There was correlation between environmental MDR *E. coli* and environmental AmpC-producing *E. coli* (r = 0. 7). In addition practice was correlated with both environmental MDR *E. coli* (r = -0.6) and environmental AmpC-producing *E. coli* (r = -0.695).

With practice ID included in the models only isolation of environmental AmpC-producing E. coli was significantly associated with the outcome of CAPA resistance. Due to the correlation between practice and environmental outcomes the effect of removal of practice was assessed. This resulted in environmental AmpC-producing E. coli becoming significant for 3GCR (OR=2.4 (1.6-4.9)), ciprofloxacin resistance (OR = 5.2 (2.0-13.1)) and AmpC producing E. coli (OR = 7.3 (3.2-16.8)).

In the multivariable models for CAPA, ESBL and 3GCR the variance for the random effect was negligible, suggesting these models explained the within dog clustering. There was some remaining clustering of outcomes in animals for ciprofloxacin resistance, MDR and AmpC-producing *E. coli*.

Table 5. Results of multivariable multilevel analysis for the outcomes of resistance to CAPA and ciprofloxacin and MDR *E. coli* in 320 faecal samples from 200 dogs and cats in 5 hospitals in North West UK.

Variable	CAPA resistance			Ciprofloxacin resistance			MDR		
	Odds ratio	**95% CI**	**P-value**	**Odds ratio**	**95% CI**	**P-value**	**Odds ratio**	**95% CI**	**P-value**
Hospital									
1				REF			3.8	1.3-11.2	0.0
2				0.21	0.05-0.98	0.047	5.0	1.3-18.3	0.02
3				0.12	0.04-0.3	<0.001	4.5	1.5-13.7	0.009
4				0.15	0.03-0.7	0.02	2.2	0.5-9.5	0.3
5				0.07	0.02-0.3	<0.001	REF		
Case Type									
Medicine	1.8	0.7-4.3	0.19	REF					
Neurosurgery	5.5	2.2-13.8	<0.001	7.3	2.3-23.4	0.001			
Soft tissue surgery	3.6	1.4-9.3	0.0078	3.8	1.2-11.8	0.02			
Orthopaedic	REF			3.0	0.9-10.4	0.08			
Other (unidentified)	4.6	1.6-12.8	0.004	3.1	0.8 -12.6	0.1			
Number days hospitalised*				1.15	1.02-1.3	0.02			
Breed									
Small	REF								
Medium	3.1	1.5-6.7	0.003						
Large	1.7	0.7-3.9	0.25						
Unknown	7.9	2.3 -27.2	0.001						
Environmental sample with AmpC that week	3.7	2.0-6.8	<0.001						
MRI or CT scan							2.2	1.2-4.2	0.01
Amoxycillin in the last 3 months							6.1	1.7-22.1	0.006
CAPA in the last 7 days	2.8	1.5-5.2	0.001						
CAPA in the last 3 months				2.1	1.01-4.6	0.05			
Fluoroquinolone in the last 3 months				8.6	2.2-34.1	0.002			
Cephalexin in the last 7 days	3.0	1.0-8.6	0.04						
Clindamycin in the last 3 months	15.8	3.4-72.9	0.001						
Metronidazole in the last 3 months							2.3	1.1-5.2	0.03
Variance (standard error)	0.0			0.8 (0.6)			0.2 (0.3)		

For antibiotic exposures the reference category is not receiving the antibiotic in the specified time period
95% CI = 95% confidence intervals; *P* values are from the Wald chi-squared test
*Piecewise fit up to 10 days

Table 6: Results of multivariable multilevel analysis for the outcomes of resistance to any third generation cephalosporin, ESBL production and AmpC production in faecal *E. coli* in 320 faecal samples from 200 dogs and cats in 5 hospitals in North West UK.

Variable	**Any resistance to third generation cephalosporins**			**ESBL producer**			**AmpC producer**		
	Odds ratio	**95% CI**	**P-value**	**Odds ratio**	**95% CI**	**P-value**	**Odds ratio**	**95% CI**	**P-value**
Hospital									
1	10.7	3.4-34.3	<0.001	14.1	1.8-108.2	0.011	27.4	5.2-144.2	<0.001
2	5.4	1.3-21.4	0.02	4.1	0.4-42.3	0.24	13.6	1.9-95.7	0.009
3	7.7	2.3-25.9	0.001	24.4	3.1-192.5	0.002	2.8	0.4-18.6	0.27
4	3.5	0.9-13.3	0.07	8.3	0.95-71.9	0.06	4.4	0.6-35.0	0.16
5	REF			REF			REF		
Case Type									
Medicine							REF		
Neurosurgery							3.9	1.2-12.3	0.020
Soft tissue surgery							2.1	0.6-6.9	0.23
Orthopaedic							3.2	0.9-10.3	0.06
							8.1	2.4-27.6	0.001
Number days hospitalised*	1.1	1.02-1.23	0.02				1.2	1.1-1.4	0.003
Breed									
Small	REF			REF					
Medium	2.2	1.1-4.5	0.03	2.8	1.2-6.2	0.01			
Large	2.9	1.4-6.0	0.006	3.4	1.4-8.0	0.005			
Other (unidentified)	3.2	1.0-10.4	0.05	5.6	1.5-20.5	0.009			
Xray							0.2	0.07-0.60	0.004
CAPA in the last 7 days	2.5	1.3-4.8	0.004	2.0	1.1-3.8	0.03	2.7	1.1-6.3	0.03
CAPA in the last 3 months									
Fluoroquinolone in the last 24 hours				9.4	2.0-45.2	0.005			
Fluoroquinolone in the last 7 days	5	1.2-21.2	0.03						
Clindamycin in the last 3 months	9.7	2.3-41.8	0.002				8.4	1.5-46.8	0.01
Variance (standard error)	0.0			0.0			0.7(0.5)		

For antibiotic exposures the reference category is not receiving the antibiotic in the specified time period
95% CI = 95% confidence intervals; *P* values are from the Wald chi-squared test
*Piecewise fit up to 10 days

DISCUSSION

This study is one of the first to investigate risk factors for carriage of antimicrobial resistance in companion hospital referral hospitals in the UK. Risk factors identified for the different resistance outcomes were similar in many cases. This is not unexpected as some resistance outcomes are very similar to one another, for example any bacteria producing an AmpC or ESBL is expected to be resistant to third generation cephalosporins. In addition resistance genes maybe co-located on the same plasmids leading to correlation of outcomes.

More than half of samples in this study were from animals which had been exposed to antimicrobials in the previous three months. High levels of exposure to antimicrobials are expected in a population of animals from a referral hospital as a number of cases will have a history of illness which has resulted in prior use of antimicrobials, as well as the use of antimicrobials once hospitalised. This represents exposure of the animal concerned up to the day before sampling only and is not representative of all the antimicrobial exposures the animal may have subsequently received. It is also possible that some exposure to antimicrobials in the prior three months may have not been recorded as in some cases the referral history was incomplete; it is therefore possible that the exposure prior to hospitalisation has been underestimated. It should also be remembered that the prevalence figures are sample level rather than animal level and animals hospitalised for longer (and therefore contributing more samples) may be more likely to be exposed to antimicrobials. However this clustering was accounted for in the multilevel modelling used to determine risk factors.

The most common antimicrobial exposure was CAPA, followed by cephalosporins and metronidazole. This is in agreement with other studies of antimicrobial use in veterinary practice (Mateus et al., 2011, Radford et al., 2011). Our study combined with these studies show there is a high exposure of companion animals to CAPA in UK veterinary practice, however the use of important antimicrobials such as fluoroquinolones and third/fourth generation cephalosporins was low in this study. The use of metronidazole was significantly associated with MDR in *E. coli* which is an interesting association given that metronidazole is not expected to have in vivo activity against *E. coli* and is therefore unlikely to exert a direct selection pressure. However this has been reported in hospitalised animals previously. In a case control study in an Australian veterinary hospital treatment with metronidazole was found to be a significant risk factor, increasing the odds

for MDR *E. coli* isolation by a factor of ten (Gibson et al., 2011). In this study we found a lower level of risk, however the same association in two studies of different design is suggestive that metronidazole is a true risk factor for MDR *E. coli* isolation. It may be that metronidazole is indirectly favouring the proliferation of *E. coli* generally in the gut by removing part of the resident microflora, which is metronidazole-sensitive and allowing *E. coli* to take its place. If MDR *E. coli* were already present this could result in an increase in numbers and an increased likelihood of detection. This could have a significant impact as metronidazole is generally viewed as a 'safe' antimicrobial to use with low levels of resistance found in obligate anaerobes from samples from dogs (Lawhon et al., 2013). It may be that this needs to be revised due to its indirect effects on populations of *E. coli* (including resistant) in the gut. Further work is required to determine the effect of different agents on the change of quantity of resistance in susceptible populations of *E. coli.*

Similarly to metronidazole the use of clindamycin would not be expected to exert a direct selection pressure on *E. coli*. High levels of clindamycin resistance have been found in other bacteria in dog faeces (Cinquepalmi et al., 2013) suggesting that the use of clindamycin may have an effect on the faecal microflora sufficient to drive the generation of resistance. It may be that there are indirect effects on *E. coli* which favour the dissemination of resistance. It is worth noting that estimates for the effect of clindamycin had wide confidence intervals, probably as a result of relatively infrequent exposure to this antimicrobial.

Use of fluoroquinolones was associated with several resistance outcomes. It is not unexpected that exposure to a fluoroquinolone would be associated with ciprofloxacin resistance as it provides a direct selection pressure. However use was also associated with general TGCR and ESBL-producer isolation. High levels of fluoroquinolone resistance have been found among ESBL-producing bacteria in humans (Balkhed et al., 2013) and use of fluoroquinolones has been identified as a risk factor for ESBL isolation (Soraas et al., 2013, Kaya et al., 2013). In a case control study of animals those exposed to enrofloxacin were more likely to have ESBL-producers isolated (Moreno et al., 2008). This link between fluoroquinolone and ESBL-producer isolation is likely to be due to co-location of bla_{ESBL} and fluoroquinolone resistance genes on the same plasmids (Hawkey and Jones, 2009). Such plasmids would be expected to confer advantageous resistance to bacteria in a clinical environment.

Compared to other antimicrobials, both in first opinion practice (Mateus et al., 2011, Radford et al., 2011) and in this study, fluoroquinolones are used relatively infrequently though the results from this study suggest that they can exert a potent selection pressure. The fact that their use is not just associated with fluoroquinolone resistance but also 3GCR is of great concern as these two classes of antimicrobial are important in human medicine (WHO, 2011). The selection for co-resistance to different antimicrobial classes has important implications, resistance to antimicrobials does not necessarily require the direct exposure to those particular antimicrobials. This is demonstrated by the high level of 3GCR seen in this study whilst no exposure to these was recorded in animals providing samples, although exposure to first and second generation cephalosporins was recorded. The results of this study highlight the particular need for cautious and judicious use of fluoroquinolones in both first opinion and referral veterinary practice.

Use of cephalexin was associated with CAPA resistance and is not unexpected given that they are both beta-lactam antimicrobials; it may be that cephalexin use drives the selection of resistance mechanisms like AmpC production which would confer resistance to CAPA though no significant association was detected in this study. More interesting is the fact that cephalosporin use was not associated with 3GCR or ESBL production. The majority of cephalosporin use in this study was cefuroxime which was always given intravenously perioperatively, either before or during general anaesthesia prior to surgery, rather than a longer course of medication, which might be expected to exert different selection pressures for resistance. It may also be that not enough animals were exposed to cephalosporins in this study making the power to detect associations low. The use of cephalosporins has been associated with: fluoroquinolone resistance in humans (Batard et al., 2013); ESBL-producing bacteria in pigs (Agerso and Aarestrup, 2013) and MDR *E. coli* from hospitalised dogs (Gibson et al., 2011). None of these were found in this study although it is likely that use of any cephalosporin will create, or add to, an overall selection pressure for mechanisms conferring resistance to third generation cephalosporins.

CAPA use was associated with every resistance outcome except MDR. Association with CAPA resistance and AmpC production is expected as CAPA use would exert a direct selection pressure for these resistances. Driving the generation of AmpC mediated resistance would also have implications for 3GCR. Use of CAPA has been shown to be associated with an increase in levels of ciprofloxacin resistance in human hospitals and was implicated as a significant driver in levels of ciprofloxacin resistance overall (Cuevas et al.,

2011). Given that exposure of animals to CAPA is frequent in both this study and first opinion practice (Mateus et al., 2011, Radford et al., 2011) the wide range of resistance associated with its use is of great concern. More than any other antimicrobial CAPA has the potential to generate resistance simply due to its widespread use in practice.

