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The Molecular Epidemiology of Multi-Drug Resistant *Escherichia coli* in Companion Animal Species

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Declaration

The research presented in this thesis is entirely my own work unless otherwise stated in the text. The material contained in this thesis has not been submitted for any other degree or professional qualification. The work presented in Chapter 2 of this thesis was published in an academic journal (Wagner et al., 2014). At the time of submission, the work presented in Chapter 3 of this thesis had been accepted for publication; and has since been published (Wagner et al., 2017). Both papers have been included in the appendix. The genomic sequences presented here may be accessed through the following sequence accession numbers: SMRT sequences PRJNA402083, Illumina sequences PRJEB11950.

Samuel Wagner
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Lu Zen, for never refusing to answer a question no matter how dumb it sounded.
Dedication

All of this is dedicated to Tom, Sally and Emma Wagner. Who needs the shoulders of giants with the three of you standing next to me.

To the memory of ‘JC’

P.S. and to Hazel and the city of Boston.
Abbreviations

AHVLA Animal Health Veterinary Laboratories Agency

AMPB AMPure® Beads

AMR antimicrobial resistance

CDC Center for Disease Control

CLSI Clinical Laboratory Standards Institute

CRE carbapenem resistant Enterobacteriaceae

DEAEC diffuse entero-aggregative E. coli

DoH Department of Health

ECDC European Center for Disease Control

EHEC enterohemorrhagic E. coli

EPEC enteropathogenic E. coli

ESAC extended-spectrum AmpC

ESBL extended-spectrum β-lactamase

ETEC enterotoxigenic E. coli

EU European Union

ExPEC extra-intestinal E. coli

HAI healthcare-acquired infections

HGEs horizontal genetic elements

HGT horizontal gene transfer

JBM Jacoby-Bush-Medeiros
kbp  kilo base pairs
LB  lysogeny broth
MDR  multi-drug resistant
MLST  multi-locus sequence-typing
MRSA  methicillin-resistant \textit{Staphylococcus aureus}
Mb  mega bases
NCBI  National Center for Biotechnology Information
NDM  New Delhi metallo-$\beta$-lactamase
NGS  next-generation sequencing
NMEC  neonatal meningitis \textit{E. coli}
OD  optical density
OIE  World Organisation for Animal Health
PBP  penicillin-binding protein
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PDR  pan drug resistant
PFGE  pulsed-field gel electrophoresis
QUAST  Quality Assessment Tool
RAST  Rapid Annotations based on Subsystem Technology
SAVSNET  Small Animal Veterinary Surveillance Network
SMRT  single-molecule real-time sequencing
SNP  single nucleotide polymorphism
SRST  short read sequence typing
ST  sequence type
UPEC  uropathogenic \textit{E. coli}
UTI urinary tract infections
VARSS Veterinary Antimicrobial Resistance and Sales Surveillance
VFDB Virulence Factor Database
VMD Veterinary Medicines Directorate
VRE vancomycin-resistant *Enterococcus*
WGS whole genome sequencing
WHO World Health Organisation
XDR extremely drug resistant
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Lay Summary

Antibiotic resistance is a growing concern in veterinary care. Not only do antibiotic resistant bacteria cause significant harm to animals, they also increase the risk of antibiotic resistant infections in humans. There is a growing body of evidence to suggest that antibiotic resistance is associated with particular types of *E. coli*; antibiotic resistant bacteria tend to associate less strongly to *E. coli* that are harmful to humans and animals. Instead they associate with *E. coli* that are commonly found in healthy humans and animals. Bacteria in general, and especially *E. coli*, are extremely adept at sharing DNA, including the DNA which encodes for antibiotic resistance.

This project utilized *E. coli* that had been collected from canine patients at the Small Animal Hospital, Royal (Dick) Veterinary School. Specifically, *E. coli* collected from urinary tract infections in dogs that were resistant to three or more different antibiotics, were compared against *E. coli* collected from urinary tract infections in dogs that were not resistant to any antibiotics. At the outset of this study it was not known how the *E. coli* had acquired their antibiotic resistance; or how easily this resistance could be transferred.

The experiments in this study were used to help define the relationship between the genes causing the antibiotic resistance and the *E. coli* carrying the resistance. Ultimately the work focused on particular molecules of DNA called plasmids which were found to be wholly responsible for spreading the antibiotic resistance in these isolates at the Small Animal Hospital, Royal (Dick) Veterinary School.
Abstract

This study focused on a group of multi-drug resistant (MDR) E. coli collected between 2002 and 2011 at the Small Animal Hospital, Royal (Dick) Veterinary School; isolated from urinary tract infections in dogs. The MDR E. coli isolates were compared against a group of fully susceptible E. coli, also collected from canine urinary tract infections.

The MDR isolates were notable for their AmpC \( \beta \)-lactamase resistance phenotype (12/18) and resistance genotype (\( \text{bla}_{\text{CMY-2}} \), 8/12). Phylogenetic comparisons between the MDR and susceptible groups of isolates showed a large degree of separation between the two groups. Susceptible isolates strongly associated the the pathogenic B2 phylogroup (67%), which was expected. The MDR group was much more mixed, with isolates more associated with commensal A (28%) and B1 (22%) phylogroups.

Virulence marker abundance, evaluated using an Identibac microarray, showed a much reduced presence of virulence markers in the MDR group, as compared with the susceptible isolates. An infection model, using Galleria mellonella larvae, was used to better test the phenotypic virulence of the MDR and susceptible E. coli isolates. An asymptomatic bacteriurea strain (ABU83972) and a virulent pyelonephritis strain (CFT073) were used as non-virulent and virulent control strains of uropathogenic E. coli. The model produced consistent and expected differences in lethality between ABU8392 (non-lethal) and CFT073 (lethal) strains. However, the model showed no differences between the MDR and susceptible isolates.

Second generation Illumina short-read sequencing and third generation single-molecule real-time sequencing were used to further dissect the genetic background of both the E. coli and the MDR genotype. Core-genomic sequence comparisons of the E. coli showed no clonal relatedness amongst the different MDR and susceptible strains. Most strikingly, sequencing revealed extensive plasmid carriage of the MDR genotype.

Plasmid mediated \( \text{bla}_{\text{CMY-2}} \) was associated with a clonal group of IncI1 plasmids. The IncI1 plasmids show favorable sequence comparisons to a plasmid backbone previously reported in dogs and other animals, with a global distribution. The remaining resistance genes were associated with a group of IncFII
plasmid sequences. This study highlights a broader range of commensal *E. coli*, which have resulted in opportunistic infections in their canine hosts. As such, they act as a potential reservoir of resistance, which is as yet uncharacterized.
Aims

- To characterize the resistance phenotype and genotype of multi-drug resistant *Escherichia coli* isolated from urinary-tract infections.

- To determine if antimicrobial resistance provides the main advantage, allowing establishment of infection in an antimicrobial environment.

- To determine if multi-drug resistant urinary-tract infection *Escherichia coli* strains are atypical of uropathogenic *E. coli*, in that they are less virulent.

- To determine if multi-drug resistance status, particularly plasmid acquisition, contributes to virulence in either a positive or negative manner.

- To determine if resistance phenotypes are co-selected with virulence factors or virulence regulation.

- To determine if multi-drug resistance characteristics are detrimental for virulence with multi-drug resistance strains generally being avirulent with expansion from a maintained low-level commensal population solely based on antimicrobial use.
Chapter 1

Introduction

1.1 Road map

A bacterial multi-drug resistant (MDR) isolate is one that is resistant to at least one antimicrobial from three or more classes of antimicrobials. Such isolates are often the result of the accumulation of separate antimicrobial resistances and pose a significant clinical challenge (Magiorakos et al., 2012). Between 2006 and 2011 a number of MDR *E. coli* infections were identified by the Veterinary Microbiology Laboratory, Royal (Dick) Veterinary School. Despite their sporadic nature, these infections were extensively MDR, and notable for their extended-spectrum β-lactamase (ESBL) resistance. These infections were isolated from a number of different animals (canine, feline and equine), and had differing clinical presentations:

- Wound abscesses
- Urinary tract infections
- Gastro-intestinal infections
• Oral infections

• Skin infections

All of the animals had been undergoing complex treatments at the time the infections had been diagnosed; and many of the patients had been prescribed prophylactic antibiotics. It was not known if they were part of an ongoing outbreak, or the consequence of modern veterinary clinical care. Neither MDR infections or ESBL infections are commonly identified. Of the 28 MDR infections initially identified, urinary tract infections (UTIs) were the largest singular group of infections (12/28). It was also the only group of infections that had a suitable comparator group of fully susceptible isolates that could be used to perform comparative studies.

Out the outset it was not known if there was a potential community-associated reservoir of resistance that the patient had been exposed to. No studies into the molecular epidemiology of UTIs, MDR or otherwise, had been conducted. MDR is a consequence of the persistence and accumulation of antimicrobial resistance (AMR) within the clinical environment. It is influenced by a number of confounding factors:

• The antibiotics prescribed routinely; which act as selection pressures promoting the spread of acquired resistances.

• The bacteria expressing the acquired resistances. Bacterial pathogens, with diverse ecological niches, affect the diversity and prevalence of AMR.

• The transferability of resistance beyond a single bacterial host, or patient. The endemic, or epidemic presence of AMR within the clinical environment is influenced by the transferability of AMR between different bacterial populations and also other mammalian hosts.
• The etiology of the infections and health status of the host prior to AMR, or MDR infection. The duration and complexity of antibiotic chemotherapy (prophylactic or not) and any underlying patient susceptibility to infection which may increase the persistence of AMR and MDR in the clinical environment.

Whereas there is a considerable body of published research detailing the effects and affects of antibiotic chemotherapy and developing resistance in human clinical care; little, if any, comparable research exists for canine veterinary AMR or any companion animals. As little is known about companion animal AMR, and almost nothing was known about the MDR *E. coli* isolates identified at the outset of the work presented here, this project focused on the following aims:

• To characterize the resistance phenotype and genotype of MDR *E. coli* isolated from the UTIs.

• To determine if AMR is the primary niche adaptation promoting the occurrence of these infections in the clinical setting.

• To determine if the MDR *E. coli* causing UTIs identified in this study are atypical of uropathogenic *E. coli* (UPEC); in that they are less virulent.

• To determine if MDR status, particularly plasmid acquisition, contributes to virulence in either a positive or negative manner.

• To determine if resistance phenotypes are co-selected with virulence or virulence regulation.

• To determine if the acquisition of MDR favor a particular *E. coli* genetic background; either from the intrinsic gut flora (commensal *E. coli*), or extrinsic pathogenic *E. coli*. 
To better frame the work laid out in this thesis, the introduction will expand on the following topics:

- The antibiotics used to treat canine UTI’s. With a particular focus on the β-lactams, given the preponderance of ESBL resistance:
  - The antibiotics used to treat companion animals.
  - The sub-set of antibiotics used to treat UTI

- The known epidemiology of AMR and MDR that arises from the clinical use of antibiotics used in veterinary care:
  - MDR and known clinical epidemiology.
  - β-lactamase and ESBL resistance.

- The intersection between AMR, MDR and UPEC; with a focus on the trade-offs between the acquisition of pathogenic and MDR conferring elements.
  - UTI
  - UPEC
  - Urovirulence and AMR

- The wider impacts of veterinary MDR isolates, including One Health.

1.2 Background

The progressive increase of world-wide incidence of AMR bacterial infections is now the biggest problem facing modern health care. The World Health Organisation (WHO) cites a growing disconnect between effective national poli-
cies, and attempts to control the spreading epidemic. WHO policy statements attach particular importance to stopping the inappropriate or blanket use of antibiotics and the concomitant stagnation of pharmaceutical development of novel antibiotics. The poor communication of the health risks posed by AMR and MDR is compounded by the academic and clinical focus aimed at containing AMR and MDR remaining at the institutional level, rather than at a national level for the majority of countries (http://www.who.int/world-health-day/2011/presskit/whd2011_fs1_natplan.pdf?ua=1).

The Center for Disease Control (CDC) now estimates that 2 million US nationals now contract a bacterial infections which is AMR; of which 23,000 will dies as a direct result. Of the 140,000 healthcare-acquired infections (HAI)’s attributable to the Enterobacteriaceae, 26,000 will harbor ESBL resistance; leading to 1,700 deaths (https://www.cdc.gov/drugresistance/biggest_threats.html).

MDR, extremely drug resistant (XDR), and pan drug resistant (PDR) bacterial isolates from across a spectrum of genera and species are of utmost concern to the international health care community (Magiorakos et al., 2012).

Notable and well published examples of the spread of AMR include: MDR tuberculosis and methicillin-resistant Staphylococcus aureus (MRSA). More recent and emerging AMR bacteria such as: Clostridium difficile, vancomycin-resistant Enterococcus (VRE), carbapenem resistant Enterobacteriaceae (CRE), and Neisseria gonorrhoeae. These organisms are no longer treatable using first-line antibiotics.

These concerns have been re-iterated by the European Center for Disease Control (ECDC). Regular resistance surveillance recorded increased prevalence of AMR in gram-negative bacteria (with emphasis on E. coli and Klebsiella pneumoniae), as well as worrying increases in the numbers of MDR ESBL pro-
Figure 1.1: Available data for the prevalence of third generation cephalosporin resistance reported to the ECDC by participating member states in *E. coli* Europe for the year 2000.
Figure 1.2: Available data for the prevalence of third generation cephalosporin resistance reported to the ECDC by participating member states in *E. coli* Europe for the year 2013
The WHO, CDC and ECDC emphasize the importance of expanding surveillance efforts in response to the developing threats of AMR and MDR; as well as improving stewardship and expanding efforts towards the pharmaceutical development of novel antibiotic compounds. Fundamentally, if not addressed, the increasing incidence of AMR can drive increases in MDR, and eventually XDR and PDR. Though still rare, there now incidences of AMR infections resistant to all available treatment options (Jain and Mondal, 2008).

The increasing prevalence of AMR infections seen in human clinical care is also influenced by the use of antibiotics in animals. The UK Department of Health (DoH) has described veterinary AMR infections as a reservoir of resistant organisms from which MDR strains can be selected for, and maintained. The transfer of AMR between humans, animals and the environment they share indicates that surveillance for resistance in just the human clinical setting is inadequate; and that national surveillance must include animals. Despite this, national AMR surveillance, particularly in companion animals, has been only partially implemented (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/447319/One_Health_Report_July2015.pdf).

Though recent efforts by the UK government, through the Veterinary Medicines Directorate (VMD), have acted to reduce the number and frequency of antibiotic prescriptions in animals; combined, food producing and companion animals antibiotic prescriptions amounted to 337 tonnes of antibiotics for 2016 (https://www.gov.uk/government/publications/veterinary-antimicrobial-resistance-and-sales-surveillance-2016).

Improved epidemiology of animal-derived AMR, in conjunction with human clinical data, provides a greater understanding of the potential future impacts of AMR to: drug discovery and pharmacological developments in antibiotic treat-
ments (Shryock, 2004); the changing effectiveness of antibiotics and the impact this has on animal welfare (Mateu and Martin, 2001, Mateus et al., 2011, Wayne et al., 2011); assess the frequency and efficiency of the transfer of AMR between humans and animals (Guardabassi et al., 2008, 2004, Phillips et al., 2004). Furthermore, the study of developing mechanisms of AMR in animals can be used as a model for improving patient outcomes in human clinical care (Mateu and Martin, 2001). The increased attention that animal derived AMR is now receiving is vital in protecting the increasingly vulnerable classes of antibiotics that form the basis for the majority of clinical interventions.

1.2.1 Companion Animal Antibiotics

Few developments in the modern health care can eclipse the impact that antibiotics have had on improving clinical treatment outcomes. Along with improved sanitation and general hygiene, the synthesis of targeted antimicrobial compounds has saved an inestimable number of lives (Aminov, 2010).

Though the continuing development of antibiotics is mostly influenced by human clinical care, the veterinary care of companion animals relies on the same classes of antibiotic compounds; with only few exceptions.

The functional diversity of the different classes of antibiotic, along with synthetic modifications made to individual antibiotics within the different classes; create a spectrum of activity against different bacterial isolates. This affects the applicability of a particular antibiotic to a bacterial infection. Inappropriate class choices, or the usage of an antibiotic whose spectrum of activity does not include the causative bacterial organism risk the development of AMR.

Despite the diversity of the available antibiotic classes, the development of
antibiotics has not kept up with developing AMR. The decreased rate of discovery of novel antibiotics compounds has coincided with an increasing prevalence of AMR; which increases the vulnerability of first and second-line antibiotics to resistance.

As the diversity of available therapeutic options has been reduced, the selective pressures which favor the aggregation of AMR into MDR have increased due to the over-use of particular antibiotics.

This process is slow and difficult to reverse, and further increases the risk of the development of AMR against newer antibiotics (Levy and Marshall, 2004). The stewardship and general prescribing practices of antibiotics used in veterinary care is not completely known.

The most recent Veterinary Antimicrobial Resistance and Sales Surveillance (VARSS) report for UK (2016) https://www.gov.uk/government/publications/veterinary-antimicrobial-resistance-and-sales-surveillance-2016 does include prescription and resistance data for companion animals (including dogs). However the report has several shortcomings which limit its use:

- Prescriptions are inferred from total antibiotic sales and are likely overestimated.

- Companion animal resistance is only recorded when the bacterial isolates are considered a direct threat to public health; estimates are therefore likely underestimated.

The VARSS report for companion animals includes data collected in a separate study (Buckland et al., 2016), indicating that 25% of total dog visits to veterinary practices resulted in the prescription of one or more antibiotics between 2012 and 2014. The most commonly prescribed antibiotics being either aminopenicillin
or cephalosporins. Though 60% of the prescriptions included antibiotics listed as vital to human clinical care (fluoroquinolones, macrolides and third-generation cephalosporins).

Along with the other classes of antimicrobials listed in the VARSS report, the β-lactams are a sizable component of the veterinary prescriptions. This is in concurrence with a previous report into companion animal prescriptions in the UK (Mateus et al., 2011), which highlighted the use of penicillins (amoxicillin-clavulanate, amoxicillin) and the cephalosporins (cephalexin and cefovecin) as being commonly prescribed to companion animals (dogs and cats).

The VARSS report along with data collected by the World Organisation for Animal Health (OIE) [https://www.oie.int/fileadmin/Home/eng/Specific_Issues/docs/pdf/OIE_list_antimicrobials.pdf](https://www.oie.int/fileadmin/Home/eng/Specific_Issues/docs/pdf/OIE_list_antimicrobials.pdf) details the extent to which veterinary care is reliant on antibiotics which have limited, or no, current alternatives:

- **Aminoglycosides**: used to treat septicemia, gastrointestinal, respiratory and urinary tract infections. Widely used and few to no alternatives.

- **Cephalosporins (all generations)**: used to treat septicemia, respiratory infections and mastitis. Therapeutic alternatives are more limited in spectrum or cannot be used due to prevalent AMR.

- **Penicillins**: used to treat septicemia, respiratory and urinary tract infections. Alternatives are limited and more expensive.

- **Fluoroquinolones**: used to treat septicemia. Widely used with few alternatives.

- **Potentiated-sulfonamides**: used to treat a wide variety of bacterial infections and can be used combinatorially. Alternatives are limited and more
expensive.

- Tetracyclines: Are applied to a broad spectrum of bacterial infections. Alternatives are limited and more expensive.

Apart from the vulnerability of several of these classes of antibiotics to developing resistance in animals; all of the antibiotics listed are also indicated for use in humans. The usage of antibiotics to treat animals that are also used to treat humans risks the selection of resistant bacteria capable of posing challenges to both human and veterinary care (de Jong et al., 2012).

While there is some debate over the direct risk to humans resulting from animal derived AMR; studies reporting such transfers exist for food producing animals (Founou et al., 2016, Lambrecht et al., 2018), and in companion animals (Schauffler et al., 2015, Somayaji et al., 2016); including direct transmission between pets and their owners (Guardabassi et al., 2004, Harada et al., 2012). These reports suggest that AMR in companion animals can directly contribute to the antibiotic resistance reservoir which may impact human health.

1.2.2 β-Lactam Antibiotics

The β-lactams are perhaps the most commercially successful antimicrobial class; represented by several hundred different compounds. The group comprises several sub-groups including the penicillins, cephalosporins, carbapenems and monobactams. Regardless of their chemical diversity, they possess the same antimicrobial property: the inhibition of cell wall synthesis by binding of the penicillin-binding protein (PBP)s.

The success of the β-lactams as therapeutic compounds, is in part, due to the properties of the molecules which they target. They do not target a single
bacterial protein, but the whole family of PBPs. This makes successful bacterial resistance by point mutation alteration of particular PBPs less likely. The PBPs are also relatively easily accessible; and cytosolic uptake of the drug compounds by the bacteria is not necessary (Schneider and Sahl, 2010).

As well as their good pharmacokinetic properties, they are well tolerated by the patient population; with low toxicity and only moderate concern for the rare development of patient hypersensitivity to them. They have a broad spectrum of activity against Gram negative and Gram positive bacteria, and are very often used empirically as first-line antimicrobials of choice to treat the majority of infections (Finch et al., 2003).

However, such use has not been without consequence. The widespread and frequent usage of the \(\beta\)-lactams has been strongly correlated with increasing resistance amongst Gram-negative bacteria, in particular. Continued clinical use of the \(\beta\)-lactams has led to a concomitant rise of acquired and vertically-inherited resistance, primarily associated with \(\beta\)-lactamase resistance genes. Such resistance (primarily recorded in humans) has been observed in the community and in health care associated infections (Davies and Davies, 2010).

1.2.3 \(\beta\)-Lactamases

\(\beta\)-lactam antibiotics inhibit bacterial cell-wall synthesis by binding to and inactivating the PBPs, which are required for the assembly and maintenance of the peptidoglycan layer (Morar and Wright, 2010). Enterobacteriaceae resistance to the \(\beta\)-lactams can occur via:

- Increased drug efflux.
- Decreased PBP binding affinity.
Enzymatic degradation of the β-lactam molecule by the β-lactamases.

Of these different resistance mechanisms, the production of β-lactamase enzymes, by the bacteria, is most important (Rubin and Pitout, 2014).

The enzymatic inactivation of the β-lactam antibiotics by the β-lactamases occurs by the hydrolytic cleavage of the chemically active β-lactam ring. This can occur via two distinct chemical pathways, which is the foundation for the classification systems used to classify and differentiate individual β-lactamase enzymes (Morar and Wright, 2010).

The Ambler classification system divides the various sub-groups of β-lactamase enzymes based on amino acid sequence (Ambler, 1980). The Ambler classification scheme identifies four main classes of β-lactamase (A, C, D and B). Classes A, C and D are separated from class B by the presence of a catalytic serine residue in the active site. The active sites of class B enzymes (also referred to as metallo-enzymes) contain a catalytically active metal ion.

A more modern and more functional system, the Jacoby-Bush-Medeiros (JBM) classification, was introduced to address the paucity of available amino acid sequence data which the Ambler classification relies on. Under the JBM, β-lactamase enzymes are grouped by their ability, or inability to hydrolyze different β-lactams and the rate by which hydrolysis occurs (Bush et al., 1995). The JBM classification is more readily capable of accommodating novel β-lactamase sequences and functional data, and incorporates the existing Ambler classifications. The most recent classification of known β-lactamases (Bush and Jacoby, 2010) identifies three major groups of β-lactamases:

- Group 1: Serine mediated hydrolysis (Class C) enzymes. Functionally defined by: cephalosporin affinity. Enzymes are not inhibited by clavulanic acid or tazobactam. Variably resistant to extended-spectrum cephalosporins.
• Group 2: Serine mediated hydrolysis (Class A and D) enzymes. Functionally defined by: variable penicillin and cephalosporin affinities. Mostly inhibited by clavulanic acid and tazobactam. Variably resistant to extended-spectrum cephalosporins.

• Group 3: Metallo-enzymes (Class B). Functionally defined by: carbapenem affinity as well as broad-spectrum $\beta$-lactam affinity. Inhibited by EDTA.

Despite comprehensive attempts to classify individual enzymes into distinct families through functional and sequence comparisons; there is considerable overlap in the inter and intra group capabilities for individual enzymes to inactivate the various $\beta$-lactams (Morar and Wright, 2010).

1.2.4 AmpC $\beta$-lactamases

Class C $\beta$-lactamases have a distinct evolutionary origin from class A and D enzymes. The archetypal class C $\beta$-lactamase, AmpC, is encoded chromosomally throughout the Enterobacteriaceae (the expression is not ineducable in E. coli).

The functional origins of chromosomal AmpC are in routine bacterial cell wall maintenance, as well as the enzymes ability to inactivate $\beta$-lactam compounds (Morar and Wright, 2010).

AmpC was first identified as being distinct from other $\beta$-lactamases in the early 1980’s (Jaurin and Grundström, 1981). AmpC enzymes have hydrolytic activity against the penicillins, cephalosporins (extended-spectrum or otherwise), the cephemycins and the monobactams. Unlike the majority of of the class A or D enzymes, AmpC enzymes are not inhibited by $\beta$-lactamase inhibitors introduced to combat the increasing prevalence of $\beta$-lactamase resistance (Bush and Jacoby, 2010).
The three dimensional structure of AmpC is analogous to class A and D enzymes. Though notably, the AmpC active site is capable of binding the larger and more complex $\beta$-lactams such as the cephalosporins and extended-spectrum cephalosporins.

As with other $\beta$-lactamases, the catalytic affinity of individual enzymes can be extended to new $\beta$-lactam compounds through structural modification of the active site, by altered amino acid sequence; to accommodate bulkier chemical side-chains associated to the $\beta$-lactam ring (Jacoby, 2009).

Chromosomal AmpC (cAmpC) mediated resistance in *E. coli* can arise through various point mutations which act to constitutively express AmpC. However, more commonly, AmpC mediated resistance is horizontally acquired via plasmid associated AmpC (pAmpC) group enzymes. Though the majority of identified $\beta$-lactamases are class A or D enzymes, novel plasmid mediated AmpC enzymes continued to be identified (Bush and Jacoby, 2010).

### 1.2.5 Extended-spectrum AmpC (ESAC) and ESBL

The rate at which novel $\beta$-lactamases have been identified has increased since their initial identification; and continues to accelerate (Bush and Jacoby, 2010). The initially well defined $\beta$-lactamase groups have become increasingly variable in their resistance profiles through the accrual of beneficial genetic mutations; expanding or altering their enzymatic affinity towards different $\beta$-lactams. The horizontal transfer of $\beta$-lactamase gene sequences to different bacterial hosts via mobile genetic elements has also increased the diversity of the $\beta$-lactamases (Livermore and Woodford, 2006). As the development of the $\beta$-lactams increased to deal with emerging resistance, so to did the rate of discovery of novel $\beta$-lactamases capable of inactivating these newer compounds.
The oxyimino-cephalosporins (extended-spectrum cephalosporins), or third and fourth generation cephalosporins, were developed to combat emerging resistance against existing $\beta$-lactams. The addition of a 7-oxyimino group to the $\beta$-lactam ring introduces steric hindrance and shields the $\beta$-lactam ring from the active site (Mammeri and Nordmann, 2007). Shortly after their introduction, the first $\beta$-lactamases capable of hydrolyzing the extended-spectrum cephalosporins; these enzymes are the ESBLs (Bradford, 2001).

The term ESBL has been applied to any $\beta$-lactamase that confers resistance against the extended-spectrum cephalosporins; and they are horizontally acquired (Cornaglia et al., 2008). However, further distinction is required to separate ESBL enzymes from the functionally separate AmpC enzymes which can also confer resistance to the extended-spectrum cephalosporins. These are referred to as extended-spectrum AmpC (ESAC) $\beta$-lactamase. ESAC enzymes remain uninhibited by clavulanic acid, but despite their characteristic extended-spectrum activity, affinities towards other $\beta$-lactams are highly varied (Mammeri and Nordmann, 2007).

1.2.6 *E. coli* and $\beta$-Lactamase Resistance

Antimicrobial resistance has been well documented as negatively impacting on patient morbidity and mortality in human clinical care. Resistance to extended-spectrum cephalosporins, in particular, has been linked to increased adverse clinical outcomes (Cosgrove et al., 2002, Cosgrove and Carmeli, 2003). Inappropriate use of $\beta$-lactams, particularly third generation cephalosporins, has been linked to increasing $\beta$-lactamase prevalence within the hospital setting (Paterson, 2004). Currently, the best preventative measures against hospital-acquired infections rely on infection control, antimicrobial stewardship, and active hospital surveil-
lance (Owens Jr. and Rice, 2006, Paterson, 2006, Weinstein, 2001). Outbreaks also impact on hospital resources and increase per-patient health care spending (Roberts et al., 2003).

The impact on human health care is not just limited to the hospital setting. AMR E. coli isolates, are also detectable in the community setting (Pitout et al., 2005, Rogers et al., 2010, Kim et al., 2017). AMR E. coli, capable of negatively impacting human health has also been detected in the wider environment (Leonard et al., 2018).

In order to effectively limit the potential impact, it is important to correctly identify the most successful AMR determinants from hospital, community and environmentally associated infections so that the may be appropriately treated and prevented from disseminating. A recent update on the prevalence of AMR in the European Union (EU) (2013-2016), issued by the ECDC (https://ecdc.europa.eu/sites/portal/files/documents/AMR-surveillance-Europe-2016.pdf), highlights concerns over the wide-spread dissemination of resistant and MDR E. coli:

- **E. coli** is the most frequent cause of bacteraemias and UTIs in Europe; and is a significant causative agent of both community and hospital-associated infections.

- 58.6% of **E. coli** isolates were resistant to at least one of the antimicrobial groups. There is a notable, increasing trends in third-generation cephalosporin resistance (and ESBL detection).

- ESBL isolates often possessed acquired resistances to other antimicrobial classes (fluoroquinolones and aminoglycosides). Some of these infections are susceptible only to the carbapenems; to which some **E. coli** isolates are resistant.
ESBL mediated resistance is a complicating factor in infections risk management and negatively impacts hospital resources and patient morbidity and mortality; though limited antibiotic chemotherapeutic options make it more difficult to assess the risk to patient welfare (Ramphal and Ambrose, 2006). The antimicrobial class of choice to treat ESBL producing infections are the carbapenems.

While they remain largely effective, increased usage of this class of antimicrobials may promote the selection for resistant isolates. Were such resistance to become acquired by MDR E. coli isolates there remains little, if any antibiotics of choice (Livermore et al., 2011).

ESAC producing E. coli isolates also present a threat to the carbapenems. Worryingly, ESAC producing isolates that are also resistant to some carbapenems (ertapenema and imipenem) have already been reported; resistance being conferred by the loss of OmpC and OmpF porins (Mammeri et al., 2008).

Despite the documented threat to human health, posted by β-lactamase resistant E. coli, it remains difficult to assess the threat that these isolates pose to veterinary care at a national level.

There is little information on the effects of infection control practices within veterinary hospitals. A survey conducted by (Benedict et al., 2008) concluded that infection outbreaks are a regular occurrence for veterinary hospitals; and have identified E. coli as an important causative organism. The authors go on to speculate that given the lack of: co-ordinated and standardized surveillance efforts, passive not active surveillance for resistance and no surveillance for environmental transmission; these are all underestimated problems in veterinary hospitals.

In general, much less is known about AMR in veterinary practice, compared to what is known about AMR in human clinical environments. While there
are regular surveys conducted on the usage of antibiotics in companion animals VARSS, Small Animal Veterinary Surveillance Network (SAVSNET) (http://www.liv.ac.uk/SAVSNET/) and GermVet (Michael et al., 2017), they do not release data on the incidence of UK wide AMR.

