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Occurrence and distribution of extended spectrum β-lactamase and AmpC-β-lactamase-producing Enterobacteriaceae and methicillin resistant *Staphylococcus aureus* in companion animals in New Zealand

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

In

Microbiology

Institute of Veterinary, Animal and Biomedical Sciences

Massey University

Palmerston North, New Zealand

Ali Karkaba

2017

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Health

Antibiotic resistance: World on cusp of 'post-antibiotic era'

By James Gallagher
Health editor, BBC News website

19 November 2015 | Health

What is a superbug?

http://www.bbc.com/news/health-34857015

WEDNESDAY, NOVEMBER 20, 2013 | THE DOMINION POST

NEWS

Science under siege from superbug

SUPERBUGS ON THE RISE

ESBL (extended-spectrum beta-lactamase)

MRSA (methicillin-resistant staphylococcus aureus)

'The problem here is that science is on the edge, we are being challenged with something we just don't know how to prevent if it broke out.'

http://www.stuff.co.nz/national/health/9418833/Science-under-siege-from-superbug

Some Alarming news during my first year in New Zealand

Abstract

The increasing incidence of infections with extended spectrum-lactamase (ESBL)- and AmpC-producing Enterobacteriaceae, and methicillin-resistant *Staphylococcus aureus* (MRSA) in humans in the last decade is a matter of concern. There is a paucity of data on the incidence of infections with these bacteria in animals, partly because veterinary diagnostic laboratories do not routinely test for these organisms in clinical specimens. The carriage rate of these bacteria by companion animals is also unknown.

This PhD project aimed to investigate the occurrence of ESBL/AmpC-producing Enterobacteriaceae and MRSA in clinical specimens from animals in New Zealand, and examine the carriage of multidrug-resistant (MDR), ESBL/AmpC-*E. coli*, and MRSA in cats and dogs in Auckland.

The results of this project indicate that ESBL/AmpC-producing *E. coli* and MRSA cause clinical infections in companion animals in New Zealand. The circulation of these bacteria is likely to be posing therapeutic challenges to unaware veterinarians. The bacteria causing infections or carried by companion animals are genetically similar to those found in humans in New Zealand, raising public health concerns about the role of carrier animals as potential sources of zoonotic infections.

General Introduction and overview of thesis

The relationship between humans and animals have changed, and pets are today considered an integral part of the family. However, this close proximity of pets is considered a potential hazard as a reservoir for antimicrobial resistant bacteria and potential zoonosis (Chomel 2011). Antimicrobial resistance is an emerging global problem, and New Zealand recognizes this threat in line with the rest of the world and is trying to reduce this burden using the 'One Health' approach (Gibbs 2014). This requires leadership and commitment from all sectors, in recognition that antimicrobial resistant bacteria abound in the different ecosystems shared by humans, food animals, and companion animals.

The widespread use and misuse of antimicrobials is considered the main factor driving the increase of antimicrobial resistance and the accelerated evolution and emergence of multi-drug resistant organisms that are difficult-to-treat (Alekshun and Levy 2006; Johns *et al.* 2012; Guo *et al.* 2015). The emergence of resistance to multiple drugs in animals decreases the efficacy of antimicrobials for the treatment of bacterial infections and may increase the rate of zoonotic resistant organisms (Yamamoto *et al.* 2014). *Escherichia coli* and *Staphylococcus aureus* are two of the most commonly isolated antimicrobial resistant bacteria in humans and animals (Yamamoto *et al.* 2014). Several studies have reported similar antimicrobial resistance profiles and genetic makeup in *E. coli* and *S. aureus* isolates recovered from both clinically affected and healthy humans and pets (Johnson *et al.* 2008; Davis *et al.* 2015; Guo *et al.* 2015; Carvalho *et al.* 2016; Loncaric *et al.* 2016).

The acquisition of antimicrobial resistance occurs mainly through mutation at the antimicrobial target sites, or by transfer of resistance genes via mobile elements, such as plasmids. In particular, a number of clinically important antimicrobial resistance mechanisms are mediated by the production of extended spectrum β-lactamases (ESBLs) or AmpC β-lactamases in Gram-negative bacteria, and by target site modification in the Gram-positive species *Staphylococcus aureus* (methicillin resistant *Staphylococcus aureus;* MRSA). Production of β-lactamase enzymes by infecting bacteria is

associated with therapy failure of β-lactam antimicrobials, prolonged and recurrent infections, and sometimes mortality (Hordijk *et al.* 2013). Furthermore, bacteria producing these enzymes are often also resistant to other antimicrobial classes, further complicating treatment (Jacoby 2005; Monecke *et al.* 2011; Bush 2012).

In New Zealand, as elsewhere, the incidence of clinical infections with ESBL and AmpC producing Enterobacteriaceae, in particular *E. coli*, and with MRSA, has increased significantly in humans in the last decade (Dyet *et al.* 2014; Heffernan and Bakker 2016). However, there is a paucity of data on antimicrobial resistant bacteria isolated from animal species, which leads to difficulty in estimating the scale of the problem and its potential impact on animal and human health. Thus, the objective of this PhD project was to study the molecular epidemiology of ESBL/AmpC producing *E. coli* (ESBL/AmpC-E) and MRSA from companion animals in New Zealand.

The thesis starts with an overview of the literature on ESBL and AmpC producing bacteria and MRSA, their resistance mechanisms and the clinical significance of the infections in humans and animals (Chapter 1). This is followed by five observational epidemiological studies of ESBL and AmpC-producing bacteria and MRSA in companion animals (Chapters 2 – 6). The thesis was written in the form of individual publications; thus, some repetitions were inevitable, especially in the introduction sections of the different chapters. A pilot study aimed at selecting the optimal method for the isolation of ESBL/AmpC-E is reported in Appendix 1 and the raw data collected in this project is in the attached CD.

The first observational study (Chapter 2) was performed in co-operation with New Zealand veterinary diagnostic laboratories, with the aims of (1) establishing whether ESBL/AmpC-E are causing clinical infections in companion animals, and (2) genetically characterise the strains. This was

followed with an epidemiological study (

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Name of Candidate: Ali Karkaba

Name/Title of Principal Supervisor: Alex Grinberg

Name of Published Research Output and full reference:

Karkaba, A. Grinberg, J. Benschop, E. Pleydell (2016). Characterisation of extended spectrum β-lactamase and AmpC β-lactamase-producing Enterobacteriaceae isolated from companion animals in New Zealand. New Zealand Veterinary Journal

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Chapter 3) performed in co-operation with veterinary practices in Auckland, to estimate the faecal carriage of these bacteria by cats and dogs (

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Chapter 3). Chapter 4 assessed the risk factors associated with the faecal carriage by cats and dogs. Phenotypic and molecular analysis of the strains

reported in Chapter 2 and

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Chapter 3 inform Chapter 5, which assesses the clonal relatedness of ESBL/AmpC-E across faecal carriage and disease.

The last study (Chapter 6) characterised MRSA identified from clinical infections in animals in New Zealand, and estimated the prevalence of MRSA carriage in dogs and cats in Auckland.

All the studies in this thesis were conducted between June 2012 and June 2013. Chapter 2 and Chapter 6 have been published in condensed form in refereed journals and were also presented at conferences.

Declaration

This thesis contains no material that has been accepted for another degree or diploma at Massey University or any other institution. To the best of my knowledge, no material previously published or written by another person has been used, except where due acknowledgment has been made in text.

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Gratitude perpetuates blessings. Words are not enough to show my gratitude for a lot of people that helped me during this journey. I would like to thank my main supervisor Alex Grinberg for his mentoring, patience, encouragement, endless hours of meetings, supervision and support. Besides my main supervisor, I would like to thank the PhD co-supervisors, Eve Pleydell, that had a major role at the beginning of this PhD studies and left for another job; Kate Hills' role at the beginning of the studies that also left; lastly but not least, Jackie Benschop that took the burden of the co-supervision, and the important role in mentoring, guidance and support during the PhD studies.

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Heartfelt thanks to my family that without them I wouldn't be here.

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List of Abbreviations

E. coli	*Escherichia coli*
ESBL	Extended spectrum β-lactamase
AmpC	AmpC β-lactamase
ESBL-E	Extended spectrum β-lactamase producing *E. coli*
AmpC-E	AmpC β-lactamase producing *E. coli*
ESBL/AmpC-E	ESBL-E and/or AmpC-E
MLST	Multilocus sequence typing
ST	Sequence type
MALDI-TOF	Matrix assisted laser desorption/ionization time of flight
AMR	Antimicrobial resistant
MDR	Multi-drug resistant
ExPEC	Extra-intestinal pathogenic *E. coli*
HGT	Horizontal gene transfer
S. aureus	*Staphylococcus aureus*
MRSA	Methicillin resistant *Staphylococcus aureus*
SCC*mec*	Staphylococcal cassette chromosome

List of publications

Chapter 2: Karkaba, A. Grinberg, K. E. Hill, J. Benschop (2017). Characterisation of methicillin resistant *Staphylococcus aureus* clinical isolates from animals in New Zealand, 2012–2013, and subclinical colonisation in dogs and cats in Auckland. *New Zealand Veterinary Journal.*

Chapter 6: Karkaba, A. Grinberg, J. Benschop, E. Pleydell (2017). Characterisation of extended spectrum β-lactamase and AmpC β-lactamase-producing Enterobacteriaceae isolated from companion animals in New Zealand. *New Zealand Veterinary Journal*.

A. Karkaba, K. Hill, J. Benschop, A. Grinberg and E. Pleydell (2013). Investigating strains of multidrug resistant Enterobacteriaceae and *Staphylococcus aureus* that are causing clinical infections in companion animals in New Zealand. *Proceedings of the New Zealand Veterinary Association Annual Conference.*

Oral conference presentations:

A. Karkaba, K. Hill, J. Benschop, A. Grinberg and E. Pleydell (2013). Investigating the strains of multidrug resistant Enterobacteriaceae and *Staphylococcus aureus* that are causing clinical infections in companion animals in New Zealand. *Proceedings of 38th Annual WSAVA Congress.*

Karkaba, A., Hill, K., Benschop, J., Grinberg, A. and Pleydell, E. (2013). Comparison between five methods for isolation of multi drug-resistant *Escherichia coli* from faeces of hospitalized and non-hospitalized cats and dogs *Queenstown Molecular Biology Week and Webster Centre for Infectious Diseases Symposium: Of Microbes and Men – Translational Medical Microbiology in the 21st Century*. 29 – 30 August, 2013 Rydges Hotel, Queenstown, New Zealand

A. Karkaba, K. Hill, J. Benschop, A. Grinberg and E. Pleydell (2012). Investigating the strains of multidrug resistant Enterobacteriaceae causing clinical infections in companion animals in New Zealand. *Proceedings of First IDReC Science Symposium.*

Chapter 1: Infections with extended-spectrum β-lactamase and AmpC β-lactamase-producing Enterobacteriaceae and methicillin resistant *Staphylococcus aureus* in companion animals – an overview

Introduction

Enterobacteriaceae, a family of Gram-negative rods, and *Staphylococcus*, a genus of Gram-positive cocci, are ubiquitous colonisers of humans and companion animals, including cats and dogs (Wieler *et al.* 2011). These organisms are also commonly identified from a broad range of infections in companion animals, including urinary tract infections (UTIs), gastrointestinal, skin and soft tissue, respiratory infection and bacteraemia, and such infections can lead to death (Moor *et al.* 2008; Pitout and Laupland 2008). The Enterobacteriaceae family, particularly the species *Escherichia coli* and *Klebsiella pneumonia*, and members of genus *Staphylococcus*, notably the species *Staphylococcus aureus*, are also a serious cause of hospital-acquired and community-onset infections in humans, and the increasing resistance of these bacteria to antimicrobials is a global problem (Thaden *et al.* 2015; van Hoek *et al.* 2015; Chang *et al.* 2016). This PhD project deals with the epidemiology of β-lactamase-resistant Enterobacteriaceae and *S. aureus* infecting companion animals in New Zealand.

The β-lactam antimicrobials are the most commonly prescribed antimicrobials for the treatment of bacterial infections in animals in New Zealand (Pleydell *et al.* 2012). This is mainly due to the wide range of compounds available and their broad antimicrobial spectrum, high safety margin, optimal pharmacokinetic properties and low cost. According to the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) reports, β-lactam antibiotics are the most utilised antimicrobial drugs in food animals also in Denmark (DANMAP 2005, 2010). In New Zealand, amoxicillin-clavulanic acid, a broad spectrum β-lactam drug

potentiated with potassium clavulanate, followed by cephalexin (1st generation cephalosporin), are the most common systemic drugs used by veterinarians for the treatment of skin and soft tissue infections and urinary tract infections (UTI) in cats and dogs (Pleydell *et al.* 2012). However, the total sales of 3rd generation cephalosporins for companion animals have increased during the period 2009-2011. In particular, the sales of cefovecin and cefpodoxime) have increased by 26% and 100%, respectively (Morris 2014).

Due to the importance of β-lactams, clinical infections with bacteria resistant to these antimicrobials, of the note methicillin resistant *Staphylococcus aureus* (MRSA) and extended spectrum β-lactamase producing Enterobacteriaceae, have a severe impact on human and veterinary medicine. Furthermore, the incidence of infections with these organisms in companion animals is not known.

This Chapter provides an overview of bacterial β-lactam resistance and of the current knowledge on the epidemiology of infections with MRSA and ESBL and AmpC-producing *E. coli* (ESBL/AmpC-E) in companion animals.

1.1 Enterobacteriaceae and *S. aureus* resistance to β-lactams-brief overview

β-lactam antimicrobials interrupt cell wall formation of Gram-negative and Gram-positive bacteria by covalently binding to enzymes involved in the terminal peptidoglycan cross-linking, known as penicillin binding proteins (PBPs) (Bush and Bradford 2016). Resistance to β-lactams is often determined by the enzymatic degradation of the drug, or an alteration of the target site, or porin deficiency. Resistance to β-lactams was first reported in *E. coli* in 1939, then in a *S. aureus* isolate in 1943 (Jevons 1961).

In Enterobacteriaceae, the main mechanism of resistance to β-lactams is enzymatic degradation, by the production of β-lactamases that inactivate the β-lactam ring (Bush and Bradford 2016). To date, more than 1300 β-lactamases have been identified and new enzymes are continually being discovered, worldwide (Livermore *et al.* 2007; Woodford *et al.* 2011; Bush 2013a). The β-lactamases are classified according to their molecular

characteristics (the so-called Ambler classification) (Ambler 1980), or functional properties (the Bush classification) (Bush *et al.* 1995). The most widely used scheme is the Ambler classification, which divides the β-lactamases into four classes: A, B, C, and D. Classes A, C, and D have a serine molecule at their active site, while class B, known as metallo β-lactamases, have an iron molecule at the active site structure. This chapter will focus on classes A (ESBL) and C (AmpC), which are the most common enzymes identified from bacterial Gram-negative human and animal infections, globally (Bush 2013a).

In *S. aureus*, the most common resistance mechanisms to β-lactams are mediated by β-lactamase production that degrades the β-lactam antimicrobial, or by binding and inactivation of penicillin binding proteins, which decreases the affinity of the β-lactams to the bacterial cell wall (Hartman and Tomasz 1984; Lyon and Skurray 1987). In addition, *S. aureus* can acquire resistance to almost every antimicrobial through horizontal gene transfer (Noble *et al.* 1992). Perhaps the most prominent example of an acquisition or resistance through horizontal gene transfer is the emergence of the methicillin resistant *Staphylococcus aureus* (MRSA) strains, which are resistant to most β-lactam drugs and may also carry resistance genes to other antimicrobial classes. MRSA cause nosocomial and community-onset infections in humans (Schulte *et al.* 2013), and are increasingly found also in animals. The common classification method for MRSA is based on the sequence of the staphylococcal cassette chromosome (SCC*mec*), the accessory gene content and the multi locus sequence type (ST) of the isolate, complemented by the sequence of the variable repeat region of the protein A gene (*spa* typing) and genes associated with the clonal complex (CC), such as *agr* (Ito *et al.* 2001; Monecke *et al.* 2011; Ito *et al.* 2014).

1.1.1 Extended spectrum β-lactamase-producing Enterobacteriaceae

The extensive use of β-lactams in general is believed to be the main force driving the evolution of new extended-spectrum cephalosporin-resistant phenotypes in Enterobacteriaceae, the ESBLs (Ghuysen 1991; Bush 2013b). ESBLs include all Ambler Class A β-lactamases with an acquired resistance

to oxyimino-cephalosporins (except carbapenems) such as CTX-M β-lactamases. These enzymes are inhibited by β-lactamase inhibitors as tazobactam and clavulanic acid (Livermore 2008). The main mode of acquisition of β-lactamases by Enterobacteriaceae is through the transfer of genes carried on plasmids.

In the 1960's, the first report of a plasmid-mediated β-lactamase with broad spectrum resistance to penicillins and first-generation cephalosporins was described in an *E. coli* strain isolated from a blood culture in Greece, and the enzyme was named Temoniera (referred to as TEM-1), after the city of its discovery (Datta and Kontomichalou 1965; Medeiros 1984). The isolate exhibited resistance to penicillins and first-generation cephalosporins. Because the TEM-type β-lactamase is plasmid and transposon-mediated, it can disseminate through horizontal gene transfer between bacterial species. In fact, the enzyme was recognised worldwide within a few years of its discovery. Mutants of the TEM-type β-lactamase (TEM-1, 2, and 13) with extended spectrum of activity were discovered in the 1980s. Another β-lactamase enzyme was discovered during the 1970s, the SHV-type (Sulfhydryl reagent variable) β-lactamase, and was first identified in an outbreak caused by β-lactam resistant *Klebsiella spp.* (Matthew *et al.* 1979). The gene encoding the SHV-type variant 1 (or SHV-1) conferred resistance to penicillins and first-generation cephalosporins.

After a series of mutations in the chromosomes of different bacterial species, through the intervention of bacteriophages and transposons, different ESBL genes were transferred onto plasmids. Resistance to β-lactam antibiotics shifted from broad spectrum (BSBL- broad-spectrum β-lactamase), constituting the classic TEM and SHV variants, to extended-spectrum resistance (ESBL - extended spectrum β-lactamase), constituting mutated TEM and SHV variants, with a much broader spectrum of activity against β-lactams, to include 2nd, 3rd and often 4th generation cephalosporins (Philippon *et al.* 2002). The first plasmid encoded resistance to an extended spectrum of cephalosporins was described in a *Klebsiella ozaenae* isolate with a mutant SHV variant early in 1985 (Kliebe *et al.* 1985b). By definition, ESBL-producing bacteria are resistant to β-lactam antimicrobials (penicillins,

1st, 2nd, 3rd and often 4th generation cephalosporins, and monobactams), but not to cephamycins (Winokur *et al.* 2001b; Bush and Jacoby 2010). To date, hundreds of ESBL enzymes have been identified in Enterobacteriaceae, and a complicated and still evolving nomenclature is used for their classification (Bush and Jacoby 2010; Bush and Bradford 2016). To the day of the writing of this chapter, 223 TEM variants and 193 SHV variants were reported in a β-lactamase online database (last accessed 28 of September 2016; https://www.lahey.org/studies/).

In the early 2000s, a major change of resistance emerged, with plasmid-mediated CTX-M-type β-lactamase ESBL emerging and becoming the dominant ESBL enzyme in clinical Enterobacteriaceae isolates, worldwide. Unlike TEM and SHV variants that emerged from a series of chromosomal mutations of the existing enzymes, CTX-M were acquired by horizontal gene transfer from *Kluyvera* species. The activity of the enzyme CTX-M against 3rd generation cephalosporin cefotaxime and ceftazidime derived the CTX abbreviation, and the M indicates the area of isolation, Munich, Germany. To date, more than 172 CTX-M β-lactamases, clustered into five groups (1, 2, 8, 9, 25) based on their founders' chronological order of identification, have been described (Bush 2013b).

ESBL-producing bacteria were initially identified as human nosocomial pathogens (Decristophoris *et al.* 2013a). However, in the last two decades, ESBL-producing bacteria emerged also in the community, and became the main cause of urinary tract infections (UTI) in humans (Pitout *et al.* 2005). The CTX-M ESBL-producing *E. coli,* now widely considered nosocomial, were introduced into health care facilities from the community (Baquero 2004). Since the year 2000, the rate of identification of ESBL producing *E. coli* (ESBL-E) in human clinical specimens, in particular carrying the CTX-M-14 and CTX-M-15 ESBLs increased dramatically (Bonnet 2004; Pitout and Laupland 2008). These bacteria have become an important cause of urinary tract and blood stream infections in many developed countries. The high prevalence of CTX-M variants is associated with the dissemination of a small group of STs, such as the pandemic ST131 and other common STs as

ST38, ST405 and ST648 (Croxall *et al.* 2011; Adams-Sapper *et al.* 2013; Riley 2014).

Bacteria harbouring ESBL genes often also carry genes encoding resistance to other antimicrobial classes, such as fluoroquinolones and aminoglycosides, and many are considered "multidrug resistant" (i.e., resistant to at least one antimicrobial agent of at least three antimicrobial classes; (Pitout 2010; Magiorakos *et al.* 2012). Infections with multidrug-resistant bacteria carrying ESBL genes are less likely to respond to first-line treatment drugs selected empirically by clinicians, than non-MDR/ESBL strain-types, resulting in prolonged infections, higher mortality rates and increased medical costs (Moor *et al.* 2008).

There are several reports of ESBL-E in clinical specimens from humans in New Zealand (Freeman *et al.* 2008; Moor *et al.* 2008; Freeman *et al.* 2012). Reports indicate an increasing incidence of infections with such bacteria, in particular urinary tract infections (UTI) (Heffernan and Woodhouse 2012; Dyet *et al.* 2014; Heffernan *et al.* 2014b). The most common ESBL genes found in New Zealand *E. coli* clinical isolates are $bla_{\text{CTX-M14}}$ and $bla_{\text{CTX-M15}}$ (Heffernan *et al.* 2014b). Moreover, the geographical distribution of infections with ESBL-producing Enterobacteriaceae is peculiar, with highest annualised incidence rates seen in Manukau, Waitemata, Auckland, and Northland counties (110-230 cases per 100,000 population), whilst the rates reported in other District Health Boards fall below the national average rate of 95.5 per 100,000 population (Heffernan *et al.* 2014b).

In animals, the first ESBL-producing Enterobacteriaceae was isolated in 1988, in the intestinal content of a laboratory dog harbouring a CTX-M ESBL-E (Matsumoto *et al.* 1988). Reports of infections with ESBL-producing Enterobacteriaceae in animals in New Zealand are lacking.

1.1.2 AmpC β-lactamase-producing Enterobacteriaceae

AmpC β-lactamases belong to Class C β-lactamases, and have the largest serine active site (*n*=360 amino acids) in comparison with classes A and D (<310 amino acids) (Jacoby 2009). The first report of an AmpC-β-lactamase dates back to 1940, when an *E. coli* producing a penicillinase was

discovered (Abraham and Chain 1940). AmpC enzymes have a wider spectrum of activity against β-lactam antimicrobials, in particular cephalosporins and cephamycins, than ESBLs. The range of activity against β-lactams include penicillins, cephalosporins (1st, 2nd, 3rd and often 4th generation), cephamycins (cefoxitin and cefotetan) and monobactams (Jacoby 2009). The AmpC β-lactamases are inhibited by cloxacillin, oxacillin and aztreonam but not by β-lactamase inhibitors as clavulanate (Jacoby 2009). In some cases, the combination of AmpC production and bacterial porin deficiency can lead to carbapenem resistance (Jacoby 2009).

Many Enterobacteriaceae species are natural producers of AmpC enzymes, and carry the coding genes in the chromosome, but differ in the level of expression of the gene, ranging from low level producers (e.g. *E. coli*, *Shigella* spp) to inducible hyper-producers (e.g. *Citrobacter freundii*, *Enterobacter cloacae* and *Morganella morganii*) (Jacoby 2009). The inducible production is complex and can be due to the exposure to a β-lactam antimicrobial (e.g. cefoxitin, clavulanate, penicillins and cephalosporins), or a mutation in the genes regulating the expression of the enzyme. Enterobacteriaceae with inducible resistance may not be detected if improperly tested *in vitro*, and this may cause therapeutic failure. When AmpC production is induced, broad spectrum resistance is conferred to 3rd and 4th generation cephalosporins, cephamycins and monobactams. Other Enterobacteriaceae lack a chromosomal AmpC gene (e.g. *Klebsiella pneumonia*, *Salmonella* spp) (Jacoby 2009).

A series of mutations led to the mobilisation of AmpC genes to plasmids, allowing it to spread among members of the Enterobacteriaceae family. Unlike chromosomal inducible AmpC producers, the plasmid-mediated AmpC genes are non-inducible, which facilitates *in vitro* detection through antimicrobial susceptibility testing. Plasmid-mediated AmpC (PAmpC) genes were firstly described in 1989, and since then they have been reported all over the world in community-onset and nosocomial isolates (Bush 2013b). To date, more than 114 PAmpC enzymes have been identified, and are categorised into groups based on minor differences in the amino acid sequences (CMY, MOX, FOX, MIR, ACT, and ACC), of which 95 belong to

the CMY enzymes (Bush 2013b). The predominant PAmpC enzyme in Enterobacteriaceae, worldwide, is the CMY-2 (Bush 2012).

In New Zealand, the results of a recent study carried out in Auckland and surrounding Health Boards indicate that infections with plasmid-mediated AmpC ß-lactamase producing Enterobacteriaceae are common in community-based patients, and the most common PAmpC gene in *E. coli* is CMY-2 (Heffernan *et al.* 2007; Drinkovic *et al.* 2015). However, several other PAmpC genes were also identified of which two novel CMY-29 and CMY-30 were first reported in New Zealand (Pope *et al.* 2009).

1.1.3 Methicillin resistant *Staphylococcus aureus* (MRSA)

1.1.3.1 *Staphylococcus aureus resistance to ß-lactams*

Staphylococcus aureus is a major human and animal pathogen and a leading cause of a broad range of skin and internal organ infections such as pneumonia, endocarditis, bacteraemia, mastitis, phlebitis, urinary tract infections and toxic shock syndrome (Lowy 1998; Baptiste *et al.* 2005; Voss *et al.* 2005; Weese *et al.* 2005; Vitale *et al.* 2006). Initially, resistance to ß-lactams was mediated by the production of staphylococcal ß-lactamases. However, about a decade after the introduction of methicillin for the treatment of penicillin-resistant *S. aureus* (ß-lactamase-producers) in human medicine in the 1950's, the first report of methicillin resistant *Staphylococcus aureus* (MRSA) strain was published in the UK (Jevons 1961). MRSA are resistant to almost all the β-lactam drugs and - as ESBL-E and AmpC-E - may also carry resistance genes to other antimicrobial classes, and be multi-drug resistant. MRSA cause nosocomial and community infections in humans (Schulte *et al.* 2013), and are increasingly found also in animals (Baptiste *et al.* 2005; Weese *et al.* 2005; Grinberg *et al.* 2008; Feingold *et al.* 2012; Couto *et al.* 2015).

Over the last two decades there has been a shift in the epidemiology of human *S. aureus* infections, worldwide, and a substantial increase in infection incidence (Tong *et al.* 2015a). Today, *S. aureus* infections are considered a significant healthcare problem (Monecke *et al.* 2011; Tong *et al.* 2015a). In parallel to the increase in incidence, in some countries there

has been an increase in the relative contribution of MRSA. According to the US Centre for Disease Control, in the USA the prevalence of MRSA increased from 2% of total *S. aureus* isolated from infection sites in 1974, to more than 60% in 2004 (Solomon 2003; NNIS 2004). It has been estimated that the *S. aureus* anterior nasal carriage rate in healthy humans is 30%, and the MRSA carriage is ~1% (Mainous *et al.* 2006; Best *et al.* 2011; Tong *et al.* 2015b). In humans, MRSA infections are of great concern as they render the use of most β-lactam antimicrobials ineffectual (Schulte *et al*. 2013).

MRSA are resistant to the β-lactam antimicrobials due to the expression of a cell-wall low-affinity penicillin-binding protein (known as PBP-2a) coded by the *mecA* gene (Ito *et al.* 2001). Horizontal gene transfer mechanisms are considered the main means of dissemination of virulence determinants and antimicrobial resistance genes within and between *S. aureus* clones (Noble *et al.* 1992). The integration, through horizontal gene transfer, of a staphylococcal cassette chromosome *mec* (SCC*mec*) spanning a *mecA* gene, into the chromosome of a methicillin-sensitive *S. aureus* (MSSA) transforms it into an MRSA. Conversely, its excision from the chromosome transforms MRSA into MSSA (Ito *et al.* 1999; Ito *et al.* 2001).

The regulation of the *mec*A gene complex is mediated by the regulatory proteins *mecI* (repressor gene), *mecR1* (signal transducer) and *IS431* (insertion sequence). *MecI* represses the transcription of *mecA* and *mecRI-mecI* genes in the absence of β-lactam antibiotics. The β-lactam antibiotic induces the cleavage of *mecRI* auto-catalytically, that leads to a metallo-protease activation (metallo-protease is positioned in the cytoplasmic area of *mecRI*). The *mecI* leads to the transcription of *mecA* gene, resulting in cleavage of the new penicillin binding protein or PBP-2 from the operator region of *mecA* by the metallo-protease. The insertion sequence *IS431* plays a role in the depression of *mecA* gene which truncates *mecI* and *mecRI*. The *orfX* is responsible for the integration and excision of *mecA* from the chromosome, whereas the *ccr* genes (cassette chromosome recombinases) truncates specific and precise excision and integration sites of SCC*mec* elements (Deurenberg and Stobberingh 2008; Ito *et al.* 2014). To date, at least 11 SCC*mec* types have been identified (Ito *et al.* 2014).

Primary detection of MRSA isolates in veterinary diagnostic laboratories can be done using the disk diffusion test using oxacillin or cefoxitin disks, and confirmation can be performed by means of PCR of the *mecA* gene (Ito *et al.* 2001; Pinho *et al.* 2001).

1.1.3.2 Epidemiology of MRSA infections in humans

Although the first case of an MRSA infection was reported in 1961 (Jevons 1961), infections with MRSA started to be reported at increasing frequency in healthcare facilities and nursing homes in the early 1970's in the UK, in particular in immune-compromised patients. Initially, MRSA infections were considered nosocomial and were designated "hospital-acquired" MRSA (HA-MRSA) (Haley *et al.* 1982). HA-MRSA infections were then reported from numerous other countries (Deurenberg *et al.* 2007; Boucher and Corey 2008). Before the PCR era, these 'hospital strains' were usually characterised by means of phenotypic methods such as antibiogram and phage-typing (Pantosti 2012). Characteristically, the HA-MRSA strain-types were multidrug resistant, expressing resistance to several antimicrobial classes, as fluoroquinolones and aminoglycosides (Deurenberg *et al.* 2007). Subsequently, the use of PCR accelerated molecular analyses, which showed HA-MRSA strain-types carried complex mobile genetic elements 'staphylococcal cassette chromosome SCC*mec* elements' of types I, II, and III (Deurenberg *et al.* 2007; Monecke *et al.* 2011). Besides being resistant to the β-lactams, HA-MRSA strain-types carrying SCC*mec* types II and III were often resistant also to ciprofloxacin, rifampicin and clindamycin (Deurenberg *et al.* 2007).

After decades of detection of MRSA infections mainly in healthcare facilities, the incidence of MRSA infections increased also in the community, and new strain-types emerged in community settings in the 1990's (the so-called "community-acquired" MRSA, or CA-MRSA). Unlike HA-MRSA, CA-MRSA usually carried the SCC*mec* elements IV and V (Deurenberg *et al.* 2007; Monecke *et al.* 2011) and did not display multi-resistance, but tended to carry more virulence determinants, such as the gene coding the Panton-Valentine Leukocidin (PVL) (Deurenberg *et al.* 2007; Monecke *et al.* 2011). Furthermore, CA-MRSA strain-types were found colonising sub-clinically

healthy people, and were a public health concern since they tended to spread person-to-person (Boucher and Corey 2008). In the last decade, however, the differentiation between CA-MRSA and HA-MRSA has become fuzzy, with strains from the community being isolated in hospitals, and vice versa (Monecke *et al.* 2011; Hetem *et al.* 2015).

MRSA also emerged in animal populations. Since 2003, MRSA carrying SCC*mec* elements IV or V have spread in livestock and were labelled as 'livestock-associated' MRSA (LA-MRSA). The first report of a LA-MRSA was from a surgical wound of a Dutch girl living close to a swine farm site, and subsequently from the nasal site of farmed pigs (Voss *et al.* 2005). In subsequent years the same strain-type, now known as the 'CC398 LA-MRSA' (LA for livestock-associated), became the predominant strain-type isolated from farm animals, especially pigs, in a number of European countries (Huber *et al.* 2010).

In 2007, during an epidemiological study of bovine mastitis in the UK, an MRSA isolate that was resistant to oxacillin and cefoxitin was phenotypically identified from a milk bulk tank. This represented a significant finding, as it was the first detection of an MRSA in the UK from dairy. The molecular DNA analysis showed PBP2a negative results, but whole genome sequencing revealed a new type of MRSA, with a mec gene type '*mec*C', that had 69% sequence homology with *mec*A and only 63% homology at the amino acid level (García-Álvarez *et al.* 2011). A retrospective analysis of archived isolates identified from cattle in the UK and Denmark identified 65 *mec*C-positive isolates, one of which dated back to 1975 (from Denmark). The *mec*C determinant has also been identified in Irish patients with SCC*mec* type XI in 2010, in strains belonging to Clonal Complex 130 (CC130).

A new potential non-*mec*A mechanism of resistance to the 5th generation cephalosporins has been recently described (Greninger *et al.* 2016). Mutations were detected upstream of the PBP4 gene that codes for the PBP4 protein in the genomes of five isolates that displayed resistance to the 5th generation cephalosporins ceftaroline and ceftobiprole, two promising recently introduced antimicrobials used for the treatment of MRSA. Other mutations were also detected in PPC2 protein phosphatase, *Rho*, and *Clpx*

endopeptidase. Although not yet confirmed in the clinical setting, these findings may suggest the emergence of new, non-*mec*A-induced β-lactam resistance mechanisms in *S. aureus.*

The increasing incidence of MRSA infections has become an international problem. The distribution of the different MRSA strain-types is diverse. Some strain types are geographically restricted while others achieved global spread and became pandemic. Some clonal complexes (CC) are present globally, such as the pandemic CC 1, 5, 8, 22 and 30 (Monecke *et al.* 2011). In New Zealand, the incidence of *S. aureus* infections is among the highest in developed countries, mostly due to skin and soft tissue infections (O'Sullivan *et al.* 2011; Ritchie *et al.* 2011; Williamson *et al.* 2014). However, most infections are caused by MSSA. Yet, the incidence of reported MRSA infections in New Zealand has also steadily increased from 1.1 to 23.8 cases per 100,000 population between 1994 and 2014 (Adhikari *et al.* 2002; Heffernan *et al.* 2014a). The predominant MRSA strain-types have changed over time, from the Western-Samoan phage pattern (WSPP, ST30 SCC*mec* type IV) in the 1990s, to the EMRSA-15 (ST22 SCC*mec* type IV) in 2000-2008, and in the last 5 years the AK3 (ST5 SCC*mec* type IV) (Heffernan *et al.* 2014a). The *S. aureus* nasal carriage rate was reported to be 18%, and the MRSA carriage rate was less than 1% (Best *et al.* 2011).

1.2 Epidemiology of infections with ESBL/AmpC-producing Enterobacteriaceae and MRSA in companion animals - general considerations

Infections with ESBL/AmpC-producing Enterobacteriaceae and MRSA are challenging as they render ineffectual use of most β-lactams. ESBL/AmpC producing Enterobacteriaceae and MRSA have been reported overseas from clinical infections and faecal carriage in companion animals. Although there is an apparent increase in the reports of these infections in companion animals (Ewers *et al.* 2010; Woodford *et al.* 2011; Garbacz *et al.* 2013; Couto *et al.* 2015), our understanding of the epidemiology of these infections is limited. Few studies assessed the prevalence of colonisation with ESBL/AmpC-producing Enterobacteriaceae and MRSA in animals, and even fewer characterised the isolates genotypically. Furthermore, studies that

characterised ESBL/AmpC producing Enterobacteriaceae and MRSA are geographically limited, and the level of colonisation with these bacteria in companion animals is poorly known.

1.2.1 Studies of ESBL/AmpC-producing Enterobacteriaceae in companion animals

Most reports describing ESBL/AmpC producing Enterobacteriaceae in companion animals dealt with *E. coli*, and reports on other Enterobacteriaceae species including, *Klebsiella pneumoniae*, *Enterobacter* spp*, Salmonella enterica* spp, *Citrobacter* spp are less common (Rubin and Pitout 2014). It is unclear whether the relatively large number of publications describing *E. coli* reflects the true burden of ESBL/AmpC-E, or a bias due to the ease of isolation and identification of *E. coli,* or a general perception that *E. coli* is more pathogenic. Another important consideration is the relatively small sample size of most studies, with the largest study assessing the prevalence of ESBL/AmpC-E faecal carriage in cats and dogs reporting sample sizes of approximately 250 animals (Albrechtova *et al.* 2012; Baede *et al.* 2015).

Studies assessing ESBL/AmpC-E faecal carriage rates in healthy cats and dogs yielded variable results. The dominant ESBL enzymes identified in these studies were CTX-M-15 (Kenya, Mexico and Algeria; Albrechtova *et al*. 2012; Rocha-Gracia *et al.* 2015; Yousfi *et al*. 2016), CTX-M-1 (Portugal, Tunisia and United Kingdom; Costa *et al*. 2008; Sallem *et al*. 2013; Schmidt *et al*. 2015), CTX-M-32 (Portugal; Belas *et al*. 2014), TEM-52B (Portugal; Costa *et al*. 2004), and SHV-12 (Japan; Harada *et al*. 2011). Other studies identified also AmpC enzymes, predominantly the CMY-2 (United Kingdom, Portugal, Tunisia, and Canada; Murphy *et al*. 2009; Wedley *et al*. 2011; Sallem *et al*. 2013; Belas *et al*. 2014; Rocha-Gracia *et al.* 2015; Schmidt *et al*. 2015), and the DHA-1 in one study (Belas *et al.* 2014). The ESBL/AmpC-E faecal carriage rate in healthy cats and dogs varied considerably between these studies. However, in addition to a true geographical difference, other factors may have contributed to this variability, such as the different culture media and sampling strategies. The risks associated with carriage of

ESBL/AmpC producing bacteria by companion animals include transmission to humans, between animals, and the risk of autoinfection.

Studies assessing ESBL/AmpC-E faecal carriage in hospitalised or sick cats and dogs have also been conducted, with variable results. For instance, the prevalence in hospitalised dogs in a South Korean study was 33% of carriage of ESBL-E carrying the CTX-M-14, and 24% of AmpC-E carrying the CMY-2 (So *et al.* 2012). In a Swedish study, 8% of the faecal samples of dogs treated with antimicrobials in surgical wards were AmpC-E positive, carrying the CMY-2 (Johard *et al.* 2015). A Dutch study found a high prevalence of ESBL and AmpC-producing Enterobacteriaceae among healthy and diarrheic cats and dogs, of 45% and 55%, respectively. The enzymes carried were mainly CTX-M-1 and CMY-2 (Hordijk *et al.* 2013). Lastly, a study in Denmark found that 8% of the dogs admitted to the hospital due to an infection were colonised with *E. coli* carrying CTX-M-1 (4%) and CMY-2 (4%) (Espinosa-Gongora *et al.* 2015). Due to the small number of studies in comparison with human studies, more research is needed to better understand the patterns of faecal carriage of ESBL/AmpC-producing Enterobacteriaceae in companion animals. Factors positively associated with the faecal carriage of ESBL/AmpC-E were previous antimicrobial treatment (Gibson *et al.* 2011; Decristophoris *et al.* 2013a; Belas *et al.* 2014; Baede *et al.* 2015; Johard *et al.* 2015), the animal residing in a breading shelter (Belas *et al.* 2014), previous hospitalisation (Gibson *et al.* 2011; Johard *et al.* 2015), and consumption of raw meat (Baede *et al.* 2015; Schmidt *et al.* 2015).

Few studies analysed ESBL/AmpC-producing Enterobacteriaceae isolates obtained from clinical infections in companion animals. As in human studies (see Section 2.2), there was variability in the ESBL/AmpC enzymes identified between the studies. The first report of an isolation of a clinical ESBL-producing Enterobacteriaceae in companion animals was from a recurrent urinary tract infection in a dog in Spain in 1998, and it was an *E. coli* carrying the SHV-12 enzyme (Teshager *et al.* 2000). Since 2000, the identification of CTX-M β-lactamases in human clinical isolates increased dramatically (Bonnet 2004; Pitout and Laupland 2008), and it has been hypothesised in a

number of papers (mentioned below), that the same ESBL/AmpC enzymes may be circulating among Enterobacteriaceae infecting companion animals (Carattoli *et al.* 2005; Li *et al.* 2007; Poeta *et al.* 2008; Bonnedahl *et al.* 2009; Guenther *et al.* 2010; Ben Said *et al.* 2016). As a matter of fact, a paper describing a study conducted between 2001 and 2003 in Italy reported the ESBL/AmpC enzymes SHV-12, CMY-2 and CTX-M-1 in *E. coli* isolates from clinical infections in cats and dogs (Carattoli *et al.* 2005). Since then, there has been an increasing number of reports describing ESBL/AmpC-producing Enterobacteriaceae in companion animals, food-producing animals, birds, wild animals, rodents, and water (Li *et al.* 2007; Poeta *et al.* 2008; Bonnedahl *et al.* 2009; Guenther *et al.* 2010; Ben Said *et al.* 2016).

ESBL/AmpC-producing Enterobacteriaceae isolates from cats and dogs were identified from various infection sites, with predominance of *E. coli* isolates from UTIs (Carattoli *et al.* 2005; O'Keefe *et al.* 2010; Schink *et al.* 2011; Shaheen *et al.* 2011; Huber *et al.* 2013; Liu *et al.* 2016). Due to the lack of proper surveillance programs, there is no substantial evidence of an increase in the incidence of such infections in companion animals during the last decade. The prevalence of ESBL/AmpC producing Enterobacteriaceae among all the Enterobacteriaceae isolated from infection sites in dogs and cats ranged between 0.8 and 7.3% in the above mentioned studies. The predominant ESBL enzymes identified in *E. coli* in companion animals were CTX-M-1,14,15, TEM-52, and SHV-12, while the predominant AmpC enzyme in *E. coli* was the CMY-2 (Ewers *et al.* 2012; Rubin and Pitout 2014).

An early study in Italy, by Carattoli *et al.* (2005), analysed 238 clinical *E. coli* isolates isolated between 2001 and 2013, and found that the predominant ESBL enzymes were SHV-12, CMY-2 and CTX-M-1, and the prevalence of ESBL/AmpC-E was 4.4%. Conversely, in one of the early studies in Germany that analysed 228 clinical *E. coli* isolates from dogs collected during 2004-2006 from various infection sites, only two ESBL/AmpC-E isolates (0.8%) were identified (from a UTI and pneumonia); the enzymes were CTX-M-15 and CTX-M-1, respectively (Schink *et al.* 2011). A Swiss study analysed 107 uropathogenic *E. coli* isolates and found that 3.3% of cats and 5% of dogs were infected with CTX-M-15 ESBL (Huber *et al.* 2013).

In another study by O'Keefe *et al.* (2010), isolated ESBL-E from UTI carrying SHV-12 and CTX-M-14, and CTX-M-15 enzymes for the first time in the United states that corresponded to 7.3% (n=11) of the 150 tested *E. coli* from cats and dogs. However, large-scale studies are few. For instance, in the Netherlands a study conducted between 2007 and 2009 collected 2700 clinical Enterobacteriaceae isolates from companion animals from various infection sites, with predominance of *E. coli* from UTI sources, and found that 2.4% of the isolates (4% from cats and 3% from dogs) were ESBL/AmpC producers. The most frequent enzymes were CTX-M 1,2, 9,14,15, TEM-52, and CMY-2 (Dierikx *et al.* 2012). A recent large-scale study in the United States collected 2443 *E. coli* isolates between 2009 and 2013 from cats and dogs from various infection sites, and found that 3.8% of the isolates were ESBL/AmpC-E carrying the CTX-M-1,14, 15 and CMY-2 enzymes (Liu *et al.* 2016).