Practice remained a significant risk factor in this study after allowing for other variables, with a reasonably consistent pattern. Practice one was associated with higher levels of risk for third generation cephalosporin resistance (3GCR), AmpC production and ciprofloxacin resistance whilst practice three was associated with higher risk for ESBL production and MDR *E. coli*. Practice five was associated with low risk for all outcomes where practice was a significant risk factor. This is broadly in agreement with the prevalence of the respective resistance outcomes detailed in chapter two. This suggests some other unmeasured variable(s) at the practice level are important. It was impossible to account for all the differences between practices within our model, for example practice size and either number of animals hospitalised or staff numbers could have an effect. Case numbers were not available for all practices and using staffing levels was not considered accurate due to practices having different levels of part time staff and variable numbers of students. Case load could also be an influence with some practices receiving predominantly routine orthopaedic cases, some seeing first opinion as well as referral cases and some seeing a wide range of tertiary referrals. In human medicine gram negative isolates from ICU departments within hospitals in Europe and the USA have been shown to have lower antimicrobial susceptibilities compared to other areas in the same hospitals (Sader et al., 2014), it may be that the differences seen between departments within hospitals are due to similar factors to those differences seen between different hospitals in this study. ICU departments are likely to see the most critically ill patients with multiple co-morbid conditions. Human spinal cord injury patients have been shown to be at higher risk of isolation of resistant bacteria in a hospitalised setting as opposed to the community (Yoon et al., 2014) which may reflect similar factors influencing practice level risk seen in this study.

Hospitalisation was a significant risk factor for the presence of ciprofloxacin resistance, 3GCR and AmpC-production, and of borderline significance (P=0.05) for ESBL production, with odds of resistance increasing between 1.1 and 1.2 per additional day hospitalised. Thus the risk of ciprofloxacin resistance, TGCR and AmpC production at day 10 of hospitalisation is 4.0, 2.6 and 6.2 respectively compared to the day of admission. Increased

duration of stay in hospital has been associated with increased risk of isolation of ESBL-producers in humans (Tham et al., 2013, Ko et al., 2013). Studies of MDR *E. coli* in hospitalised horses have shown an increased burden of MDR organisms over time hospitalised (Williams et al., 2013, Maddox et al., 2011). In companion animals hospitalisation for more than six days has been implicated as a risk factor for the isolation of MDR organisms in a case-control study in an Australian veterinary hospital (Gibson et al., 2011). Studies of hospitalised companion animals tend to show higher prevalence of antimicrobial resistance compared to community studies in similar areas (Nam et al., 2010, So et al., 2012, Sun et al., 2010), suggesting that there are factors involved in hospitalisation which increase risk of AMR acquisition. The acquisition of a nosocomial MDR *E. coli* urinary infection has been demonstrated in a cat (Hernandez et al., 2014) which demonstrates the consequences of increased risk of acquisition of AMR bacteria in hospitalised patients. This increase in risk of hospitalisation could reflect increased likelihood and duration of exposure to antimicrobials, although individual exposure was included we were unable to estimate total antimicrobial usage in the hospital at the time and this may also represent a source of exposure to the hospitalised animal. Increased duration of hospitalisation also represents increased duration of exposure to an environment contaminated with resistant bacteria, and of exposure to other patients which may be carriers of resistant bacteria. Furthermore it may also reflect more debilitated patients which are likely to hospitalised for longer periods. In reality it is likely to be a combination of multiple factors however if the duration of hospitalisation can be minimised, without compromising patient welfare, then this could have an impact on the rates of carriage of some antimicrobial resistance.

Environmental AmpC-producing bacteria detection in the same week as sample collection was associated with an increase in risk for CAPA resistance in faecal *E. coli*, after removing practice from the final models with the same observation for ciprofloxacin and AmpC producer outcomes. AmpC-producing *E. coli* have been isolated from the environment in an Australian veterinary hospital (Sidjabat et al., 2006). Initial contamination of the practice environment is likely to be of animal (or human) origin, however it is likely that exchange can occur in both directions and the fact that AmpC-producers in the environment appear to be associated with resistance outcomes in animals hospitalised in the environment in this study is suggestive that this is the case.

Where breed was significant it was generally consistent with small breeds being associated with low risk and medium and large breeds being associated with higher risk. It may be that this is a reflection of different case types being more common in different breeds and the inclusion of a different species (cats) in the small breed group compared to the other groups may be skewing these results, although the number of cats in the study was low. It was not possible to specify the breed more precisely than small, medium or large and we did not have data on what the dogs were fed, or their home environment, therefore we are unable to speculate whether larger breeds have different resistance levels due to these factors.

The case type was a significant risk factor for some resistance outcomes in this study with neurosurgery cases generally being associated with a high level of risk compared to orthopaedic and medical cases. The high risk for neurosurgery cases could be a reflection of the often high functional dependence of these cases. Studies in humans have shown that a high degree of functional dependence is a risk factor for the isolation of important resistance phenotypes in *E. coli* (Nicolas-Chanoine et al., 2012, Hayakawa et al., 2013). Placement of a urinary catheter is a common part of treatment of neurosurgery cases where the bladder is affected, this has frequently been identified as a risk factor in human studies (Hayakawa et al., 2013, Pitout and Laupland, 2008) and is likely to contribute to the overall increased risk among these patients. It would be interesting to determine the proportion of these patients which get urinary tract infections, and the proportion of those with resistant *E. coli* as this might give a better understanding of the degree of effect that urinary catheterisation has on these patients. Neurosurgery often has long duration of surgery and they may receive more preoperative antibiotics, or different regimes. Although we included previous antimicrobials there may be some subtleties of antimicrobial administration that were not captured in this study. Further longitudinal studies would be useful in this subset of patients. Soft tissue surgery cases were at increased risk for some resistance outcomes compared to medical and orthopaedic cases. In humans spending time in the surgical department of a hospital was shown to be a risk factor (Tham et al., 2013). It may be that the higher risk of soft tissue and neurosurgery cases represents sub-populations of animals with greater morbidity, greater likelihood to stay in higher risk environments, longer anaesthetics and other unidentified factors compared to orthopaedic and medical cases. Certainly among orthopaedic cases it would be expected that there would be a low level of exposure to antimicrobials and a higher proportion would be expected to be routine cases with low functional dependency

compared to neurosurgical cases. Medical cases in this study included a varied caseload including gastroenterology and cardiology patients and within this there is a great deal of variation. The identification of cases which may be at increased risk of acquiring *E. coli* with important AMR types is useful to guide infection control measures, allowing for specific high level infection control procedures to be put in place with these animals which might not be feasible to apply to the whole population of hospitalised animals.

Receiving an MRI or CT scan was associated with an increased risk of isolation of MDR *E. coli*. It is unlikely that the act of such a scan is likely to increase risk inherently, however in all practices the policy for MRI or CT scanning was to maintain animals under general anaesthetic for the duration of the scan, it is possible this may be associated with more of a risk. It also may reflect the type of case which is likely to receive an MRI or CT scan although case type was assessed in the model. Interestingly radiography was associated with a lower risk of AmpC-producer isolation. Again there is no obvious biological explanation for a direct influence of this and it may reflect an unidentified confounding factor.

This study has demonstrated the association of several different risk factors with important resistance outcomes in *E. coli*. The principle among these is exposure to various antimicrobials, which has been reported previously in companion animals. It is worth noting that several associations were found in this project which suggests co-selection for resistance to different antimicrobial classes can occur with antimicrobial use in companion animal practice. This implies that reduction of resistance to a certain antimicrobial may not be achieved by simply reducing the use of that antimicrobial and a more wide ranging approach to antimicrobial stewardship is necessary. Hospitalisation and case type were also significantly associated with some outcomes, with implications for infection control. If hospitalisation times can be minimised and high risk cases identified and specific infection control measures implemented then the incidence of important AMR among *E. coli* in companion animals may be reduced.

Chapter 4

Concluding discussion

Antimicrobial resistance has become an issue of major importance globally in both human and veterinary medicine. Multidrug-resistant (MDR) bacterial infections are becoming more common in veterinary hospitals and the incidence of these infections is expected to increase. This along with the emergence of specific resistance mechanisms, such as ESBL or AmpC production (Donati et al., 2014, Belas, 2014), in companion animals make this a major concern for animal welfare as there are limited therapeutic options to manage such infections and an increased risk of treatment failure. In addition companion animals may play an important role as a reservoir of resistant bacteria or resistance genes due to their frequent exposure to antimicrobials and their close contact with human beings

The overall aim of this project was to further our understanding of the epidemiology and microbiology of antimicrobial resistant *E. coli* in hospitalised companion animals, concentrating particularly on several important antimicrobial resistant (AMR) phenotypes and genotypes, such as multidrug resistance, third generation cephalosporin resistance (including resistance via ESBL or AmpC production), fluoroquinolone resistance and resistance to the most commonly used antimicrobial in veterinary practice, clavulanic acid potentiated amoxicillin (CAPA). Specific objectives included determining the prevalence and risk factor for carriage of resistance and determining the frequency of environmental contamination with resistant *E coli*. Finally, further characterisation of genes associated with resistance was performed to allow comparisons between hopsitals and with other published work in animals and humans.

These objectives were met by conducting longitudinal studies in five referral hospital practices in the North West UK with repeated sampling of hospitalised animals and the hospital environment, resulting in a total of 333 faecal samples and 257 environmental samples.

Overall resistance in *E. coli* to one or more of the antimicrobial classes was high (50.1%) in faecal samples, however perhaps of more concern is the high levels, in some cases, of resistance to critically important antimicrobials.

Resistance to CAPA was a significant finding throughout this project with a high prevalence of resistance found. CAPA was the most common antimicrobial to which animals providing samples were exposed, this is in agreement with other studies in the UK (Mateus et al., 2011, Radford et al., 2011). It is likely that this high level of exposure is a significant driver of the high levels of resistance seen. Most isolates demonstrating resistance to CAPA were also identified as producers of AmpC directly implicating the production of AmpC with high levels of clinically relevant AMR in UK veterinary practice. Previous exposure to CAPA was associated with every resistance outcome except MDR, in some cases this can be attributed to a direct selection pressure exerted by the antimicrobial (CAPA resistance, AmpC production) while in other cases (ciprofloxacin) it is likely to be due to co-carriage of different resistance genes on the same plasmid. This is of great concern as the impact of this is likely to be high given both the widespread use of this antimicrobial in UK companion animal practice and therefore the large numbers of animals exposed to it and the significant resistance outcomes that appear to be associated with its use. This study indicates that use of CAPA is likely to be a significant contributor to the burden of AMR in animals in UK referral hospitals and likely more widely. Neurological and soft tissue surgery cases were more likely to have CAPA resistant isolates, this may reflect a high degree of functional dependence, intra-operative use of antimicrobials or other factors. Further work is required to identify levels of similar resistance in pathogenic isolates in these animals (for example uropathogenic bacteria) as opposed to the commensal organisms in this study. Urinary catheterisation has been identified as a significant risk factor in humans (Pitout and Laupland, 2008, Hayakawa et al., 2013) and this may go some way to explaining the particularly high levels seen in neurosurgery cases.

High levels of AmpC production were identified in this study among faecal *E. coli*, but there was large between practice variation. In addition there was clustering of outcomes within animals, with some animals providing 2 and 3 samples all positive for AmpC-producing *E. coli*. Once within animal and within practice clustering was taken into account the prevalence of AmpC producing *E. coli* was lower, with wider confidence intervals, demonstrating the need to take this into account when analysing results from similar projects, particularly when disparate numbers of samples are collected from different

practices, which is often inevitable as some hospitals are larger and busier than others. Ideally to get a good estimate of the prevalence of AmpC production among *E. coli* in UK referral practices samples would be collected from a wider range of practices to maximise the applicability of results to practices as a whole. This was not logistically possible in this project however analytical methods have accounted for the issue of clustering. The prevalence of AmpC production in this project was 4.3% (CI 1.1, 15.6) which still indicates a significant overall contribution to resistance, with potentially more of a contribution to resistance in some practices. It would be of great interest to study the dynamics of AmpC levels in practice one over time. It is possible that these high levels do not reflect the usual situation. This project was only carried out over six weeks spaced out over a few months, constant monitoring over a longer period may provide a more accurate picture. It has been shown that in environmental *E. coli* isolates from human hospitals AmpC production is reduced in favour of ESBL production over time (Starlander et al., 2014), it would be interesting to discover if this is similar in veterinary practice. Multivariable analysis did not account for the differences seen in AmpC levels between practices and further identification of the practice level factors would be very helpful both for addressing the problem in this practice specifically and mitigation of risk more generally.

Levels of resistance to ciprofloxacin were high in this study, again there was significant practice variation with practice remaining a significant risk factor in final multivariable models. In comparison to levels in the UK community (Wedley et al., 2011) levels in this study were high, which may be expected when comparing a population of hospitalised animals to community animals as disease and hospitalisation are significant risk factors for carriage of resistance (Nicolas-Chanoine et al., 2012, Hayakawa et al., 2013, Gibson et al., 2011). However once adjusted for clustering the levels of resistance found in this study are significantly lower than those found in a similar study of hospitalised animals carried out in China indicating that levels of resistance are likely to have significant variation by geographic location, this is similar to the situation described in humans (Thomson, 1999). Ciprofloxacin resistance frequently coexisted with MDR and AmpC production and just under half of ciprofloxacin resistant samples also produced ESBLs. This is probably a reflection of co-selection for multiple resistances. Ciprofloxacin resistance was also more likely to be found alongside resistance to other, unrelated antimicrobials, which is of great concern as it is an indicator for general fluoroquinolone resistance. Fluoroquinolone resistance alone presents a challenge to treatment in both humans and animals, co-resistance to other important antimicrobial classes will only reduce treatment options. The

mechanism for ciprofloxacin resistance was not identified in this study with qnr genes being found at low levels, it may be of interest for further studies to identify the primary mechanisms involved.