Similar to reports originating from the EU and the UK, a national survey of veterinary hospital inpatient prescribing practices in the USA (Baker et al., 2012) indicated the $\beta$-lactams as the most significantly prescribed antimicrobials (72.7% of all surveyed dogs). Interestingly, the authors also indicated a significant proportion of the dogs surveyed (55.6%) had been prescribed at least one antibiotic within 12 months of admission; which the authors conclude contributes to the co-selection of AMR traits and propagation of MDR.

Despite the frequency and scale of reports investigating the epidemiology of AMR in food producing animals, less is known about the presence of AMR in companion animals. Most of the the reports of the incidence of veterinary AMR and MDR are from individual institutions.

### 1.2.7 MDR and $\beta$-Lactam Resistance in Dogs

AmpC andor MDR *E. coli* both present clinical challenges in the care of dogs. However, the degree to which either is a problem is difficult to assess. AmpC and ESAC mediated AMR functionally overlap (in terms of their resistance profile) with many of the other ESBL and $\beta$-lactamases. Though ESBL mediated resistance is reported, not all of the reports attempt to distinguish ESBL from ESAC resistance. A large proportion of the published literature of either AMR prevalence, or AmpC/ESBL resistance comprises reports of either clinical outbreaks of MDR or passive surveillance from hospital bacteriology departments (table 1.1).
<table>
<thead>
<tr>
<th>Location</th>
<th>Setting</th>
<th>Isolates</th>
<th>Size</th>
<th>Summary</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hungary</td>
<td>Clinical</td>
<td>E. coli Enterobacter Klebsiella Proteus Providencia Morganella</td>
<td></td>
<td>Enterobacteriaceae: 48-69% resistant to amoxycillin and ampicillin. E. coli 11-17% resistant to 2nd Gen cephalosporins 3-8% 3rd Gen cephalosporins</td>
<td>(Gal et al., 2000)</td>
</tr>
<tr>
<td>Denmark</td>
<td>Clinical</td>
<td>E. coli</td>
<td>24 (0.4%) isolates positive for AmpC. Hyperproduction in 23/24 isolates. pAmpC and other ESBLs detected</td>
<td></td>
<td>(Jorgensen et al., 2010)</td>
</tr>
<tr>
<td>Australia</td>
<td>Clinical</td>
<td>E. coli</td>
<td>13/55 isolates were AmpC positive. Identification of CMY2 CMY7 and OXA10.</td>
<td></td>
<td>(Gibson et al., 2010)</td>
</tr>
<tr>
<td>UK</td>
<td>Community</td>
<td>E. coli</td>
<td>7 CMY2 isolates detected amongst 183 healthy dogs</td>
<td></td>
<td>(Wedley et al., 2011a)</td>
</tr>
<tr>
<td>Portugal</td>
<td>Clinical</td>
<td>E. coli</td>
<td>Ampicillin associated β-lactamase production in 26/72 isolates. TEM1 most prevalent (77%). AmpC present in (31%).</td>
<td></td>
<td>(Féria et al., 2002)</td>
</tr>
<tr>
<td>Italy</td>
<td>Clinical / Community</td>
<td>E. coli</td>
<td>204 dogs</td>
<td>16 CTXM-1 isolates (large number CTXM-1 and TEM positive). 3 isolates with CMY2</td>
<td>(Carattoli et al., 2005b)</td>
</tr>
<tr>
<td>Country</td>
<td>Type</td>
<td>Year Range</td>
<td>Organisms</td>
<td>Isolates</td>
<td>Resistance Notes</td>
</tr>
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<tr>
<td>Canada</td>
<td>Clinical</td>
<td>2002 - 2007</td>
<td>E. coli, S. intermedius, Enterococcus spp., Proteus spp., Staphylococcus spp., Enterobacter spp., Pseudomonas spp., Klebsiella spp. etc</td>
<td>473 UTI isolates from dogs</td>
<td>Increased resistance in E. coli isolates from dogs (decreased resistance to 1st and 2nd Gen cephalosporins).</td>
</tr>
<tr>
<td>Canada</td>
<td>Clinical</td>
<td>2005</td>
<td>E. coli, Salmonella enterica, Clostridium difficile, Enterococcus spp., Staphylococcus aureus, Staphylococcus pseudintermedius</td>
<td>Microbiological survey of 101 Veterinary Hospitals</td>
<td>Low prevalence of resistance for E. coli; 9 hospitals reported isolates with CMY2; no reported ESBLs</td>
</tr>
<tr>
<td>Portugal</td>
<td>Community</td>
<td>2003</td>
<td>E. coli</td>
<td>39 dogs 36 cats</td>
<td>Marked resistance to tetracycline and ampicillin/streptomycin overall resistance was low; TEM gene detected in 12/17 ampicillin resistant isolates and CTX-M1 and OXA30 detected in the other two isolates</td>
</tr>
<tr>
<td>Australia</td>
<td>Clinical</td>
<td>E. coli</td>
<td>11 isolates</td>
<td>Clinical ICU cases; isolates were selected based on multi-drug resistant profile. Isolates showed resistance to 10 different antibiotics as well as weakened susceptibility to ceftriaxone, ceftazidime, cefotaxime and cefoxitin. TEM1B and CMY7 found in all isolates</td>
<td>(Sidjabat et al., 2006)</td>
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<tr>
<td>Australia</td>
<td>Clinical</td>
<td>E. coli</td>
<td>10 dogs</td>
<td>Isolates were notable for fluoroquinolone and 3rd Gen cephalosporin resistance. Isolates with similar profiles were isolated from hospital environment and other clinical patients</td>
<td>(Warren et al., 2001)</td>
</tr>
<tr>
<td>Denmark</td>
<td>Clinical</td>
<td>E. coli Staphylococcus intermedius Streplococcus canis Pseudomonas aeruginosa Pasteurella multocida Proteus spp.</td>
<td>780 isolates (449 E. coli)</td>
<td>Notable E. coli resistance for sulphamides streptomycin ampicillin trimethoprim and tetracycline.</td>
<td>(Pedersen et al., 2007)</td>
</tr>
<tr>
<td>Location</td>
<td>Setting</td>
<td>Organisms</td>
<td>Isolates</td>
<td>Resistance/Antimicrobial Activity</td>
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<tr>
<td>Holland 2007 - 2009</td>
<td>Clinical</td>
<td><em>Enterobacteriaceae</em></td>
<td>65 isolates</td>
<td>Isolates selected for ESBL and AmpC phenotype. CTX-M1 was the predominant genotype - CMY2 and CMY39 were also identified. (Dierikx et al., 2012a)</td>
<td></td>
</tr>
<tr>
<td>Canada 2002</td>
<td>Community</td>
<td><em>E. coli</em>, <em>Salmonella spp.</em>, <em>Staphylococcus aureus</em>, <em>Staphylococcus pseudintermedius</em></td>
<td>188 dogs, 39 cats</td>
<td>E. coli resistance low (intermediate resistance to all classes was detected in different isolates). 3 dog isolates possessed CMY2; 2 isolates contained plasmid mediated CMY2 no plasmid was detected in the other isolate. (Murphy et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>Korea 2006 - 2007</td>
<td>Clinical / Community</td>
<td><em>E. coli</em></td>
<td>628 isolates</td>
<td>CTX-M and AmpC (mostly CMY-2) found on large plasmids. Element mediated transfer for both genes. (Tamang et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>UK 2016</td>
<td>Clinical</td>
<td><em>E. coli</em></td>
<td>5 veterinary hospitals</td>
<td>Variable levels of third-generation cephalosporins, MDR, ESBL and AmpC isolates between different hospitals. High prevalence of CMY-2 and CTX-M-15. (Tuerena et al., 2016)</td>
<td></td>
</tr>
<tr>
<td>Country</td>
<td>Setting</td>
<td>Species</td>
<td>Sample Size</td>
<td>Description</td>
<td>Reference</td>
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<tr>
<td>Portugal</td>
<td>Community</td>
<td>E. coli</td>
<td>151 rectal swabs</td>
<td>Presence of a number of CTX-M and CMY genes in otherwise healthy dogs. One dog positive for DHA-1; previously only associated with humans.</td>
<td>Belas et al. (2014)</td>
</tr>
<tr>
<td>Tunisia</td>
<td>Community</td>
<td>E. coli</td>
<td>80 faecal samples</td>
<td>14 samples variously positive for CTX-M, TEM and CMY-2 resistance genes.</td>
<td>Sallem et al. (2013)</td>
</tr>
<tr>
<td>EU 2008 - 2011</td>
<td>Clinical</td>
<td>E. coli</td>
<td>1152 isolates (669 from dogs)</td>
<td>Study examining ESBL resistance and sequence type in E. coli isolated from various infections. Isolates of focused sequence type contained various CTX-M, OXA, TEM and SHV resistance genes.</td>
<td>Ewers et al. (2014)</td>
</tr>
<tr>
<td>Switzerland</td>
<td>Community</td>
<td>Enterobacteriaceae</td>
<td>376 rectal swabs (dogs and cats)</td>
<td>Pets from care homes surveillance for ESBL resistance. 2.5% prevalence of ESBL resistance amongst the isolates.</td>
<td>Gandolfi-Decristophoris et al. (2013)</td>
</tr>
<tr>
<td>USA 2013</td>
<td>Clinical</td>
<td>Enterococci, Staphylococci and E. Coli</td>
<td>622 dogs</td>
<td>Longitudinal study; employing disk-diffusion and population study methods (MLST and PFGE). Decreasing trend over time for MDR E. Coli</td>
<td>Hamilton et al. (2013)</td>
</tr>
</tbody>
</table>
Some of the reports summarized here do not specifically address the issue of AmpC resistance in dogs. Earlier studies tend to report only the results of antibiogram susceptibility testing, which provides only basic phenotypic resistance of the isolates. Where the reports have included clinical breakpoints, and more extensive phenotypic screening of suspected ESBL isolates; further diagnostic testing may not have been conducted to confirm either ESBL or ESAC resistance determinants. Also, the studies tend to be small (either in isolates, or numbers of dogs surveyed), isolated from each other (spatially and temporally) and biased towards isolates that have been clinically selected for resistance. Despite this, the correlation of incidence and recurrence of *E. coli* UTI, with regards to increasing AMR, is notable (Ball et al., 2008).

Throughout the reports, *E. coli* resistance to various β-lactams was highly variable. Resistance to the cephalosporins (all generations) was not highly prevalent.

The largest survey of clinically detected *E. coli* resistance was carried out in Canada (Murphy et al., 2010). Whilst the prevalence of *E. coli* was low, no antibiotic class was without detectable resistance. MDR isolates were also detectable, mediated by the possession of pAmpC resistance genes (*bla*<sub>CMY-2</sub>).

Within the UK, the only canine based resistance surveillance with national cov-
verage was carried out by (Normand et al., 2000). The authors conducted a longitudinal, retrospective evaluation of resistance phenotypes (using clinical records) from across the UK, over an eight year period. The report does not specifically address *E. coli*, but identified rising trends in resistance to amoxicillin-clavulanate and streptomycin in *E. coli*. The method of reporting is proportionality of resistance phenotypes only, no elucidation of resistance mechanisms is possible from the data as presented.

More recent studies from the UK also highlight an underlying prevalence of AMR. A cross-sectional community based evaluation of canine *E. coli* isolate resistance in Cheshire (Wedley et al., 2011b), confirmed the presence of multiple ESBL (\(\text{bla}_{\text{SHV}}, \text{bla}_{\text{TEM}}, \text{and } \text{bla}_{\text{CTX-M}}\)) and AmpC isolates; correlated to high levels of resistance to ampicillin, tetracycline, trimethoprim and overall MDR status.

A survey of nosocomial *E. coli* isolates (patients and clinical environment) from several veterinary hospitals (Tuerena et al., 2016), also identified a high proportion of MDR *E. coli* endemic to both patient (13.1%) and environmental (8.9%) samples collected. These isolates were also notable for high carriage rates of ESBL and AmpC resistance; commonly mediated by \(\text{bla}_{\text{CTX-M}}, \text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}\) and \(\text{bla}_{\text{CMY}}\) resistance genes.

Despite the relatively low number of reports, \(\text{bla}_{\text{CMY-2}}\) and \(\text{bla}_{\text{CTX-M}}\) resistance genes have continued to be identified in isolates globally (Belas et al., 2014, Ewers et al., 2014, Gandolfi-Decristophoris et al., 2013, Hamilton et al., 2013, Huber et al., 2013, Sallem et al., 2013).

Though mostly focused on nosocomial resistance, there have been reports of community-associated MDR *E. coli* outbreaks which have extensive resistance towards the fluoroquinolones (important third-line antimicrobials) and the β-
lactams; and that such resistant isolates may be capable of zoonotic transfer and persistence in the community or the clinical setting (Warren et al., 2001). But reports of AmpC resistance amongst *E. coli* isolates in companion animals within the community remain rare. An extensive literature search using EMBASE returned only five reports of β-lactamase resistance in companion animal or canine *E. coli* isolates.

Reports from Portugal (Costa et al., 2008) and Italy (Carattoli et al., 2005a) confirm that AmpC resistance and ESBLs are isolated from community samples. Many of the isolates had extensive resistance to multiple antimicrobial classes; and some isolates possessed both AmpC (*bla*<sub>CMY-2</sub>) and the ESBL resistance gene *bla*<sub>CTX-M</sub>. Though (Costa et al., 2008) found no AmpC resistance markers in the isolates they sampled; AMR was still detected against a number of antibiotic classes including tetracycline, ampicillin and streptomycin and various ESBL genes were detected (*bla*<sub>CTX-M</sub>, *bla*<sub>OXA</sub> and *bla*<sub>TEM</sub>).

The reports highlight different infectious aspects of MDR *E. coli* such as clonality, horizontal gene transfer of plasmid and integron mediated AmpC, and chromosomal mutations of AmpC; important to the dissemination of MDR *E. coli*.

### 1.2.8 Urinary Tract Infections

UTIs are ascending infections of the genito-urinary tract epithelium. Bacterial colonization of the urinary tract occurs via the periurethral epithelium, and may be caused by a number of different bacterial species. The predominant organism isolated from UTIs is *E. coli* in both humans (Nicolle, 2008) and dogs (Ball et al., 2008).

It is estimated that 14% of dogs contract a UTI within their lifetime. As
well as increased age and sex, there are many predisposing factors (Beutin, 1999, Dunning and Stonehewer, 2002):

- Urinary incontinence
- Anatomical or physiological abnormalities
- Underlying disease
- Catheterization

UTI infections are diagnostically confirmed by the direct observation and culture of bacteria in urine samples obtained by free-catch or cytocentesis. Significant bacterial presence in the urine, indicative of an active UTI, is greater than $10^3$ bacteria per ml. Such bacterial growth can present as an asymptomatic infection, or when presenting with other clinical signs, as cystitis or pyelonephritis (Dunning and Stonehewer, 2002).

Asymptomatic UTI, cystitis and pyelonephritis are defined separately (Mobley and Warren, 1996) as:

- Asymptomatic bacteriuria: where bacteria are present in urine at greater than $10^5$ detectable organisms per ml, but there are no presenting symptoms.

- Cystitis: infection of the bladder (possibly the urethra), presenting with clinical signs (dysuria, pain, frequent urination). Cystitis is confirmed with bacterial counts in urine of greater than $10^9$ organisms per ml.

- Pyelonephritis: infection of the kidney, though it can present as a lower UTI; associated with more severe clinical signs (pyrexia, nausea, vomiting and pain). If left untreated pyelonephritis can progress into kidney failure and septicemia. Pyelonephritis is confirmed with bacterial counts in the
urine greater than $10^4$ organisms per ml.

Each clinical presentation of UTI requires different treatment protocols, as laid out by the International Society for Companion Animal Infectious Diseases (Scott Weese, 2008). UTIs are often treated empirically, before culture and sensitivity panels are carried out to identify the causative organism or potential resistance.

Amoxicillin and trimethoprim-sulfonamide combination therapy are both suggested as possible empirical treatments. Amoxicillin-clavulanic acid is acceptable for use as an empiric treatment, however, it is important to maintain as narrow a spectrum before an informed decision about potential resistance can be made. It is recommended with complicated UTIs, that treatment by withheld if at all possible, until culture and sensitivity panels have been performed. The recommendation for cases of pyelonephritis is an immediate course of an appropriate fluoroquinolone, to be re-evaluated once culture and sensitivity data become available.

In all potential presentations of UTI, the guidelines recommend that empirical treatment decisions are made by taking local information into account, regardless of clinical severity. For example, the bacterial organisms most prevalent to the setting and local resistance surveillance data. AMR and MDR may limit the choice of appropriate antimicrobials; as can other factors (cost, risk of adverse reaction, sub-optimal pharmacokinetic properties in the urinary tract).

The prevention of relapse, or recurrence of a UTI, is dependent on efficient diagnosis and treatment using antimicrobials. Which is contingent on the confirmation of both the causative organism and its antibiotic susceptibility (Dunning and Stonehewer, 2002).
1.2.9 Uropathogenic *E. coli*

UPEC are a heterogeneous collection of *E. coli* isolates capable of causing infection of the urinary tract in humans and in animals; and are just one of several established groups of pathogenic *E. coli*, distinguished by a pathotypic combination of virulence factors and distinctive pathology. More broadly, UPEC is part of the larger group of pathogenic *E. coli* capable of causing extra-intestinal infection; referred to as extra-intestinal *E. coli* (ExPEC) (Kaper et al., 2004).

UPEC are derived from the host gut flora, and progressively colonize the perineum and urethra before ascending to the bladder. Recurrent or persistent UTIs are caused by the host failing to clear UPEC from the urinary tract; and are more likely in hosts with underlying factors (underlying disease and abnormal physiology) which predispose the host to UTI (Johnson and Russo, 2005).

Two hypotheses have been proposed for the way in which UPEC strains transfer between the gastro-intestinal and urinary tracts. The prevalence hypothesis (Turck and Petersdorf, 1962) proposes that colonization of the urinary tract is caused by dominant *E. coli* clones present in the intestinal flora; which have an increased chance of colonizing the urinary tract because of their greater prevalence. The specialist pathogen hypothesis (Plos et al., 1995), which identified the P fimbriae as an important UPEC virulence marker, suggests that UPEC are separate population of *E. coli* within the gut flora which are better defined by their virulence potential.

Despite a variable developmental background, UPEC are described as having a defined basal number of virulence factors; type I and P pili adhesins, and array of siderophores (iron acquisition), and various cytotoxins such as CNF1. These make up the UPEC pathotype (Wiles et al., 2008), though the degree to which the various markers are present can vary amongst different UPEC strains.
This variability is thought to be representative of the different backgrounds under which UPEC strains may be selected from (Marrs et al., 2005). For example, the acquisition of virulence genes is more favorable in genetic backgrounds associated with pathogenic *E. coli* (Tramuta et al., 2011, Wirth et al., 2006).

The distribution of virulence across the global *E. coli* population is not straightforward. The plastic nature of the *E. coli* genome, which may be greatly expanded by horizontal transfer, as well as being specific to the niche that particular isolates may occupy, favors different virulence factor distributions. Virulence factors may be broadly divided into three groups, those that are transferable and may be expressed in different genetic backgrounds (such as the various enteropathogenic *E. coli*), virulence factors that are widely dispersed by individually associated with various pathotypes (as in the specialist pathogen hypothesis), and virulence factors only associated with a very restricted genetic background (such as the Shiga toxin producing *E. coli*) (Escobar-Páramo et al., 2004, Escobar-Páramo et al., 2009).

The host response to UPEC infections is through the innate immune system; which includes neutrophil recruitment via the activation of Toll-like receptors (such as TLR4), and the excretion of chemokines (Svanborg et al., 2006). Other defense mechanisms include exfoliation of the uroepithelium and secretion of Tamm-Horsfall protein, both of which disrupt the binding of invading bacteria to the epithelium (Mulvey et al., 2000). UPEC are also thought to be capable of immune-modulation, which increases the likelihood of developing recurrent UTI. Moreover, the bacteria are also capable of invading the uroepithelial cells and forming quiescent intracellular reservoirs to prevent their removal by exfoliation (Mysorekar and Hultgren, 2006). Otherwise, the environmental conditions of a healthy urinary tract (regular urine flow, pH and osmotic pressure) act to prevent the colonization of the tract by bacteria.
The basic epidemiology of UTIs in dogs is similar to that in humans. *E. coli* is the predominant bacterial isolate collected from canine UTI, including bacteriuria and pyelonephritis. UPEC isolates have been identified as originating from a number of different clonal lineages, identifiable by their O, K and H serological groups; and the clones frequently identified in the faecal flora (Beutin, 1999). As well as sharing many core virulence factors, human and canine UPEC isolates also share similar phylogenetic and serological distributions; whilst it has been assumed that canine and human UPEC populations overlap (Johnson et al., 2001, 2003), far fewer reports exist for the characterization of canine UPEC isolates than human isolates.

### 1.2.10 Uropathogenesis and AMR

Work done towards detecting the potential associations between resistance and phylogenetic group in *E. coli* remains largely incomplete. Many reports of companion animal resistance surveillance fail to attempt such population descriptions. Where studies have tried to identify a link, reports have been mixed; despite strong assertions of association in human clinical isolates. Ultimately, the success of any particular resistance gene is reflected in the stochastic nature of host infection; the more successful an acquired or inherited resistance marker is at integrating into a bacterial host, the more likely it is to continue to be disseminated (Martinez et al., 2009).

*E. coli* AMR mediated by chromosomal ApmC has been indicated to be more associated with phylogenetic group A isolates (Guillouzouic et al., 2009); and has also been correlated to a loss of virulence expression (Corvec et al., 2007). Although, this is certainly not the case amongst human clinical isolates positive for pAmpC; where β-lactamase resistance has been associated with pathogenic
phylogroups B2 and D (Oteo et al., 2010). One of the more difficult obstacles in dealing with developing resistance in the Enterobacteriaceae is the development of resistance amongst strains associated with the commensal flora. Commensal isolates are abundant, but clinically silent, despite the potential for them to harbor and transmit AMR; becoming noticeable only when they become associated with an infection. Such an association, between commensal E. coli and fluoroquinolone resistance has been reported (Alekshun and Levy, 2006).

The degree to which a particular E. coli isolate expresses a virulence or resistance phenotype is likely representative of its life history and the context of its environment (Martínez and Baquero, 2002). The bias towards the collection of clinical isolates, which may introduce confounding variables such as host health and host immunity, may obscure the complexities of the interaction between virulence and resistance. So far little has been done to compare resistance and virulence in animal models. The data that is available does suggest that MDR E. coli isolated in the clinic are less virulent than fully susceptible isolates; confirmed in a mouse model by (Sidjabat et al., 2009).

However, studies addressing this in companion animals are hard to come by. That MDR may be stably maintained within community populations by E. coli isolates belonging to successful sequence type (ST)s which may also be virulent, suggests that there is an overlap. Furthermore, the detection of antibiotic resistance genes such as pAmpC and the ESBLs indicates that the long-term impacts on clinical and community-associated E. coli populations should be investigated (Pitout, 2012).
Chapter 2

Molecular Typing of Multi-Drug Resistant \textit{Escherichia coli}

2.1 Thesis Introduction

The successful treatment of a bacterial infection requires the matching of the appropriate treatment to a particular infectious organism. Though many infections are initially treated empirically. The genesis of antimicrobial resistance (AMR), and it’s progression to multi-drug resistant (MDR), is in the inappropriate application of non-optimal antimicrobial compounds to the infectious organism. The rate of progression of a population of infectious bacteria from fully susceptible to AMR can be influenced by a number of different processes:

- The misuse of antibiotics (lack of appropriate stewardship) acts to select and promote particular resistance elements capable of acting against one or more antimicrobial compounds.

- The predominance of a successful bacterial clonal lineage, which may be
more closely associated with antimicrobial resistance.

- Reservoirs of transferable resistance elements within the clinical environment which the animal may become colonized with during, or after, treatment.

- Reservoirs of resistance elements without the clinical environment which the animal may become colonized with before treatment.

To further complicate this, the confluence of selection pressures which promote the spread of AMR and MDR can influence and be influenced by selection pressures affecting different sub-populations of *E. coli*. As described in the general introduction, *E. coli* which are MDR tend to be derived from the commensal flora. uropathogenic *E. coli* (UPEC) have a definable virulence profile which may act as a barrier toward the acquisition of AMR and MDR. The work presented in this chapter seeks to define the association between the *E. coli* and their recorded MDR phenotypes. The primary questions addressed in this chapter were:

- What resistance genotype is conferring phenotypic extended-spectrum AmpC (ESAC) resistance?

- Do the MDR *E. coli* share a common genetic background, and is this genetic background different from a group of fully susceptible *E. coli*?

- Does the MDR group constitute of *E. coli* isolates that are virulent with respects to their ability to cause a urinary tract infections (UTI)? Or is there evidence to suggest that an MDR phenotype is detrimental to a more virulent phenotype?

- Is there any evidence to suggest that the resistance genotype is horizontally transferable?
The work presented here has been published in an academic journal (Wagner et al., 2014), the full paper is included in the appendix. A reference table with the strain identification numbers is included in the appendix as strain identification numbers were changed in the published paper to conform to submission guidelines.

2.2 Paper Introduction

Increasing numbers of reports have documented the emergence of *Escherichia coli* capable of producing broad-spectrum \( \beta \)-lactamases. This is significant since the \( \beta \)-lactam antimicrobials are of therapeutic importance in humans and many domestic animals. Furthermore, many isolates are resistant to additional antimicrobial classes and therefore MDR.

Carriage of extended-spectrum \( \beta \)-lactamase (ESBL) and AmpC producing *E. coli* has been documented in many species (Bortolaia et al., 2011). Antimicrobial use has been reported as a risk factor (Damborg et al., 2011, 2012, Maddox et al., 2012) and there is also evidence of sharing of organisms between species living in close proximity to each other (Dolejska et al., 2011). Considering the physical closeness in which many humans live with their pet companions, sharing of these organisms between humans and pets could pose a significant mutual risk.

Clinical disease associated with these organisms is well documented in humans. Initially the pattern was of hospital-acquired infection but community-acquired infection has become increasingly important. ESBL *E. coli* are associated with a variety of clinical diseases, in particular UTI, neonatal septicemia and wound infections (Ben-Ami et al., 2009, Pitout, 2010). Reports of similar clinical disease associated with AmpC-producing *E. coli* are far fewer than those pertaining to
ESBLs, however these also appear to an emerging problem (Oteo et al., 2010).

Although most animal studies have focused on the zoonotic risk posed by carriage, there are increasing reports demonstrating the involvement of these organisms in clinical disease in domestic species in a variety of locations including Europe, North America, Asia and Australia. For example, in the United States AmpC (CMY-2) and ESBL-producing \textit{E. coli} (O’Keefe, A.a, Hutton, T.A.b, Schifferli, D.M.a, Rankin, 2010, Sanchez et al., 2002, Shaheen et al., 2011) have been reported from canine clinical isolates. Clinical disease associated with AmpC-producing \textit{E. coli} in dogs in Australia, was first reported in 2006 (Sidjabat et al., 2006). More recently, a survey of clinical isolates from dogs and horses in the Netherlands demonstrated a 2% prevalence of ESBL and AmpC-producing isolates (Dierikx et al., 2012a).

The aim of this study was to evaluate the association of ESBL or AmpC production with MDR \textit{E. coli} isolated from clinical cases of UTI in dogs from a local patient population, over a period of time ranging from 2002 to 2011. Isolates were further characterized in terms of phylogenetic grouping, sequence type (ST) and virulence genotype. Plasmid replicon typing was also performed to identify the type and diversity of plasmids involved. Comparisons were made to a group of susceptible \textit{E. coli} isolates, also associated with canine UTI and collected over a similar time frame.
2.3 Methods

2.3.1 Source of Clinical Isolates

All 15 susceptible and 17/18 MDR isolates were identified in clinical cases seen at the Hospital for Small Animals, University of Edinburgh. One MDR isolate (1223) came from a local practice serviced by the University of Edinburgh’s diagnostic microbiology service.

2.3.2 *E. coli* Identification

A total of 33 clinical isolates from canine UTI were cultured on Blood and MacConkey agar. Any lactose fermenting colonies were confirmed as *E. coli* utilizing biochemical testing (API 10S™strip bioMerieux).

2.3.3 Antimicrobial susceptibility testing

Susceptibility testing was performed using the disc diffusion method in accordance with Clinical Laboratory Standards Institute (CLSI) guidelines. The following discs were used: Co-trimoxazole (25 µg); ciprofloxacin (1 µg); amoxicillin clavulanate (30 µg); cephalexin (30 µg); gentamicin (10 µg); tetracycline (10 µg); cefotaxime (30 µg). All discs were sourced from Mast group Ltd.

2.3.4 Control strains

The following control strains were utilized in both the phenotypic combination disc testing and for polymerase chain reaction (PCR). ATCC 25922™(negative
control); ATCC BA-199™ (SHV-3 positive control); NCTC 13353™ (CTX-M-15 positive control), and NCTC 13351™ (TEM-3 positive control).

2.3.5 Combination disc method for plasmid-mediated AmpC and ESBL detection

A commercially available AmpC and ESBL detection set (Mast group Ltd.) was utilized. This comprised a set of 4 discs containing cefpodoxime plus or minus AmpC and ESBL inhibitors. Interpretation was made following the manufacturer’s instructions.