Few studies have described the clonal relatedness and STs of the ESBL/AmpC-E in cats and dogs. The STs identified varied between studies from different countries, but in general, and as in humans there is a small number of 'universal' STs, and these are: ST131, ST38, ST10, ST405 and ST648 (Albrechtova *et al.* 2012; Tamang *et al.* 2012; Liu *et al.* 2016). Among other Enterobacteriaceae species, the number of studies that reported the STs of the ESBL/AmpC producers in companion animals is even smaller, such as studies from France and Germany identified *K. pneumoniae* CTX-M-15 ST15 from cats and dogs (Stolle *et al.* 2013).

Multidrug-resistant bacteria are also isolated from companion animals in New Zealand. A 2008 master's thesis at Massey University showed that bacteria isolated from clinically overt infections in companion animals in Auckland and the surrounding regions were more likely to exhibit resistance to multiple antibiotics than in other regions (Kimaro 2009, pers. comm.). In 2011, two PAmpC-E isolates from urine samples from a dog and a cat were described for the first time in animals in New Zealand (Darling 2012). These isolates had *in vitro* resistance to all the cephalosporins (except the 4th generation) and to all the antimicrobials used in the veterinary panel tested, but were susceptible to imipenem and gentamicin.

1.2.2 Studies of MRSA in companion animals

The first report of an overt MRSA infection in an animal found in the literature dates back to 1972 and described a case of bovine mastitis (Devriese *et al.* 1972). Subsequently, cases were also described in horses and dogs (Hartmann *et al.* 1997; Gortel *et al.* 1999; Tomlin *et al.* 1999). The most common MRSA infection sites reported in cats and dogs are skin and soft tissue infections, surgical sites, and urinary tract (Weese and van Duijkeren 2010; Vincze *et al.* 2014a). However, the epidemiology of clinical MRSA infections in animals, in particular companion animals, is not well characterised due to the lack of surveillance efforts. The genetics of MRSA isolates found in companion animals has been studied in detail only in the last decade (Baptiste *et al.* 2005; Grinberg *et al.* 2008; Davis *et al.* 2015; Wipf and Perreten 2016). However, the understanding of the dynamics of MRSA colonisation in cats and dogs is still limited. Another issue was the sample size of the studies assessing MRSA colonisation from various anatomical sites in cats and dogs, with the two larger studies in cats sampling 415 and 540 animals (Loeffler *et al.* 2010; Bierowiec *et al.* 2016) and in dogs sampling 704-724 animals (Loeffler *et al.* 2010; Wedley *et al.* 2014). The colonisation of pets with MRSA is perceived to be important, due to the possible risk of an auto-infection, transmission to other animals, and to humans (Weese 2010).

A number of studies have assessed MRSA colonisation in healthy cats and dogs (Kottler *et al.* 2010; Loeffler *et al.* 2010; Decristophoris *et al.* 2013b; Garbacz *et al.* 2013; Muniz *et al.* 2013; Davis *et al.* 2014; Schmidt *et al.* 2014; Wedley *et al.* 2014; Gharsa *et al.* 2015; Bierowiec *et al.* 2016). A common result was the relatively low prevalence of colonisation. A large-scale study in the UK sampled the nostrils of 724 dogs from 87 veterinary practices and found an MRSA prevalence of 1% (Wedley *et al.* 2014). The only strain-type identified was the EMRSA-15 (ST22 SCC*mec*IV, with different *spa* types). Another study in the UK sampled 540 healthy cats and 704 dogs, and found a prevalence of 0.37% (Loeffler *et al.* 2010). One study in the US conducted by Davis *et al.* (2014) sampled 121 cats and 155 dogs, and found a prevalence of 0% in cats and 7% in dogs. The only strain-type

identified was ST5 SCC*mec* II, Spa type t002. Another study sampled cats (n=160) and dogs (n=258) of veterinarians (n=171), and found an MRSA colonisation rate of 2.5% among cats, 0.7% among dogs and 3.5% among the veterinarians (Morris *et al.* 2010). A Polish study by Bierowiec *et al.* (2016) swabbed 415 cats from 224 households and found a prevalence of 6.3%, but the isolates were not further analysed. While few studies have identified MRSA from healthy cats or dogs, some did not detect them (Garbacz *et al.* 2013; Muniz *et al.* 2013; Schmidt *et al.* 2014; Gharsa *et al.* 2015; Mouney *et al.* 2015; Katakweba *et al.* 2016). The low MRSA colonisation prevalence found in animals might be due to the presence of colonising competitive bacteria, in particular methicillin resistant *Staphylococcus pseudointermedius*.

A few studies assessed the risk factors for colonisation or infection with MRSA in companion animals (Loeffler *et al.* 2010; Magalhaes *et al.* 2010; Decristophoris *et al.* 2013b; Bierowiec *et al.* 2016). Risk factors for colonisation were: owner working in the healthcare industry (human or veterinary sectors) (Bierowiec *et al.* 2016), previous treatment with antimicrobials or chemotherapeutics (Bierowiec *et al.* 2016), presenting to a veterinary treatment or staying in the clinic (Loeffler *et al.* 2010; Decristophoris *et al.* 2013b). Risk factors for clinical infection were assessed in two studies using matched case-control designs, where MRSA infected companion animals were cases and MSSA-infected animals were controls (Magalhaes *et al.* 2010; Vincze *et al.* 2014a). The significant risk factors for infection with MRSA were previous antimicrobial treatment (Magalhaes *et al.* 2010; Vincze *et al.* 2014a), length of hospitalisation (Magalhaes *et al.* 2010), receiving a surgical implant or surgical site infection (Magalhaes *et al.* 2010; Vincze *et al.* 2014a), contact with humans that were ill and admitted to hospitals (Magalhaes *et al.* 2010), and number of veterinary staff working at the clinic that increases the risk of carriage and nosocomial transmission of the bacteria (Vincze *et al.* 2014a).

A few studies also described the occurrence of MRSA in companion animal skin wound samples (Abraham *et al.* 2007a; Garbacz *et al.* 2013; Vincze *et al.* 2014b). A large-scale German study by Vincze *et al.* (2014b) sampled

wound swabs from 1146 cats and 3479 dogs and found a prevalence 5.7% among cats and 3.6% among dog samples. In this study, MRSA accounted for 46% of cat and 62% of dog *S. aureus* samples. A small scale US study identified MRSA isolates from 4% of the wound samples of 50 cats (Abraham *et al.* 2007a). Conversely, a Polish study by Garbacz *et al.* (2013) did not isolate MRSA in wound samples obtained from 140 dogs.

Genetic characterisation of MRSA obtained from infection sites in companion animals have also been reported. Bacteria belonging to clonal complexes (CC) CC5, CC8 and CC22, in particular STs 5, 8, and 22, are the most frequently isolated strain types from infections in cats and dogs (Baptiste *et al.* 2005; Ishihara *et al.* 2014; Loncaric *et al.* 2014; Vincze *et al.* 2014b; Couto *et al.* 2015; Couto *et al.* 2016; Wipf and Perreten 2016). In New Zealand, the only published study in veterinary medicine identified MRSA CC22 (strain-type EMRSA-15) from cats and dogs (Grinberg *et al.* 2008). Increasingly reported is the CC398 livestock associate (LA) MRSA that emerged in the last decade in farm animals and is currently frequently isolated from infection sites in cats and dogs. It has been postulated that the CC398 LA-MRSA is now among the most common MRSA strain-type isolated from companion animals in some European countries (Vincze *et al.* 2014b; Couto *et al.* 2015; Wipf and Perreten 2016). Other STs that were identified were either geographically restricted, or sporadic. For instance, ST239 was identified from diseased dogs in Australia (Malik *et al.* 2006). Lastly, a new *mec*C (*mec*A_{LGA251}) MRSA variant has been identified recently from bulk milk in the UK, is not restricted to ruminants and has been isolated from in infections in cats and dogs in Germany (Walther *et al.* 2012b). The isolates belonged to ST130 and ST599.

1.3 Interspecies transmission of ESBL/AmpC producing Enterobacteriaceae and MRSA, and associated public health concerns

Pets may become colonised or infected by ESBL/AmpC Enterobacteriaceae and MRSA that are genetically similar to the strains causing clinical infections in humans (Harrison *et al.* 2014; Carvalho *et al.* 2016). However, the pathway of transmission of ESBL/AmpC producing bacteria and MRSA

between humans and animals is likely to be bidirectional (Davis *et al.* 2012; Rubin and Pitout 2014). The differentiation between transient and long term carriers of these bacteria is challenging for studies applying cross sectional sampling. Similarly to the situation in humans, reports showed that animals can become infected through nosocomial transmission, either in human nursing homes or veterinary clinics (Johard *et al.* 2015; Kaspar *et al.* 2015; Drougka *et al.* 2016). In New Zealand, 68% of the households have at least one pet (Anonymous 2011), which presents a potential risk for the transmission of different bacterial species between people and their pets, which is yet to be determined.

1.3.1 Interspecies transmission of ESBL/AmpC producing Enterobacteriaceae

The occurrence of ESBL/AmpC producing Enterobacteriaceae in animal populations has only been studied relatively recently (Ewers *et al.* 2011; Schaufler *et al.* 2015; Liu *et al.* 2016). Efficient national or regional surveillance programs have been established to monitor the presence of these bacteria in food animals, such as the ‘DANMAP’ in Denmark, ‘NARMS’ in the USA, and ‘CIPARS’ in Canada. These programs are driven mainly by the need to address food-borne transmission of antimicrobial resistant organisms. Conversely, very little is known about the situation in companion animals (Ewers *et al.* 2012), in spite of the fact that pets co-exist with humans in the household. Information about the occurrence of ESBL/AmpC producing bacteria in companion animals is mainly based on academic research, and is mostly preliminary and descriptive and lacking statistical power to be generalised.

Evidence of transmission of *E. coli* between animals and humans is available (Johnson *et al.* 2008; Carvalho *et al.* 2016). In a longitudinal study by Johnson *et al.* (2008), faecal samples from five family members and their dog were taken on different occasions over a period of three years and the *E. coli* isolates were typed by O and F typing, pulsed field gel electrophoresis, multilocus sequence typing and presence of selected virulence genes. In the study period, the family mother suffered from UTI from which an *E. coli* was isolated, and the same strain-type was found in the

faeces of the four family members and the dog. Moreover, the dog had a UTI caused by a different clone. In a recent study, Carvalho *et al.* (2016) analysed for the genetic relatedness of *E. coli* isolates obtained from healthy dogs and owners that did not receive antimicrobials in the previous three months. In 6% (9/134) of the sampled dogs and owners, similar *E. coli* clones were identified by PFGE. A study by Guo *et al.* (2015) compared *E. coli* isolates from faeces of hospitalised dogs and clinical isolates from diagnostic laboratories, and found strong evidence that the dogs were carrying or were infected with similar strain-types.

While these cited papers provided evidence of a transmission between humans and animals, the majority of published studies only concluded a possible bidirectional pathway, without finding the same clones in clinical infections and faeces of animals and humans. Moreover, the direction of transmission of ESBL/AmpC producing bacteria between humans and animals has not been established. One review that analysed data from previous studies reported the isolation of the same pandemic STs of ESBL producing isolates from animals and humans (Ewers *et al.* 2012). One study by Nam *et al.* (2013) investigated canine *E.coli* isolates identified from UTI, and showed that these isolates have virulence factors that interact with the human bladder epithelial cells.

Indirect evidence for a transmission between humans and animals can be extrapolated from the findings of some risk factor studies. For instance, the study by Meyer *et al.* (2012) that sampled attendees at an infection control conference, found that attendees owning pets had increased odds (OR=6.7) of colonisation with ESBL-E. Another study by Huijbers *et al.* (2013) that assessed the risk factors for human proximity to broiler farms and the risk for colonisation of ESBL producing Enterobacteriaceae, found that owning a horse was a significant risk factor (OR=4.69).

1.3.2 Interspecies transmission of MRSA

In the last decade, there has been an increase in the incidence of MRSA infections in humans worldwide (although not so in New Zealand), and an increasing number of reports of transmission of MRSA between humans and

companion animals (Sing *et al.* 2008; Morris *et al.* 2010; Ferreira *et al.* 2011; Morris *et al.* 2012; Harrison *et al.* 2014; Ishihara *et al.* 2014; Davis *et al.* 2015; Drougka *et al.* 2016; Loncaric *et al.* 2016), which were corroborated by molecular analyses of the isolates. However, the direction of transmission often remained unknown (Weese 2010; Davis *et al.* 2012). In a review published in 2012, the main MRSA sequence types were shared between humans and companion animals, worldwide, and were ST5, ST8 and ST22, belonging to CCs 5, 8 and 22 (Pantosti 2012).

The first report of MRSA transmission between humans and companion animals dates back to 1988, following an outbreak in a rehabilitation geriatric ward. The study reported a high rate of carriage of MRSA in staff, and a ward cat was also a carrier (Scott *et al.* 1988). The removal of the infected cat and other infection control measures led to the resolution of the outbreak. Subsequently, a number of studies reported possible transmission between humans and pets (Weese *et al.* 2005; Hanselman *et al.* 2006; Vitale *et al.* 2006). It has been hypothesised that the contaminated environment of commonly touched sites may represent a source of infection for co-residing pets and humans. In particular, pillows, bedding, hand towels, toys, etc have been implicated (Davis *et al.* 2012). Recent studies used high resolution molecular tools, such as whole genome sequencing, to compare isolates from different sources (Harrison *et al.* 2014; Drougka *et al.* 2016), while earlier studies used low-resolution tools, or basic antimicrobial susceptibility patterns that only suggested a transmission between humans and pets (Vitale *et al.* 2006; Couto *et al.* 2015).

In 2005, a UK study by Loeffler *et al.* (2005) isolated indistinguishable patterns or closely related EMRSA-15 isolates from nasal and oral mucosa swabs of pets and veterinary staff, and from environmental surfaces. The same strain-type was carried by pets and veterinary staff in veterinary clinics throughout Ireland (O'Mahony *et al.* 2005). A US study by Vitale *et al.* (2006) documented the pandemic MRSA strain-type USA300 from a skin infection of a cat, and the same strain colonised the anterior nares of the owner. A recent UK study by Harrison *et al.* (2014) sequenced the genomes of 46 EMRSA-15 isolates from cats and dogs. Genomic comparison of the MRSA

isolates sourced from the study (cats and dogs) and MRSA isolates sourced from humans indicated that humans and companion animals share the same MRSA strain-types. Furthermore, the data showed that there were no animal or human host-specific genes, which is concordant with previously described data indicating that companion animals are not natural hosts, but transient carriers of MRSA (Loeffler *et al.* 2013; Vincze *et al.* 2013). A recent report by Iverson *et al.* (2015) found colonisation of cats and dogs with the same MRSA strain-types obtained from infected household members. The study collected samples from pets at the beginning of the observation and three months later, and found that the pets in infected households were 11 times more likely to carry MRSA after a three-month interval than pets in non-infected households.

Veterinary clinics and staff are often colonised or infected with MRSA strain-types that are identified from animals visiting the clinics for treatment or surgery (Hanselman *et al.* 2006). This is mainly due to a close contact with colonised and infected animals on a daily basis, and to poor infection control measures (Davis *et al.* 2012). One of the early studies by Weese *et al.* (2006) identified EMRSA-2 (ST5 SCC*mec*II) from cats, dogs, veterinary staff and household members. A subsequent study by Hanselman *et al.* (2006) identified indistinguishable Canadian epidemic MRSA-2 from anterior nares of veterinarians of a small animals clinic. In New Zealand, ST22 EMRSA-15 with indistinguishable PFGE patterns were isolated from a post-operative infection site of a dog and the anterior nares of one of the surgeons (Grinberg *et al.* 2008). A recent Greek study collected 224 samples from colonised and infected sites of 10 cats and 92 dogs, and 18 veterinary personal (Drougka *et al.* 2016). A comparison of genetic markers showed very similarly or identical MRSA, strain-type ST80, among three veterinary personnel and nine companion animals. A Swedish study by (Grönlund Andersson *et al.* 2014) reported an outbreak of MRSA in three veterinary hospitals with 18%, 17% and 2% of the staff carrying MRSA with *spa* type t032 and PFGE patterns indistinguishable from MRSA identified from the infected dogs. While a Jordanian study by Tarazi *et al.* (2015) identified MRSA isolates from the nares of dogs at rearing centres and from the care-taking personnel, and PFGE analysis showed an 80-90% homology between

the isolates. In a study in a small animal hospital (van Balen *et al.* 2013) that analysed 81 environmental and 37 canine MRSA isolates during a year-long monthly sampling, most of the isolates belonged to MRSA SCC*mec* type II and USA100.

New MRSA strain-types have emerged in the last decade (as LA-MRSA and the new *mec*C MRSA), and have also been reported from human-animal transmission events. A study by Nienhoff *et al.* (2009) reported the isolation of ST398 *spa* t034 MRSA from a dog admitted to a small animal clinic in Germany for teeth extraction. The owner was a specialist veterinarian in swine diseases that had access to pig farms colonised with ST398. The PFGE analysis revealed similar banding patterns in the isolates identified from the anterior nares of the owner and the dog. Recently, the CC398 LA-MRSA has been identified from three human infections in New Zealand with no clear evidence of the source of the infection (Williamson *et al.* 2013). Lastly, a new *mec*C ($mecA_{LGA251}$) MRSA variant has been identified from humans and companion animal infections (Walther *et al.* 2012b; Petersen *et al.* 2013). Further studies are needed to assess interspecies transmission of this strain-type.

Lastly, MRSA infections in companion animals, as in humans, may result in skin and a soft tissue infection, in particular post-surgical infections. Evidence from studies using genotyping indicates that the MRSA strain-types causing clinical infections in companion animals are often the same types causing infections in the local human population (Baptiste *et al.* 2005; Grinberg *et al.* 2008; Wipf and Perreten 2016). However, the dominant strain-types in humans often vary over time and between geographical regions.

1.4 Population genetics

1.4.1 *Escherichia coli*

1.4.1.1 The population genetic structure of *E. coli*

Bacteria diversify by mutation and recombination. The balance between mutation and recombination is largely defined as the population structure, which ranges from panmicitic, where every strain can recombine with any other and recombination occurs at a high rate, to clonal, where recombination is rare or inexistent, and mutation drives most of the diversification (Wirth *et al.* 2006; Tenaillon *et al.* 2010). In a completely panmictic population, alleles at different genetic loci exist in 'linkage equilibrium', i.e., the frequencies of different associations between these alleles are similar to the frequencies expected under 'random sprinkling' theory. Conversely, in clonal populations, allelic association frequencies are in 'linkage disequilibrium'. The null hypothesis of linkage equilibrium can be tested statistically. When this hypothesis is rejected, the population is said to be in 'linkage disequilibrium' (Wirth *et al.* 2006; Tenaillon *et al.* 2010).

The average *E. coli* genome contains 4,721 genes, of which less than half (n= 2,000) are conserved with high homology among all strains (that constitutes the core genome). The *E. coli* genome has a highly dynamic structure, with constant deletions and insertions, which allows the bacterium to adapt to different environments. However, the genome keeps a strong permanent structure, with rare genome rearrangement occurrences (Tenaillon *et al.* 2010). In fact, most of the horizontal gene transfers occur in few hotspots of integration, as tRNA and phage spots. Recombination has a significant role in the *E. coli*'s genome evolution, but the rate of recombination is insufficient to determine linkage equilibrium. Moreover, since most recombination events occur in short fragments, alterations in the phylogeny topology may rarely be observed (Tenaillon *et al.* 2010).

1.4.1.2 Molecular techniques used for the study of the population genetic structure of E. coli

Several molecular tools have been developed for the universal classification of *E. coli* into clones, clonal complexes, and phylo-groups, and each one has advantages and disadvantages. A triplex PCR commonly known as the 'Clermont method' was developed to assign isolates into phylo-groups based on a combination of presence/absence of genetic markers: *chuA*, *yjaA* and TSPE4.C2 (*chuA* gene encodes for the outer membrane hemin receptor, the *yjaA* encodes an uncharacterised protein, and TSPE4.C2 encodes for putative lipase esterase gene) (Clermont *et al.* 2000; Clermont *et al.* 2013). This allowed for classification into four major phylo-groups: A, B1, B2 and D. This scheme was found to show high, but not complete congruence (80-85%) with multilocus sequence typing (MLST) data.

Phylo-grouping is highly correlated to pathogenicity and antimicrobial resistance. In fact, the various phylo-groups are not randomly distributed among the phenotypes, and, strains belonging to different phylo-groups often differ in phenotypic characteristics such as sugar fermentation, antibiotic resistance profiles, and growth rate (Gordon *et al.* 2008). For instance, phylo-groups B2 and D are more likely related to each other and to carry similar virulence genes. Furthermore, phylo-groups B2 and D are significantly associated with extra-intestinal infections. Whereas phylo-groups A and B1 are closely related with each other, these are more commonly isolated from the environment and less likely to be found in extra-intestinal infections. Group C is closely related to group B1; group E is related to group D, and finally, group F is related to group B2 (Clermont *et al.* 2013). Subsequently, the use of whole genome sequencing made clear there are more than four phylo-groups. Later, the same authors designed a quadruplex PCR with the addition of *arpA* gene, that allowed delineation to three new phylo-groups: C, E, and F, to define seven *E. coli* phylo-groups *sensu stricto* and one closely related *Escherichia* clade I, with more than 95% confidence of congruence between the MLST and the PCR (Clermont *et al.* 2013).

The MLST is one of the most used typing methods for *E. coli.* However, the information derived is not enough to assign phylo-groups. There are

currently three multilocus sequence typing (MLST) schemes based on three different genetic combinations, with the housekeeping gene *icd* in common. The three schemes maintain independent databases, the *Ec*MLST (Michigan University, US), Warwick data base (Warwick medical school, UK) and Pasteur database (Pasteur Institute, France). However, congruence analysis of concatenated sequence data derived from MLST schemes and phylogenies derived from whole genome sequencing showed that the Warwick scheme has optimal performance (Clermont *et al.* 2015).

1.4.2 *Staphylococcus aureus*

1.4.2.1 Molecular techniques used for the study of the population genetic structure of S. aureus

The *S. aureus* population structure was defined using a number of established molecular techniques that can be divided into band-based or sequence based. However, these techniques vary considerably in their discriminatory power and in their utility, cost and reproducibility (Chua *et al.* 2014). Among the band-based techniques, the traditional gold standard typing method is pulsed field gel electrophoresis (PFGE), which involves digestion of the genomic DNA using rare cutter restriction enzymes such as *Sma*I, loaded in an agarose gel, and running by an electrical current in zig zag to resolve DNA fragments between 8-20 bands (Tenover *et al.* 1994). However, this technique is labour intensive and difficult to standardise and be used to study the global epidemiology (Chua *et al.* 2014).

Among the sequence-based typing techniques, the MLST in combination with SCC*mec* typing and *spa* typing is one of the most widely accepted approaches for the description of the epidemiology of *S. aureus*. The MLST involves the sequencing of seven fragments of housekeeping genes that are present in all the strains. The advantage of using the MLST is portability and the presence of a robust and widely used online sequence data base (http://saureus.mlst.net/) (Enright *et al.* 2000). The SCC*mec* typing is often carried out in MRSA isolates, and is based on the identification of the *mec*A gene that encodes for methicillin resistance through PBP2a, ccr genes and *mec*A regulators (Ito *et al.* 1999). The difference in orientation of the ccr and

mec genes are used to classify the SCC*mec* into eleven allotypes (Ito *et al.* 2014). The different allotypes were described in the previous section. Instead, *spa* typing is based on the sequencing of the 24 base pairs variable tandem repeat region of the polymorphic C region of the *S. aureus* protein A gene (*spa*) (Harmsen et al. 2003). The *spa* can generally predict the ST, and in few cases can narrow down the list of possible STs (Cookson *et al.* 2007). *Spa* typing as an epidemiological tool for epidemics is proved to have high discriminatory power, with Simpson's index of diversity ranging between 0.97 and 0.98, and there is high concordance between spa typing, PGFE and MLST, reaching 96-98% (Strommenger *et al.* 2008). In addition to high typeability and reproducibility, *spa* typing is the cheapest among the mentioned techniques, and requires a minimal time (Strommenger *et al.* 2008). However, a major drawback of the use of single locus typing is that the population structure might be erroneously inferred due to homoplasy, and variations between closely related isolates during epidemics are difficult to be inferred (Chua *et al.* 2014).

S. aureus genomes may be also studied in more detail using a robust commercial DNA microarray (Monecke *et al.* 2011). The array contains probes that hybridize to markers for the identification of *S. aureus* species, antibiotic resistance, microbial surface components recognizing adhesive molecules (MSCRAMMs), various enzymes and pathogenicity markers. The microarray assigns *S. aureus* isolates to known clonal complex or multilocus-sequence types (MLST) based on the presence/absence of specific markers.

Lastly, the advent of cheap whole genome sequencing (WGS) presents new opportunities for the characterisation of *S. aureus* populations. Whole genome sequencing platforms are continuously improving (Thomsen *et al.* 2016).

1.4.2.2 S. aureus population structure

The *S. aureus* genome comprises a highly conserved core genome and a non-conserved "accessory' genome not present in all the isolates. The core genome comprises more than 80% of *S. aureus* genome, and is approximately 2.3 Mbp in size (Chua *et al.* 2014). It contains the genes

required for the bacteria to sustain growth, housekeeping genes, and a number of virulence determinants. In contrast, the accessory genome encompasses a number of mobile genetic elements that may have a significant impact on the phenotypic behaviour of the strain. These are insertion sequences, plasmids, transposons, bacteriophages, and pathogenicity islands (Chua *et al.* 2014). Furthermore, the accessory genome may encode antimicrobial resistance determinants, super-antigens, exotoxins, and more (Chua *et al.* 2014).

In *S. aureus*, recombination events occur rarely, and the multi-locus sequence typing analysis revealed that the organisms is fundamentally clonal (Chua *et al.* 2014). None the less, large recombination events lead from time to time to the emergence of new strains, for example the emergence of the well-characterised MRSA ST239 (so called Akh4) (Monecke *et al.* 2011; Chua *et al.* 2014). Thus, it is important to explore the evolutionary history of *S. aureus*. A common algorithm used for the visualisation of the phylogenetic relationships between strains is based upon related multi locus sequence types (BURST), that groups the isolates based on related genotypes and clonal complexes, and predicts the ancestor clone and recent evolutionary history (Chua *et al.* 2014; Aanensen *et al.* 2016).. For instance, most of the so called 'healthcare-associated' MRSA strains cluster into five clonal complexes (CC5, 8, 22, 30, and 45). In contrast, the community-associated MRSA strains lineages are more diverse (Monecke *et al.* 2011).

1.5 Concluding Remarks

Despite the relatively small number of studies describing colonisation and infections with ESBL/AmpC producing Enterobacteriaceae and MRSA in companion animals, the number of reports is increasing and so the public health concerns. Most reports agree in indicating a lack of strict host-specificity, and the same bacteria infecting humans are often found in animals. The carriage rate in companion animals is not well known, with different rates reported from different countries. The worrying aspects of faecal carriage are the potential for zoonosis and the risk of auto-infection in animals. β-lactam antimicrobials are important in veterinary medicine and an

increase in the prevalence of these bacteria poses a risk of losing the efficacy of these drugs in veterinary practice. This requires a better understanding of the epidemiology of ESBL/AmpC producing bacteria and MRSA in companion animals.

Chapter 2: Characterisation of extended spectrum β-lactamase and AmpC β-lactamase-producing Enterobacteriaceae isolated from companion animals in New Zealand

Preamble

This is the first study of thesis that describes the occurrence and characterisation of ESBL/AmpC producing Enterobacteriaceae from clinical infections of companion animals in New Zealand. To the best of the author's knowledge, this is the first paper of ESBL/AmpC producing bacteria in animals in New Zealand in the peer reviewed scientific literature. This chapter is presented as an extended version of the paper published in the New Zealand Veterinary Journal as:

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Summary

Multidrug resistant (MDR) bacteria, in particular bacteria producing extended spectrum (ESBL) and AmpC β-lactamases, cause clinically-overt infections in humans and there is increasing concern regarding the possibility of zoonotic transmission of such bacteria. Over the last decade there has been a significant increase in the incidence of infections with ESBL and AmpC-producing bacteria in humans in New Zealand, and abroad. Comparatively little is known about the epidemiology of infections with ESBL and AmpC-producing bacteria in animals, in particular in New Zealand, as veterinary diagnostic laboratories do not always test for the ESBL or AmpC phenotype in Enterobacteriaceae isolates.

This study aimed to assess the occurrence of, and characterise extended-spectrum-β-lactamase (ESBL) and AmpC β-lactamase-producing Enterobacteriaceae in infection sites in companion animals in New Zealand.

The Enterobacteriaceae isolates resistant to amoxicillin-clavulanic acid, fluoroquinolones, or any combination of two or more antimicrobials, isolated from infection sites in companion animals between June 2012 and June 2013 by New Zealand veterinary diagnostic laboratories, were analysed. The isolates were phenotypically tested for ESBL/AmpC-production and the ESBL/AmpC genes were characterised by means of PCR-sequencing. *Escherichia coli* isolates were also subtyped by multilocus sequence typing (MLST).

115 isolates were obtained from the participating laboratories. Most isolates originated from dogs (n=74; 64%) and cats (n=29; 25%). Seven Enterobacteriaceae species were identified, of which *Escherichia coli* was the most common (n=87; 76%). Sixty-four (56%) Enterobacteriaceae isolates originated from urinary tract infections. Of the 115 isolates, 10 (9%) displayed the ESBL phenotype, 43 (37%) the AmpC phenotype, and 7 (6%) both ESBL and AmpC phenotypes. Thirty-six ESBL/AmpC-positive *E. coli* were identified. The ESBL/AmpC-positive isolates were more likely to be multidrug-resistant (MDR) than the non-ESBL/AmpC ($P<0.01$). The ESBL/AmpC-positive accounted for <5% of the Enterobacteriaceae isolated by one laboratory network over the study period, but their prevalence among the amoxicillin-clavulanic acid-resistant isolates was 63%. The predominant ESBL genes were the $bla_{CTX-M-14}$ and $bla_{CTX-M-15}$, and the dominant plasmid mediated AmpC gene was the bla_{CMY-2}. Thirty-eight *E. coli* sequence types (ST) were identified, and the most prevalent were ST12, ST131, ST648 and ST744, all of which are also common ST causing infections in humans in New Zealand and abroad.

ESBL and AmpC-producing Enterobacteriaceae cause clinical infections in animals in New Zealand. Whereas these organisms accounted for <5% of all Enterobacteriaceae isolated from infection sites, their prevalence among the amoxicillin-clavulanic acid-resistant isolates was >60%. As ESBL/AmpC producing Enterobacteriaceae are resistant to a broad spectrum of β-lactams

and are often also MDR, routine secondary testing for the expression of ESBL/AmpC phenotypes, of Enterobacteriaceae that are resistant to amoxicillin-clavulanic acid, could improve the accuracy of definitive antimicrobial therapy in companion animal practice.

2.1 Introduction

Soon after the introduction of extended-spectrum cephalosporins in clinical practice in the 1980s, β-lactamase enzymes able to hydrolyse and reduce the clinical efficacy of a broad range of β-lactam antibiotics (the 'extended spectrum β-lactamases', or ESBL), were discovered in Gram-negative bacteria (Kliebe *et al.* 1985a; Bush 2012). To date, more than 100 different ESBL enzymes have been identified, and a complicated and evolving nomenclature is used for their classification (Bush 2013a). ESBL-producing Gram-negative bacteria are often resistant to other antimicrobials classes such as aminoglycosides, fluoroquinolones and tetracyclines (Winokur *et al.* 2001a; Jacoby 2005; Nakai *et al.* 2016). Infections with ESBL-producing bacteria, in particular Enterobacteriaceae, are associated with therapeutic failure and increased mortality in human hospital settings (Cantón and Coque 2006; Pitout and Laupland 2008; MacVane *et al.* 2014). Enterobacteriaceae expressing another class of broad-spectrum β-lactamases, the AmpC β-lactamases, have also been recently identified as a significant cause of infection in hospitals (Matsumura *et al.* 2013). Unlike ESBL, the expression of AmpC genes confers resistance to cephamycins. The predominant ESBL, globally, are the $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$, while $bla_{\text{CMY-2}}$ is the dominant AmpC (Bush 2013a).

The incidence of community-acquired infections with ESBL/AmpC-producing Enterobacteriaceae in humans, in particular urinary tract infections (UTI), is also increasing (Heffernan *et al.* 2014b; MacVane *et al.* 2014; Drinkovic *et al.* 2015). A worrisome aspect of the rise of these pathogens is their ability to exchange genes with co-residing bacteria in the gut through horizontal gene transfer, with the emergence of multidrug-resistant (MDR) strains (Magiorakos *et al.* 2012; van Hoek *et al.* 2015). Human studies showed higher rates of resistance to non-β-lactams in ESBL/AmpC-producing Enterobacteriaceae, compared with non-ESBL-producing strains in both

hospital (Schwaber *et al.* 2005; Matsumura *et al.* 2013; Nakai *et al.* 2016), and community settings (Dayan *et al.* 2013; Nakai *et al.* 2016), suggesting a propensity of the former to accumulate resistance genes under certain selective forces.

ESBL/AmpC-producing Enterobacteriaceae have also been isolated from infection sites in companion animals in various countries (Nebbia *et al.* 2014; Guo *et al.* 2015; Rzewuska *et al.* 2015; Liu *et al.* 2016). In 2011, two AmpC-producing *E. coli* (AmpC-E) were identified for the first time in animals in New Zealand, from urine samples from a dog and a cat (Darling 2012). Routine identification of the ESBL and AmpC phenotypes requires the use of specific diagnostic tests, which are not commonly used in commercial veterinary diagnostic laboratories in New Zealand, partly due to the lack of basic knowledge on the prevalence of these bacteria in clinical specimens submitted for analysis. Hence, the aim of this study was to estimate the occurrence of, and characterise the ESBL and AmpC-β-lactamase-producing Enterobacteriaceae isolated by diagnostic laboratories from infection sites in companion animals.

2.2 Materials and methods

2.2.1 Collection of Enterobacteriaceae isolates

Enterobacteriaceae isolates were obtained from seven New Zealand veterinary diagnostic laboratories covering the North and South Islands (Gribbles: Christchurch, Palmerston North, Auckland, Hamilton, Dunedin branches; New Zealand Veterinary Pathology: Palmerston North and Hamilton branches). The isolates were collected prospectively between June 2012 and June 2013, and sent weekly to Massey University. At that time, the commercial laboratories were not testing clinical isolates for the expression of ESBL or AmpC phenotypes. Hence, we requested receipt of all the Enterobacteriaceae isolates identified from companion animals, exhibiting *in vitro* resistance to amoxicillin-clavulanic acid. This sampling strategy was implemented because many ESBL and most AmpC-producing isolates are resistant to this drug combination (Bush and Jacoby 2010). The laboratories were also asked to send isolates resistant to fluoroquinolones, or to any

combination of two or more antimicrobials in the companion animal susceptibility testing panel for Gram-negative bacteria. This enhanced the capture of ESBL-producing Enterobacteriaceae susceptible to amoxicillin-clavulanic acid, as ESBL-producing Enterobacteriaceae are likely to carry resistance determinants to non-β-lactam antimicrobials (Bush and Jacoby 2010). This included cephalothin, amoxicillin-clavulanic acid, enrofloxacin, doxycycline, and trimethoprim-sulphamethoxazole. In addition, 12 *E. coli* isolates from infections in companion animals, fully susceptible to the susceptibility testing panel, were supplied for comparison. Isolates of the type requested were submitted on nutrient agar slopes, along with information about the host species and the infection site. To enable an estimation of the relative occurrence of ESBL and AmpC-producing isolates, the total number of Enterobacteriaceae isolated from infection sites in companion animals during the study period was extracted from the database of one laboratory network.

2.2.2 Bacterial identification and antimicrobial susceptibility testing

At Massey University, the bacteria were subcultured onto MacConkey agar plates (Fort Richards, Auckland, New Zealand) and incubated aerobically overnight at 37°C. To ensure a pure culture, a single colony was subcultured onto a nutrient agar plate and incubated as above. Conventional phenotypic tests were used for species identification (Winn and Koneman 2006) and were: Gram-stain; oxidase test (Mast ID, Mast Group Ltd., Merseyside, UK); urease activity and indole production; citrate utilisation; Kligler and lysine iron agar reactions; phenylalanine deaminase activity; and combined methyl red and Voges-Proskauer tests (Fort Richards, Auckland, NZ). Multi-test diagnostic kits were used when the species could not be unambiguously determined (API-20E; API, BioMérieux, Marcy l'Etoile, France). A subset of 29 isolates selected at random was sent to Middlemore Hospital Laboratory, Auckland, for confirmation of species by matrix-assisted laser-desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (MYLA Version 3.2.0_5. Sys. Compliance: VITEK MS1.1.0).

Antimicrobial susceptibility testing was conducted using the disk diffusion test, according to the most current version of the veterinary guidelines of the Clinical and Laboratory Standards Institute (CLSI 2013). Using a 1μL sterile loop, well separated colonies were picked and inoculated into a 0.9% saline tube and vortex to achieve a suspension of 0.5 McFarland turbidity, and a new swab was used to inoculate the suspension onto the surfaces of two Mueller Hinton agar plates (Fort Richard, Auckland, New Zealand), as recommended by the guideline. Disks used were cephalothin (30 μg); amoxicillin-clavulanic acid (30 μg); cefoxitin (30 μg); cefotaxime (30 μg); ceftazidime (30μg); imipenem (10 μg); tetracycline (30 μg); gentamicin (10 μg); trimethoprim/ sulphamethoxazole (25 μg); enrofloxacin (5 μg) and aztreonam (30 μg) (Oxoid, Basingstoke, UK). Susceptibility to cefovecin (30 μg; Oxoid, Basingstoke, UK), a 3rd generation cephalosporin used in dogs and cats, was tested using the breakpoints which according to the CLSI guidelines, were recommended by the manufacturer (≤20 mm: resistant; 21-23 mm: intermediate resistance; ≥24 mm: susceptible). These breakpoints were reported in the guidelines, but not endorsed in it (CLSI 2013).

All the Enterobacteriaceae isolates were tested for ESBL production using the combination of cefotaxime, ceftazidime and amoxicillin-clavulanic acid (CLSI 2013). Synergy is seen in ESBL-producers as a distortion of the zone of no growth, with a ≥5 mm increase in the zone diameter for cefotaxime or ceftazidime in the proximity of amoxicillin-clavulanic acid. For the identification of AmpC-production, we used the double disk synergy test of cefoxitin, with and without 3-aminophenyl boronic acid (Yagi et al. 2005). AmpC-producers show an increase of ⩾5 mm in the zone diameter for cefoxitin and cefotaxime in the presence of boronic acid. *E. coli* ATCC25922 and *K. pneumoniae* ATCC700603 were used as quality control for every batch testing (supplied by the Institute of Environmental Science and Research Ltd, Porirua, New Zealand). Enterobacteriaceae exhibiting non-intrinsic non-susceptibility to at least one antimicrobial from three or more classes were defined as MDR (Magiorakos et al. 2012). In consideration of the renewed interest in polymyxins as an option for treatment of human infections with MDR Gram-negative bacteria (Michalopoulos et al. 2005), all the ESBL/AmpC-producers were tested for susceptibility to colistin using 10

μg disks (BD BBLTM, New Jersey, USA). After the antimicrobial susceptibility testing was conducted, the isolates were frozen at -80°C in glycerol broth for future reference.

2.2.3 Genotyping

Isolates displaying ESBL or AmpC phenotypes were tested for the presence of β-lactamase genes by PCR and subsequent DNA sequencing. Briefly, 2–3 colonies obtained from frozen isolates on 5% sheep blood agar plates were suspended in a 2mL conical tube containing 100 μL of 2% chelating resin (Chelex; Thermo Fisher Scientific, Auckland, NZ) with the addition of H_2SO_4 to a final pH of 7.4. Suspensions were mixed with a vortex mixer, heated to 95°C in a heat block for 5 minutes then centrifuged at 14,000g for 3 minutes. Supernatants were transferred to a 2mL tube containing 200 μL DNA/RNA-free water, and the DNA was quantified using spectrophotometry (NanoDrop, Thermo Fisher Scientific, Wilmington, DE, USA). The minimal acceptable DNA concentration was >15 ng/μL.

Primers and conditions for amplification of the ESBL variants *bla*$_{TEM}$ and *bla*$_{SHV}$ by PCR, and sequencing of the resulting products have been previously described by Rayamajhi *et al.* (2008). For detection and amplification of *bla*$_{CTX-M}$, the following sets of primers were used in series: consensus primer pair MA1 and MA2 for amplification of *bla*$_{CTX-M}$ gene family members (Saladin *et al.* 2002), followed by a multiplex PCR for detection and differentiation of CTX-M variants in the isolates positive for *bla*$_{CTX-M}$ (Woodford 2010b). Additional primer pairs were then used to amplify and sequence specific *bla*CTX-M genes in the isolates positive for *bla*$_{CTX-M}$ (Jeong *et al.* 2005; Costa *et al.* 2006). Positive control strains (NIL05/26 and ARL03/641) were supplied by the Institute of Environmental Science and Research.

Primers and conditions for amplification of the six plasmid-encoded AmpC groups (*bla*$_{EBC}$, *bla*$_{FOX}$, *bla*$_{DHA}$, *bla*$_{ACC}$, *bla*$_{CIT}$, and *bla*$_{MOX}$) have been previously described (Pérez-Pérez and Hanson 2002). The AmpC phenotype can be determined by mutations in the promoter/attenuator region of the chromosomal *ampC* gene, as well as plasmid-encoded

AmpC genes, so isolates displaying the AmpC phenotype that were negative for the six plasmid-encoded AmpC groups by PCR were tested by PCR-sequencing of the chromosomal *ampC* promoter/attenuator region to detect mutations, as described by Caroff *et al.* (2000). The bla_{CMY-2} in Citrobacter freundii (bla_{CIT} group) and $bla_{ACT/MIR-1}$ in Enterobacter spp. (bla_{EBC} group) were not considered for further analysis, as they were considered intrinsic (van Hoek *et al.* 2015).

Products of the PCR were separated by agarose gel electrophoresis and stained with 2% ethidium bromide. Bands of the appropriate size were excised from the gel and the DNA extracted on a column (PureLink PCR Purification Kit, Thermo Fisher Scientific). Purified products were bi-directionally sequenced using an ABI 377 DNA sequencer (Massey University Genomic Service Unit, Palmerston North, NZ). Forward and reverse sequences were aligned and manually edited to generate consensus sequences using commercial software (Geneious Pro 5.6.7; Biomatters Ltd, Auckland, NZ). Consensus sequences were mapped to an online ß-lactamase database (http://www.lahey.org/Studies/).

To investigate the population structure of *E. coli*, all the *E. coli* isolates in this collection were analysed by multilocus sequence typing (MLST) (Wirth *et al.* 2006). These included 36 ESBL or AmpC-producing isolates, nine MDR isolates, 32 non-ESBL or AmpC-producing and non-MDR isolates that displayed resistance to any combination of two or more antimicrobials, as well as the 12 fully susceptible *E. coli* isolates. PCR amplification of seven housekeeping genes for MLST was performed by the Institute of Environmental Science and Research Ltd, as previously described (Wirth *et al.* 2006). The ST obtained were compared to human global *E. coli* ST deposited in a public database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/).

2.2.4 Data analysis

Two-tailed Fisher's exact tests were used to compare proportions between ESBL and AmpC-producing and non-producing isolates.

Rarefaction curves were produced using the package "vegan" in R (Oksanen *et al.* 2015), to compare sequence types (ST) diversities of the clinical

isolates between ESBL-E, AmpC-E and non-ESBL-E. Rarefaction is a statistical method used for the estimation of the number of taxa expected to be present in a random sample of any size taken from a given collection. Similarly to diversity indices such as Shannon and Simpson's indices of diversity, rarefaction allows to compare taxonomic richness between samples of unequal sample sizes (in fact, observed taxonomic richness can fluctuate stochastically due to sampling variation and is sample-size dependent) (Hughes and Bohannan 2008). These calculations were performed using the "vegan" package in R (Oksanen *et al.* 2015) (version 3.0.3, 2014; The R Foundation for Statistical Computing, Vienna, Austria).

To visualise clustering of STs, a minimum spanning tree was produced using the Hamming distances between the ST (ranging from 0 for any pair of isolates belonging to the same ST, to 7 for isolates differing at all loci) and based on the calculations by Wirth *et al.* (2006) (BioNumerics® software 7.5; Applied Maths NV. Available from http://www.applied-maths.com). The algorithm represents each ST as a circle which diameter is proportional to the number of isolates belonging to it, and links between ST by lines that indicate the number of loci by which the STs differ. Minimum spanning trees are model-based algorithms, but throughout this thesis they are used as visualisation tools, and not to infer phylogeny.