Case types were identified as a significant risk factor with a similar pattern, again the exact reason for this is unclear and further study is indicated for this. Duration of hospitalisation was a significant risk factor with risk increasing over the time spent in hospital. This could reflect a longer time spent in a contaminated environment, increased contact with other animals or could reflect animals with more severe illnesses with higher functional dependence/multiple conditions, or more likely a combination of these factors. Antimicrobial use was a significant risk factor for ciprofloxacin resistance with fluoroquinolone use being associated with an eight-fold increase in risk. This is understandable from a biological point of view as exposure to a fluoroquinolone will exert a direct selection pressure for ciprofloxacin resistance, though the degree of increase in risk is high and indicates the use of fluoroquinolones in practice is strongly associated with resistance development. It is also worth noting that it was use of a fluoroquinolone in the three months prior to the date of sample collection which was the most significant fluoroquinolone exposure; this time period included the use in first opinion practice in many cases (as opposed to use within referral hospitals). Further study to look at the changes in resistance over time after antimicrobial exposure is indicated.

It is worth emphasising the difference between animals included in this study and both the general small animal population and those attending first opinion practice. The vast majority of animals in this study were cases referred to specialist referral centres (though a very small number were first opinion cases seen at these centres), these animals are more likely to have had recent treatment, possibly including hospitalisation prior to referral. As a population they may have had greater exposure to antimicrobials for longer periods, be hospitalised for longer periods and in some cases be more debilitated than a population of animals in a first opinion setting. Due to these factors it is possible that the results in this study are not directly applicable to first opinion practice, hospitalisation is likely to occur less frequently in a first opinion setting however the use of antimicrobials is frequent in first opinion practice (Radford et al., 2011, Mateus et al., 2011) and indeed the total number of animals exposed to antimicrobials in first opinion practice is likely to greatly exceed that in a referral setting purely due to the number of animals involved. The levels of exposure to different antimicrobials in this project were broadly similar to those in the

two studies of antimicrobial use in first opinion practices mentioned previously, this suggests resistance patterns seen in first opinion practice may not be very different to those seen in this project due to similar antimicrobial exposures. Therefore it is likely that while there are differences in the study population in this study to that in a study of first opinion animals it is likely that this study has at least a fair degree of relevance to first opinion practice as well as referral practice.

Fluoroquinolones are an essential antimicrobial class in human medicine and are useful in veterinary medicine. Animals were relatively infrequently exposed to a fluoroquinolone in this study which concurs with other studies (Mateus et al., 2011, Radford et al., 2011), however it was still an important risk factor not just for ciprofloxacin resistance but also for resistance to other important antimicrobials. There is an urgent need for effective stewardship of these antimicrobials in practice.

The prevalence of ESBL producing *E. coli* was lower in this study compared to other studies of hospitalised animals in China (Sun et al., 2010) and Korea (So et al., 2012) and higher than comparable studies of community animals. There was significant masking of ESBL-producer phenotype due to AmpC production in this study. This demonstrates the limitations of using phenotypic methods alone for the detection of ESBL-producers, particularly where high levels of AmpC production are suspected, molecular methods are essential for confirmation. The most frequently detected type of ESBL in this study was CTX-M which is a common finding in both human and animal studies. The most common ESBL found was CTX-M-15 which is commonly found in human studies but is also commonly reported in studies of animals in Europe (Ewers et al., 2012). Importantly this may reflect human to animal transmission of ESBL-producers and it is logical to assume that transmission can occur in both directions, suggesting a possible public health risk for AMR in companion animals. Linking AMR in humans and animals means that effective control of the problem in either is likely to require effective control in the other, highlighting the need for a 'One Health' approach to this important issue. The finding of TEM-158 in this project is important, it was the second most frequently identified ESBL in this project and it is worth noting that all TEM-158 positive samples were from the same practice. Further study would be useful to determine what factors may be responsible for this, including examining the referring population of animals in the community. It may simply be due to chance, however this hospital also had the highest levels of ciprofloxacin resistance and AmpC producing *E. coli*. Further study of hospitals with higher levels of resistance, particularly

looking at initial antimicrobial burdens and how they change over time is indicated. There are no other studies reporting $bla_{TEM-158}$ in companion animals but it has been reported occasionally in humans (Kiiru et al., 2012, Jacquier et al., 2013, Robin et al., 2007). TEM-158 is a complex mutant TEM (CMT) with inhibitor resistant properties and is of concern as the resistance to inhibitors means that there is both a wider spectrum of resistance to common antimicrobial treatments and the potential for masking of ESBL presence. Molecular methods including sequencing are required to distinguish TEM-158 production from that of AmpC and other ESBL variants.

Fluoroquinolone and CAPA use were associated with increased risk of isolation of ESBL-producing *E. coli* from faeces. A fluoroquinolone would not be expected to directly select for ESBL production and it is likely this is due to co-selection for multiple resistance genes located on the same plasmid which is well described (Hawkey and Jones, 2009). Further analysis looking at plasmids to identify both the plasmid types and the resistance genes located on them would be interesting and it would be expected from these results that multiple resistance genes conferring resistance to a range of important antimicrobials would be identified.

3GCR was detected at relatively high rates in this study, which is a significant concern given the importance of these antimicrobials in human medicine. It is particularly interesting to note that the prevalence of 3GCR was higher in this study than that of CAPA resistance, given the much higher levels of exposure of animals to CAPA than 3GC's, it would be expected that a more potent selection pressure would exist for CAPA resistance. In this project no exposure to third or fourth generation cephalosporins was recorded however there was significant exposure to first and second generation cephalosporins, it may be that these are exerting a selection pressure for general cephalosporin resistance. Other significant contributors could be the use of fluoroquinolones and CAPA which are significant risk factors for mechanisms which would confer resistance to 3GC's. This is important as it demonstrates the potential for resistance to develop to a specific antimicrobial even when an animal has not been directly exposed to that antimicrobial.

There were many different MDR profiles identified in this study, as MDR is simply an umbrella term for many different resistance combinations. The clinical relevance generally depends on which classes of antimicrobial there is resistance to. A large number of the MDR isolates in this study featured resistance to CAPA and ciprofloxacin which is of concern as treatment options for these samples are likely to be limited. This probably

reflects the fact that the samples in this project came from a population of hospitalised animals in a referral environment. By their nature referral cases are likely to be non-routine and may have had previous hospitalisation and been exposured to antimicrobials. Comparison of MDR profiles from this project to a similar project (Wedley et al., 2011) looking at community based animals in northwest England show a stark difference in the MDR profiles in the community. This is expected however further study between more directly comparable animals is indicated: for example dogs in the same household where one is hospitalised and the other not. MDR samples with resistance to all classes of antimicrobial were found at a reasonably high frequency in this project. In some cases certain MDR profiles were prevalent in single practices compared to others, suggesting that these may be acquired within the hospital rather than the community, though we cannot rule out a geographical or case type based influence. It is interesting that the only antimicrobial exposures associated with increased risk of MDR were metronidazole and amoxicillin, this may simply reflect a lack of power in this project as antimicrobial exposure is a key risk factor for AMR and it would be expected that exposure to many antimicrobials would favour MDR development. However many antimicrobial exposures were significant in univariable analysis but were not significant in our final model. Further study directly comparing animals in the community, first opinion practice and referral practice is indicated, it would be hoped that this could provide information on the differences between MDR profiles in these populations, and some idea of the drivers of these differences, which could go some way to informing measures to mitigate the impact of MDR infections.

All types of AMR examined in this study were found in both faecal and environmental samples. Indeed patterns across practices were similar between the two sample types which is suggestive of transfer in one or both directions between the two. Invariably the prevalence was lower in environmental samples compared to faecal samples, perhaps reflecting lower survivability of these bacteria in the environment, perhaps due to hygiene measures or environmental conditions. Useful future studies could look directly at the transfer of resistant bacteria between animals, their hospital environment and humans to try to identify transmission dynamics. This could greatly inform infection control measures. Only one MDR phenotype was found in environmental samples which was not found in faecal samples, the source for this contamination is likely to be either human or animal, faecal sample coverage of hospitalised animals was not 100%, if complete coverage had been achieved then it is possible this MDR phenotype would have been identified. Results

of genotype analysis were similar to that in faecal samples with CTX-M-15 the most common ESBL and TEM-158 also found in the environment of practice one. It is interesting to note that SHV-12 was only found in the environment, this probably reflects incomplete coverage of faecal sample collections.

The origin of the environmental sample seemed to have an influence, resistance consistently seemed more likely from outside walking areas. This may reflect mixing of patients in this area, increased likelihood of defaecation, reduced disinfection or some unidentified factor. This is useful information for infection control measures as changes such as use of artificial surfaces which are easier to disinfect, or reduced mixing of animals from different wards, may have a beneficial effect. Study of the exact dynamics of bacteria populations in animals and their environment is indicated.

Although the different timescales are not directly comparable between practices (samples collected on different dates with different intervals between collection periods), it is interesting that the pattern of contamination levels appeared to vary between practices. One practice seemed to have a consistently moderate level of contamination and two practices a consistently low level of contamination while the two remaining practices generally had low levels of contamination but had transient periods of high contamination. This difference in patterns suggests different dynamics due to practice level factors, further study to confirm this and identify reasons is indicated. There were several limitations encountered for environmental sampling in this study, it was not possible to time sample collection around cleaning as timing was often variable due to workload and it was not possible for this to be fitted around sampling. Environmental disinfection is likely to have a significant effect of bacterial isolation rates from environmental samples. Also sampling timing was made as regular as possible but there was some variation between practices and even for the same practices between different sampling blocks, it is possible this has introduced some bias to the results.

Probably the main limitations of this project are the number of samples collected and the restriction to five hospitals in northwest England. As a result of this some results are imprecise with wide confidence intervals both for prevalence and risk factor analysis. Future similar projects should consider the difficulty of faecal sample collection as it is likely to be done by members of practice staff who have multiple other duties. Sample collection was greatly enhanced by collection from multiple practices at the same time and improving communication. As far as breed was concerned cats were included in the small breed

category. This may be a limitation for this study as cats have a very different lifestyle to dogs and indeed a small breed dog is likely to be much more similar to a large breed dog in terms of lifestyle, illnesses for which veterinary treatment might be required and the type of treatment (including different antimicrobial agents) which may be given. This are all potentially relevant factors which may influence outcomes in this study, in future studies it would be better to include cats in their own breed category. It is worth re-iterating the fact that cats contributed only a very small proportion of the samples in this project and for this reason any effect is likely to be small.

In conclusion this study has identified that companion animals are carriers of antimicrobial resistant bacteria, in some cases at high levels. The level of carriage appears to be associated with hospitalisation, reason for hospitalisation and antimicrobial use among other factors. This information will help inform measures to tackle what is probably the most important issue facing medicine and veterinary medicine in the future. Further studies are needed to determine the effect that antimicrobial stewardship or specific infection control measures have on the rates of carriage of resistance and hence infection in these populations.

Chapter 5

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APPENDICES

Appendix I – In house sequencing protocol

CONTENTS

PCR - reactions

Reagents/Equipment required -

PCR reagents (dNTPs [10mM], 10x buffer, $MgCl_2$ [25mM], *Taq* DNA polymerase [5Uμl^{-1}], primers [10µM], molecular grade H_2O)
96-well non-skirted microtitre plates (AbGene)
Adhesive PCR film (AbGene)
DNA extracts
Thermal cycler
Centrifuge with microtitre plate rotor
Plate vortexer

1. In Excel create a spreadsheet to indicate which DNA isolate will be in each well of the half-microtitre plate. This sheet will act as a sample tracking sheet throughout the MLST process. Remember to include a negative control. 2.
2. Mix PCR reagents together (Master mix) in the following quantities;

	1x Master Mix	52x Master Mix (for 1 full plate)
Sigma molecular grade H_2O	37.25µl	1936µl
10x buffer	5.0µl	260µl
$MgCl_2$ (25mM)	3.0µl	156µl
dNTPs (20mM stock)	0.5µl	26µl
Forward primer (10µM stock)	1.0µl	52µl
Reverse primer (10µM stock)	1.0µl	52µl
Taq polymerase (5 units/µl)	0.25µl	13µl

3. Aliquot 48 µl master mix per microtitre well and tap plate gently to ensure liquid is in the bottom of the well. Pipette 2 µl DNA onto the side of each well as per plate layout created in Excel.

4. Gently tap plate to move DNA to well bottom and carefully seal the plate. Vortex and spin plate briefly at 500 rpm.

5. Place plate in thermal cycler and load program.

6. Once PCR is finished, mix 5 µl of each sample (or a selection of samples) with 1 µl 6x loading buffer and load into wells of a 2% agarose gel containing ethidium bromide 0.5 µg / ml. Electrophorese at about 120 V for 20 min and visualise DNA on a U.V. transilluminator.

The method can be halted here indefinitely, with products being stored at 4°C for up to 2 weeks, or at -20°C for indefinite storage.