2.3.6 PCR for ESBL and AmpC gene detection

DNA from single colonies of each isolate was prepared using the lysis method as previously described (Perez-Perez and Hanson, 2002). Primers for the genes \( \text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}, \text{bla}_{\text{CTX}}, \text{bla}_{\text{CMY-1}} \) group, \( \text{bla}_{\text{CMY-2}} \) group, \( \text{bla}_{\text{OXA-1}} \) group and \( \text{bla}_{\text{OXA-2}} \) group were derived from a previously established assay (Hasman et al., 2005) (table 2.1). For additional detection of AmpC \( \beta \)-lactamase genes, multiplex PCR was performed on all samples using the methodology and primers previously described (Perez-Perez and Hanson, 2002). PCR products were electrophoresed in a 1% agarose gel; gel bands were excised and sequences were compared to the NCBI database to confirm identity.
Table 2.1: Table of ESBL and AmpC PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene Target</th>
<th>Primer Sequence</th>
<th>Product Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>757</td>
<td>blaTEM</td>
<td>GCGGAACCCCTATTGG</td>
<td>964</td>
<td>(Perez-Perez and Hanson, 2002)</td>
</tr>
<tr>
<td>821</td>
<td></td>
<td>TCTAAAGTATATATGAGTAAACTTTGCTTGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1113</td>
<td>PampC</td>
<td>GTGAATACAGAGCCAGACGC</td>
<td>343</td>
<td>(Hasman et al., 2005)</td>
</tr>
<tr>
<td>796</td>
<td></td>
<td>GTTGTGTCCCGGTGTGATGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1436</td>
<td>blaSHV</td>
<td>TTCG CCTGTGTATTATCTCCCTG</td>
<td>854</td>
<td>(Hasman et al., 2005)</td>
</tr>
<tr>
<td>1437</td>
<td></td>
<td>TTAGCGTGTGCCAGTGYTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1354</td>
<td>blaCTX</td>
<td>ATGTGCAGYACCAGTAARGTKATGCC</td>
<td>593</td>
<td>(Perez-Perez and Hanson, 2002)</td>
</tr>
<tr>
<td>1355</td>
<td></td>
<td>TGGGTRAARTARGTSACCAGAAAYCGCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1004</td>
<td>blaCMY-1 group</td>
<td>GTGGTGAGATGCCAGCATCC</td>
<td>915</td>
<td>(Hasman et al., 2005)</td>
</tr>
<tr>
<td>1005</td>
<td></td>
<td>GGTGCGAGCCGTCTTGTTGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1006</td>
<td>blaCMY-2 group</td>
<td>GCACCTTAGCCACCTATACCGCAG</td>
<td>758</td>
<td>(Hasman et al., 2005)</td>
</tr>
<tr>
<td>1007</td>
<td></td>
<td>GCTTTTCAAGAATGCGCCAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1062</td>
<td>blaOXA-1</td>
<td>ATGAAAAACACAATACATACACTTCGC</td>
<td>820</td>
<td>(Perez-Perez and Hanson, 2002)</td>
</tr>
<tr>
<td>1063</td>
<td></td>
<td>GTGTTGTTTAGAATGTGTGATCGCATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1420</td>
<td>blaOXA-2</td>
<td>ACGATAGTTGTGGCACAGAAGAC</td>
<td>602</td>
<td>(Hasman et al., 2005)</td>
</tr>
<tr>
<td>1421</td>
<td></td>
<td>ATYCTGTGTTGGCGATATCATTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1359</td>
<td>blaACC-1</td>
<td>AGCCTCAGACGCGGTTAC</td>
<td>818</td>
<td>(Hasman et al., 2005)</td>
</tr>
<tr>
<td>1360</td>
<td></td>
<td>GAAGCCGTAGTTGTGATCAGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3.7 Multiplex PCR pyhlogenetic grouping of clinical isolates

DNA from single colonies was prepared using the Qiagen DNeasy blood and tissue extraction kit according to the manufacturer’s instructions (Qiagen, UK). Multiplex PCR methodology (table 2.2) was employed to assign the clinical isolates to one of four phylogenetic groups (A, B1, B2 or D). Primers and methodology have been described previously (Doumith et al., 2012a).
Table 2.2: Table of Phylotyping PCR primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Direction</th>
<th>Primer Sequence</th>
<th>Product Size</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>gadA</td>
<td>Forward</td>
<td>GATGAAATGGCGTTGGCGCAAG</td>
<td>373</td>
<td>(Doumith et al., 2012a)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGCGGAAGTCCCAGACGATATCC</td>
<td></td>
<td>(Doumith et al., 2012a)</td>
</tr>
<tr>
<td>chuA</td>
<td>Forward</td>
<td>ATGATCATCGCGGCGGTGCTG</td>
<td>281</td>
<td>(Doumith et al., 2012a)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAACGCGCTCGCGCCTAAT</td>
<td></td>
<td>(Doumith et al., 2012a)</td>
</tr>
<tr>
<td>yjaA</td>
<td>Forward</td>
<td>TGTTCGCGATCTTGAAAGCAACGT</td>
<td>216</td>
<td>(Doumith et al., 2012a)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACCTGTGACAAACCCTCACTCA</td>
<td></td>
<td>(Doumith et al., 2012a)</td>
</tr>
<tr>
<td>TSPE4.C2</td>
<td>Forward</td>
<td>GCGGGTGAAGACAGAAACGCG</td>
<td>152</td>
<td>(Doumith et al., 2012a)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTGTCGAGTGGCGAACC CGG</td>
<td></td>
<td>(Doumith et al., 2012a)</td>
</tr>
</tbody>
</table>
2.3.8 PCR-based plasmid replicon typing of clinical isolates

DNA was isolated as described above for the phylogenetic grouping. Methodology involved the use of the 8 multiplex reactions (table 2.3.8) in a commercial kit (Diatheva, Italy) based on methodology described previously (Carattoli et al., 2005a).
Table 2.3: Table of Replotyping PCR primers

<table>
<thead>
<tr>
<th>Name</th>
<th>DNA sequence</th>
<th>Target Gene</th>
<th>Product Size</th>
<th>Reference</th>
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<tbody>
<tr>
<td>hi1 FW</td>
<td>ggagcgatggattacttcagtac</td>
<td>parA-parB</td>
<td>471</td>
<td>(Carattoli et al., 2005a)</td>
</tr>
<tr>
<td>hi1 RV</td>
<td>tgccgtttcacctgtgagta</td>
<td></td>
<td></td>
<td>(Carattoli et al., 2005a)</td>
</tr>
<tr>
<td>hi2 FW</td>
<td>tttctctgagtcacctgtaaacac</td>
<td>iterons</td>
<td>644</td>
<td>(Carattoli et al., 2005a)</td>
</tr>
<tr>
<td>hi2 RV</td>
<td>ggctcactacggtgtcatct</td>
<td></td>
<td></td>
<td>(Carattoli et al., 2005a)</td>
</tr>
<tr>
<td>I1 FW</td>
<td>cgaaagccgacgacgcaaaga</td>
<td>RNAI</td>
<td>139</td>
<td>(Carattoli et al., 2005a)</td>
</tr>
<tr>
<td>I1 RV</td>
<td>tcgctgctcgcacagttcg</td>
<td></td>
<td></td>
<td>(Carattoli et al., 2005a)</td>
</tr>
<tr>
<td>X FW</td>
<td>aaccttagagctatattaagttgctgat</td>
<td>ori</td>
<td>376</td>
<td>(Carattoli et al., 2005a)</td>
</tr>
<tr>
<td>Primer Type</td>
<td>Sequence</td>
<td>Function</td>
<td>Position</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>---------------------------------</td>
<td>--------------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>X RV</td>
<td>tgagagtcatttttatctctgattttagc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/M FW</td>
<td>ggtcgaaactatcagcatctgaa</td>
<td>repA,B,C</td>
<td>785</td>
<td></td>
</tr>
<tr>
<td>L/M RV</td>
<td>cttcgagggcgttctgtagg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N FW</td>
<td>gtctaacgaggtacccgaa</td>
<td>repA</td>
<td>559</td>
<td></td>
</tr>
<tr>
<td>N RV</td>
<td>gtttcaactctgcacagttc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fia FW</td>
<td>ccatgctggttcagagaggg</td>
<td>iterons</td>
<td>462</td>
<td></td>
</tr>
<tr>
<td>fia RV</td>
<td>gttatcttactgtcctgcgag</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>fib FW</td>
<td>ggagttctgacacagatgatttcg</td>
<td>repA</td>
<td>702</td>
<td></td>
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</table>

(Carattoli et al., 2005a)
<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Sequence</th>
<th>Gene</th>
<th>Position</th>
</tr>
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<tbody>
<tr>
<td>fib RV</td>
<td>ctccggtgettcagggcatt</td>
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</tr>
<tr>
<td>W FW</td>
<td>cctaagaacaaacaagccccgc</td>
<td>repA</td>
<td>242</td>
</tr>
<tr>
<td>W RV</td>
<td>ggtgcgcggcatagaacgtcgt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y FW</td>
<td>aattcaaaacaaactgtgcagctg</td>
<td>repA</td>
<td>765</td>
</tr>
<tr>
<td>Y RV</td>
<td>gcgagaatggacgaattacaaaaactttn</td>
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<tr>
<td>P FW</td>
<td>ctatggccctgcaacggtgcagaaa</td>
<td>iterons</td>
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<tr>
<td>P RV</td>
<td>tcacgggcaggggcaggccaggcc</td>
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<tr>
<td>fic FW</td>
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<td>Gene Target</td>
<td>Fold Change</td>
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<tr>
<td>------------</td>
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<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>fic RV</td>
<td>ttctctctgctgccaactagat</td>
<td></td>
<td></td>
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<tr>
<td>A/C FW</td>
<td>gagaaccaaagaccaagactgga</td>
<td>repA</td>
<td>465</td>
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<tr>
<td>A/C RV</td>
<td>acgacaaacctgaattgcctcctt</td>
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<tr>
<td>T FW</td>
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<td>repA</td>
<td>750</td>
</tr>
<tr>
<td>T RV</td>
<td>cgttgattcactttagctttggac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fiis FW</td>
<td>ctgtctgaagctgatgge</td>
<td>repA</td>
<td>270</td>
</tr>
<tr>
<td>fiis RV</td>
<td>ctctgccaaacttcage</td>
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<td></td>
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<tr>
<td>frepb FW</td>
<td>tgatcgctttaaggaattttg</td>
<td>RNAI/repA</td>
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</table>

(Carattoli et al., 2005a)
<table>
<thead>
<tr>
<th>FrepB RV</th>
<th>gaagatcagtcacacccc</th>
<th></th>
<th>(Carattoli et al., 2005a)</th>
</tr>
</thead>
<tbody>
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<td>K/B FW</td>
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<td>RNAI</td>
<td>160</td>
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<tr>
<td>K RV</td>
<td>tctttcacgagccgcca</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/O RV</td>
<td>tctggttcgcaagtctga</td>
<td>RNAI</td>
<td>159</td>
</tr>
</tbody>
</table>
2.3.9 Identibac® micro-array analysis

A micro-array assay developed and carried out by the Animal Health Veterinary Laboratories Agency (AHVLA) was used (Batchelor et al., 2008). The micro-array contained a selection of oligonucleotide probes mapping to a range of resistance and virulence-associated genes. Probe hybridizations resulting in signal intensities greater than 0.4 were considered positive indicating the presence of the gene.

2.3.10 MLST methodology

DNA was extracted using a DNeasy extraction kit (Qiagen) and performed as to the manufacturer’s instructions. Sequencing of the DNA was carried out on an Illumina MiSeq (ARK Genomics). Raw sequence reads were aligned to two reference sequences: *E. coli* ABU83972 (NC_017631.1) and *E. coli* MG1655 (NC_000913.2) using BWA and Samtools (Li et al., 2009). Sequence type calling (for multi-locus sequence-typing (MLST)) was performed using short read sequence typing (SRST) (Inouye et al., 2012). Isolates which could not be typed using SRST were called manually using sequences mapped to MG1655. Sequences aligning to the MLST genes in MG1655 were extracted using VCFtools (Danecek et al., 2011) and Extractseq (Rice et al., 2000) and entered manually into the MLST Database, hosted by University College Cork (http://mlst.ucc.ie/mlst/dbs/Ecoli).

2.3.11 Statistical Methods

Comparisons were tested using Fisher’s exact test. The criterion for statistical significance was taken to be $P < 0.05$. 51
2.4 Results

2.4.1 Culture and Sensitivity

Between 2002 and 2011, 18 *E. coli* isolates associated with UTI in 16 dogs were identified as MDR. Results of culture and sensitivity testing for MDR isolates are displayed in table 2.4. Two of the dogs had recurrent UTI. One case recurred 1 month later (565 and 737) and the second case 6 months later (136 and 1088). The criterion used to make the MDR determination was resistance to 3 or more classes of antimicrobial on routine culture and sensitivity testing (Magiorakos et al., 2012). All MDR isolates were resistant to amoxicillin clavulanate and tetracycline, 83% (15/18) were resistant to cephalexin, 78% (14/18) were resistant to co-trimoxazole, 56% (10/18) were resistant to ciprofloxacin, 22% (4/18) were resistant to the 3rd generation cephalosporin cefotaxime. The latter was used as an indicator of broad-spectrum β-lactamase production.

For comparison, 15 *E. coli* isolates were selected for study, based on the criteria that they were susceptible to all 7 antimicrobial classes listed above. These isolates were associated with UTI in dogs.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Breed</th>
<th>Age</th>
<th>Clinical Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>Dachshund</td>
<td>10</td>
<td>Surgery to treat intervertebral disc prolapse. Prescribed amoxicillin/clavulanate for surgical prophylaxis</td>
</tr>
<tr>
<td>R2</td>
<td>Weimeraner</td>
<td>9</td>
<td>Prostatic abscess. Surgical drainage and antibiotics (enrofloxacin and clindamycin).</td>
</tr>
<tr>
<td>R4</td>
<td>Cocker Spaniel</td>
<td>4</td>
<td>Immune mediated haemolytic anaemia, which was treated with immunosuppressant drugs.</td>
</tr>
<tr>
<td>R5</td>
<td>Cross Breed</td>
<td>11</td>
<td>Hyperadrenocorticism. Amoxicillin/clavulanate administered for several months to manage concurrent liver disease.</td>
</tr>
<tr>
<td>R6</td>
<td>Boxer</td>
<td>9</td>
<td>Multiple mast cell tumours. Resection followed by cancer chemotherapy.</td>
</tr>
<tr>
<td>R7</td>
<td>Schnauzer</td>
<td>6</td>
<td>Mast cell tumour. Surgical management followed by cancer chemotherapy.</td>
</tr>
<tr>
<td>R8</td>
<td>Labrador</td>
<td>10</td>
<td>Surgery to resect intestinal adenocarcinoma. Post surgery developed pneumonia and treated with multiple antibiotics</td>
</tr>
<tr>
<td>R9</td>
<td>Collie</td>
<td>10</td>
<td>Diabetes mellitus, which was poorly controlled.</td>
</tr>
<tr>
<td>R10</td>
<td>German Shepherd</td>
<td>8</td>
<td>Prostatic infection and a perineal hernia accompanied by recurrent cystitis.</td>
</tr>
<tr>
<td>R11</td>
<td>Bouvier de Flandres</td>
<td>6</td>
<td>Placement of gastrostomy tube. Stoma became infected requiring antibiotics. Subsequent development of recurrent urinary tract infections.</td>
</tr>
<tr>
<td>R12</td>
<td>Bearded Collie</td>
<td>1</td>
<td>Pyelonephritis; initially treated with amoxicillin/clavulanate.</td>
</tr>
<tr>
<td>R13</td>
<td>Cocker Spaniel</td>
<td>7</td>
<td>Pancreatitis.</td>
</tr>
<tr>
<td>R14</td>
<td>Golden Retriever</td>
<td>5</td>
<td>Ectopic ureters possibly acting as a predisposing factor for urinary tract infection.</td>
</tr>
<tr>
<td>R15</td>
<td>Yorkshire Terrier</td>
<td>11</td>
<td>Concurrent urolithiasis.</td>
</tr>
<tr>
<td>R16</td>
<td>Boxer Cross</td>
<td>7</td>
<td>Detrusor muscle atony decreasing the ability to empty the bladder during urination.</td>
</tr>
</tbody>
</table>

Table 2.4: MDR case summaries: This table details the available clinical information for the dogs from which the MDR isolates were obtained; including age, dog breed and a brief synopsis of the clinical treatment at time of infection.
2.4.2 AmpC β-lactamase phenotype and genotype

To identify the contribution of either ESBL or AmpC β-lactamase to the MDR phenotype, all isolates (both MDR and susceptible groups) were tested using the 4 disc test as described in materials and methods. All susceptible isolates were negative (data not shown). A total of 67% (12/18) of MDR isolates were positive for AmpC production. ESBL production was not detected.

Using ampC multiplex PCR, 9/12 isolates phenotypically AmpC positive were genotypically positive for pAmpC (CITM group). Further simplex PCR identified 8 of these to be specifically associated with CMY-2 gene. Identibac micro-array also detected a CMY gene signal in 8/9 isolates tested (1283, 127, 1223, 1428, 746, 144, 1943, 1201 and 317) (table 2.5).
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Isolation Date</th>
<th>Resistance Profile</th>
<th>3rd Gen. Resistance</th>
<th>Mast Pheno.</th>
<th>pAmpC PCR</th>
<th>Phylotype</th>
<th>Sequence Type</th>
<th>Replicon Type</th>
</tr>
</thead>
<tbody>
<tr>
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<td>AMC / CEF / COT / CIP / TET / GEN</td>
<td>R</td>
<td>AmpC</td>
<td>CMY-2</td>
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<td>10</td>
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</tr>
<tr>
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<td>n/t</td>
<td>46</td>
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</tr>
<tr>
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<td>AmpC</td>
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</tr>
<tr>
<td>1428</td>
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<td>R</td>
<td>AmpC</td>
<td>CMY-2</td>
<td>D</td>
<td>648</td>
<td>FII I1</td>
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<tr>
<td>746</td>
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<td>AMC / CEF / TET</td>
<td>R</td>
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<td>963</td>
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</tr>
<tr>
<td>144</td>
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<td>R</td>
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<td>n/t</td>
<td>FII I1</td>
</tr>
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<td>12/2007</td>
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<td>D</td>
<td>10</td>
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<td>AMC / CEF / TET</td>
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<td>-</td>
<td>D</td>
<td>372</td>
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<td>D</td>
<td>372</td>
<td>I2</td>
</tr>
<tr>
<td>1258</td>
<td>8/2009</td>
<td>AMC / COT / CIP / TET</td>
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<td>FII FIA</td>
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<td>AMC / CEF / CIP / TET / GEN</td>
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<td>-</td>
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<td>AMC / COT / CIP / TET / GEN</td>
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<td>-</td>
<td>n/d</td>
<td>B2</td>
<td>23</td>
<td>FII FIB</td>
</tr>
</tbody>
</table>

Table 2.5: Incorporates the resistance typing of the MDR isolates: Kirby-Bauer disk typing (Resistance Typing), MAST 4-disk testing (Mast Pheno), plasmid-mediated AmpC resistance gene typing PCR (pAmpC PCR), and phenotypic extended-spectrum resistance (3rd. Gen. Resistance). Also descriptive genotyping for E. coli population segregation and plasmid burden: phylotyping PCR (Phylotype), multi-locus sequence-typing scheme (Sequence Type), plasmid-based replicon typing (Replicon Type).
2.4.3 Phylogenetic group and ST designation

PCR-based phylogenetic grouping was performed to assign the isolates to one of 4 phylogenetic groups, namely A, B1, B2 or D. Phylotypes A and B1 are considered to be associated with commensal status or intestinal pathotypes, while B2 and D are more commonly associated with strains causing extraintestinal infections (Tenaillon et al., 2010). Among the susceptible isolates B2 was the predominant phylogenetic group (10 isolates, 67%) with no isolates in group A, 2 isolates (13%) in the B1 grouping and 3 isolates (20%) in group D. In contrast, the MDR group showed a more even distribution among all 4 phylogenetic groups (1 isolate could not be typed), with B2 comprising the smallest category. The distribution for A, B1, B2 or D was 28%, 22%, 11% and 33% respectively. The proportion of MDR isolates identified as phylotype B2 differed significantly from the proportion of susceptible isolates identified as B2 ($P < 0.001$).

MLST identified 18 ST types among *E. coli* isolates. Within the MDR group these were: ST10 (n=4); ST23 (n=3); ST372 (n=2); ST46 (n=1); ST744 (n=1); ST648 (n=1); ST963 (n=1); ST539 (n=1); ST101 (n=1); ST167 (n=1); and ST998 (n=1). The two MDR isolates belonging to phylogenetic B2 belonged to ST 167 and 23. The overall association of ST and phylogenetic group is listed in table 2.5.

Within susceptible group STs identified were: ST73 (n=4); ST12 (n=2); ST641 (n=1); ST127 (n=1); ST10 (n=1); ST625 (n=1); ST929 (n=1); and ST3005 (n=1). A total of 3 isolates from the susceptible group and 1 from the MDR group did not map to existing STs (table 2.6. None of the isolates belonged to ST131.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Isolation Date</th>
<th>Phylotype</th>
<th>Sequence Type</th>
<th>Replicon Type</th>
</tr>
</thead>
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<tr>
<td>162</td>
<td>1/2003</td>
<td>B2</td>
<td>73</td>
<td>-</td>
</tr>
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<td>1365</td>
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<td>B2</td>
<td>73</td>
<td>-</td>
</tr>
<tr>
<td>1568</td>
<td>11/2001</td>
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<td>12</td>
<td>-</td>
</tr>
<tr>
<td>1660</td>
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<td>73</td>
<td>-</td>
</tr>
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<td>1711</td>
<td>11/2001</td>
<td>B2</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>73</td>
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<td>B1</td>
<td>641</td>
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<td>I2 R</td>
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<td>D</td>
<td>N</td>
<td>FIA</td>
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<td>78</td>
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<td>625</td>
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<td>1105</td>
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<td>1190</td>
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<td>B1</td>
<td>3005</td>
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</table>

Table 2.6: Descriptive genotyping for *E. coli* population segregation and plasmid burden: phylotyping PCR (Phylotype), multi-locus sequence-typing scheme (Sequence Type), plasmid-based replicon typing (Replicon Type) for the Susceptible isolates.
2.4.4 Identibac micro-array analysis

A panel of 11 specific probes was extracted from a much larger panel. These represent probes for which any isolate, whether susceptible or MDR, demonstrated a positive result. The full list of probes against which isolates were tested can be found in the supplementary materials.

The virulence marker panel results are summarized in table 2.7. Although the number of isolates examined was limited, there were significantly higher ($P < 0.05$) levels of carriage demonstrated for 7/11 specific virulence markers in the susceptible group compared to the MDR group.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Phylogroup</th>
<th>ireA</th>
<th>iroN</th>
<th>iss</th>
<th>mchB</th>
<th>mchC</th>
<th>mchF</th>
<th>mcm</th>
<th>perA</th>
<th>prfB</th>
<th>senB</th>
<th>sfaS</th>
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</thead>
<tbody>
<tr>
<td>1283</td>
<td>R</td>
<td>A</td>
<td></td>
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Table 2.7: Identibac virulence micro-array results for the MDR and susceptible isolates. R = MDR isolate, S = susceptible isolate; n/t = non-typable; X = presence of gene; reA = siderophore receptor; iroN = enterobactin siderophore receptor; iss = increased serum survival; mchB = microcin H47 (colicin H); mchC = MchC protein; mchF = ABC transporter protein; mcm = microcin M (colicin H); perA = enteropathogenic *E. coli* adherence factor; prfB = P-fimbriae regulation; senB = plasmid encoded enterotoxin; sfaS = S-fimbriae subunit; FE Sig. = Significant difference for MDR versus susceptible by Fisher’s Exact test.
2.4.5 Plasmid Replicon Typing

In the susceptible group (table 2.6) plasmid replicons could not be identified in 5 of the isolates. In the remaining 10 isolates, 6 isolates had 2 or more replicons and 4 isolates carried single replicons. The FII replicon was present in 5 isolates and the FIB replicon was present in 4 isolates. Overall 8 different replicon types were identified in this group.

In the MDR group (table 2.5), plasmid replicons could not be identified in 4 of the isolates. A total of 12 of the remaining 14 carried 2 or more replicons and only 2 isolates carried single replicons. The FII replicon was present in 11 isolates and the I1 replicon was present in 9 isolates. A total of 6 isolates carried the FII and I1 replicons together (these 6 isolates were all phenotypically and genotypically positive for pAmpC). Overall 6 different replicon types were identified in this group.

2.5 Discussion

These findings demonstrate clinically significant MDR E. coli in canine UTI. The antibiogram phenotype of isolates (table 2.5) shows that treatment options are limited. All MDR isolates were resistant to the recommended first line treatment amoxicillin clavulanate and more than half of the isolates were resistant to fluoroquinolones, a third line option (Weese et al., 2011).

MDR strains were analyzed in some detail. Comparisons were made to a susceptible group of canine UTI isolates from the same locality and indirectly to significant human clonal lineages. The latter is particularly important in light of concerns regarding the transfer of organisms between humans and domestic animals, and the potential for either to act as a reservoir of infection for the
other.

AmpC rather than ESBL-producing *E. coli* were commonly identified among the MDR isolates. This is interesting because, in human UTIs associated with MDR *E. coli*, ESBLs (particularly the CTX-M group) seem to be the predominant enzymes responsible for broad-spectrum resistance to β-lactams. Although this study has a low number of isolates, other studies have also identified the presence of AmpC producing *E. coli* in dogs associated with both fecal carriage and clinical disease (Damborg et al., 2012, Dierikx et al., 2012b, Murphy et al., 2009, Shaheen et al., 2011, Sidjabat et al., 2006, Tamang et al., 2012, Wedley et al., 2011a). Furthermore, routine screening in our laboratory has continued to identify AmpC-producing isolates causing UTI (10 isolates from January 2012 to November 2013). Although this represents a relatively low local incidence, these cases are still clinically significant and therapeutically challenging.

The phylogenetic profile differed between the two groups of isolates. The susceptible group of UTI isolates predominantly belonged to the B2 phylogenetic group, as predicted from previous studies in humans and dogs (Johnson et al., 2003, Mao et al., 2012, Thompson et al., 2011). The MDR group meanwhile demonstrated a more even distribution across all four phylo-groups, with significantly less representation of the B2 phylo-group. It is worth noting that a more recent methodology has been able to assign *E. coli* to 8 rather than 4 phylo-groups (Clermont et al., 2013), which could have altered the profile of the isolates in this study, and future work should employ the revised methodology to provide greater detail and depth of characterization. In the context of the results from this study, isolates already assigned to B1 or B2 would be unlikely to change classification, so the difference between groups in the proportions of isolates in the phylotype B2 should still be valid.

The virulence marker profile also differed between the two groups, suggesting
a reduced virulence genotype in the MDR isolates compared to the susceptible ones. Previous studies have indicated that, in MDR isolates associated with UTI in both humans and dogs, there may be a shift away from the dominance of the B2 phylogenetic group and a decrease in certain virulence genes (Moreno et al., 2006, Vila et al., 2002). The reason for this pattern is unclear. Other researchers have speculated that less pathogenic phylogenetic groups are more receptive to the acquisition of the MDR phenotype (Johnson et al., 2004), or that acquisition of the MDR phenotype results in a trade off, with a loss of virulence traits. Whatever the order of events, it would seem logical that less pathogenic phylogenetic groups, with an MDR phenotype, would require certain conditions under which to cause clinical disease.

Sequence typing did not provide evidence for clonal spread of isolates in either group. Considering the extended sampling time this is probably not surprising. More pertinent perhaps was the fact that ST131 was not identified amongst any of our isolates. The O25b-ST131 clonal lineage is one of the most important uropathogenic E. coli groups in humans. It belongs to the B2 phylogenetic group, is a MDR (almost always resistant to fluoroquinolones and often resistant to 3rd generation cephalosporins), is often, but no always, an ESBL producer (CTX-M-15) and is highly virulent (Oteo et al., 2010, Thompson et al., 2011). Total reports of ST131 in domestic animals are still extremely low and although there is some support for interspecies transfer of ST131, it is unclear if animals are a major reservoir or incidental host of this extra-intestinal E. coli (ExPEC) clonal lineage, or indeed if humans act as an animal reservoir (Platell et al., 2011). Certainly within the limitations of our study we found no evidence for this.

One MDR isolate (1428) was typed as ST648 phylogenetic group D. Strains of this clonal lineage that carry ESBLs, have been associated with bacteraemias in human patients in the Netherlands and New Delhi metallo-β-lactamase (NDM)
carbapenamases in human patients in the United Kingdom and Pakistan (Tamang et al., 2012).

Plasmid replicon typing was performed in order to establish the range and diversity of plasmids amongst both the MDR and susceptible isolates. Most of the plasmid replicons correspond to incompatibility groups. In the susceptible group replicon types could not be assigned to 5 of the isolates. This is perhaps not surprising, as the assay is designed to detect resistance plasmids. In the MDR group there were 4 isolates that were not assigned replicon types. Interestingly 2 of these (136 and 1088) were phenotypically AmpC-producing, but it was not possible to detect plasmid-associated genes. We speculate that AmpC production in these isolates could be attributed to chromosomal mutations in the ampC promoter. Further analysis of these isolates will be required to confirm this. In the remainder of the MDR isolates the FII and I1 replicon types were the most highly represented. We observe a cluster comprising 6 isolates (1283, 127, 1223, 1428, 746, 144) (table 2.5) all AmpC-producing and carrying IncFII/IncI1 plasmids. Since IncFII and IncI1 plasmids are two of a number of plasmid types that are particularly successful in their ability to spread MDR (Carattoli, 2011), further characterization and comparison of the plasmids from these isolates would be of particular interest.

With the exception of one case for which we have no history, all cases caused by MDR E. coli had underlying disease involving suppression of the immune system (e.g. hyperadrenocorticism, cancer chemotherapy), an anatomical abnormality of the genitourinary tract (e.g. detrusor muscle atony, ectopic ureters), and/or a history of prior antimicrobial treatment. Clinical details are summarized in table 2.4. This is not a surprising finding since it can be envisaged that such factors will increase the potential of isolates, which we speculate may be less virulent, to cause clinical disease. What will be of interest is to follow the natural
history of infections caused by these organisms in companion animals. It is highly probable that future changes in the epidemiology of MDR *E. coli* infections in dogs, will reflect those seen in the human population, where there has been a shift from hospital-acquired infection, analogous to what we have observed in this study in dogs, to a community-acquired infection, where they are associated with uncomplicated urinary tract disease.

2.6 Thesis Discussion

The methods applied in this chapter are powerful descriptive tools in identifying genotypic and phenotypic commonalities amongst *E. coli* in general. PCR typing of AmpC β-lactamase resistance markers identified a common genotype for the MDR isolates; with a clear bias towards plasmid mediated ESAC resistance. *bla*_{CMY-2} was the predominant detected β-lactamase resistance marker. The presence of the *bla*_{CMY-2} genotype also correlated strongly with an IncI1/IncFII plasmid replicon type; not significantly detectable in the susceptible isolates. This strongly suggests that both the *bla*_{CMY-2} genotype and MDR phenotype may be horizontally acquired amongst the MDR *E. coli*.

However, neither method provides a basis to link the presence of *bla*_{CMY-2} or MDR phenotype, with one of the detected replicon types; or whether either is present chromosomally in the MDR isolates. More fundamentally, it is also not desirable to perform PCR analysis to dissect the genotypic basis of the MDR phenotype as has been done for the ESAC resistance.

Any attempt to fully describe the resistance genotype for the MDR by PCR alone would be hindered by the large and still growing number of resistance genes and resistance gene families; some of which may not be reliably typeable.
by PCR. In a similar manner, the degree of genetic relatedness between the MDR and susceptible can only be partially defined by the work presented in this chapter. Phylotyping indicated a shift towards a more common commensal (A/B1) background in the MDR group, and a strong shift to pathogenic (B2/D) phylotypes in the susceptible group. Both trends were further emphasized by the presence and absence of the limited set of *E. coli* virulence markers included in the Identibac micro-array. However, both are limited by the small number of markers used, and the low numbers of isolates in both the susceptible and MDR groups, to extrapolate the phenotypic potential of the *E. coli* isolates; and both are surrogate measures of overall virulence and uropathogenic potential. There is no experimental knowledge of a potential virulence phenotype for isolates in either group; beyond that they were isolated from active urinary tract infections. A virulence phenotype for the MDR and susceptible isolates would increase the validity of the association of either group with a virulent or avirulent background; and to correlate against recorded phylotypes.

MLST is capable of providing a detailed measure of clonal relatedness between sub-populations of *E. coli*. However, there is no obvious clonal relationship between many of the isolates in either group; despite having been collected at the same location (the Small Animal Hospital). Not only were some of the isolates un-typable by this method, intra and inter-group detected STs were equally variable. Given such variation, which is compounded by the relatively low sample size of the project, very few comparisons can be made between the isolates in this study to the STs of *E. coli* recorded from other studies.

Though not talked about in the paper it is interesting to note that several (4/15) susceptible isolates (162, 1365, 1660 and 1489) were typed as ST73 by MLST (table 2.6). The ST73 clonal group, along with other pandemic clonal ExPEC lineage ST131 and ST69, is associated with virulent UTIs in humans.
(Tartof et al., 2005, Wirth et al., 2006); and is also prevalent in the UK (Lau et al., 2008). ST73 isolates are associated with expanded virulence profile (not in the case of isolate 1489) and is also associated with ESBL resistance (Fam et al., 2011, Oteo et al., 2009) and the is linked with the dissemination of $\text{bal}_{\text{CTX-M}}$ resistance genes. ST73 strains have been isolated from animal sources (Manges et al., 2015) and the discovery of ST73 isolates in the susceptible group (which may indicate a local endemism in the outpatient dog population at the Small Animal Hospital) certainly warrants further inquiry.

To address the shortcomings of the work presented so far, a more detailed genetic understanding of the MDR and susceptible isolates is needed. This would ideally include a greater diversity of samples from different locations (i.e. other hospitals) and greater depth of sampling (i.e. more samples or improved longitudinal sampling); though this was not achieved during this project.