2.3 Results

2.3.1 Identified bacterial species

A total of 115 Enterobacteriaceae isolates fulfilling the inclusion criteria were submitted by the participating laboratories. Of these isolates, 93 originated from the North and 22 from the South Island, 74 from dogs, 29 from cats, six from birds, one each from a horse, a lizard, a rabbit, a monkey, and two from unknown species. The bacterial species identified and the source material are reported in Table 2.1. The most common species was *E. coli* (87/115, 75.6%). Biochemical and MALDI-TOF MS results were discordant for 4/29 isolates, and these species were designated based on the MALDI-TOF MS result.

Table 2.1: Number (%) of Enterobacteriaceae isolates (n=115) sourced from companion animals and submitted by seven veterinary diagnostic laboratories between June 2012 and June 2013 that met selection criteria[a] by bacterial species and source material.

	Source material[b]							
Organism	Urine	Tissue	Wound	Fluid	Blood	Abscess	'Other'	Total
Escherichia coli	54 (47)	5 (4)	4 (3)	5 (4)	1 (1)	1 (1)	17 (15)	87 (75)
Enterobacter cloacae	5 (4)	3 (3)	1 (1)	1 (1)	1 (1)		3 (3)	14 (12)
Serratia marcescens		2 (2)	2 (1)				1 (1)	5 (4)
Klebsiella pneumoniae	2 (2)		1 (1)				1 (1)	4 (3)
Citrobacter freundii							2 (2)	2 (2)
Enterobacter aerogenes	2 (2)							2 (2)
Citrobacter braakii	1 (1)							1 (1)

[a] Enterobacteriaceae that were resistant to amoxycillin-clavulanic acid or fluoroquinolone or the combination of two or more antimicrobials
[b] Reported in the laboratory submission form

2.3.2 Antimicrobial susceptibility test results

Comprehensive antimicrobial resistance results are reported in section 2.6, Table S 2.3 and summarised in Table 2.2. Of the 115 isolates, 95 were resistant to amoxicillin-clavulanic acid. Among these, there were 10 (11%) ESBL, 42 (44%) AmpC, six (6%) ESBL and AmpC, and 37 (39%) non-ESBL or AmpC-producers. Among the 20 isolates that were susceptible to amoxicillin-clavulanic acid, two intrinsic inducible AmpC producing isolates, *E. cloacae* and *S. marcescens*, were detected. Of the 60 ESBL and AmpC-producing isolates, 36 (60%) were *E. coli*.

Forty-two (35%) of 115 Enterobacteriaceae isolates were resistant to at least one compound from three or more antimicrobial classes and so were classified as MDR. Of the 60 ESBL and AmpC-producing isolates 27 (45%) were classified MDR, compared with 15/55 (27%) non-ESBL or AmpC-producing isolates ($p<0.01$). Nine of the 10 ESBL-producing isolates and 54/55 (98%) non-ESBL or non-AmpC-producing isolates were susceptible to ceftazidime according to the CLSI zone diameter interpretive criteria. However, note that these guidelines may not adequately detect ceftazidime resistance in areas where $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ ESBL-E genes are highly prevalent (Williamson *et al.* 2012). Amongst all isolates, the most commonly detected resistance to non-β-lactam antimicrobials was against tetracycline, which was observed in 74/115 (64%) isolates. Resistance to imipenem was not observed, whilst 14/115 (12%) isolates were resistant to gentamicin.

Of the 60 ESBL or AmpC-producing isolates 26 (43%) were resistant to enrofloxacin. Twenty-eight non-ESBL-E or AmpC-E and two non-ESBL or AmpC-producing *K. pneumoniae* exhibited resistance to cefovecin, based on the breakpoints provided by the manufacturers. All the ESBL and AmpC-producing isolates were susceptible to colistin. During the study period, one laboratory network reported the isolation of 1,082 Enterobacteriaceae from companion animals, and this network contributed 35 (3.2%) isolates to this study that were found to be ESBL or AmpC-producing.

Table 2.2: The number and proportion of Enterobacteriaceae isolates resistant to commonly used antibiotics sourced from companion animals between June 2012 and June 2013 that met selection criteria [a], categorised by β-lactamase genotype

	Genotype					
Antimicrobial	**ESBL**	**PAmpC**	***ampC***	**ESBL +AmpC**	**Non-ESBL/ AmpC**	**Total resistant**
Cephalothin	10 (100)	17 (100)	26 (100)	7 (100)	45 (82)	105 (91)
Amoxicillin-clavulanic acid	10 (100)	17 (100)	26 (100)	7 (100)	37 (66)	97 (84)
Cefoxitin	2 (20)	17 (100)	23 (88)	6 (86)	12 (22)	60 (52)
Cefovecin	10 (100)	17 (100)	16 (62)	7 (100)	30 (55)	80 (70)
Cefotaxime	8 (80)	17 (100)	7 (27)	3 (43)	3 (5)	38 (33)
Ceftazidime	1 (10)	17 (100)	7 (27)	3 (43)	1 (2)	29 (25)
Aztreonam	6 (60)	16 (94)	12 (46)	5 (71)	1 (2)	40 (35)
Gentamicin	3 (30)	1 (6)	3 (12)	4 (57)	3 (5)	14 (12)
Enrofloxacin	8 (80)	8 (47)	7 (27)	3 (43)	4 (7)	30 (26)
Sulfamethoxazole/ Trimethoprim	8 (80)	7 (41)	6 (23)	4 (57)	24 (43)	49 (42)
Tetracycline	9 (90)	12 (70)	17 (65)	5 (71)	31 (56)	74 (64)
Total isolates	10	17	26	7	55	115

ESBL: Extended spectrum β-lactamase; PAmpC: plasmid mediated AmpC-β-lactamase; *ampC*: isolate with a chromosomal *ampC* mutation; ESBL+ AmpC: isolates that have both ESBL and either PAmpC or *ampC.* Resistant and intermediately resistant isolates are merged into one group.

[a] Enterobacteriaceae that were resistant to amoxicillin-clavulanic acid or fluoroquinolone or the combination of two or more antimicrobials

2.3.3 Distribution of ESBL and AmpC genes

Specific ESBL and AmpC genes were identified by means of PCR sequencing and complete results are reported in section 2.6, Table S 2.1,Table S 2.2 and Table S 2.3. A summary of the genes identified in the ESBL and AmpC-producing isolates is presented in Table 2.3. Among the 10 ESBL-producing isolates $bla_{CTX\text{-}M\text{-}14}$ was found in four isolates; $bla_{CTX\text{-}M\text{-}14}$ and $bla_{TEM\text{-}1}$ in three isolates; $bla_{CTX\text{-}M\text{-}14}$ and $bla_{SHV\text{-}12}$ in two isolates and $bla_{CTX\text{-}M\text{-}15}$ in a single isolate. Among the 43 AmpC-producing isolates

bla$_{CMY-2}$ was found in 12 isolates and *bla*$_{CMY-2}$ and *bla*$_{TEM-1}$ in five isolates. The remaining 26 isolates exhibiting the AmpC phenotype were negative for the six plasmid-encoded AmpC groups by PCR. Of these 26 isolates, nine were *E. coli*, and further analysis by PCR-sequencing revealed mutations in the *ampC* promoter/attenuator regions of all these nine isolates, as detailed in section 2.6, Table S 2.1. In the 17 AmpC-producing isolates that were not *E. coli*, the PCR for detection of mutations in the promoter/attenuator regions of the *ampC* gene was not performed as these isolates belonged to species for which such mutations are considered intrinsic.

Among the seven Enterobacteriaceae isolates producing both ESBL and AmpC, *bla*$_{CTX-M-15}$ was found in three isolates, *bla*$_{CTX-M-14}$ in one isolate, *bla*$_{SHV-12}$ in two isolates, *bla*$_{SHV-12}$ and *bla*$_{TEM-1}$ in one isolate, and *bla*$_{SHV-2a}$ in one isolate. All of these isolates were negative for the six AmpC groups by PCR, indicating the presence of chromosomal *ampC* mutations, although PCR for *ampC* mutations was not performed on these isolates.

2.3.3.1 Analysis of E. coli multilocus sequence types

A total of 89 *E. coli* isolates were characterised by MLST; these isolates included 10 ESBL-producers, 17 isolates with plasmid-encoded production of AmpC, seven isolates with chromosomal *ampC* mutations, 41 isolates producing neither ESBL nor AmpC, and 12 fully susceptible *E. coli* isolates. Results for the ESBL and AmpC-producing isolates are summarised in Table 2.3, and complete results for all 89 isolates are provided in section 2.6, Table S 2.3.

Overall there were 38 ST that clustered into 17 clonal complexes, and 21 ST that did not cluster with known clonal complexes. The most frequent ST were ST12 (12/89, 13%), ST131 (6/89, 7%) and ST648 (6/89, 7%). Five novel ST (ST4166, ST4167, ST4200, ST4201, ST4202) were identified in five isolates. The novel ST4200 was an ESBL-producing isolate carrying the *bla*$_{CTX-M-14}$ gene, and the novel ST4166 was an AmpC-producing isolate carrying the *bla*$_{CMY-2}$ gene.

Rarefaction analysis indicated the ST richness of ESBL-E was smaller than that of non-ESBL-E (Figure 2.1). Sixteen ST were represented by multiple

isolates; seven of these isolates differed from each other in their β-lactamase gene composition (Figure 2.2). No obvious patterns of host-specificity were observed among the ST (Figure 2.2). However, ST744 was represented by multiple isolates from cats only and ST648 was represented by multiple isolates from dogs only. The minimum spanning tree did not reveal single central nodes that could indicate the presence of central founders, and most ST were connected as double, triple, or quadruple locus variants (Figure 2.2).

Table 2.3: Characteristics of ESBL and/or AmpC-producing Enterobacteriaceae isolates (n=60)

Phenotype	*bla* genes detected (No of isolates)	Species	*E. coli* ST	Source	Material
ESBL (n=10)	CTX-M-14 (n=4)	*E. coli*	648, 131 (n=2), 12	canine (n=3), feline (n=1)	ear swab, urine (n=3)
	CTX-M-14 + TEM-1 (n=3)	*E. coli*	744 (n=2), 648	canine (n=3)	catheter, fluid, urine
	CTX-M-14 + SHV-12 (n=2)	*E. coli*	648, 4200*	canine, feline	urine (n=2)
	CTX-M-15 (n=1)	*E. coli*	131	canine	urine
AmpC *E. coli* (n=24)	CMY-2 (n=12)	*E. coli*	101, 1485, 155, 372, 46, 156 (n=2), 4166*, 648, 457 (n=2), 744	canine (n=8), feline (n=4)	abdominal fluid, ear swab, nail swab, pus, urine (n=6), wound (n=2)
	CMY-2 + TEM-1 (n=5)	*E. coli*	23, 46, 156, 205, 90	canine (n=4), feline	blood, swab, urine (n=3)
	chromosomal *ampC* mutations (n=4)	*E. coli*	38, 58, 88, 517	canine (n=2), feline (n=2)	urine (n=4)
	chromosomal *ampC* mutations + TEM-1 (n=2)	*E. coli*	88 ,648	canine (n=2)	urine (n=2)
	chromosomal *ampC* mutations + TEM-1b (n=1)	*E. coli*	744	unknown	unknown
ESBL + AmpC (n=7)	CTX-M-15 (n=2)	*E. cloacae*	NA	canine (n=2)	tissue, lung
	CTX-M-15 + TEM-1 (n=1)	*E. cloacae*	NA	bird	joint swab
	CTX-M-14 + chromosomal *ampC* mutations (n=1)	*E. coli*	648	canine	urine
	SHV-12 + chromosomal *ampC* mutations (n=1)	*E. coli*	12	bird	blood
	SHV-12 + TEM-1 (n=1)	*E. cloacae*	NA	canine	urine
	SHV-2a (n=1)	*E. cloacae*	NA	monkey	lung tissue
AmpC non-*E. coli* (n=19)	Not detected (n=9)	*E. cloacae*	NA	canine (n=5), feline (n=3), bird (n=1)	bronchial washing, ear swab, urine (n=5), wound
	Not detected (n=5)	*S. marcescens*	NA	canine (n=5)	ear swab, implant, tissue, wound
	Not detected (n=2)	*C. freundii*	NA	canine, lizard	nail, swab
	Not detected (n=2)	*E. aerogenes*	NA	canine (n=2)	urine (n=2)
	Not detected (n=1)	*C. braakii*	NA	feline	urine

bla: β-lactamase genes; ESBL: Extended spectrum β-lactamase; AmpC: AmpC β-lactamase; ESBL+ AmpC: isolates that have both ESBL and either PAmpC or *ampC;* ST: multilocus sequence types; NA: not applicable; *: novel ST.

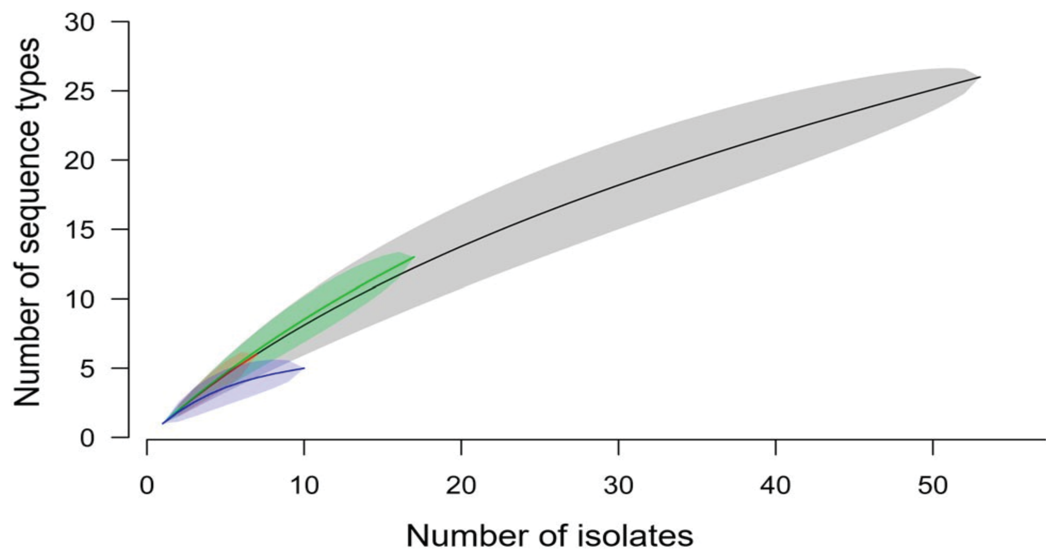

Figure 2.1: Rarefaction curves for multilocus sequence types of *Escherichia coli* isolates sourced from companion animals in New Zealand between June 2012 and June 2013 carrying extended spectrum β-lactamase (blue line), plasmid-encoded AmpC β-lactamase (green line) genes, or a chromosomal ampC mutation (red line), or that did not carry those genes (black line). The shaded areas around the curves represent 95% CI.

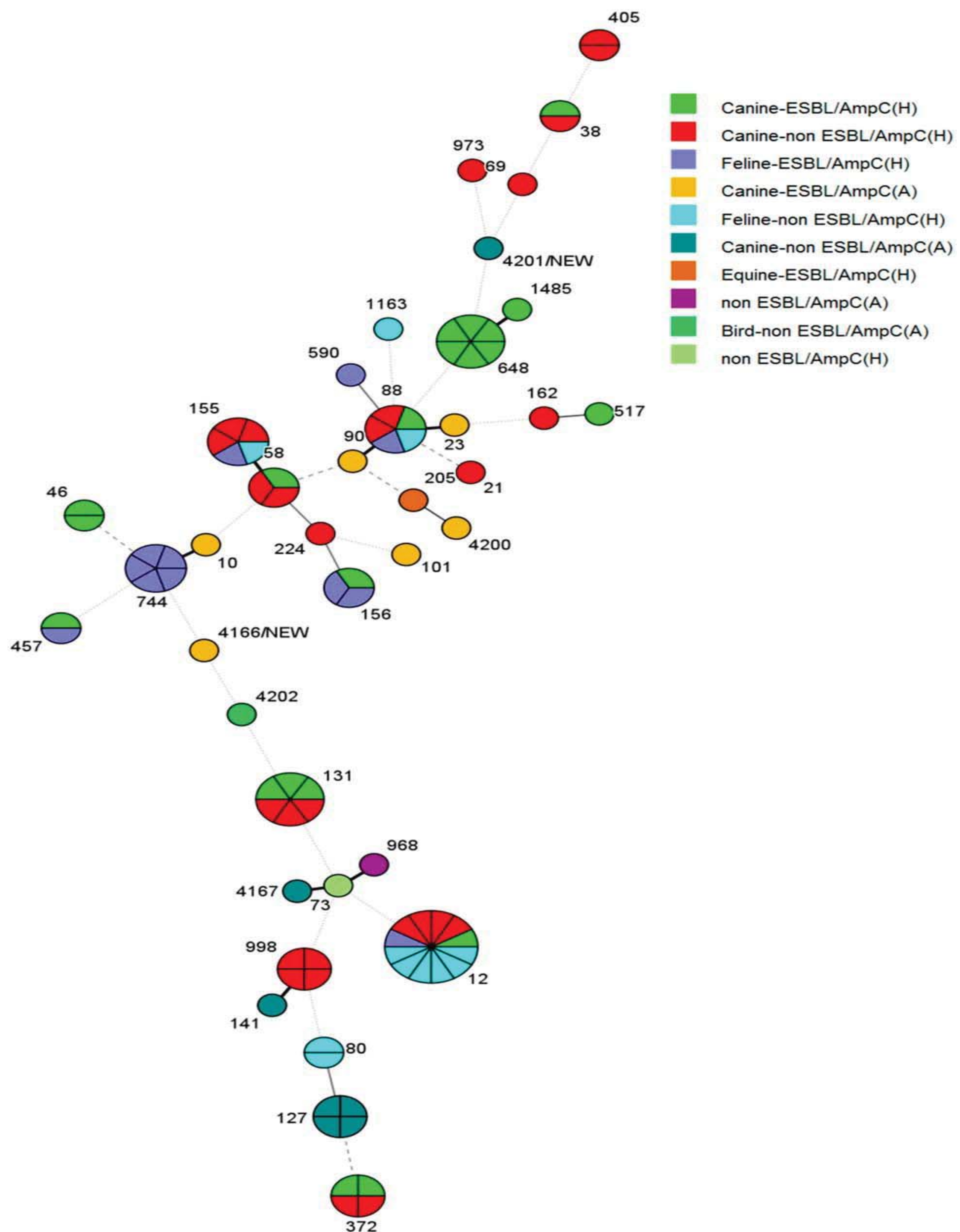

Figure 2.2: Minimum spanning tree of the multilocus sequence types (ST) of 89 *Escherichia coli* isolates sourced from companion animals in New Zealand between June 2012 and June 2013.

Each circle represents one numbered ST (according to http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/). The number of partitions indicate the number of isolates for each ST. Isolates are categorised by source species, and by isolates carrying extended spectrum β-lactamase (ESBL), plasmid-encoded AmpC β-lactamase (AmpC) or chromosomal ampC mutations, or isolates that did not carry those genes, with ST first reported in humans indicated by (H) and in animals by (A). Connecting lines between circles indicate a single locus variant (———), double locus variant (———), triple locus variant (———) quadruple locus variant (-----).

2.4 Discussion

Extended spectrum β-lactamase and AmpC-producing Enterobacteriaceae represent a serious problem in human medicine, due to their resistance to β-lactam antimicrobials and the high prevalence of co-resistance to other antimicrobial classes. The impact of these bacteria on animal health is poorly understood. In our study, 60/115 (52%) of the Enterobacteriaceae isolates that matched the inclusion criteria of resistance to amoxicillin-clavulanic acid or fluoroquinolones, or to any combination of two or more antimicrobials in the testing panel, exhibited ESBL or AmpC phenotypes and carried either plasmid-encoded genes or chromosomal mutations. This result indicates that Enterobacteriaceae producing ESBL or AmpC are a non-negligible cause of clinical infections in companion animals in New Zealand. It was estimated that ESBL or AmpC-producing isolates represented 3.2% of the Enterobacteriaceae isolated over the same study period by one participating laboratory network. This does not differ substantially from the results obtained in the Netherlands, where 2% of the isolates were ESBL or AmpC-producers (Dierikx *et al.* 2012). It is possible that some ESBL-producing isolates that did not fulfill the inclusion criteria of this study remained undetected. At this point in time, testing of all the Enterobacteriaceae isolated from companion animals for the presence of the ESBL or AmpC phenotypes may not be cost effective in New Zealand. Of the 95 isolates resistant to amoxicillin-clavulanic acid, 58 (61%) were ESBL or AmpC-producing. Therefore, secondary testing of amoxicillin-clavulanic acid-resistant Enterobacteriaceae for the ESBL and AmpC phenotypes could be justified, as it could prevent complications associated with inadequate treatment with other β-lactam antimicrobials.

Interestingly, only 12% of all the isolates expressed resistance to gentamicin, and this may be the result of the low usage of gentamicin by companion animal veterinarians in New Zealand (Pleydell *et al.* 2012). Conversely, 43% of the ESBL and AmpC-producing isolates were resistant to enrofloxacin (Table 2.2), which is in agreement with the high resistance to this compound observed in Enterobacteriaceae causing human infections in New Zealand and overseas (Matsumura *et al.* 2013; Woerther *et al.* 2013; Heffernan *et al.* 2014b).

Comparative data of multidrug-resistance in ESBL and AmpC-producing and non-producing Enterobacteriaceae affecting humans in community settings, are scarce. In this study, we found a higher proportion of co-resistant and MDR isolates in ESBL and AmpC-producing isolates than in the non-producing isolates (Table 2.2), consistent with previous studies (Schwaber *et al.* 2005; Dayan *et al.* 2013). Co-resistance and multidrug-resistance adds complexity to the management of infections caused by ESBL and AmpC-producers. Due to multidrug-resistance, first-line antimicrobial treatments may not result in clinical resolution, and an antimicrobial considered critically important for human treatment (use of which should be limited) may be required to achieve a favourable outcome. On the other hand, most non- ESBL or AmpC-producers in our study were susceptible to non-β-lactam antimicrobials (Table 2.2), leaving a range of drug choices that are likely to result in effective treatment for most Enterobacteriaceae infections in companion animals in New Zealand.

A variety of ESBL genes were identified in this study. The majority of isolates carried $bla_{CTX\text{-}M\text{-}14}$, whereas among the isolates carrying plasmid-encoded AmpC, only $bla_{CMY\text{-}2}$ was identified. All the ESBL and AmpC genes identified in this study have been previously identified in human clinical isolates in New Zealand (Heffernan *et al.* 2007; Heffernan *et al.* 2009). In some isolates carrying $bla_{CTX\text{-}M}$ or bla_{CMY}, we also detected bla_{TEM} or bla_{SHV}, which is consistent with previous observations indicating ESBL or AmpC-producing strains may carry more than one β-lactamase-encoding gene (Woodford *et al.* 2009). Yet 37/55 (67%) isolates did not exhibit the ESBL or AmpC phenotypes but were resistant to amoxicillin-clavulanic acid, and may have harboured other genes conferring resistance that were not analysed in this study (Wedley *et al.* 2011).

Most ST found in the *E. coli* isolates analysed in this study have been reported in human and animal infections in different studies overseas, suggesting cross transmission of these STs between human and animals (Rogers *et al.* 2011; Dierikx *et al.* 2012; Nebbia *et al.* 2014; Guo *et al.* 2015; van Hoek *et al.* 2015). Furthermore, some isolates belonged to ST considered human pandemic lineages, such as ST131, ST73 and ST648

(Adams-Sapper *et al.* 2013). MLST analysis revealed a relatively high abundance of ST12 (n=12), ST131 (n=6), and ST648 (n=6). The isolation of abundant ST from different locations in New Zealand during the study period, and from different host species, underlines the success of these ST in the dissemination of ESBL or AmpC genes in the community. Interestingly, the isolates varied between each ST in terms of the production, or lack of production, of ESBL and AmpC, and by genotyping results, which likely reflected extensive horizontal gene transfer of resistance determinants (Borjesson et al. 2013).

We identified six *E. coli* isolates belonging to the human pandemic ST131 (Adams-Sapper *et al.* 2013; Riley 2014) (Table 2.3; Supplementary material). The isolates were ESBL-producers (n=3) and non-ESBL-producers (n=3). In a previous study, ST131 contributed to more than half of the ESBL-E isolates identified from human infections in New Zealand (Heffernan *et al.* 2014b), and overseas (Rogers *et al.* 2011), and this ST has also been often isolated from animal infection sites overseas (Nebbia *et al.* 2014; Bogaerts *et al.* 2015). Conversely, ST131 was not identified among the AmpC-E, consistent with the low prevalence of this ST among plasmid mediated AmpC-E reported in Auckland (Drinkovic *et al.* 2015). Together, these results suggest that, at least at the level of genetic resolution used in this study (MLST), infections with ESBL/AmpC-E in animals in New Zealand are largely caused by the same lineages described in animals overseas, and in humans in New Zealand. What makes these lineages so prevalent in different countries, is not completely understood.

Rarefaction analysis indicated that non-ESBL/AmpC-E isolates had higher ST diversity than ESBL/AmpC-E isolates. Many factors could have contributed to this difference. Firstly, combination of virulence factors and antimicrobial resistance genes may be carried by some STs and confer advantage to some uropathogenic STs such as ST131 (Croxall *et al.* 2011; Chung *et al.* 2012). In fact, it is estimated that 10-20% of UTI cases are caused by a small group of STs (Manges *et al.* 2008; Banerjee *et al.* 2013; Nebbia *et al.* 2014). Second, and this is a limitation of this analysis,

ESBL/AmpC-E isolates were selected using specific criteria of antimicrobial resistance, which may have selected for specific clones.

The visualisation of the minimum spanning tree revealed several clusters of STs, but there was no single central node that could be interpreted as a founder, and most connections between STs were weak (double, triple or quadruple locus variants), as expected from a highly recombining population (Turner & Feil, 2007). The five new STs identified in this study did not cluster together but were found in association with other established STs.

Lastly, cefovecin is a relatively new compound for use in dogs and cats, categorised as a long-acting third generation cephalosporin (Stegemann *et al.* 2006). Twenty-eight *E. coli* and two *K. pneumoniae* isolates were classified as non-susceptible to cefovecin based on the breakpoint zone diameter provided by the manufacturer (CLSI 2013). All these isolates were non-ESBL or AmpC- producers. This discrepancy between the disk diffusion test results and the lack of ESBL or AmpC production may be due to the presence of unidentified enzymes, or a different resistance mechanism. Alternatively, the interpretive breakpoint may need revision. We note that no breakpoint diameter is reported for cefovecin in the latest CLSI guidelines published in 2015 (CLSI 2015).

2.5 Conclusion

In conclusion, this is the first study characterising clinical ESBL and AmpC-producing Enterobacteriaceae in animals in New Zealand. Our results suggest that ESBL and AmpC-producing isolates may account for approximately 60% of the isolates resistant to amoxicillin-clavulanic acid obtained from infection sites in companion animals in New Zealand. Multidrug resistance was detected in 45% of ESBL and AmpC-producers. MLST analysis indicated that many ESBL-E or AmpC-E from infected animals in New Zealand belong to common human endemic and pandemic ST, suggesting cross transmission of these strains between humans and animals. Further studies are needed in order to assess the prevalence of infection with ESBL and AmpC-producing Enterobacteriaceae in New Zealand.

2.6 Supplementary material

Table S 2.1: Details of the mutations in the *ampC* promoter sequences in the nine bla_{AmpC}-negative *E. coli* isolates displaying the AmpC phenotype

Isolate number	Mutations in the *ampC* promoter
L6 and L221	-18 (G to A), -1 (C to T), +58 (C to T)
L9	-8 (A) –10 (box), +69 (C to T)
L48	+3 (T), +54 (G to A), +58 (C to T), +63 (T to C),
L59	+3 (T), +58 (C to T), +81 (G to A)
L69	-10 (box), -12 (A to T), -11 (C to G), -10 (A to T), +58 (C to T), +77 (G to A)
L87	-9 (A to T), -1 (C to T), +58 (C to T)
L89	-18 (G to A), -1 (C to T), +32 (G to A), +58 (C to T), +74 (G to T)
L126	-10 (box) -12 (A to T), -11 (C to A), -10 (A to C), -7 (C to T), -1 (C to T), +58 (C to T)

Table S 2.2: Multilocus sequence type allele designation of the five new sequence types found in this study

	MLST loci								
Isolate Number	adk	fumC	gyrB	icd	mdh	purA	recA	MLST	MLST CC
L19	83	331	42	12	1	2	2	4166/New	None
L219	36	24	9	13	17	11	287/New	4167/New	None
L35	62	100	17	31	5	58	4	4201/New	None
L97	83	93	136	30	1	162	29	4202/New	None
L241	6	23	32	449	9	8	7	4200/New	None

MLST: multi locus sequence typing, MLST CC: multi locus sequence typing clonal complex

Table S 2.3: Antibiograms and details of the 115 Enterobacteriaceae and 12 fully susceptible *E. coli* isolates used in this study

Number	Source	Organism	β-lactamase identified	MLST	MLST CC	Cep	Amc	Fox	Ctx	Caz	Cvn	Cn	Enr	Sxt	Te	Atm	MDR
L1	Urine	*E. coli*	TEM-1, CMY-2	46	ST46 Cplx	R	R	R	I	I	R	S	R	S	R	S	Y
L6	Urine	*E. coli*	TEM-1, *ampC* Mutation	88	ST23 Cplx	R	R	I	S	S	R	S	S	S	R	S	N
L7	Ear Swab	*E. coli*		998		I	S	S	S	S	S	S	S	R	S	S	N
L9	Urine	*E. coli*	*ampC* Mutation	38	ST38 Cplx	R	R	I	S	S	I	R	R	R	R	S	Y
L10	Ear Swab	*E. coli*	CMY-2	744		R	R	R	I	R	R	S	R	R	R	S	Y
L14	Urine	*E. coli*	SHV-12, CTXM-14	648		R	R	S	R	S	R	S	R	R	R	R	Y
L15	Urine	*E. coli*	TEM-1, CTXM-14	648		R	I	I	R	S	R	R	R	R	R	R	Y
L17	Urine	*E. coli*		973		R	S	S	S	S	S	R	R	R	R	S	Y
L19	Urine	*E. coli*	CMY-2	4166/New		R	R	R	I	R	R	S	S	S	S	S	N
L24	Urine	*E. coli*		88	ST23 Cplx	R	I	S	S	S	S	S	S	S	R	S	N
L30	Swab	*E. coli*		58	ST155 Cplx	S	S	S	S	S	S	S	S	R	R	S	N
L32	Urine	*E. coli*	CTXM-14	648		R	R	S	R	S	R	S	R	R	R	R	Y
L43	Catheter	*E. coli*	TEM-1, CTXM-14	744		R	R	S	R	S	R	R	R	R	R	R	Y
L45	Urine	*E. coli*		12	ST12 Cplx	R	R	I	S	S	I	S	S	S	S	S	N
L48	Urine	*E. coli*	TEM-1, SHV-12, *ampC* Mutation	12	ST12 Cplx	R	R	I	S	S	R	S	S	R	S	S	N
L49	Swab	*E. coli*	TEM-1 CMY-2	90	ST23 Cplx	R	R	R	I	I	R	S	R	S	S	I	N
L51	Milk	*E. coli*		162	ST469 Cplx	R	R	S	S	S	R	S	S	S	S	S	N
L53	Pus	*E. coli*	CMY-2	156	ST156 Cplx	R	R	R	R	R	R	S	R	R	R	R	Y
L57	Urine	*E. coli*		590	ST590 Cplx	R	S	S	S	S	R	S	S	S	R	S	N
L58	Urine	*E. coli*		12	ST12 Cplx	R	R	I	S	S	I	S	S	S	S	S	N
L59	Urine	*E. coli*	CTXM-14, *ampC* Mutation	648		R	I	I	R	S	R	R	R	R	R	R	Y
L62	Swab	*E. coli*		21	ST29 Cplx	I	S	S	S	S	S	S	S	S	R	S	N
L63	Urine	*E. coli*		155	ST155 Cplx	I	S	S	S	S	S	S	S	R	R	S	N
L65	Urine	*E. coli*	TEM-1, CMY-2	23	ST23 Cplx	R	R	R	I	R	R	S	S	R	R	I	Y
L66	Ear Swab	*E. coli*	CTXM-14	131		R	I	S	R	S	R	S	R	R	R	S	Y
L69	Urine	*E. coli*	TEM-1, *ampC* Mutation	648		R	I	R	R	S	R	R	R	R	R	I	Y
L73	Nasal swab	*E. coli*		1163		S	S	S	S	S	S	S	S	R	R	S	N
L78	Urine	*E. coli*	CMY-2	457		R	R	R	R	R	R	S	S	R	I	R	Y

Number	Source	Organism	β-lactamase identified	MLST	MLST CC	Cep	Amc	Fox	Ctx	Caz	Cvn	Cn	Enr	Sxt	Te	Atm	MDR
L83	Urine	*E. coli*	CTXM-14	12	ST12 Cplx	R	R	I	S	S	R	S	S	S	S	I	N
L84	Ear Swab	*E. coli*		12	ST12 Cplx	R	R	I	S	S	I	S	S	S	S	S	N
L85	Wound	*E. coli*	CTXM-14	131		R	I	S	R	I	R	S	R	R	R	I	Y
L86	Urine	*E. coli*	TEM-1, CMY-2	156	ST156 Cplx	R	R	R	I	R	R	S	R	R	R	I	Y
L87	Foetus	*E. coli*	TEM-1b, *ampC* Mutation	744		R	R	I	S	S	R	S	S	S	R	I	N
L88	Urine	*E. coli*		131		I	R	S	S	S	S	R	R	I	S	S	Y
L89	Urine	*E. coli*	*ampC* Mutation	58	ST155 Cplx	R	R	R	S	S	R	S	S	S	I	I	N
L90	Blood	*E. coli*	TEM-1, CMY-2	205	ST205 Cplx	R	R	R	R	R	R	R	R	R	R	I	Y
L93	Nail Swab	*E. coli*	CMY-2	1485		R	R	R	R	R	R	S	S	S	S	I	N
L94	Swab	*E. coli*		968		R	I	S	I	S	I	S	S	S	S	S	N
L95	Urine	*E. coli*	TEM-1	10	ST10 Cplx	R	I	S	S	S	R	S	R	S	R	S	Y
L97	Liver	*E. coli*		4202/New		I	S	S	S	S	I	S	S	S	I	S	N
L98	Fluid	*E. coli*	TEM-1	58	ST155 Cplx	R	I	S	S	S	R	S	S	R	S	S	N
L100	Wound	*E. coli*	CMY-2	46	ST46 Cplx	R	R	R	I	I	R	S	R	S	S	I	Y
L101	Valval Swab	*E. coli*		155	ST155 Cplx	R	R	S	S	S	R	S	S	S	S	S	N
L103	Wound	*E. coli*		405	ST405 Cplx	R	I	S	S	S	S	S	R	R	R	S	Y
L104	Urine	*E. coli*	CMY-2	156	ST156 Cplx	R	R	R	I	R	R	S	R	R	R	I	Y
L105	Urine	*E. coli*		127		I	S	S	S	S	S	S	S	R	R	S	N
L112	Ear Swab	*E. coli*		141		R	I	S	I	I	R	S	S	S	I	S	N
L114	Urine	*E. coli*		12	ST12 Cplx	R	R	I	S	S	R	S	S	I	R	S	N
L124	Wound	*E. coli*	CMY-2	457		R	R	R	R	R	R	S	S	S	I	R	N
L125	Urine	*E. coli*		998		R	I	S	S	S	S	S	S	R	S	S	N
L126	Urine	*E. coli*	*ampC* Mutation	517		R	R	I	S	S	R	S	S	S	S	S	N
L128	Vaginal swab	*E. coli*		405	ST405 Cplx	R	I	S	S	S	S	S	S	S	I	S	N
L130	Ear Swab	*E. coli*		38	ST38 Cplx	S	S	S	S	S	S	S	S	R	S	S	N
L134	Urine	*E. coli*		69	ST69 Cplx	S	R	S	S	S	S	S	S	S	R	S	N
L135	Urine	*E. coli*		12	ST12 Cplx	R	R	S	S	S	I	S	S	S	S	S	N
L71	Fluid	*E. coli*	TEM-1, CTXM-14	744		R	R	S	I	S	R	R	R	R	R	S	Y
L138	Urine	*E. coli*	TEM-1	131		R	R	S	S	S	I	S	R	R	S	S	Y

Number	Source	Organism	β-lactamase identified	MLST	MLST CC	Cep	Amc	Fox	Ctx	Caz	Cvn	Cn	Enr	Sxt	Te	Atm	MDR
L137	Urine	*E. coli*		12	ST12 Cplx	R	R	I	S	S	I	S	S	S	S	S	N
L139	Urine	*E. coli*		80	ST568 Cplx	R	R	S	S	S	I	S	S	S	S	S	N
L140	Urine	*E. coli*		127		R	R	S	S	S	R	S	S	S	S	S	N
L147	Fluid	*E. coli*		127		S	S	S	S	S	S	S	S	S	R	S	N
L150	Urine	*E. coli*		12	ST12 Cplx	R	R	I	S	S	I	S	S	S	S	S	N
L155	Urine	*E. coli*	CMY-2	372		R	R	R	I	I	R	S	S	S	S	S	N
L174	Urine	*E. coli*		12	ST12 Cplx	R	R	S	S	S	I	S	S	S	S	S	N
L175	Urine	*E. coli*	CTXM-15	131		R	R	S	S	S	R	S	R	R	R	S	Y
L179	Urine	*E. coli*		12	ST12 Cplx	R	R	I	S	S	I	S	S	S	S	S	N
L183	Urine	*E. coli*	TEM-1	155	ST155 Cplx	R	I	S	S	S	R	S	S	R	R	S	Y
L184	Prostate-Fluid	*E. coli*		131		R	R	S	I	S	R	S	R	R	R	S	Y
L185	Unknown	*E. coli*		73	ST73 Cplx	R	R	S	S	S	I	S	S	S	S	S	N
L188	Urine	*E. coli*	TEM-1	88	ST23 Cplx	R	R	I	S	S	R	S	S	R	R	S	Y
L190	Urine	*E. coli*	CMY-2	648		R	R	R	R	R	R	S	S	S	I	I	N
L210	Kidney	*E. coli*		998		R	I	S	S	S	S	S	S	R	S	S	N
L221	Urine	*E. coli*	*ampC* Mutation	88	ST23 Cplx	R	R	R	R	R	R	S	S	R	R	I	Y
L227	Urine	*E. coli*		224		I	I	S	S	S	I	S	R	R	R	S	Y
L234	Abdominal Fluid	*E. coli*	CMY-2	101	ST101 Cplx	R	R	R	I	R	R	S	S	S	I	I	N
L235	Urine	*E. coli*	CMY-2	155	ST155 Cplx	R	R	R	I	R	R	S	S	S	R	I	N
L241	Urine	*E. coli*	SHV-12, CTXM-14	4200/New		R	I	S	I	S	R	S	S	S	R	S	N
L29	Urine	*E. coli*		12	ST12 Cplx	R	I	S	S	S	S	S	S	S	S		N
L40	Urine	*E. coli*		127		S	S	S	S	S	S	S	S	S	R		N
L43B	Urine	*E. coli*	CTXM-14	744		R	R	S	I	S	R	R	R	R	R		Y
L64	Uterine Swab	*E. coli*		372		R	I	S	S	S	S	S	I	S	S	S	N
L200	Urine	*E. coli*		155	ST155 Cplx	R	R	I	S	S	R	S	S	S	S	S	N
L35	Ear Swab	*E. coli*		4201/New		S	S	S	S	S	S	S	S	S	S	S	N
L36	Ear Swab	*E. coli*		372		S	S	S	S	S	S	S	S	S	S	S	N
L111	Ear Swab	*E. coli*		372		S	S	S	S	S	S	S	S	S	S	S	N
L219	Pus	*E. coli*		4167/New		S	S	S	S	S	S	S	S	S	S	S	N

Number	Source	Organism	β-lactamase identified	MLST	MLST CC	Cep	Amc	Fox	Ctx	Caz	Cvn	Cn	Enr	Sxt	Te	Atm	MDR
L220	Nail	*E. coli*		88	ST23 Cplx	S	S	S	S	S	S	S	S	S	S	S	N
L228	Abdominal Fluid	*E. coli*		80	ST568 Cplx	S	S	S	S	S	S	S	S	S	S	S	N
L120	Unknown	*E. coli*		998		S	S	S	S	S	S	S	S	S	S	S	N
L16	Urine	*E. coli*				S	S	S	S	S	S	S	R	R	S	S	N
L22	Urine	*E. coli*				S	S	S	S	S	S	R	S	R	S	S	N
L25	Abscess	*K. pneumoniae*				R	I	I	S	S	S	I	R	R	S	S	Y
L31	Bronchial Washing	*E. cloacae*	*ampC* Mutation			R	R	R	R	R	R	R	R	R	S	S	Y
L33	Tissue	*E. cloacae*	CTXM-15			R	R	R	S	S	R	S	S	I	S	S	N
L38	Urine	*K. pneumoniae*				R	S	I	S	S	R	I	S	R	S	S	Y
L39	Swab	*K. pneumoniae*				R	S	R	S	S	R	I	S	R	S	S	Y
L50	Swab	*C.freundii*	*ampC* Mutation			R	R	R	S	S	R	S	S	S	S	S	N
L74	Blood	*E. cloacae*	SHV-12			R	R	R	S	I	R	I	S	I	I	S	Y
L76	Urine	*E. coli*				R	S	S	I	R	R	S	S	S	S	S	N
L82	Urine	*E. coli*				R	R	S	S	S	R	S	S	R	S	S	N
L91	Abdominal Fluid	*E. cloacae*	CTX-M-15, *ampC* Mutation			R	R	R	I	R	R	I	R	R	R	S	Y
L102	Urine	*E. coli*				R	R	R	R	S	R	S	R	S	S	S	N
L106	Ear Swab	*S. marcescens*	*ampC* Mutation			R	R	R	I	R	S	S	S	I	S	R	N
L109	Urine	*E. aerogenes*	*ampC* Mutation			R	R	R	R	R	R	I	S	R	I	S	N
L110	Nail	*C. freundii*	*ampC* Mutation			R	R	R	S	S	S	R	R	R	S	S	Y
L113	Urine	*E. aerogenes*	*ampC* Mutation			R	I	I	S	S	S	I	S	R	R	S	N
L115	Urine	*E. coli*				R	R	R	R	R	R	S	S	S	S	S	N
L116	Urine	*E. cloacae*	*ampC* Mutation			R	R	R	S	S	S	S	S	S	R	S	N
L118	Wound	*E. cloacae*	*ampC* Mutation			R	S	I	S	S	S	S	S	S	S	S	N
L122	Joint Swab	*E. cloacae*	TEM-1, CTXM-15			R	R	R	R	R	R	I	R	R	R	S	Y
L123	Faecal Swab	*E. coli*				R	R	R	S	S	S	R	S	I	S	S	Y
L127	Wound	*S. marcescens*	*ampC* Mutation			R	R	R	S	S	R	S	S	R	S	R	N
L129	Urine	*E. coli*				R	R	S	S	S	S	S	R	I	S	S	N
L131	Swab	*E. cloacae*	*ampC* Mutation			R	R	R	I	R	R	S	S	I	S	S	N

Number	Source	Organism	β-lactamase identified	MLST	MLST CC	Cep	Amc	Fox	Ctx	Caz	Cvn	Cn	Enr	Sxt	Te	Atm	MDR
L136	Tissue	*S. marcescens*	*ampC* Mutation			R	R	R	S	S	S	S	S	R	S	R	N
L141	Urine	*C.braakii*	*ampC* Mutation			R	R	R	S	S	S	S	S	S	I	S	N
L142	Ear Swab	*E. cloacae*	*ampC* Mutation			R	R	I	S	S	S	S	S	I	S	S	N
L162	Urine	*E. cloacae*	*ampC* Mutation			R	R	R	S	S	S	S	S	S	R	S	N
L163	Beak Swab	*E. coli*				R	R	R	S	S	R	R	R	R	R	S	Y
L177	Wound	*S. marcescens*	*ampC* Mutation			R	S	S	S	S	S	S	S	S	S	R	N
L189	Urine	*E. cloacae*	*ampC* Mutation			R	R	R	S	S	R	S	S	S	S	S	N
L191	Lung	*E. cloacae*	SHV-2a			R	R	R	S	S	S	S	S	S	S	S	N
L197	Implant	*S. marcescens*	*ampC* Mutation			R	R	S	S	S	S	S	S	R	S	R	N
L199	Urine	*E. cloacae*	*ampC* Mutation			R	R	R	R	R	R	R	R	R	S	S	Y
L201	Urine	*E. cloacae*	*ampC* Mutation			R	R	R	S	S	S	S	S	S	S	S	N
L236	Urine	*E. coli*				R	R	R	S	S	R	S	S	I	S	S	N
L243	Urine	*K. pneumoniae*				R	R	S	S	S	S	S	R	R	S	S	Y

MDR: multidrug-resistant; N: non multidrug-resistant; Cep: Cephalothin; Amc: Amoxicillin-clavulanic acid; Fox: Cefoxitin; Ctx: Cefotaxime; Caz: Ceftazidime; Cvn: Cefovecin; Te: Tetracycline; Cn: Gentamicin; Sxt: Sulphamethoxazole-Trimethoprim; Enr: Enrofloxacin; Atm: Aztreonam; MLST: multi locus sequence typing; MLST CC: multi locus sequence typing clonal complex; R: resistance; I: intermediate-resistance; S: susceptibility

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DRC 16

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Ali Karkaba

Name/Title of Principal Supervisor: Alex Grinberg

Name of Published Research Output and full reference:

Karkaba, A. Grinberg, J. Benschop, E. Pleydell (2016). Characterisation of extended spectrum β-lactamase and AmpC β-lactamase-producing Enterobacteriaceae isolated from companion animals in New Zealand. New Zealand Veterinary Journal

In which Chapter is the Published Work: Chapter 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed the study, collected the samples and processed them at Massey University. He wrote the first draft of the paper and responded to the co-authors' feedback and qualified as first and correspondent author.