PCR product clean-up (PEG precipitation)

1. Aliquot 60 µl 20% (w/v) PEG_{8000}, 2.5M NaCl per well, using a multichannel pipette, seal wells with adhesive film, vortex and briefly spin the plate at 500 rcf to ensure mix is at the bottom of the wells. Incubate the plates for either 15 min at 37 °C, 30 min at 20 °C or overnight at 4 °C. (Longer incubations do not have a detrimental effect on the clean up procedure).

2. Spin at 2750 rcf at 4 °C for 60 min.

3. To remove PEG, place folded blue tissue into the bottom of the centrifuge plate holders and gently invert the plate onto blue tissue. Spin at 500 rpm for 60 sec.

4. Wash pellet twice with 150 µl 70% ice-cold ethanol. i.e. add 150 µl per well and spin at 2750 rcf for 10 min. Remove ethanol by inversion of plate onto blue tissues, and then spin inverted plate on folded clean blue at 500 rpm for 60 sec. Repeat.

5. Air dry plate on bench for 10 min.

6. Re-suspend pellet in STERILE water. Re-suspension volume is dependent on intensity of PCR product observed following PCR e.g. Barely visible products are re-suspended in 5 µl with more intense products re-suspended in volumes up to 50 µl. Volumes for each locus batch are determined with reference to intensity of product band on gel image. Seal lid carefully, vortex and spin briefly.

7. Resuspended products can be stored long-term at -20°C, or short-term at 4°C.

Sequencing reactions

1. Create a spreadsheet in Excel to indicate which isolate/primers will be in which wells, such that the PCR product from well A1 will be in A1 and A2, the forward primer will be A1 and the reverse in A2. PCR product from A2 in A3 and A4 etc, according to the sequence plate pipetting guide sheet in Appendix VII.

	1	2	3	4	5	6	7	8	9	10	11	12
A	806.aspA1	806.aspA2	869.aspA1	869.aspA2	1030.aspA1	1030.aspA2	1200.aspA1	1200.aspA2	1267.aspA1	1267.aspA2	1431.aspA1	1431.aspA2
B	808.aspA1	808.aspA2	875.aspA1	875.aspA2	1062.aspA1	1062.aspA2	1202.aspA1	1202.aspA2	1280.aspA1	1280.aspA2	1434.aspA1	1434.aspA2
C	809.aspA1	809.aspA2	882.aspA1	882.aspA2	1075.aspA1	1075.aspA2	1209.aspA1	1209.aspA2	1291.aspA1	1291.aspA2	1491.aspA1	1491.aspA2
D	815.aspA1	815.aspA2	892.aspA1	892.aspA2	1079.aspA1	1079.aspA2	1210.aspA1	1210.aspA2	1293.aspA1	1293.aspA2	1495.aspA1	1495.aspA2
E	818.aspA1	818.aspA2	912.aspA1	912.aspA2	1094.aspA1	1094.aspA2	1212.aspA1	1212.aspA2	1310.aspA1	1310.aspA2	1506.aspA1	1506.aspA2
F	825.aspA1	825.aspA2	920.aspA1	920.aspA2	1190.aspA1	1190.aspA2	1218.aspA1	1218.aspA2	1417.aspA1	1417.aspA2	1540.aspA1	1540.aspA2
G	834.aspA1	834.aspA2	923.aspA1	923.aspA2	1192.aspA1	1192.aspA2	1219.aspA1	1219.aspA2	1418.aspA1	1418.aspA2	1558.aspA1	1558.aspA2
H	850.aspA1	850.aspA2	935.aspA1	935.aspA2	1196.aspA1	1196.aspA2	1221.aspA1	1221.aspA2	1423.aspA1	1423.aspA1	blank	blank

2. Make up master mix in required volume. Make two batches of 50 aliquots per sequencing plate :

	1x Master Mix	50x Master Mix
Molecular grade H_2O 119 µl	2.38 µl	
5x buffer 93.5 µl	1.87 µl	
Big Dye	0.25 µl	12.5 µl
Primer (forward OR reverse) 200 µl	[0.67µM] 4 µl	

N.B. Sequencing primers are at 0.67µM i.e. 1:15 dilution of PCR primer concentration (see Appendix I). Sequencing primers are not necessarily the same as the PCR primers.

3. Add 8.5µl of master mix containing forward primer to wells of columns 1,3,5,7,9 and 11; 8.5µl of master mix containing reverse primer to wells of columns 2,4,6,8,10 and 12

4. Pipette 1.5 µl of the first PCR product onto the side of wells A1 and A2. Repeat for remainder of wells as per plate layout. Spin briefly to move DNA template to bottom of wells.

5. Place plate in thermal cycler and load program with the following conditions;

96 °C for 10 sec
50 °C for 5 sec
60 °C for 2 min
} X 30
4 °C forever.

7. Do not stop at this point. Proceed immediately to precipitation unless sequencing reaction runs overnight.

Sequencing reaction clean-up (ethanol precipitation)

a. Per plate mix 7000 µl 100% ethanol and 280 µl 3M sodium acetate and aliquot 52 µl per well.

b. Replace adhesive film, vortex and briefly spin (500 rpm). Incubate at room temp for 45 min and spin at 2750 rcf (4 °C) for 1 h.

c. Remove adhesive film and gently invert plate onto absorbent tissue. Spin inverted plate on fresh tissue (500 rpm) for < 1min.

d. Wash pellet once by addition of 150 µl ice-cold 70% ethanol per well, cover plate with film and spin at 2750 rcf for 10 min.

e. Remove adhesive film, invert plate onto absorbent tissue and give a final short inverted spin at 500 rpm.

f. Air dry at room temp for 10 minutes. Recover plate with adhesive film and store at –20 °C prior to sending away.

Reactions in tubes (repeats)

PCR

1. To carry out MLST PCR in tubes use a 0.2 ml thin-walled tube and use the same reaction mixture, quantity and thermal cycler conditions as for a 96-well plate.

2. Run 5 µl out on a gel.

3. To PEG precipitate; add 50 µl water to each tube and transfer total volume to a 1.5 ml tube. Then add 60 µl PEG / NaCl, vortex, incubate as for plates and spin at 13000g for 30 min.

4. Pipette off PEG and wash once with 500 µl 70% ethanol (13000g 10 min). Air dry and resuspend as per usual.

SEQUENCING

1. Use 0.2 ml tubes and set up sequencing reactions as per 96 well plate.

2. Add 10 µl water per tube and transfer reactions to1.5 ml tubes. Add 52 µl ethanol/Na acetate, incubate as per plates and spin 13000g for 30 min.

3. Wash once with 70% ethanol as above.
Stock primers are kept at 100 µM (100 pmol/µl) and diluted 1:10 for use in PCR and further diluted 1:15 for use in sequencing reactions (0.67 µM).

Appendix II: Figures showing AMR phenotype prevalence in environmental and faecal samples collected from practices

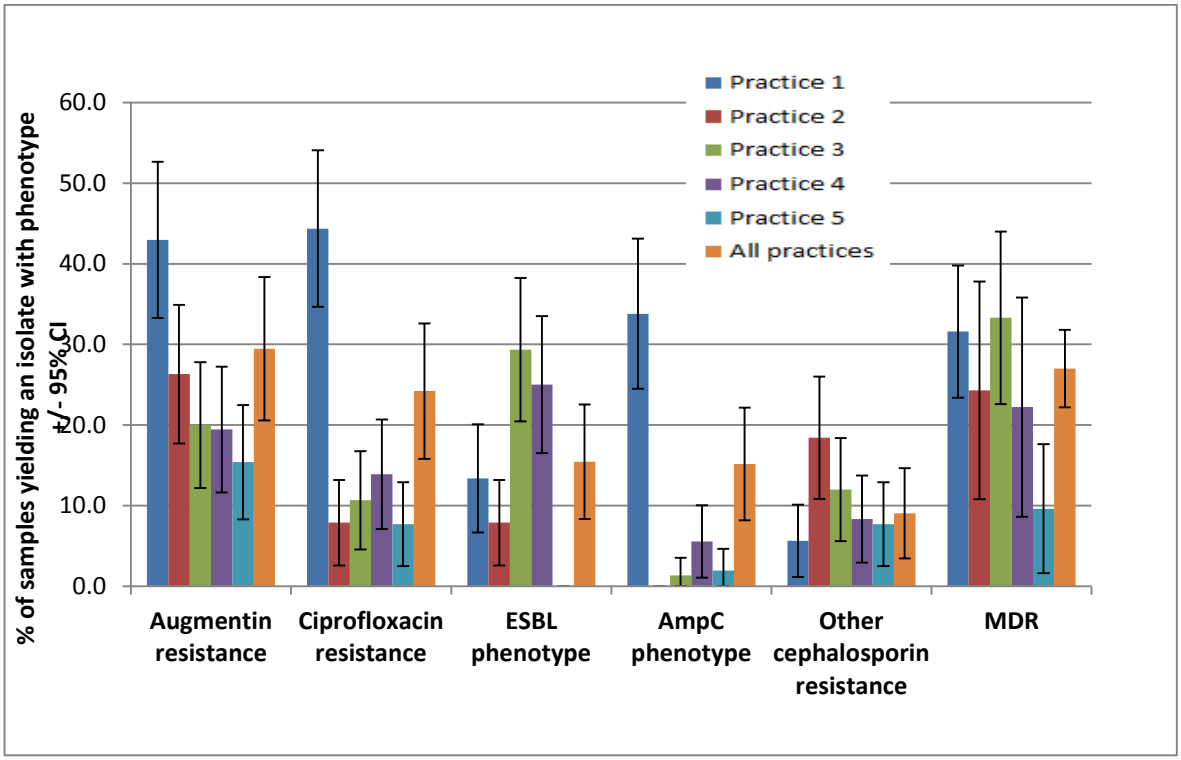


Figure 1: The prevalence of each important AMR type found in faecal samples from each practice and in total

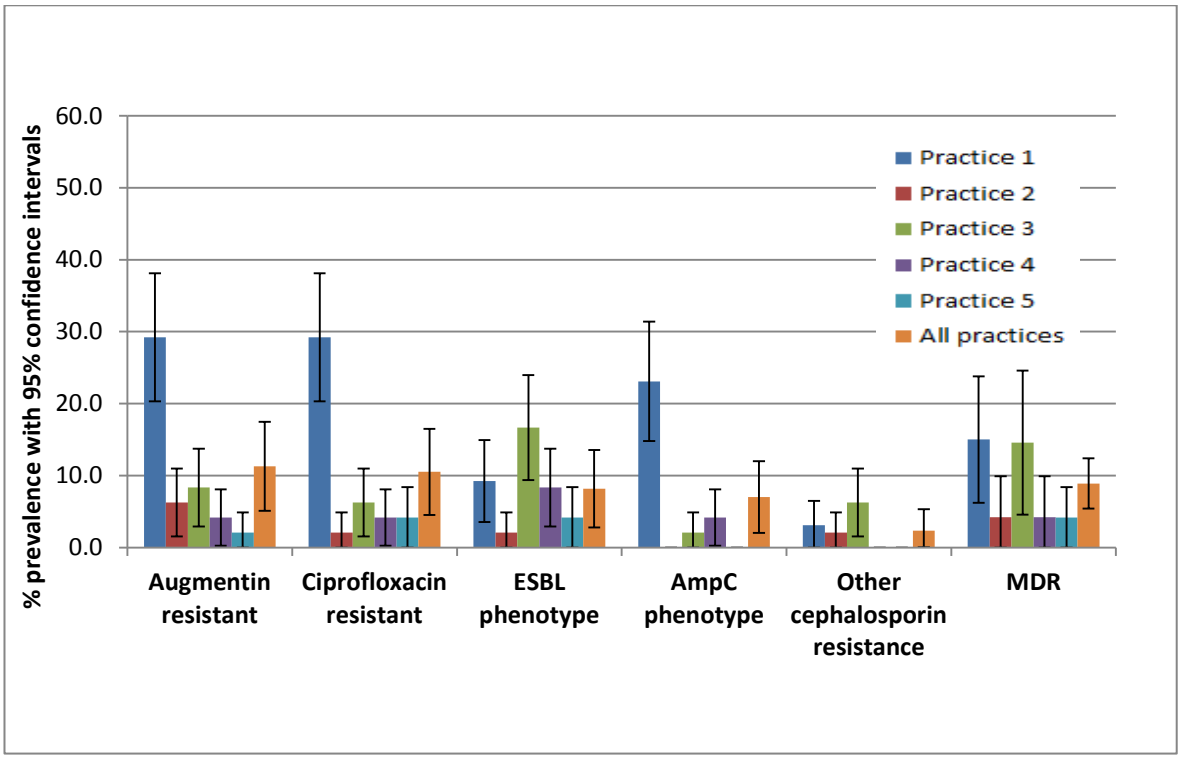


Figure 2: The prevalence of each important AMR type found in faecal samples from each practice and in total