First the MDR and susceptible isolates will be sequenced to allow for more accurate genotypic comparisons. With the wide-spread availability of next-generation sequencing, it is now possible to sequence and assemble large numbers of bacterial genomes. Short-read, whole-genome sequencing allows for the MDR and susceptible strains to be compared with a much higher degree of accuracy. whole genome sequencing (WGS) provides an opportunity to assess both core-genomic sequences (single nucleotide polymorphism (SNP) phylogeny) and pan-genomic sequences in silico; which is entirely impractical by other molecular techniques. The large number of high-quality assembled and published $E. \text{coli}$ genomes, which can be easily included in such comparisons, can add greater context to any inferences arising from sequence data; and may help to address the limitations of a narrow (geographically and chronologically) dataset. WGS would also help assess the genetic linkage of identified resistance markers to either transferable genetic elements (horizontally acquired), or with the $E. \text{coli}$ chromosome (vertical
In order to better correlate broad differences in virulence marker presence an infection model capable of testing virulence in a larger number of *E. coli* isolates will provide a more adequate assessment of phenotypic virulence. Detailed infection models do exist for uropathogenic *E. coli*, but are not easily applied to more than a few strains of *E. coli*. *Galleria mellonella* larvae have successfully been used as an infection model using a number of different pathogenic bacteria. Variations in lethality and innate immune responses elicited by the larvae against bacterial challenge provide strong *in vivo* phenotypes that can be to evaluate difference in virulence potential. The infection model has been applied to UPEC previously (Alghoribi et al., 2014); but like with WGS data there is inherent comparative value in applying the model to previously untested *E. coli* strains. The infection model will be evaluated using previously well characterized UPEC strains, as well as the MDR and susceptible isolates.
Chapter 3

Comparative Genomics of
Multi-Drug Resistant
Escherichia coli

3.1 Introduction

Work in the preceding chapter identified a strong commensal association to the
multi-drug resistant (MDR) group of isolates, but no strong clonal relatedness
amongst any of the isolates in either the susceptible or MDR groups. Work in
this chapter is focused on refining the population structure of both groups of E. coli.

Since the first whole-genome shotgun sequencing of Haemophilus influenzae
in 1995 (Fleischmann et al., 1995), the continued development of next-generation
sequencing (NGS) techniques has allowed increasingly large scale sequencing
projects of prokaryotic and eukaryotic organisms. E. coli is the paradigm model
organism in microbiology. Because of this, NGS has been applied to numerous
different strains from a variety of environmental backgrounds. Short-read NGS is both more powerful than other molecular epidemiological tools and can also be applied more efficiently to a far greater number of samples.

The development of bioinformatic tools to help process the growing abundance of sequencing data is useful in both clinical and research settings. The progression of sequencing technology has steadily replaced molecular epidemiological tools such as multi-locus sequence-typing (MLST), pulsed-field gel electrophoresis (PFGE) and 16s rRNA gene sequence analysis with bioinformatic tools that are both more tolerant and more flexible to genetic variation; and are more easily adapted to incorporate novel genomic loci (Laing et al., 2011). Such flexibility is particularly desirable with _E. coli_; where MLST (Noller et al., 2003) and PFGE (Pei et al., 2008) have been found to underestimate sequence diversity amongst _E. coli_ strains.

Whole-genome comparisons of _E. coli_ from different environmental backgrounds indicate that the core set of gene sequences, common to all _E. coli_, account for no more than 20% of the total genome. By contrast, the remaining 80% of the identifiable gene content is variably associated to _E. coli_ strains of different environmental niches; and is strongly linked to horizontal gene transfer (HGT) and genetic recombination (Touchon et al., 2009, Lukjancenko et al., 2010). Such genetic diversity is not unexpected given that _E. coli_ is a widely distributed, yet niche driven pathogenic and commensal component of the microbiota in a diverse range of hosts.

Pathogenic _E. coli_ are capable of causing a spectrum of diseases which include intestinal infections: enterohaemorrhagic _E. coli_ (EHEC), enterotoxigenic _E. coli_ (ETEC), enteropathogenic _E. coli_ (EPEC) and diffuse entero-aggregative _E. coli_ (DEAEC); as well as extra-intestinal infections (extra-intestinal _E. coli_ (ExPEC)): uropathogenic _E. coli_ (UPEC) and neonatal meningitis _E. coli_ (NMEC)
infections. The partitioning of *E. coli* into these various pathotypes is done by the detection of virulence factors that are characteristic of a particular pathology (Kaper et al., 2004). Because these virulence factors are exclusively pan-genomic, and not core to all *E. coli*, whole genome sequencing (WGS) can be used to predict the pathotype and pathogenic potential of an *E. coli* isolate *in silico*.

Despite a moderately conserved core genome, it has been proposed that ExPEC lineages are independently derived from the convergence of different clonal lineages of *E. coli* (Kaas et al., 2012). To date, there are multiple complete assembled genomes for UPEC isolates. These included UPEC strains: CFT073, UTI89, and 536; the asymptomatic UPEC strain: ABU83972; and the MDR pathogenic UPEC strain JJ1886. With the exception of CFT073 and ABU83972, which are highly related strains of *E. coli* (Wassenaar et al., 2015), these UPEC strains have been isolated from differing genetic backgrounds.

As discussed in the general introduction, the aetiology of urinary tract infections (UTI)s is strongly influenced by a number of different environmental factors, such as: host health status and age; which cannot be accounted for by sequencing *E. coli*. Despite this, core-genomic phylogeny provides another means of predicting, *in silico*, the pathogenic potential, as well as the genetic lineage of previously unsequenced *E. coli* strains.

Based on the work in the previous chapter, it was hypothesized that there is a clear difference in the pathogenic potential of the MDR and susceptible isolates. WGS allows these strains to now be directly compared to *E. coli* isolates from across a range of pathotypes and niches; by comparing both the core and pan-genome of different *E. coli* isolates.

As well as using *in silico* WGS comparison studies of *E. coli* to obtain estimates of potential virulence it is desirable to have a means to test the virulence of
previously uncharacterized \textit{E. coli} \textit{in vivo}. \textit{Galleria mellonella} larvae have been used as an infection model for a number of different bacterial pathogens, including EPEC, UPEC and commensal \textit{E. coli} (Alghoribi et al., 2014). The \textit{Galleria mellonella} model can also be ethically applied to a much larger number of samples than other infection models; and larvae can be incubated at 37° C, which is optimal for \textit{E. coli}.

\section*{3.2 Methods}

\subsection*{3.2.1 DNA Extraction}

Illumina sequencing was performed by Edinburgh Genomics, using an Illumina Hi-Seq 2500 sequencer. Nextera XT libraries were prepared by Edinburgh Genomics, yielding 100bp paired-end reads. \textit{E. coli} strains were grown overnight at 37°C, 170 rpm, in lysogeny broth (LB). DNA was extracted using a Qiagen DNeasy extraction kit. DNA was isolated from 1ml of bacterial culture, and performed to the manufacturer’s specifications.

\subsection*{3.2.2 Sequence Assembly}

Raw sequence reads were assessed using FastQC (Andrews, 2010) before assembly to ensure successful sequencing. Sequence reads were then assembled using the velvetOptimiser script, from Velvet (v1.2.08) (Zerbino and Birney, 2008). A kmer size range of 47 to 67, with size increments of 2, was used. Reads were filtered before assembly using Sickle (Joshi and Fass, 2011), with a minimum read quality score of 30, and a minimum read length of 50bp. The sequence reads were then re-shuffled, using the shuffleSequences\_fastq.pl script. \textit{De novo} assembly used both
paired reads and singletons. The quality of assembled genomes was evaluated using Quality Assessment Tool (QUAST) (Gurevich et al., 2013).

### 3.2.3 Sequence Annotation and Analysis

Sequence annotation was performed with both the Rapid Annotations based on Subsystem Technology (RAST) annotation server (Aziz et al., 2008), Prokka (Seemann, 2014) and the Sanger genome pipeline: Roary (Page et al., 2015). AMR and virulence marker genes were detected using tools developed by the Center for Genomic Epidemiology: ResFinder (Zankari et al., 2012) and VirulenceFinder (Joensen et al., 2014). SeqFinder (Stanton-Cook et al.) was used to identify virulence gene sequences in the assembled genomes. Virulence gene sequences were obtained from Virulence Factor Database (VFDB) (Chen et al., 2012).

Phylogenetic analysis was performed using RealPhy (Bertels et al., 2014). ClonalFrameML (Didelot and Wilson, 2015) was used to estimate core sequence phylogeny and recombination estimation analysis. *E. coli* reference sequences were obtained from the NCBI RefSeq collection. Sequence alignment input files and the guide-tree were generated suing the Harvest suite (Treangen et al., 2014). Roary was used to generate maximum-parsimony core and pan-genomic sequence comparisons.

### 3.2.4 *Galleria mellonella* infection model

*E. coli* strains were grown aerobically, overnight in 10 ml of LB at 37°C, and 170 rpm. Cultures were re-suspended in phosphate buffered saline (PBS) and standardized to an optical density (OD) of 1.0 at 600nm. Strains were then further diluted ten-fold in PBS. Moth larvae were injected with μL of bacterial
suspension or PBS control. *Galleria mellonella* larvae were obtained from a local pet supply store.

PBS sham injection and no-injection control groups of 10 larvae were used to control for adverse outcomes due to injection methodologies. CFT073 (pyelonephritis) and ABu83972 (asymptomatic) *E. coli* strains were used to assess baseline virulence thresholds for the model.

Each injection group consisted of 10 individual larvae. Once successfully inoculated, larvae were incubated at 37°C for the remainder of the experiment. Larvae were evaluated for melanisation and death at 24 hour intervals, for a period of four days post-injection.

### 3.3 Results

#### 3.3.1 Sequence Assembly

FastQC indicated successful sequencing runs for all of the isolates; all sequences exceeded minimum quality scores for the metrics assessed by FastQC. Genome coverage was estimated at roughly 100 fold for all of the isolates. A strain key is provided to allow for the conversion between sequencing ID numbers and isolate ID numbers (table 3.1).

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Table 3.1: The table shows the sequence identification number, strain ID and group (MDR or susceptible) for each of the isolates sequenced in this study.

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*E. coli* is notorious for being difficult to sequence; with many large repeat regions which prevent contig assembly from short-read sequencing (Koren et al., 2013). This was reflected in the assemblies for some of the isolates in both groups.
(table 3.2 and table 3.3).
Table 3.2: MDR Isolate assembly statistics

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Multi-drug resistant isolates Illumina sequence assembly statistics. Isolates were sequenced using and Illumina Hi-Seq, with read lengths of 100bp. All statistics are based on contigs of size $\geq$200 bp, except for contig number (which includes all assembled contigs). N50: the length (N) of the assembled genome which is accounted for by 50% of all bases; i.e the median length of a genome assembly. L50: The number (L) of assembled sequences, whose total length is $\geq$50% of the total length of all assembled sequences. Misassemblies: Identified base positions (breakpoints) within assembled contigs that i) contain flanking sequences that are greater than 1kb apart, ii) contain flanking sequences that align to opposing strands to one another, iii) contain flanking sequences that overlap by more than 1kb. Assembly metric calculated by Quast using GeneMarkS, GeneMark-ES and GlimmerHMM.
Table 3.3: **Susceptible isolate assembly statistics**

<table>
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<tr>
<th>Assembly</th>
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Susceptible isolates Illumina sequence assembly statistics. Isolates were sequenced using and Illumina Hi-Seq, with read lengths of 100bp. All statistics are based on contigs of size ≥200 bp, except for contig number (which includes all assembled contigs). N50: the length (N) of the assembled genome which is accounted for by 50% of all bases; i.e the median length of a genome assembly. L50: The number (L) of assembled sequences, whose total length is ≥50% of the total length of all assembled sequences. Miss-assemblies: Identified base positions (breakpoints) within assembled contigs that i) contain flanking sequences that are greater than 1kb apart, ii) contain flanking sequences that align to opposing strands to one another, iii) contain flanking sequences that overlap by more than 1kb. Assembly metric calculated by Quast using GeneMarkS, GeneMark-ES and GlimmerHMM.
The National Center for Biotechnology Information (NCBI) RefSeq collection of completed *E. coli* genomes provides a rough benchmark to compare genome assemblies against. *E. coli* has a median total genome length of 5.17 mega bases (Mb) (min 3.98Mb, max 5.87Mb), with a median number of 5040 annotated protein coding sequences (min 4017, max 6377) and a median guanidine-cytosine (GC) content of 50.6%. The draft assemblies for the isolates sequenced in this project broadly conform to these values; with a few exceptions (MDR: fig. 3.1, fig. 3.3. Susceptible: fig. 3.2, fig. 3.4).
Figure 3.1: The cumulative length of all assembled contigs for each sequenced multi-drug resistance isolate. Contigs are plotted from largest to smallest on the x-axis. The baseline genome length used for this plot is the reference sequence *Escherichia coli* MG1655, which has a chromosome length of 4.64Mb. The current size range for listed NCBI RefSeq *E. coli* genomes is between 4.48Mb and 5.87Mb.
Figure 3.2: The cumulative length of all assembled contigs for each sequenced susceptible isolate. Contigs are plotted from largest to smallest on the x-axis. The baseline genome length used for this plot is the reference sequence *Escherichia coli* MG1655, which has a chromosome length of 4.64Mb. The current size range for listed NCBI RefSeq *E. coli* genomes is between 4.48Mb and 5.87Mb.
Figure 3.3: The percentage guanidine, cytosine nucleotide composition of the assembled multi-drug resistance isolate genomes. GC% is plotted on the x-axis. The assembled genomes are divided into non-overlapping 100bp windows, and the number of windows for each GC% is plotted on the y-axis. The reference genome *Escherichia coli* MG1655 is indicated on the plot by the dashed line. *E. coli* isolates typically have a GC% of 50%. Monoculture sequences (i.e. uncontaminated samples) typically display a Gaussian distribution about the mean. Contaminated samples show multiple peaks.
Figure 3.4: The percentage guanidine, cytosine nucleotide composition of the assembled susceptible isolate genomes. GC% is plotted on the x-axis. The assembled genomes are divided into non-overlapping 100bp windows, and the number of windows for each GC% is plotted on the y-axis. The reference genome *Escherichia coli* MG1655 is indicated on the plot by the dashed line. *E. coli* isolates typically have a GC% of 50%. Monoculture sequences (i.e. uncontaminated samples) typically display a Gaussian distribution about the mean. Contaminated samples show multiple peaks.
From the MDR group (table 3.2), isolate 1176 (0863N0002) has a projected cumulative genome length of greater than 6Mb. Along with an increased projected genome length, the contigs contain 7175 predicted genes; also much higher than expected. Much of this is expected to be a result of a poorer than average sequence assembly. The assembled contigs contain a ten-fold higher number of un-sequenced bases (1216.24 N’s per 100kilo base pairs (kbp)), along with a comparatively poor N50 score (13298) and much higher number of assembled contigs (1458).

Isolate 1428 (0863N0005) also contains an expanded number of predicted genes (6195), without being an outlier in terms of estimated cumulative genome length (5.8Mb). Like 1176, the N50 (97177), N’s per kbp (1053.61) and total number of contigs (1458) indicate that this is likely due to sequence assembly error.

Isolates 1943 (0863N0006) (predicted genes: 6914, total length: 6.9Mb) and 317 (0863N0030) (predicted genes: 6243, total length: 5.6Mb) also show small projected expansions of genomic content, also with relatively poor assembly metrics: N50 (11713, 41339) and number of assembled contigs (2481, 1617).

Isolate 1271 (0863N0007) shows a particularly large predicted gene content (9217) and genome length (6.7 Mb). However, the N50 score (4613) and total number of contigs (4548) indicate an exceptionally fragmented assembly; compared to the other isolates. Though estimated mis-assemblies (56) and N’s per 100 kbp are not much higher than the other isolates.

The remaining MDR isolates have assemblies which are not worse than expected for *E. coli* (Koren et al., 2013); and compare favorably to the *E. coli* RefSeq sequences.

The susceptible isolates were less variable, in terms of assembly metrics and compared favorably to expected genome size and predicted genome content (table
3.3. However, isolate 58 (0863N0015) is a significant outlier in almost every assessed metric. Total number of assembled contigs (15813, hundred fold higher than other isolates), N50 (939, excessively short for 200bp paired-end reads) and L50 (2072) all point to a poor genome assembly. This is expected to account for the drastically expanded predicted genome (total length: 8.8Mb, predicted genes: 15294).

3.3.2 Assembly Mapping to Reference Genome

In concurrence with relatively good assembly metrics for the majority of the isolates in both the MDR and susceptible groups, and favorable comparisons to published RefSeq sequences; the sequenced isolates show good sequence alignment to *E. coli* MG1655. Both the MDR (fig. 3.5) and susceptible alignments (fig. 3.6) show aligned contiguous sequences along the majority reference sequence; with few gaps. This includes isolate 58 (0863N0015), which has the strongest mapping of contigs to the reference genome despite a poor assembly.
Figure 3.5: Multi-Drug Resistance isolate genome assemblies, as aligned to the reference genome of *Escherichia coli* MG1655 (NCBI accession:NC000913.3). Green blocks identify contigs that are aligned to the reference genome. Red blocks identify contigs that are aligned to the reference genome that contain ≥1 missassemblies. Blank space indicates an area of the reference genome to which none of the assembled contigs have aligned.
Figure 3.6: Susceptible isolate genome assemblies, as aligned to the reference genome of *Escherichia coli* MG1655 (NCBI accession:NC000913.3). Green blocks identify contigs that are aligned to the reference genome. Red blocks identify contigs that are aligned to the reference genome that contain $\geq 1$ mis-assemblies. Blank space indicates an area of the reference genome to which none of the assembled contigs have aligned.
3.3.3 Resistance Markers

The MDR isolates (table. 3.4) showed strong mapping to a variety of resistance gene markers. The β-lactam and extended-spectrum cephalosporin resistance phenotype is largely attributable to a $\text{bla}_{\text{CMY}-2}$ resistance genotype across the MDR isolates; with only a couple of exceptions.

Isolate 317 (0863N0030) possesses a $\text{bla}_{\text{CMY}-7}$ resistance gene which accounts for the extended-spectrum AmpC (ESAC) resistance. An additional β-lactamase resistance maker $\text{bla}_{\text{TEM}-1B}$ was also detected in the 317 assembly.

Isolate 565 (0863N0031), also phenotypically positive for ESAC resistance, contained no detectable ESAC resistance genotype.

Isolate 127 (0863N0033) was positive for $\text{bla}_{\text{TEM-33}}$ resistance marker; which is an extended-spectrum β-lactamase (ESBL) rather than an ESAC resistance gene.

Isolate 1088 (0863N0009), despite showing phenotypic resistance to cefalexin, ampicillin, tetracycline and extended-spectrum resistance, contained no detectable resistance markers.

Aminoglycoside resistance markers were detected in 8 of the MDR isolates; despite only 4 of the isolates being phenotypically positive for gentamycin resistance. Though many of the detected resistance markers convey resistance against other aminoglycosides ($\text{strA strB, aad}$ (streptomycin), $\text{aph}$ (hygromycin)) and variably against gentamycin ($\text{aac}$); the isolates were only tested for gentamycin resistance so detectable resistance cannot necessarily correlated with phenotypic resistance.

Phenotypic resistance to co-trimoxazole (potentiared-sulfonamide) was preva-
lent amongst the MDR isolates (14/18). Correspondingly, resistance markers were identified in the majority of the MDR isolates. Only one MDR isolate, 565 (0863N0031) (sul1, dfra1) showed positive identification of potentiated-sulfonamide resistance markers with no evidence of phenotypic resistance.

Tetracycline was the second most common resistance (15/18). With few exceptions, the resistance phenotype can be attributed to the widespread identification of tetA and tetB resistance marker genes. Though 1943 (0863N0006), 1088 (0863N0009), 1201 (0863N0027) and 127 (0863N0033) were all positive for phenotypic resistance, but contained no detectable resistance markers in their sequence assemblies.

Phenotypic resistance to chloramphenicol resistance markers (cat, floR) were identified in 8/18 of the isolates; though as the isolates were not tested for phenotypic resistance the two cannot be correlated.

Similarly, isolates 1176 (0863N0002), 1223 (0863N0013), 565 (0863N0031) and 737 (0863N0035) all contain a singular resistance marker (mphA or mphB) targeting macrolide antibiotics; but no phenotypic resistance testing was carried out for any of the macrolides.

Despite 8/18 isolates being positive for phenotypic resistance against ciprofloxacin, no fluoroquinolone resistance markers were detected in any of the MDR isolates. Fluoroquinolone resistance is primarily achieved through point mutation to DNA gyrase and topoisomerase genes; though resistance can be acquired by plasmid mediated qnr genes (Jacoby, 2005). ResFinder cannot identify such point mutations from the sequence data; and is only capable of identifying the acquired qnr resistance genes. No work was done to attempt to identify any point mutations in the isolates that may contribute towards the observed ciprofloxacin resistance (which is the most likely explanation).
The susceptible isolates contained almost no detected resistance markers. With the exception of isolate 58 (0863N0015) and 1190 (0863N0024) (table 3.4). The β-lactamase resistance gene \( \text{bla}_{\text{TEM-1D}} \) was detected in the sequence assembly for 1190, despite no detectable phenotypic resistance to either cefalexin or amoxicillin.

Isolate 58 possessed an extensive resistance genotype. Aminoglycoside (\( \text{aadA1} \), \( \text{aph(3')-lc} \), \( \text{strA} \), \( \text{strB} \)), macrolide (\( \text{mphB} \)), sulfonamide (\( \text{sul2} \)) and trimethoprim (\( \text{dfrA1} \)) resistance markers were all detected in the sequence assembly. The isolate had been previously tested against a standard panel of antibiotics (not carried out in this project) and found to be fully susceptible. The detectable resistance markers are assumed to be an artifact of poor sequence assembly and the isolate has not been re-typed.

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<th>Resistance Genes</th>
<th>Resistance Phenotype</th>
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<td>3rd/2nd gen. CEF AMC</td>
</tr>
</tbody>
</table>
0863N0002  MDR  aph(4)-1a  aac(3)-Iva  aph(3)-1c  aadA1  blaTEM-1A  mph(B)  catA1  floR  sul1  tet(A)  dfrA1  1st gen. CEF AMC CO-TRIM CIP TET GENTA

0863N0003  MDR  sul2  tet(B)  3rd/1st gen. CEF AMC COT TET

0863N0005  MDR  blaCMY-2  tet(B)  3rd/1st gen. CEF AMC TET

0863N0006  MDR  blaCMY-2  catB3  dfrA1  3rd/1st gen. CEF AMC CO-TRIM CIP TET

0863N0007  MDR  strA  strB  aadA1  blaTEM-1A  floR  sul2  tet(B)  dfrA1  1st gen. CEF AMC CO-TRIM TET

0863N0009  MDR  aph(3')-1a  aadA5  blaCMY-2  mph(A)  catA1  sul1  tet(B)  dfrA17  3rd/1st gen. CEF AMC TET

0863N0027  MDR  blaCMY-2  dfrA5  3rd/1st gen. CEF AMC CO-TRIM TET

0863N0030  MDR  aadA2  strA  strB  aadA2  blaTEM-1B  blaCMY-7  sul1  sul2  tet(A)  dfrA12  dfrA1  3rd/1st gen. CEF AMC CO-TRIM CIP TET

0863N0031  MDR  aac(3)-Iva  aph(4)-1a  aadA1  aph(3')-1c  blaTEM-78  mph(B)  catA1  floR  sul1  tet(A)  dfrA1  3rd/1st gen. CEF AMC CIP TET GEN

0863N0032  MDR  strA  strB  blaCMY-2  sul2  tet(A)  dfrA14  3rd/1st gen. CEF AMC CO-TRIM CIP TET

0863N0033  MDR  aadA5  aph(3')-1a  strA  strB  blaTEM-33  catA1  sul2  sul1  dfrA1  dfrA17  3rd/1st gen. CEF AMC CO-TRIM TET

0863N0035  MDR  aadA1  aph(3')-1c  blaTEM-1A  mph(B)  catA1  sul1  tet(A)  dfrA1  AMC CO-TRIM CIP TET GEN

0863N0039  MDR  aadA1  strB  strA  blaTEM-1A  floR  sul2  tet(B)  dfrA1  1st gen. CEF AMC CO-TRIM CIP TET

0863N0036  MDR  aadA1  strB  strA  blaTEM-1A  floR  sul2  tet(B)  dfrA1  1st gen. CEF AMC CO-TRIM CIP TET

0863N0028  MDR  aadA1  strB  strA  blaTEM-1A  floR  sul2  tet(B)  dfrA1  1st gen. CEF AMC CO-TRIM CIP TET

0863N0037  MDR  aadA1  strB  strA  blaTEM-1A  floR  sul2  tet(B)  dfrA1  1st gen. CEF AMC CO-TRIM CIP TET

90
Shows the detected *in silico* resistance markers for both the susceptible and multi-drug resistance isolate assemblies. Resistance alleles include beta-lactamase (blaCMY, blaTEM), tetracycline resistance (sul, dfrA, tet), fluoroquinolone (floR, catA, catB), aminoglycoside (aadA, aph, strA, strB), and macrolide (mphB) resistances. The *in silico* resistance profile is then compared with the recorded phenotypic resistance: 3rd gen (extended-spectrum AmpC phenotype), AMC (ampicillin), CEF (cefalexin), COT (co-trimoxazole), CIP (ciprofloxacin), TET (tetracycline), GEN (gentamycin).

### 3.3.4 Virulence Markers

Two different *in silico* methods were used to assess the presence of different virulence genes in the MDR and susceptible isolates. VirFinder uses reference virulence gene sequences from *E. coli* from across the different pathotypes (table 3.5). It does not provide an opportunity to expand the curated databases of virulence markers beyond a limited selection; but can be used to confirm the Identibac results from Chapter 2.

SeqFinder was used to better identify urovirulence genes from a more comprehensive database of *E. coli* virulence markers from VFDB. SeqFinder provides a more complete set of genetic markers to help differentiate the susceptible and MDR isolates.

In general, the attempted separation of the isolates by presence or absence of virulence markers was not successful. Initial typing using VirFinder (table 3.5) and Identibac suggested that there was an increased presence of virulence markers in the susceptible isolates. Whilst the result produced by SeqFinder (fig. 3.7) is concurrent with some of the results, increasing the number of virulence markers used to type the isolates does indicate more general inter and intra-group differences amongst the isolates.
Overall, SeqFinder identified a much stronger toxin presence than either VirFinder or Identibac. Haemolysin (hly) (14/31) and vacuolating toxin (vat) were identified in both susceptible and MDR isolates. SeqFinder does identify two sub-groups of isolates, based on their virulence profile. The first group (11/31) is comprised mostly of susceptible isolates (7/11). These isolates do possess a strong urovirulence profile; including: fimbrial proteins (pap, fim (type-1 fimbriae) and foc (F1C fimbriae)), hly (haemolysin), and multiple siderophore gene families (iro, ybt, chu). The second group (12/31) is almost exclusively MDR (10/12) and is mostly bereft of virulence markers, with the exception of: fim, ecp (E. coli common pillus) and some siderophores (iut, ybt, sit).

With both groups trending towards either susceptible or MDR status, the remaining small group of isolates do not cluster; and have more unique virulence profiles (presence of toxins vat, sat and pic). Though the inability of SeqFinder to successfully cluster these isolates is better explained by the inconsistent identification of genes that better define the two clusters.

Table 3.5: VirFinder detected virulence markers

<table>
<thead>
<tr>
<th>ID</th>
<th>Group</th>
<th>Virulence Genes</th>
<th>Galleria melonella lethality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0863N0004</td>
<td>Susceptible</td>
<td>iss mchB mchC lpfA mchF mcmA</td>
<td>90-100%</td>
</tr>
<tr>
<td>0863N0008</td>
<td>Susceptible</td>
<td>pic vat iss cnf1 iroN mchC mcmA mchB prfB</td>
<td>90-100%</td>
</tr>
<tr>
<td>0863N0014</td>
<td>Susceptible</td>
<td>ireA mcmA vat astA mcmA mchB mchC sfaS prfB iroN cnf1</td>
<td>70-80%</td>
</tr>
<tr>
<td>0863N0015</td>
<td>Susceptible</td>
<td>senB mchB mchC iroN mcmA mchF iss f17G cnf1 mchF prfB lpfA astA</td>
<td></td>
</tr>
<tr>
<td>0863N0016</td>
<td>Susceptible</td>
<td>mcmA iss mchF pic iroN vat mchC prfB ireA mchB cnf1</td>
<td>20-90%</td>
</tr>
<tr>
<td>0863N0018</td>
<td>Susceptible</td>
<td>ehxA cnf1 mcmA mchF f17A iroN iss mchC vat prfB mchB senB</td>
<td>0-10%</td>
</tr>
<tr>
<td>0863N0021</td>
<td>Susceptible</td>
<td>mchB iroN mchC vat iss gad prfB mchF mcmA cnf1</td>
<td>90-100%</td>
</tr>
<tr>
<td>0863N0022</td>
<td>Susceptible</td>
<td>vat iss mcmA ireA prfB mchC cnf1 mchB iroN sfaS astA</td>
<td>90-100%</td>
</tr>
<tr>
<td>0863N0024</td>
<td>Susceptible</td>
<td>prfB</td>
<td>80-100%</td>
</tr>
<tr>
<td>Code</td>
<td>Type</td>
<td>Markers</td>
<td>Percentages</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
<td>-------------------------------------------------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>0863N0025</td>
<td>Susceptible</td>
<td>iss vat sfaS mcmA iroN prfB cnf1</td>
<td>90-100%</td>
</tr>
<tr>
<td>0863N0026</td>
<td>Susceptible</td>
<td>prfB pic iss sat vat iha senB</td>
<td>20%</td>
</tr>
<tr>
<td>0863N0029</td>
<td>Susceptible</td>
<td>iroN sfaS mcmA ireA vat mchF gad iss mchC cma mchB cba cnf1 prfB</td>
<td>100%</td>
</tr>
<tr>
<td>0863N0034</td>
<td>Susceptible</td>
<td>mchC iroN pic mchF iss senB mcmA prfB iha vat gad</td>
<td></td>
</tr>
<tr>
<td>0863N0038</td>
<td>Susceptible</td>
<td>astA iss mcmA sfaS vat iroN prfB</td>
<td>70-100%</td>
</tr>
<tr>
<td>0863N0001</td>
<td>MDR</td>
<td>cba cma prfB gad</td>
<td>75-100%</td>
</tr>
<tr>
<td>0863N0002</td>
<td>MDR</td>
<td>mchF mcmA prfB f17G astA iss tsh lpfA iroN</td>
<td>90-100%</td>
</tr>
<tr>
<td>0863N0003</td>
<td>MDR</td>
<td>ireA iss prfB</td>
<td>50-75%</td>
</tr>
<tr>
<td>0863N0005</td>
<td>MDR</td>
<td>iss prfB lpfA gad iss</td>
<td>100%</td>
</tr>
<tr>
<td>0863N0006</td>
<td>MDR</td>
<td>lpfA iss prfB mcmA</td>
<td>75-80%</td>
</tr>
<tr>
<td>0863N0007</td>
<td>MDR</td>
<td>mcmA prfB mchC mchB cnf1 iss</td>
<td>100%</td>
</tr>
<tr>
<td>0863N0009</td>
<td>MDR</td>
<td>mcmA vat iroN iss mchB mchC mchF prfB</td>
<td></td>
</tr>
<tr>
<td>0863N0013</td>
<td>MDR</td>
<td>mchF iss iroN prfB</td>
<td>10-30%</td>
</tr>
<tr>
<td>0863N0027</td>
<td>MDR</td>
<td>lpfA iss prfB mchF iroN iss</td>
<td>0%</td>
</tr>
<tr>
<td>0863N0030</td>
<td>MDR</td>
<td>prfB gad iss</td>
<td>0-25%</td>
</tr>
<tr>
<td>0863N0031</td>
<td>MDR</td>
<td>mchF astA mcmA lpfA prfB tsh iss f17G iroN</td>
<td>100%</td>
</tr>
<tr>
<td>0863N0032</td>
<td>MDR</td>
<td>prfB</td>
<td>0-100%</td>
</tr>
<tr>
<td>0863N0033</td>
<td>MDR</td>
<td>iss prfB</td>
<td>0-25%</td>
</tr>
<tr>
<td>0863N0035</td>
<td>MDR</td>
<td>iroN tsh iss astA mchF mcmA lpfA prfB</td>
<td>100%</td>
</tr>
<tr>
<td>0863N0039</td>
<td>MDR</td>
<td>mcmA prfB</td>
<td>100%</td>
</tr>
<tr>
<td>0863N0036</td>
<td>MDR</td>
<td></td>
<td>60-90%</td>
</tr>
<tr>
<td>0863N0028</td>
<td>MDR</td>
<td></td>
<td>10%</td>
</tr>
<tr>
<td>0863N0037</td>
<td>MDR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Shows the detected *in silico* virulence markers for both the susceptible and multi-drug resistance isolate assemblies. Virulence markers detected include: microcins (mchA, mchB, mchC, mcmA), toxins (pic, vat, cnf1, senB, astA), fimbrial proteins (lpfa, sfaS, f17A), metabolic related proteins (prfB, gad, tsh), cellular adherence (iha), haemolysis (ehxA), colicins (cba, cma), innate immune evasion (iss) and haem-utilization (iroN, ireA). The *in silico* virulence profile is then compared to *in vivo* resistance in the *Galleria mellonella* infection model; as equated by the proportion of the moth larvae killed by the final day of infection.
Figure 3.7: SeqFinder analysis of urovirulence gene presence using CFT073 virulence genes obtained from VFDB. Black = gene presence, green square = susceptible isolate, red square = MDR isolate. Included virulence genes: P-fimbriae (pap), type 1 fimbriae (fim), FIC (foc), vat (toxin), sat (toxin), pic(toxin), haemolysin (hly), enterobactin (iro), Yersiniabactin (ybt), sit (siderophore), chu (siderophore), E. coli common pillus (ecp). SeqFinder identified two clusters of similar virulence gene profiles. Group 1 are highlighted in red on the cladogram. Group 2 are highlighted in green on the cladogram. Blue branches on the cladogram indicate strains with unique virulence gene profiles.
3.3.5 *Galleria mellonella* Virulence Model

The *Galleria mellonella* infection model does not show a trend towards more virulence, or more asymptomatic isolates in either the MDR or susceptible groups. The number of virulence markers detected did not correlate at all with moth larvae lethality (table 3.5).