Ali Karkaba Digitally signed by Ali Karkaba Date: 2017.03.02 17:13:35 +13'00' | 02/03/2017

Candidate's Signature | Date

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Chapter 3: Cross sectional study of prevalence of faecal carriage of multi-drug resistant, and extended spectrum β-lactamase and AmpC β-lactamase-producing *Escherichia coli* in cats and dogs in the Auckland region

Preamble

This Chapter describes a cross sectional period-prevalence study of faecal carriage of multi-drug resistant and ESBL/AmpC-producing *E. coli* in cats and dogs in Auckland. Isolation of these bacteria from faeces by culture is challenging because faecal matrices contain hundreds of competing bacterial species. Hence, it is necessary to enrich and then select the multi-drug resistant and ESBL/AmpC-producing *E. coli* in order to isolate them from the faeces by culture. A preliminary literature search found several methods in the literature, but no gold standard for the isolation of multi-drug resistant and ESBL/AmpC-producing *E. coli.* Hence, it was necessary to assess the utility of different methods and select one method for use in this prevalence study. The assessment of the different available methods is described in Appendix 1.

Summary

Infections with multi-drug resistant *E. coli* (MDR-E) in humans and companion animals in New Zealand have been reported (Kimaro 2009; Darling 2012; Heffernan *et al.* 2014b; Williamson and Heffernan 2014; McMeekin *et al.* 2016). In particular, infections due to extended spectrum β-lactamase (ESBL) and AmpC β-lactamase (AmpC)-producing *E. coli* (ESBL/AmpC-E) are increasing, raising significant health concerns. In addition, Chapter 2 showed that pets can be infected with MDR-E and ESBL/AmpC-E belonging to the same multilocus sequence types, and carrying the same enzymes, found in human isolates, suggesting cross transmission of strains between these host species. However, there is a

paucity of data in New Zealand about the faecal carriage by pets of these bacteria. Therefore, the aim of the present study was to assess the faecal carriage rate of MDR-E and ESBL/AmpC-E in cats and dogs in Auckland, New Zealand.

A total of 362 dogs and 225 cats were sampled at 29 veterinary practices in the Auckland region. A faecal swab was taken from the animals and a short questionnaire was delivered to the owner at the time of sampling. Swabs were cultured to isolate MDR-E and ESBL/AmpC-E using a previously assessed isolation method (Appendix 1). Isolates displaying MDR-E and/or ESBL/AmpC-E phenotypes were genotypically characterised by multilocus sequence typing (MLST) and the ESBL/AmpC genes were identified using polymerase chain reaction (PCR), followed by DNA sequencing.

The prevalence of MDR-E, ESBL-E and AmpC-E isolates in the sample was 2% (95% CI 1%-3.5%), 0.34% (95% CI 0.04%-1.2%) and 6.1% (95%CI 4.3%-8.4%), respectively. The predominant β-lactamase genes detected in ESBL/AmpC-E were the $bla_{\text{CTX-M-14}}$ (n=2) and $bla_{\text{CMY-2}}$ (n=34). Multiple isolates (n=24), sharing the same sequence type by MLST displayed different antimicrobial phenotypes and carried different β-lactamase genes, reflecting the occurrence of extensive horizontal gene transfer between and within clonal lineages. The study identified six novel *E. coli* multi-locus sequence types not previously reported in the public MLST databases.

To the authors' knowledge, this study is one of the largest cross-sectional surveys of faecal carriage of MDR-E and ESBL/AmpC-E in companion animals, which includes a genetic characterisation of the isolates. The results indicate that several ESBL/AmpC-E sequence types circulating internationally in humans and animals are part of the intestinal bacterial flora of cats and dogs in New Zealand.

3.1 Introduction

Antimicrobial resistance is a global problem in both human and veterinary medicine, and it is associated with a significant economic burden and increased morbidity and mortality (Paladino *et al.* 2002; Maragakis *et al.* 2008; Bogaerts *et al.* 2015). There are increasing concerns that companion

animals could be potential reservoirs for antimicrobial resistant bacteria, in particular multidrug-resistant (MDR), and that these may cause endogenous auto-infections and be transferred to humans (Boerlin and Reid-Smith 2008; Szmolka and Nagy 2013).

Escherichia coli is part of the intestinal microbiota of most of humans and animals, and one of the most frequently isolated and studied antimicrobial resistant bacterial species (Yamamoto *et al.* 2014). The antimicrobial resistance profiles of *E. coli* isolated from both healthy and clinically infected humans and pets have been described in several studies overseas (Feria *et al.* 2002; Machado *et al.* 2004; Pena *et al.* 2004; Machado *et al.* 2006; Mendonca *et al.* 2006; Costa *et al.* 2008; Bogaerts *et al.* 2015; Schmidt *et al.* 2015). A recent report by McMeekin *et al.* (2016) analysed existing diagnostic laboratory bacteria isolates cultured from canine urine samples submitted to five commercial veterinary diagnostic laboratory in New Zealand, from January 2005 and December 2012. The analysis of 5786 urine samples showed, that *E. coli* was the most commonly isolated (35%; 1104/5786) and the percentage of resistant isolates to cephalothin, amoxicillin-clavulanic acid and enrofloxacin increased significantly between 2005 and 2012.

Of particular concern are ESBL- and AmpC- producing *E. coli* (ESBL-E, AmpC-E) strains that cause infections that are difficult to treat due to the production of β-lactamase enzymes that confer resistance to a wide range of β-lactams. Furthermore, ESBL/AmpC-E are more likely to carry genes that confer resistance to other antimicrobial families and be multi-drug resistant (MDR-E) (Jacoby 2005), than non-ESBL/AmpC-E. This phenomenon was observed in the study presented in Chapter 2. Bacterial multi-drug resistance has been defined as resistance to more than two antimicrobial families (Magiorakos *et al.* 2012). The ESBL-E and AmpC-E can be differentiated phenotypically, as ESBL-E are susceptible to cephamycins, whereas the AmpC-E are resistant, and this is due to the carriage of the correspondent genetic determinants (Jacoby 2009; Bush 2013b). The ESBL/AmpC phenotypes can be acquired via horizontal gene transfer (HGT) of plasmids

coding the enzymes, or through chromosomal mutation leading to *ampC* gene overexpression (Matsumura *et al.* 2013).

It was demonstrated that ESBL/AmpC-E strains causing clinical infections in humans were carried within the same household by humans and their pets (Lefebvre *et al.* 2006; Seiffert *et al.* 2013). Studies have reported faecal carriage of ESBL/AmpC-E, lasting from 132 days (Zahar *et al.* 2010), up to 58 months (Alsterlund *et al.* 2012) in previously clinically infected humans, and for more than six months in infected dogs treated with antimicrobials (Baede *et al.* 2015). Moreover, intestinal carriage has been associated with ascending urinary tract auto-infections in humans (Gopinath *et al.* 2014). Hence, the role of intestinal carriage in the dissemination of MDR-E, ESBL/AmpC-E strains should not be underestimated.

ESBL/AmpC-E have been described in New Zealand from clinical cases in humans (Freeman *et al.* 2008; Pope *et al.* 2009; Freeman *et al.* 2012; Drinkovic *et al.* 2015). Surveillance reports indicate an increasing annualised incidence rate of clinical infections with ESBL Enterobacteriaceae, predominantly ESBL-E, in humans in New Zealand (Dyet *et al.* 2014). For example, an increase of ~95% was observed between July 2007 (100 cases per 100,000 population) and July 2014 (195.7 cases per 100,000 population). In the last decade, the highest notification rates of infections with ESBL-E have been observed in the Auckland region (Waitemata, Auckland and Manukau District Health Boards). A community study in Auckland found a prevalence of intestinal colonisation with ESBL-E in humans of approximately 5.1% (92/1799) (Upton *et al.* 2011). In animals in New Zealand, ESBL/AmpC-E genes were reported from a clinical urine specimen of a cat and a dog (Darling 2012), and from a myriad of clinical infections in animals (Chapter 2).

A report of MDR-E that analysed existing diagnostic laboratory data from seven commercial veterinary diagnostic laboratories in New Zealand, from July 2008 and November 2008, found that 15% (15/100) of clinical *E. coli* isolates were MDR, and the highest rates of MDR were observed in Auckland, Northland and Waikato (Kimaro 2009). However, virtually no data exist on the faecal carriage rates of ESBL-E, AmpC-E and MDR-E.

Therefore, the main aim of this study was to estimate the prevalence of faecal carriage of MDR-E and ESBL/AmpC-E in cats and dogs in the Auckland region. Subsequently, we used molecular methods to characterise phenotypically confirmed isolates.

3.2 Materials and methods

3.2.1 Study population and recruitment of veterinary clinics

The target population was pet cats and dogs attending veterinary clinics in the greater Auckland region of New Zealand between June 2012 and June 2013. The study was introduced to potential participating veterinarians by an oral presentation during a veterinary meeting held in Auckland in May 2012. After receiving Massey University human and animal ethical approval for the study in June 2012, veterinary clinics in the study area were recruited from an online publicly available list provided by the Veterinary Council of New Zealand (VCNZ- http://www.vetcouncil.org.nz; accessed June 2012). The recruitment was as follows: initially, emails were sent to all 131 clinics, then a staggered recruitment of the 121 clinics on the list that did not respond by emails was attempted by direct phone calls or email. Clinics were recruited at a rate of approximately two clinics per week until September 2013.

The New Zealand census published in 2006 indicated that there were 437,985 households in the Auckland region, and around 68% of New Zealand households owned at least one pet animal. Thus, we estimated the number of households having pets in Auckland to be 297,830 (Anonymous 2011) and assumed that all would attend one of the 131 veterinary practices (http://www.vetcouncil.org.nz). Studies conducted overseas in healthy cats and dogs indicated a faecal carriage prevalence of ESBL-E ranging between 1.3 % and 5% (Costa *et al.* 2008; Harada *et al.* 2011; Wedley *et al.* 2011). However, the sample size used in these studies was small. Human carriage studies of ESBL-E in healthy populations suggested a prevalence ranging between 5% and 7% (Stromdahl *et al.* 2011; Ben Sallem *et al.* 2012; Geser *et al.* 2012). We compromised with an estimate of 6% prevalence of faecal carriage in dogs and cats. Using a cluster-sample survey design, to estimate the ESBL-E faecal carriage prevalence in healthy cats and dogs in the

Auckland region with 95% confidence of a precision of 10%, we would have needed 31 samples from each of 61 practices, for a total of 1891 samples. This was calculated using an estimated cluster design effect of 2, corresponding to a low intra-cluster correlation coefficient (Otte and Gumm 1997). Budget was calculated according to this sample size.

3.2.2 Sample collection

Clinics were provided with a kit with all the material for sample collection, and were asked to follow a standardised protocol to obtain rectal swabs from cats and dogs. The kit contained charcoal Amies sampling swabs (Transystem®, Copan, Brescia, Italy) with labels to allow the identification of the swab, vials containing sterile normal saline solution, an instructions sheet, a questionnaire and prepaid courier envelopes for the delivery of the samples to Massey University (MU). Samplers were instructed to immerse the swab in the sterile saline and remove excess moisture prior to the insertion of the swab into the rectum of the cat or dog for 3-5 seconds (to allow a thorough contact of the swab with the rectal wall). The samplers were asked to submit the samples to MU by overnight courier delivery from Monday to Thursday, to prevent samples from deteriorating on route or in storage over the weekend. Frequent follow-up calls were used to improve the submission process.

To minimise selection bias, the recruited clinics were asked to attempt the sampling of the first five animals presenting during the course of a normal working day, over a period of 2-3 weeks, for a maximum of 31 animals. To be eligible for inclusion, the animals' owners must have been 18 years of age or older and signed a consent agreement to participate. In addition, owners were asked to complete a short questionnaire to ascertain potential risk factor information for ESBL/AmpC-E and MDR-E carriage (see Chapter 4). The email correspondence, forms and questionnaire used in this study are presented in Section 0.

No identifying information, such as name or house address was provided, but the suburb of residence was recorded. Only one animal per owner was eligible for sampling and the decision of which animal to sample was

arbitrarily made by the sampling personnel. Owners were asked if they would like to receive the results of the testing and free retesting of positive animals was offered to the owner via clinic staff. The researchers were available for phone consultation by the veterinarians. An information sheet (Section 0) explaining the precautions to be taken during sampling and the potential risks for animal and human health associated with ESBL/AmpC carriage was also provided to the clinics and the owners.

3.2.3 Isolation of MDR-E and ESBL/AmpC-E and genotyping of the isolates

Culture for the isolation of MDR-E and ESBL/AmpC-E from faeces was performed using enrichment method M1 described in Appendix 1, with one modification, i.e. only a single loopful was obtained from the bacterial growth around the antimicrobial disks, and only one colony was subcultured. This modification applied in preliminary trials indicated that subculture of more than one colony did not increase the rate of isolation of ESBL/AmpC-E or MDR-E in faeces, in comparison with the isolation of several colonies from positive ESBL/AmpC faecal specimens Appendix 1. The presumptive *E. coli* isolates were confirmed at Middlemore Hospital Laboratory, Auckland New Zealand, using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF, MYLA Version 3.2.0_5. Sys. Compliance: BioMérieux VITEK MS1.1.0).

The identification and characterisation of ESBL/AmpC genes by PCR-sequencing and MLST of the *E. coli* isolates was performed as described in Chapter 2 and Appendix 1. To visualise clustering in the data, a minimum spanning tree was produced using the Hamming distances between the ST (ranging from 0 for any pair of isolates belonging to the same ST, to 7 for isolates differing at all loci), using commercial software (BioNumerics 7.5; Applied Maths NV), as described in Chapter 2.

3.2.4 Data Handling and Statistical Analysis

A predesigned electronic database IRIS (Incident Response and Disease Management Information; http://www.epimanager.com/Epicentre/en-nz/LogOn) was used to store demographic data and questionnaire and

microbiological results. The final dataset is provided as an appendix in an attached CD. Descriptive statistics were calculated using the R statistical software (R version 3.0.3, Copyright© 2014 The R Foundation for Statistical Computing). As animals were clustered within veterinary clinics, the crude prevalence of MDR-E and ESBL/AmpC-E faecal carriage was adjusted for clustering within clinic using the 'gee' package in R (Carey 1998). An analysis for risk factors for carriage is described in Chapter 4.

3.2.5 Ethical Approval

The Massey University Human Ethics committee granted the institutional High Risk Notification approval to administer the owner's questionnaire (HEC: Southern A Application – 12/28) and the Animal Ethics committee granted approval to sample the dogs and cats (MUAEC application 12/46).

3.3 Results

3.3.1 Characteristics of the study population

All the 131 veterinary practices within the Auckland region were contacted by email of which 10 did respond and agreed to participate, and a subset of the clinics (n=50) that did not respond by email were contacted by telephone. A total of 29 clinics agreed to participate in the study. In total, 586 faecal swabs were collected from dogs (n=361) and cats (n=225). There were 572/586 usable questionnaires (351 questionnaires from dog owners and 221 from cat owners), with a median of 16 questionnaires per clinic (range: 1 to 31). The analysis of the questionnaires is described in the risk factor analysis reported in Chapter 4.

3.3.2 Isolation of MDR-E and ESBL/AmpC-E

MDR-E were isolated from 43/586 (7.3%) faecal swabs, of which 38/43 (88%) were ESBL/AmpC-E. Of the 38 ESBL/AmpC-E, 36 were AmpC producers, one displayed both AmpC and ESBL production, and one was an ESBL-producer (Table 3.2). ESBL/AmpC-E were identified in 4.7% (12/225) in cats and 7.2% (26/361) in dogs (two-tailed Fisher's exact test for the difference between the species: P=0.12).

Among the 38 ESBL/AmpC-E, 31% (n=12) were resistant only to β-lactam antimicrobials, 50% (n=19) to one additional antimicrobial class and 18% (n=7) were MDR (i.e., resistant to compounds belonging to three or more antimicrobial groups). The highest prevalence of resistance to the non-β-lactams was to tetracycline 65% (n=25), then to enrofloxacin 26% (n=10). None of the isolates was resistant to imipenem or gentamicin (Table 3.2). The overall adjusted prevalence of MDR-E, ESBL-E and AmpC-E was 2%, 0.2% and 6.1%. The number and sample prevalence, adjusted for clustering within clinics, and classified by the different resistance groups are presented in Table 3.1.

Table 3.1: Number of *Escherichia coli* isolates isolated from faecal samples, classified by the resistance groups, obtained in a cross-sectional study of 586 cats and dogs in Auckland, New Zealand (confidence intervals were adjusted for clustering within clinics)

Outcome	Number of positive samples (%; 95% confidence interval)
MDR	12/586 (2; 0.77-7.8)
ESBL and AmpC	38/586 (6.5; 2.1-18.2)
ESBL	2/586 (0.34; 0.02-1.1)
AmpC (total)	36/586 (6.1; 2-17.8)
PAmpC	34/586 (5.9; 1.9-17.1)
ampC mutation	3/586 (0.5; 0.12-1.91)

MDR: multidrug resistant; ESBL: Extended spectrum β-lactamase; PAmpC: plasmid-mediated AmpC-β-lactamase; *ampC*: isolate with a chromosomal *ampC* mutation.

Table 3.2: Antimicrobial susceptibility results of faeces and of *Escherichia coli* isolates obtained in a cross-sectional survey of 586 cats and dogs in Auckland, New Zealand.

Antimicrobial Agent	**Faeces**	***E. coli* Isolates**			
	% of resistance, out of 586 faeces]	**PAmpC-E [% of resistance, out of 34 isolates]**	***ampC*-E [% of resistance, out of 3 isolates]**	**ESBL-E [% of resistance, out of 2 isolates]**	**MDR-E and ESBL/AmpC-E [% of resistance, out of 43 isolates]**
Cephalothin	42.6	100	100	100	97
Amoxicillin Clavulanic Acid	22.9	100	100	100	93
Cefoxitin	-	100	100	50	88
Cefovecin	21.3	100	100	100	88
Cefotaxime	10.9	97	66	100	83
Ceftazidime	-	94	66	50	79
Gentamycin	3	0	0	0	0
Enrofloxacin	7.7	18	33	50	27
Sulphamethoxazole-Trimethoprim	31.4	8.8	33	1	23.2
Tetracycline	53.1	62	66	1	65
Aztreonam	-	91	66	1	79

MDR: multidrug resistant; ESBL: Extended spectrum β-lactamase; PAmpC: plasmid mediated AmpC-β-lactamase; *ampC*: isolate with a chromosomal *ampC* mutation; ESBL+ AmpC: isolates that have both ESBL and either PAmpC or *ampC*. Resistant and intermediately resistant isolates are merged into one group.

3.3.3 Molecular Characterisation of MDR-E and ESBL/AmpC positive-E

The *bla*$_{CMY-2}$ gene was detected in 34/37 AmpC-E (Table 3.3Table 3.3). The remaining three AmpC-producing isolates were PCR-negative for the six AmpC-β-lactamase genes, and were therefore tested by a PCR designed for the detection of chromosomal mutations within the 191 bp region of the promoter and attenuator regions of the *ampC* gene (Caroff et al., 1999). Sequenced PCR amplicons were compared with sequences of the *E. coli* K-12 reference strain (GenBank accession U00096), and a number of mutations were observed in the promoter region. Isolate R14 contained mutations at positions +89 (T to A), +91 (A to T), +92 (C to A), +94 (G to C), +95 (C to G),+97 (T to C), +99 (T to C); isolate R58 contained mutations at positions -18 (G to A), -1 (C to T),+58 (C to T); and isolate R60 at positions +1 (A to G),+7 (A to C),19 (A to T),+22 (C to T),+26 (T to G),+27 (A to T), +32 (G to A),+42 (G to T),+47 (G to A),+54 (G to T).

Two ESBL-E carried *bla*$_{CTX-M}$ which was further identified by sequencing as the *bla*$_{CTX-M-14}$ gene. Of the remaining five MDR-E (non ESBL/AmpC-E), one isolate harboured *bla*$_{TEM-1}$ (Table 3.3Table 3.3). Table 3.3None of the isolates were positive for the *bla*$_{SHV}$.

Table 3.3: ESBL and AmpC β-lactamase genes of the 43 *Escherichia coli* isolates included in the study

β-lactamase gene	MDR (n=12)	MR (n=19)	N (n=12)
bla$_{CMY-2}$	4	17	10
bla$_{CMY-2}$ + *bla*$_{TEM-1}$	-	1	1
bla$_{CMY-2}$ + *bla*$_{CTX-M14}$	1	-	-
bla$_{CTX-M14}$	1	-	-
ampC mutation + *bla*$_{TEM-1}$	-	-	1
ampC mutation	1	1	-
bla$_{TEM-1}$	1	-	-
None	4	-	-

MDR: multi drug resistance, MR: resistance to one antimicrobial family in addition to the β-lactam family, N: resistance to β-lactam family only

3.3.4 Multilocus sequence typing (MLST)

Multi-locus sequence typing was performed on the 43 MDR-E and ESBL/AmpC-E isolates, and a subset of 14 non-MDR/ESBL/AmpC-E isolated from randomly selected non-resistant faecal swabs from cats and dogs for comparison. The 57 isolates belonged to 30 sequence types (ST). Sixteen STs belonged to clonal complexes (CCs) that have been previously reported in the Warwick database (Table 3.4). The remaining 14 ST were assigned as singletons (clonal complex "none"), of which six were novel ST. These novel sequences were submitted to the Warwick MLST database (http://EnteroBase.warwick.ac.uk) and were assigned as clonal complex "none" by the curators. These are: ST4390 (new recA locus type 305), ST4391 (new recA locus type 306), ST4392 (new purA locus type 335), ST4393 (new gyrB locus type 399), ST4395 (new icd locus type 212), and ST4406 (new adk locus type 373, recA 304).

Nearly half of the ST's found in this study (n=15) were defined in the Warwick MLST database as 'human associated' STs. Conversely, 13 MLSTs were 'animal associated', one was 'environmental-associated' and one was 'water-associated'. Six of the 43 MDR-E and ESBL/AmpC-E isolates displayed novel STs and harboured *bla*$_{CMY-2}$ genes. In all six cases, the PCR-sequencing of the unique alleles were repeated with similar results, precluding PCR or sequencing error. Table 3.4 shows the STs identified in *E. coli*. Note that 7/30 ST's (ST10, ST88, ST162, ST372, ST405, ST540, ST973) were represented by multiple isolates with different β-lactamase gene profiles. The different *E. coli* ST's, categorised according to the host species and β-lactamase production are represented in a minimum spanning tree (MST) in Figure 3.1. Consistent with a recombining population, the minimum spanning tree (MST) revealed that most ST were connected as double, triple, or quadruple locus variants (Didelot *et al.* 2012).

Further details on the 43 MDR-E, ESBL/AmpC-E and 14 non-MDR/ESBL/AmpC-E, including their antimicrobial susceptibility profiles, β-lactamase enzymes, ST's are presented in Section 0, Table S 3.1.

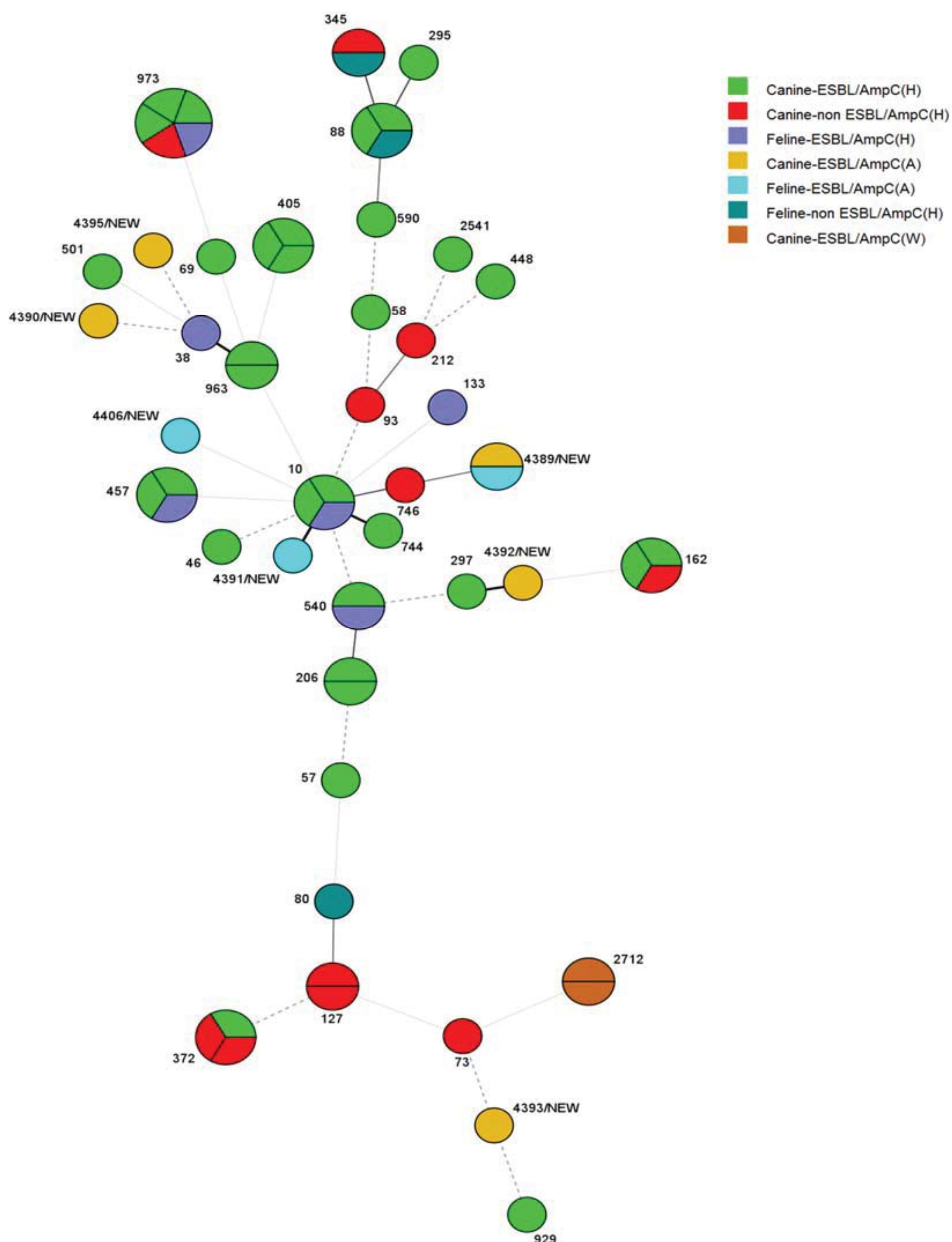

Figure 3.1: Minimum spanning tree showing the associations between the different *E. coli* multilocus genotypes (ST) found in this study.

Each circle represents an ST, flanked by its numerical designation according to the Warwick database. The number of partitions indicate the number of isolates found for each ST. Feline and canine *E. coli* isolates appear in different colours. The letters H, A and W indicate that the STs were first reported in humans, animals only and water according to the Warwick database

▬▬ link between single locus variant; ——— link between double locus variants; ——— link between triple locus variants; ----- link between quadruple locus variants.

Table 3.4: Classification of the MDR/ESBL/AmpC-E and 14 non-MDR/ ESBL/AmpC-E isolates found in this study according to their multilocus sequence types (ST) and clonal complexes, as defined in the Warwick database

	E. coli Multilocus Sequence Type					
Clonal complex	PAmpC (n=33)	*ampC* mutation (n=3)	PAmpC + ESBL (n=1)	ESBL (n=1)	MDR (non-ESBL/AmpC) (n=5)	Non-ESBL/AmpC (n=14)
469	ST162^{2}					ST162^{1}
10	ST10^{2}				ST10^{1}	
23					ST88^{1}	ST88^{3}
405		ST405^{1}	ST405^{1}	ST405^{1}		
57	ST57^{1}					
206		ST206^{1}				
155	ST58^{1}					
38	ST38^{1}					
568						ST80^{1}
73						ST73^{1}
590		ST590^{1}				
168						ST93^{1}
46	ST46^{1}					
448					ST448^{1}	
Singletons	ST133^{1},295^{1},372^{1},457^{3},501^{1},540^{3}, 929^{1},963^{2},973^{4},2541^{1},2712^{1}, *4390^{1}, *4391^{1}, *4392^{1}, *4393^{1*}, *4395^{1}, *4406^{1}				ST744^{1}	ST127^{2},212^{1}, 297^{1},345^{2},372^{2},746^{1}, 973^{1}

ESBL: Extended spectrum β-lactamase; PAmpC: plasmid mediated AmpC-β-lactamase; *ampC*: isolate with a chromosomal *ampC* mutation; ESBL+ AmpC: isolates that have both ESBL and either PAmpC or *ampC*; Singletons: multilocus sequence types not associated with any known clonal complex; superscripts$^{1-5}$ indicate the frequency of isolates; *: new MLSTs, not previously reported in the Warwick database

3.4 Discussion

This study investigated the prevalence of faecal carriage of MDR-E and ESBL/AmpC-E in 586 cats and dogs presenting to veterinarians in Auckland, New Zealand. The adjusted prevalence of faecal carriage of MDR-E, AmpC-E and ESBL-E was 7.3%, 6.3% (5.8% PAmpC and 0.5% *ampC* chromosomal mutation), and 0.3%, respectively. Of the 43 MDR-E and ESBL/AmpC-E isolates, almost half belonged to known clonal complexes, and the majority of the STs found have been previously detected overseas.

The most common resistances observed among MDR-E (n=12), ESBL/AmpC-E (n=38) was to tetracycline, cephalothin and enrofloxacin. More than half of the MDR-E isolates in this study (n=7/12, 58%) were ESBL/AmpC producers. As previously described (Jacoby 2005), and in the previous study in Chapter 2, a high proportion (25/38,65%) of ESBL/AmpC-E isolates carried resistance determinants to other antimicrobials, in addition to the β-lactam antimicrobials.

The molecular characterisation of the 38 ESBL/AmpC-E isolates revealed the predominance of the $bla_{CTXM-14}$ and bla_{CMY-2} β-lactamase genes. Both genes were previously detected in *E. coli* from animals and humans worldwide, and a review of the published literature indicated that $bla_{CTXM-14}$ is one of the three most frequently detected ESBL genes, while bla_{CMY-2} is the most frequently detected PAmpC gene (Ewers *et al.* 2012; Bush 2013b). Both $bla_{CTXM-14}$ and the bla_{CMY-2} β-lactamase genes were also detected in *E. coli* isolated from clinical infections of animals in New Zealand (Chapter 2), hospitalised (n=5) and non-hospitalised (n=2) cats and dogs in Palmerston North in the pilot study (Appendix 1), and also in *E. coli* isolated from clinical infections in humans in New Zealand (Heffernan *et al.* 2007; Heffernan *et al.* 2009), indicating the strains carrying these genes are present in human and pets in New Zealand.

MLST analysis identified no dominant CC or ST in the sample (n=57). Many STs identified in this study are associated with human and animal infections overseas (Corvec *et al.* 2010; Ewers *et al.* 2012; Hasan *et al.* 2012; Stoesser *et al.* 2012; Adams-Sapper *et al.* 2013; Voets *et al.* 2013; Hansen *et al.* 2014; Riley 2014) (Section 0, Table S 3.1). The most abundant ST among

the PAmpC isolates was ST973 (n=4). Seven STs were detected (ST's10, 88, 162, 372, 405, 540, and 973) from multiple isolates with different antimicrobial resistant profiles and β-lactamase gene profiles (Table 3.4). An interesting finding was the presence of two different β-lactamase enzymes (CTX-M-14+CMY-2, CMY-2), and one *ampC* chromosomal mutation in three isolates identified as ST405. This finding is consistent with the literature (Borjesson *et al.* 2013), and is due to horizontal gene transfer. Interestingly, ST131 the predominant human pandemic ESBL-producing lineage causing urinary tract infections worldwide (Ewers *et al.* 2012) was not found in this study, although it has been identified in human infections in New Zealand and also in clinical infections in animals in the study presented in Chapter 2. This study also found six new STs carrying the $bla_{\text{CMY-2}}$ (of which one was a MDR).

The prevalence of MDR-E carriage of 2% found in this study was similar to the prevalence reported in healthy pets in Portugal using less than 100 faecal samples (Costa *et al.* 2008), but lower than the 31% and 15% prevalence found in two studies in healthy dogs in the UK where the sample size was 73 and 183 respectively (Wedley *et al.* 2011; Schmidt *et al.* 2015). The 0.3% prevalence of ESBL-E in this study is comparable with the results of two studies in dogs, where the prevalence was 0.8% in the US and 0.5% in the UK, respectively (Murphy *et al.* 2009; Wedley *et al.* 2011), and lower than the detected ESBL-E prevalence in numerous other studies in cats and dogs [in Portugal 1.3% (Costa *et al.* 2008); in UK 4.5% (Wedley *et al.* 2011) and 5.4% (Schmidt *et al.* 2015); in Japan 6.9% (Harada *et al.* 2011); in Algeria 11% (Yousfi *et al.* 2016); 16% in two studies in Tunisia and Brazil (Sallem *et al.* 2013; Rocha-Gracia *et al.* 2015); and in Nigeria 19% (Albrechtova *et al.* 2012)]. The 6.1% prevalence of AmpC-E is comparable with a study in the Netherland that sampled faeces of healthy cats and dogs where the prevalence was 7.5% (Hordijk *et al.* 2013), lower than the result of a study in Mexico that sampled only healthy dogs where the prevalence of AmpC-E was 15% (Rocha-Gracia *et al.* 2015), and higher than then the results of two studies in healthy dogs in the UK, where the prevalence was 3.8% and 4.1% respectively (Wedley *et al.* 2011; Schmidt *et al.* 2015). The difference in prevalence between the different studies, could be explained by

the sampled population, predisposing risk factors as hospitalisation and antimicrobial treatment, urban versus rural sampled animals, culturing methods, animal hygiene, etc.

Studies assessing the faecal ESBL-E prevalence in healthy humans ranged between 5 and 7% (Stromdahl *et al.* 2011; Ben Sallem *et al.* 2012; Geser *et al.* 2012). In New Zealand the human faecal carriage of ESBL-E was 5.1% (Upton *et al.* 2011). Although comparisons between human and animal studies might not be valid, due to the different hosts, but insight on the prevalence of ESBL-E of healthy humans and other animals' species (Guenther *et al.* 2010; Maddox *et al.* 2012) showed that the prevalence of ESBL-E detected in this study was relatively low.

Forty-five % (n=17/38) of the AmpC-E isolates identified in this study harboured the bla_{CMY-2} and were isolated from animals (n=395) that did not receive antimicrobials or had not undergone surgery or hospitalisation in the six month prior to the sampling. No ESBL-E were isolated from these 395 animals. The prevalence of AmpC-E in the non-treated subgroup of animals was 4.3%. This finding is comparable with the results of two studies in the UK (Wedley *et al.* 2011; Schmidt *et al.* 2015) that found a faecal carriage rate of 4.3% (95% CI: 3.3-10.7) and 4.1% (95% CI 0.1-6.94) of AmpC-E from healthy dogs.

Fifty-five percent (n=21/38) of the ESBL/AmpC-E isolates were identified from cats and dogs (n=177) that received antimicrobial treatment and/or hospitalisation and/or surgery in the last six months prior to sampling. The prevalence of ESBL/AmpC-E in the treated subgroup is 12% (as will be seen in Chapter 4, in this study, animals that had antimicrobial treatment/hospitalised/surgery were more likely to carry ESBL/AmpC-E than those that did not have these exposures). This is consistent with literature that assessed faecal carriage in cats and dogs in Denmark, UK and Switzerland (Damborg *et al.* 2011; Damborg *et al.* 2012; Johns *et al.* 2012; Decristophoris *et al.* 2013a). Comparison of the studies of prevalence of MDR-E or ESBL/AmpC-E between different countries should take into consideration due to the different culturing methods, sampling strategies and inclusion criteria used. The risk factors for shedding of ESBL/AmpC-E will be

further analysed using multivariable approaches in the study presented in Chapter 4.

As in Chapter 2, the minimum spanning tree revealed several clusters of STs, there was no single central node that could be interpreted as a founder and most connections between STs were weak (double, triple or quadruple locus variants), as expected from a highly recombining population (Turner & Feil, 2007).

No accepted gold standard method for the isolation of MDR-E or ESBL/AmpC-E bacteria from faecal matrixes existed at the time of this study, the culture method used was that described in Appendix 1. The subculture of only one colony by this study could be seen as a limitation, as sub-culturing more than one colony could in theory capture different *E. coli* strains, and more ESBL/AmpC-producing strains (Vieira *et al.* 2008; Leonard *et al.* 2012). Another limitation was the recruitment of 29 clinics instead of 61 clinics, in spite of the considerable efforts made to increase participation. Increasing the number of sampled animals would have increased the precision of the prevalence estimate. Nevertheless, with 587 faecal samples from 29 practices this is the largest prevalence study performed in cats and dogs, worldwide, to the best of the author's knowledge.

3.5 Conclusions

This study provides an estimate of the prevalence of MDR-E and ESBL/AmpC-E faecal carriage in cats and dogs in Auckland, New Zealand, as obtained by culture. There was no predominant ST or clonal complexes among the MDR-E or ESBL/AmpC-E carried in the study population. The isolation of different β-lactamase genes from the same STs reflects extensive horizontal gene transfer. The study contributed six novel *E. coli* STs harbouring bla_{CMY-2} to the Warwick database. Deeper genetic comparison may elucidate the relationships between faecal ESBL/AmpC-E and pathogenicity in New Zealand strains, and this will be further investigated in the study presented in Chapter 5. Chapter 4. describes an epidemiological study of risk factors for faecal carriage of MDR-E, ESBL/AmpC-E.

Supplementary material

Owners' questionnaire

Institute of Veterinary, Animal and Biomedical Sciences

Study to investigate the carriage of multidrug resistant bacteria by cats and dogs in Auckland

QUESTIONNAIRE FOR OWNERS OF PARTICIPATING PETS

Page 1

Key-code:

The date your pet was sampled: [ ]

Do you wish to receive a copy of your cat's / dog's test results from your vet?
(Circle the appropriate answer) Yes / No

1. Details regarding the animal that was sampled for this study.
   a. Species: (circle the appropriate answer) Cat / Dog
   b. Breed:
   c. Sex:
   d. Has your cat / dog been desexed? (circle the appropriate answer) Yes / No
   e. Age: [ ] Years [ ] Months
   f. Have you owned this cat / dog for at least 6 months? Yes / No

If no, where did your cat / dog come from?

	✔	
A breeder		
A friend / family member		
Animal shelter		
Other (please state):		

2. In which suburb or town do you and your cat / dog live?

[ ]

3. How many other animals and people live in the same house as your cat / dog?

	The total number	Their ages in years
Adults		
Children under 16y		
Cats		
Dogs		
Other animals (state species)		

4. Has the cat / dog that was sampled today had antibiotics in the last 6 months?

Yes / No

If yes, tick all that apply and provide some details	✔	Number of courses	Name of antibiotic/s
Yes – ear/eye drops			
Yes – oral tablets /powder/ liquid			
Yes – injection			

5. Has the cat / dog that was sampled today been hospitalised for over 24 hours in the past 6 months?

Yes / No

If yes for how long were they in hospital?

[ ] Weeks [ ] Days

Page 3

6. Has the cat / dog that was sampled today had a surgical operation in the past 6 months?

Yes / No

If yes, what operation did they have?

7. If there are other animals living in the house, over the last 6 months have any of them:

	✔
Joined the household?	
Been given oral antibiotics?	
Been hospitalised for over 24 hours?	
Had a surgical operation?	

8. Do you, or any other people living in the house work in a healthcare industry?

Yes / No

If yes, please indicate which sector/s people work in:

	✔
Veterinary practice	
Human hospital	
Human medical practice	
Other (e.g residential care facility, dental practice). Please state:	

9. Have you, or any other people living in the house, had to take a course of antibiotics in the last 6 months?

Yes / No

10. Have you, or any other people living in the house, been hospitalised for over 24 hours in the last 6 months?

Yes / No

11. Have you, or any other people living in the house, had a surgical operation in the last 6 months?

Yes / No

12. Have you, or any other people living in the house, travelled overseas during the last 6 months?

Yes / No

If yes, which country was visited?

Thank you for completing this questionnaire. ☺
The information you have provided will be treated as confidential and it will not be used in any way that would allow other people to identify you.
Please put the completed questionnaire into the envelope provided, seal the envelope and hand it to the person who took the samples from your cat/ dog.

Institute of Veterinary, Animal and Biomedical Sciences

Study to investigate the carriage of multidrug resistant bacteria by cats and dogs in Auckland

INFORMATION SHEET FOR VETERINARIANS

Dear

We are a group of veterinary clinicians, microbiologists and epidemiologists from Massey University starting a research project into multidrug resistant *E. coli* and methicillin-resistant *Staphylococcus aureus* (MRSA) in companion animals in the Auckland region and we're looking to recruit veterinary surgeons in clinical practice to assist with this work.

The global rise in multidrug resistant bacteria forms the background to this work, and such strains are causing increasing problems in human and veterinary medicine around the world. The picture in New Zealand is (currently) slightly better than in some other countries, but over the last decade there has been a dramatic rise in the number of multidrug resistant bacteria, such as extended-spectrum beta-lactamase producing *E. coli* or ESBL *E. coli*, that are being isolated from human clinical cases. Furthermore, this rise is particularly pronounced in Auckland and the surrounding regions. Some strains of ESBL producers are capable of colonising and causing infection in both humans and animals, and they have been demonstrated to be able to pass between humans and animals living in the same house.

ESBL *E. coli* cause extra-intestinal infections such as urinary tract, wound or post-operative infections. Typically, these infections will occur in elderly animals and in animals with concurrent medical problems. These bacteria can also persist in a hospital environment and become a cause of nosocomial (i.e. hospital acquired) infections, with animals undergoing extensive surgical procedures or numerous medical interventions (such as urinary catheterisation) being at primary risk of contracting infection.

In recent months, the Auckland Gribbles laboratory has reported three cases of ESBL-type *E. coli* in cats and dogs in the Auckland region. These were AmpC producing *E. coli* showing identical patterns of resistance and providing extremely limited treatment options. We're currently working with the seven veterinary diagnostic laboratories to monitor the number of infections with multidrug resistant *E. coli* and MRSA, across New Zealand.

We'd also like to gain more information about how frequently cats and dogs without clinical infections are acting as carriers of multidrug resistant *E. coli* and MRSA, or in other words – what is coming through your practice door? This information is important for designing infection control programmes within a vet practice, and also on a wider scale within the New Zealand community in general.

We would like to recruit 61 vets in the Auckland region to work with us to take nasal / perianal (dog / cat) swabs to screen for MRSA and rectal swabs (dog and cat) to screen for multidrug resistant *E. coli*. We would ask each participating veterinarian

to sample 30 cats and dogs coming into their clinic over a 2-3 week period. The vets will be required to explain the study to their owners and to obtain their written approval before taking the samples. There is also a short (3 page, 12 questions), confidential questionnaire for the owner to complete that asks non-specific questions about the recent medical and travel histories of the animals and people living in their house. We will screen the swab samples for the presence of multidrug resistant bacteria, test the bacteria isolated against a large number of antibiotics, and use PCR and other genetic methods to ascertain which resistance genes the strains are carrying and whether they are identical strains to those causing problems in human medicine.