Appendix III: Origin and timing of environmental samples

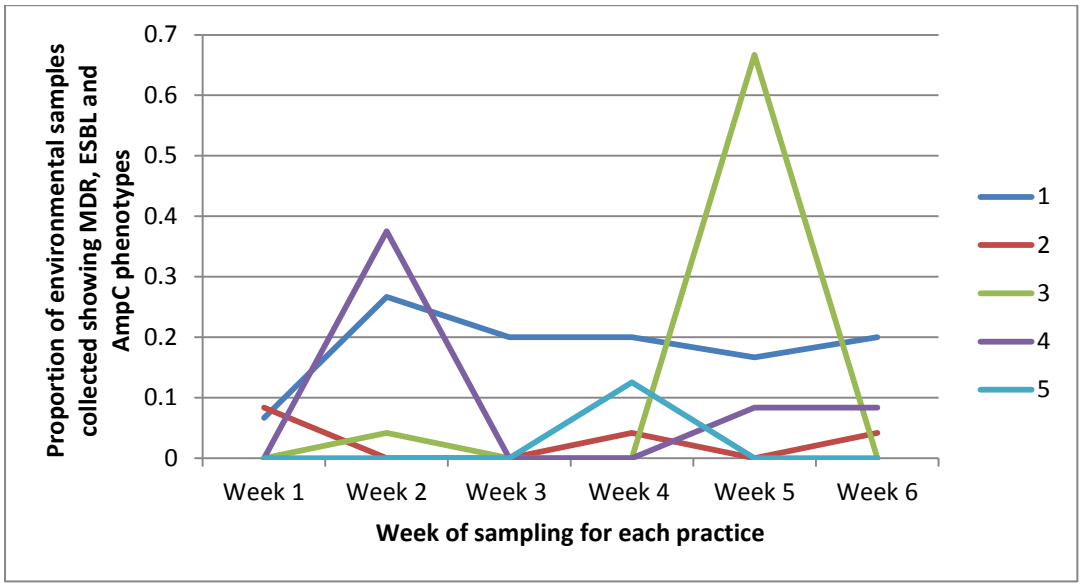


Figure 1: The proportion of potential ESBL, AmpC and MDR samples which were positive from each practice in each sampling week (X axis) giving a crude indication of the overall resistance burden in the environment in each week of sampling for each practice (practices are represented by different coloured lines) and changes in this burden between sampling weeks. For example: if all samples collected from a practice in a week were positive for ESBL production, AmpC production and MDR then the proportion (Y axis) would be one, if all samples were positive for one of the three outcomes only (but no others) the proportion would be 0.33. It is important to note that the gaps between sample weeks 2 and 3 and 4 and 5 are variable, there is no gap between weeks 1 and 2, 3 and 4 and 5 and 6.

Appendix IV: Univariable analysis results

Table 1 Results of univariable multilevel analysis for the outcome of MDR in 320 faecal samples from 200 dogs and cats in 5 hospitals in North West UK.

variable		B	SD	z ratio	odds ratio	lower95oddsr	upper95oddsratio	P value
Practice (Compared to 1)								0.068
	2	-0.022	0.505000	-0.0430	0.98	0.36	2.64	0.966
	3	0.182	0.3780	0.4810	1.20	0.57	2.52	0.630
	4	-1.076	0.6290	-1.710	0.34	0.10	1.17	0.087
	5	-1.297	0.5940	-2.182	0.27	0.09	0.88	0.029
Days hospitalised		0.054	0.038	1.427	1.055	0.98	1.137	0.154
Days hospitalised10		0.094	0.046	2.016	1.098	1.003	1.203	0.044
Environment contaminated with MDR		0.31	0.299	1.036	1.363	0.758	2.449	0.300
Environment contaminated with ESBL		0.134	0.304	0.442	1.144	0.63	2.075	0.659
Environment contaminated with AmpC		0.032	0.307	0.105	1.033	0.566	1.885	0.916
Age		-0.033	0.037	-0.903	0.967	0.9	1.04	0.366
Breed (compared to small breed)								0.110
	Medium	0.953	0.409	2.331	2.593	1.164	5.777	0.020
	Large	0.831	0.424	1.958	2.295	0.999	5.273	0.050
	Unidentified	0.927	0.697	1.331	2.527	0.645	9.898	0.183
Gender (compared to mn)								0.115
	m	0.584	0.382	1.53	1.794	0.849	3.792	0.126
	f	-0.342	0.567	-0.603	0.71	0.234	2.159	0.547
	fn	-0.215	0.436	-0.492	0.807	0.343	1.898	0.623
Previous hospitalisation		-0.12	0.32	-0.375	0.887	0.473	1.661	0.708
Case type (compared to neuro)								0.050
	Ortho	-0.747	0.436	-1.713	0.474	0.201	1.114	0.087
	STS	0.024	0.462	0.052	1.024	0.414	2.535	0.958
	Medical	-1.11	0.459	-2.416	0.33	0.134	0.811	0.016
	Unidentified	-0.131	0.543	-0.241	0.877	0.303	2.54	0.809
Xray performed		-0.186	0.361	-0.516	0.83	0.409	1.684	0.606
Ultrasound performed		-0.391	0.489	-0.801	0.676	0.26	1.762	0.423
MRI or CT performed		0.587	0.332	1.77	1.798	0.939	3.444	0.077
Any antimicrobial given in the last 3 months		0.427	0.302	1.411	1.532	0.847	2.772	0.158
Cefuroxime given in the last 3m		0.034	0.408	0.084	1.035	0.465	2.302	0.933
Amoxycillin given in the last 3m		1.457	0.694	2.099	4.293	1.101	16.741	0.036
CAPA given in the last 3m		0.186	0.306	0.608	1.204	0.661	2.194	0.543
Metronidazole given in the last 3m		0.902	0.422	2.138	2.465	1.078	5.638	0.033
Fluoroquinolone given in the last 3m		0.255	0.636	0.401	1.29	0.371	4.489	0.688
Cephalexin given in the last 3m		0.412	0.496	0.832	1.511	0.572	3.992	0.405
Clindamycin given in the last 3m		x	x	x	x	x	x	x
Cefuroxime given in the last 24h		-1.222	1.224	-0.999	0.295	0.027	3.244	0.318
Cefuroxime given in the last 48h		-0.659	0.733	-0.9	0.517	0.123	2.174	0.368
Cefuroxime given in the last 7d		-0.573	0.549	-1.043	0.564	0.192	1.655	0.297
CAPA given in the last 24h		0.227	0.363	0.624	1.254	0.615	2.557	0.533
CAPA given in the last 48h		0.144	0.357	0.402	1.154	0.573	2.325	0.688
CAPA given in the last 7d		0.214	0.333	0.641	1.238	0.644	2.379	0.521
Metronidazole given in the last 24h		0.23	0.573	0.402	1.259	0.41	3.872	0.688
Metronidazole given in the last 48h		0.23	0.573	0.402	1.259	0.41	3.872	0.688
Metronidazole given in the last 7d		0.163	0.532	0.307	1.178	0.415	3.339	0.759
Fluoroquinolone given in the last 24h		-0.272	0.925	-0.294	0.762	0.124	4.673	0.769
Fluoroquinolone given in the last 48h		0.241	0.789	0.305	1.272	0.271	5.978	0.760
Fluoroquinolone given in the last 7d		-0.234	0.773	-0.303	0.791	0.174	3.597	0.762
Cephalexin given in the last 24h		-0.322	0.759	-0.424	0.725	0.164	3.21	0.672
Cephalexin given in the last 48h		0.012	0.69	0.017	1.012	0.262	3.91	0.987
Cephalexin given in the last 7d		0.69	0.58	1.19	1.994	0.64	6.213	0.234
Any antimicrobial given in the last 24h		0.023	0.314	0.073	1.023	0.553	1.893	0.941
Any antimicrobial given in the last 48h		0.117	0.304	0.385	1.124	0.619	2.04	0.701
Any antimicrobial given in the last 7d		0.248	0.297	0.833	1.281	0.715	2.295	0.405

Table 2 Results of univariable multilevel analysis for the outcome of CITM in 320 faecal samples from 200 dogs and cats in 5 hospitals in North West UK.

variable		B	SD	z ratio	odds ratio	lower95oddsr	upper95oddsratio	P value
Practice (Compared to 1)	Overall							0.003
	2	-1.207	0.518	-2.33	0.299	0.108	0.825	0.020
	3	-1.615	0.414	-3.9	0.199	0.088	0.448	0.000
	4	-1.535	0.561	-2.738	0.215	0.072	0.646	0.006
	5	-2.588	0.743	-3.482	0.075	0.017	0.323	0.000
Days hospitalised		0.156	0.037	4.216	1.169	1.087	1.257	0.000
Days hospitalised10		0.215	0.044	4.843	1.240	1.137	1.353	0.000
Environment contaminated with MDR		2.029	0.35	5.797	7.606	3.831	15.105	0.000
Environment contaminated with ESBL								
Environment contaminated with AmpC		2.317	0.418	5.549	10.150	4.477	23.012	0.000
Age		0.027	0.032	0.844	1.027	0.965	1.094	0.407
Breed (compared to small breed)	Overall							0.006
	medium	0.493	0.342	1.440	1.637	0.837	3.202	0.150
	large	-0.176	0.388	-0.453	0.839	0.392	1.796	0.651
	unidentified	1.628	0.555	2.935	5.096	1.717	15.119	0.003
Gender (compared to mn)	Overall							0.047
	m	-0.168	0.331	-0.506	0.846	0.442	1.619	0.613
	f	-0.077	0.444	-0.174	0.926	0.388	2.210	0.862
	fn	-1.206	0.447	-2.696	0.300	0.125	0.719	0.007
Previous hospitalisation		0.498	0.277	1.801	1.646	0.957	2.831	0.072
Case type (compared to ortho)								0.000
	Neurosurgery	1.562	0.443	3.530	4.770	2.003	11.356	0.000
	STS	1.445	0.455	3.178	4.244	1.740	10.349	0.001
	Medical	0.305	0.464	0.657	1.357	0.546	3.371	0.511
	Unspecified	1.644	0.502	3.274	5.178	1.935	13.859	0.001
Xray performed		-1.135	0.426	-2.664	0.321	0.139	0.741	0.008
Ultrasound performed		-0.115	0.422	-0.272	0.891	0.390	2.039	0.785
MRI or CT performed		0.359	0.303	1.185	1.431	0.791	2.590	0.236
Any antimicrobial given in the last 3 months		0.87	0.297	2.933	2.387	1.335	4.269	0.003
Cefuroxime given in the last 3m		0.872	0.327	2.670	2.392	1.261	4.536	0.008
Amoxycillin given in the last 3m		1.488	0.574	2.594	4.430	1.439	13.638	0.009
CAPA given in the last 3m		0.629	0.272	2.309	1.876	1.100	3.199	0.021
Metronidazole given in the last 3m		0.6	0.38	1.578	1.822	0.865	3.840	0.115
Fluoroquinolone given in the last 3m		1.11	0.495	2.243	3.035	1.150	8.006	0.025
Cephalexin given in the last 3m		0.002	0.484	0.005	1.002	0.388	2.587	0.996
Clindamycin given in the last 3m		1.835	0.593	3.096	6.266	1.961	20.024	0.002
Cefuroxime given in the last 24h		x	x	x	x	x	x	x
Cefuroxime given in the last 48h		-0.443	0.645	-0.688	0.642	0.181	2.271	0.492
Cefuroxime given in the last 7d		-0.515	0.506	-1.018	0.598	0.222	1.610	0.309
CAPA given in the last 24h		0.647	0.319	2.028	1.909	1.022	3.566	0.043
CAPA given in the last 48h		0.603	0.312	1.933	1.827	0.992	3.366	0.053
CAPA given in the last 7d		0.914	0.286	3.192	2.493	1.423	4.370	0.001
Metronidazole given in the last 24h		0.919	0.478	1.924	2.508	0.983	6.398	0.054
Metronidazole given in the last 48h		0.919	0.478	1.924	2.508	0.983	6.398	0.054
Metronidazole given in the last 7d		0.749	0.44	1.700	2.114	0.892	5.011	0.089
Fluoroquinolone given in the last 24h		1.308	0.647	2.019	3.697	1.039	13.151	0.043
Fluoroquinolone given in the last 48h		1.505	0.621	2.422	4.505	1.333	15.225	0.015
Fluoroquinolone given in the last 7d		1.479	0.536	2.758	4.390	1.534	12.563	0.006
Cephalexin given in the last 24h		-0.047	0.666	-0.070	0.955	0.259	3.519	0.944
Cephalexin given in the last 48h		-0.138	0.66	-0.209	0.871	0.239	3.177	0.835
Cephalexin given in the last 7d		-0.071	0.58	-0.123	0.931	0.299	2.900	0.902
Any antimicrobial given in the last 24h		0.57	0.282	2.024	1.769	1.018	3.073	0.043
Any antimicrobial given in the last 48h		0.682	0.276	2.470	1.978	1.151	3.399	0.014
Any antimicrobial given in the last 7d		0.934	0.275	3.402	2.545	1.486	4.359	0.001

Table 3 Results of univariable multilevel analysis for the outcome of ciprofloxacin resistant in 320 faecal samples from 200 dogs and cats in 5 hospitals in North West UK.