Sham injected (PBS) and non-injected (NI) control group larvae showed no lethality throughout. The asymptomatic bacteriurea control strain ABU83972 showed no lethality. In contrast the urovirulence control strain (CFT073) showed consistently high mortality of 80% (8/10) (fig. 3.8, fig. 3.9).

In general there was good consistency across both the MDR (fig. 3.10, 3.11) and susceptible (fig. 3.8, fig. 3.9). Only 1283 (MDR) and 162 (susceptible) showed large inconsistencies.

1283 showed complete lethality (100%) in the first round of infections, but showed no larvae lethality in the second round of infections.

162 showed a large increase in lethality within the first 24 hours post-injection of the second infection. This resulted in a 3 fold increase in the proportion of larvae killed by the end of the infection (90%) in comparison to the first round of infections (30%).

As well as showing no increased or decreased virulence between the two groups of isolates, when assessed collectively (fig. 3.12); there was a lack of increasing lethality across the time period of infection for any of the isolates. With few exceptions of minor increases in proportion of larvae dead across the time period of infection; the proportion of larvae dead did not generally increase after the first 24 hours post-injection.
Figure 3.8: First repeat of the *Galleria mellonella* larvae infection model for the susceptible group isolates. NI = no injection control, PBS = PBS sham injection control, ABU = asymptomatic bacteriurea strain, CFT = virulent UPEC strain.
Figure 3.9: Second repeat of the *Galleria mellonella* larvae infection model for the susceptible group isolates. NI = no injection control, PBS = PBS sham injection control, ABU = asymptomatic bacteriurea strain, CFT = virulent UPEC strain.
Figure 3.10: First repeat of the *Galleria mellonella* larvae infection model for the MDR group isolates. NI = no injection control, PBS = PBS sham injection control.
Figure 3.11: Second repeat of the *Galleria mellonella* larvae infection model for the MDR group isolates. NI = no injection control, PBS = PBS sham injection control.
Figure 3.12: Combined *Galleria mellonella* survival curves for the MDR and susceptible groups. Moth mortality across the 4 day time points is calculated as the proportion of larvae alive across all isolates within the group.
3.3.6 Core and Pan Genome Analysis

Core-genomic and pan-genomic sequence constructions do indicate different genetic backgrounds for the MDR and susceptible group isolates; though it is important to note the genetic variability between isolates is still high. Core-genome single nucleotide polymorphism (SNP) comparisons, combined with pan-genomic sequence presence or absence does show distinct backgrounds for the susceptible and MDR groups; though the isolates are far from clonal (fig. 3.13).
Figure 3.13: Core genomic phylogenetic estimation of MDR, susceptible and RefSeq *E. coli* sequences. *E. fergusonii* was used to root the tree as the closest common ancestor. Pathogenic (EHEC, UPEC, EPEC, APEC, and ExPEC) and non-pathogenic (Environmental and Commensal/Probiotic) *E. coli* RefSeq sequences were included in the sequence comparisons.
Phylogenetic analysis of the MDR isolates, susceptible isolates and RefSeq sequences for *E. coli* of different pathotypes (fig. 3.13) suggest a wide ranging variability in genetic background. Which is concurrent with previous estimates of genetic relatedness with MLST. There is some closer relatedness to UPEC RefSeq sequences ABU83972 and CFT073 with a small group of susceptible isolates (1489, 1365, 1660 and 162); but the remainder of the isolates sequenced in this project are widely dispersed and form no distinct grouping.

Given the dispersal of the strains across the tree, it is not possible to correlate many MDR or susceptible strains to any one pathotype. Even amongst the UPEC RefSeq sequences (JJ1886, 536, CFT073, ABU83972, UTI89 and IAI39) there is no clustering, which reflects the more heterogeneous background of UPEC in general. Though there are exceptions; most notably EHEC and K-12 derived sequences.

A potential source of variability in the phylogenetic reconstruction is likely recombination. Genomic recombination (the transfer of genetic material between different bacteria) can greatly impact phylogenetic estimations and generate spurious relationships between isolates; based on regions of recently shared DNA (Didelot and Wilson, 2015).

There is significant recombination between all the isolates sequenced in this project (fig. 3.14); and this may be, in part due to sub-optimal assemblies. Sites of recombination frequently include repeat regions of DNA, which sequence assembly is vulnerable to; and *E. coli* commonly contains very large repeat regions (Koren et al., 2013, Tenaillon et al., 2010).
Figure 3.14: ClonalFrameML core genome phylogeny and recombination estimation analysis. Red circle = MDR isolate, green circle = susceptible isolate. Sites of recombination identified by ClonalFrameML are noted by horizontal dark blue bars.
3.4 Discussion

3.4.1 Next-generation Sequence Assembly

The differences in the MDR and susceptible strains from the results of chapter 2 required further exploration. Illumina NGS was used as it allows for better contextualisation of both the MDR and susceptible isolates. The de novo assembly of the sequenced MDR and susceptible isolates also allowed more detailed analysis of virulence genotype, antimicrobial resistance (AMR) genotype, core and pan-genomic sequence comparisons; but also highlighted the vulnerabilities of NGS to the complexities of the *E. coli* genome.

While the quality of the sequence reads (unassembled) met expected quality standards (FastQC); assembly of those sequencing reads into contiguous sequences was less straightforward. None of the assemblies, for either MDR or susceptible isolates, is likely to contain the whole genome; many remain highly fragmented and probably without regions of potential interest. Critically, short-read NGS methods are particularly vulnerable to repetitive regions (often associated with horizontal genetic elements (HGEs)) of the genome that are longer than the maximum insert size of the sequence read pairs; that therefore cannot be assembled (Koren et al., 2013). These repeat regions can often contain the genomic sequences of interest, and collectively drive much of the functional adaptation and variation seen across different *E. coli* (Welch et al., 2002). Moreover, repetitive sequences can also introduce miss-assemblies and sequence errors which may prevent the correct annotation of genes and other downstream sequence analysis.

For the MDR isolates, sequence assembly and annotation quickly identified a range of AMR gene which potentially contribute to the MDR phenotype. This had not been achievable with the results of the AMR gene Identibac panel (Chap-
ter 2). The identified AMR genes mapped well to the phenotypic resistance observed for the MDR isolates (table 3.4) and confirmed that the observed ESAC phenotype was attributable to CMY-2.

NGS methods cannot easily assemble plasmid and other horizontally-transferable DNA sequences. Like chromosomal sequences, phages, transposable elements and plasmids contain large repetitive regions of DNA sequence. HGEs, which are important vectors of transmission for both virulence and AMR genes, may be detectable but cannot be fully assembled from NGS data alone (Koren et al., 2013, Lanza et al., 2014). The annotation of protein sequences, from the assembled genomes (data not shown), confirmed the presence of IncI1 and IncF plasmid replicon sequences; as predicted using the replicon typing PCR in Chapter 2. RAST annotation showed a number of plasmid contigs, as well as transposable element sequences throughout the MDR isolates. However, contigs that could be identified as being of potential plasmid origin contained only sequences associated with the plasmid replicon. Because of this (i.e. fragmented assemblies), it was not possible to associate annotated virulence or AMR genes to any of the plasmid replicons.

Identibac virulence typing (Chapter 2) showed a greater number of virulence markers present in the susceptible isolates, as compared to the MDR. This was confirmed in the sequence assembly data by the virulence genes detected by Identibac in both groups (microcins and haem-utilization). Neither VirFinder, or Identibac specifically assess potential uropathogenesis; but instead include more general E. coli virulence markers. To expand on these results, sequence assembly data for the MDR and susceptible isolates was compared using a larger collection of UPEC specific virulence genes present in the genome of CFT073 (VFDB, fig. 3.7). Primarily, the results show a greater variability between the MDR and susceptible isolates; than indicated by VirFinder or Identibac. However, for both
the susceptible and MDR isolates, few of the virulence gene families have been fully annotated; particularly *fim*, *chu* and *ybt* genes.

The detection of a virulence gene in an assembled contig relies on: 1) the presence of the virulence gene sequence in an assembled contig, and 2) correct identification of the gene sequence in an assembled contig. Failure to detect virulence genes could be the result of 1) the virulence gene is not present in the assembled *E. coli* genome; 2) the virulence gene sequence cannot be assembled; 3) the sequence assembly contains a virulence but contains too many miss-assembly errors to be identified; and 4) the virulence gene is present but is too divergent from the reference gene sequence to be detected. Without a complete genome assembly it is not trivial to attribute the absence of a particular virulence gene to any one of those scenarios. This is a significant vulnerability in declaring any of the isolates in this study uropathogens, or otherwise.

The genetic flexibility of *E. coli* further complicates the prediction of pathogenic potential and the hypothesis proposed; that because the susceptible isolates had not been exposed to antibiotic chemotherapy they therefore may have a greater pathogenic potential due to a difference in genetic background. SeqFinder (fig. 3.7) and VirFinder (fig. 3.5) virulence typing do suggest that susceptible isolates carry a somewhat higher burden of virulence markers; which would certainly aid in the colonization of the urinary tract. However, some of the key UPEC virulence genes are absent from susceptible and MDR isolates. Given the highly plastic nature of the *E. coli* genome, a large degree of variability is reasonably expected. Much of the published literature characterizing and exploring the *E. coli* genome has relied on the limited collection of *E. coli* isolates that have been sequenced to date. Efforts to characterize new strains of *E. coli* have focused predominantly on strains of clinical and academic focus; and there is undoubtedly a much greater diversity amongst wild-type *E. coli* isolates than has been
There are many definable pathotypes of *E. coli*, characterizable by: their clinical presentation, genetic relatedness to other pathogenic strains and possession of canonical virulence makers. ExPEC, and more specifically UPEC, remain difficult to adequately define. Moreover, pathogenic *E. coli* and their virulence markers, often defined by their absence from healthy gastro-intestinal flora; may actually make up a sizable component of healthy flora. The focus on disease causing strains of *E. coli* may overemphasize the contribution of certain virulence markers and limit their usefulness as true predictors of pathogenicity (Dobrindt, 2005, Dobrindt and Hacker, 2008).

Ultimately, because of the small number of isolates and the incomplete nature of the sequence assembly presented here, the SeqFinder and VirFinder results merely expand the descriptive data available for the susceptible and MDR isolates. It is unlikely that the NGS data is predictive of uropathogenic potential.

### 3.4.2 Core and Pan Genomic Analysis

Attempts to classify diversity amongst pathogenic *E. coli* have traditionally only focused on a very small component of the wider genomic diversity. Pathotypic typing schemes have focused predominantly on virulence makers as a surrogate measure of the pathological differences exhibited by discrete sub-lineages of *E. coli*. Much of the generated diversity amongst pathogenic *E. coli* is conferred via horizontal acquisition of these virulence markers into favorable genetic backgrounds; via plasmids, genomic islands, transposons or bacteriophages (Chen, 2003, Groisman and Ochman, 1996, Hochhut et al., 2006, Kaper et al., 2004).

Estimation of pathotypic tendency using core-genome sequencing doesn’t seem
to be capable of indicating the virulence or pathogenic background of a given \textit{E. coli} isolate (fig. 3.5). Work done to detail genomic comparisons between CFT073 (pyelonephritis causing strain), ABU83972 (asymptomatic bacteriurea strain) and Nissle 1917 (a gut commensal with probiotic potential) revealed a startling genomic similarity; with all three strains grossly varying in their infectious phenotype. Despite both ABU83972 and Nissle 1917 possessing the majority of the virulence markers present in the CFT073 genome (including toxins and adhesive fimbriae), both ABU83972 and Nissle 1917 are completely asymptomatic (Dobrindt et al., 2010). Even with accurate and detailed annotation and phylogeny, detailed modeling of \textit{in vivo} expression of virulence highlights the inability virulence markers alone to be completely predictive pathogenicity (Leimbach et al., 2013, Vejborg et al., 2010).

This is certainly not an observation that is confined to UPEC. Much of the pathotypic characterizations require the assumption that typable strains are obligate, and not facultative pathogens; and such a scheme does not adequately encompass the spectrum of niche distribution that \textit{E. coli} is capable of. Therefore, commensal or pathogenic specialism is not just a divergent genotypic trait, but also a convergent one (Vejborg et al., 2010, Leimbach et al., 2013). In all likelihood any classification system capable of predicting the pathogenic potential would need to encompass a much richer list of genetic markers than any of the current schemes. Even typing schemes with expanded panels of markers have been able to identify greater heterogeneity, beyond commensal and pathogenic behavior.

Along with the paucity of available commensal \textit{E. coli} and the lack of a clear definition of what an \textit{E. coli} commensal is; these bias any pathogenic prediction from sequence information towards an overly narrow definition of pathogenic \textit{E. coli}. Especially as there is a prevalence of many virulence factors from across
the different pathotypes, in commensal isolates (Touchon et al., 2009). There may be no such thing as an obligate commensal; particularly in conjunction with antimicrobial chemotherapy, where persistence and growth in an environment can be considered virulence in their own right (Blango and Mulvey, 2010, Escobar-Páramo et al., 2004, Leimbach et al., 2013, Narciso et al., 2012).

Ultimately the sequence assembly data generated here is not sufficient for such detailed comparisons between the susceptible and MDR isolates; there is simply is not enough data. Moreover, no effort was made to isolate commensal E. coli isolates (i.e. faecal) from healthy dogs, which would have greatly strengthened the comparisons between susceptible and MDR isolates.

Furthermore, the degree of recombination and sequence disparity identified by ClonalFrameML (fig. 3.14) limits the confidence with which conclusions of genetic relatedness can be drawn. While both the mapping of sequence contigs (fig. 3.5, fig. 3.6) and ClonalFrameML analysis indicate good coverage across both a reference genome (MG1655) and amongst the MDR and susceptible isolates (fig. 3.14), it is very likely that some of the sequence diversity used to calculate phylogenetic relationships has been influenced by ambiguous base calls made during assembly. Though it is still likely these isolates are not closely related (MLST and maximum-likelihood analysis are in agreement), it would be desirable to re-sequence these isolates to gain more confidence in the results presented here.

### 3.4.3 Galleria mellonella Infection Model

The *Galleria mellonella* larvae infection model has been used to compare general virulence of pathogenic *E. coli* (Alghoribi et al., 2014), as well as detailed comparisons of host immune responses to virulent and attenuated strains of *E. coli*. The work published by Alghoribi et al. (2014), looked at a group of 71
uncharacterized UPEC strains of mixed genetic background. By correlating phenotypic virulence against detected virulence genes they observed greater killing with increased virulence gene presence. Though they did not detect significant difference between strains of different phylogroups.

That even the more overtly virulent markers, such as the cytotoxins do not necessarily correlate to increased virulence in the moth infection model presented here, could be a product of the expressed phenotype of the *E. coli* isolate, or an aberration of the model. Whilst there may be a slight overall trend of increased detected virulence markers in the susceptible isolates, this does not translate into increased virulence in the *Galleria mellonella* infection model (fig. 3.5).

Moreover, the heterogeneity of canine host status prior to, and throughout the course of infection, cannot be accounted for with the isolates. It is assumed that the MDR group isolates will have been isolated from hosts with decreased susceptibility to UPEC infection in comparison to the susceptible group. But this has not been quantified in any way; any of the MDR or susceptible infections could have been asymptomatic or more virulent but this data was not available.

As has been stated previously, the canine patients from which the MDR isolates were obtained from received a significant level of clinical intervention. It is assumed that these hosts are likely to have had increased susceptibility towards infection given their general health state. Though this does not seem to have had a discernible impact on the phenotypic virulence of the isolates in the *Galleria mellonella* model.
3.4.4 Summary

The designation of any _E. coli_ isolate as pathogenic or commensal is greatly complicated by the evolutionary selective pressures exerted by antimicrobial chemotherapy. There is not enough data to conclusively say whether the combined cost of acquired virulence and acquired AMR are mutually exclusive phenotypes for the isolates sequenced in this study. Even though, in terms of a reduced virulence genotype (VirFinder, SeqFinder) do point to a separate background for the MDR and susceptible isolates; the variability in the phylogenetic reconstructions (Re-alphy, Clonalframe) and _Galleria mellonella_ infection model make any assertion difficult.

Work published by others has speculated that the increased metabolic cost of expanded virulence and resistance, without concerted adaptation towards a more metabolically efficient genotype was presumed is too costly to be optimal for any given bacterial isolate. However, this view has shifted somewhat as the mechanisms by which AMR can be inherited have come to be better defined (Martínez and Baquero, 2002). Recently, focus has shifted towards host microbial flora. Indeed some of the most notorious MDR bacteria, such as community-associated methicillin-resistant _Staphylococcus aureus_ (MRSA), vancomycin-resistant _Enterococcus_ (VRE) are derived from the gut flora.

Phylogenetic evaluation of resistance acquisition in _E. coli_ suggests that phyllogroup A isolates are more closely linked to the acquisition of horizontally transferable resistance (Tenaillon et al., 2010). This a trend that has been readily identified in a number of clinical reports of veterinary MDR (Gibson et al., 2010, 2011).

Despite the various assertions that MDR favors more commensal type _E. coli_, there are also reports of MDR associated with more pathogenic _E. coli_. This in-
cludes 025b:H4-ST131, belonging to phylogroup B2, which has a well documented virulence phenotype and genotype (Platell et al., 2011).
Chapter 4

Genomic Contribution of Horizontally Transferable Resistance

4.1 Introduction

The work outlined in this chapter seeks to investigate the genetic basis of plasmid mediated multi-drug resistant (MDR) and extended-spectrum $\beta$-lactamase (ESBL) or extended-spectrum AmpC (ESAC) carriage; using single-molecule real-time sequencing (SMRT) sequencing with MDR ESBL resistant and fully susceptible community-associated E. coli. This work builds upon the correlation between $bla_{CMY-2}$ resistance and IncI1 plasmid incompatibility types identified in the previous chapters and in (Wagner et al., 2014).

MDR is an increasingly prevalent, global phenomenon. MDR Enterobacteriaceae producing ESBL infections pose a particular challenge. Such infections are often resistant to first and second line $\beta$-lactams, as well as third-line extended-
spectrum cephalosporins. The prevalence of ESBL resistance is increasing in both nosocomial and community-acquired infections (Giske et al., 2008, Paterson, 2006). ESBL infections increase both patient morbidity and mortality. Successful treatments against such infections increasingly rely on last-line carbapenem treatments (Livermore et al., 2011).

*E. coli* is an important member of the microbial flora as a commensal, but also causes a number of intestinal and extra-intestinal infections (Kaper et al., 2004). Clinically, ESBL resistant *E. coli* is seen in both emerging epidemics, as seen globally with *E. coli* ST131 (Petty et al., 2014), as well as antibiotic usage selecting for endemic, opportunistic infections associated with the commensal flora (Alekshun and Levy, 2006).

The mobilisation of ESBL resistance to horizontal genetic elements (HGEs), such as plasmids and transposable elements, has greatly affected the dissemination of ESBL’s and MDR (Pitout, 2012). Plasmids and transposable elements have a diverse bacterial host range, conferring resistance markers between bacterial species; generating a large potential resistance reservoir allowing anthroponotic and zoonotic transfer (Guardabassi et al., 2004).

As sequencing technologies have developed, an increasing number of resistant *E. coli* strains have been sequenced and analyzed. To date, much of the sequence reconstruction has relied upon read mapping and short-read *de novo* assembly. In reality, both methods are unlikely to resolve HGEs successfully without prior isolation and subsequent re-sequencing. As a consequence, the availability of high quality sequence data relating to horizontally acquired resistance remains poor. Given the close contact of companion animals to other animals and humans, a more in depth understanding of *E. coli* strains is of great importance.
4.2 Methods

4.2.1 PacBio Sequencing

MDR isolates 127, 1223, 1283, 1428, 144, 317, 746 and 1943 (all positive for ESAC (bla\textsubscript{CMY-2}) and the IncI1/IncFII replicon type) and a susceptible isolates 1190 and 1105 (also positive for the IncI1 replicon type) were selected for SMRT sequencing; performed using the PacBio platform.

Briefly, \textit{E. coli} were inoculated into 10 ml of lysogeny broth (LB) and incubated overnight at 37°C, 170 rpm. Extracted DNA was prepared for PacBio sequencing using AMPure\textsuperscript{®}Beads (AMPB), with a target insert size range of 10kb or greater. DNA was sheared using a Covaris\textsuperscript{®}g-TUBE\textsuperscript{®}. Sheared DNA was purified and concentrated using AMPBs. Samples were eluted from the AMPBs using PacBio Elution Buffer, and single stranded fragments were removed with ExoVII restriction enzyme. DNA was repaired using the PacBio DNA Damage and Repair Ends buffers, as per the PacBio protocol.

Processed DNA was further purified using AMPBs; then blunt end sequence adapters were ligated, and ExoIII and ExoVII restrictions enzymes were used to removed failed ligation products. Successfully ligated sequences fragments were then concentrated by three successive AMPB purification steps. Final DNA concentration for all of the samples exceeded 5\(\mu\)g.

DNA was sequenced on the PacBio RSII sequencing platform.
4.2.2 Plasmid Sequence Comparison

SMRT analysis was used to generate a fastq file from the PacBio reads and error-corrected reads were adjusted using PCcR, with self-correction (Koren et al., 2013). The longest 20x coverage reads were assembled with Celera Assembler 8.1 and polished using Quiver (Chin et al., 2013).

Annotated genomes (Do-It-Yourself-Annotator (DIYA) (Stewart et al., 2009)) were imported into Geneious (Biomatters LTD., Auckland, New Zealand) (Kearse et al., 2012) and duplicated sequences removed from the 5’ and 3’ ends to generate the circularized chromosomes and plasmids.

The origin of replication was approximated using Ori-Finder (Luo et al., 2014) and the chromosome reoriented; using as base 1.

4.2.3 Plasmid Sequence Comparison

Broad sequence comparisons between the SMRT sequenced plasmids were performed using CCTViewer (Grant et al., 2012), and progressiveMauve (Darling et al., 2010). CCTViewer was used to visualize both sequence and coding domain sequence BLAST, using isolate 1428 plasmid sequence p96 as a reference sequence for the IncI1 plasmid comparisons, and isolate 144 plasmid sequence p134 as the reference sequences for the IncF plasmid comparisons. ProgressiveMauve alignment of genbank sequences was performed with default parameters.

Plasmid multi-locus sequence-typing (MLST) was performed using the plasmid MLST website (https://pubmlst.org/plasmid/) and sited at the University of Oxford (Jolley and Maiden, 2010). Resistance genes and virulence genes were identified using ResFinder (Zankari et al., 2012) and BLAST Koala (Kanehisa et al., 2016).
4.2.4 Plasmid Core and Pan Genomes

Core sequence homology between the plasmid sequences was detected with get_homologues (Contreras-Moreira and Vinuesa, 2013). Homologous clusters of coding domain sequences were detected using bi-directional best-hits (BDBHs), clusters of orthologous groups (COGs), and orthoMCL algorithms; through the get_homologues script.

Clusters were aligned by amino acid sequence with Muscle aligner. Amino acid alignments were then translated back into nucleotide sequences using the pal2nal script (Suyama et al., 2006). Fasta file headers were modified to provide unique sequence identification for each of the individual sequences, using an in-house script.

The homologous clusters were then concatenated to provide an aligned sequence in fasta format. The Galaxy Web Server fasta width formatting, set to width 0, was used to format the fasta file before converting sequence formats. The catafasta2phyl.pl script (https://github.com/nylander/catfasta2phml) was used to generate a phylm format input for RaXML phylogenetic estimation.

RaXML (Stamatakis, 2014) was used to reconstruct a phylogenetic estimation (with a GTRGAMMA model, with 100 bootstrap replicates).

The get_homologues clusters were also used for the pan genome analysis. The PARS program from the PHYLIP package was used to perform maximum-parsimony analysis.
4.2.5 Conjugation Recipient Strains

Spontaneous rifampicin resistant MG1655 *E. coli* mutants were generated as marked recipient strains for plasmid conjugation experiments. MG1655 mutants were isolated from overnight cultures on LB agar plates containing 100µg/ml rifampicin at 37°C. A single resistant colony was re-streaked onto rifampicin plates and re-cultured overnight at 37°C, to ensure purity of culture. The mutants were then compared to wild-type strains grown on LB agar in the absence of the antibiotic to ensure comparable strain fitness.

4.2.6 Plasmid Conjugation

Donor and recipient strains were inoculated into 10ml of LB and incubated overnight at 37°C, 170rpm. 500µL of donor and recipient cultures were inoculated into 5ml of 10mM MgSO₄. Cultures were then filtered (Millipore, HAWP02500) and the filter paper placed onto an LB agar plate and incubated overnight at 37°C. The filter paper was then vortexed in 550µL of LB, and serial dilutions (10, 100, and 1000 fold) dilutions made of the bacterial suspension. 100µL of the 100 and 1000 fold dilutions was plated, in triplicate, onto LB agar and incubated overnight.

Candidate colonies were then streaked onto LB plates (100µg/ml rifampicin/100µg/ml ampicillin) and incubated overnight at 37°C. Colonies resistant to rifampicin and ampicillin were then incubated in 10ml of 100µg/ml rifampicin, 100µg/ml ampicillin LB at 37°C, 170 rpm for glycerol stocks and storage at −70°C storage.

Conjugants were also screened using antibiotic sensitivity testing, replicon typing PCR (Carattoli et al., 2005a) and phylotyping PCR (Doumith et al., 2012b).
4.2.7 Antibiotic Sensitivity Testing

Kirby-Bauer disk diffusion was used to detect phenotypic antimicrobial resistance for both conjugant and wild-type strains. Briefly, *E. coli* strains were incubated in nutrient broth (Oxoid) at 37°C, and then diluted to a MacFarland standard in sterile saline solution. This was then plated on to Mueller-Hinton agar (Oxoid), and incubated overnight at 37°C. Antibiotics used were: amoxicillin-clavulanic acid 30µg (Oxoid CT0223B), cephalexin 30µg (Oxoid CT0007B), ciprofloxacin 5µg (Oxoid CT0425B), gentamicin 10µg (Oxoid CT0024B), doxycycline 30µg (Oxoid CT0018B) and tetracycline 30µg (Oxoid CT0054B).

4.3 Results

The aim of the work in this chapter is to understand the genetic context of antibiotic resistant alleles in the collection of MDR *E. coli* clinical isolates; defined by ESAC *bla*\textsubscript{CMY-2}. Previous work had correlated this resistance with carriage of an incI1-FII replicon type plasmid.

4.3.1 Sequencing and Plasmid Carriage

In order to try and obtain plasmid sequences, the Illumina sequence data was filtered against reference *E. coli* sequences to remove chromosomal sequence (assembled *de novo*) and resulting contigs compared against published plasmid sequences using BLAST. However, this was largely unsuccessful as was direct *de novo* assembly of unfiltered sequence reads. To resolve this, a subset of MDR isolates, focusing on the IncI1-FII were selected for sequencing by SMRT. These included 8 MDR isolates and 2 susceptible isolates.
SMRT sequencing of the MDR isolates revealed multiple extra-chromosomal contigs per isolate, with good agreement in relation to the initial replicon typing PCR; total detected plasmid carriage per isolate is depicted in (fig. 4.1). Sequencing revealed the complex and multi-replicon capacity of the majority of the MDR isolates, explaining the inability of short-read sequencing to uncover this plasmid architecture.
Figure 4.1: Schematic representation of the plasmid carriage of the *E. coli* isolates in this study. Boxes represent each isolate and referred to by their isolate ID. Depicted sequences are not to scale. Plasmid sequences are identified and distinguished by their sequence length. Plasmid encoded resistance, where detected by BLAST search, is indicated by color coding on the plasmids. Plasmid incompatibility type, where typable, is indicated (IncF or IncI1). Chromosomal sequences are not depicted.
While 7/8 MDR strains had the anticipated replicons, isolate 317 only contained an InCF plasmid according to the SMRT sequencing, despite both PCR and Illumina sequence data indicating the presence of IncF and IncI1 replicons.

One of the susceptible isolates, 1105, was PCR positive for the incI1 genotype, but neither Illumina or SMRT sequence data detected nucleotide sequences associated with the replicon type.

MDR isolate 746 contained an IncI1 contig, but potentially two IncF contigs. While this should not be possible, both sequences show marked similarity, despite discrepancies with their size (10kb difference).

SMRT sequencing also detected numerous extra-chromosomal contigs with no discernible replicon type. Some of these sequences carry resistance markers, and are of clinical relevance. These sequences would not have been detectable without SMRT sequencing.

In the MDR isolates, the IncI1 plasmid associated exclusively with the $bla_{CMY-2}$ ESAC resistance gene. The resistance maker is associated with an insertional element (IS91) and contains the backbone of a resistance cassette ($bla_{CMY-2}$-$blc$-$sugE$) that is associated with the dissemination of resistance in *E. coli* (Fang et al., 2015) and *Salmonella enterica* (Fricke et al., 2009).