We'd like you to consider joining this study and we will telephone you within the next fortnight to discuss this with you. We're also keen to hear about any clinical experiences you may have had with multidrug resistant bacteria. In the meantime, if you have any questions about this work please feel free to contact us.

With best wishes

Dr Eve Pleydell BVSc BSc PhD MRCVS
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MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

Institute of Veterinary, Animal and Biomedical Sciences

Study to investigate the carriage of multidrug resistant bacteria by cats and dogs in Auckland

Sampling Methods

METHODS

Each participating vet is being asked to recruit the animals belonging to 30 (thirty) owners over a 3 (three) week period. To prevent selection bias of owners, please recruit the first 5 (five) consenting owners of the day. We'd recommend sampling on Mondays, Tuesday and Wednesdays – to avoid the possibility of samples getting stuck in the postal system over the weekend.

There is an owner information sheet that can used to help inform owners about the study. For ethical reasons we need each participating owner to sign a written consent form. However, we do not need to know the identities of the owners, so please don't send the consent forms to us, but keep them safely filed in your practice.

We have ethical approval for vets or trained vet nurses to actually collect the swabs and to ask the owner to complete the anonymous questionnaire. Two swab samples are to be collected from each animal – one for MRSA and one for ESBL-producing Gram negatives.

SWABS REQUESTED FROM DOGS

1. Anterior nares

Staphylococcus aureus colonises skin and mucous membranes. So we require swabs of the inside surfaces of the nostrils – not deep nasal swabs or lumps of mucus.

Using aseptic technique, moisten the swab in sterile saline and gently roll it around the inside surface of the nostrils for 3-5 seconds. If you can swab both nostrils that's great, if not – well, we understand!
In the case of a very fractious dog, the perianal area could be sampled instead.

2. Faeces / Rectum

We're looking to detect faecal carriage of ESBL-producing *E. coli* and other Enterobacteriaceae.

Moisten the swab with sterile saline, insert it into the rectum and rotate it gently for 3-5 seconds.

SWABS REQUESTED FROM CATS

1. Perianal skin

Staphylococcus aureus also colonises the skin and mucous membranes around the anus. Roll a swab moistened in sterile saline right around the perianal skin for 3-5 seconds. Try not to contaminate the perianal swab with large amounts of faeces as this may decrease the uptake of surface bacteria.

2. Faeces / Rectum

As per the instructions for dogs, but please take the rectal swab after the perianal swab to minimise the possibility of contaminating the perianal skin with excess faecal material.

Please use a fresh vial of sterile saline for each animal you sample.

Institute of Veterinary, Animal and Biomedical Sciences

This is to minimise the possibility of contaminating the saline between animals

Submitting the swabs:

Label both swabs with the same ID number as the corresponding owner questionnaire; and indicate on each swab the site from which it was collected (i.e. nose, anal and rectal).

Please send the swabs to us as soon as possible after sampling (using the pre-addressed, prepaid, packaging materials provided); although swabs could be stored in the fridge for up to 48 hours if necessary. If you could avoid sending swabs on a Friday we' d appreciate it as they are likely to sit in the postal system until Monday, which could decrease or prevent successful bacterial culture.

If you have any questions, or need extra materials, then please contact us.

MANY THANKS!

Eve, Kate and Ali

Eve Pleydell — Eve: 06 356 9099 ext 81843

Kate Hill — Kate: 06 356 9099 ext 7448

Ali Karkaba (PhD candidate) — Ali: 06 356 0900 ext 5366

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Table S 3.1: Details of the 43 characterised MDR-E, and ESBL/AmpC-E and 14 Non-MDR/ESBL/AmpC-E

Isolate Number	Species	MLST ST	MLST CC	First Detected	β-lactamase identified	AMR	Cep	Amc	Fox	Ctx	Caz	Cvn	Cn	Enr	Sxt	Te	Atm
R41	C	10	ST10 Cplx	Bovine, Canada, 1982		MDR	r	r	s	s	s	s	s	r	r	r	s
R59	C	10	ST10 Cplx	Bovine, Canada, 1982	CMY-2	Y	r	r	r	i	r	r	s	s	s	s	i
R65	C	10	ST10 Cplx	Bovine, Canada, 1982	CMY-2	MDR	r	r	r	r	r	r	s	s	r	r	r
R64	C	38	ST38 Cplx	Human, Nigeria, 1995	CMY-2	MDR	r	r	r	r	r	r	s	r	s	r	i
R38	C	46	ST46 Cplx	Human, Nigeria, 1995	CMY-2	MDR	r	r	r	i	r	r	s	r	s	r	r
R67	C	57	ST57 Cplx	Chicken, Germany, 2004	CMY-2	MR	r	r	r	i	r	r	s	i	s	s	i
R55	F	58	ST155 Cplx	Bovine, Germany, 1998	CMY-2	MR	r	r	r	i	r	r	s	s	s	r	r
R13	F	73	ST73 Cplx	Human, Germany, 1917		N	r	r	i	s	s	r	s	s	s	s	s
R33	C	80	ST568 Cplx	Dog, Germany, 2004		N	r	r	s	s	s	s	s	s	s	s	s
R26	C	88	ST23 Cplx	Chicken, Germany,1998		MR	i	r	s	s	s	i	s	s	s	r	s
R27	C	88	ST23 Cplx	Chicken, Germany,1998		MR	i	s	s	s	s	s	s	s	s	r	s
R35	C	88	ST23 Cplx	Chicken, Germany,1998	TEM-1	MDR	r	s	s	s	s	s	s	s	r	r	s
R24	C	93	ST168 Cplx	Human, Ghana, 1995		N	s	s	s	s	s	s	s	s	s	r	s
R12	C	127		Horse, Germany, 1996/Dog, USA,1996		MR	r	s	s	s	s	s	s	s	s	r	s
R22	C	127		Horse, Germany, 1996/Dog, USA,1996		MR	r	r	i	s	s	r	s	s	s	r	s
R37	C	133		Dog, Germany, 1996	CMY-2	MR	r	r	r	i	r	r	s	s	s	i	i
R36	C	162	ST469 Cplx	Human, Ghana,1996	CMY-2	N	r	r	r	i	r	r	s	s	s	s	i
R63	C	162	ST469 Cplx	Human, Ghana,1996		N	s	s	s	s	s	s	s	r	s	s	s
R70	F	162	ST469 Cplx	Human, Ghana,1996	CMY-2	N	r	r	r	r	s	r	s	s	s	s	i
R14	C	206	ST206 Cplx	Human, Ghana,1996	*ampC* mutation	MDR	r	r	r	s	s	r	s	i	r	r	s
R48	F	212		Human, Ghana, 1996		MR	r	r	r	i	r	r	s	s	s	r	i
R61	C	295		Human, USA, 1956	CMY-2	MR	r	r	r	i	r	r	s	s	s	i	i
R69	C	297		Human, peru, 1970	CMY-2	N	r	r	r	r	r	r	s	s	s	s	r
R05	C	345		Cat, Germany, 2009		MR	i	s	s	s	s	s	s	s	s	r	s
R42	C	345		Cat, Germany, 2009		MR	i	s	s	s	s	s	s	r	s	r	s
R11	C	372		Chicken, Germany, 1985		N	s	s	s	s	s	s	s	s	r	s	i
R40	C	372		Chicken, Germany, 1985		MR	s	s	s	s	s	s	s	s	r	r	s
R62	C	372		Chicken, Germany, 1985	CMY-2	MR	r	r	r	i	r	r	s	s	s	r	i
R18	C	405	ST405 Cplx	Human, USA, 1999	CTX-M14	MDR	r	i	s	r	s	r	s	r	r	r	i

Isolate Number	Species	MLST ST	MLST CC	First Detected	β-lactamase identified	AMR	Cep	Amc	Fox	Ctx	Caz	Cvn	Cn	Enr	Sxt	Te	Atm
R60	F	405	ST405 Cplx	Human, USA, 1999	*ampC* mutation, TEM-1	N	r	r	r	r	r	r	s	s	s	s	i
R66	C	405	ST405 Cplx	Human, USA, 1999	CMY-2, CTX-M14	MDR	r	r	r	r	i	r	s	r	r	r	r
R74	C	448	ST448 Cplx	Human, Nigeria, 2005		MDR	s	s	s	s	s	s	s	r	r	r	s
R54	C	457		Human, UK	CMY-2	MR	r	r	r	r	r	r	s	s	s	i	r
R56	C	457		Human, UK	CMY-2	MR	r	r	r	r	r	r	s	s	s	r	i
R75	C	457		Human, UK	CMY-2	MR	r	r	r	r	r	r	s	s	s	i	r
R51	C	501		Human, Nigeria, 1995	CMY-2	MR	r	r	r	i	r	r	s	s	s	i	i
R49	F	540		Human, Germany, 2001	CMY-2, TEM-1	MR	r	r	r	r	r	r	s	s	s	r	i
R78	C	540		Human, Germany, 2001	CMY-2	N	r	r	i	i	r	r	s	s	s	s	s
R58	F	590	ST590 Cplx	Human, Norway, 2002	*ampC* mutation	MR	r	r	r	i	r	r	s	s	s	r	r
R72	C	744		Human, France, 2006		MDR	i	s	s	s	s	s	s	r	r	r	s
R30	C	746		Human, Egypt 2001		N	s	s	s	s	s	s	s	s	s	r	s
R34	F	929		Human, UK	CMY-2	N	r	r	r	i	r	r	s	s	s	s	s
R47	C	963		Environment, France, 2011	CMY-2	MR	r	r	r	r	r	r	s	s	s	r	i
R53	C	963		Environment, France, 2011	CMY-2	MR	r	r	r	r	r	r	s	s	s	i	i
R39	F	973		Chicken, Germany, 2002	CMY-2, TEM-1	N	r	r	r	r	r	r	s	s	s	s	i
R44	C	973		Chicken, Germany, 2002	CMY-2	MR	r	r	r	i	r	r	s	s	s	i	i
R45	C	973		Chicken, Germany, 2002		N	r	r	s	s	s	s	s	s	s	s	s
R50	F	973		Chicken, Germany, 2002	CMY-2	MR	r	r	r	i	r	r	s	s	s	i	i
R68	C	973		Chicken, Germany, 2002	CMY-2	N	r	r	r	i	r	r	s	s	s	s	i
R71	F	2541		Human, USA	CMY-2	N	r	r	r	r	r	r	s	s	s	s	i
R32	C	2712		Water, Australia	CMY-2	N	r	r	r	i	r	r	s	s	s	s	i
R46	C	4390/New		New Zealand, 2013	CMY-2	MR	r	r	r	r	r	r	s	s	s	i	i
R52	C	4391/New		New Zealand, 2013	CMY-2	MR	r	r	r	i	r	r	s	s	s	i	i
R57	C	4392/New		New Zealand, 2013	CMY-2	MDR	r	i	r	s	s	i	s	r	r	r	s
R76	C	4393/New		New Zealand, 2013	CMY-2	MR	r	r	r	r	r	r	s	r	s	s	i
R77	C	4395/New		New Zealand, 2013	CMY-2	MR	r	r	r	r	r	r	s	s	s	r	i

Isolate Number	Species	MLST ST	MLST CC	First Detected	β-lactamase identified	AMR	Cep	Amc	Fox	Ctx	Caz	Cvn	Cn	Enr	Sxt	Te	Atm
R43	F	4406/New		New Zealand, 2013	CMY-2	N	r	r	r	i	r	r	s	s	s	s	i

C: Canine; F: Feline; AMR: Antimicrobial resistance; MDR: multidrug resistant; MR: isolates that are resistant to two antimicrobial families;
N: isolates that are resistant to one antimicrobial family; F: totally susceptible; E: no-β-lactamase gene was detected by the PCR protocols used; MLST: multi locus sequence type

CEP: Cephalothin; AMC: Amoxicillin-clavulanic acid; FOX: Cefoxitin; CTX: Cefotaxime; CAZ: Ceftazidime; CVN: Cefovecin; TE: Tetracycline; CN: Gentamicin; SXT: Sulphamethoxazole-Trimethoprim;
ENR: Enrofloxacin; ATM: Aztreonam.
Six New MLST strains 4390-92,4393,4395 and 4406

Chapter 4: Risk factors for faecal carriage of multidrug resistant, extended spectrum β-lactamase and AmpC β-lactamase-producing *Escherichia coli* in cats and dogs in Auckland, New Zealand

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Preamble

The previous chapter reported the prevalence of ESBL/AmpC-E in cats and dogs in Auckland, New Zealand. The microbiological data obtained combined with the questionnaire-derived data obtained from the animal owners were used to assess the risk factors associated with the faecal carriage of ESBL/AmpC-E in cats and dogs in the study.

Summary

Companion animals could be reservoirs for antimicrobial-resistant bacteria, and faecal shedding of resistant bacteria may represent a risk for animal and human health. Moreover, as dogs and cats co-exist with humans in the same households, dogs and cats may serve as sentinels of antimicrobial resistance.

Pets are integral parts of modern households, and it is well known that pets may be colonised by the same organisms colonising or causing infections in humans. Hence, studying the factors associated with carriage of antimicrobial resistant bacteria in pets may indirectly elucidate ecological selective pressures exerted on the normal flora. Yet, studies assessing the factors associated with faecal carriage of ESBL-E and AmpC-E in cats and dogs are few. In the study presented here, we aimed to assess the risk factors associated with faecal carriage of multi-drug resistant *Escherichia coli* (MDR-E) and ESBL/AmpC-producing *E. coli* (ESBL/AmpC-E) in cats and dogs presenting to veterinarians in Auckland, New Zealand. A cross-sectional study of 225 cats and 361 dogs was performed, with the participation of 29 veterinary clinics. Faecal samples were cultured to isolate

MDR-E, ESBL-E and AmpC-E. Data collected from the owners by questionnaire were analysed by multivariable statistical models to investigate the factors associated with the presence of MDR-E and ESBL/AmpC-E.

A total of 572 completed questionnaires were used for the analysis. Variables independently associated with increased odds of carriage of MDR-E/ESBL-E/AmpC-E were: receiving an antimicrobial treatment in the previous six months (versus untreated animals: OR=1.5, 95% CI: 1.2-1.9, p=0.001), a household member working in a veterinary clinic (versus not working: OR=2.87, 95% CI: 1.1-7.6, p=0.03), and a household member having travelled overseas in the previous six months (versus didn't travelled: OR=2.3, 95% CI: 1.1-4.8, p=0.02). No differences were detected between cats and dogs, or between the sex of the animals. These results corroborate previous observations indicating travelling and working in the healthcare setting as risk factors for MDR-E/ESBL-E/AmpC-E carriage also in humans. Further studies are needed to investigate transmission of antimicrobial-resistant bacteria between pets and their owners.

4.1 Introduction

Antimicrobial resistance is an emerging and complex problem in both human and veterinary medicine. It can lead to therapeutic failure and increased morbidity and mortality associated with a high economic and social burden (Wedley *et al.* 2011). The use of antimicrobials may exert selection pressure on normal intestinal flora, and therefore select for antimicrobial resistant (AMR) bacteria (Wedley *et al.* 2011). Horizontal gene transfer (HGT) of antimicrobial resistance determinants through plasmids, integrons and transposons between various bacterial species are integral to the dissemination of antimicrobial resistance (van Hoek *et al.* 2015). Due to the clonal relatedness between antimicrobial resistant *E. coli* (AMR-E) strains colonising the gut of companion animals and humans found in the study

presented in

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Chapter 3, and overseas (Johnson and Russo 2002; Guo *et al.* 2015), and the close physical interaction between companion animals and humans in the households, there is a risk of transmission of resistance genes between pathogens and commensals of both hosts (Guardabassi *et al.* 2004; Procter *et al.* 2014).

MDR-E and ESBL/AmpC-E have been identified in faeces of healthy and clinical cats, dogs, and food producing animals (Ho *et al.* 2011). Faecal carriage and shedding of ESBL/AmpC-E in dogs can last more than six months (Baede *et al.* 2015; Espinosa-Gongora *et al.* 2015). Human faecal carriage of ESBL-E producing bacteria has been associated with increased risk of endogenous urinary tract infections in women (Niki *et al.* 2011). A study by Alsterlund *et al.* (2012) assessing the ESBL-E faecal carriage of hospitalised patients detected ESBL-E in the faeces of 12% of the 42 patients over a period of 30 months after hospitalisation. In animals, a recent study investigating faecal carriage of ESBL-E for six months in dogs with clinical signs of infection found that that 30% of the dogs shed ESBL-E over the entire study period (Baede *et al.* 2015). Other studies showed that hospitalised dogs affected with diarrhoea or undergoing antimicrobial treatment were more likely to shed ESBL/AmpC-E in faeces than other dogs (Gibson *et al.* 2011; Wedley *et al.* 2011; Belas *et al.* 2014).

Pets are integral parts of modern households. Hence, understanding intestinal colonisation with antimicrobial resistant bacteria in pets may enhance our understanding of the selective pressures determining increasing antimicrobial resistance in bacteria colonising the human gut. Moreover, studying the factors associated with carriage of antimicrobial resistant bacteria in pets may be a valid surrogate to studies in humans, which pose serious ethical challenges associated with the collection of the samples, and privacy. The carriage of MDR-E and ESBL/AmpC-E in the faeces of pet also poses a public health risk from pet ownership, given that in New Zealand 68% of households own at least one pet animal (Anonymous 2011). Hence, in the study described in this chapter we aimed to determine the risk factors for carriage of multidrug resistant *E. coli* (MDR-E) and ESBL/AmpC-E in cats and dogs presenting to veterinary clinics in the

Auckland region, using multivariable statistical modeling of data elicited by the questionnaire delivered during the faecal sampling described in

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4.2 Materials and methods

4.2.1 Data collection, sample processing and questionnaire analysis

A cross-sectional study assessing the carriage rate of MDR-E, ESBL/AmpC-E in cats and dogs in Auckland was conducted during the period June 2012-

June 2013, and has been presented in

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Chapter 3. Twenty-nine veterinary clinics sampled 586 cats and dogs from different households. A single faecal swab was obtained from animals attending the veterinary clinic, and the owners completed a short questionnaire. The administration of the questionnaire, microbiological and

molecular methods and results are described in

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In brief, faecal swabs were cultured at Massey University for the isolation of MDR-E, ESBL-E and AmpC-E. Antimicrobial susceptibility was determined to 12 antimicrobials according to the CLSI guidelines (CLSI 2013). The following antimicrobials were tested: cephalothin, amoxicillin-clavulanic acid, cefoxitin, cefotaxime, ceftazidime, cefovecin, imipenem, tetracycline, gentamicin, sulphamethoxazole/trimethoprim, enrofloxacin, and aztreonam. This testing was followed by a molecular characterisation of the ESBL-E and AmpC-E isolates by multilocus sequence typing (MLST) and by PCR-

sequencing of the ESBL and AmpC genes (

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Chapter 3).

The information elicited by the questionnaire and the microbiological results were entered into a predesigned electronic database "IRIS", and were extracted in an Excel format. Questionnaire responses were coded into variables using an electronic spreadsheet in IRIS (http://www.epimanager.com/Epicentre/en-nz/LogOn). An audit for data entry was performed by a third person by random sampling of 170 (29.6%) questionnaires, and minor errors (such as spelling mistakes) were detected in five (2.9%) questionnaires. The data were analysed using R statistical software (R version 3.0.3, Copyright© 2014, The R Foundation for Statistical Computing).

4.2.2 Multivariable statistical modeling

In this study, the data from cats and dogs were initially analysed together after dropping host-specific variables such as breed, similarly to a study by Decristophoris *et al.* (2013a). The data on cats and dogs were subsequently analysed separately, and in these analyses the host-specific variables were maintained.

Data elicited by the questionnaire were coded into dichotomous or categorical variables using standardised definitions. The animals' age was defined as a continuous variable in years and fractions of years. For example, the age of one year and three months was coded as 1.25. The age of the people living in the household was categorised into five categories in years (<16; 16-30; 31-45; 46-65; >65). Three routes of antimicrobial administration were defined in the questionnaire (topical ear and/or eye treatment; oral treatment; treatment by injection). All the variables included in the analysis of Q1 and Q2 are described in Table 4.1 and Table 4.2, respectively.

We used logistic regression models to address the following main research questions.

Q1- Analysis of risk factors for faecal carriage of ESBL and/or AmpC-E. This analysis assessed the presence of independent associations of explanatory variables with faecal carriage of ESBL/AmpC-E in cats and dogs.

Q2-Analysis of risk factors for faecal carriage of MDR-E and/or ESBL and/or AmpC-E (MDR/ESBL/AmpC-E): This analysis assessed the presence of independent associations of explanatory variables with faecal carriage of MDR/ESBL/AmpC-E (including any MDR, ESBL and/or AmpC-E) in cats and dogs.

The analysis was step-wise approach with an initial screening for associations between single variables and the outcome variables of presence of ESBL-E and/or AmpC-E (for Q1); or presence of MDR/ESBL/AmpC-E (for Q2). The analysis continued with multivariable modeling using the same outcome variables of Q1 and Q2, with veterinary clinic modeled as random effect to account for within-clinic correlation.

The initial univariate screening was performed using Laplace approximation and adaptive Gauss-Hermite quadrature to adjust for the lack of independence of observations coming from the same clinic, using “glmer” function in ‘lme4’ package (Bates *et al.* 2014). All the variables resulting in p-value<0.25 were included in the next modeling step, where mixed-effects logistic regression models were built. The biologically important variables ‘age’, ‘sex’ and species (cat/dog) were also included and kept in all the models and in the final model, regardless of their p-value. The explanatory variables of interest were included in the models as fixed effects, with clustering of animals within the veterinary clinics accounted for, using a second level random intercept, as in the following model:

$$\boldsymbol{Y_i = \beta_0 + \beta_1 x_{1j} + \cdots + \beta_n x_{nj} + u_{clinic(i)} + \varepsilon_i} \qquad (4.1)$$

where:

Y = Outcome variable

β = *regression coefficient* (β_0 = intercept; $\beta_{1 \ldots n}$ = variable coefficients)

$x_{1i}, \ldots, x_{ki}$ = explanatory variables

u_i = random effect of clinic (which contains the jth animal), defined with as a normal distribution of a mean of zero and a variance of $\pi^2/3$

ε_{i} = residual, defined as $\sim Normal(0, \sigma^2)$

σ^2 = Variance of residuals

Manual backward stepwise elimination was used for model building. Initially, some variables with large standard errors and p-values had to be removed manually in order to allow the model to converge (Table 4.3). Then, the process progressed by elimination of the variable with the highest p-value, re-introduction of the previously eliminated variable and selection of the model (between the two) with the smallest Akaike Information Criterion score (AIC) (Müller *et al.* 2013). This modeling progressed until only variables with $p<0.05$ remained. The Wald test (function “waldtest” in ‘aod’ package; (Lesnoff and Lancelot 2012) was used to assess model fitness in the last iterations. Caterpillar plots were produced to visualise the random effects of clinics, using the function “ranef” in the ‘lattice’ package of R (Sarkar 2008). The plots display the difference between the statistical estimate of the effect of each clinic, and the mean effect of ‘clinic’ in the sample, and show the 95% confidence of this difference.

4.3 Results

4.3.1 Prevalence of MDR-E and ESBL/AmpC-E

A total of 586 owners agreed to participate and have their cat or dog sampled. A total of 361/586 (61%) dogs and 225/586 (39%) cats were sampled. The animals were recruited from 29 veterinary clinics. In total, 572/586 (97%) questionnaires were completed from 28 clinics. The prevalence of faecal carriage of MDR-E, ESBL-E and AmpC-E was 7.3% (43/586), 0.3% (2/586) and 6.4% (36/586), respectively. Among cats, the prevalence of faecal carriage of MDR and ESBL/AmpC-E was 6.2% (14/225) and 4.7% (12/225), respectively. Among dogs, it was 8% (29/361) and 7.2% (26/361), respectively.

4.3.2 Data analysis

4.3.2.1 Demographic characteristics of the sampled cats and dogs

The median age of the sampled animals was 5 years (range: 3 months - 13.5 years for dogs; 4 months - 17 years for cats). The majority of cats and dogs were neutered (n=515, 90%), and were owned by the same owner for at least six months prior to the sampling (n=487; 85%). A total of 151 breeds

were represented in the dataset (129 dog breeds; 23 cat breeds). Dogs breeds were grouped into seven categories (terriers, gundogs, hounds, working, utility, non-sporting and cross breeds) used by the New Zealand kennel club (http://www.nzkc.org.nz/;accessed on June 2014**).** For cats, the breed classification was based on the owners' response in the questionnaire, which included the options: 1, domestic short hair (DSH) breeds; 2, pure breeds (any); 3, other breed. Gundogs (Group 3) was the most prevalent group of dog breeds (n=70; 20%), while DSH was the most prevalent group among cats (n=123; 56%). The characteristics of the sampled animals are presented in Table S 4.1Table S 4.2 in Section 4.6.

Most households (n=548; 95%) included at least two adults (>16 years old). The age of the people living in the households ranged between 1 month and 82 years old, of which the 50-55 years-old was the most common age group. Nearly 32% (n=183) of the owners reported at least one child living in the same house. In addition to the animal sampled, 72% (n=412) of the owners had at least one more animal. There were other species, such as horses, chicken, pigs, lizards, and birds in ~13% (n=74) of the households. The number of people and animals living in the same household or within the household environment are reported in Table S 4.3 (Section 4.6). Further details about the age of the people and animals living in the same household are reported in the CD in Appendix.

According to the responses to the questionnaires, 31% (n=177/572) of the animals (dogs=122/351; cats=55/221) had at least one antimicrobial treatment in the 6 months preceding the sampling. Most of these treated animals had a single course of antimicrobials (Table S 4.4; Section 4.6). Systemic antimicrobials (that is, injectable and oral treatments) were prescribed to 26% (n=147) of the sampled animals, of which oral treatments were more common, and were administered to 112 (20%) animals, compared with 75 (13%) animals receiving injections. Also, oral antimicrobials were more commonly prescribed for dogs (n=80*;* 25%) than cats (n=24; 11*%*), whereas there was a similar rate of treatment by injection in dogs (n=42; 12%) and cats (n=27; 12.2%). Dogs had a higher frequency of non-systemic antimicrobial treatments through ear and eye drops than

cats. Details of the frequencies of the different routes of antimicrobial administration are described in Table S 4.4, Section 4.6.

Most of the antimicrobials used for systemic treatment belonged to the β-lactam family (n=108/147; 74%) (Table S 4.4 and Table S 4.5, Section 4.6). Amoxicillin-clavulanic acid (63%, n=92/147) was the predominant oral compound and cefovecin (15%, n=22/147) was the predominant injected compound. There was also difference between the prescription patterns for cats and dogs. For instance, amoxicillin-clavulanic acid was more commonly prescribed for dogs as oral and injectable preparations, whilst cefovecin injections were more commonly prescribed for cats. On the contrary, most non-systemic antimicrobials prescribed belonged to aminoglycosides (38%, n=16/42) and polymixin B (28.5%, n=12/42). A list of the antimicrobial drugs used for the treatment of the sampled animals grouped by the antimicrobial family are described in (Table S 4.5, Section 4.6).

Overall, 6% (n=34/572) of the animals were hospitalised for more than 24 hours and 13.6% (n=78/572) underwent surgery in the six months preceding the sampling. Surgeries performed were classified into four categories: dental, de-sexing, orthopaedics, and soft-tissue. Soft tissue surgery was the most frequent among dogs (43%; n=23/53) whilst de-sexing surgery was the most frequent among cats (40%; n=8/25). Antimicrobial treatment was administered to 4.5% (n=26/572) of the sampled animals, but to 72% (n=56/78) of the animals that underwent surgery. The highest frequency of antimicrobial prescriptions by surgery type was associated with soft tissue surgery (90.3%; n=28/31). De-sexing had the least prescription frequency among all surgery types, with 50% of the de-sexed animals (n=13/26) receiving antimicrobial treatment. Further details about the hospitalisation and surgeries and antimicrobial prescription patterns are described in Table S 4.6 (Section 4.6).

Other information, such as co-habitation of other animals in the same household and other antimicrobial uses in the household, travel patterns, etc., was used for the risk factor analysis presented below and are reported in Table S 4.7 and Table S 4.8 (Section 4.6).

4.3.3 Analysis of results:

4.3.3.1 Factors for faecal carriage of ESBL/AmpC-E (Q1) and MDR-E (Q2) in cats and dogs

Univariate analysis identified nine variables associated with ESBL/AmpC-E carriage with a $p<0.25$ (Table 4.1). Three of these remained in the final model after backwards elimination and were independently associated with the odds of faecal carriage of ESBL/AmpC-E in cats and dogs. These were: 'antimicrobial treatment (systemic treatment) in the six months prior to sampling' (treated animals: OR=1.5, 95% CI: 1.2-1.9, p=0.001); people living in the household 'working in a veterinary clinic' (working: OR=2.87, 95% CI: 1.1-7.6, p=0.03); owner or household members travelled/did not travel overseas (travelled: OR=2.3, 95% CI: 1.1-4.8, p=0.02). The variables 'species' 'sex' and 'age' were not significant (Table 4.3). The caterpillar plot did not identify significant variations between random effects of most of the clinics, except for two clinics "SC" and "CH" (Figure 4.1). Further investigation indicated that these clinics contributed more positive samples than any other clinic ('SC' contributed 7/38 and "CH" 6/38 ESBL/AmpC-positive animals). Moreover, 7/7 and 5/6 ESBL/AmpC-positive animals from clinics 'SC' and "CH" belonged to owners working in veterinary clinics.

Subsequently, the binary variable 'antimicrobial treatment' in the six months prior to sampling' was replaced by a new three-level categorical variable, 'antimicrobial family' (values: β-lactam; any other treatment; no treatment). In this model, the odds of administering a β-lactam antimicrobial or any other treatment were significantly higher than in non-treated animals (Table 4.3). A number of biologically meaningful interaction terms were iteratively fitted in the final model, but these were not significant.

For Q2, the variables that remained in the final model were the same as in Q1 (Table 4.4). The caterpillar plot did not identify significant variations between random effects of most of the clinics (Figure 4.2). When the variable 'animal treated with an antimicrobial in the six months prior to sampling treatment' was replaced with 'antimicrobial family', the model did not converge.

4.3.3.2 Factors associated with faecal carriage of ESBL/AmpC-E (Q1) and MDR-E (Q2) in cats

Univariate analysis identified seven variables associated with ESBL/AmpC-E carriage with a p<0.25 in the sampled cats (Table 4.1). Three of these variables had a p<0.05 in the final model after backwards variable elimination and were independently associated with increased odds for faecal carriage of ESBL/AmpC-E in cats. These were: cat received antimicrobial treatment (OR=0.8; 95% CI: 0.3-2.2, p=0.02); cat was hospitalised and/or had surgery in the last six months (OR=8.7; 95% CI:1.3-55, p=0.02); people living in the household 'working in a veterinary clinic' (working; OR=5.2, 95% CI: 0.8-32, p=0.06). The variables 'sex' and 'age' were not significant (Table 4.3). Biologically plausible interaction terms were fitted in the final model. The interaction age*antimicrobial treatment was significant (OR= 1.1; 95% CI: 1.02-1.2; p<0.01), suggesting that the effect of antimicrobial treatment on carriage increased with the age of cats. The final model with this interaction had a better fit than the model without it (AIC= 87; lower deviance; p=0.02 by ANOVA).

To assess the odds of different antimicrobial treatments for the faecal carriage of ESBL/AmpC-E, the variable 'antimicrobial treatment' was withdrawn from the final model and a new categorical variable, 'antimicrobial family' (of values: β-lactam; any other treatment; no treatment) was introduced (Table 4.3). The models did not converge due to a low number of positive faecal samples from cats treated with non-β-lactam antimicrobials, and among non-treated cats.

The caterpillar plot did not indicate significant variations between random effects of the clinics, suggesting that most factors were captured in the final model. However, the effect of the top eight clinics in the graph was slightly shifted towards the right (

Figure 4.3). This suggests that some of the determinants of faecal carriage of ESBL/AmpC-E by cats may have not been captured by the final model.

The variables that remained significant in the final model for Q1 were also significant in the model for Q2. However, the interaction age*antimicrobial treatment was not significant. The caterpillar plot did not identify significant

variations between random effects of most of the clinics, except for clinics "TM" (Figure 4.4). Further investigation showed that clinic "TM" contributed more positive samples (3/12 MDR/ESBL/AmpC-positive animals) than any other clinic.

4.3.3.3 Factors associated with faecal carriage of ESBL/AmpC-E (Q1) and MDR-E (Q2) in dogs

Univariate analysis identified seven variables associated with ESBL/AmpC-E carriage with a p<0.25 in dogs (Table 4.1). Three of these variables had a p<0.05 in the final model after backwards variable elimination. These were: 1, sex of dog (female entire /male neutered, OR = 6.5, 95% CI:1.7-25.2, p=0.009); 2, antimicrobial treatment in the previous six months (treated: OR=1.5, 95% CI: 1.1-2.2, p=0.03); international travel of household member (travelled: OR=2.2, 95% CI: 0.8-5.6, p=0.08) (Table 4.3). Biologically plausible interaction terms were fitted and were not significant. The caterpillar plot indicated that there were variations between the random effects of the clinics as in the model for cats and dogs (Figure 4.5).

To assess the odds of different antimicrobial treatments with the faecal carriage of ESBL/AmpC-E in dogs, the variable 'antimicrobial treatment' was withdrawn from the final model and a new categorical variable: 'antimicrobial family' (β-lactam; any other treatment; no treatment) was introduced as in the models for cats and dogs (Table 4.3). In this model, the odds of faecal carriage of ESBL/AmpC-E were higher in dogs receiving a β-lactam antimicrobial than in non-treated or in treated with non-β-lactams (Table 4.3).

For Q2, the only variables that remained significant were 'dog hospitalised and/or had surgery in the last six months' (hospitalised, OR=2.92, 95% CI: 0.9-9.3, p=0.01<); sex of dog (female entire/male neutered, OR = 3.9, 95% CI: 1.1-14, p=0.04); 2, antimicrobial treatment in the previous six months (treated, OR=1.9, 95% CI:1.4-2.6, p=<0.01).

4.4 Discussion

In this chapter, we assessed the factors associated with faecal carriage of MDR-E and ESBL/AmpC-E in cats and dogs presenting to veterinarians in Auckland. The multivariable analysis that analysed cats and dogs together

indicated that a cat/dog had increased odds of carriage of MDR and/or ESBL/AmpC-E if it lived in a household with people working in a veterinary clinic or with people that had travelled overseas in the six months prior to the cat/dog being sampled. The cat/dog receiving an antimicrobial treatment in the six months prior to the sampling was also associated with increased odds of carriage. When cats and dogs were analysed separately, some of these associations became non-significant, mainly due to sample size reduction. The significant variables were the same in Q1 (associations with presence of ESBL/AmpC-E) and Q2 (associations with presence of MDR/ESBL/AmpC-E), mainly to the small difference between the two groups (38 positives in Q1 versus 43 in Q2).

In our study, 25% of animals received antimicrobial treatment in the six months preceding the sampling. Almost 55% (21/38) of ESBL/AmpC-E positive animals had antimicrobial treatment (n=21/147;14.2%) in the previous six months versus 45% (n=17/38) among the systemically treated (n=17/426; 3.9%). The univariate analysis indicated that cats and dogs that had antimicrobial treatment were more likely to carry MDR-E and ESBL/AmpC-E (OR=3.8, 95% CI: 1.8-7.7). After adjusting for the main effects, confounding variables and the clinic random effect in the multivariable analysis, the odds of faecal carriage of MDR-E and ESBL/AmpC-E in cats and dogs decreased, but remained significant (OR=1.36, 95% CI: 1.1-1.7). This is consistent with previous findings in humans, cats and dogs where antimicrobial treatment was associated with the acquisition, faecal carriage and shedding of AMR-E (Decristophoris *et al.* 2013a; Espinosa-Gongora *et al.* 2015; Johard *et al.* 2015; Zhao *et al.* 2016). A study by Damborg *et al.* (2011) found that treatment of dogs with cephalexin selected for strains carrying CMY-2 (an AmpC β-lactamase) genes in intestinal bacteria.

The most commonly prescribed antimicrobials in the study population were amoxicillin-clavulanic acid among oral treatments and cefovecin among injections, followed by oral fluoroquinolones (predominantly enrofloxacin) (Table 5, Section 4.6). This is similar to the results of a survey of antimicrobial usage by veterinarians in New Zealand for the treatment of

skin, ear and urinary tract infections (Pleydell *et al.* 2012), where β-lactams (amoxicillin-clavulanic acid and cephalexin) were the most widely used, followed by fluoroquinolones. The effect of a single antimicrobial families could not be analysed separately due to low sample size within each group. However, the odds of shedding ESBL/AmpC-E in animals treated with β-lactam (compared with the non-treated), was similar to that of animals treated with non- β-lactams (OR=2.5 and 2.9, respectively). Although hospitalisation is a known risk factor for faecal carriage of ESBL/AmpC producing bacteria in humans and dogs (Gibson *et al.* 2011; Nakai *et al.* 2016), in this study this factor was only significant in the cats models (Q1 and Q2). A break through the numbers showed that there was a low number of hospitalised animals in general among cats and dogs, and most of the hospitalised stayed less than a week.

Reports have indicated that human healthcare workers have an approximate 30% faecal carriage rate of ESBL/AmpC-E (Adler *et al.* 2014; Bassyouni *et al.* 2015) in comparison with the rate among people in the community, that ranged between 5-7% (Stromdahl *et al.* 2011; Ben Sallem *et al.* 2012; Geser *et al.* 2012). This might be due to the frequent contact with infected individuals and the selection of resistant organisms in the hospital environment due to extensive use of antimicrobials. Healthcare workers may transmit ESBL/AmpC-E to the hospitalised patients (Adler *et al.* 2014) and vice versa. Interestingly, in our study the presence of household members working in a human healthcare system, whether in hospitals, medical or dental clinics, was not associated with increased odds of isolation of ESBL/AmpC-E and MDR/ESBL/AmpC-E in cats and dogs. On the contrary, we found that cats and dogs originating from households where at least one person worked in a veterinary clinic had increased odds of ESBL/AmpC-E and MDR/ESBL/AmpC-E carriage (OR=2.94, 95% CI: 1.2-7.2). To our knowledge, this is the first report of this risk factor for ESBL/AmpC-E and MDR/ESBL/AmpC-E colonisation in dogs and cats. An explanation of this risk factor could be the repeated visits of veterinary staff pets to the clinics, posing a high risk of contact with different animals, nosocomial transmission of pathogens, and contact with the veterinary clinic personnel. In fact, a study by Decristophoris *et al.* (2013a) found that cats and dogs visiting

veterinary clinics were at a higher risk of faecal carriage (OR=4.4) of ESBL-producing Enterobacteriaceae than cats and dogs visiting nursing homes of people. An Australian study by Sidjabat *et al.* (2006) described the spread of clonal MDR-E in a veterinary teaching hospital. The study identified a distinct clonal MDR-E from the faeces of hospitalised dogs and in two veterinary staff. This could suggest that veterinary personnel may also be at a higher risk of MDR-E carriage than the general population. It is important to note that in our study the number of owners that worked in veterinary clinics was relatively high at (n=52; 9%), and this may have introduced bias.

International travel (in particular to the Indian subcontinent), has been associated with increased risk of intestinal colonisation and infection with ESBL-producing Enterobacteriaceae (Tängdén *et al.* 2010; Kaspar *et al.* 2015; Valverde *et al.* 2015). Travel-associated factors linked with increased risk of human faecal carriage of ESBL/AmpC-E include the median length of the trip (longer visits increase the risk), poor sanitation in the visited country, visitor suffering from gastroenteritis during the visit, use of antimicrobials during the visit, and surface contact of toilets in aeroplanes and airports (Tängdén *et al.* 2010). Likewise, in New Zealand, a study by Freeman *et al.* (2008) reviewed cases of community-onset urinary tract infections of females due to ESBL-E, and found that some cases had a recent travel to Indian subcontinent without previous hospitalisation in New Zealand. In the current study, travelling overseas of any person inside the household was associated with increased odds of faecal carriage of MDR/ESBL/AmpC-E of the cat or dog (OR=1.69, 95% CI: 0.8-3.4). Most of the travelling was to Australia (n=105/182; 57%), followed by Asia (n=28/182; 15%).

There were no differences in the risk factors associated with faecal carriage of MDR and ESBL/AmpC-E between cats and dogs. This is consistent with the results of a study that assessed colonisation of ESBL-producing Enterobacteriaceae in cats and dogs (Decristophoris *et al.* 2013a). Females (neutered and entire) were at higher risk of carrying MDR, ESBL/AmpC-E than males (neutered and entire) in both dogs and cats (OR=3.9, 95% CI: 1.1-14). The reason behind this higher risk is not fully understood. Female sex has been recognised as a risk factor for faecal carriage of ESBL/AmpC-

E in dogs (Belas *et al.* 2014), and ESBL-producing Enterobacteriaceae in humans (Friedmann *et al.* 2009). A hypothesis is that females have a higher incidence of bacterial infections as urinary tract infections, thus, are more prone to antimicrobial treatment, which leads to the selection of antimicrobial resistant bacteria in the gut (Belas *et al.* 2014). In this study, the frequency of MDR/ESBL/AmpC-E positive animals did not differ between males and females among treated and non-treated animals (14.1% of females and 13.5% of males among treated group, versus 4.3% of females and 3% of males among non-treated group). However, this rate of MDR/ESBL/AmpC-E positive animals differs substantially between gender groups (among treated group: male neutered 13.8%, female neutered 8.8%, male entire 4.1%, female entire 66%; versus among non-treated group: male neutered 2.8%, female neutered 4%, male entire 0%, female entire 4.1%).

Interestingly, the number of humans in the household, number of animals in the household beside the recruited one, animals visiting the household and/or animals treated with antimicrobials or animals had surgery were not significant in the models. This is consistent with a study by Procter *et al.* (2014) that assessed risk factors for AMR-E faecal carriage in domestic dogs

In an attempt to assess if there were differences in the risk of the faecal MDR/ ESBL/AmpC-E carriage in cats and dogs, the data were analysed separately by species. The classification of cats and dogs breeds was challenging due to the presence of many cross breeds. We used the owners' response to categorise cat breeds and New Zealand Kennel club classification of breeds for dogs Table 4.1. No association was found between either cat or dogs breeds with faecal MDR-E/ESBL/AmpC-E carriage in the separate multivariable models. The classification we used was different from the classification used by a study by Procter *et al.* (2014) that sampled dog faeces from parks in Canada. The study classified breeds according to the size as large mix, large pure, small mix and small pure found that dogs with large mixed breeds were more likely to shed MDR-E.

The variables that were associated with increased odds of MDR/ESBL/AmpC-E carriage in cats were cat was hospitalised and/or had surgery in the last six months (OR=10.2), people living in the household

working in a veterinary clinic (OR=5.2) and cat received antimicrobial treatment (OR=1.7). The interaction of age of the cat and antimicrobial treatment was significant in Q1 model (OR=1.1), suggesting that age modifies the effect of treatment on the outcome. The variables that were associated with the increased risk in dogs were sex of dog (female entire/male neutered; OR=3.9), antimicrobial treatment in the previous six months (OR=1.9), travelling overseas of a household member (OR=2.2) in Q1 model, and dog hospitalised and/or had surgery in the last six months and animal in Q2 model (OR=2.9). The differences in the significant risk factors for carriage between cats and dogs are maybe due to the sample size issue which requires further assessment.

Although a number of findings in our study are consistent with the results of previous studies there are some ways limitations to our work. As in any questionnaire-based study, differential recall bias may have occurred with some of the questions. Gathering information about where the dog/cat stayed during the owner's travelling period would add to this study. Previous work has shown that animals staying at breeding kennels or dog day care centres are at a greater risk for faecal carriage of antimicrobial resistant *E. coli* bacteria (Harada *et al.* 2011; Procter *et al.* 2014). Without this information it is challenging to make inference about the role of owner travel *per se*. The normal number of travellers of a normal population ranges between 5-10%, while in this study the owners or any persons inside the household that travelled overseas was 31% (n=182). The high numbers of travellers are of households that financially afford to treat their pets at good veterinary clinics. Thus, the household income may have introduced a differential bias to the sampled population. Another limitation of this survey was the lack of information about the diet of the cats and dogs. For instance, consumption of raw meat in dogs was associated with faecal carriage of AMR-E (OR=9.7) in comparison with dry food (Schmidt *et al.* 2015).