variable		beta	se	z-ratio	odds ratio	CI95low	CI95hi	p-value
Practice (Compared to 1)	Overall							0.000
	2	-2.073	0.647	-3.205	0.126	0.035	0.447	0.001
	3	-1.861	0.417	-4.462	0.156	0.069	0.352	0.000
	4	-1.775	0.564	-3.150	0.169	0.056	0.511	0.002
	5	-2.078	0.554	-3.748	0.125	0.042	0.371	0.000
Days hospitalised		0.154	0.037	4.21	1.167	1.086	1.253	0.000
Days hospitalised10		0.209	0.044	4.755	1.233	1.131	1.344	0.000
Environment contaminated with MDR		1.491	0.315	4.736	4.442	2.396	8.234	0.000
Environment contaminated with ESBL								
Environment contaminated with AmpC		1.513	0.303	4.994	4.541	2.507	8.225	0.000
Age		-0.025	0.033	-0.771	0.975	0.914	1.04	0.441
Breed (compared to small breed)	Overall							0.350
	medium	0.469	0.336	1.395	1.599	0.827	3.092	0.163
	large	0.078	0.363	0.215	1.081	0.531	2.202	0.830
	unidentified	0.73	0.586	1.247	2.076	0.658	6.546	0.213
Gender (compared to mn)	Overall							0.255
	m	0.037	0.327	0.114	1.038	0.546	1.972	0.909
	f	-0.612	0.496	-1.232	0.542	0.205	1.435	0.218
	fn	-0.536	0.382	-1.403	0.585	0.277	1.237	0.161
Previous hospitalisation		0.283	0.276	1.027	1.328	0.773	2.28	0.304
Case type (compared to ortho)	Overall							0.000
		1.619	0.41	3.949	5.05	2.261	11.282	0.000
		1.303	0.425	3.069	3.682	1.602	8.462	0.002
		0.346	0.415	0.833	1.413	0.626	3.19	0.405
		0.377	0.549	0.686	1.458	0.497	4.28	0.493
Xray performed		-0.239	0.333	-0.718	0.788	0.41	1.512	0.473
Ultrasound performed		0.167	0.399	0.419	1.182	0.541	2.583	0.675
MRI or CT performed		0.636	0.298	2.135	1.888	1.054	3.385	0.033
Any antimicrobial given in the last 3 months		1.148	0.3	3.83	3.153	1.752	5.675	0.000
Cefuroxime given in the last 3m		0.766	0.328	2.333	2.15	1.13	4.091	0.020
Amoxycillin given in the last 3m		1.64	0.599	2.74	5.156	1.595	16.664	0.006
CAPA given in the last 3m		0.933	0.272	3.435	2.542	1.493	4.329	0.001
Metronidazole given in the last 3m		1.088	0.369	2.951	2.967	1.441	6.111	0.003
Fluoroquinolone given in the last 3m		1.926	0.527	3.653	6.859	2.441	19.274	0.000
Cephalexin given in the last 3m		-0.728	0.577	-1.261	0.483	0.156	1.497	0.207
Clindamycin given in the last 3m		0.704	0.601	1.171	2.022	0.622	6.572	0.242
Cefuroxime given in the last 24h		x	x	x	x	x	x	x
Cefuroxime given in the last 48h		-0.613	0.661	-0.928	0.541	0.148	1.979	0.354
Cefuroxime given in the last 7d		-0.686	0.517	-1.327	0.504	0.183	1.387	0.184
CAPA given in the last 24h		0.659	0.318	2.07	1.932	1.036	3.604	0.038
CAPA given in the last 48h		0.604	0.311	1.94	1.829	0.994	3.365	0.052
CAPA given in the last 7d		1.002	0.285	3.521	2.723	1.559	4.756	0.000
Metronidazole given in the last 24h		0.969	0.483	2.007	2.636	1.023	6.791	0.045
Metronidazole given in the last 48h		0.969	0.483	2.007	2.636	1.023	6.791	0.045
Metronidazole given in the last 7d		0.937	0.438	2.141	2.553	1.083	6.021	0.032
Fluoroquinolone given in the last 24h		2.568	0.808	3.179	13.041	2.677	63.524	0.001
Fluoroquinolone given in the last 48h		2.698	0.794	3.396	14.847	3.129	70.451	0.001
Fluoroquinolone given in the last 7d		2.238	0.606	3.692	9.374	2.857	30.758	0.000
Cephalexin given in the last 24h		-1.493	1.057	-1.412	0.225	0.028	1.785	0.158
Cephalexin given in the last 48h		-1.572	1.054	-1.491	0.208	0.026	1.64	0.136
Cephalexin given in the last 7d		-1.874	1.061	-1.766	0.154	0.019	1.228	0.077
Any antimicrobial given in the last 24h		0.576	0.279	2.065	1.779	1.03	3.073	0.039
Any antimicrobial given in the last 48h		0.576	0.279	2.065	1.779	1.03	3.073	0.039
Any antimicrobial given in the last 7d		0.836	0.267	3.128	2.307	1.366	3.895	0.002

Table 4 Results of univariable multilevel analysis for the outcome of CAPA resistance in 320 faecal samples from 200 dogs and cats in 5 hospitals in North West UK.

variable		beta	se	z-ratio	odds ratio	CI95low	CI95hi	p-value
Practice (Compared to 1)	Overall							0.001
	2	-0.695	0.448	-1.552	0.499	0.207	1.201	0.121
	3	-1.043	0.345	-3.025	0.352	0.179	0.693	0.002
	4	-1.263	0.493	-2.561	0.283	0.108	0.743	0.010
	5	-1.381	0.455	-3.034	0.251	0.103	0.613	0.002
Days hospitalised		0.117	0.035	3.369	1.124	1.05	1.203	0.001
Days hospitalised10		0.034	0.011	3.183	1.035	1.013	1.056	0.001
Environment contaminated with MDR		1.382	0.282	4.904	3.981	2.292	6.916	0.000
Environment contaminated with ESBL		1.584	0.3	5.281	4.876	2.708	8.78	0.000
Environment contaminated with AmpC								
Age		0.035	0.03	1.149	1.035	0.976	1.098	0.251
Breed (compared to small breed)	Overall							0.008
	medium	0.725	0.325	2.227	2.064	1.091	3.905	0.026
	large	0.179	0.352	0.509	1.196	0.6	2.383	0.611
	unidentified	1.633	0.568	2.877	5.119	1.683	15.568	0.004
Gender (compared to mn)	Overall							0.060
	m	-0.208	0.315	-0.661	0.812	0.438	1.505	0.509
	f	-0.399	0.439	-0.908	0.671	0.284	1.587	0.364
	fn	-1.005	0.381	-2.638	0.366	0.174	0.772	0.008
Previous hospitalisation		0.234	0.263	0.891	1.264	0.755	2.116	0.373
Case type (compared to ortho)	Overall							0.000
	Neurosurgery	1.628	0.42	3.874	5.095	2.235	11.614	0.000
	STS	1.675	0.429	3.905	5.337	2.303	12.369	0.000
	Medical	0.637	0.41	1.552	1.891	0.846	4.226	0.121
	unidentified	1.615	0.483	3.341	5.03	1.95	12.976	0.001
Xray performed		-1.005	0.363	-2.771	0.366	0.18	0.745	0.006
Ultrasound performed		-0.122	0.398	-0.307	0.885	0.406	1.929	0.759
MRI or CT performed		0.511	0.287	1.783	1.667	0.951	2.924	0.075
Any antimicrobial given in the last 3 months		0.892	0.269	3.318	2.44	1.441	4.131	0.001
Cefuroxime given in the last 3m		0.543	0.323	1.679	1.721	0.913	3.244	0.093
Amoxycillin given in the last 3m		1.056	0.587	1.798	2.876	0.909	9.094	0.072
CAPA given in the last 3m		0.514	0.256	2.006	1.672	1.012	2.762	0.045
Metronidazole given in the last 3m		0.774	0.367	2.11	2.168	1.056	4.447	0.035
Fluoroquinolone given in the last 3m		0.878	0.505	1.739	2.405	0.895	6.468	0.082
Cephalexin given in the last 3m		0.675	0.42	1.605	1.964	0.861	4.477	0.108
Clindamycin given in the last 3m		2.186	0.69	3.17	8.901	2.304	34.386	0.002
Cefuroxime given in the last 24h		x	x	x	x	x	x	x
Cefuroxime given in the last 48h		-0.535	0.599	-0.893	0.586	0.181	1.895	0.372
Cefuroxime given in the last 7d		-0.514	0.457	-1.125	0.598	0.244	1.465	0.261
CAPA given in the last 24h		0.504	0.309	1.631	1.655	0.903	3.033	0.103
CAPA given in the last 48h		0.436	0.302	1.443	1.546	0.856	2.792	0.149
CAPA given in the last 7d		0.75	0.274	2.737	2.118	1.237	3.625	0.006
Metronidazole given in the last 24h		0.479	0.488	0.981	1.614	0.62	4.201	0.326
Metronidazole given in the last 48h		0.479	0.488	0.981	1.614	0.62	4.201	0.326
Metronidazole given in the last 7d		0.269	0.451	0.597	1.309	0.541	3.171	0.551
Fluoroquinolone given in the last 24h		0.845	0.666	1.27	2.329	0.632	8.583	0.204
Fluoroquinolone given in the last 48h		1.032	0.635	1.625	2.807	0.808	9.749	0.104
Fluoroquinolone given in the last 7d		1.003	0.55	1.824	2.725	0.928	8.005	0.068
Cephalexin given in the last 24h		0.304	0.588	0.517	1.355	0.428	4.286	0.605
Cephalexin given in the last 48h		0.496	0.558	0.889	1.642	0.55	4.905	0.374
Cephalexin given in the last 7d		0.802	0.492	1.63	2.231	0.85	5.853	0.103
Any antimicrobial given in the last 24h		0.334	0.269	1.244	1.397	0.825	2.365	0.213
Any antimicrobial given in the last 48h		0.439	0.261	1.68	1.551	0.929	2.587	0.093
Any antimicrobial given in the last 7d		0.656	0.253	2.592	1.927	1.173	3.163	0.010

Table 5 Results of univariable multilevel analysis for the outcome of cephalosporin resistance in 320 faecal samples from 200 dogs and cats in 5 hospitals in North West UK.

variable		beta	se	z-ratio	odds ratio	CI95low	CI95hi	p-value
Practice (Compared to 1)	Overall							0.000
	2	-0.951	0.436	-2.183	0.386	0.165	0.907	0.029
	3	-0.356	0.297	-1.201	0.7	0.391	1.252	0.230
	4	-0.741	0.409	-1.814	0.477	0.214	1.062	0.070
	5	-2.323	0.554	-4.193	0.098	0.033	0.29	0.000
Days hospitalised		0.107	0.034	3.158	1.113	1.041	1.189	0.002
Days hospitalised10		0.14	0.04	3.514	1.15	1.064	1.244	0.000
Environment contaminated with MDR		1.065	0.248	4.293	2.9	1.784	4.715	0.000
Environment contaminated with ESBL		1.171	0.255	4.587	3.224	1.955	5.316	0.000
Environment contaminated with AmpC								
Age		0.001	0.028	0.031	1.001	0.947	1.058	0.975
Breed (compared to small breed)	Overall							0.030
	medium	0.607	0.305	1.99	1.835	1.009	3.338	0.047
	large	0.519	0.317	1.638	1.681	0.903	3.127	0.101
	unidentified	1.544	0.569	2.714	4.682	1.535	14.277	0.007
Gender (compared to mn)	Overall							0.170
	m	0.254	0.303	0.838	1.289	0.712	2.333	0.402
	f	-0.019	0.413	-0.046	0.981	0.437	2.204	0.963
	fn	-0.465	0.343	-1.355	0.628	0.32	1.231	0.175
Previous hospitalisation		0.115	0.249	0.463	1.122	0.689	1.827	0.644
Case type (compared to ortho)	Overall							0.005
	Neurosurgery	0.872	0.375	2.325	2.392	1.147	4.99	0.020
	STS	1.246	0.385	3.237	3.477	1.635	7.393	0.001
	Medical	0.323	0.346	0.933	1.381	0.701	2.722	0.351
	Unidentified	1.114	0.443	2.515	3.045	1.278	7.254	0.012
Xray performed		-0.465	0.298	-1.558	0.628	0.35	1.127	0.119
Ultrasound performed		0.222	0.36	0.615	1.248	0.616	2.528	0.538
MRI or CT performed		0.34	0.275	1.238	1.406	0.82	2.41	0.216
Any antimicrobial given in the last 3 months		0.856	0.249	3.443	2.354	1.446	3.833	0.001
Cefuroxime given in the last 3m		0.461	0.313	1.47	1.585	0.858	2.93	0.142
Amoxycillin given in the last 3m		1.338	0.625	2.142	3.811	1.12	12.968	0.032
CAPA given in the last 3m		0.743	0.245	3.036	2.102	1.301	3.396	0.002
Metronidazole given in the last 3m		0.721	0.363	1.985	2.056	1.009	4.189	0.047
Fluoroquinolone given in the last 3m		1.515	0.552	2.744	4.551	1.542	13.433	0.006
Cephalexin given in the last 3m		0.592	0.414	1.428	1.807	0.802	4.07	0.153
Clindamycin given in the last 3m		1.809	0.692	2.613	6.105	1.572	23.713	0.009
Cefuroxime given in the last 24h		-1.749	1.071	-1.633	0.174	0.021	1.42	0.102
Cefuroxime given in the last 48h		-0.331	0.524	-0.632	0.718	0.257	2.005	0.527
Cefuroxime given in the last 7d		-0.404	0.409	-0.987	0.668	0.299	1.489	0.323
CAPA given in the last 24h		0.797	0.299	2.67	2.219	1.236	3.984	0.008
CAPA given in the last 48h		0.679	0.289	2.347	1.972	1.118	3.476	0.019
CAPA given in the last 7d		0.957	0.267	3.587	2.603	1.543	4.391	0.000
Metronidazole given in the last 24h		0.737	0.478	1.542	2.09	0.819	5.336	0.123
Metronidazole given in the last 48h		0.737	0.478	1.542	2.09	0.819	5.336	0.123
Metronidazole given in the last 7d		0.61	0.431	1.417	1.841	0.791	4.283	0.156
Fluoroquinolone given in the last 24h		2.747	1.068	2.573	15.601	1.924	126.507	0.010
Fluoroquinolone given in the last 48h		2.85	1.055	2.702	17.286	2.187	136.61	0.007
Fluoroquinolone given in the last 7d		1.948	0.668	2.918	7.016	1.895	25.968	0.004
Cephalexin given in the last 24h		-0.109	0.585	-0.187	0.896	0.285	2.822	0.852
Cephalexin given in the last 48h		0.081	0.555	0.147	1.085	0.366	3.219	0.883
Cephalexin given in the last 7d		0.389	0.488	0.796	1.475	0.566	3.84	0.426
Any antimicrobial given in the last 24h		0.606	0.255	2.376	1.833	1.112	3.022	0.017
Any antimicrobial given in the last 48h		0.638	0.248	2.569	1.893	1.163	3.081	0.010
Any antimicrobial given in the last 7d		0.767	0.241	3.186	2.153	1.343	3.45	0.001