Plasmid sequence sizes ranged from 85-96kb, with between 106-113 coding domain sequences; many of which have no homology with proteins of identified function and therefore designated hypothetical proteins (table 4.1).

All but two of the IncI1 plasmids belong to the same MLST clonal complex (CC-2), determined *in silico*, based on the presence and sequence similarity of $repI1$, $ardA$, $trbA$, $sogS$ and $pilL$ genes to previously published IncI1 pMLST profiles. Remarkably, despite their considerable size and number of coding regions,
it is not possible to discern from the nucleotide sequence any further functional contribution of the plasmids to the host strain beyond the \(\text{bla}_{\text{CMY-2}}\) resistance gene cassette.

The IncF plasmids were much more heterogeneous in size (100-147kb), number of coding regions (105-150) and the number and classifications of various resistance markers (table. 4.1). Aminoglycoside, chloramphenicol, potentiated-sulfonamide and \(\beta\)-lactamase (not AmpC or ESAC associated) resistance markers were all linked with IncF type plasmids.

<table>
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<tr>
<th>Name</th>
<th>CDS</th>
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<th>Resistance Genes</th>
<th>Virulence Genes</th>
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<td>CMY-2</td>
<td>-</td>
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<td>(CC 2)</td>
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<tr>
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<td></td>
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<td>tet(A)</td>
<td>TC.FEV.OM3, sit</td>
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<td>101</td>
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<td>CMY-2</td>
<td>dot</td>
</tr>
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Table:

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<th>CMY-2 Strain</th>
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<th>Virulence Genes</th>
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<td></td>
<td>p66</td>
<td>108</td>
<td>FII:4</td>
<td>tet(B)</td>
</tr>
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<td></td>
<td>p48</td>
<td>65</td>
<td>nt</td>
<td></td>
</tr>
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<td>142</td>
<td>FII:1</td>
<td>strA, strB, iroC, sit, iuc, FII:2</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>TEM-1b, sul2, dfrA5</td>
<td>iuc, mer</td>
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<td>p62</td>
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<td>p80</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>virB</td>
</tr>
</tbody>
</table>

Basic descriptive information on plasmid sequences derived from this study. nt = not typable, CC = clonal complex, - = none detected, ** = probable split contig

Despite the greater number of resistance markers shared amongst the plasmids, four of the plasmids contained no detectable resistance markers. Virulence genes, mostly associated with iron uptake and metabolism, were detected on many of the IncF plasmids. This suggests a possible metabolic contribution towards bacterial host function.
By predicted *in silico* replicon typing, the IncF plasmids are of mixed lineage. 127 p123, 1223 p147, 1283 p109, 1428 p111 and 144 p134 all possess IncFII and IncFIB replicon type sequences. The remaining strains containing replicon type sequences were: 317 p100, 1428 p66, 746 p62 and 746 p72, which were all exclusively IncFII; 1953 p80 was of the IncFIA replicon type.

### 4.3.2 IncI1 Comparative Analysis

Sequence comparisons of the strains indicates a strong underlying genetic relationship between the IncI1 plasmids. Nucleotide and coding domain sequence BLAST comparisons show greater than 90% sequence similarity for the bulk of the sequences. Multiple co-linear blocks, with high synteny and sequence similarity and large-scale rearrangements were identified using progressiveMauve (fig. 4.2).
Figure 4.2: Core sequence alignment of IncI1 replicon type plasmid sequences, using 1428 p96 as the index sequence. Comparisons were performed using BLAST Atlas searches using the CCT comparison tool. Both the whole nucleotide sequence (smaller ring) and coding domain specific (larger ring) BLAST comparisons were carried out. ProgressiveMauve was also used to compare the plasmids. Local co-linear blocks, detected by progressiveMauve have been annotated onto the BLAST comparisons.
The IncI1 plasmids were compared against 42 plasmid sequences obtained through GenBank, which were identified by BLAST as being similar to the plasmids sequenced in this study. Pan-genomic analysis detected 305 homologous gene clusters across all sequences. Wagner parsimony phylogenetic estimations of a binary matrix of homologous cluster presence or absence indicates a close phylogenetic relationship between all of the IncI1 plasmids sequenced in this study; and places the IncI1 plasmid from the susceptible isolate 1190 ancestrally to the MDR isolate plasmids.

Pan-genomic analysis showed a region of conservation amongst the MDR, susceptible and GenBank plasmid sequences. Ten homologous gene clusters: \( \text{traL}, \ M, \ nikB, \ \text{traO}, \ \text{traJ}, \ \text{traF}, \ \text{traE}, \ \text{traT} \) and \( \text{traX} \); all relating to plasmid transfer and replication were core to all plasmid sequences (fig. 4.3).
Figure 4.3: Maximum-parsimony analysis using detected presence or absence of homologous gene clusters from the pan genome of PacBio IncI1 replicon sequences and comparator IncI1 sequences obtained from the NCBI nucleotide database. Plasmid sequences from this study lie with the red box. Conserved genes (used for maximum-likelihood analysis) are highlighted in the blue box.
These sequences were extracted from the pan-genomic analysis and aligned, for maximum-likelihood core genome phylogenetic analysis (fig. 4.4). The analysis of these core genes also indicates close phylogenetic relationships between the majority of the IncI1 plasmids; with moderate bootstrap support for the majority of the identified clades. Unlike the maximum parsimony tree, the plasmids from 1943 and 1428 are no longer monophyletic to the remaining canine IncI1 plasmids. There is considerable genetic distance between susceptible isolate 1190’s plasmid and the MDR group plasmids. Despite only moderate bootstrap support overall, the phylogenetic tree is congruent with the predicted in silico MLST groups; though 1190 and KJ484640 could not be typed by in silico IncI1 MLST (fig. 4.4).
Figure 4.4: Alignment and maximum-likelihood phylogenetic analysis of core IncI1 plasmid sequences. IncI1 replicon PacBio sequences were used to identify other putative IncI1 plasmids from the NCBI nucleotide database. The sequences have been annotated with predicted plasmid multi-locus sequence types (clonal groups). The bacterial and animal hosts, from which the plasmid sequences were extracted, have also been annotated where available from submitted sequence metadata. Isolates from this study are identified by black squares.
Clades show multiple bacterial and mammalian hosts for the IncI1 sequences. IncI1 plasmids are notable for their extensive host-range within the *Enterobacteriaceae*. However, human-associated sequences are largely absent from the tree; suggesting limited zoonotic potential for the plasmids.

The majority of the canine sequences form a monophyletic clade, with chicken and porcine associated plasmid sequences being the closest ancestrally. Of note, CP009566 and KF434766 are two reference IncI1 plasmids are both associated with canine *E. coli* infections; the first isolated in the USA (Bortolaia et al., 2014) and the second in Scandinavia (Bogaerts et al., 2015). These are clonally related to the plasmids sequenced in this study despite their isolation being geographically and temporally distinct. While CP009566 was isolated from and *E. coli isolate* in Arizona, KF434766 was isolated from a *Salmonella* Newport strain in Denmark.

Despite the multiplicity of *E. coli* sequences which dominate the tree, multiple bacterial hosts are present within each clade on the tree. This suggest that intra-species transmission of the IncI1 plasmids present on the tree is a common occurrence; which has been previously reported for the IncI1 plasmids (Carattoli, 2011).

Where the information was available, the animal host of origin has also been annotated on the tree with the noted comparative absence of human hosts despite the heavy bias of human isolate plasmid sequences available on the GenBank database.

### 4.3.3 IncF Comparative Analysis

In comparison to the IncI1 plasmids, much less sequence similarity was detected by BLAST coding domain sequence, nucleotide alignments, or by progressive-
Mauve sequence comparisons. The single region conserved amongst these plasmids is related to plasmid replication machinery. The remaining regions of local-colinearity have little synteny, or support across all the plasmid sequences (fig. 4.5).
Figure 4.5: Core sequence alignment of IncF replicon type plasmid sequences, using 1428 p96 as the index sequence. Comparisons were performed using BLAST Atlas searches using the CCT comparison tool. Both the whole nucleotide sequence (smaller ring) and coding domain specific (larger ring) BLAST comparisons were carried out. ProgressiveMauve was also used to compare the plasmids. Local co-linear blocks, detected by progressiveMauve have been annotated onto the BLAST comparisons.
GenBank BLAST searches identified 9 sequences similar to the IncFII/IncFIB plasmids. Pan-genomic maximum-parsimony analysis of both reference and Inc-FII/IncFIB plasmids indicates a mixed phylogeny (fig. 4.6). Many of the plasmids are individually monophyletic, with few clades describing more than a couple of plasmid sequences. Most of the plasmids show a mixed predicted replicon type, with FII/FIA the most commonly identified. All of the reference sequences used for the pan-genomic and core-genome comparisons were also IncFII/IncFIB.
Figure 4.6: Top: Maximum-parsimony analysis of pan-genomic homologous gene clusters for IncFII replion sequences. Similar NCBI nucleotide sequences, with detectably diverse replicon types were included. Isolates sequenced in this study are indicated by the red boxes. Bottom: Maximum-likelihood analysis of aligned core homologous sequences. Sequences have been annotated with their detected replicon type.
A singular clade of plasmids, exclusively IncFII, show greater sequence homology than the remaining plasmid sequences. Despite the closeness of these plasmid sequences to one another, they cannot be compared by maximum-likelihood analysis. 1428 p66, 746 p62, 746 p72 and 1943 p80 could not be included as no homologous clusters, between all of the plasmid sequences, could be detected when they were included in the analysis. All other IncF type plasmid sequences were used to generate a core genome of 8 homologous gene clusters.

Like the IncI1 plasmid comparisons, these genes were mostly associated with plasmid maintenance and replication: \textit{traA}, \textit{traL}, \textit{traE}, \textit{traB}, \textit{traX} and X-polypeptide. Maximum likelihood phylogenetic analysis of the IncFII, IncFIA and IncFIB replicon types show a dispersal of all sequences of different replicon types throughout the tree (fig. 4.6). None of the PCR typed IncFII plasmids sequenced in this study are members of the same clade; moreover, 317 p100 is a significant outlier from the rest of the IncF-type plasmid sequences.

Given that the \textit{E. coli} isolates included in the work presented in this thesis were chosen based their extended-spectrum \textit{β}-lactamase (ESAC) phenotype (associated only with IncI1 plasmids); a lack of distinct genetic relatedness amongst the IncF-type plasmids is not surprising.

746 p62 and 746 p72 are somewhat baffling. Plasmids incompatibility should dictates that no two plasmids of the same type should exist in the same isolate. Pan-genomic analysis indicates mostly similar gene content between, which does not necessarily indicate mis-assembly (i.e. that the contigs are both part of a larger contiguous sequence). Similarly, 1428 p111 and 1428 p66 share a predicted IncFII replicon type.

There are multiple potential explanations for the contigs both being present in the same isolate. Firstly, their may be two plasmids within the same bacterial
population that are split between bacterial hosts. Secondly, it is possible that the
10 kb difference in sequence length represents an insertion sequence that cannot be
sequenced across using the SMRT sequencing protocols used here. Finally, these
sequences could represent the mis-annotation of the plasmid replicon sequences.

4.3.4 Other Plasmids

Numerous other extra-chromosomal contigs were detected by PacBio sequencing.
IncI1, IncFII, IncFIA and IncFIB replicon types only account for a fraction of the
plasmid sequences. The nature of SMRT sequencing and analysis make it unlikely
that these sequences are part of the main chromosome, or the plasmids with well
defined replicon-types. Whilst many of these sequences do not contain identifiable
resistance markers, some do. Many of these sequences do not have established
replication and transfer machinery encoded onto them, so their transfer capacity
is unknown; but may represent small replicons that can be co-inherited with other
transferred plasmids or possibly other methods.

Though these plasmid sequences contain few coding regions, the majority of
which are of unknown function (hypothetical), it was not assessed as to whether
any of these regions may play a role in the inheritance of the plasmids. Such other
methods of transfer include: partition systems that contribute towards replication,
incompatibility and long-term stability (and may be encoded for on the
chromosome), genetic addiction and post-segregational killing of daughter cells
which do not contain a plasmid (though no toxin/anti-toxin systems were an-
notated), DNA site-specific resolution systems which involve chromosomal genes
encoded in all _E. coli_ (such as _OriC_ ) and the increased likelihood of transmission
based on plasmid copy number (not assessed in this work), and even the native
advantage that smaller DNA molecules associate more closely together (super-
coiling) which increases the likelihood of replication events during normal cellular function (Barbara and Gregory, 2004).

4.3.5 Plasmid Conjugation

Successful conjugation of the IncI1 plasmids was achieved from wild-type strains to *E. coli* MG1655. Conjugants were confirmed using PCR and Kirby-Bauer disk diffusion to type donor and recipient strains. Phenotypic resistance to amoxicillin-clavulanic acid, cephalexin and extended-spectrum cephalosporin resistance was conferred to conjugants in line with transfer of the IncI1 plasmids containing the CMY-2 resistance gene.

4.4 Discussion

It is critical to understand the genetic context and reservoirs of antimicrobial resistance genes in order to stem the selection of MDR bacteria. In this study both Illumina and SMRT sequencing, of a defined set of clinical isolates, was applied to investigate resistance gene alleles and their context in the reservoir organism.

Illumina data from both MDR and susceptible *E. coli* strains allowed their phylogenies to be determined and enabled sub-selection of strains for SMRT sequencing (8 MDR and 2 susceptible) and this revealed the extensive repertoire of plasmids that can be present within individual isolates; with a subset shown to contribute to the resistance phenotypes.

A focus of this study was the ESAC resistance; encoded predominantly by *bla_{CMY-2}* in this set of strains; though interestingly isolate 127 contained a plas-
mid, p43, encoding \textit{bla}_{TEM-33} which also confers ESAC resistance (Bush and Jacoby, 2010). CMY-2 was shown to be encoded on IncI1 replicon plasmids and these formed a closely related phylogenetic cluster despite the strains having been collected over a 10 year period. The association of IncI1 plasmids with extended-spectrum $\beta$-lactamase resistance has been documented in members of the \textit{Enterobacteriaceae}; but it is more unusual for them to be associated with \textit{bla}_{CMY-2} (ESAC) resistance (Bortolaia et al., 2011, Diestra et al., 2009, Folster et al., 2010, Hradecka et al., 2008, Leverstein-van Hall et al., 2011, Liu et al., 2007, Marcade et al., 2008, Su et al., 2008, Tato et al., 2007). The strains possessed an \textit{bla}_{CMY-2} harboring IncI1 plasmid in combination with many other plasmids contributing to an MDR genotype; in particular IncF replicons that harbored multiple resistances.

As indicated from our preliminary studies, the majority of the MDR strains containing these plasmid combinations (IncI1/IncF) are associated with commensal phylogroups. The IncI1 plasmids indicate a level of endemism within the commensal population of the urinary tract infections (UTI) isolates. There is currently little information in the published literature describing the prevalence of plasmid incompatibility groups present in nosocomial, or community-associated canine populations. Interestingly, in this study, extended-spectrum $\beta$-lactam resistance has not been detected in a bacterial isolate that is not also MDR. This would suggest that the acquisition of other resistance alleles, in particular on IncF type plasmids, concertedly to the acquisition of the IncI1 ESAC plasmids.

The MDR stains in this study often associated with animals that have had a complex treatment history; and all they strains were isolated after they had successfully invaded the urinary tract. It is interesting to note that the IncF plasmids carry a much higher proportion of the resistance alleles and putative virulence-associated genes. It is therefore interesting to speculate that the acqui-
sition of these plasmids, in part due to antibiotic use, then drive the emergence of strains that may be more virulent.

The *E. coli* isolates do represent a model of the genetic and phenotypic responses of *E. coli* to the current clinical practices in modern care. A combination of prophylactic use of antibiotics, for surgical or other treatments, combined with patient vulnerability has provided an environment for the selection of at least two plasmid replicon groups; with numerous other horizontally-transferable DNA molecules also under selection.

In contrast to this study, previous comparisons of IncI1 resistance plasmids isolated from enterotoxigenic *E. coli* (ETEC) showed greater sequence diversity, rather than sequence conservation (Johnson et al., 2011). The majority of the MDR isolate IncI1 plasmids sequenced in this study share a closest common ancestor. This may be due to: i) limited sequence divergence or ii) sequence convergence. Given the genetic distance between the susceptible IncI plasmids, the absence of any dominant *E. coli* clone associated with the IncI1 plasmids, the lack of any indication of potential bacterial host range of the plasmids (other than *E. coli*) and the discordance of sequence similarity with the chronological sequence of the *E. coli* isolates; it becomes very difficult to approximate either scenario with the work presented here. The reliability of any estimation of rates of divergence between different plasmids is questionable; though the identification of a similar plasmid backbone in Denmark and the USA (Bogaerts et al. (2015); (Bortolaia et al., 2011)) does suggest an underlying core plasmid sequence stability capable of maintaining the *bla*<sub>CMY-2</sub> resistance cassette.

Fecal carriage of β-lactamase resistance markers in healthy canines has been reported in relatively high abundance (Baede et al., 2015, Okubo et al., 2014, Rocha-Gracia et al., 2015); and IncI1 plasmids sharing the pMLST clonal type have also been detected in healthy canines (Haenni et al., 2014). The genetic
similarity, despite geographic dispersion of the canine specific IncI1 clade, is highly suggestive of stable persistence of horizontally-transferable extended-spectrum \( \beta \)-lactam resistance in community associated canine \textit{E. coli} strains; with global circulation.

In comparison with the IncI1 plasmids, the IncF plasmids are much more disparate in both core and pan-genomic sequence content. This may, in part, be a consequence of experimental design. The isolates for this study were collected based on their status as being MDR including an ESAC phenotype; the ESAC resistance is not encoded by the IncF plasmids. This may over emphasize the relationship between circulating IncI1 plasmids and underestimate the degree of relationship between circulating IncF plasmids. However, fewer sequences could be identified in the NCBI database sharing nucleotide similarity and very few of the resistance markers appear to be conserved between one IncF plasmid and another. This may be an indication that vertical inheritance may be a more important means of plasmid transfer for this group of plasmids. It is difficult to consider a strong hypothesis as to whether sequences reflect progressive divergence, or weak convergence. They are at least much more capable of undergoing extensive genetic rearrangement.

Detailed sequence comparisons and descriptions for plasmids harboring clinically relevant antimicrobial resistance (AMR) are currently rare. Despite detailed knowledge of the mechanisms of replication, inheritance and transfer; much has been extrapolated from small-scale studies (Barbara and Gregory, 2004). Successful infection control practices rely on appropriate and targeted surveillance measures. Whilst PCR and short-read sequencing have provided a means for estimating the underlying contribution of plasmids to AMR; their impact has been limited.

Much of the resistance phenotype of the bacterial isolates sequenced here can
be attributed to the multiplicity of the resistance markers found on the plasmids. The dispersion of resistance genes across different plasmids, in many of the isolates, suggests sequential acquisition by the bacterial isolates; it is unlikely that the transfer of multiple plasmids would occur as a singular event. It is likely that these MDR strains have emerged in the flora of the gastrointestinal tract during treatments; and this also helps explain the genetic diversity of the isolates backgrounds. Commensal strains have been implicated in other studies as being a significant component of the AMR reservoir (Alekshun and Levy, 2006, Bailey et al., 2010, Marshall et al., 2009).

As sequencing techniques have developed it has become apparent that it is not unusual for *E. coli* isolates to contain multiple plasmid sequences simultaneously. A simple search of RefSeq *E. coli* genomes reveals numerous examples. But the long-term stability of the plasmids, within their host bacterial genomes, is unknown. It is assumed that the acquisition of so much extra-chromosomal DNA is energetically costly to the bacterial host; and because of this many of the plasmids may not be stably maintained without antibiotic selective pressure, or active plasmid maintenance systems (such as toxin/antitoxin plasmid addiction systems).

Despite this *bla*\textsubscript{CMY-2}, which accounts for a small proportion of the total coding domain sequences encoded on the IncI1 plasmids, appears to be a sufficiently strong selectable marker to allow the plasmid to remain endemic across a range of *E. coli* isolates affecting canine patients.

Fully contiguous sequences for each plasmid within each *E. coli* isolate would likely not have been achievable with SMRT sequencing. This includes plasmids that do not contain a marker for either incompatibility or replicon typing schemes, and are not detectable by PCR or sequence annotation. While some of these sequences are devoid of any immediately obvious contribution to either the MDR
phenotype, or pathogenic potential of the *E. coli* host; it is possible that they may still play a role. Though basic sequence comparisons reveal some similarities amongst this group of plasmids; their functional contribution has not been assessed in anyway in this project. More over, the abundance of smaller plasmids (with no typeable replicon) remains completely unknown in the wider MDR *E. coli* group (other than the 8 isolates sequenced in this chapter). SMRT sequencing and core genome comparisons also revealed numerous plasmids with mixed replicon sequences; which was not detected by PCR, or by Illumina sequencing.

Zoonotic transmission of antibiotic resistance from companion animals has been previously reported (Damborg et al., 2009, Guardabassi et al., 2004). The zoonotic potential of the MDR genotypes, by transmission of the plasmids (IncI1, IncF or other), is not known for any of the sequences described here. Maximum-likelihood and parsimony analysis of the plasmid sequences did not indicate a strong evolutionary relationship between the sequenced plasmids and any reference sequence plasmids associated with human infections. However, there has been much concern in the scientific literature over the global spread of MDR *E. coli* clones, such as ST131 (Petty et al., 2014). The identification of a resistance plasmid with wide dispersion with *E. coli* and an unknown host-range is also concerning. With a strong match between plasmids located in Edinburgh and found in dogs, to plasmids seen in the USA and Denmark, suggests that dispersal and maintenance of closely related plasmid lineages is a possibility.

The concern over the usage of multiple antibiotics in high-risk groups, leading to MDR, is not a new one. But the potential for change and the scale of such transition, from fully susceptible to MDR, is alarming. Population analysis of the *E. coli* isolates has not indicated a particular genetic background associated with any of the plasmids. It seems more likely that the concerted selective pressure of changing antibiotic treatments has acted to select particular plasmids, rather
than particular strains of *E. coli*.

Little is known about the fundamental interaction between members of a complex bacterial community upon challenge with multiple antibiotics. SMRT sequencing suggests that not only are there complex interactions between host flora and antibiotic challenge; the mobilization of multiple plasmids and other HGEs to this diverse background of strains, creates a daunting genetic complexity.

These isolates remain a rarity and MDR is not seen in uncomplicated UTIs. Neither the clinical environment, or the canine patients can be ruled out as potential reservoirs for the horizontal elements carrying the resistance gene markers. The *E. coli* from the set of UTIs, presented in this thesis, was isolated from canine patients that had been provided a significant level of clinical care. Evolving MDR, resulting from longer-term clinical interventions, appears provides a selective environment for the development of multiple DNA elements with the potential to be stably inherited and maintained within a clinical population; with remarkably few resistance markers.

While the order in which the plasmids were acquired cannot be stated without a more detailed understanding of the chronology of the treatments each patient received; it is suggested that the mobilization of multiple resistance markers on the IncF plasmids allows for better persistence in a complex clinical environment, and more aggressive treatment protocols (including extended-spectrum β-lactams and combinatorial β-lactam/inhibitor chemotherapies) have selected for a singular, clinically associated IncI1 plasmid.

In this regard, the findings here indicate the importance and relevance of the attention to MDR in companion animals.
Chapter 5

Discussion

5.1 Veterinary Care and Derived antimicrobial resistance (AMR)

Experimental findings:

- The prescription of antibiotics in complex veterinary care does lead to the selection of multi-drug resistant (MDR) isolates similar to those seen in human clinical settings.

This research project was undertaken to gain a better understanding of the differences in MDR strains of *E. coli* in dogs. Much of the work presented here is focused on basic descriptive data, applied to previously unknown susceptible and MDR *E. coli* isolates.

The primary aim was to evaluate MDR, characterized by extended-spectrum AmpC (ESAC) resistance, and provide insight into the carriage of this resistance in multiple strains of *E. coli*. 

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Fortunately such infections remain rare and the Small Animal Hospital, University of Edinburgh; though the low number of isolates that could be included in this project greatly limits the interpretation of the results presented in early chapters.

The continued isolation of MDR *E. coli* at the Small Animal Hospital is the result of the level of clinical intervention being applied to the animals in care. This closely mirrors what is being recorded in the human clinical setting; even though the resistance profiles of the *E. coli* isolates is different.

Beyond the locality of the Small Animal Hospital, the paucity of available information on the prevalence of MDR in veterinary clinical settings creates a bias of focus towards infection and resistance control measures and how these apply to human clinical care.

The isolates described in the work presented here make a limited contribution towards addressing the comparative lack of available data on the prevalence of MDR in companion animal veterinary care. These isolates do confirm that dogs undergoing complex, long-term treatments may be may act as a reservoir for potentially transferable MDR (IncF plasmid mediated) and ESAC (IncI1, *bla_{CMY-2}* mediated) resistance; which is capable of persisting. Unfortunately, the scale of the project, with only 18 MDR isolates (all from the same hospital), prevents the findings of the work being extrapolated beyond a very limited geographical setting (the Small Animal Hospital); even to the local community (Edinburgh).

Long-term antibiotic chemotherapy, exposure to multiple antibiotics and poor host health status are all known selective pressures which favor the dissemination of MDR. Unfortunately the available clinical records did not contain enough information to allow the prescription of antibiotics to be correlated to the MDR *E. coli*; which were all isolated as secondary urinary tract infections (UTI)s.
Therefore, it was not possible to assess the progression of AMR to MDR in the individual patients. Enhanced communication between clinicians and researchers could enable future studies on AMR infection progression, and acquisition of horizontally-acquired resistance by *E. coli*.

Future work could also include studies into determine whether the detected AMR or MDR persists within the wider clinical environment; for example, are patients colonized with resistance before treatment, or is there an external reservoir for the MDR, or ESAC phenotype that can be identified?

Such information is necessary to better understand how the transmission of AMR between *E. coli*, and between dogs, may be occurring. There is evidence that suggest that MDR and ESAC, derived from infections in animals may pose a threat to the control of AMR. This was beyond the scope of the current project to address. It is not known what the long term stability of the MDR *E. coli* is when not continuously exposed to antibiotic treatment.

**Future Work:**

- Is MDR resistance detectable in *E. coli* and other bacteria in healthy dogs?
- Is MDR resistance detectable only in a clinical environment?
- Is AMR/MDR maintained in the clinical environment?
- How does the MDR profile of the isolates in this study compare to the resistance seen in humans?
- How does the use of antibiotics at the Small Animal Hospital influence the incidence of MDR?

In order to best address these questions a better epidemiological understanding of AMR and MDR a longitudinal study (i.e active surveillance); tracking in-
patients from point of admission, with sentinel samples from healthy dogs in the community (no just fully susceptible).

With the genetic resolution afforded by next-generation sequencing (NGS) techniques, coupled with a more accurate sampling and treatment time line, a much more accurate model of transmission for these MDR isolates could be developed; and answer questions over the source of resistance (clinical or community) and the longer term stability of the various resistance markers.

NGS and detailed sampling have already been used to detail hospital and community transmission in *Staphylococcus aureus* (Harris et al., 2013, Holden et al., 2004), and large scale intra-city and inter-continental transmission studies in *Klebsiella pneumoniae* (Holt et al., 2015); to great effect.

The sampling could also be expanded to include human samples, to better understand the zoonotic potential of the resistances detailed.

## 5.2 Extended-Spectrum AmpC and MDR

**Experimental Findings**

- $\text{bla}_{\text{CMY-2}}$ derived ESAC phenotype

- Strong genetic linkage of $\text{bla}_{\text{CMY-2}}$ mediated resistance to a well conserved plasmid backbone.

- Mixed MDR genotypic profile.

- Genetic linkage of remaining MDR resistance markers to a separate plasmid backbone.
Despite the lack of an active surveillance program, the results presented in this thesis have captured the successful propagation of ESAC resistance amongst very different MDR *E. coli*.

The focus of this project has been on the ESAC (*bla*<sub>CMY-2</sub>) mediated resistance, yet it is clear for many of the isolates that consistent use of multiple antibiotics (not just the β-lactams), over a variable time-frame has selected for two, or more, significant horizontal acquisition events (IncF and IncI1 plasmids).

While the use of particular β-lactam antibiotics has selected for an IncI1 with a very targeted resistance, the majority of the MDR phenotype is carried by other IncF-type plasmids.

It would be interesting to know if either of the IncI1 or IncF plasmids are community associated or limited to the clinical environment where adequate antibiotic selective pressure is maintained. From a veterinary care perspective, this is important as both the IncF and IncI1 are capable of impacting the effective treatment of infections. However, it was beyond the scope of this project to determine the wider prevalence of either the MDR (IncF) or ESAC (*bla*<sub>CMY-2</sub>) resistance beyond the small number of isolates already collected from the clinical setting.

**Future work:**

- How common is the incidence of plasmid-linked ESAC and MDR resistance beyond the Small Animal Hospital?

- Is there evidence for a reservoir, or increased prevalence for either ESAC or MDR, in dogs or other animals (community or clinical)?

It was postulated in Chapter 4 that the acquisition of the resistance plasmids was likely to be sequential; and that the order in which the IncI1 and IncF
plasmids are acquired and transmitted has implications for better infection control at the Small Animal Hospital.

As suggested previously, a more comprehensive and active surveillance program, targeted towards the resistance genotypes described in this thesis would help to answer these questions.

Now that there is a much better descriptive understanding of the ESAC and MDR genotypes circulating, a much more rigorous epidemiological study can be implemented.

5.3 Strain Backgrounds

Experimental Findings:

- Classical urovirulence markers do correlate with pathogenic-type isolates.

- MDR *E. coli* are more associated with commensal phylogroups; and susceptible *E. coli* more associated with pathogenic phylogroups.

- MDR and susceptible *E. coli* strains do not share underlying genetic relatedness.

A further aim of the project was to attempt to contextualize MDR isolates against a background of more routine out-patient *E. coli* infections. It was not known if the MDR isolates were of nosocomial origin, or whether there was any evidence of community-derived infections and resistance elements.

Furthermore, it was not known if virulence phenotype, or genotype could discern whether or not the MDR isolates had a distinctive genetic background of commensal origin.

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The pathotypic variability of *E. coli* is well defined for a number of disease states. Each pathogenic group is defined using canonical strains of *E. coli* which produce obvious and characteristic infections from which controlled experiments can be conducted to elucidate the genetic basis of the observed virulence phenotype (Kaper et al., 2004, Wirth et al., 2006).

Such typing schemes have focused on sets of virulence genes, which by their presence or absence can be used to infer pathogenic ability. However, the application of such predictive pathotyping to the isolates in this study was not so straightforward.

The results of virulence genotyping (Identibac, SeqFinder, VirFinder) and the comparisons of the MDR and susceptible isolates to archetypal uropathogenic *E. coli* (UPEC) strains (phylotyping, MLST and phylogeny) may or, may not suggest a trend towards greater uropathogenicity in the susceptible isolates.

Many of the virulence factors that define UPEC are missing from the susceptible and MDR isolates. While some of the variability may be a product of incomplete annotation, and sub-optimal sequence assemblies; extra-intestinal *E. coli* (ExPEC) and UPEC are highly flexible in terms of their virulence (Hill, 2012, Wirth et al., 2006). Either way, it is not likely that pathogenic potential can be reliably predicted for some of the isolates in this study.

Even for isolates which possess a more full complement of urovirulence markers, the actual expression of these markers has not been quantified in any way. To do so would require more detailed and *in vitro* work to assay particular groups of virulence markers to try and discern potential differences; which is well beyond the scope of this project.