Finally, a number of potential risk factors such as age of the owners and age of the animals were dropped from the final model, to allow the model to converge. However, most of the caterpillar plots suggested that most factors associated with carriage were probably captured in the final model (see

Figures 4.1 to 4.6), although a few clinics deviated marginally from the overall pattern, which warrants further investigation.

4.5 Conclusion

In summary, this study has identified several factors associated with increased odds of faecal carriage of MDR/ESBL/AmpC-E in cats and dogs in Auckland. In general, faecal carriage of these bacteria may increase the risk of infection due to auto-infection, and represent a source of infection to other animals and contact humans. However, in order to assess the impact of faecal carriage on morbidity, it is necessary to perform molecular epidemiological studies comparing genetically isolates from faeces and infection sites. Such a study is presented in Chapter 5. Further studies are needed to assess the impact of MDR/ESBL/AmpC producing bacteria on the public health.

Table 4.1: Univariate screening of the variables elicited by the questionnaire against the binary outcome of faecal carriage (yes/no) of extended spectrum β-lactamase (ESBL) and AmpC-β-lactamase-producing *Escherichia coli,* with the clinic as random effect

Variable	Categories	Odds Ratio (95% Confidence Interval), P-value		
		Dogs (n=352)	Cats (n=225)	Cats and dogs (n=572)
Animal-level variables				
Species	Dogs	Reference Category		
	Cats			0.7 (0.3-1.4), 0.3
Cat Breeds	Domestic Short Hair	Reference Category		
	Pure Breed		0.7 (0.16-3.1), 0.6	
	Other + cross-breed		0.9 (0.16-5), 0.9	
Sex	Male	Reference Category		
	Female	1.5 (0.6-3.4), 0.4	0.7 (0.2-2.3), 0.5	1.2 (0.6-2.3), 0.6
Animal sex	Male neutered	Reference Category		
	Female neutered	1 (0.02-0.1), 0.9	0.5 (0.1-2.5), 0.4	0.8 (0.3-1.8), 0.6
	Male entire	0.9 (0.09-8.2), 0.8	1	0.8 (0.09-6.9), 0.6
	Female entire	5.4 (1.47-20.4), 0.02	4.9 (0.3-7.7), 0.2	6.2 (1.9-20.3), 0.002
Age of the animals sampled	0.08-1 year	Reference Category		
	>1-10 years	0.3 (0.09-0.9), 0.03	0.5 (0.08-3.5), 0.52	0.3 (0.15-0.9), 0.04
	>10 years	0.8 (0.2-2.7), 0.7	1.8 (0.3-10.9), 0.45	0.9 (0.3-2.5), 0.89
Cat/dog was owned by the owner in the last six months	Yes/No	0.49 (0.2-1.4), 0.1	0.6 (0.1-3.6), 0.6	0.5 (0.2-1.2), 0.1
Household Information				
Adults >16yrs (not including the owner) living in the household	Yes/No	1.4 (0.2-10.7), 0.8	0.46 (0.04-4.5), 0.5	1.3 (0.2-10.7), 0.8
Total number of adults living in the household	0	Reference Category		
	1	1	1	1
	2	1	1	1
	3	1	1	1
	>3	1	1	1
Children living in the household	Yes/No	1.7 (0.7-4.1), 0.2	2.1 (0.5-8.4), 0.3	1.6 (0.8-3.4), 0.1
Total number of children living in the household	0	Reference Category		
	1	2.5 (0.9-6.6), 0.06	1.9 (0.4-8.9), 0.4	2.1 (0.9-4.6), 0.07
	>1	0.92 (0.2-3.4), 0.9	2.7 (0.4-19.5), 0.3	1.2 (0.4-3.9), 0.7
Cats (not including the sampled cat/dog) living in the household	Yes/No	1.7 (0.7-4.5), 0.2	1.3 (0.26-6.1), 0.7	1.5 (0.7-3.2), 0.3
Total number of cats living in the household	0	Reference Category		
	1	0.4 (0.1-1.7), 0.3	1.2 (0.26-5.3), 0.8	0.7 (0.3-1.7), 0.4
	>1	1.7 (0.6-4.3), 0.3	1.3 (0.29-5.9), 0.7	1.4 (0.7-3.2), 0.3
Dogs (not including the sampled cat/dog) living in the household	Yes/No	0.7 (0.2-2.7), 0.6	1.2 (0.3-6.4), 0.8	0.9 (0.3-2.6), 0.9
Total number of dogs living in the household	0	Reference Category		
	1	0.9 (0.4-2.5), 0.9	0.9 (0.2-5), 0.9	1.3 (0.4-2.3), 0.9
	>1	0.7 (0.2-2.7), 0.6	1.5 (0.3-8), 0.6	0.9 (0.3-2.7), 0.9
Other animal species living in the household	Yes/No	1	1	1
Antimicrobial Treatment/ Hospitalisation/ Surgery of the animal (in the last six months)				
Antimicrobial treatment	Yes/No	3.4 (1.4-8.4), 0.01	6.2 (1.5-22.6), 0.01	3.8 (1.8-7.7), 0.0001
Had ear, eye drops	Yes/No	1.6 (0.5-5.2), 0.4	2.2 (0.2-22), 0.5	1.8 (0.6-5), 0.3
Had Oral tablets/powder/liquid	Yes/No	2.9 (1.2-6.8), 0.02	1.9 (0.4-9.9), 0.4	2.5 (1.3-5.3), 0.01
Number of courses	0	Reference Category		
	1	1.9 (0.7-5.5), 0.2	-	1.9 (0.8-4.5), 0.1
	>1	4.3 (1.1-18), 0.03	-	3.7 (1-13.7), 0.04
Antimicrobial family	None	Reference Category		
	β -lactam	2.9 (1.1-8.3), 0.04	-	2.3 (0.9-6), 0.07
	Non-β-lactam	2.5 (0.7-9.7), 0.1	-	3.1 (1-8.9), 0.03
Had an injection	Yes/No	2.9 (1.1-7.7), 0.02	5.6 (1.5-21.5), 0.01	3.5 (1.6-7.5), 0.001
	None	Reference Category		
	1	2.9 (0.9-8.4), 0.05	4.2 (0.9-20.2), 0.07	3 (1.2-7), 0.01
	>1	5 (0.8-31), 0.08	21.6 (1.8-24.6), 0.01	8.8 (2.2-34.5), 0.001
Systemic Treatment (oral and injection	None	Reference Category		
	Systemic treatment	3.3 (1.4-7.9), 0.005	4.5 (1.2-15.8), 0.02	3.5 (1.7-7), 0.0004
Hospitalised	Yes/No	0.8 (0.09-6.8), 0.8	5 (0.7-34.4), 0.09	1.5 (0.4-5.4), 0.5
Surgery	Yes/No	1.7 (0.6-4.7), 0.3	1.7 (0.6-4.7), 0.9	1.5 (0.4-2.7), 0.9
Hospitalised and Surgery	Yes/No	1.5 (0.5-4.1), 0.4	1.3 (0.2-7.2), 0.7	1.3 (0.5-3.2), 0.5
Animals (not including the sampled cat/dog) living in the household (in the last six months)				
Joined the household	Yes/No	2.5 (0.8-7.7), 0.1	1.5 (0.15-13.8), 0.7	2.3 (0.8-6.3), 0.08
Been given oral antibiotics	Yes/No	2.2 (0.8-6.3), 0.1	6.3 (1.4-30.8), 0.02	2.7 (1.2-6.3), 0.02
Been hospitalised for over 24 hours or/and had surgical operation	Yes/No	1.6 (0.4-5.3), 0.4	6 (1.2-29), 0.02	2.4 (0.9-6.1), 0.06

Table 4.1 (Continued): Univariate screening of the variables elicited by the questionnaire against the binary outcome of faecal carriage (yes/no) of extended spectrum β-lactamase (ESBL) and AmpC-β-lactamase-producing *E. coli,* with the clinic as random effect

Variable	Categories	Odds Ratio (95% Confidence Interval), P-value		
		Dogs (n=352)	**Cats (n=225)**	**Cats and dogs (n=572)**
Information about people living in the same household (in the last six months)				
Work in healthcare industry	Yes/No	1.5 (0.6-4.1), 0.3	1.3 (0.3-5.7), 0.6	1.4 (0.7-3.3), 0.3
Work in a veterinary clinic	Yes/No	1.5 (0.4-5), 0.5	6.5 (1.2-30.4), 0.02	2.5 (0.9-6.5), 0.05
Work in human medical Clinic	Yes/No	0.7 (0.15-3.3), 0.6	0.6 (0.07-5.7), 0.7	0.6 (0.2-2.4), 0.5
Had an antimicrobial	Yes/No	0.8 (0.4-2.1), 0.7	1.3 (0.4-4.9), 0.6	1 (0.5-2), 0.9
Been Hospitalised >24hrs	Yes/No	1	1	1
Had surgery	Yes/No	0.49 (0.1-2.3), 0.3	1	0.43 (0.09-1.8), 0.2
Travelled overseas	Yes/No	2.1 (0.9-5.1), 0.08	1.6 (0.4-5.5), 0.4	1.8 (0.9-3.8), 0.08
Number of Countries visited	None	Reference Category		
	1	1.4 (0.02-4.5), 0.4	0.6 (0.6-9), 0.2	1.6 (0.7-3.8), 0.2
	>1	2 (0.7-6), 0.1	1	1.5 (0.5-3.9), 0.4

- Model did not converge.

Table 4.2: Univariate screening of the variables elicited by the questionnaire against the binary outcome of faecal carriage (yes/no) of multi drug resistant (MDR), and/or extended spectrum β-lactamase (ESBL) and/or AmpC-β-lactamase-producing *Escherichia coli,* with the clinic as random effect

Variable	Categories	Odds Ratio (95% Confidence Interval), P-value		
		Dogs (n=352)	**Cats (n=225)**	**Cats and dogs (n=572)**
Animal-level variables				
Species	Dogs	Reference Category		
	Cats			0.8 (0.4-1.5), 0.4
Dog Breeds	Toy	Reference Category		
	Terrier	-		
	Gun Dog	-		
	Hound	-		
	Working	-		
	Utility	-		
	Non-Sporting	-		
	Cross-breed	-		
Cat Breeds	Domestic Short Hair	Reference Category		
	Pure Breed		0.7 (0.16-3.1), 0.6	
	Other + cross-breed		0.9 (0.16-5), 0.9	
Sex	Male	Reference Category		
	Female	0.8 (0.6-3.4), 0.7	0.7 (0.2-2.3), 0.5	0.9 (0.5-1.8), 0.9
Animal sex	Male neutered	Reference Category		
	Female neutered	0.6 (0.3-1.6), 0.4	-	0.7 (0.03-0.1), 0.3
	Male entire	1.3 (0.3-6.9), 0.8	-	0.6 (0.07-4.7), 0.6
	Female entire	2.4 (0.7-8.8), 0.2	-	3.1 (1.1-9.6), 0.04
Age of the animals sampled	0.08-1 year	Reference Category		
	>1-10 years	0.3 (0.2-1.7), 0.6	0.5 (0.08-3.6), 0.5	0.8 (0.3-2), 0.6
	>10 years	0.8 (0.3-2.5), 0.8	1.9 (0.3-11), 0.5	1.4 (0.5-3.8), 0.5
Cat/dog was owned by the owner in the last six months	Yes/No	0.4 (0.2-1.7), 0.6	0.6 (0.1-3.6), 0.6	0.9 (0.3-2.4), 0.9
Household Information				
Adults >16yrs (not including the owner) living in the household	Yes/No	-	0.5 (0.04-4.6), 0.5	1.6 (0.2-13.1), 0.6
Total number of adults living in the household	0	Reference Category		
	1	-	-	-
	2	-	-	-
	3	-	-	-
	>3	-	-	-
Children living in the household	Yes/No	1.3 (0.6-3), 0.5	2 (0.5-8.4), 0.3	0.7 (0.4-1.5), 0.4
Total number of children living in the household	0	Reference Category		
	1	1.7 (0.6-4.3), 0.3	1.9 (0.4-8.9), 0.4	0.8 (0.4-2), 0.7
	>1	0.9 (0.3-3.1), 0.8	2.7 (0.4-19.5), 0.3	0.6 (0.2-1.8), 0.4
Cats (not including the sampled cat/dog) living in the household	Yes/No	1.4 (0.6-3.6), 0.5	1.3 (0.26-6.1), 0.8	1.3 (0.6-2.7), 0.6
Total number of cats living in the household	0	Reference Category		
	1	1.2 (0.4-3), 0.8	1.2 (0.3-5.3), 0.8	1.1 (0.5-2.3), 0.9
	>1	1.5 (0.6-3.6), 0.5	1.3 (0.3-5.9), 0.7	1.2 (0.6-2.7), 0.6
Dogs (not including the sampled cat/dog) living in the household	Yes/No	0.9 (0.3-2.8), 0.8	1.3 (0.3-6.4), 0.8	1.1 (0.4-2.8), 0.9
Total number of dogs living in the household	0	Reference Category		
	1	1 (0.4-2.4), 0.9	0.9 (0.2-5), 0.9	1.2 (0.6-2.5), 0.7
	>1	0.9 (0.3-2.8), 0.6	1.5 (0.3-8), 0.7	1.1 (0.4-2.9), 0.9
Other animal species living in the household	Yes/No	-	-	-
Antimicrobial Treatment/ Hospitalisation/ Surgery of the animal (in the last six months)				
Antimicrobial treatment	Yes/No	5.6 (2.3-13.5), 0.01	6 (1.6-22.6), 0.01	2.7 (1.4-5.2), 0.01
Had ear, eye drops	Yes/No	4.4 (1.7-11.4), 0.01	2.2 (0.2-22), 0.5	2.6 (1.1-6.7), 0.04
Had Oral tablets/powder/liquid	Yes/No	5.7 (2.5-13), 0.02	1.9 (0.4-9.9), 0.5	2.5 (1.3-4.9), 0.01
Number of courses	0	Reference Category		
	1	5.2 (2.1-12.9), 0.01	-	2 (0.9-4.5), 0.08
	>1	4.3 (0.9-19), 0.05	-	2.3 (0.6-9.3), 0.2
Antimicrobial family	None	Reference Category		
	β -lactam	5.4 (1.1-8.3), 0.01	-	2.5 (1.1-6.1), 0.04
	Non-β-lactam	6.7 (0.7-9.7), 0.01	-	2.2 (0.7-6.7), 0.2
Had an injection	Yes/No	2.7 (1.1-6.8), 0.03	5.6 (1.5-21.5), 0.01	1.6 (0.7-3.6), 0.02
	None	Reference Category		
	1	2.9 (0.9-7.4), 0.06	4.2 (0.9-20.2), 0.07	1.3 (0.5-3.5), 0.5
	>1	5 (0.2-17.9), 0.6	21.6 (1.8-24.6), 0.01	2.3 (0.5-11.7), 0.3
Antimicrobial family	None	Reference Category		
	β -lactam	-	-	-
	Non-β-lactam	-	-	-
Systemic Treatment (oral and injection	None	Reference Category		
	Systemic treatment	5.4 (2.4-12.4), 0.01	4.5 (1.2-15.8), 0.02	2.5 (1.3-5), 0.001
Hospitalised	Yes/No	0.6 (0.08-5.1), 0.7	5 (0.7-34.4), 0.1	1.3 (0.4-4.6), 0.7
Surgery	Yes/No	1.7 (0.6-4.7), 0.4	-	0.7 (0.4-2.7), 0.9
Hospitalised and Surgery	Yes/No	1.6 (0.6-4), 0.3	1.4 (0.3-7.2), 0.7	1.2 (0.5-2.7), 0.7

Table 4.2 (Continued): Univariate screening of the variables elicited by the questionnaire against the binary outcome of faecal carriage (yes/no) of multi drug resistant (MDR), and/or extended spectrum β-lactamase (ESBL) and/or AmpC-β-lactamase-producing *E. coli,* with the clinic as random effect

Variable	**Categories**	**Odds Ratio (95% Confidence Interval), P-value**		
		Dogs (n=352)	**Cats (n=225)**	**Cats and dogs (n=572)**
Animals (not including the sampled cat/dog) living in the household (in the last six months)				
Joined the household	Yes/No	2.1 (0.7-6.3), 0.2	1.5 (0.16-13.8), 0.7	2.5 (1.16-2), 0.049
Been given oral antibiotics	Yes/No	1.9 (0.7-5.2), 0.1	6.6 (1.4-30.8), 0.02	2.3 (1.1-5.2), 0.03
Been hospitalised for over 24 hours or/and had surgical operation	Yes/No	2.1 (0.7-6.2), 0.2	6 (1.2-29), 0.03	2.2 (0.8-5.4), 0.09
Information about people living in the same household (in the last six months)				
Work in healthcare industry	Yes/No	0.9 (0.4-2.5), 0.9	1.4 (0.3-5.7), 0.6	1.1 (0.5-2.3), 0.8
Work in a veterinary clinic	Yes/No	1.9 (0.6-5.5), 0.25	6.5 (1.2-30.4), 0.025	2.7 (1.1-6.5), 0.026
Work in human medical Clinic	Yes/No	0.5 (0.12-2.4), 0.4	0.6 (0.07-5.7), 0.7	0.6 (0.2-1.9), 0.37
Had an antimicrobial	Yes/No	0.8 (0.4-2.1), 0.29	1.3 (0.4-4.9), 0.6	1 (0.5-2), 0.045
Been Hospitalised >24hrs	Yes/No	0.3 (0.04-2.7),0.3	-	0.6 (0.1-2.5), 0.4
Had surgery	Yes/No	1.1 (0.1-2.3), 0.8	-	1.5 (0.6-3.8), 0.4
Travelled overseas	Yes/No	1.2 (0.5-2.8), 0.6	1.4 (0.7-2.7), 0.36	1.4 (0.7-2.7), 0.3
Number of Countries visited	None	Reference Category		
	1	1.4 (0.02-4.5), 0.4	-	1.6 (0.7-3.8), 0.2
	>1	2 (0.7-6), 0.1	-	1.5 (0.5-3.9), 0.4

- Model did not converge.

Table 4.3: Variables associated with faecal carriage of ESBL/AmpC producing *E. coli* in the final multivariable model with random clinic effect

Variable description	**Outcomes**	**Odds Ratio (95%CI)**	**P-value**
Cats			
Sex	Male (Reference)		
	Female	0.7 (0.2-2.4)	0.7
Age in months (continuous)		0.8 (0.9-1.1)	0.25
Antimicrobial treatment	No (Reference)		
	Yes	0.8 (0.3-2.2)	0.6
Interaction of age of the cat and antimicrobial treatment	Not treated (reference)		
	Treated across age	1.1 (1.02-1.2)	0.04
Animal hospitalised and/or had surgery	No (Reference)		
	Yes	8.7 (1.3-55)	0.02
work in a veterinary clinic	No (Reference)		
	Yes	5.2 (0.8-32)	0.06
Dogs			
Sex	Male neutered (reference)		
	Female neutered	1 (0.3-2.7)	0.9
	Male entire	0.8 (0.08-8.3)	0.9
	Female entire	6.5 (1.7-25.2)	<0.01
Age of the animal		1.02 (0.9-1.1)	0.7
Antimicrobial treatment	No (reference)		
	Yes	1.5 (1.1-2.2)	0.003
Travelled overseas	No (reference)		
	Yes	2.2 (0.8-5.59)	0.1
Antibiotic oral/liquid family	Not treated (reference)		
	β-lactam	3 (0.9-9.13)	0.05
	non-β-lactam	2.4 (0.6-9.9)	0.2
Both (Cats and Dogs)			
Species	Cat (reference)		
	Dog	0.8 (0.4-1.77)	0.6
Sex	Male (reference)		
	Female	1.2 (0.6-2.5)	0.5
Age of the animal		0.9 (0.9-1.06)	0.99
Antimicrobial treatment	No (reference)		
	Yes	1.52 (1.2-1.94)	<0.01
Owner work in a veterinary clinic	No (Reference)		
	Yes	2.87 (1.1-7.6)	0.03
Owner travelled overseas	No (reference)		
	Yes	2.3 (1.1-4.8)	0.02
Antibiotic oral/liquid family	Not treated (reference)		
	β-lactam	2.5 (0.96-6.7)	0.05
	Combination of treatments	2.9 (1.02-8.6)	0.045

Table 4.4: Variables associated with faecal carriage of MDR and/or ESBL and/or AmpC producing E. coli in the final multivariable model with random clinic effect

Variable description	Outcomes	Odds Ratio (95%CI)	P-value
Cats			
Sex	Male (Reference)		
	Female	0.7 (0.2-2.5)	0.6
Age (continuous)		1 (0.9-1.1)	0.6
Antimicrobial treatment	No (Reference)		
	Yes	1.7 (1.1-2.6)	0.02
Animal hospitalised and/or had surgery	No (Reference)		
	Yes	10.2 (1.9-54)	<0.01
Work in a veterinary clinic	No (Reference)		
	Yes	5.2 (1.1-24.9)	0.04
Dogs			
Animal sex	Male neutered (reference)		
	Female neutered	0.5 (0.2-1.5)	0.2
	Male entire	1.7 (0.3-10.1)	0.5
	Female entire	3.9 (1.1-14)	0.04
Age of the animal		1.02 (0.9-1.1)	0.67
Antimicrobial treatment	No (reference)		
	Yes	1.9 (1.4-2.6)	<0.01
Animal hospitalised and/or had surgery	No (Reference)		
	Yes	2.92 (0.9-9.3)	<0.01
Antibiotic oral/liquid family	Not treated (reference)		
	β-lactam	6 (2.1-17)	<0.01
	non-β-lactam	6.4 (1.9-21)	<0.01
Both (Cats and Dogs)			
Species	Cat (reference)		
	Dog	0.8 (0.4-1.77)	0.65
Animal sex	Male (reference)		
	Female	1 (0.5-1.9)	0.9
Age of the animal		1 (0.9-1.12)	0.16
Antimicrobial treatment	No (reference)		
	Yes	1.36 (1.1-1.7)	<0.01
Owner work in a veterinary clinic	No (Reference)		
	Yes	2.94 (1.2-7.2)	0.01
Owner travelled overseas	No (reference)		
	Yes	1.69 (0.8-3.4)	0.14

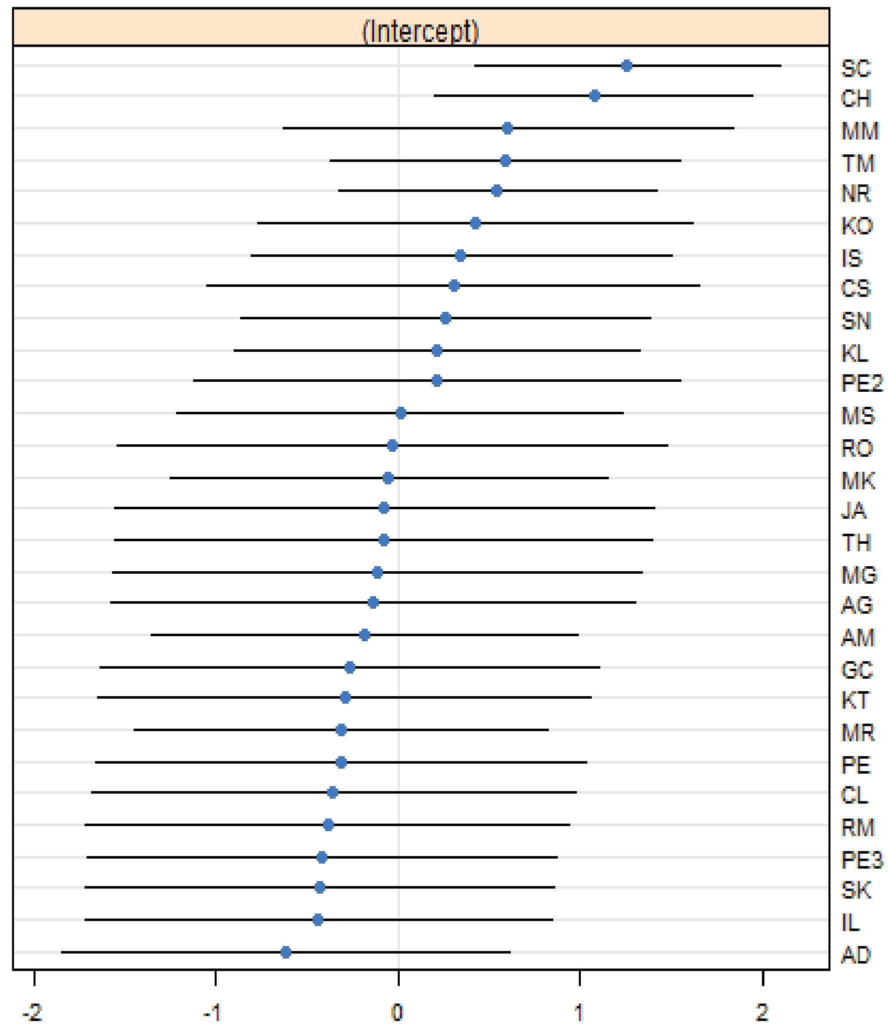

Figure 4.1: Caterpillar plot of random clinic effect in the final model faecal carriage of ESBL and/or AmpC-E in cats and dogs. The symbols on the y-axis are the anonymous identifier codes of the clinics

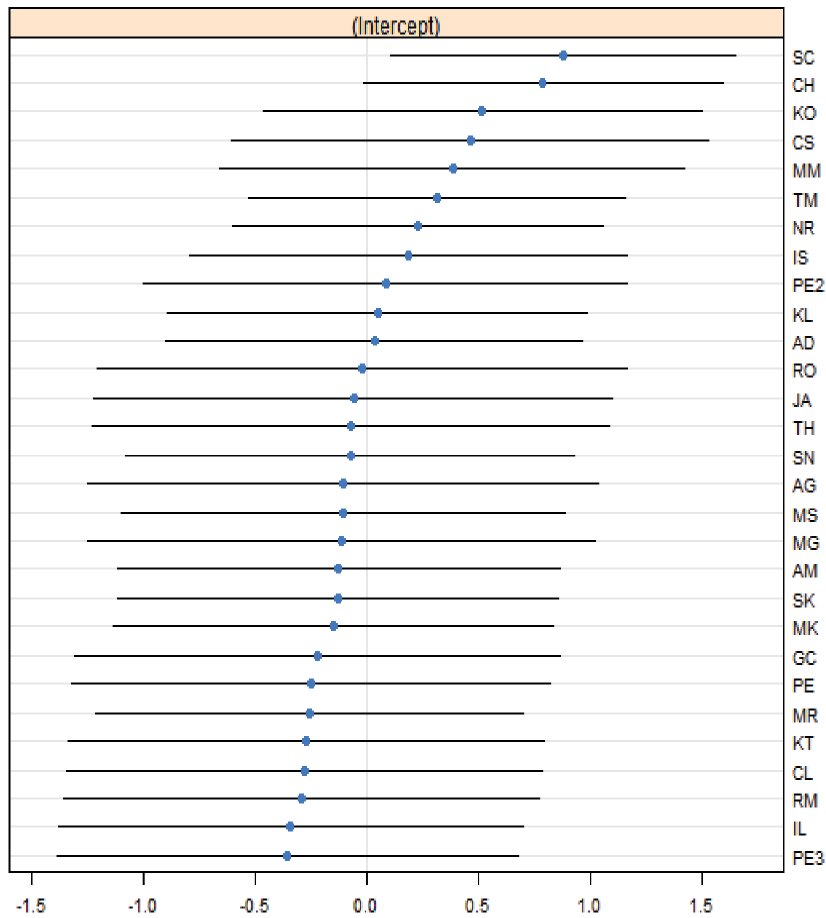

Figure 4.2: Caterpillar plot of random clinic effect in the final model faecal carriage of MDR-E and/or ESBL and/or AmpC-E in cats and dogs. The symbols on the y-axis are the anonymous identifier codes of the clinics

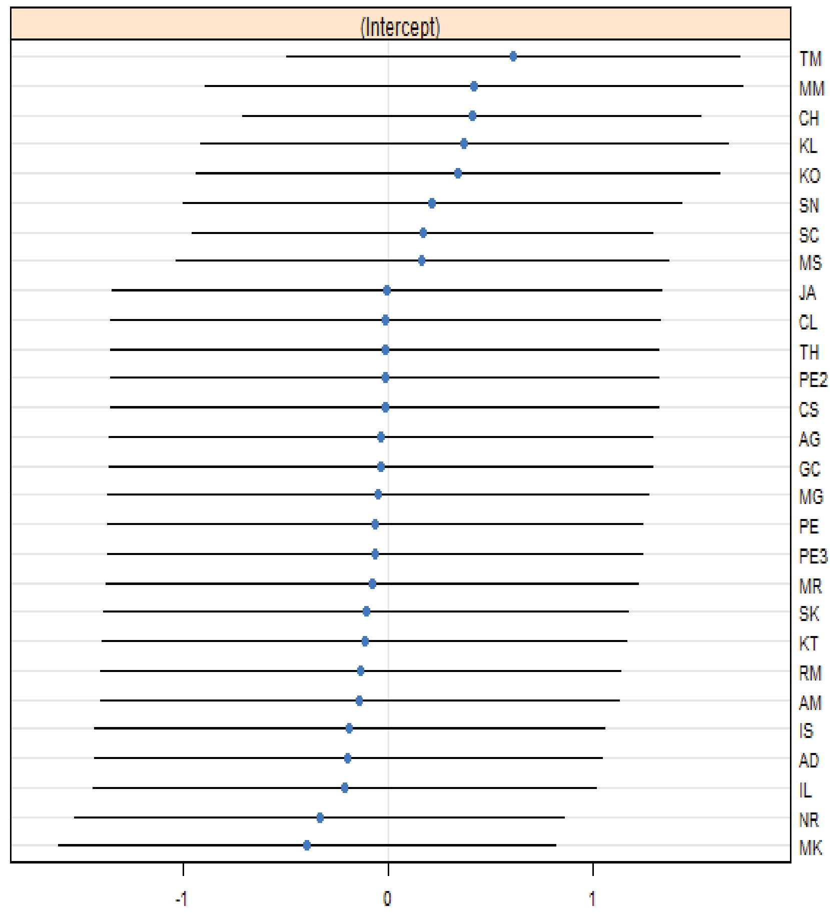

Figure 4.3: Caterpillar plot of random clinic effect of final model faecal carriage of ESBL/AmpC-E in cats. The symbols on the y-axis are anonymous identifier codes of the clinics

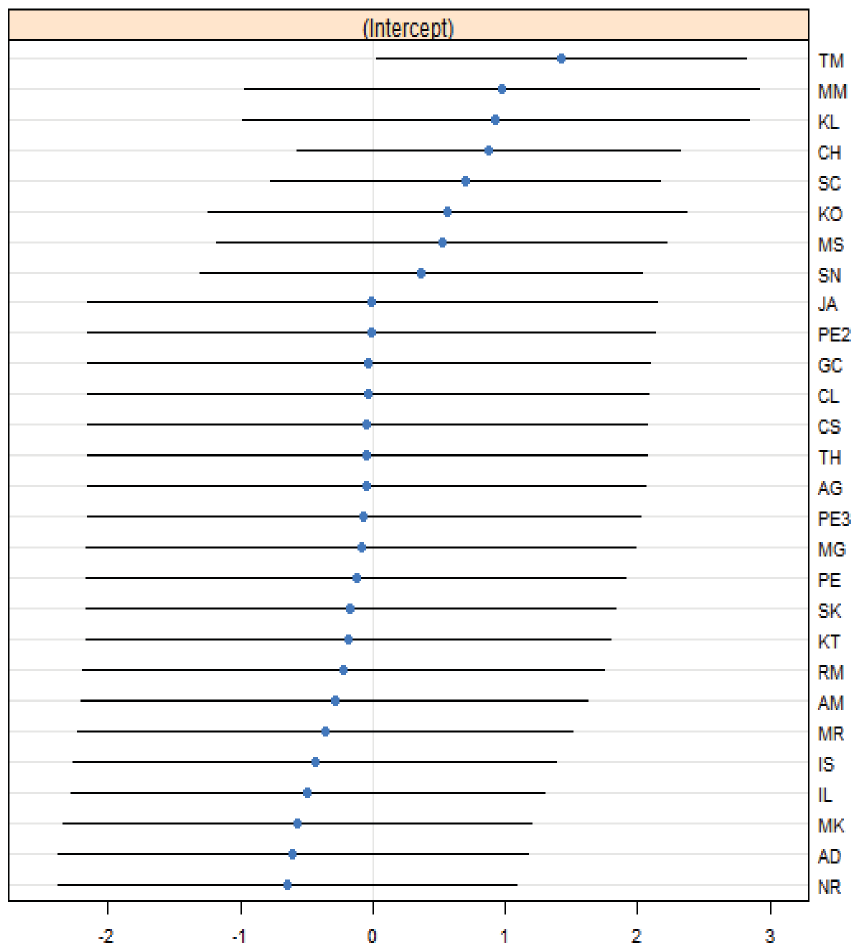

Figure 4.4: Caterpillar plot of random clinic effect of final model faecal carriage of MDR/ESBL/AmpC-E in cats. The symbols on the y-axis are anonymous identifier codes of the clinics

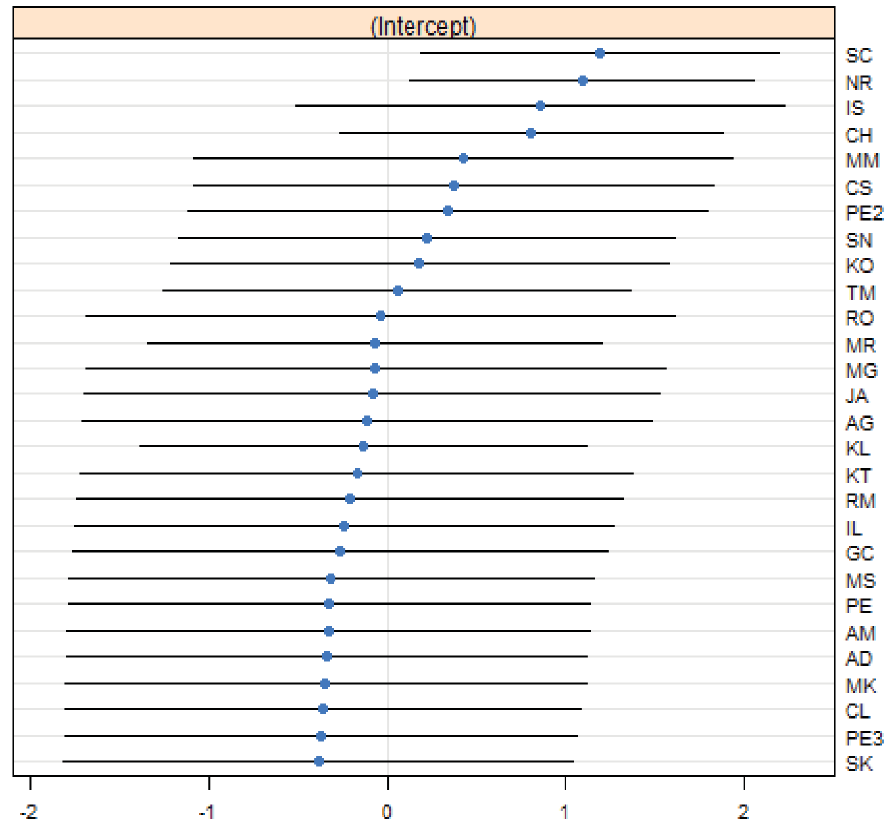

Figure 4.5: Caterpillar plot of random clinic effect in the final model of the risk of faecal carriage of ESBL/AmpC-E in dogs. The symbols on the y-axis are anonymous identifier codes of the clinics

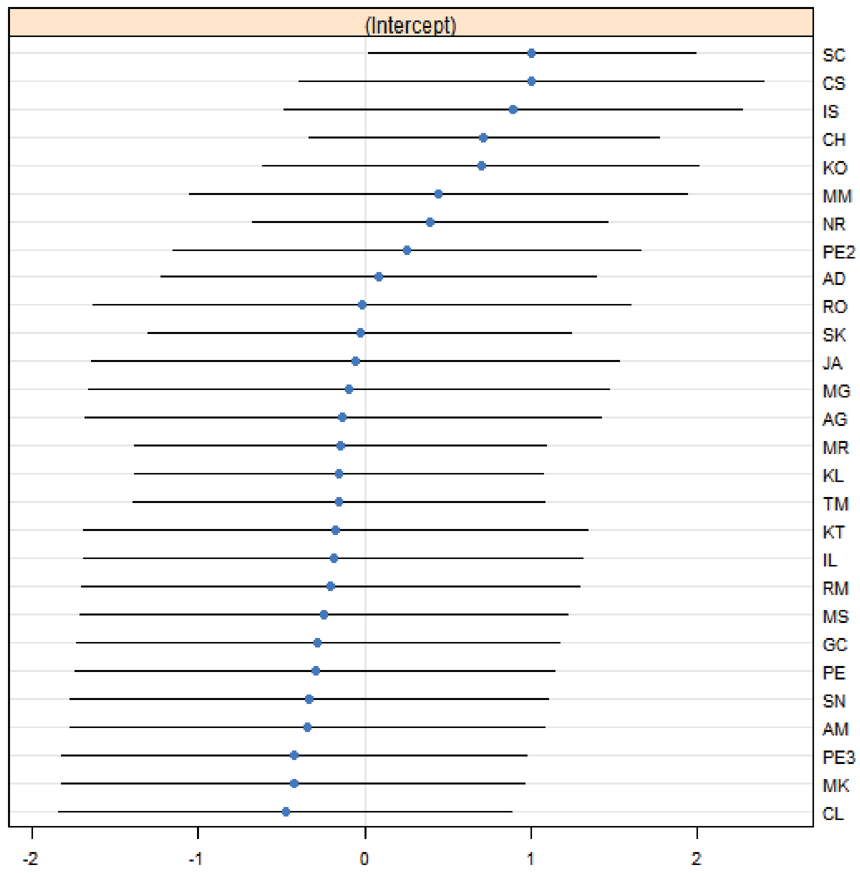

Figure 4.6: Caterpillar plot of random clinic effect of final model of the risk of faecal carriage of MDR and/or ESBL and/or AmpC-E in dogs. The symbols on the y-axis are anonymous identifier codes of the clinics

4.6 Supplementary Material

Table S 4.1: Characteristics of 572 cats and dogs for which completed questionnaires were available

		Dogs n=351 (%)	Cats n=221 (%)	Total n=572 (%)
Sex	Feline, entire	28 (9)	5 (2)	33 (6)
	Male, entire	19 (6)	5 (2)	23 (4)
	Female, neutered	143 (44)	111 (56)	254 (49)
	Male, neutered	134 (41)	80 (40)	214 (41)
	Sex information missing	28 (8)	20(9)	48 (8)
Animal origin (within 6 months)				
Owned by owner		288 (85)	181 (86)	469 (85)
Own by a breeder		25 (7.1)	6 (2.7)	31 (5.4)
Own by a friend / family member		6 (1.7)	4 (1.8)	10 (1.7)
Owned by an animal shelter		5 (1.4)	4 (1.8)	8 (1.5)
Ownership information Missing		28 (8)	26 (12)	54 (9.4)

Table S 4.2: Breeds of 572 cats and dogs for which completed questionnaires were available

Canine breeds	Dogs n=351(%)	Feline breeds	Cats n=221 (%)
Toys	34 (11)	Domestic Short Hair (DSH)	110 (56)
Terriers	59 (18)	Pure Breed	59 (30)
Gundogs	64 (20)	Other	29 (15)
Hounds	13 (4)	Missing	23(10)
Working	37 (12)		
Utility	17 (5)		
Non Sporting	42 (13)		
Cross Breed	55 (17)		
Missing	30 (8.5)		

Table S 4.3: Household demographics of the sampled animals

		Dogs n=351(%)	Cats n=221 (%)	Total n=572 (%)
Adults ≥16 yrs (not including the owner)	0	13 (3.8)	11 (4.8)	24 (4.3)
	1	49 (13.5)	36 (16)	85 (14.8)
	2	203 (56.3)	122 (54.2)	325 (56.8)
	3	53 (14.6)	35 (15.5)	88 (15.3)
	>4	33 (9.11)	17 (7.5)	50 (8.7)
Children <16 yrs	0	236 (65.7)	148 (65.8)	384 (67.3)
	1	59 (16.2)	43 (19.1)	102 (17.8)
	≥2	56 (15.4)	30 (13.3)	86 (15)
Dogs	0	184 (51.3)	147 (65.3)	331 (58)
	1	112 (30.9)	45 (20)	157 (27.3)
	≥2	55 (24.4)	29 (12.8)	84 (14.6)
Cats	0	189 (52.4)	82 (36.4)	271 (47.4)
	1	85 (23.4)	70 (31.1)	155 (27)
	≥2	77 (21.5)	69 (30.6)	146 (25.6)
Other species	0	315 (87.5)	196 (87.1)	511 (89.5)
	1	10 (2.7)	8 (3.5)	18 (3.1)
	≥2	26 (7.1)	17 (7.5)	43 (7.5)
Total Number of animals/house (not including the sampled animal)	0	99 (27.6)	59 (26.2)	158 (27.7)
	1	102 (28.1)	65 (28.8)	167 (29.1)
	2	55 (15.4)	34 (15.1)	89 (15.7)
	≥3	95 (26.2)	63 (28)	158 (27.5)

Table S 4.4: Types of antimicrobial prescriptions in cats and dogs in the 6 month preceding the sampling

		Dogs n=351 (%)	Cats n=221 (%)	Total n=572 (%)
Antimicrobial treatment in the last 6 months		122 (35)	55 (25)	177 (31)
Topical Ear/ Eye treatment		34 (10)	9 (4)	43 (8)
Number of courses	1	22 (6)	5 (2)	27 (5)
	≥2	8 (2)	2 (1)	10 (2)
	Missing	4 (1)	2 (0.9)	6 (1)
Antimicrobial Family				
	β-lactam	0	0	0
	Other classes	25 (7)	4 (2)	29 (5)
	Missing	9 (2.5)	5 (2)	13 (2)
Oral Tablets/liquid		88 (25)	24 (11)	112 (20)
Number of Courses	1	66 (19)	21 (10)	87 (15)
	≥2	15 (4)	2 (1)	17 (3)
	Missing	7 (2)	2 (0.9)	9 (1.5)
Antimicrobial Family				
	β-lactam	45 (14)	12 (6)	57 (10)
	Other classes	25 (8)	9 (4)	34 (5.8)
	Missing	18 (5)	3 (1)	21 (4)
Injection		45 (13)	30(14)	75 (13)
Number of courses	1	34 (10)	23(10)	57 (10)
	≥2	7 (2)	6(3)	13 (2)
	Missing	4 (1)	1(0.5)	5 (0.8)
Antimicrobial Family				
	β-lactam	39 (11)	23 (10)	62 (11)
	Other classes	1 (0.2)	1 (0.4)	2 (0.3)
	Missing	5 (1)	6 (3)	11 (2)
Combination of therapy	0	237 (68)	170 (77)	407 (68.9)
	1	20 (6)	4 (2)	24 (4)
	2	14 (4)	5 (2.2)	19 (3)
	3	80 (22)	42 (19)	122 (21)

0- No treatment
1-All treatments (ear and eye treatment + oral tablets/liquid and injections)
2-Ear, eye treatment
3-Systematic treatment without eye or ear treatment

N.B In parts a, b, and c the percentages don't add to 100% because some of the cases had more than one treatment at the same time and due to missing data.