Table 6 Results of univariable multilevel analysis for the outcome of ESBL- producer in 320 faecal samples from 200 dogs and cats in 5 hospitals in North West UK.

variable		beta	se	z-ratio	odds ratio	CI95low	CI95hi	p-value
Practice (Compared to 1)	Overall							0.030
	2	-1.247	0.637	-1.958	0.287	0.082	1.001	0.050
	3	0.369	0.314	1.176	1.447	0.782	2.677	0.240
	4	-0.157	0.449	-0.35	0.855	0.355	2.059	0.726
	5	-2.762	1.023	-2.701	0.063	0.009	0.469	0.007
Days hospitalised		0.088	0.034	2.59	1.092	1.022	1.167	0.010
Days hospitalised10		0.116	0.042	2.746	1.123	1.034	1.22	0.006
Environment contaminated with MDR		0.408	0.276	1.479	1.504	0.876	2.584	0.139
Environment contaminated with ESBL		0.478	0.276	1.735	1.613	0.94	2.768	0.083
Environment contaminated with AmpC		0.496	0.28	1.768	1.642	0.948	2.845	0.077
Age		-0.009	0.033	-0.262	0.991	0.929	1.058	0.793
Breed (compared to small breed)	Overall							0.048
	medium	0.903	0.381	2.372	2.467	1.17	5.201	0.018
	large	0.886	0.392	2.261	2.425	1.125	5.224	0.024
	unidentified	1.336	0.6	2.226	3.803	1.173	12.332	0.026
Gender (compared to mn)	Overall							0.630
	m	-0.007	0.336	-0.02	0.993	0.514	1.92	0.984
	f	-0.248	0.474	-0.523	0.78	0.308	1.976	0.601
	fn	-0.437	0.39	-1.121	0.646	0.3	1.388	0.263
Previous hospitalisation		0.507	0.273	1.858	1.66	0.973	2.832	0.063
Case type (compared to ortho)	Overall							0.195
	Neurosurgery	-0.101	0.394	-0.256	0.904	0.418	1.956	0.798
	STS	0.405	0.429	0.945	1.5	0.647	3.477	0.345
	Medical	-0.627	0.432	-1.45	0.534	0.229	1.247	0.147
	Unidentified	-0.174	0.529	-0.33	0.84	0.298	2.367	0.742
Xray performed		0.113	0.324	0.35	1.12	0.594	2.114	0.726
Ultrasound performed		-0.146	0.431	-0.34	0.864	0.372	2.008	0.734
MRI or CT performed		0.474	0.295	1.604	1.606	0.9	2.864	0.109
Any antimicrobial given in the last 3 months		0.596	0.287	2.077	1.815	1.034	3.185	0.038
Cefuroxime given in the last 3m		0.39	0.348	1.122	1.478	0.747	2.923	0.262
Amoxycillin given in the last 3m		0.432	0.639	0.677	1.541	0.441	5.388	0.499
CAPA given in the last 3m		0.573	0.275	2.082	1.773	1.034	3.042	0.037
Metronidazole given in the last 3m		0.092	0.42	0.22	1.097	0.482	2.497	0.826
Fluoroquinolone given in the last 3m		1.761	0.527	3.344	5.818	2.073	16.333	0.001
Cephalexin given in the last 3m		0.179	0.471	0.381	1.197	0.476	3.011	0.703
Clindamycin given in the last 3m		0.792	0.596	1.328	2.208	0.686	7.106	0.184
Cefuroxime given in the last 24h		-1.009	1.085	-0.93	0.364	0.043	3.058	0.352
Cefuroxime given in the last 48h		0.218	0.549	0.397	1.243	0.424	3.644	0.692
Cefuroxime given in the last 7d		0.098	0.439	0.223	1.103	0.466	2.61	0.823
CAPA given in the last 24h		0.28	0.334	0.839	1.323	0.688	2.544	0.402
CAPA given in the last 48h		0.249	0.326	0.765	1.283	0.677	2.43	0.444
CAPA given in the last 7d		0.784	0.289	2.712	2.19	1.243	3.859	0.007
Metronidazole given in the last 24h		-0.168	0.587	-0.287	0.845	0.268	2.67	0.774
Metronidazole given in the last 48h		-0.168	0.587	-0.287	0.845	0.268	2.67	0.774
Metronidazole given in the last 7d		-0.171	0.53	-0.323	0.843	0.299	2.38	0.747
Fluoroquinolone given in the last 24h		2.335	0.733	3.185	10.326	2.455	43.434	0.001
Fluoroquinolone given in the last 48h		2.022	0.666	3.036	7.556	2.048	27.881	0.002
Fluoroquinolone given in the last 7d		1.606	0.566	2.838	4.983	1.644	15.107	0.005
Cephalexin given in the last 24h		-0.602	0.792	-0.76	0.548	0.116	2.588	0.448
Cephalexin given in the last 48h		-0.693	0.79	-0.877	0.5	0.106	2.352	0.380
Cephalexin given in the last 7d		-0.112	0.594	-0.189	0.894	0.279	2.864	0.850
Any antimicrobial given in the last 24h		0.192	0.291	0.661	1.212	0.685	2.143	0.509
Any antimicrobial given in the last 48h		0.24	0.283	0.849	1.272	0.73	2.215	0.396
Any antimicrobial given in the last 7d		0.651	0.274	2.373	1.917	1.12	3.281	0.018

Appendix V: GAM plots

Fig. 1a to 1e. Graphs representing the functional forms of the continuous variable days hospitalised modelled in a generalised additive model (where the continuous fixed effects are fitted using smoothers) to determine the shape of the relationship between the predictor variable and the outcome (log odds of different resistance outcomes). The plots show the fitted curves with 95% confidence intervals (dashed lines). The rug plots along the x-axis represent the number of data points. The P-value is a chi-square test for non-linearity.

Fig 1a. MDR

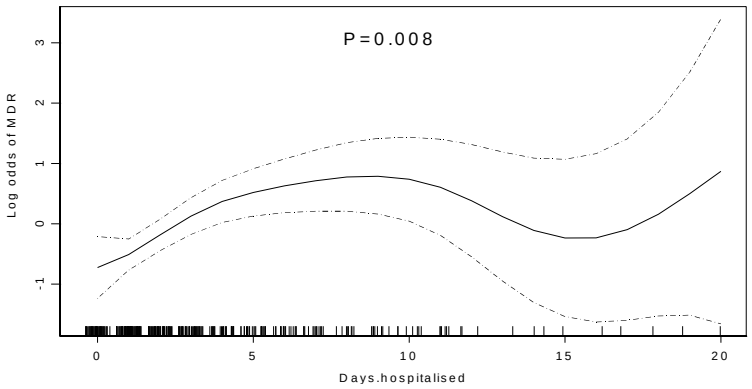


Fig 1b Ciprofloxacin

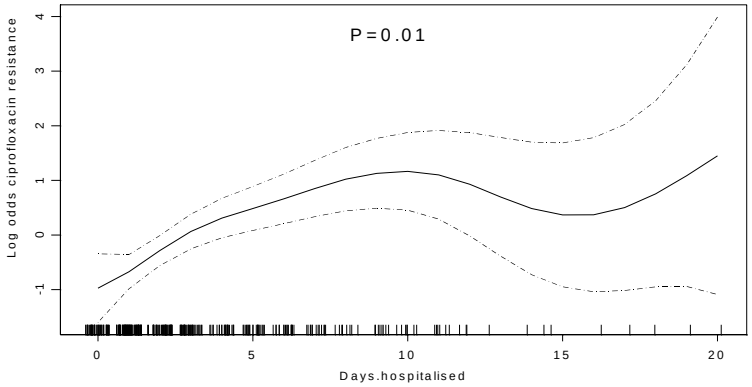


Fig 1c. 3rd/4th Generation Cephalosporin Resistance

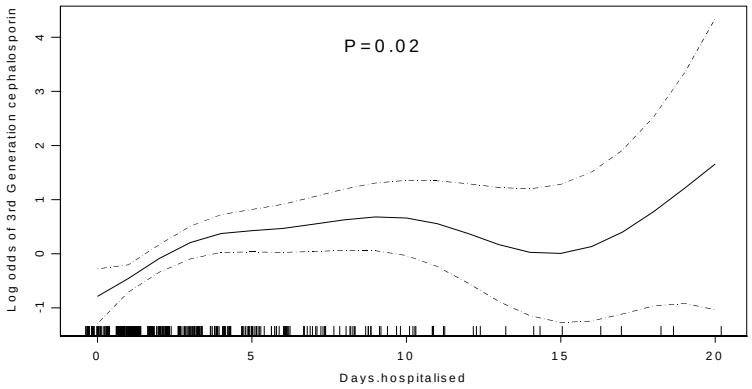


Fig 1d. ESBL Phenotypic resistance

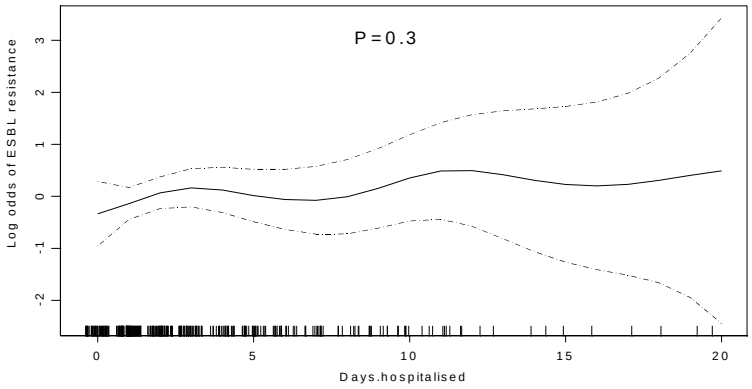


Fig 1e. AmpC (by PCR)

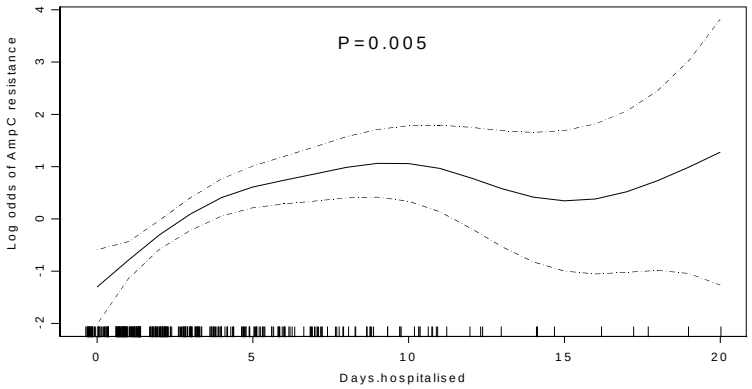


Fig 1f. CAPA Resistance

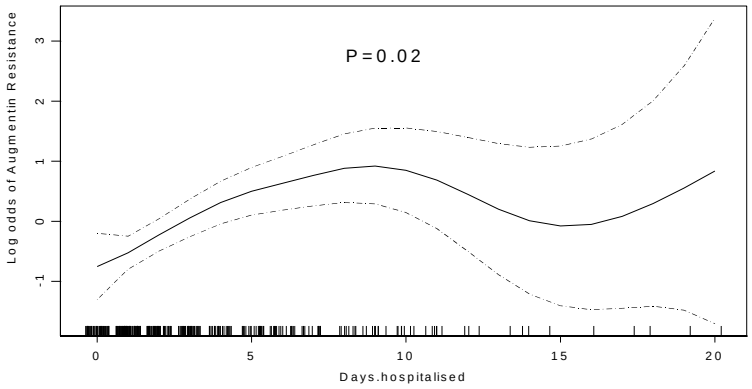


Fig. 2a to 2f . Graphs representing the functional forms of the continuous variable age modelled in a generalised additive model (where the continuous fixed effects are fitted using smoothers) to determine the shape of the relationship between the predictor variable and the outcome (log odds of different resistance outcomes). The plots show the fitted curves with 95% confidence intervals (dashed lines). The rug plots along the x-axis represent the number of data points. The P-value is a chi-square test for non-linearity.