Uropathogenesis has been defined as a distinct pathotype, yet there is still large variability in the presence and expression of many core virulence genes;
even amongst very closely related UPEC strains (Vejborg et al., 2010). CFT073 is a highly virulent uropathogen, expressing a number of virulence factors (Dobrindt et al., 2016). Despite this highly pathogenic genetic background CFT073 is the ancestral parent strain for both ABU83972 (asymptomatic) and Nissle 1917 (probiotic) E. coli strains. All three strains belong to the B2 phylogroup, possess a number of urovirulence genes and yet are highly variable in their ability to cause disease. Only detailed in vitro comparisons between CFT073 and ABU83972 revealed other mechanisms such as immuno-modulation, persistence and metabolic growth advantages targeted to the urinary and gastrointestinal tracts that could better account for the pathogenic variability seen in these strains (Dobrindt and Hacker, 2008, Dobrindt et al., 2016, Vejborg et al., 2010).

This spectrum of virulence and pathogenesis is also observed in MDR UPEC. The ST131 clonal lineage is closely associated with rapid expansion of the extended-spectrum β-lactamase (ESBL) blaCTX-M-15 and uropathogenesis (Petty et al., 2014). Despite their clonal relatedness and pathogenic phylogrouping (B2), there is variability of virulence between ST131 isolates; which is attributable to variable carriage of ExPEC virulence markers as well as loss or acquisition of genomic loci associated to virulence markers not specific to a particular pathovars (Alghoribi et al., 2014, Petty et al., 2014).

The small number of isolates, combined with a lack of underlying genetic relatedness to one another make such detailed comparisons of virulence very difficult. Should such disparate E. coli isolates be expected to be uniform in their virulence types? Or, do the results reflect the overall heterogeneity of the ExPEC and UPEC pathotypes?

Both questions require both a broadening of the number and type of isolates sequenced here, coupled with more detailed in vitro assays of virulence capable of discerning genuine differences of urovirulence beyond just the presence, or absence
of a small list of virulence factors.

To complicate the interpretation of any results further; assessing only the pathogenic potential by virulence genes may also underestimate the effect of commensal traits which may contribute towards the colonization of the urinary tract. Persistence in an otherwise sterile and immune-controlled environment, metabolic advantages (superior growth rates) and the ability to progressively colonize the gastrointestinal tract, as well as the urinary tract are often neglected in virulence typing schemes (Hill, 2012).

Even resistance itself may be considered a virulence factor as long as the selection pressure is strong enough to overcome other environmental conditions; like resource competition from other gut flora bacteria.

Just as pathotype and commensal niche adaptation drive genomic diversity in \textit{E. coli}, so to do antimicrobials. Many of the IncF sequences detailed in this body of work contained virulence markers as well as resistance markers; suggesting multiple selection pressures favoring the maintenance of the plasmid (including expanded virulence).

The prescription of an antibiotic, though targeted at a single bacterial population, impacts all bacterial populations which the antibiotic is active against. Therapeutic compounds that are active against pathogenic \textit{E. coli} are active against all other populations of \textit{E. coli} which the host is colonized by; whether they are commensal, asymptomatic, or pathogenic. By providing novel evolutionary pressure, such treatments may augment existing pathogens such as ST131, or drive opportunistic acquisition of AMR in commensal populations (Alekshun and Levy, 2006).

\textit{E. coli} commensalism is not easily defined by the presence or absence of virulence markers. Commensalism is better defined and contextualised by core-
genomic sequence comparisons. As such, commensalism and its relationship to the isolates focused on in this study, is likely a convenient place holder label for an isolate who’s pathogenic potential, or environmental niche, has not been fully evaluated under the right conditions (Tenaillon et al., 2010).

Future Work:

- What is the minimal requirement for uropathogenesis?
- What is the distinction between an opportunistic *E. coli* isolate and a commensal *E. coli* isolate?
- Are core genomic sequences adequate for determining the uropathogenic potential of *E. coli* isolates?
- How much does the pan-genome contribute to determining uropathogenic potential?

The variability in the predicted virulence markers seen in the work presented here may well represent real differences between the strains; but ultimately this is a far from complete understanding of the adaptation of the MDR and susceptible isolates to either a pathogenic or commensal niche background.

The strongest indicator of uropathogenic potential for the isolates, presented in this body of work, is their presence and isolation from an active UTI; but this observation is not without complications.

For the MDR isolates, it is likely that there was a greater underlying susceptibility to infection given the level of treatment the dogs were receiving; compared to the susceptible isolates, due to the disease status of the host. To confirm this, the host immune status prior to infection, and host response to an active UTI between the dogs in both groups would be required.
A more detailed understanding of the changes to the gut flora, in response to antibiotic treatment would help to answer the question of whether the observed MDR strains are more likely to originate from commensal or pathogenic populations of *E. coli*. The change in *E. coli* populations, post antibiotic treatment, would indicate which populations (commensal or pathogenic) persist better during antibiotic chemotherapy; which are then more likely to develop AMR and MDR.

Questions of minimal virulence requirement and commensal trait markers are better answered by expanding the current work to include a much more diverse range of *E. coli*. As previously stated, UPEC isolates display a wide-spectrum of virulence (including avirulence); defined not just by the presence of virulence markers, but also each strains unique expression of these markers. While this may not be completely predictive, as long as enough is known about the isolate (i.e. was it isolated from an active UTI), this will provide a better approximation of what baseline virulence markers are required to invade the urinary tract.

The work should also be expanded to include faecal isolates to better understand commensal type *E. coli* in a healthy dog. Colonization of the gut, which is a key stage in the development of a UTI, needs to be better understood so that the genetic markers which favor colonization can also be used to better distinguish potential virulence between the MDR and susceptible isolates.

Finally, it is also important to expand and improve the work started on defining the pan-genomic sequences which distinguish the various isolates from one another. Core-genome phylogeny, whilst undoubtedly useful in providing more accurate evolutionary estimations, relies on only a fraction of the total genome of *E. coli* (20%) (Tenaillon et al., 2010).

At the most basic level (how it is used here), it provides another means to
test the evolutionary relatedness amongst different strains. By expanding it, and dissection the pan-genome further, it is a powerful tool for identifying genes that may be positively selected for in the different populations of *E. coli* of interest here (commensal, MDR, UPEC etc.) (Mainda et al., 2016).

### 5.4 Phenotypic Virulence

**Experimental Findings**

- *Galleria mellonella* may be valuable for comparing virulence between UPEC strains.

- Urovirulence markers do not correlate with greater virulence in an infection model.

- MDR phenotypes did not correlate with reduced virulence in an infection model.

*Galleria mellonella* is a promising infection model which has already been applied to *E. coli* (Alghoribi et al., 2014), and a number of other pathogenic bacteria (Aperis et al., 2007, Jarrett and Stephenson, 1990, Mylonakis and Moreno, 2005, Peleg and Jara, 2009, Seed and Dennis, 2008).

The application of the infection is validated by the reproducibility of the difference in lethality between asymptomatic ABU83972 and pathogenic CFT073 control strains. Though there is clearly much scope for refinement of the model.

For example, ABU83972 and CFT073 are closely related strains of UPEC and it would be desirable to expand the current infection model to include more reference UPEC strains; to better assay the potential variability of both UPEC
strains and the infection model. The model currently only uses larvae death as a surrogate value to summarize all virulence. The probability of larval death is much more likely to be the result of complex multi-factorial influences of various virulence genes (such as toxins); which should be assessed.

It is also unlikely that individual virulence gene families contribute equally to the killing phenotype. In order to generate a more robust model, it would be useful to determine which urovirulence genes contribute the most towards virulence in the larvae.

Furthermore, the larvae in this work were exposed to a single infectious dose. While this was optimal for reproducing the effects seen in the control strains, it is very probable that individual *E. coli* strains will be more or less virulent when this concentration is altered; and concentration-dependent killing provides another useful metric to measure variability in virulence. Titrating the infectious dose of *E. coli* and quantifying virulence in terms of both number of bacteria injected, as well as the proportion of moth larvae killed, would allow much more powerful comparisons to be made between strains.

When using the model to investigate the virulence of MDR strains, it would also be desirable to expand the model to investigate the effects of antibiotic dosing during an infection. By introducing antibiotics, it may be possible to quantify the effect of the expression of antibiotic resistance, positive or negative, on the expression of virulence during infection.

Overall, the results produced here did not show any correlation between the number of virulence factors and moth lethality. This incongruence might result as a by-product of the infection model; though the strong link to an asymptomatic bacteriurea strain and no lethality, and a virulent UPEC strain to higher lethality does make this less likely. This may also be due to a stronger nascent pathogenic
potential amongst commensal type *E. coli* than has been previously estimated.

**Future work:**

- How many virulence makers illicit an immune response in a *Galleria mellonella* infection model?
- Are reference UPEC strains virulent in *Galleria mellonella* virulent in a *Galleria mellonella* infection model?
- Are reference commensal and probiotic *E. coli* strains virulent in a *Galleria mellonella* infection model?
- Can antibiotics be used in the infection model?

### 5.5 Plasmids and MDR

**Experimental Findings:**

- IncI1 associated with singular *bla*CMY-2 resistance.
- IncFII associated with various virulence and resistance.

The focus of the project has been on ESAC resistance, which creates a bias in the reported genotypes for both resistance markers and the plasmids carrying these resistance markers. However, there is no doubt of their contribution towards the MDR status of the isolates.

If the IncF plasmid sequence variation seen in the isolates in this study is a true reflection of the wider *E. coli* population-level genetic background, it would suggest a closer relationship between the IncF plasmids and their host isolates.
Such a relationship would be important in helping to determine the progression of the resistance state of the isolates.

Does the acquisition of an IncI1 plasmid carrying a singular resistance marker drive the acquisition of further resistance markers carried by the IncF plasmid?

Or does the acquisition of a broader MDR phenotype allow persistence and a progressive adaptation towards and extended-spectrum resistance?

Also important in determining the relationship between the plasmids and their *E. coli* hosts; what is the long-term stability of resistance markers and elements, in the wider pool of resistance?

The indication of phylogenetic comparisons to other plasmid backbones suggests a tendency towards persistence and conservation of these markers in isolates with wide geographic distributions. Furthermore, that the plasmids, which are in temporally isolated infections at the Small Animal Hospital, would suggest that transferability between disparate bacterial populations is certainly possible. This would also be helpful in asserting whether a potential transfer of resistance markers between the clinic and the community is driven by one or the other.

Currently the observed differences between the genetic backgrounds of the susceptible and MDR isolates, and the much closer genetic relatedness between the plasmids conferring resistance, suggests that it is the conservation of horizontal genetic elements (HGEs) that is driving ESAC resistance at the Small Animal Hospital.

Rather than a clonal expansion of particularly virulent, or infectious *E. coli* isolates; it is likely that the dissemination of resistance is the product of the transferability of the plasmids. Given the mixed results of the phenotypic and genotypic virulence typing of the MDR isolates, this may indicate a reduced impact
of ESAC and MDR acquisition. This may be due to acquisition in a favourable *E. coli* host background (commensal, not pathogen), a functional contribution of the plasmids that extends beyond just resistance phenotype (metabolic, virulence etc.), or finally an artifact of canine host status (reduced host immune pressure on the *E. coli* isolates).

Given the persistence of the IncI1 *bla*CMY-*2* plasmid it would be of considerable value to know its distribution amongst *E. coli*, or indeed other bacterial species, at the Small Animal Hospital. Is it being actively maintained by antibiotic selective pressure in a clinical setting? Or, given the reported presence of the plasmid backbone internationally, is it distributed in the wider community?

Due to the focus on UTIs (which are overwhelmingly caused by *E. coli*), the focus on *E. coli* may be disproportionately biased; especially given the indication that the plasmid backbone is found in a number of other *Enterobacteriaceae*.

These questions would be answered with an expanded active surveillance program (as suggested earlier); and answering would greatly improve the understanding of the transmission dynamics of ESAC and MDR within the Small Animal Hospital.

### 5.6 Work in Progress

**Further Work:**

- Is AMR plasmid acquisition favorable in all *E. coli* genetic backgrounds?
- Expression and control of AMR phenotype.
- Expression and control of virulence profile.
• Ascribing further function to the hypothetical coding information.

• What is the long-term stability of the resistance elements?

One of the more striking differences between the MDR and susceptible isolates is their plasmid burden. Whereas the MDR isolates are characterizable by a consistent IncI1 and IncFII replicon type, the susceptible isolates show little to no such consistency and less replicon detection overall.

Very little is known about the baseline plasmid burden for *E. coli*, other than it varies widely across a broad range of isolates. No information exists about the expected range of plasmid replicon types associated with either pathogenic, or commensal populations beyond well characterized strains.

For the IncI1 plasmids, the maintenance of 90kb (and larger) plasmids by a single resistance marker, is either a product of either strong antibiotic selection pressure, or through the mitigation of the metabolic expense of the acquisition of such a large amount of genetic material. While strong antibiotic selection pressure is likely in a clinical setting, the widespread presence of the IncI1 plasmid backbone does suggest some competitive advantage or selection pressure beyond just ESAC resistance.

This is also likely true of the IncF plasmids, which contain not just resistance markers (some do not possess any), but virulence markers as well. In light of this potential functional diversity, it would be desirable to know the capacity of a plasmid to impact regular cellular function in the presence or absence of antibiotics. Similarly, to be able to quantify the ease of transfer of these plasmids to *E. coli* isolates of different genetic backgrounds may highlight further impacts to various environmental niches.

If the plasmids cannot be transferred to highly adapted, virulent UPEC isolates
it would confirm whether or not the resistance plasmids favor commensal *E. coli*; and would be suggestive of a negative impact of resistance carriage towards virulence.

The conjugation of the plasmids into other reference strains allows for the investigation of the effects the resistance plasmids may have in a different host background.

Due to time constraints, further work to assess the effect of resistance expression on normal *E. coli* cellular function has since been started (but cannot be included).

Briefly, conjugants and wild-type strains were grown in dog urine in the presence and absence of a β-lactam antibiotic and allowed to reach mid exponential growth phase. RNA was extracted and sequenced using Illumina sequencing in the anticipation of performing differential RNA-Seq expression analysis.

In such a way, the effect of resistance expression can by measured in both an MDR, UPEC and K-12 background.

Looking beyond the contribution of plasmid-mediated resistance; there is very little data available on the nature of the various populations of *E. coli* which form a component of the host flora. Less information still, is available about the baseline presence of virulence or AMR markers that are present in a healthy animal host; and the degree to which the presence or absence of either is affected during clinical intervention.

The observation that AMR is more likely to be associated with commensal-type *E. coli* may be a product of greater susceptibility of more pathogenic *E. coli* to antibiotics pressure. In other words, is there a selective advantage to be gained by plasmids re-introducing virulence markers (UPEC specific or otherwise) into a
more commensal type population; which go onto cause opportunistic infections?

Another, perhaps more likely scenario, is that commensal *E. coli* simply present
the lowest energetic barrier in expressing the acquired antibiotic resistance.

Despite the small-scale (the biggest shortcoming of this project), the results
shown here do suggest that virulence and antibiotic resistance are not mutually
exclusive. Despite the commensal associations with AMR observed here and in
other studies; there are well published examples of AMR associated with out-
breaks of virulent *E. coli*.

To better contextualize these questions in terms of the wider impacts of plasmid-
mediated MDR, is it necessary to attempt to control the derivation of AMR
plasmids from otherwise neutral plasmids within the flora? Or, does therapeutic
intervention need to target the transfer of nascent AMR plasmids from the wider
pool of AMR organisms to other bacteria?

Either scenario is a daunting challenge and unlikely to be completely achieved,
even within the clinical environment (Williams and Hergenrother, 2008).
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Appendices
Appendix A

Publications
Multidrug-resistant *Escherichia coli* from canine urinary tract infections tend to have commensal phylotypes, lower prevalence of virulence determinants and *ampC*-replicons

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**Abstract**

Multidrug-resistant *Escherichia coli* is an emerging clinical challenge in domestic species. Treatment options in many cases are limited. This study characterized MDR *E. coli* isolates from urinary tract infections in dogs, collected between 2002 and 2011. Isolates were evaluated in terms of β-lactamase production, phylogenetic group, ST type, replicon type and virulence marker profile. Comparisons were made with antibiotic susceptible isolates also collected from dogs with urinary tract infections. *ampC* β-lactamase was produced in 67% of the MDR isolates (12/18). Of these, 8 could be specifically attributed to the CMY-2 gene. None of the isolates tested in either group expressed ESBLs. Phylo-group distribution was as expected in the susceptible isolates, with an over representation of the pathogenic B2 phylo-group (67%). In contrast, the phylogenetic background for the MDR group was mixed, with representation of commensal phylo-groups A and B1. The B2 phylo-group represented the smallest proportion (A, B1, B2 or D was 28%, 22%, 11% and 33%, respectively). Virulence marker profiles, evaluated using Identibac™ microarray, discriminated between the two groups. Marker sequences for a core panel of virulence determinants were identified in most of the susceptible isolates, but not in most of the MDR isolates. These findings indicate that for MDR isolates, plasmid-mediated *ampC* is an important resistance mechanism, and while still capable of causing clinical disease, there is evidence for a shift towards phylogenetic groups of reduced inferred virulence potential. There was no evidence of zoonotic potential in either the susceptible or MDR urinary tract isolates in this study.

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1. **Introduction**

Increasing numbers of reports have documented the emergence of *Escherichia coli* capable of producing broad-spectrum β-lactamases. This is significant since the β-lactam antimicrobials are of therapeutic importance in humans and many domestic animals. Furthermore, many isolates are resistant to additional antimicrobial classes and are therefore multidrug-resistant (MDR).

Carriage of ESBL- and AmpC-producing *E. coli* has been documented in many species (Bortolaia et al., 2011). Antimicrobial use has been reported as a risk factor (Damborg et al., 2011, 2012; Maddox et al., 2012) and there is also evidence of sharing of organisms between species living in close proximity to each other (Dolejska et al., 2011). Considering the physical closeness in which many humans live with their pet companions, sharing of these organisms between humans and pets could pose a significant mutual risk.
Clinical disease associated with these organisms is well documented in humans. Initially the pattern was of hospital-acquired infection but community-acquired infection has become increasingly important. ESBL E. coli are associated with a variety of clinical diseases, in particular urinary tract infections, neonatal septicemia and wound infections (Ben-Ami et al., 2009; Pitout, 2010). Reports of similar clinical disease associated with AmpC-producing E. coli are far fewer than those pertaining to ESBLs, however these also appear to be an emerging problem (Oteo et al., 2010).

Although most animal studies have focused on the zoonotic risk posed by carriage, there are increasing reports demonstrating the involvement of these organisms in clinical disease in domestic species in a variety of locations including Europe, North America, Asia and Australia. For example, in the United States AmpC (CMY-2) and ESBL-producing E. coli (O’Keefe et al., 2010; Sanchez et al., 2002; Shaheen et al., 2011) have been reported from canine clinical isolates. Clinical disease associated with AmpC-producing E. coli in dogs in Australia, was first reported in 2006 (Sidjabat et al., 2006). More recently, a survey of clinical isolates from dogs and horses in the Netherlands demonstrated a 2% prevalence of ESBL and AmpC-producing isolates (Dierikx et al., 2012).

The aim of this study was to evaluate the association of ESBL or AmpC production with multidrug-resistant E. coli isolated from canine cases of urinary tract infection in dogs from a local patient population, over a period of time ranging from 2002 to 2011. Isolates were further characterized in terms of phylogenetic grouping, sequence type and virulence genotype. Plasmid replicon typing was also performed to identify the type and diversity of plasmids involved. Comparisons were made to a group of susceptible E. coli isolates, also associated with canine urinary tract infection and collected over a similar time frame.

2. Materials and methods

2.1. Source of clinical isolates

All 15 susceptible and 17/18 MDR isolates were identified in clinical cases seen at the Hospital for Small Animals, University of Edinburgh. One MDR isolate (R3) came from a local practice serviced by the University of Edinburgh’s diagnostic microbiology service.

2.2. E. coli identification

A total of 33 clinical isolates from canine urinary tract infections were cultured onblood and MacConkey agar. Any lactose fermenting colonies were confirmed as E. coli utilizing biochemical testing (API 10S™ strip bioMerieux).

2.3. Antimicrobial susceptibility testing

Susceptibility testing was performed using the disc diffusion method in accordance with Clinical Laboratory Standards Institute (CLSI) guidelines. The following discs were used: Co-trimoxazole (25 μg); ciprofloxacin (1 μg); amoxicillin clavulanate (30 μg); cephalexin (30 μg); gentamicin (10 μg); tetracycline (10 μg); cefotaxime (30 μg). All discs were sourced from Mast group Ltd.

2.4. Control strains

The following control strains were utilized in both the phenotypic combination disc testing and for polymerase chain reaction, ATCC 25922™ (negative control); ATCC BA-190™ (SHV-3 positive control); NCTC 13353™ (CTX-M-15 positive control), and NCTC 13351™ (TEM-3 positive control).

2.5. Combination disc method for plasmid-mediated AmpC and ESBL detection

A commercially available AmpC and ESBL detection set (Mast group Ltd) was utilized. This comprised a set of 4 discs containing cefpodoxime plus or minus AmpC and ESBL inhibitors. Interpretation was made following the manufacturer’s instructions.

2.6. PCR for ESBL and AmpC gene detection

DNA from single colonies of each isolate was prepared using the lysis method as previously described (Pérez-Pérez and Hanson, 2002). Primers for the genes blatem, blashv, blactx, blcmv, 1 group, blcmv, 2 group, bloxa, 1 group and bloxa, 2 group were derived from a previously established assay (Hasman et al., 2005). For additional detection of AmpC β-lactamase genes, multiplex PCR was performed on all samples using the methodology and primers previously described (Pérez-Pérez and Hanson, 2002). PCR products were electrophoresed in a 1% agarose gel, gel bands were excised, and sequences were compared to the NCBI database to confirm identity.

2.7. Multiplex PCR phylogenetic grouping of clinical isolates

DNA from single colonies was prepared using the Qiagen DNeasy blood and tissue extraction kit according to the manufacturer’s instructions (Qiagen, UK). Multiplex PCR methodology was employed to assign the clinical isolates to one of four phylogenetic groups (A, B1, B2 or D). Primers and methodology have been described previously (Doumith et al., 2012).

2.8. PCR-based plasmid replicon typing of clinical isolates

DNA was isolated as described above for the phylogenetic grouping. Methodology involved the use of 8 multiplex reactions in a commercial kit (Diathera, Italy) based on methodology described previously (Carattoli et al., 2005).

2.9. Identibac™ microarray analysis

A microarray assay developed and carried out by the Animal Health Veterinary Laboratories Agency (AHVLA) was used (Batchelor et al., 2008). The microarray contained a selection of oligonucleotide probes mapping to a range of
resistance and virulence-associated genes. Probe hybridizations resulting in signal intensities greater than 0.4 were considered positive indicating the presence of the gene.

2.10. MLST methodology

DNA was extracted using a DNeasy extraction kit (Qiagen) and performed as to the manufacturer’s instructions. Sequencing of the DNA was carried out on an Illumina MiSeq (ARK Genomics). Raw sequence reads were aligned to two reference sequences, *E. coli* MG1655 (Accession numbers NC_017631.1 and NC_000913.2), using BWA and Samtools (Li and Durbin, 2009). Sequence type calling (for multi-locus sequence typing) was performed using SRST (Inouye et al., 2012). Isolates which could not be typed using SRST were called manually using sequences mapped to MG1655. Sequences aligning to the MLST genes in MG1655 were extracted using VCFTools (Danecek et al., 2011) and Extractseq, (Rice, 2006) and entered manually into the MLST Database, hosted by University College Cork (http://mlst.ucc.ie/mlst/dbs/Ecoli).

2.11. Statistical methods

Comparisons were tested using Fisher’s exact test. The criterion for statistical significance was taken to be $P < 0.05$.

3. Results

3.1. Culture and sensitivity

Between 2002 and 2011, 18 *E. coli* isolates associated with urinary tract infections (UTIs) in 16 dogs were identified as multidrug-resistant (MDR). Results of culture and sensitivity testing for the MDR isolates (R1-16) are displayed in Table 1. Two of the dogs had recurrent UTI infection. One case recurred 1 month later (R16a and b) and the second case 6 months later (R11a and b). The criterion used to make the MDR determination was resistance to 3 or more classes of antimicrobial on routine culture and sensitivity testing. All MDR isolates were resistant to amoxicillin clavulanate and tetracycline, 83% (15/18) were resistant to cephalaxin, 78% (14/18) were resistant to co-trimoxazole, 56% (10/18) were resistant to ciprofloxacin, 22% (4/18) were resistant to gentamicin and 67% (12/18) were resistant to the 3rd generation cephalosporin cefotaxime. The latter was used as an indicator of broad-spectrum β-lactamase production.

For comparison, 15 *E. coli* isolates were selected for study, based on the criteria that they were susceptible to all 7 antimicrobial classes listed above. These isolates were associated with urinary tract infections in dogs.

3.2. AmpC β-lactamase phenotype and genotype

To identify the contribution of either ESBL or AmpC β-lactamases to the MDR phenotype, all isolates (both MDR and susceptible groups) were tested using the 4 disc test as described in materials and methods. All susceptible isolates were negative (data not shown). A total of 67% (12/18) of MDR isolates were positive for AmpC production. ESBL production was not detected.

Using *ampC* multiplex PCR, 9/12 isolates phenotypically AmpC positive were genotypically positive for pAmpC (CITM group). Further simplex PCR identified 8 of these to be specifically associated with the CMY-2 gene. Identibac® microarray also detected a CMY gene signal in 8/9 isolates tested (isolates relate to R1–R7, R9 and R10 in Table 1).

| Isolate | Isolation date (month/year) | Resistance pattern | 3rd Gen | AmpC Phenotype | pAmpC PCR | Phylotype | ST | Replicon type |
|---------|-----------------------------|--------------------|---------|----------------|-----------|-----------|    |---------------|
| R1      | 9/2006                      | AMC CEF COT CIP    | TET     | R              | +         | A         | 10  | FII 11       |
| R2      | 1/2008                      | AMC CEF COT CIP    | TET     | R              | +         | A         | 46  | FII 11       |
| R3      | 9/2010                      | AMC CEF COT CIP    | TET     | R              | +         | A         | 744 | FII 11       |
| R4      | 10/2010                     | AMC CEF CIP TET    |         | R              | +         | D         | 648 | FII 11       |
| R5      | 3/2010                      | AMC CEF CIP TET    |         | R              | +         | D         | 963 | FII 11       |
| R6      | 2/2011                      | AMC CEF CIP TET    |         | R              | +         | D         |     | FII 11       |
| R7      | 12/2007                     | AMC CEF CIP TET    |         | R              | +         | B1        | 539 | B/O 11       |
| R8      | 9/2011                      | AMC CEF COT CIP TET| S       | +              |           | B1        | 23  | FII HB       |
| R9      | 9/2011                      | AMC CEF COT TET    |         | R              | +         | B1        | 101 | I1           |
| R10     | 3/2002                      | AMC CEF COT CIP    | TET     | R              | +         | B2        | 167 | FII HA I1    |
| R11a    | 2/2010                      | AMC CEF COT TET    |         | R              | +         | D         | 10  | –             |
| R11b    | 8/2010                      | AMC CEF CIP TET    |         | R              | +         | D         | 372 | –             |
| R12     | 7/2006                      | AMC CEF COT CIP TET| S       | –              | n/d       | D         | 372 | I2           |
| R13     | 8/2009                      | AMC COT CIP TET    |         | S              | n/d       | A         | 10  | FII HA       |
| R14     | 8/2008                      | AMC COT CIP TET    |         | S              | n/d       | A         | 10  | FII HA       |
| R15     | 4/2009                      | AMC CEF CIP TET    |         | S              | n/d       | A         | 988 | –             |
| R16a    | 4/2011                      | AMC CEF CIP TET    |         | R              | n/d       | B1        | 23  | FII B/O      |
| R16b    | 5/2011                      | AMC CEF COT TET    |         | S              | n/d       | B2        | 23  | FII HB       |

Resistance pattern identifies antimicrobials to which organisms were resistant: AMC = Amoxicillin clavulanate; CEF = Cephalaxin; COT = co-trimoxazole; CIP = ciprofloxacin; TET = tetracycline; GEN = gentamicin. 3rd Gen identifies which organisms were resistant (R) or sensitive (S) to the 3rd generation cephalosporin cefotaxime. AmpC Phenotype identifies which isolates were positive or negative for AmpC on the 4 disc phenotypic test. AmpC PCR identifies those isolates where plasmid associated AmpC genes could be identified by PCR (n/d designates not done). Phylotype represents phylogenetic group, ST represents sequence type and N represents a novel ST type.
3.3. Phylogenetic group and ST designation

PCR-based phylogenetic grouping was performed to assign the isolates to one of 4 phylogenetic groups, namely A, B1, B2 or D. Phylogenotypes A and B1 are considered to be associated with commensal status or intestinal pathotypes, while B2 and D are more commonly associated with strains causing extraintestinal infections (Tenaillon et al., 2010). Among the susceptible isolates B2 was the predominant phylogenetic group (10 isolates, 67%) with no isolates in group A, 2 isolates (13%) in the B1 grouping and 3 isolates (20%) in group D. In contrast, the MDR group showed a more even distribution among all 4 phylogenetic groups (1 isolate could not be typed), with B2 comprising the smallest category. The distribution for A, B1, B2 or D was 28%, 22%, 11% and 33% respectively. The proportion of MDR isolates identified as phyotype B2 differed significantly from the proportion of susceptible isolates identified as B2 (P < 0.001).

MLST identified 18 ST types among the E. coli isolates. Within the MDR group these were: ST10 (n = 4); ST 23 (n = 3); ST372 (n = 2); ST46 (n = 1); ST744 (n = 1); ST648 (n = 1); ST963 (n = 1); ST539 (n = 1); ST101 (n = 1); ST167 (n = 1); and ST 998 (n = 1). The two MDR isolates belonging to phylogenetic group B2 belonged to ST types 167 and 23. The overall association of ST type and phylogenetic group is listed in Table 1. Within the susceptible group ST types identified were: ST73 (n = 4); ST12 (n = 2); ST641 (n = 1); ST127 (n = 1); ST10 (n = 1); ST625 (n = 1); ST929 (n = 1); and ST3005 (n = 1). A total of 3 isolates from the susceptible group and 1 from the MDR group did not map to existing ST types. None of the isolates belonged to ST131.

3.4. Identibac® microarray analysis

A panel of 11 specific probes was extracted from a much larger panel. These represent probes for which any isolate, either susceptible or MDR, demonstrated a positive result. The full list of probes against which isolates were tested can be found in the supplementary materials.

The virulence marker panel results are summarized in Table 3. Although the number of isolates examined was limited, there were significantly higher (P < 0.05) levels of carriage demonstrated for 7/11 specific virulence markers in the susceptible group compared to the MDR group.

3.5. Plasmid replicon typing

In the susceptible group (Table 2) plasmid replicons could not be identified in 5 of the isolates. In the remaining 10 isolates, 6 isolates had 2 or more replicons and 4 isolates carried single replicons. The FII replicon was present in 5 isolates and the FIB replicon was present in 4 isolates. Overall 8 different replicon types were identified in this group. In the MDR group (Table 1), plasmid replicons could not be identified in 4 of the isolates. A total of 12 of the remaining 14 carried 2 or more replicons and only 2 isolates carried single replicons. The FII replicon was present in 11 isolates and the I1 replicon was present in 9 isolates. A total of 6 isolates carried the FII and I1 replicons together (these 6 isolates were all phenotypically and genotypically positive for pAmpC). Overall 6 different replicon types were identified in this group.