Table S 4.5: List of antimicrobials administered to cats and dogs in the last six months

Antibiotic class	Antimicrobial	Ear, Eye drops		Oral tablets and liquids		Injections	
		Dogs n (%)	Cats n (%)	Dogs n (%)	Cats n (%)	Dogs n (%)	Cats n (%)
Penicillins	Penicillin G					1 (0.3)	
	Amoxicillin clavulanic acid			42 (12)	13 (5.9)	27 (7.7)	10 (4.5)
	Bacitracin	1 (0.3)					
Cephalosporins	Cephalexin			10 (2.8)		2 (0.6)	
	Cephazolin					4 (1.1)	
	Cefovecin					7 (2)	15 (6.8)
	Cefpodoxime			7 (2)			
Aminoglycosides	Gentamycin	2 (0.6)					
	Neomycin	8 (2.3)	1 (0.45)				
	Framycetin	4 (1.1)	1 (0.45)				
Fluoroquinolones	Enrofloxacin	1 (0.3)		7 (2)	1 (0.45)	2 (0.6)	1 (0.5)
Trimethoprim-Sulphonamides	Sulfamethoxazole-Trimethoprim			2 (0.6)			
	Chloramphenicol						
	Spiramycin			7 (2)			
	Fusidic acid	4 (1.1)					
Polymixin	Polymyxin B	11 (3.1)	1 (0.45)				
	Metronidazole			12 (3.4)		1 (0.3)	
Macrolide	Erythromycin				1 (0.5)		
Lincosamides	Clindamycin	5 (1.4)	3 (1.4)		1 (0.5)		1 (0.45)
Tetracyclines	Doxycycline			4 (1.1)	6 (2.7)		
	Tigacyline				1 (0.5)		

Dogs n=351, Cats n=221

Table S 4.6 : Frequency of hospitalisation and/or surgery six months prior to sampling, among the sampled cats and dogs

		Dogs n=351(%)	Cats n=221 (%)	Total n=572 (%)
Hospitalised >24hrs		19 (5)	15 (7)	34 (6)
Surgery		53 (15)	25 (11)	78 (14)
Type of Surgery	Dental	1 (0.3)	4 (2)	5 (1)
	De-sexing	16 (5)	10 (4)	26 (4)
	Orthopaedics	7 (2)	1 (0.5)	8 (1)
	Soft Tissue	23 (7)	8 (4)	31 (5)
	Missing	6 (2)	2 (1)	8 (1)
Antimicrobial treatment with hospitalisation and surgery	Antimicrobial Treatment	78 (22)	34 (15)	112 (20)
	Hospitalised + Surgery	12 (3)	9 (4)	21 (4)
	Hospitalised +Antimicrobial	2 (1)	7 (3)	9 (2)
	Surgery +Antimicrobial	29 (8)	10 (5)	39 (7)
	Hospitalised + Surgery+ Antimicrobial	10 (3)	7 (3)	17 (3)

Table S 4.7: Information about animals living in the same house over the last 6 months

Animals present in the same house over the 6 months prior to the sampling	Dogs n=351(%)	Cats n=221 (%)	Total n=572 (%)
Joined the household	34 (10)	15 (7)	49 (9)
Given oral antibiotic	42 (12)	24 (11)	66 (12)
Hospitalised for over 24 hours	14 (4)	3 (1)	17 (3)
Had a surgical operation	28 (8)	14 (6)	42 (7)

Table S 4.8: Information about people living in the same house over the last 6 months

Owner or people living in the house the last 6 months		Dogs n=351(%)	Cats n=221 (%)	Total n=572 (%)
Work in healthcare industry		75 (21)	42 (19)	117 (20)
Veterinary Clinic (includes veterinary staff)		37 (11)	15 (7)	52 (9)
Human Medical Clinic		42 (2)	26 (12)	68 (12)
Had an antimicrobial		37 (131)	70 (32)	201 (35)
Been Hospitalised >24hrs		30 (9)	15 (7)	45 (8)
Had surgery		44 (13)	18 (8)	62 (11)
Travelled overseas		108 (30)	74 (32)	182 (31)
Number of Countries visited	1	62 (18)	48 (22)	110 (19)
	>1	46 (13)	26 (12)	71 (13)
Countries/Regions visited	Australia	60 (17)	45 (20)	105 (18)
	Australasia	4 (1)	3 (1)	14 (2)
	Asia	38 (11)	38 (17)	28 (5)
	Europe	40 (11)	23 (10)	16 (3)
	North America	30 (9)	10 (5)	14 (2)
	South America	20 (6)	10 (5)	2 (0.3)
	Africa	4 (1)	0	3 (0.5)

Chapter 5: Genetic comparison of extended-spectrum β-Lactamase and AmpC-producing *Escherichia coli* strains from faecal carriage and clinical infections in cats and dogs in New Zealand

Preamble

The study presented in this chapter compares genetically the extended spectrum β-lactamase (ESBL) and AmpC-producing *Escherichia coli* (ESBL-E/AmpC-E) faecal isolates obtained in this PhD project from faeces of cats

and dogs (

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Chapter 3 and Appendix 1) with the clinical isolates obtained from the New Zealand diagnostic laboratories (Chapter 2), with the aim of assessing the potential role of faecal carriage in the dissemination of pathogenic bacteria, and the role of carriage in the pathogenesis of auto-infection.

Summary

In animals and humans, ESBL/AmpC-E faecal shedding can last more than six months, posing a potential risk of environmental contamination with these strains, of infections of new hosts and auto-infection, in particular ascending urinary tract infections (UTI). The recognition of body excretions, or specific body colonisation sites as significant epidemiological reservoirs of pathogenic bacteria requires the identification of genetic similarity between the isolates obtained from the reservoir and those obtained from infection sites. Most of the ESBL/AmpC-E STs found in the faeces of cats and dogs and in extra-intestinal infection sites, were previously described in faeces and infections in humans. This supports the notion that animal faeces may be important reservoirs of infections in humans. The epidemiological reservoirs of pathogenic ESBL/AmpC-E infecting cats and dogs has not been sufficiently studied. The availability of contemporaneous ESBL/AmpC-E from infection sites and faeces of dogs and cats in New Zealand provided an opportunity to assess their similarity.

In the study presented here, we assessed the clonal relatedness of faecal and clinical ESBL/AmpC-E isolates identified from cats and dogs in New Zealand. The isolates were identified from faeces of hospitalised and community companion animals (n=45), and from clinical infections of companion animals obtained New Zealand veterinary diagnostic laboratories (n=36). The isolates were genotyped by MLST and their ESBL/AmpC genes characterised by PCR-sequencing.

In total, 43 sequence types (STs) were identified among faecal (31 STs) and clinical (19 STs) ESBL/AmpC-E isolates, of which five were shared by both groups (STs 46, 372, 457, 155, 744). The shared STs corresponded to well characterised human extra-intestinal pathogenic *E. coli* strains (ExPEC). The predominant ESBL and AmpC genes within the faecal and clinical isolates were *bla*$_{CTX\text{-}M\text{-}14}$ and *bla*$_{CMY\text{-}2}$, respectively. Interestingly, the leading

pandemic clone ST131 was only identified among clinical isolates. In order to better understand the auto-infection route, future studies should compare between faecal and infection site isolates in the same animals.

5.1 Introduction

Escherichia coli is a commensal inhabitant of the intestinal tract of humans and mammals (Clermont *et al.* 2000; Ruppe *et al.* 2013). In humans, intestinal colonisation with pathogenic *E. coli* strains, termed extra-intestinal pathogenic *E. coli* (ExPEC), serves as a reservoir for a heterogeneous group of infections, in particular urinary tract infections (UTI) through the ascending faecal-perineal-urethral route (Yamamoto *et al.* 1997; Niki *et al.* 2011; Chen *et al.* 2013; Ruppe *et al.* 2013). Urinary tract infections caused by *E. coli* is a significant morbidity (Johnson and Russo 2002; Nakamura *et al.* 2016), and in recent years there has been a significant increase in UTI caused by ESBL/AmpC-producing *E. coli* (ESBL/AmpC-E), which is a major concern in both human medicine (Cantón and Coque 2006; Livermore *et al.* 2007; Ben Sallem *et al.* 2012; Nakamura *et al.* 2016; Philippon *et al.* 2016).

In the last decade there has been an increase in the incidence of ESBL-E infections in humans also in New Zealand, in particular UTIs (Dyet *et al.* 2014). The most frequent ESBL-E strain isolated from cases of UTI in New Zealand belongs to ST131 (Heffernan *et al.* 2014b). The faecal ESBL-E carriage rate in humans in Auckland, New Zealand, has been estimated at 5%, but there is no published record of the molecular characterisation of the isolates to determine the predominant STs in faeces, and compare them with the strains obtained from UTI or other extra-intestinal infections (Upton *et al.* 2011).

ESBL/AmpC-E strains have also been detected in the faeces of healthy and infected companion animals in this study and abroad (Guo *et al.* 2015; Rocha-Gracia *et al.* 2015). In animals, characterisation of isolates obtained in New Zealand in 2012 and 2013 in Chapter 2 revealed that the prevalent ESBL-E STs isolated from clinical infections were ST131 and ST648, and the most prevalent AmpC-E ST was ST156. The adjusted faecal ESBL-E and

AmpC-E prevalence apparently healthy cats and dogs in the Auckland region was 0.34% (95% CI 0.04-1.2%) and 6.1% (95%CI 4.3-8.4%), respectively (

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Chapter 3). Thus, companion animals can shed ESBL/AmpC-E in faeces and can also be clinically infected with ESBL/AmpC-E (Chapter 2 and

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Chapter 3).

This chapter analyses the clonal relatedness of ESBL/AmpC-E isolated from faeces and clinical infections in cats and dogs in the studies presented in

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Chapter 3 and Appendix 1, in order to assess the potential role of faecal carriage as a source of extra intestinal infections.

5.2 Materials and methods

5.2.1 *E. coli* isolates from faeces and clinical samples

The faecal ESBL/AmpC-E isolates (n=45) were identified from faeces or rectal swabs of cats and dogs in Auckland (n=38) and Palmerston North (n=7), New Zealand, between June 2012 and June 2013. In this Chapter these isolates are referred to as 'faecal isolates'. The *E. coli* isolates identified from infection sites (n=36) are described in Chapter 2. These isolates are referred to as 'clinical isolates'. The isolates were subjected to antimicrobial susceptibility testing (AST), and genotyped by MLST and

sequencing of the *bla* genes (Chapter 2 and

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Chapter 3 and Appendix 1). The MLST analysis defined the ST of each isolate.

5.2.2 Data analysis

Rarefaction curves were produced as in described in Chapter 2, to compare ST diversities of the faecal and clinical isolates.

To visualise clustering in the data, a minimum spanning tree was produced using the Hamming distances between the ST (ranging from 0 for any pair of isolates belonging to the same ST, to 7 for isolates differing at all loci), using commercial software (BioNumerics 7.5; Applied Maths NV), as described in Chapter 2.

Differences between proportions of interest were statistically assessed by means of two tailed Fisher's exact tests using R (version 3.0.3, Copyright (C) 2014; The R Foundation for Statistical Computing).

5.3 Results

A total of 45 faecal *E. coli* isolates were obtained from hospitalised and healthy cats and dogs in Palmerston North (n=8; cats=3, dogs=5; Appendix

1), and Auckland region (n=37; cats=7, dogs=30;

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Chapter 3). Thirty-six clinical isolates originating from different New Zealand locations (based on the submission forms) were provided by diagnostic laboratories (n=36; cats=8, dogs=28; Chapter 2)

5.3.1 Antimicrobial susceptibility profiles of analysed *E. coli* isolates

The classification of the 81 *E. coli* isolates included in this chapter according to their ESBL/AmpC status and their antimicrobial susceptibility profiles are reported in Table 5.1 and Table 5.2.

Table 5.1: Classification of 81 *Escherichia coli* isolates included in this chapter according to their β-lactamase production

Phenotype	Faecal isolates	Clinical isolates
ESBL	5	11
PAmpC	35	16
ampC	4	7
ESBL/AmpC	1	2
Total	45	36

ESBL: extended spectrum β-lactamase,
PAmpC: plasmid mediated AmpC β-lactamase,
ampC: chromosomal AmpC mutation

Table 5.2: The antimicrobial susceptibility testing results of the 81 *Escherichia coli* isolates analysed in this study; No (%)

	Faecal isolates (n=45)				**Clinical isolates (n=36)**			
Antimicrobials	ESBL	PAmpC	*ampC*	ESBL + AmpC	ESBL	PAmpC	*ampC*	ESBL+ AmpC
Cephalothin	5 (100)	35 (100)	4 (100)	1 (100)	11 (100)	16 (100)	7 (100)	2 (100)
Amoxicillin-Clavulanic acid	5 (100)	35 (100)	4 (100)	1 (100)	11 (100)	16 (100)	7 (100)	2 (100)
Cefoxitin	0 (100)	35 (100)	4 (100)	1 (100)	2 (18)	16 (100)	7 (100)	2 (100)
Cefovecin	5 (100)	35 (100)	4 (100)	1 (100)	11 (100)	16 (100)	7 (100)	2 (100)
Cefotaxime	5 (100)	34 (97)	4 (50)	1 (100)	9 (82)	16 (100)	7 (100)	1 (50)
Ceftazidime	0 (0)	33 (94)	2 (50)	1 (100)	1 (9)	16 (100)	7 (100)	0 (0)
Aztreonam	5 (100)	29 (86)	2 (50)	1 (100)	2 (18)	15 (94)	7 (100)	1 (50)
Gentamicin	4 (80)	0 (0)	0 (0)	0 (0)	4 (36)	0 (0)	2 (29)	1 (50)
Enrofloxacin	3 (80)	5 (14)	1 (25)	1 (100)	9 (82)	7 (44)	2 (29)	1 (50)
Sulfamethoxazole/ Trimethoprim	5 (100)	3 (9)	1 (25)	1 (100)	9 (82)	6 (38)	3 (43)	2 (100)
Tetracycline	5 (100)	21 (59)	2 (50)	1 (100)	10 (91)	11 (69)	6 (86)	1 (50)

The resistance and intermediate resistance were categorised together into one group in the cells. ESBL: Extended spectrum β-lactamase, PAmpC: plasmid mediated β-lactamase, *ampC*: chromosomal *ampC* mutation.

Faecal isolates: ESBL n=5, PAmpC n=35, *ampC* n=4, ESBL +AmpC n=1
Clinical isolates: ESBL n=11, PAmpC n=16, *ampC* n=7, ESBL +AmpC n=2

5.3.2 Genotyping results

5.3.2.1 Genes for ESBL/AmpC production

Among the faecal and clinical isolates, the predominant ESBL gene was *bla*$_{CTX-M-14}$ and the predominant AmpC was the *bla*$_{CMY-2}$. Ten isolates that displayed the AmpC phenotype did not amplify any plasmid-mediated AmpC genes (PAmpC) but had mutations in the chromosomal *amp*C promoter/attenuator region. Details of the ESBL/AmpC enzymes and *ampC* chromosomal mutations observed in this study are reported in Table 5.3.

Table 5.3: β-lactamase genes identified in *E. coli* isolates

	Faecal isolates		Clinical Isolates	
ESBL (n=16)	CTX-M-14, TEM-1	4	CTX-M-14	5
	CTX-M14	1	CTX-M-14, TEM-1	3
			CTX-M-14, SHV-12	2
			CTX-M-15	1
PAmpC (n= 51)	CMY-2	32	CMY-2	12
	CMY-2, TEM-1	3	CMY-2, TEM-1	4
ampC (n= 11)	chromosomal *ampC* mutations	3	chromosomal *ampC* mutations	4
	chromosomal *ampC* mutations, TEM-1	1	chromosomal *ampC* mutations, TEM-1	2
			chromosomal *ampC* mutations, TEM-1b	1
ESBL and AmpC (n=3)	CMY-2, CTX-M14	1	CTX-M-14, chromosomal *ampC* mutations	1
			SHV-12, chromosomal *ampC* mutations	1
Total		45		36

ESBL: extended spectrum β-lactamase, PAmpC: plasmid-mediated AmpC β-lactamase, *ampC*: chromosomal AmpC mutation

5.3.2.2 Results of multilocus sequence typing (MLST)

MLST of 81 *E. coli* isolates revealed 43 STs belonging to 14 clonal complexes (CCs) (Table 5.4). There were 31 STs among the faecal (n=45) and 19 among the clinical isolates (n=36), and seven STs (38, 46, 58, 155, 372, 457, 744) were present in both faecal and clinical isolates. The majority of STs were represented by multiple isolates (Table 5.5). There were 24 STs among the PAmpC-E isolates, with the most common STs being ST973 (n=4). There were 14 STs among the ESBL-E, the most common STs being ST744 (n=5) and ST131 (n=3). There were no obvious regional patterns. For instance, ST744 was identified in ESBL-E from the faeces of healthy dogs in Palmerston North and cats and dogs in Auckland, and from clinical samples from cats and dogs received from laboratories in Christchurch and Palmerston North. Rarefaction curves constructed to compare the ST richness between clinical and faecal ESBL/PAmpC-E indicated a greater ST richness of the faecal isolates (Figure 5.1). The subset of the *E. coli* identified from UTI (n=24/36) had five common STs with isolates from faecal carriage (Table 5.5).

Table 5.4: Sequence types (STs) of the *E. coli* isolates included in this study categorised by multi drug resistance (MDR) and β-lactamase production.

	E. coli Multi Locus Sequence Type			
β-lactamase production	Faecal isolates (n=45)		Clinical isolates (n=36)	
	MDR	Non-MDR	MDR	Non-MDR
ESBL (n=16)	155, 744 (n=3), 405		648 (n=3),131 (n=3), 744 (n=3)	12, 4200*
PAmpC (n=51)	162 (n=2), 2541, 372, 58, 973	10 (n=2), 133, 2712, 295, 155, 297, 4390*, 4391*, 4392*, 4393*, 4394*, 4395*, 4406*, 457 (n=3), 46, 501, 540 (n=2), 57, 929, 963 (n=2), 973 (n=3)	156 (n=3), 23, 457, 46 (n=2), 205, 744	101,1485, 457, 90, 155, 372, 4166*, 648
ampC mutation (n=11)		206, 2175, 590, 405	38, 648, 88	744, 517, 58, 88
ESBL/AmpC (n=3)		405	648	12
Total number of isolates	11	34	21	15

MLST: multilocus sequence type; UTI: urinary tract infection source; superscript* indicates a new MLST not previously reported in the Warwick database ESBL: Extended spectrum β-lactamase, PAmpC: plasmid mediated AmpC β-lactamase.

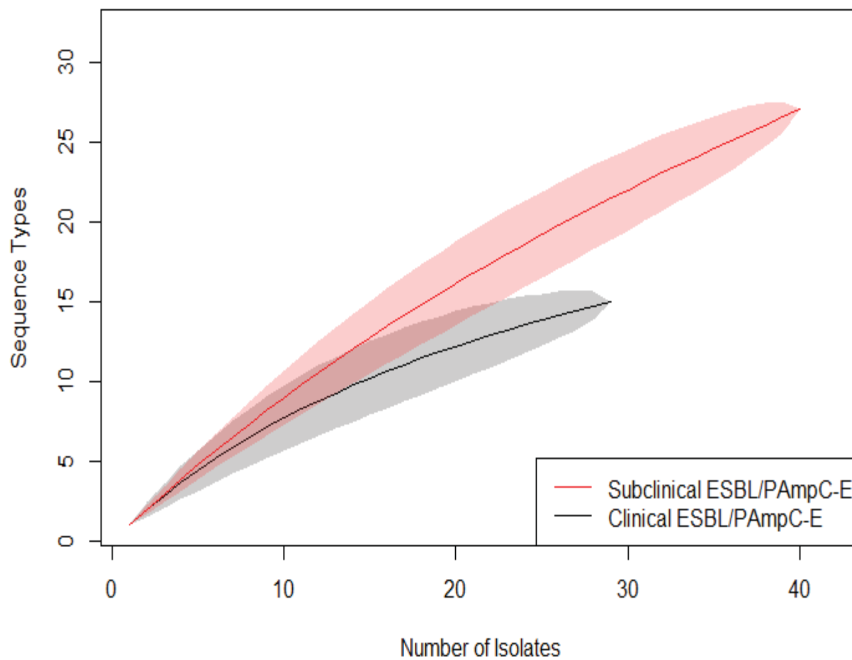

Figure 5.1: Rarefaction analysis of sequence types (STs) of ESBL/PAmpC-producing *E. coli* isolates.

Rarefaction curves for STs of the faecal and clinical *E. coli* isolates are shown separately. The shadings around the lines represent upper and lower 95% confidence intervals. Note that at maximum sampling size the lower boundary of the faecal isolates does not overlap the number of STs of the clinical isolates, indicating the faecal isolates had a greater of ST richness.

Table 5.5: *E. coli* sequence types (STs) identified in ESBL/AmpC-E (both faecal and clinical isolates)			
	Count of isolates		
ST denomination	Faecal isolates	Clinical isolates	
	ESBL/AmpC	ESBL/AmpC	UTI isolates
ST 155	2	1	4
ST 372	1	1	1
ST 457	3	2	1
ST 46	1	2	1
ST 744	3	4	1
Total	10	10	8

ST: sequence type, ESBL: extended spectrum β-lactamase, AmpC: AmpC-β-lactamase,
UTI: urinary tract infections

5.3.2.2.1 Minimum spanning tree (MST)

A MST was constructed to visualise the relatedness between clinical and faecal isolates (Figure 5.2). The tree showed three abundant STs (155, 457 and 744) that were represented in both cats and dogs. Another feature was the relatively small number of single locus variant STs (n=5) suggesting a highly recombinant population, or alternatively, a high rate of mutation.

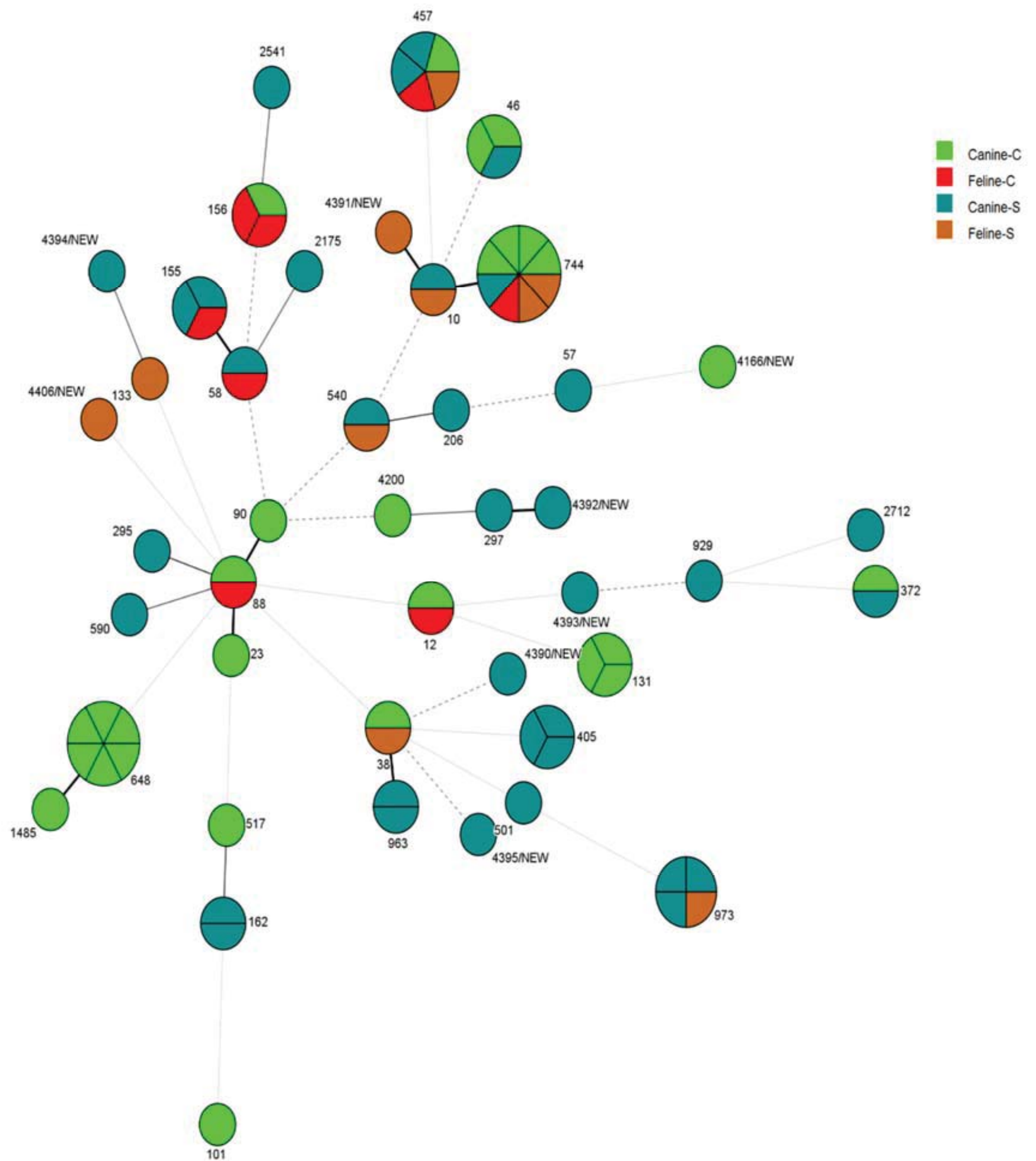

Figure 5.2: Minimum spanning tree showing the associations between the different *E. coli* multilocus sequence types (ST) analysed in this study.

Each circle represents an ST, flanked by its numerical designation according to the Warwick database. The number of partitions indicate the number of isolates found for each MLST. Feline and canine *E. coli* isolates are depicted in different colours. The letters C and S indicate that the MLSTs were reported from clinical specimens and faecal samples

single locus variant; double locus variants; triple locus variants; quadruple locus variants.

5.4 Discussion

In this chapter, we assessed the genetic relatedness of faecal and clinical ESBL/AmpC-E isolates identified from companion animals in New Zealand. The significance of such comparison lies in the possible role of the intestinal reservoir as a source of exogenous infections to others, or auto-infections.

Despite the diverse geographical origin of the *E. coli* isolates, the clinical and faecal groups of ESBL-E and PAmpC-E shared five STs, and most isolates belonging to the same ST had identical ESBL/AmpC genes. The ESBL genes *bla*$_{CTX\text{-}M\text{-}14}$, *bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{SHV\text{-}12}$ were identified only among the clinical ESBL-E isolates. Conversely, a single ESBL gene, *bla*$_{CTX\text{-}M\text{-}14}$, was identified only among the faecal ESBL-E. This may be due to the low number of faecal ESBL-E isolates analysed (n=5). A single AmpC gene (*bla*$_{CMY\text{-}2}$) was detected among both faecal and clinical isolates. The *bla*$_{CMY\text{-}2}$ is recognised as the predominant PAmpC gene, worldwide (Bush 2013b).

When only UTI isolates are considered among the clinical isolates, five STs are shared with faecal isolates (Table 5.5). This result suggests that the intestinal reservoir may play a central role as a source of auto-infection, or exogenous UTI. The majority of *E. coli* STs observed in this study correspond to leading STs identified overseas as 'uropathogenic', such as the ST405, ST38 and ST131 (Alghoribi *et al.* 2015). These STs have also been identified from a range of extra-intestinal infections in humans and companion animals (Alghoribi *et al.* 2015; Schaufler *et al.* 2015; Nakamura *et al.* 2016). The presence of the same STs in different months (data not shown) and geographical regions highlights the successful dissemination of these STs in the community. Interestingly, some STs considered human 'pandemic' (e.g. ST131 and 648) were only identified from clinical infections. Further investigation of these isolates using whole genome sequencing (WGS) could elucidate their virulome. Several abundant STs were found in faeces only (see ST973 and ST405, Table 5.4). Comparative genomic analysis of carriage and invasive isolates using WGS could shed light on the role of the different virulence factors of *E. coli*.

Rarefaction analysis indicated that faecal ESBL/AmpC-E isolates had higher ST diversity than clinical isolates. Many factors could have contributed to this

difference. Firstly, this study may have only revealed a small fraction of the diversity of ESBL/AmpC-E existing in the gut, as only one colony was selected per culture of faecal specimen, and in the gut microbiota, a multitude of mobile genetic elements and a high rate of horizontal gene transfer of antimicrobial resistance determinants occurs (Broaders *et al.* 2013). Hence, it is likely that we only picked the dominant variants (Niki *et al.* 2011). On the contrary, a lower number of STs was observed among the clinical isolates, although the isolates were identified from animals in different geographical regions. This underlines the success of certain ST's in causing extra intestinal infections (Alghoribi *et al.* 2015; Kim *et al.* 2016). Interestingly, some pandemic STs as ST131 and ST648 were only identified from clinical infections.

Auto-infection through the ascending faecal-urinary route is well established pathogenetic mechanism of UTI in humans, (Yamamoto *et al.* 1997; Niki *et al.* 2011; Ruppe *et al.* 2013; Schaufler *et al.* 2015). Furthermore, it has been postulated that the increasing selective pressure due to the use of antimicrobials is driving a rise in the ESBL-E intestinal carriage prevalence, and this is determining an increase in the incidence of urinary tract auto-infections caused by ESBL-E, through the ascending faecal-urethral route (Ruppe *et al.* 2013). To the author's knowledge, the faecal-urethral auto-infection theory has not been established in animals. Finding the same STs from clinical infections and faeces could indicate faeces as a source of infections endogenous, animal-to-animal transmission or human-to-animal transmission (Ewers *et al.* 2012; Schaufler *et al.* 2015). The overlap between the faecal carriage and UTI STs observed in the current study suggests the endogenous ascending faecal-urethral route should be taken into consideration whenever recurrent UTI due to ESBL/AmpC-E are diagnosed in cats and dogs.

As in the previous chapters, minimum spanning tree revealed several clusters of STs, and there was no single central node that could be interpreted as a founder and most connections between STs were weak (double, triple or quadruple locus variants).

5.5 Conclusion

The results of this comparison between faecal and clinical ESBL/AmpC-E indicate a high degree of genetic similarity between the two groups in terms of clonal affiliation, based on MLST and ESBL/AmpC gene composition. This suggests auto-infection, in particular the ascending faecal-urethral route of infection, may play an important role in the pathogenesis of ESBL/AmpC-E infections in animals. The identification of several widespread ESBL/AmpC-E STs underlines their high transmissibility in the community. Future longitudinal studies assessing the impact of the faecal-urethral auto-infection route in companion animals, and the risk of zoonotic transmission of ESBL/AmpC-E, are warranted.

Chapter 6: Characterisation of methicillin resistant *Staphylococcus aureus* isolated from animals in New Zealand, 2012–2013, and MRSA colonisation in cats and dogs in Auckland

Preamble

Previous sections of this thesis (Chapter 2,

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(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

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Name of Candidate: Ali Karkaba

Name/Title of Principal Supervisor: Alex Grinberg

Name of Published Research Output and full reference:

Karkaba, A. Grinberg, J. Benschop, E. Pleydell (2016). Characterisation of extended spectrum β-lactamase and AmpC β-lactamase-producing Enterobacteriaceae isolated from companion animals in New Zealand. New Zealand Veterinary Journal

In which Chapter is the Published Work: Chapter 2

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  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed the study, collected the samples and processed them at Massey University. He wrote the first draft of the paper and responded to the co-authors' feedback and qualified as first and correspondent author.

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Candidate's Signature

02/03/2017
Date

Alex Grinberg Digitally signed by Alex Grinberg Date: 2017.03.03 10:55:04 +13'00'
Principal Supervisor's signature

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Date

Chapter 3 and Chapter 5) focused on ESBL/AmpC-producing Gram-negative bacteria. This Chapter describes a study of methicillin resistant *Staphylococcus aureus* (MRSA). To the best of the author's knowledge, this is the second paper of MRSA in animals in New Zealand published in the peer reviewed scientific literature.

The Chapter is presented as an extended version of a paper published in the New Zealand Veterinary Journal as:

Karkaba A, Benschop J, Hill KE, Grinberg A. Characterisation of methicillin-resistant *Staphylococcus aureus* clinical isolates from animals in New Zealand, 2012-2013, and subclinical colonisation in cats and dogs in Auckland. *New Zealand Veterinary Journal*, 65, 78-83, 2017.

Summary

Methicillin resistant *Staphylococcus aureus* (MRSA) is a major human clinical pathogen that has been identified as a cause of infections also in animals. In the past decade, the incidence of human infections with MRSA in New Zealand has steadily increased, and this increase has been accompanied by changes in the relative contribution of the different MRSA clones. Yet, little is known about the MRSA infection incidence and colonisation in animals in New Zealand.

The aim of the present study was to characterise methicillin-resistant *S. aureus* (MRSA) isolated from infection sites in animals in New Zealand and assess the prevalence of MRSA colonisation in cats and dogs attending veterinary clinics in Auckland.

MRSA isolated from clinical samples by the main New Zealand veterinary diagnostic laboratories between June 2012 and June 2013 were genotypically characterised by DNA microarray hybridisation analysis and spa typing. Nasal or perianal skin swabs were collected from a cross-sectional sample of dogs (n=361) and cats (n=225) attending 29 veterinary clinics in Auckland during the same period, and were analysed for MRSA by culture.

A total of eight MRSA clinical isolates were submitted by the participating laboratories during the study period. The isolates originated from five dogs, including two isolates from the same dog, one foal, and one isolate had no

identification of the host. The strain-types identified were AK3 (SCCmecIV t045; n=1), USA500 (SCCmecIV t064; n=1), WSPP (SCCmecIV t019; n=1), Rhine Hesse (SCCmecII t002; n=2), and EMRSA-15 (SCCmecIV t032; n=3). No MRSA were isolated from 586 cultured swabs. Methicillin-susceptible *S. aureus* (MSSA) was detected in 9/257 (3.5%) swabs and non-aureus staphylococci in 22/257 (8.5%) swabs. The estimated true MRSA colonisation prevalence was 0%, with an upper 95% CI boundary of 1.5% for cats and 1.1% for dogs.

The modest number of MRSA isolates submitted for this study by the participating laboratories suggests clinical MRSA infection in animals in New Zealand continues to be sporadic. The wide variety of strain-types found mirrored the evolving strain-type diversity observed in humans in New Zealand. We cannot rule out bias due to the non-random sampling of cats and dogs, but the apparent colonisation prevalence of 0% was consistent with the low prevalence of colonisation reported in humans in New Zealand. These similarities indicate the epidemiology of animal and human MRSA infections are linked.

6.1 Introduction

Staphylococcus aureus is a Gram-positive bacterium that colonises healthy human skin and mucous membranes, but it is also commonly isolated from skin, internal organ and blood stream infections in humans and animals (Abraham *et al.* 2007a; Garbacz *et al.* 2013). It is estimated that the nasal colonisation rate of *S. aureus* in healthy humans ranges between 20-30% (Mainous *et al.* 2006), while in New Zealand, was reported to be 18% (Best *et al.* 2011).

Horizontal gene transfer mechanisms are considered the main means of dissemination of virulence determinants and antimicrobial resistance genes within and between *S. aureus* clones (Noble *et al.* 1992). The integration, through horizontal gene transfer, of a staphylococcal cassette chromosome *mec* (SCC*mec*) spanning a *mecA* gene, into the chromosome of a methicillin-sensitive *S. aureus* (MSSA) transforms it into a methicillin-resistant *S. aureus* (MRSA) (Ito *et al.* 1999; Ito *et al.* 2001). MRSA are resistant to the β-lactam antimicrobials and are of great concern as they

render the use of most β-lactam antimicrobials ineffectual (Schulte *et al.* 2013).

Descriptions of clinically overt MRSA infections in animals are sporadic. The first overt infection with an MRSA in an animal found in the literature dates back to 1972, and it described a case of bovine mastitis in Belgium (Devriese *et al.* 1972). Subsequent cases were described in hospitalised dogs in Korea (Gortel *et al.* 1999; Tomlin *et al.* 1999) and post-operative wound infections in equine (Hartmann *et al.* 1997). Isolates from overt infections in animals started to be genetically characterised in the last decade (Baptiste *et al.* 2005; Weese *et al.* 2005; Grinberg *et al.* 2008).

Reports in dogs, cats and horses suggest that the prevalence of *S. aureus* colonisation is lower than in humans, and this has been attributed to the presence on the skin of these hosts of competing organisms, such as *Staphylococcus pseudintermedius* (Kottler *et al.* 2010). The prevalence of nasal *S. aureus* colonisation in dogs ranged between 4.3 and 12% in the different studies (Loeffler *et al.* 2005; Loeffler *et al.* 2010; Schmidt *et al.* 2014), and in horses it was 3.7% (Maddox *et al.* 2012). The reported MRSA nasal colonisation prevalence is much lower, and is between 0 and 5% in cats and dogs (Baptiste *et al.* 2005; Loeffler *et al.* 2005; Abraham *et al.* 2007b; Garbacz *et al.* 2013; Muniz *et al.* 2013; Davis *et al.* 2014), and between 0.6 and 16% in horses (Maddox *et al.* 2012; Maddox *et al.* 2015). Interestingly, human medical workers and veterinary personnel seem to carry MRSA at a higher rate than the general population. Furthermore, the strain-types carried by medical and veterinary personnel tend to be similar to those isolated from their respective patients, suggesting possible cross-transmission between personnel and patients, in both environments (Loeffler *et al.* 2005; Perry and Henderson 2008; Ishihara *et al.* 2014).

The distribution of the different MRSA strains in humans appears to vary between regions and in time. For example, currently, the USA300 CA-MRSA (ST8 SCC*mec* IV) strain is the dominant strain in North America, and the ST93 (Queensland clone) is the most common strain found in Australia (Monecke *et al.* 2011). In New Zealand, the situation is dynamic, and in the last decade there have been significant changes in the proportion of the

different strains isolated from humans, and new strain-type not previously reported in the country were identified. In 2005, the predominant strains were the EMRSA-15 and the Western Samoan Phage Pattern strains 1 and 2 (WSPP), and the USA300 clone was isolated in a small number of cases for the first time. Year 2006 saw the first report of the AK3 (ST5 SCC*mec*IV), but by 2013, the AK3 (ST5 SCC*mec*IV) was the dominant strain, accounting for 52% of the isolates, whereas the USA300 strain-type accounted for 6.5%, the EMRSA-15 8.1% and the WSPP 7.9% of the cases (Heffernan *et al.* 2013). The only study that estimated nasal MRSA colonisation rate in New Zealand found a prevalence of <1% (Best *et al.* 2011). In New Zealand, ST22-MRSA-IV (or EMRSA-15) was the most common strain-type identified in humans in 2006, and in a molecular analysis of MRSA isolated from infection sites in animals in the same year, EMRSA-15 was the only strain-type identified in the sample (Grinberg *et al.* 2008). No other published studies of MRSA in animals in New Zealand are available.

In the last decade, there have been major changes in the relative occurrence of the different MRSA strain-types found in humans in New Zealand (Heffernan *et al.* 2014). Therefore, almost a decade after the first characterisation of MRSA in animals in New Zealand, the aim of the present study was to genetically characterise the MRSA isolated from infection sites in animals in 2012–2013. In humans, skin or nasal MRSA colonisation is considered a risk factor for infection from the endogenous source and a potential infection source to others (Kluytmans *et al.* 1997). Hence, a second aim was to assess the prevalence of MRSA in nasal swabs from dogs and perianal swabs from cats attending veterinary clinics in Auckland.

6.2 Materials and methods

6.2.1 Study population and phenotypic MRSA detection

Between June 2012 and June 2013, all commercial veterinary diagnostic laboratories operating in the country were asked to submit to Massey University (MU) the clinical *S. aureus* isolated from animals (except *S. aureus* isolated from bovine milk samples, unless showing resistance to oxacillin), along with the specimen submission form. The participating

laboratories were Gribbles (Auckland, Hamilton, Palmerston North, Christchurch and Dunedin) and New Zealand Veterinary Pathology (NZVP) (Palmerston North and Hamilton). The isolates were submitted by courier on nutrient agar slopes. Upon arrival to MU, a bacterial loopful was subcultured on a nutrient agar plate (Fort Richards, Auckland, New Zealand) and incubated at 37ºC in aerobic conditions for 24hr, and a single colony was subsequently subcultured on a new 5% blood agar plate, to obtain a pure culture. This was followed by a phenotypic testing for the confirmation of *S. aureus,* which included a Gram-stain, colony morphology and haemolysis pattern verification, a catalase test, a fast clumping factor and/or protein A card test (BactiStaph®, Remel, Lenexa, KS), and the acetoin production test, as previously described (Grinberg *et al.* 2008).

Isolates exhibiting the *S. aureus* phenotype were subjected to antimicrobial susceptibility testing (AST) using methods compliant with the Clinical and Laboratory Standards Institute (CLSI) guidelines for *S.aureus* and MRSA (CLSI 2013). Antimicrobials used were penicillin G (10 U); oxacillin (1 μg); cefoxitin (30 μg); erythromycin (10 μg); clindamycin (2 μg); vancomycin (30 μg); gentamycin (10 μg), enrofloxacin (5 μg); trimethoprim/sulphamethoxazole (25 μg) tetracycline (30 μg); polymixin B (300 U); (Oxoid, Wade Road, Basingstoke, UK). The D-test for the detection of inducible clindamycin resistance was performed, as previously described (Fiebelkorn *et al.* 2003). When available, animal-specific zone diameter interpretive criteria were used. If these were unavailable, the human interpretive criteria were applied. A quality control test using *S. aureus* ATCC25923 (supplied by Institute of Environmental Science and Research Ltd, Porirua, New Zealand) was applied for each batch of isolates tested. Isolates showing oxacillin and/or cefoxitin resistance were tested for the expression of penicillin binding protein PBP-2 using a latex agglutination test kit (Denka Seiken, Co Ltd, Japan). All the isolates identified as *S. aureus* were frozen at -80ºC in glycerol broth (15%) for future reference. The *S. aureus* isolates were re-identified by matrix-assisted laser desorption/ionization (MALDI-TOF, MYLA Version 3.2.0_5. Sys. Compliance:

VITEK MS1.1.0) in 2014, at the Middlemore Hospital Laboratory, Auckland New Zealand.

The second part of the study aimed at estimating the prevalence of MRSA colonisation in dogs and cats in Auckland. It was performed as part of a survey primarily designed to estimate the prevalence of ESBL/AmpC-E in

rectal swabs from cats and dogs in Auckland (see

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Name/Title of Principal Supervisor: Alex Grinberg

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  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed the study, collected the samples and processed them at Massey University. He wrote the first draft of the paper and responded to the co-authors' feedback and qualified as first and correspondent author.

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Candidate's Signature

02/03/2017
Date

Alex Grinberg Digitally signed by Alex Grinberg Date: 2017.03.03 10:55:04 +13'00'
Principal Supervisor's signature

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Chapter 3 `andChapter 4). For the assessment of the MRSA prevalence, the clinics' personnel were asked to follow a uniform sampling protocol which included the insertion of a sterile swab moistened with normal sterile saline into one or two nostrils of the dog, and gentle rotation to allow contact with the nasal mucosa. Due to their smaller nostrils, swabs were rubbed on the perianal skin area of cats (Kottler *et al.* 2010). The swabs were placed in charcoal Amies transport medium (Transystem®, Copan, Brescia, Italy). Staff were asked to send the swabs as soon as possible after collection to MU by courier delivery.

For the first 329 swabs (213 dogs and 143 cats), the isolation of MRSA isolates from swabs was carried using a previously described protocol (Grinberg et al., 2008). Briefly, upon arrival to MU, the tip of the swab was cut and immersed in 1 ml of phosphate buffered saline pH 7.4 (PBS) prepared in house, and vortexed for 3-5 seconds. A 100µl of suspension was transferred into 7% NaCl broth (Fort Richards, Auckland, New Zealand), and incubated at 37°C for *Staphylococcus spp* enrichment. A loopful of vortexed broth was subcultured every 24 hours on MRSA agar (containing 4mg/L oxacillin, Fort Richards, Auckland, New Zealand) and incubated as above, for a total of four subcultures.

As no growth was identified on MRSA agar plates in the first 329 swabs, the following 257 swabs (148 dogs and 109 cats) were processed by the same protocol except for the use of mannitol salt agar without oxacillin. This modification was introduced to enable the growth of MSSA, as well as MRSA, because no Gram-positive, catalase positive, clumping factor-positive cocci were isolated from the first 329 swabs, calling into question the sensitivity of the first method. We reasoned that a successful identification of MSSA at the expected prevalence on plates without oxacillin would have provided indirect validation of the ability to detect MRSA, if these were present in the sample. This modification underpinned the isolation of MSSA, as well as MRSA. One colony growing on mannitol salt agar was isolated on a new nutrient agar plate and the organism was identified using the phenotypic tests described above. All the presumably identified *S. aureus* isolates were re-identified by MALDI-TOF.

6.2.2 MRSA strain-type definition and nomenclature

The terms “clone” and “strain” have been widely used interchangeably to describe *S. aureus* isolates in the literature over the last two decades. A *S. aureus* strain has been defined as “an isolate or group of isolates that can be distinguished from other isolates of the same genus and species by phenotypic characteristics or genotypic characteristics or both”, whereas a “clone” as “isolates that are indistinguishable from each other by a variety of genetic tests” (Tenover *et al.* 1995; Dijkshoorn *et al.* 2000). These definitions are problematic and have been questioned by different authors, as different molecular typing tools may delineate different ‘strains’ or clonal groups. For example, isolates displaying the same pulsed-field gel electrophoresis (PFGE) banding pattern might show differences in their microarray hybridisation profiles due to horizontal gene transfer phenomena, or vice-versa, different banding patterns may delineate isolates with very similar microarray profiles. Indeed, according to some authors, *S. aureus* could be defined as a ‘‘quasi-species’’, where the genome “cannot be described as a defined structure, but rather as a weighted average of a large number of individual sequences’’ (Domingo *et al.* 1978; Monecke *et al.* 2011). Therefore, the vernacular strain-type nomenclature used in official New Zealand reports is used in this study. The implication is that in some cases, what could be considered by some authors as different ‘clones’, in this thesis may be lumped in under a single strain-type.