Fig 2a. MDR

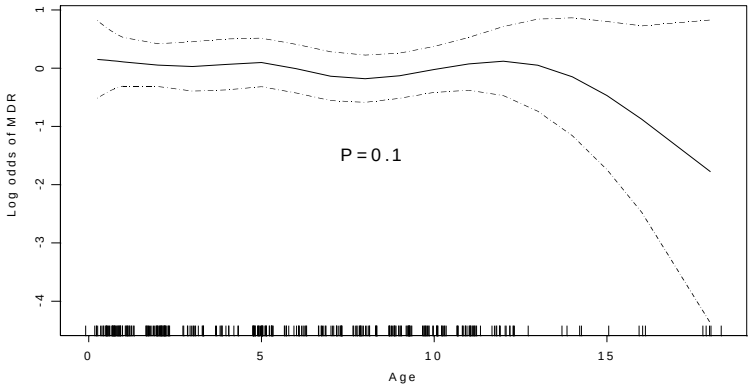


Fig 2b Ciprofloxacin

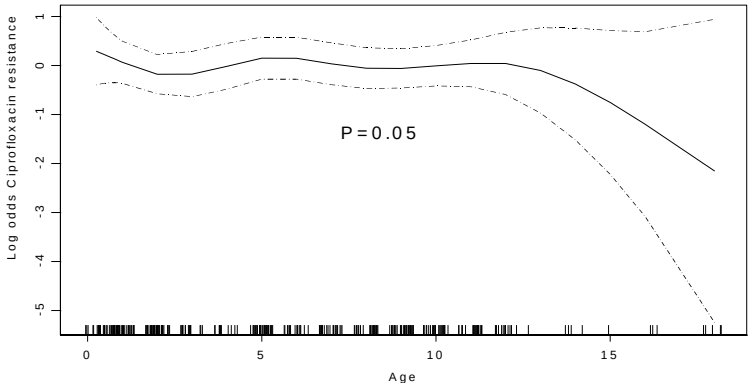


Fig 2c. 3rd/4th Generation Cephalosporin Resistance

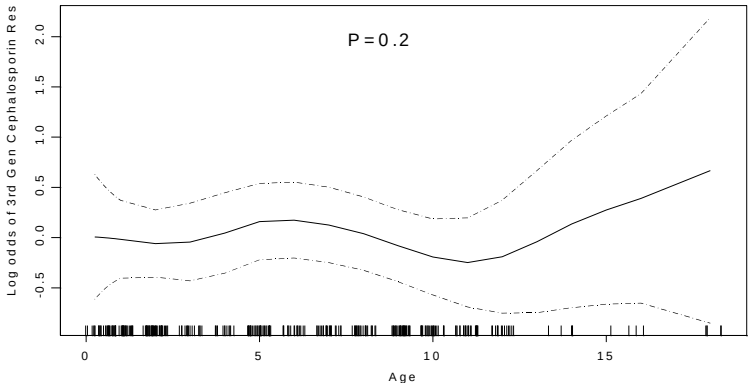


Fig 2d. ESBL Phenotypic resistance

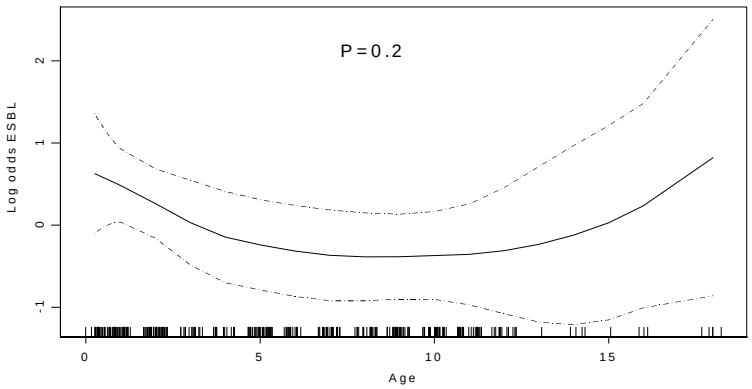


Fig 2e. AmpC (by PCR)

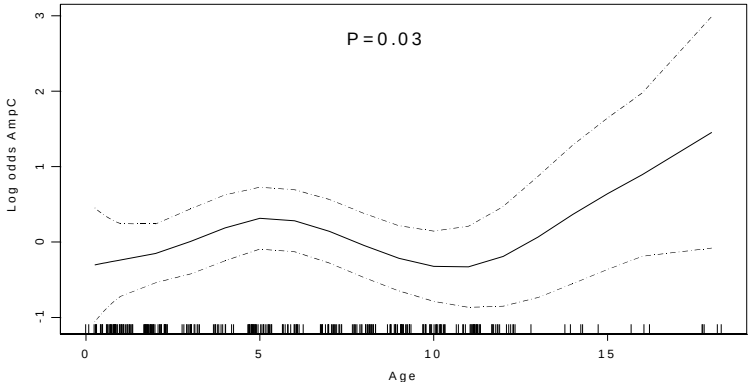
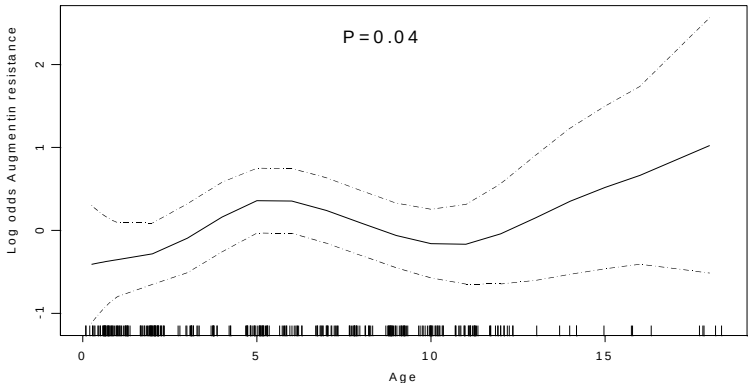


Fig 2f. CAPA Resistance



Appendix VI – Sampling guidelines for practices

Sampling Plan – preliminary information for practices

Thank-you for your assistance in taking samples for this study, your help is very much appreciated. Our study aims to help understand the factors affecting the prevalence of some antibiotic resistant bacteria in referral hospitals in the North-West. By participating you are helping increase our understanding of the development and spread of antibiotic resistance within veterinary practice. We will also be taking some samples from your hospital, which will be used to give your practice some information about the potential for colonisation with antibiotic resistant bacteria. However, your practice will not be identified in any subsequent publications.

We aim to keep sampling as simple as possible and minimise the amount of time it takes. Please let us know if you have any problems as we want to make this as easy as possible for you.

We plan to carry out sampling over several periods, which we will arrange with your practice. Our sampling plan is:

- On sampling days we would like you to collect a faecal sample from ALL cats and dogs that have passed faeces (where the owners have consented) while hospitalised in your practice (for more than 24 hours) and place it in the bags provided. Please use a different bag for each sample using the labels provided to identify the individual animal (by name and case number) and the date it was collected.

- Please only collect one sample per day from each animal that passes faeces.

- We appreciate many animals will only be staying for a short period and thus may only provide one sample but collection of daily samples from animals hospitalised over longer periods is also required.

- Please store samples in a fridge or cool box which can be provided

- During each sampling period we will visit your hospital twice/three times weekly to both collect the faecal samples and take the environmental samples.

Once again thank-you for participating, your assistance is very much appreciated and without it this study would not be possible. Please contact us if there are any problems

Ian Tuerena MRCVS (Ian.Tuerena@liv.ac.uk)

Dr Gina Pinchbeck MRCVS (ginap@liv.ac.uk)

Dr Tim Nuttall MRCVS (timn@liv.ac.uk)

Other contact details:

Mobile: 07872315340 (weekdays 9-5)

Address: The University of Liverpool School of Veterinary Science, Leahurst Campus , Chester High Rd, Neston, CH64 7TE

Independent Complaints Procedure

If you have any concerns about the study or its conduct that we cannot satisfactorily resolve or you feel that you cannot ask us, you can use the university complaints procedure. Complaints should be addressed to the Research Governance Officer in Research and Business Services (RBS) – email: ethics@liv.ac.uk; tel.: 0151 794 8727. The information on this sheet will enable them to identify the study and the investigators.

Appendix VII – Combined information and consent form for owners

Detection of antibiotic resistant bacteria in animal faecal samples: Participant Information Sheet and informed consent form

Please read the following information carefully. You may also request a copy for yourself.

Dear dog or cat owner,

Your veterinary surgeon has kindly agreed to help The University of Liverpool Veterinary School with a new study looking at antibiotic resistant bacteria. As part of this study, you and your animal(s) are invited to take part. We would be very grateful if you would allow us to take some samples from your dog or cat whilst it is in hospital, and allow us to record some data on your animal and its treatment.

Please read the following information carefully and please ask if you would like more information or if there is anything you do not understand. Your vet may be able to answer some questions; otherwise my contact details are at the end of this letter. We would like to emphasise that you do not have to accept this invitation and you should only agree to take part if you want to. If you decide not to participate this will **not** affect the care and treatment of your animal

Why are we getting these samples?
All animals carry bugs (such as bacteria) in their guts (and other places too). Most of them cause no problem, but some cause infections that need treatment with antibiotics. Bacteria that are not killed by antibiotics (antibiotic resistant bacteria) are now becoming more of a concern in animal and human medicine, especially in hospitals. We are trying to see how much antibiotic resistance there is in the normal bacteria that animals carry. This will give us a greater understanding of how antibiotic resistance occurs in hospitals and hopefully can lead to the development of new ways to combat the problem. This is especially important for our animals, as increasing antibiotic resistance could result in real problems treating infections in the future.

What samples are we collecting?
For this study we are interested in obtaining samples of the poo (faecal samples) that your cat or dog does whilst in hospital.

What does this involve?
A faecal sample (poo sample) will be collected from your cat or dogs kennel (if available), or picked up off the ground when your dog is taken outside, and collected or posted back to us at the University of Liverpool. If your animal is hospitalised for several days we may collect more than one sample. We would also like obtain data on your dog or cat from the records your veterinary surgeon holds, such as the reason for hospitalisation and what treatment your animal has had. Your dog or cat will not be affected by the collection of their faeces in any way.

Further information
Samples and the information obtained may be retained for up to seven years and possibly used in future projects. All data will be kept strictly confidential and will be stored in a secured database accessible only by people working on the project. If you decide you want to withdraw from the study you may do so without explanation, and any information you have given can be destroyed.

Results from the study will be printed in veterinary journals and the non-veterinary animal press, but no-one will be identifiable from any published work.

Is there an independent complaints procedure?
Yes - if you have any concerns about the study, its conduct or the investigators that we cannot satisfactorily resolve or you feel that you cannot ask us, you can use the university complaints procedure. Complaints should be addressed to the Research Governance Officer in Research and Business Services (RBS) – email: ethics@liv.ac.uk; tel.: 0151 794 8727. The information on this sheet will enable them to identify the study and the investigators.
The Research Governance Officer will document the complaint and refer it to the Chair of the relevant sub-committee or departmental committee within two working days. The Chair is responsible for investigating the complaint and for responding to you within two weeks.

What next?
If you are happy to allow your dog or cat to become involved, then please read and sign the consent form, and the vet can start getting the samples. Please note that due to the large number of samples involved in this project we will not be able to give you back any individual results from your animal.

Many thanks,

Dr Gina Pinchbeck MRCVS

Consent form: "Detection of antibiotic resistant bacteria in hospitalised animal faecal samples."

Researchers: Gina Pinchbeck/Ian Tuerena/Tim Nuttall/Nicola Williams

Please initial box

1. I confirm that I have read and have understood the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected. If I do not participate this will **not** affect the care and treatment of my animal. ☐

3. I understand that, under the Data Protection Act, I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish. ☐

4. I allow participation of my animal in the above study. ☐

If you agree with the above-stated conditions please sign below:-

Participant Name	Date	Signature
Name of Person taking consent	Date	Signature
Researcher	Date	Signature

The contact details of lead Researcher (Principal Investigator) are: [Contact: Gina Pinchbeck, Leahurst campus, University of Liverpool, Neston, Wirral, CH64 7TE, telephone: 0151 794 6195, email: ginap@liverpool.ac.uk]. If there are any problems, please let us know and we will try to help.