4. Discussion

These findings demonstrate clinically significant MDR E. coli in canine urinary tract infections. The antibiogram phenotype of isolates (Table 1) shows that treatment options are limited. All MDR isolates were resistant to the recommended first line treatment amoxicillin clavulanate and more than half of the isolates were resistant to fluoroquinolones, a third line option (Weese et al., 2011). MDR strains were analyzed in some detail. Comparisons were made to a susceptible group of canine UTI isolates from the same locality and indirectly to significant human clonal lineages. The latter is particularly important in light of concerns regarding the transfer of organisms between humans and domestic animals, and the potential for either to act as a reservoir of infection for the other.

AmpC rather than ESBL-producing E. coli were commonly identified among the MDR isolates. This is interesting because, in human UTIs associated with MDR E. coli, ESBLs (particularly the CTX-M group) seem to be the predominant enzymes responsible for broad-spectrum resistance to β-lactams. Although this study has a low number of isolates, other studies have also identified the presence of AmpC producing E. coli in dogs associated with both faecal carriage and clinical disease (Damorg et al., 2011; Dierikx et al., 2012; Murphy et al., 2009; Shaheen et al., 2011; Sidjabat et al., 2006; Tamang et al., 2012; Wedley et al., 2011). Furthermore, routine screening in our laboratory has continued to identify AmpC-producing isolates causing urinary tract infection (10 isolates from January 2012 to November 2013). Although this represents a relatively low local incidence, these cases are still clinically significant and therapeutically challenging.

The phylogenetic profiles differed between the two groups of isolates. The susceptible group of UTI isolates predominantly belonged to the B2 phylogenetic group, as predicted from previous studies in humans and dogs.
Table 3
Identification of virulence markers in the MDR and susceptible E. coli isolates. X indicates presence of the gene.

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R = MDR isolate; S = susceptible isolate. Phyl = phylogroup; ireA = siderophore receptor; iroN = enterobactin siderophore receptor; iss = increased serum survival; mcbB = microcin H47 part of colicin H; mcbC = MchC protein; mchF = ABC transporter protein; mcm = microcin M part of colicin H; perA = EPEC adherence factor; prfB = F-related fimbriae regulatory gene; senB = plasmid encoded enterotoxin; sfaS = S fimbriae minor subunit.

* Identifies significant difference for MDR versus susceptible.

The virulence marker proﬁle also differed between the two groups, suggesting a reduced virulence genotype in the MDR isolates compared to the susceptible ones. Previous studies have indicated that, in MDR isolates associated with UTI in both humans and dogs, there may be a shift away from the dominance of the B2 phylogenetic group and a decrease in certain virulence genes (Moreno et al., 2006; Vila et al., 2002). The reason for this pattern is unclear. Other researchers have speculated that less pathogenic phylogenetic groups are more receptive to the acquisition of the MDR phenotype (Johnson et al., 2004), or that acquisition of the MDR phenotype results in a trade off, with a loss of virulence traits. Whatever the order of events, it would seem logical that less pathogenic phylogenetic groups, with an MDR phenotype, would require certain conditions under which to cause clinical disease.

Sequence typing did not provide evidence for clonal spread of isolates in either group. Considering the extended sampling time this is probably not surprising. More pertinent perhaps was the fact that ST131 was not identified amongst any of our isolates. The O25b-ST131clonal lineage is one of the most important uropathogenic E. coli groups in humans. It belongs to the B2 phylogenetic group, is multidrug-resistant (almost always resistant to the fluoroquinolones and often resistant to 3rd generation cephalosporins), is often, but not always, an ESBL producer (CTX-M-15) and is highly virulent (Oteo et al., 2010; Thompson et al., 2011). Total reports of ST131 in domestic animals are still extremely low and, although there is some support for interspecies transfer of ST131, it is unclear if animals are a major
reservoir or incidental host of this ExPEC clonal lineage, or indeed if humans act as an animal reservoir (Platell et al., 2011). Certainly within the limitations of our study we found no evidence for this.

One MDR isolate (R4) was typed as ST648 phylogenetic group D. Strains of this clonal lineage that carry ESBLs, have been associated with bacteraemias in human patients in the Netherlands and NDM carbapenemases in human patients in the United Kingdom and Pakistan (Tamang et al., 2012).

Plasmid replicon typing was performed in order to establish the range and diversity of plasmids amongst both the MDR and susceptible isolates. Most of the plasmid replicons correspond to incompatibility groups. In the susceptible group replicon types could not be assigned to 5 of the isolates. This is perhaps not surprising, as the assay is designed to detect resistance plasmids. In the MDR group there were 4 isolates that were not assigned replicon types. Interestingly 2 of these (R11a and b) were phenotypically AmpC-producing, but it was not possible to detect plasmid-associated genes. We speculate that AmpC production in these isolates could be attributed to chromosomal mutations in the ampC promoter. Further analysis of these isolates will be required to confirm this. In the remainder of the MDR isolates the F1 and I1 replicon types were the most highly represented. We observed a cluster comprising 6 isolates (R1–R6 Table 1) all AmpC-producing and carrying IncFII/IncI1 plasmids. Since IncFII and IncI1 plasmids are two of a number of plasmid types that are particularly successful in their ability to spread multidrug resistance (Carattoli, 2011), further characterization and comparison of the plasmids from these isolates would be of particular interest.

With the exception of one case for which we have no history, all cases caused by MDR E. coli had underlying disease involving suppression of the immune system (e.g. hyperadrenocorticism, cancer chemotherapy), an anatomical abnormality of the genitourinary tract (e.g. detrusor muscle atony, ectopic ureters), and/or a history of prior antimicrobial treatment. Clinical details are summarized in Table 4. This is not a surprising finding since it can be envisaged that such factors will increase the potential of isolates, which we speculate may be less virulent, to cause clinical disease. What will be of interest is to follow the natural history of infections caused by these organisms in companion animals. It is highly probable that future changes in the epidemiology of MDR E. coli infections in dogs, will reflect those seen in the human population, where there has been a shift from hospital-acquired infection, analogous to what we have observed in this study in dogs, to a community-acquired infection, where they are associated with uncomplicated urinary tract disease.

The main limitations of this study are the low number of MDR isolates, and the relatively extended time over which samples were collected. However, this reflects the relative scarcity of such MDR isolates associated with routine community-acquired canine urinary tract infections tested over the last decade at our laboratory, with the majority of the MDR isolates arising from more complex clinical cases at the small animal hospital.

### 5. Conclusion

MDR E. coli are a cause of a small but clinically significant number of urinary tract infections in dogs serviced by the veterinary microbiology service at Edinburgh University.

Broad-spectrum β-lactamase production is an important resistance mechanism and, unlike in human infections, AmpC rather than ESBL production predominates. This may reflect the gut carriage of AmpC E. coli in the dog. Amongst our isolates MDR E. coli are skewed towards less pathogenic phylogenetic groups and have a reduced virulence genotype. The variety of ST types does not support clonal spread; horizontal transfer of certain plasmid types may be a more important mechanism for the transmission of the MDR phenotype, judging by the frequency of the IncFII and IncI1 plasmid types. Most importantly, ST type analysis of both susceptible and MDR
isolates suggests that dogs with urinary tract infection are not acting as a reservoir for zoonotic spread. Neither does it provide evidence for reverse zoonosis from the human population.

Conflict of interest

The authors have no financial or personal relationships with organizations or people that could inappropriately influence or bias their work.

Acknowledgements

This work is jointly funded by Zoetis and the Biotechnology and Biological Sciences Research Council (BBSRC). This work has been submitted for publication with the approval of Zoetis. We would like to thank Ms Lorna Hume, Ms Jennifer Harris and Dr James Gibbons for technical assistance; the clinical staff at the Hospital for Small Animals, University of Edinburgh for allowing access to clinical material; the AHVLA for carrying out the Identiba® assay and Dr Darren Shaw for statistical advice.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.jvetmic.2014.01.003.

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disease in dogs and cats: antimicrobial guidelines working group of
the international society for companion animal infectious diseases.
Convergence of plasmid architectures drives emergence of multi-drug resistance in a clonally diverse Escherichia coli population from a veterinary clinical care setting

Sam Wagner, Nadejda Lupolova, David L. Gally, Sally A. Argyle

The Royal (Dick) School of Veterinary Studies and The Roslin Institute, The University of Edinburgh, Roslin, Midlothian, United Kingdom

ABSTRACT

The purpose of this study was to determine the plasmid architecture and context of resistance genes in multi-drug resistant (MDR) Escherichia coli strains isolated from urinary tract infections in dogs. Illumina and single-molecule real-time (SMRT) sequencing were applied to assemble the complete genomes of E. coli strains associated with clinical urinary tract infections, which were either phenotypically MDR or drug susceptible. This revealed that multiple distinct families of plasmids were associated with building an MDR phenotype. Plasmid-mediated AmpC (CMY-2) beta-lactamase resistance was associated with a clonal group of IncI1 plasmids that has remained stable in isolates collected up to a decade apart. Other plasmids, in particular those with an IncF replicon type, contained other resistance gene markers, so that the emergence of these MDR strains was driven by the accumulation of multiple plasmids, up to 5 replicons in specific cases. This study indicates that vulnerable patients, often with complex clinical histories provide a setting leading to the emergence of MDR E. coli strains in clonally distinct commensal backgrounds. While it is known that horizontally-transferred resistance supplements uropathogenic strains of E. coli such as ST131, our study demonstrates that the selection of an MDR phenotype in commensal E. coli strains can result in opportunistic infections in vulnerable patient populations. These strains provide a reservoir for the onward transfer of resistance alleles into more typically pathogenic strains and provide opportunities for the coalition of resistance and virulence determinants on plasmids as evidenced by the IncF replicons characterised in this study.

1. Introduction

E. coli is an important commensal organism and a significant pathogen. It is associated with intestinal and extra-intestinal infections and is a leading cause of urinary tract infections (UTIs) and bacteraemia leading to sepsis (Pitout, 2010).

Beta-lactam antimicrobials are widely used in both human and animal medicine. Due to their spectrum of activity, pharmacokinetic characteristics and good safety profile, members of the group are a good choice in the treatment of UTIs associated with E. coli. However, there is increasing resistance to these antimicrobials, mediated by the ability of Enterobacteriaceae such as E. coli, to produce plasmid mediated AmpC (pAmpC) and extended spectrum beta-lactamase enzymes (ESBLs). This imparts resistance to most of the Beta-lactam antimicrobials, including the later generation cephalosporins. In addition, many are also resistant to other antimicrobial classes rendering them multi-drug resistant (MDR), significantly increasing morbidity and mortality (Harris, 2015). The prevalence of pAmpC and ESBL resistance is increasing in both hospital and community acquired infections (Nakai et al., 2016). Less information is available for companion animals, but studies that have evaluated this report a resistance epidemiology similar to that observed in human clinical care (Dierikx et al., 2012; Gibson et al., 2010; Murphy et al., 2010; Tamang et al., 2012)

Successful dissemination of resistance within MDR E. coli is attributable to the fact that they are mostly located on horizontally transferable elements (HTEs) such as plasmids and transposable elements. HTEs are often promiscuous with a diverse bacterial host range, conferring resistance not just within but also between bacterial species, generating a large potential resistance reservoir (Bortolaia et al., 2011). It is important to determine the genetic context of resistance alleles to understand co-inheritance of traits that may drive selection and the potential of resistances to coalesce in single isolate backgrounds.

As sequencing technologies have advanced, an increasing number of resistant E. coli strains, have been sequenced. Initially, much of the sequence comparison relied upon read mapping and short read de novo assembly. These methods often fail to accurately resolve complete
chromosomes and other replicons due to multiple repeat regions which are often present in mobile genetic elements. As a consequence, there is a relative lack of availability of high quality sequence data relating to complete bacterial plasmids encoding antibiotic resistance. The advent of long read sequencing technologies is now leading to increased representation of these sequences in public databases.

The aim of this study was to characterise MDR E. coli associated with urinary tract infections in dogs focusing on the use of Illumina sequencing combined with Pacific Biosciences single molecule real time (SMRT) sequencing to elucidate the plasmid repertoires that have been assembled to generate MDR isolates.

2. Materials and methods

2.1. Bacterial strains

All isolates (16 MDR and 14 antibiotic susceptible (AS)) were previously characterized in terms of bacterial identification, phylogenetic group, plasmid replicon type, MDR phenotype and ST type (Wagner et al., 2014).

2.2. Illumina sequencing

E. coli isolates were cultured overnight at 37°C, 170 rpm, in lysogeny broth (LB). DNA was extracted using QiaGen DNAeasy extraction kit (Qiagen U.K.). DNA was isolated from 1 ml of bacterial culture, according to the manufacturer's specifications. Following integrity assessment on agarose gels and quantification/quality determination by spectrophotometry (including absorbance 260:280 nm), Nextera XT libraries were prepared by Edinburgh Genomics. Paired-end Illumina sequencing was performed using an Illumina Hi-Seq 2500 sequencer libraries were prepared by Edinburgh Genomics. Paired-end Illumina sequencing was performed using an Illumina Hi-Seq 2500 sequencer with read lengths of 100 bp to achieve an average depth of 60×.

2.3. SMRT sequencing

SMRT sequencing was performed using the PacBio platform (Pacific Biosciences). E. coli were inoculated into 10 ml LB and incubated overnight at 37°C, 170 rpm. DNA was extracted using the QiaGen 100/ G extraction kit, according to the manufacturer's instructions. Extracted DNA was prepared for sequencing using AMPure Beads (Beckman Coulter), with a target insert size range of 10 kb or greater. DNA was sheared using a g-TUBE™ (Covaris). Sheared DNA was purified and concentrated using AMPure beads. Samples were eluted using PacBio Elution Buffer, and single stranded fragments were removed with ExoVII DNA. Fragments were then repaired using the PacBio DNA Damage and Repair Ends buffers, as per the protocol. Processed DNA was further purified using AMPure beads; then blunt ended sequence adapters were ligated, and ExoIII and ExoVII restriction enzymes used to remove any failed ligation products. Successfully ligated sequence fragments were concentrated using three successive AMPure bead purification steps. Final DNA concentration for all of the samples exceeded 5 μg. DNA was sequenced on the PacBio RSII sequencing platform.

2.4. Sequence assembly and annotation

Illumina sequence reads were filtered using Sickle (Joshi and Fass, 2011) with a minimum read quality score of 30, and a read length of 50 bp. Reads were re-shuffled with shuffleSequences fastq.pl script (Zerbino, 2010). De novo assembly used both paired reads and singletons. Illumina sequence reads were assembled with the VelvetOptimiser script, from Velvet 1.2.08 (Zerbino and Birney, 2008) using a kmer size range of 47 to 67.

SMRT analysis was used to generate a fastq file from the PacBio reads and error-corrected reads were adjusted using PbcR with self-correction (Koren et al., 2013). Then the longest 20× coverage reads were assembled with Celera Assembler 8.1 and polished using Quiver (Chin et al., 2013). Annotated genomes (Do-It-Yourself Annotator (DIYA) (Stewart et al., 2009)) were imported into Geneious (Biomatters LTD., Auckland, New Zealand) (Kearse et al., 2012) and duplicated sequence removed from the 5′ and 3′ ends to generate the circularized chromosomes/plasmids. Origin of replication was approximated using Ori-Finder (Liao et al., 2014) and the chromosome reoriented using the origin as base 1.

2.5. Core genome analysis

To construct core SNPs trees short reads were aligned with BWA http://www.ncbi.nlm.nih.gov/pubmed/19451168 (Li and Durbin, 2009) to a reference E. coli O157:H7 Sakai strain (RefSeq accession: GCF\_000008865). Consensus sequence for each alignment of 5,590,092 bp was produced using the majority rule and then used to detect and remove recombination regions with Gubbins http://nar.oxfordjournals.org/content/early/2014/11/20/nar.gku1196.short (Croucher et al., 2015).

Resulting sequences were used to construct a Maximum Likelihood (ML) tree with RAxML http://www.ncbi.nlm.nih.gov/pubmed/24451623 (Stamatakis, 2014) under a GAMMA model of heterogeneity with 500 bootstrap replicates.

2.6. Plasmid sequence comparison

CCTViewer (Grant et al., 2012) was used to visualise SMRT sequenced plasmids, using 1428 p96 as a reference sequence for the IncP plasmids (Accession no CP023370) and 144 p134 (Accession no CP023363) as the reference for the IncF plasmid (Table 2). ProgressiveMauve (Darling et al., 2010) alignment was performed using default parameters. ResFinder (Zankari et al., 2012) was used to confirm annotated antimicrobial resistance markers. BlastKOALA (Kanehisa et al., 2016) was used to identify putative virulence factor sequences on the plasmid.

2.7. Analysis of plasmid core and pan genomes

Plasmid replicon and clonal complex types were determined using the pubMLST database (Jolley and Maiden, 2010). Core sequence homology between the plasmid sequences was detected with GET_HOMOLOGUES (Altschul et al., 1997) using bi-directional best-hits (BDBHs) and orthoMCL algorithms. Protein clusters were aligned with Muscle (Edgar, 2004). Amino acid alignments were then translated back into nucleotide sequences using the PAL2NAL (Suyama et al., 2006), concatenated and transformed into phylm format with castsataplymph/castsataplymph.pl script to use for RaXML phylogenetic estimation under a GTR model and 100 bootstrap replicates (Stamatakis, 2014). GET_HOMOLOGUES was also used for the pan genome analysis, using the PARS program from the PHYLIP package (Contreras-Moreira).

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3. Results

3.1. Comparative analysis of E. coli strains

Core SNP-based phylogenetic analysis of the 30 clinical UTI associated isolates (16 MDR and 14 AS) plus 10 reference strains (Table 1) was carried out, and identified at least two distinct clusters that mostly correlates well with the isolates’ MDR status (Fig. 1). Isolates were ancestrally diverse, however there was a trend for susceptible isolates to be more closely associated to human UPEC E. coli reference sequences and MDR isolates clustered together with commensal E. coli reference sequences. Whole genome sequencing (WGS) analysis of the MDR strains confirmed previous standard phylotyping results regarding their diverse commensal backgrounds. Isolates that possess the IncI1 plasmid genotype were well dispersed throughout the tree. Tree clades were largely independent with respect to date of isolation.

3.2. Sequencing and plasmid carriage

Plasmid sequences could not be assembled using Illumina short read sequence data. Therefore, 8 MDR isolates (127, 1223, 1283, 1428, 144, 317, 746 and 1943) were sequenced by SMRT, these were selected to examine the IncI1 replicon context of the CMY-2 (AmpC) beta-lactamase. Two sensitive isolates (1190 and 1105, Supplementary Table S2) but carrying an IncI1 replicon were also sequenced by SMRT. The plasmid combinations present in the MDR strains are described in Table 2 and depicted in Fig. 2 along with their contribution to MDR as defined by carriage of specific resistance alleles.

While 7/8 MDR strains had the anticipated replicons, one isolate (317) only carried an IncF plasmid, despite PCR and illumina sequence data indicating the presence of IncF and IncI1 replicons. It is possible that the plasmid was lost during the experimental process. One susceptible isolate, 1105, was PCR positive for the IncI1 genotype, but this replicon could not be assembled from the SMRT sequence data.

3.3. IncI1 comparative analysis

In the MDR isolates, the IncI1 plasmid sequence sizes ranged from 85 to 96 kb, with between 106 and 113 coding domain sequences; many of which still had no ascribed function. All but two of the IncI1 plasmids belonged to the same clonal complex (CC-2), determined in silico, based on the presence and sequence similarity of repII, ardA, traB, sosF, and pilL to previously published IncI1 pMLST profiles. As anticipated, the IncI1 plasmids associated exclusively with the CMY-2 encoded pAmpC beta-lactamase resistance gene; this was located within a resistance cassette associated with a mobile element (Figs. 2 and 3). Significant periods of time separate isolation of the sequenced strains; the first collected in 2001, the most recent 2011. This makes the comparison of the IncI1 replicons unique as it provides insight into the evolution of this resistance-encoding replicon in our local context over this time period.

BLAST Atlas search using the CCT comparison tool for analysis of the IncI1 replicons show greater than 90% sequence similarity for most of the sequences. Multiple collarine blocks, with high synteny and sequence similarity and limited large-scale rearrangements were identified using progressive Mauve (Fig. 3). IncI1 plasmids were compared against 42 plasmid sequences obtained through GenBank, which were identified by BLAST as being similar (Supplementary Fig. 1). Pan-genomic analysis detected 305 homologous gene clusters across all sequences. Phylogenetic estimations using homologous cluster presence or absence indicated a close phylogenetic relationship between all IncI1 plasmids sequenced in this study including the IncI1 plasmid from the susceptible E. coli isolate in this study (1190/01 Accession no CP023387). The resistance cassette conferring CMY-2 mediated pAmpC resistance although not exclusive to the canine resistance plasmids, was not detected in many other IncI1 sequences available on databases. Ten of the homologous gene clusters: tral, trAM, nickB, troD, troA, traL, trAF, traJ, and trX; all relating to plasmid transfer and replication were core to all the IncI1 plasmid sequences (Fig. 4). These were extracted from the pan-genomic analysis and aligned for maximum likelihood core genome phylogenetic estimation (Supplementary Fig. 1). Despite only weak bootstrap support overall, the phylogenetic tree is congruent with the predicted in silico MLST plasmid groups. The IncI1 plasmids included in the analysis were from different Salmonella serovars and E. coli isolated from different animal hosts suggesting that interspecies transmission of the IncI1 plasmids occurs. However, IncI1 sequences associated with bacteria isolated from humans were largely absent, despite the over representation of human isolates in databases in general. The majority of canine sequences form a sub-cluster, with chicken- and porcine-associated plasmid sequences being the most similar. Of note, CP090563 and KP434766 are the only two reference IncI1 plasmids associated with canine clinical infections; isolated in Salmonella enterica serovar Newport in Arizona in 2015 (Cao et al., 2015) and E. coli in Denmark in 2008 (Hansen et al., 2016) respectively. These were closely related to the canine E. coli plasmids sequenced in this study even though their host bacterial strains were obtained from different geographical areas and at different times (Supplementary Fig. 1).
Fig. 1. Maximum-parsimony tree of E. coli chromosomal sequence alignment. Isolates from this study are listed in red (MDR) or green (AS). Ref-Seq sequences are listed in black. Plasmid replicon types are designated from left to right by the colour coded squares: FII , FIA , FIB , I1 , I2 , B/O , × 1 , R , × 2 . Phylogroup designations are colour coded in the right hand column as follows, A = yellow, B1 = blue, B2 = red and D = green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Fig. 2. Graphical representation of the plasmid carriage of the E. coli isolates sequenced in this study. Boxes represent each isolate referred to by their isolate ID. Depicted sequences are not to scale. Plasmid sequences are identified and distinguished by their sequence length. Plasmid encoded resistance, where detected by BLAST search, is indicated by colour coding on the plasmids. Plasmid incompatibility type, where typable, is indicated (either IncF or IncI1). Chromosomal sequences are not depicted.
3.4. **IncF comparative analysis**

The IncF plasmids were more heterogeneous in size (100–147 kb), the number of coding regions (105–150), and the number and classifications of various resistance markers. Aminoglycoside, chloramphenicol, potentiated-sulfonamide, and beta-lactam resistance markers (other than CMY-2) were associated with IncF or smaller untyped plasmids (Fig. 2).

Despite the greater variety and number of resistance markers shared amongst some of the IncFII plasmids, five contained no detectable resistance markers. Virulence genes, mostly associated with iron uptake, and genes associated with metabolism were detected on many of the IncF plasmids (Fig. 2). In silico replicon typing indicates that many of these plasmids were of mixed lineage (Table 2). IncF plasmids showed less sequence similarity when compared by BLAST or by progressiveMauve sequence analysis. With one exception, all regions of local co-linearity had little synteny, or support across all the plasmid sequences (Fig. 5). GenBank BLAST searches identified 9 sequences similar to the IncFII/IncFIB plasmids. Pan-genomic maximum parsimony analysis indicated diverse content (Fig. 6a). No core genes could be identified for all the plasmid sequences, although a subset, excluding 1428 p96, 746 p62, 746 p72, and 1943 p80, could be compared using 8 homologous gene clusters; as with the IncI1 plasmids these genes were mostly associated with plasmid maintenance and replication, including traA, traL, traE, traB and traX. The different replicon sub-types are depicted in Fig. 6b, with FII/FIB the most commonly identified. A singular clade of plasmids, exclusively IncFII, showed greater sequence homology than the remaining plasmid sequences (Fig. 6a & b). Maximum likelihood phylogenetic analysis of IncFII, IncFIA, and IncFIB replicon types showed dispersal of all sequences of different replicon types throughout the tree (Fig. 6b). None of the PCR typed IncFII plasmids sequenced in this study were members of the same clade and 317 p100 was a significant outlier from the rest of the plasmid sequences.

3.5. **Other plasmids**

Numerous other extra-chromosomal contigs were detected from SMRT sequencing. IncI1, IncFII, IncFIA, and IncFIB, were the only
detected incompatibility types, yet account for a fraction of plasmid sequences. The nature of the SMRT sequencing and the analysis means detected incompatibility types, yet account for a fraction of plasmid sequences. The nature of the SMRT sequencing and the analysis means identified by *. The CMY-2 resistance cassette, common to the IncI1 plasmid sequences isolated in this study, is highlighted by the blue box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Maximum-parsimony analysis using detected presence or absence of homologous gene clusters from the pan genome of PacBio IncI1 replicon sequences, and comparator IncI1 sequences obtained from the NCBI nucleotide sequence database. Plasmid sequences from this study lie within the red box. Core genome sequences (used for maximum-likelihood analysis) are indicated by △. The CMY-2 resistance cassette, common to the IncI1 plasmid sequences isolated in this study, is highlighted by the blue box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Core sequence alignment of IncFII replicon type plasmid sequences, using 1223 p147 as the index sequence. Comparisons were performed using a BLAST Atlas search using the CCT comparison tool. Both the whole nucleotide sequence (smaller ring) and coding domain sequence specific (larger ring) BLAST comparisons were carried out. ProgressiveMauve was also used to compare the plasmids. Local co-linear blocks detected by progressiveMauve have been annotated onto the BLAST comparisons.

4. Discussion

MDR E. coli isolates are relatively rarely isolated from in and outpatient samples presented for testing at the University of Edinburgh’s small animal hospital. As previously described (Wagner et al., 2014) MDR isolates are often associated with animals with complex medical histories and understanding their emergence in the context of generally sensitive E. coli isolates (AS) provides an important opportunity to develop our understanding of MDR emergence in a clinical setting.

Combined use of Illumina and SMRT sequencing has allowed detailed examination of individual plasmids and strain background, as well as an overview of plasmid carriage in the context of individual isolates.

Phenotypic beta-lactamase resistance is attributed to pAmpC, encoded exclusively by the CMY-2 allele on IncI1 replicon plasmids, forming a notably closely related phylogenetic cluster, with high levels of homogeneity in the IncI1 plasmid sequence, despite the strains having been collected over a 10 year period. The CMY-2 allele was the only identifiable resistance gene present on this subset of IncI1 plasmids. This has been reported previously in CMY-2 carrying IncI1 plasmids isolated from canine, feline and human hosts (Bortolaia et al., 2014; Sidjabat et al., 2014). The majority of the MDR IncI1 plasmids share a closest common ancestor, using maximum-likelihood analysis of the core genome. This is either due to i) limited sequence divergence or ii) sequence convergence. Given the genetic distance between the susceptible IncI1 plasmids, the absence of any dominant E. coli clone associated with the IncI1 plasmids, the lack of any indication of potential bacterial host range of the plasmids (other than E. coli), and the discordance of sequence similarity with the chronological sequence of the E. coli isolates; it is difficult to identify which. The reliability of any estimation of rates of divergence between the different plasmids is questionable; the identification of a similar plasmid backbone in canine clinical isolates CP009566 and KF434766 from the USA and Scandinavia does suggest underlying core genome stability (Bogaerts et al., 2015; Bortolaia et al., 2014). CMY-2 has also been identified from faeces of healthy dogs in a number of geographical locations including the Netherlands, Mexico, France and Japan, (Baede et al., 2015; Haenni et al., 2014; Ökubo et al., 2014; Rocha-Gracia et al., 2015) and where determined, the plasmid context is predominantly IncI1. This may indicate that the IncI1-CMY-2 is endemic to the commensal population especially in the dog. The common use of cephalosporins such as cephalxin in companion animal practice has been implicated in high prevalence carriage particularly in canine isolates (Haenni et al., 2014).

In comparison to the IncI1 plasmids, the IncF plasmids are more disparate, in core and pan-genome sequence content. This could be a consequence of experimental design, as the isolates for this study were collected based on their pAmpC production, the gene for which is present on the IncI1 plasmid. However other studies also provide evidence for the relative heterogeneity of the IncF plasmids (Villa et al., 2010), and relatively few sequences could be identified in the NCBI database, sharing nucleotide similarity with the IncF sequences in this study; with little conservation of resistance markers between one IncF plasmid and another. Another contrasting feature of the IncF plasmids, supported by other studies, was their greater compliment of resistance alleles and putative virulence-associated genes compared to the IncI1 plasmids. As most of the strains containing the IncI1/IncF plasmid combination specifically were associated with commensal phylogroups, we speculate that acquisition of IncF plasmids in combination with IncI drives emergence of normally commensal E. coli strains resulting in clinical disease in vulnerable patients, especially in the presence of antibiotic selection. All patients from which MDR isolates were identified could without exception fall into this categorisation having significant underlying disease, often multiple antibiotic treatments and some receiving immunosuppressant therapies (Wagner et al., 2014). The dispersion of resistance genes across different plasmids, in many of the isolates, suggests sequential acquisition perhaps whilst part of the
normal flora of the gastrointestinal tract; as it is unlikely that the transfer of multiple plasmids would occur as a singular event. Commensal strains have previously been implicated as a significant component of the resistance reservoir (Marshall and Levy, 2011) whilst other studies have clearly identified the carriage of CMY-2 in more typical UPEC strains such as ST 131 (Dashti et al., 2014).

Fig. 6. Maximum parsimony and maximum likelihood analysis for IncFII. a) Maximum parsimony analysis of pan-genomic homologous gene clusters for IncFII replicon sequences. Similar NCBI nucleotide sequences, with detectably diverse replicon types were included. Isolates sequenced in this study are indicated by the red boxes. b) Maximum-likelihood analysis of aligned core homologous sequences. Sequences have been annotated with their detected replicon type. FII ➕ FIA ➖ FIB. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
SMRT sequencing identified strains containing up to 5 plasmids contributing up to 300KB of additional genetic information. Much of the function of these coding regions is unknown. Sequencing also revealed the presence of small non-typable plasmids of which we were previously unaware. It is assumed that the acquisition of so much extra-chromosomal DNA may be energetically costly to the bacterial host, and many of the plasmids may not be stably maintained in this combinatorial manner in these specific backgrounds without antibiotic selective pressure. The long-term stability of these plasmids within their host bacterial genomes is currently unknown, but investigation of this in future work would be of significant value.

The MDR E. coli strains characterised in this study were isolated from clinical cases with significant underlying disease, which had received often multiple courses of antimicrobial chemotherapy. This represents a model of the genetic and phenotypic adaptation of E. coli to current clinical practices in both the human and veterinary setting. Patient vulnerability and antibiotic selective pressures provide an environment for the emergence of opportunistic MDR resistant E. coli based on the acquisition of at least two plasmid replicon groups, with numerous other horizontal DNA molecules also under selection. It will be of interest to evaluate the long-term stability of extra-chromosomal DNA in relation to antibiotic selective pressure and individual plasmid and resistance gene effects. In addition, it would also be of value to establish the reservoir potential of isolates as a source of resistance. It could be argued that the commensal background of the isolates requires a confounding several factors for disease to occur, but should the horizontal transfer of plasmids to more pathogenic E. coli occur then the clinical significance of this increases exponentially.

Transparency declarations
None to declare.

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Appendix A: Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2017.09.016.

References