6.2.3 Genotyping of MRSA isolates

Positive phenotypic MRSA isolates were first confirmed by polymerase chain reaction (PCR) at MU using a previously described protocol for the detection of the *mecA* gene (Oliveira and de Lencastre 2002). Subsequently, the isolates were analysed by DNA microarray analysis, using a kit composed of 336 probes (CLONDIAG *S. aureus* Genotyping Kit 2.0, Alere Technologies, Jena, Germany). The probes identified species markers, markers of antimicrobial resistance, microbial surface components recognizing adhesive molecules (MSCRAMMs), various enzymes, pathogenicity markers, and markers allowing assignment of unknown *S. aureus* isolates to known strains. Since the DNA Microarray hybridisation patterns cannot discriminate

between sequence types that differ by a single nucleotide and only affiliates to clonal complex, the isolates were characterised at the same centre by means of PCR-sequencing for of the hypervariable segment of the protein A gene (*spa* typing) as previously described (Harmsen *et al.* 2003), in order to identify or narrow down the number of possible sequence types (ST). *spa* sequence types were aligned with previously reported sequences using the Ridom database (http://spa.ridom.de/spatypes.shtml). Furthermore, three isolates were not narrowed to one MLST type, thus whole genome sequencing (WGS) was performed formed using Illumina Miseq (San Diego, USA). Genomic DNA library was prepared using the Nextera XT kit (Illumina) and sequenced on the 250-bp pair-ended chemistry. DNA sequences collated at http://saureus.mlst.net/ belonging to 3044 ST types were downloaded in FASTA format and mapped to the Illumina reads using the pipeline described by Inouye *et al.* (2014). The molecular analysis of the isolates was performed at the Australian Collaborating Centre for Enterococcus and *Staphylococcus* Species Typing and Research, School of Biomedical Sciences, Curtin University, Australia (Direct contact: Prof. Geoffrey Coombs).

Massey University Animal Ethics committee, New Zealand granted the institutional approval to obtain samples from cats and dogs (MUAEC Protocol 12/46).

6.2.4 Data Analysis

The estimated true prevalence of MRSA colonisation and 95% confidence intervals (CI) were calculated separately for cats and dogs. The sensitivity of MRSA culture was estimated to be 90% and 80% (Safdar *et al.* 2003) for cats and dogs respectively, with a specificity fixed at 100% (any MRSA was confirmed by genotyping). To account for uncertainty of the estimate, six different deterministic models of prevalence provided by an online calculator were calculated (http://epitools.ausvet.com.au/content.php?page=TruePrevalence&SampleSize)

6.3 Results

6.3.1 Characteristics and antimicrobial susceptibility of *S. aureus* clinical isolates submitted by diagnostic laboratories

A total of 30 *S. aureus* were submitted from the seven veterinary participating laboratories. Eight isolates presumptively identified as MRSA were received (Table 6.1 and Table 6.2). The isolates were re-identified by biochemical tests and MALDI-TOF as *S. aureus.* All the MSSA were susceptible and the eight MRSA were resistant to oxacillin at MU.

A presumptive MRSA isolate originating from a milk sample from a cow with mastitis was resistant to penicillin, but was intermediate to oxacillin and susceptible to cefoxitin. Further testing using the E-test indicated an oxacillin MIC of 1.5µg/ml, which categorised it as oxacillin-susceptible according to the CLSI guidelines. Furthermore, the isolate was negative for the *mecA* gene by PCR, and for the PBP2a by latex agglutination. Further genotyping using DNA microarray, MLST and *spa* typing indicated the isolate was negative for *mecA*, belonged to clonal complex 1, ST1 and harboured lukF-P83/lukM+ ruminant leucocidins. This isolate was not further analysed.

6.3.2 Characteristics of the MRSA clinical isolates

Eight presumptively identified MRSA and 22 MSSA isolates were submitted by the participating laboratories. The MRSA isolates originated from five dogs, one foal, and one isolate had no identification of the source (Table 6.1). Four isolates originated from the North Island and four from the South Island. Two isolates were sourced four months apart from a post-operative chronic wound of the same dog (Table 6.1).

Only 51 of 2,688 (1.9%) microarray probes returned ambiguous hybridisation results. Microarray results indicated six MRSA carried SCC*mec*IV and two carried SCC*mec*II (Table 6.2). The MRSA strain-types and ST could be predicted with high certainly in 5/8 isolates by combining microarray and *spa* typing results, and whole genome sequencing was required to define the ST of three isolates. The MRSA strain-types identified were the AK3 (ST-5 SCC*mec*IV t045), EMRSA-15 (ST22 SCC*mec*IV t032), WSSP (ST30

SCC*mec*IV t019), USA500 (ST8 SCC*mec*IV t064), and Rhine Hesse (ST5 SCC*mec*II t002) (Table 6.2).

In all the MRSA, the resistome microarray results matched the disk diffusion test results (Table 6.2). Fosfomycin antimicrobial was not included in the disc diffusion testing but the microarray probe for fosfomycin detected resistance (resistance genotype fosB) in 5/8 isolates (Table 6.4). Three MRSA isolates were found to have *in vitro* inducible clindamycin resistance (Table 6.2). Full microarray and *spa* typing results of the MRSA are presented in Section 6.6. The 22 MSSA were oxacillin and cefoxitin-susceptible.

Table 6.1: Details of the sources of eight methicillin resistant *Staphylococcus aureus* isolates submitted by veterinary diagnostic laboratories in New Zealand between June 2012 and June 2013.

Isolate number	Location	Host species	Signalment	Infection site [a]
1	North Island	Canine	German Shepherd, 2 years old, M	Skin
2	North Island	Canine	Cavalier King Charles Spaniel, 10 months old, F	Throat
3	North Island	Canine	Spaniel, 11 years old, F	Throat
4	South Island	Unknown	Unknown	Unknown
5 [b]	South Island	Canine	Doberman, 8-months-old, F	Surgical wound
5.1 [b]	South Island	Canine	Doberman, 1-year-old, F	Surgical wound
6	South Island	Canine	Chinese Crested dog, 8-years-old, F	Wound
7	South Island	Equine	Standardbred, 5-months-old, F	Wound

[a] Stated on submission form

[b] Isolates 5 and 5.1 were obtained from the same dog four months apart, from a chronic wound following femoral head excision.

F= female; M= Male

6.3.3 Prevalence of MRSA in the samples from cats and dogs

A total of 586 swabs were cultured (225 from cats, 361 from dogs). The submission forms indicated that 177 (31%; 55 from cats and 122 from dogs) were treated with antimicrobials at the time of sampling. No MRSA were isolated from the 586 cultured swabs. When the culture protocol was changed, MSSA were detected in 9/257 (3.5%) swabs (from 1 perianal cat swab and 8 nasal dog swabs; all these isolates were susceptible to oxacillin and cefoxitin), and non-*aureus* staphylococci in 22/257 (8.5%) swabs. The isolates were re-identified by biochemical tests and MALDI-TOF. The estimated true MRSA prevalence of the six deterministic models (assuming 90% and 80% culture sensitivity) was 0%, with 95% CI ranges of 0 to 2.1% for cats and 0 to 1.4% for dogs. The 95% CI of MSSA prevalence ranged between 0 and 4% for cats and 0 and 5% for dogs.

Table 6.2: *In vitro* antimicrobial susceptibility and molecular characterisation of the eight-methicillin resistant *Staphylococcus aureus* isolates isolated by veterinary diagnostic laboratories in New Zealand between June 2012 and June 2013.

	Antimicrobial susceptibility [a]								Microarray analysis		Type [d]		
Isolate	E	CL	IC	CN	ENR	SXT	TE	PVL	Antimicrobial resistance genes	SCC*mec* [b]	*spa* [c]	ST	Strain-type
1	S	S	neg	S	S	S	S	-	*bla*Z/I/R; fosB; sdrM	IV	t045	ST5	AK3
2	R	R	pos	S	R	S	S	-	*bla*Z/I/R; *erm*A; *fos*B; sdrM	II	t002	ST5	Rhine Hesse
3	R	R	pos	S	R	S	S	-	*bla*Z/I/R; *erm*A; *fos*B; sdrM	II	t002	ST5	Rhine Hesse
4	S	S	neg	S	S	S	S	+	*bla*Z/I/R; *fos*B; sdrM	IV	t019	ST30	WSSP
5 [e]	S	S	neg	S	R	S	S	-	*bla*Z/I/R	IV	t032	ST22	EMRSA-15
5.1 [e]	S	S	neg	S	R	S	S	-	*bla*Z/I/R	IV	t032	ST22	EMRSA-15
6	S	S	neg	S	R	S	S	-	*bla*Z/I/R	IV	t032	ST22	EMRSA-15
7	R	I	pos	R	S	R	R	-	*bla*Z/I/R; *erm*C; aacA-aphD; *dfr*S1; *tet*M; *fos*B; *sdr*M	IV	t064	ST8	USA500

[a] Results for erythromycin (E), clindamycin (CL), inducible clindamycin resistance (IC), gentamicin (CN), enrofloxacin (ENR), sulphamethoxazole-trimethoprim (SXT) and tetracycline (TE),
[b] Staphylococcal cassette chromosome *mec* type
[c] *Spa*-type as described in Harmson *et al.* (2003)
[d] Multilocus sequence type (ST) and strain type indirectly inferred from the combined results of *spa* typing and microarray profile. Possible new STs were not ascertained. [e] Isolates 5 and 5.1 were obtained from the same dog, four months apart
I=Intermediate; neg=negative; pos=positive; R=resistant; S=susceptible; PVL: presence (+) or absence (-) of the Panton Valentine Leukocidin gene
Detailed DNA microarray and *spa* typing results are presented in Section 6.6.

6.4 Discussion

In this study, we characterised the MRSA isolated by the main New Zealand veterinary diagnostic laboratories over one year and assessed the prevalence of MRSA in nasal swabs from dogs and perianal swabs of cats attending veterinary clinics in Auckland. Eight MRSA isolates from infection sites were obtained from the participating laboratories, whilst no MRSA were isolated from the sampled dogs and cats. The previous survey of MRSA in animals performed in New Zealand obtained seven isolates (Grinberg *et al.* 2008). We cannot rule out the occurrence of other cases not referred to us, but the modest number of isolates provided for the present study by the laboratories indicates MRSA infection in animals in New Zealand remain sporadic.

All the MRSA found in this study belonged to strain-types previously isolated from human infections in New Zealand, of which three, AK3 (SCC*mec*IV t045), EMRSA-15 (SCC*mec*IV t032), and WSSP (SCC*mec*IV t019), were among the six predominant strains, accounting for >90% of the human clinical isolates in the study period (Heffernan *et al.* 2014). In a previous New Zealand study from 2006, all the isolates from infection sites in animals were EMRSA-15, then the most prevalent strain-type in humans (Grinberg *et al.* 2008). In the current study, in addition to EMRSA-15, AK3, and WSSP, we found the less common USA500 (SCC*mec*IV t064) and Rhine Hesse (SCC*mec*II, t002) strain-types. Interestingly, AK3 is the predominant community-associated MRSA strain in New Zealand and was first reported in the country in 2005 (Williamson *et al.* 2013). Instead, the USA500 has been identified more recently (Helen Heffernan, pers. comm.). Together, these results are consistent with data from the United States and Canada indicating MRSA causing overt infections in animals are often the same strain-types found in humans (Faires *et al.* 2009; Harrison *et al.* 2014)

The Rhine Hesse is multidrug resistant and has been reported in many countries (Monecke *et al.* 2011). In animals, the Rhine Hesse has been isolated from healthy dogs in the USA (Davis *et al.* 2014) and from clinical infections in cats and dogs in Austria (Loncaric *et al.* 2014). The USA500 strain-type identified from a foal displayed a microarray hybridisation pattern

consistent with the 'equine' USA500 MRSA variant. Our search indicated this variant was first found in horses and in-contact humans in Ireland (Kinnevey *et al.* 2010). The strain differs from the so-called 'human' USA500 variant by the absence of the lysogenic β-haemolysin-converting phages, *sea, sak, chp*, and *scn* genes (Monecke *et al.* 2011). Based on previous *spa* typing results, it is likely that the USA500 strain has been identified previously in horses in New Zealand (Helen Heffernan, pers. comm.;(D'Amours 2011) but the author was unable to retrieve further published information about the presence of this strain in New Zealand.

The anterior nares are among the most widely studied MRSA colonisation sites in humans and in animals. In our study no MRSA was isolated from the 587 sampled dogs and cats, and the estimated true prevalence was 0% with an upper 95% CI of <3% in all cases. Some limitations of the study could have led to underestimation of the colonisation prevalence. This study was not designed to assess MRSA prevalence. In fact, we used a non-random sample of animals visiting veterinary clinics, and 31% of dogs and cats were treated with antimicrobials at the time of sampling. Sampling an additional anatomical site may have enhanced detection of MRSA (Fang and Hedin 2006). However, MSSA were detected in 8/148 (6%) of the swabs from dogs cultured on oxacillin-free media, which is higher than the 4% MSSA prevalence reported in dogs in the UK (Schmidt *et al.* 2014) and 1.8% reported in Germany (Walther *et al.* 2012a). The prevalence of MSSA in the current study indirectly validated the method used for MRSA culture, suggesting the true prevalence of MRSA colonisation in dogs and cats in Auckland, was low. Moreover, our results are consistent with a relatively low prevalence of MRSA nasal colonisation reported in humans in Auckland, with one study reporting <1% among healthy people and 4% among healthcare workers (Perry and Henderson 2008).

Finally, the bovine isolate was eventually found to be an MSSA. To our knowledge, this is first identification of bovine MSSA CC1 ST1 carrying the ruminant bicomponent lukF-P83/lukM+ (Vrieling *et al.* 2015) in New Zealand. Some authors have identified the lukF-P83/lukM+ as a major contributor to the pathogenicity of bovine mastitis-causing strains (Monecke *et al.* 2007;

Schlotter *et al.* 2012). The lukF-P83/lukM has been detected in other bovine lineages, as the CC151, CC479 and CC133 (Schlotter *et al.* 2012).

6.5 Conclusion

The results suggest overt MRSA infection in animals in New Zealand continues to be sporadic. As in humans, the prevalence of MRSA nasal colonisation in dogs and cats attending veterinary clinics in Auckland was also low. The MRSA strain-type diversity reflected the diversification observed in humans in New Zealand over the same period. Taken together, these results suggest that the epidemiologies of MRSA infections in dogs, cats and humans in New Zealand are linked.

6.6 Supplementary Material

Table S 6.1 :Summary of the DNA microarray and *spa* results of the MRSA isolates

Isolate	1	2 & 3	4	5 & 5.1	6	a
TYPING SUMMARY						
strain	CC5-MRSA-IV, Paediatric clone	ST5/ST225-MRSA-II, Rhine-Hesse EMRSA/ New York-Japan Clone	CC30-MRSA-IV [PVL+], Southwest Pacific Clone	CC22-MRSA-IV [fnbB-,sec/l+], UK-EMRSA-15/Barnim EMRSA	CC8-MRSA-IV, USA500	CC1-MSSA [lukF-P83/lukM+]
strain synonyms	WA MRSA-03/25/50/71/74/82/105/111, USA800, Spanish PFGE type E7/8, Marseille CF clone	USA100, Canadian MRSA-2, Irish AR7.3/AR7.4, Finland E1, JH1/JH9	USA 1100, West Samoan Phage Pattern (WSPP) Clone	Irish AR06, Canadian MRSA-8, Spanish PFGE type E13, "ST22-A" clade of UK-ERMSA-15	WA MRSA-20/58	
MLST clonal complex affiliation	CC5	CC5	CC30	CC22	CC8	CC1
sequence types associated with this strain	ST5, ST225	ST5, ST231	ST30	ST22	ST8	
spa type associated with this strain	t045	t002	t019	t032	t064	t144
assignment score for CC identification	96.83%	97.66%	97.52%	96.97%	94.21%	95.04%
MRSA (mecA)	positive	positive	positive	positive	positive	negative
MRSA (mecC)	negative	negative	negative	negative	negative	negative
PVL	negative	negative	positive	negative	negative	negative

SPECIES MARKER						
Target Gene	Result	Result	Result	Result	Result	Result
rrnD1 (S. aureus)	positive	positive	positive	positive	positive	positive
gapA	positive	positive	positive	positive	positive	positive
katA	positive	positive	positive	positive	positive	positive
coA	positive	positive	positive	positive	positive	positive
nuc1	positive	positive	positive	positive	positive	positive
spa	positive	positive	positive	positive	positive	positive
sbi	positive	positive	positive	positive	positive	positive
REGULATORY GENES						
Target Gene	Result	Result	Result	Result	Result	Result
sarA	positive	positive	positive	positive	positive	positive
saeS	positive	positive	positive	positive	positive	positive
vraS	positive	positive	positive	positive	positive	positive
agrI (total)	negative	negative	negative	positive	positive	negative
agrB-I	negative	negative	negative	positive	positive	negative
agrC-I	negative	negative	negative	ambiguous	positive	negative
agrD-I	negative	negative	negative	positive	positive	negative
agrII (total)	positive	positive	negative	negative	negative	negative
agrB-II	positive	positive	negative	negative	negative	negative
agrC-II	positive	positive	negative	negative	negative	negative
agrD-II	positive	positive	negative	negative	negative	negative
agrIII (total)	negative	negative	positive	negative	negative	positive
agrB-III	negative	negative	positive	negative	negative	positive
agrC-III	negative	negative	positive	negative	negative	positive
agrD-III	negative	negative	positive	negative	negative	positive
agrIV (total)	negative	negative	negative	negative	negative	negative
agrB-IV	negative	negative	negative	ambiguous	ambiguous	negative
agrC-IV	negative	negative	negative	negative	negative	negative
hld	positive	positive	positive	positive	positive	positive
METHICILLIN RESISTANCE GENOTYPE AND SCCmec TYPING						
Target Gene	Result	Result	Result	Result	Result	Result
mecA	positive	positive	positive	positive	positive	negative
mecC	negative	negative	negative	negative	negative	Negative

Isolate	1	2 & 3	4	5 & 5.1	6	a
delta_mecR	positive	positive	positive	positive	positive	negative
ugpQ	positive	positive	positive	positive	positive	negative
ccrA-1	negative	negative	negative	negative	negative	negative
ccrB-1	negative	negative	negative	negative	negative	negative
plsSCC (COL)	negative	negative	negative	negative	negative	negative
Q9XB68-dcs	positive	positive	positive	positive	positive	negative
ccrA-2	positive	positive	positive	positive	positive	negative
ccrB-2	positive	positive	positive	positive	positive	negative
kdpA-SCC	negative	positive	negative	negative	negative	negative
kdpB-SCC	negative	positive	negative	negative	negative	negative
kdpC-SCC	negative	positive	negative	negative	negative	negative
kdpD-SCC	negative	positive	negative	negative	negative	negative
kdpE-SCC	negative	positive	negative	negative	negative	negative
mecI	negative	positive	negative	negative	negative	negative
mecR	negative	positive	negative	negative	negative	negative
xylR	negative	positive	negative	negative	negative	negative
ccrA-3	negative	negative	negative	negative	negative	negative
ccrB-3	negative	negative	negative	negative	negative	negative
merA	negative	negative	negative	negative	negative	negative
merB	negative	negative	negative	negative	negative	negative
ccrAA (MRSAZH47)_probe 1	negative	negative	negative	negative	negative	negative
ccrAA (MRSAZH47)_probe 2	negative	negative	negative	negative	negative	negative
ccrC (85-2082)	negative	negative	negative	negative	negative	negative
ccrA-4	negative	negative	negative	negative	negative	negative
ccrB-4	negative	negative	negative	negative	negative	negative
RESISTANCE GENOTYPE : PENICILLINASE						
Target Gene	Result	Result	Result	Result	Result	Result
blaZ	positive	positive	positive	positive	positive	positive
blaZ-SCCmec XI	negative	negative	negative	negative	negative	negative
blaI	positive	positive	positive	positive	positive	positive
blaR	positive	positive	positive	positive	positive	positive
RESISTANCE GENOTYPE : MLS-ANTIBIOTICS						
Target Gene	Result	Result	Result	Result	Result	Result
erm(A)	negative	positive	negative	negative	negative	negative
erm(B)	negative	negative	negative	negative	negative	negative
erm(C)	negative	negative	negative	negative	positive	negative
lnu(A)	negative	negative	negative	negative	negative	negative
msr(A)	negative	negative	negative	negative	negative	negative
mef(A)	negative	negative	negative	negative	negative	negative
mph(C)	negative	negative	negative	negative	negative	negative
vat(A)	negative	negative	negative	negative	negative	negative
vat(B)	negative	negative	negative	negative	negative	negative
vga(A)	negative	negative	negative	negative	negative	negative
vga(A) (BM 3327)	negative	negative	negative	negative	negative	negative
vgB(A)	negative	negative	negative	negative	negative	negative
RESISTANCE GENOTYPE : AMINOGLYCOSIDES						
Target Gene	Result	Result	Result	Result	Result	Result
aacA-aphD	negative	negative	negative	negative	positive	negative
aadD	negative	negative	negative	negative	negative	negative
aphA3	negative	negative	negative	negative	negative	negative
RESISTANCE GENOTYPE : MISCELLANEOUS GENES						
Target Gene	Result	Result	Result	Result	Result	Result
sat	negative	negative	negative	negative	negative	negative
dfrS1	negative	negative	negative	negative	positive	negative
fusB	negative	negative	negative	negative	negative	negative

Isolate	1	2 & 3	4	5 & 5.1	6	a
fusC	negative	negative	negative	negative	negative	negative
mupA	negative	negative	negative	negative	negative	negative
tet(K)	negative	negative	negative	negative	negative	negative
tet(M)	negative	negative	negative	negative	positive	negative
cat (total)	negative	negative	negative	negative	negative	negative
cat (pC221)	negative	negative	negative	negative	negative	negative
cat (pc223)	negative	negative	negative	negative	negative	negative
cat (pMC524)	negative	negative	negative	negative	negative	negative
cat (pSBK203R)	negative	negative	negative	negative	negative	negative
cfr	negative	negative	negative	negative	negative	negative
fexA	negative	negative	negative	negative	negative	negative
fosB	positive	positive	positive	negative	positive	negative
fosB (plasmid)	negative	negative	negative	negative	negative	negative
RESISTANCE GENOTYPE : EFFLUX SYSTEMS						
Target Gene	Result	Result	Result	Result	Result	Result
qacA	negative	negative	negative	negative	negative	positive
qacC (total)	negative	negative	negative	negative	negative	negative
qacC (consensus)	negative	negative	negative	negative	negative	negative
qacC (equine)	negative	negative	negative	negative	negative	negative
qacC (SA5)	negative	negative	negative	negative	negative	negative
qacC (Ssap)	negative	negative	negative	negative	negative	negative
qacC (ST94)	negative	negative	negative	negative	negative	negative
sdrM	positive	positive	positive	negative	positive	positive
RESISTANCE GENOTYPE : GLYCOPEPTIDES						
Target Gene	Result	Result	Result	Result	Result	Result
vanA	negative	negative	negative	negative	negative	negative
vanB	negative	negative	negative	negative	negative	negative
vanZ	negative	negative	negative	negative	negative	negative
VIRULENCE: TOXIC SHOCK TOXIN						
Target Gene	Result	Result	Result	Result	Result	Result
tst1 (consensus)	negative	negative	negative	negative	negative	negative
tst1 ("human" allele)	negative	negative	negative	negative	negative	negative
tst1 ("bovine" allele, from RF122)	negative	negative	negative	negative	negative	negative
VIRULENCE: ENTEROTOXINS						
Target Gene	Result	Result	Result	Result	Result	Result
sea	negative	negative	negative	negative	negative	negative
sea (320E)	negative	negative	negative	negative	negative	negative
sea (N315)	positive	negative	negative	negative	negative	negative
seb	negative	negative	negative	negative	positive	negative
sec	positive	negative	negative	positive	negative	negative
sed	negative	positive	negative	negative	negative	negative
see	negative	negative	negative	negative	negative	negative
seg	positive	positive	positive	positive	negative	negative
seh	negative	negative	negative	negative	negative	positive
sei	positive	positive	positive	positive	negative	negative
sej	negative	positive	negative	negative	negative	negative
sek	negative	negative	negative	negative	positive	negative
sel	positive	negative	negative	positive	negative	negative
selm	positive	positive	positive	positive	negative	negative
seln (consensus)	positive	positive	positive	positive	negative	negative
seln (other than RF122)	positive	positive	positive	positive	negative	negative
selo	positive	positive	positive	positive	negative	negative
egc	positive	positive	positive	positive	negative	negative
seq	negative	negative	negative	negative	positive	negative
ser	negative	positive	negative	negative	negative	negative
selu	positive	positive	positive	positive	negative	negative
Isolate	1	2 & 3	4	5 & 5.1	6	a

ORF CM14_ probe1	negative	negative	negative	negative	negative	negative
ORF CM14_ probe2	negative	negative	negative	negative	negative	negative
VIRULENCE: HLG AND LEUKOCIDINS						
Target Gene	Result	Result	Result	Result	Result	Result
lukF	positive	positive	positive	positive	positive	positive
lukS	positive	positive	positive	ambiguous	positive	positive
lukS (ST22+ST45)	positive	positive	positive	positive	ambiguous	positive
hlgA	positive	positive	positive	positive	positive	positive
lukF-PV	negative	negative	positive	negative	negative	negative
lukS-PV	negative	negative	positive	negative	negative	negative
lukF-PV (P83)	negative	negative	negative	negative	negative	positive
lukM	negative	negative	negative	negative	negative	positive
lukD	positive	positive	negative	negative	positive	positive
lukE	positive	positive	negative	negative	positive	positive
lukX	positive	positive	positive	positive	positive	positive
lukY	positive	positive	negative	positive	positive	positive
lukY (ST30+ST45)	negative	negative	positive	negative	negative	negative
VIRULENCE: HAEMOLYSINS						
Target Gene	Result	Result	Result	Result	Result	Result
hl	positive	positive	positive	positive	positive	positive
hla	positive	positive	positive	positive	positive	positive
hlIII (consensus)	positive	positive	positive	negative	positive	positive
hlIII (other than RF122)	positive	positive	positive	negative	positive	positive
hlb_probe 1	positive	positive	positive	positive	positive	positive
hlb_probe 2	positive	positive	positive	positive	positive	positive
hlb_probe 3	ambiguous	positive	ambiguous	positive	positive	positive
un-disrupted hlb	negative	negative	negative	negative	positive	positive
VIRULENCE: HLB-CONVERTING PHAGES						
Target Gene	Result	Result	Result	Result	Result	Result
sak	positive	positive	positive	positive	negative	negative
chp	positive	positive	positive	positive	negative	negative
scn	positive	positive	positive	positive	negative	negative
VIRULENCE: EXFOLIATIVE TOXINS						
Target Gene	Result	Result	Result	Result	Result	Result
etA	negative	negative	negative	negative	negative	negative
etB	negative	negative	negative	negative	negative	negative
etD	negative	negative	negative	negative	negative	negative
VIRULENCE: EPIDERMAL CELL DIFFERENTION INHIBITORS						
Target Gene	Result	Result	Result	Result	Result	Result
edinA	negative	negative	negative	negative	negative	negative
edinB	negative	negative	negative	negative	negative	negative
edinC	negative	negative	negative	negative	negative	negative
VIRULENCE: ACME LOCUS						
Target Gene	Result	Result	Result	Result	Result	Result
ACME cluster	negative	negative	negative	negative	negative	negative
arcA-SCC	negative	negative	negative	negative	negative	negative
arcB-SCC	negative	negative	negative	negative	negative	negative
arcC-SCC	negative	negative	negative	negative	negative	negative
arcD-SCC	negative	negative	negative	negative	negative	negative
VIRULENCE: PROTEASES						
Target Gene	Result	Result	Result	Result	Result	Result
aur (consensus)	positive	positive	positive	positive	positive	positive
aur (other than MRSA252)	positive	positive	negative	negative	positive	positive
aur (MRSA252)	negative	negative	positive	positive	negative	negative
splA	positive	positive	negative	negative	positive	positive
splB	positive	positive	negative	negative	positive	positive
splE	negative	negative	positive	negative	positive	positive
Isolate	1	2 & 3	4	5 & 5.1	6	a

sspA	positive	positive	positive	positive	positive	positive
sspB	positive	positive	positive	positive	positive	positive
sspP (consensus)	positive	positive	positive	positive	positive	positive
sspP (other than ST93)	positive	positive	positive	positive	positive	positive
VIRULENCE: STAPHYLOCOCCAL SUPERANTIGEN/ENTEROTOXIN-LIKE GENES (SET/SSL)						
Target Gene	Result	Result	Result	Result	Result	Result
setC/selx	positive	positive	negative	positive	positive	positive
ssl01/set6_probe1_11	positive	positive	negative	negative	positive	negative
ssl01/set6_probe2_11	negative	negative	positive	positive	negative	positive
ssl01/set6_probe1_12	positive	positive	positive	positive	negative	negative
ssl01/set6_probe2_12	negative	negative	negative	negative	negative	positive
ssl01/set6_probe4_11	positive	positive	positive	negative	positive	negative
ssl01/set6_probeRF122	negative	negative	negative	negative	negative	negative
ssl01/set6 (COL)	negative	negative	negative	negative	positive	negative
ssl01/set6 (Mu50+N315)	positive	positive	negative	negative	ambiguous	negative
ssl01/set6 (MW2+MSSA476)	negative	negative	negative	negative	negative	positive
ssl01/set6 (MRSA252)	negative	negative	positive	positive	negative	negative
ssl01/set6 (RF122)	negative	negative	negative	negative	negative	negative
ssl01/set6 (other alleles)	negative	negative	negative	negative	negative	negative
ssl02/set7	positive	positive	negative	ambiguous	positive	positive
ssl02/set7 (MRSA252)	negative	ambiguous	positive	positive	negative	negative
ssl03/set8_probe 1	positive	positive	negative	negative	positive	positive
ssl03/set8_probe 2	positive	positive	negative	negative	positive	positive
ssl03/set8 (MRSA252, SAR0424)	negative	negative	positive	negative	negative	negative
ssl04/set9	negative	positive	negative	negative	positive	positive
ssl04/set9 (MRSA252, SAR0425)	negative	negative	positive	positive	negative	negative
ssl05/set3_probe 1	positive	positive	negative	positive	positive	positive
ssl05/set3 (RF122, probe-611)	ambiguous	ambiguous	negative	negative	negative	ambiguous
ssl05/set3_probe 2 (612)	positive	positive	negative	negative	positive	positive
ssl05/set3 (MRSA252)	negative	negative	positive	positive	negative	negative
ssl06/set21	negative	negative	negative	negative	positive	positive
ssl06 (NCTC8325+MW2)	negative	negative	negative	negative	positive	positive
ssl07/set1	positive	positive	negative	ambiguous	positive	positive
ssl07/set1 (MRSA252)	ambiguous	ambiguous	positive	ambiguous	ambiguous	ambiguous
ssl07/set1 (AF188836)	negative	negative	negative	positive	negative	negative
ssl08/set12_probe 1	positive	positive	negative	negative	positive	positive
ssl08/set12_probe 2	positive	positive	negative	negative	positive	positive
ssl09/set5_probe 1	positive	positive	negative	positive	positive	positive
ssl09/set5_probe 2	positive	positive	negative	positive	positive	positive
ssl09/set5 (MRSA252)	negative	negative	positive	negative	negative	negative
ssl10/set4	positive	positive	ambiguous	positive	positive	positive
ssl10 (RF122)	negative	negative	negative	negative	negative	negative
ssl10/set4 (MRSA252)	ambiguous	ambiguous	positive	negative	ambiguous	ambiguous
ssl11/set2 (COL)	negative	negative	negative	negative	positive	negative
ssl11+set2(Mu50+N315)	positive	positive	negative	negative	negative	negative
ssl11+set2(MW2+MSSA476)	negative	negative	negative	negative	negative	positive
ssl11/set2 (MRSA252)	negative	negative	positive	negative	negative	negative
setB3	positive	positive	negative	negative	positive	positive
setB3 (MRSA252)	negative	negative	positive	negative	negative	negative
setB2	positive	positive	negative	negative	positive	positive
setB2 (MRSA252)	negative	negative	positive	negative	negative	negative
setB1	positive	positive	positive	negative	positive	positive
CAPSULE- AND BIOFILM-ASSOCIATED GENES						
Target Gene	Result	Result	Result	Result	Result	Result
cap 1 (total)	negative	negative	negative	negative	negative	negative
Isolate	1	2 & 3	4	5 & 5.1	6	a
Isolate	1	2 & 3	4	5 & 5.1	6	a

capH1	negative	negative	negative	negative	negative	negative
capJ1	negative	negative	negative	negative	negative	negative
capK1	negative	negative	negative	negative	negative	negative
cap 5 (total)	positive	positive	negative	positive	positive	negative
capH5	positive	positive	negative	positive	positive	negative
capJ5	positive	positive	negative	positive	positive	negative
capK5	positive	positive	negative	positive	positive	negative
cap 8 (total)	negative	negative	positive	negative	negative	positive
capH8	negative	negative	positive	negative	negative	positive
capI8	negative	negative	positive	negative	negative	positive
capJ8	negative	negative	positive	negative	negative	positive
capK8	negative	negative	positive	negative	negative	positive
icaA	positive	positive	positive	positive	positive	positive
icaC	positive	positive	positive	positive	positive	positive
icaD	positive	positive	positive	positive	positive	positive
bap	negative	negative	negative	negative	negative	negative
ADHAESION FACTORS / GENES ENCODING MICROBIAL SURFACE COMPONENTS RECOGNIZING ADHESIVE MATRIX MOLECULES (MSCRAMM GENES)						
Target Gene	Result	Result	Result	Result	Result	Result
bbp (total)	positive	positive	positive	positive	positive	positive
bbp (consensus)	positive	positive	positive	positive	positive	positive
bbp (COL+MW2)	negative	negative	negative	negative	positive	positive
bbp (MRSA252)	negative	negative	positive	negative	negative	negative
bbp (Mu50)	positive	positive	negative	negative	negative	negative
bbp (RF122)	negative	negative	negative	negative	negative	negative
bbp (ST45)	negative	negative	negative	negative	negative	negative
clfA (total)	positive	positive	positive	positive	positive	positive
clfA (consensus)	positive	positive	positive	positive	positive	positive
clfA (COL+RF122)	negative	negative	ambiguous	negative	positive	negative
clfA (MRSA252)	negative	negative	positive	negative	ambiguous	negative
clfA (Mu50+MW2)	positive	positive	negative	positive	ambiguous	positive
clfB (total)	positive	positive	positive	positive	positive	positive
clfB (consensus)	positive	positive	positive	positive	positive	positive
clfB (COL+Mu50)	positive	positive	negative	negative	positive	negative
clfB (MW2)	negative	negative	negative	negative	negative	positive
clfB (RF122)	negative	negative	negative	negative	negative	ambiguous
cna	negative	negative	positive	positive	negative	positive
ebh (consensus)	positive	positive	positive	negative	positive	positive
ebpS (total)	positive	positive	positive	positive	positive	positive
ebpS_probe 612	positive	positive	positive	positive	positive	positive
ebpS_probe 614	positive	positive	positive	positive	positive	positive
ebpS (01-1111)	negative	negative	negative	negative	negative	negative
ebpS (COL)	negative	negative	negative	positive	positive	negative
eno	positive	positive	positive	positive	positive	positive
fib	positive	positive	negative	negative	positive	positive
fib (MRSA252)	negative	negative	positive	positive	negative	negative
fnbA (total)	positive	positive	positive	positive	positive	positive
fnbA (consensus)	positive	positive	positive	positive	positive	positive
fnbA (COL)	negative	negative	negative	negative	positive	negative
fnbA (MRSA252)	negative	negative	positive	negative	negative	negative
fnbA (Mu50+MW2)	positive	positive	negative	positive	negative	positive
fnbA (RF122)	negative	negative	negative	negative	negative	negative
fnbB (total)	positive	positive	positive	negative	positive	positive
fnbB (COL)	negative	negative	negative	negative	positive	negative
fnbB (COL+Mu50+MW2)	positive	ambiguous	ambiguous	negative	ambiguous	ambiguous
fnbB (Mu50)	ambiguous	positive	positive	negative	negative	negative
fnbB (MW2)	negative	negative	negative	negative	negative	positive
Isolate	1	2 & 3	4	5 & 5.1	6	a

fnbB (ST15)	negative	negative	negative	negative	negative	negative
fnbB (ST45-2)	negative	negative	negative	negative	negative	negative
map (total)	positive	positive	positive	positive	positive	positive
map (COL)	ambiguous	positive	negative	negative	positive	negative
map (MRSA252)	negative	negative	positive	negative	negative	negative
map (Mu50+MW2)	positive	positive	negative	positive	negative	positive
sasG (total)	positive	positive	negative	positive	positive	positive
sasG (COL+Mu50)	positive	positive	negative	negative	positive	negative
sasG (MW2)	negative	negative	negative	positive	negative	positive
sasG (other than MRSA252+RF122)	positive	positive	negative	positive	positive	positive
sdrC (total)	positive	positive	positive	positive	positive	positive
sdrC (consensus)	positive	positive	positive	positive	positive	positive
sdrC (B1)	negative	negative	negative	negative	negative	negative
sdrC (COL)	negative	negative	negative	negative	positive	negative
sdrC (Mu50)	positive	positive	negative	positive	negative	negative
sdrC (MW2+MRSA252+RF122)	negative	negative	negative	negative	negative	positive
sdrC (other than MRSA252+RF122)	positive	positive	negative	positive	positive	positive
sdrD (total)	positive	positive	positive	positive	positive	positive
sdrD (consensus)	positive	positive	positive	positive	positive	positive
sdrD (COL+MW2)	negative	negative	negative	negative	positive	positive
sdrD (Mu50)	positive	positive	negative	negative	negative	negative
sdrD (other)	negative	negative	positive	positive	negative	negative
vwb (total)	positive	positive	positive	positive	positive	positive
vwb (consensus)	positive	positive	positive	positive	positive	positive
vwb (COL+MW2)	negative	negative	negative	negative	positive	positive
vwb (MRSA252)	negative	negative	positive	negative	negative	negative
vwb (Mu50)	positive	positive	negative	negative	negative	negative
vwb (RF122)	negative	negative	negative	positive	negative	negative
IMMUNODOMINANT ANTIGEN B						
Target Gene	Result	Result	Result	Result	Result	Result
isaB	positive	positive	negative	negative	positive	positive
isaB (MRSA252)	ambiguous	ambiguous	positive	positive	ambiguous	ambiguous
DEFENSIN RESISTANCE GENOTYPE						
Target Gene	Result	Result	Result	Result	Result	Result
mprF (COL+MW2)	ambiguous	ambiguous	ambiguous	ambiguous	positive	positive
mprF (Mu50+MRSA252)	positive	positive	positive	positive	ambiguous	ambiguous
TRANSFERRIN BINDING PROTEIN						
Target Gene	Result	Result	Result	Result	Result	Result
isdA (consensus)	positive	positive	positive	positive	positive	positive
isdA (MRSA252)	negative	negative	positive	negative	negative	negative
isdA (other than MRSA252)	positive	positive	negative	positive	positive	positive
PUTATIVE TRANSPORTER						
Target Gene	Result	Result	Result	Result	Result	Result
lmrP (other than RF122)_probe1	positive	positive	positive	positive	positive	positive
lmrP (other than RF122)_probe2	positive	positive	positive	positive	positive	positive
lmrP (RF122)_probe1	negative	negative	negative	negative	negative	negative
lmrP (RF122)_probe2	negative	negative	negative	negative	negative	negative
TYPE I RESTRICTION-MODIFICATION SYSTEM, SINGLE SEQUENCE SPECIFICITY PROTEIN						
Target Gene	Result	Result	Result	Result	Result	Result
hsdS1 (RF122)	negative	negative	negative	negative	negative	negative
hsdS2 (Mu50+N315+COL+USA300+NCTC 8325)	positive	positive	negative	positive	positive	negative
hsdS2 (MW2+MSSA476)	ambiguous	negative	negative	negative	negative	positive
hsdS2 (RF122)	negative	negative	negative	negative	negative	negative
hsdS2 (MRSA252)	negative	negative	positive	negative	negative	negative
hsdS3 (all other than RF122+ MRSA252)	positive	positive	negative	negative	negative	positive
Isolate	1	2 & 3	4	5 & 5.1	6	a

hsdS3 (COL+USA300+NCTC8325+MW2+ MSSA476+RF122)	negative	negative	negative	negative	negative	positive
hsdS3 (Mu50+N315)	positive	positive	negative	negative	negative	negative
hsdS3 (CC51+ MRSA252)	negative	negative	positive	negative	negative	negative
hsdS3 (MRSA252)	negative	negative	positive	negative	negative	negative
hsdSx (CC25)	positive	positive	positive	positive	ambiguous	positive
hsdSx (CC15)	negative	negative	negative	negative	negative	negative
hsdSx (etd)	negative	negative	negative	negative	negative	negative
MISCELLANEOUS GENES						
Target Gene	Result	Result	Result	Result	Result	Result
Q2FXC0	negative	negative	negative	negative	positive	positive
Q2YUB3	negative	negative	negative	negative	negative	positive
Q7A4X2	positive	positive	positive	positive	negative	negative
HYALURONATE LYASE						
Target Gene	Result	Result	Result	Result	Result	Result
hysA1 (MRSA252)	negative	negative	positive	negative	negative	negative
hysA1 (MRSA252+RF122) and/or hysA2 (consensus)	positive	positive	positive	positive	positive	positive
hysA1 (MRSA252+RF122) and/or hysA2 (COL+USA300)	negative	negative	positive	negative	positive	negative
hysA2 (all other than MRSA252)	positive	positive	negative	negative	positive	positive
hysA2 (COL+USA300+NCTC8325)	negative	negative	positive	negative	positive	negative
hysA2 (all other than COL+USA300+NCTC8325)_probe1	positive	positive	positive	positive	negative	positive
hysA2 (all other than COL+USA300+NCTC8325)_probe2	positive	positive	positive	positive	negative	positive
hysA2 (MRSA252)	negative	negative	positive	negative	negative	negative

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Chapter 7: General Discussion

This work represents the first systematic effort to collect representative data about the prevalence and genetic makeup of ESBL/AmpC producing Enterobacteriaceae and MRSA in companion animals in New Zealand. Studies of this nature in veterinary medicine have increased in the past decade, as a reflection of the increasing concerns of companion animals potentially acting as reservoirs of antimicrobial resistant bacteria, given their close relationship with humans (Walther *et al.* 2012a; Rubin and Pitout 2014). When this PhD project started in 2012, only one short communication of MRSA infections and a non-peer reviewed report of two AmpC-E infections in companion animals in New Zealand were found in the literature (Grinberg *et al.* 2008; Darling 2012). In addition, there were no official reporting schemes for animal ESBL/AmpC producing Enterobacteriaceae and MRSA, and the commercial veterinary diagnostic laboratories did not routinely test for the presence of these organisms in clinical specimens. This lack of background data posed many challenges, but also opportunities to make substantial contributions to the assessment of the occurrence of these organisms in companion animals. The molecular analysis of the organisms isolated from carriage and infection sites in animals revealed their similarity to human pandemic and endemic strains. This highlights the necessity of collaborative actions to combat these pathogens in both human and veterinary medicine, under the “One Health” concept (Priest *et al.* 2016).

In this thesis, ESBL/AmpC producing Enterobacteriaceae isolates from infection sites (Chapter 2) and faeces (

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Chapter 3 and Appendix 1) of companion animals were characterised. The majority of the ESBL producing isolates carried the blaCTX-M-14, while only *bla*$_{CMY-2}$ was identified among the PAmpC producing isolates. In some isolates carrying *bla*$_{CTX-M}$ and/or *bla*$_{CMY}$, we also detected *bla*$_{TEM}$ and/or *bla*$_{SHV}$, consistent with previous observations indicating each ESBL or AmpC-producing strain may carry various β-lactamases (Woodford *et al.* 2009). These enzymes have been widely reported internationally and in New Zealand in human clinical infections, and in faecal carriage overseas (Lau *et al.* 2008; Freeman *et al.* 2012; Stoesser *et al.* 2012; Riley 2014; Drinkovic *et al.* 2015).They have also been identified in companion animals overseas, from clinical infections and faecal carriage (Hasan *et al.* 2012; Borjesson *et al.* 2013).

Overall, 63% of the Enterobacteriaceae submitted by the diagnostic laboratories (Chapter 2) on the basis of resistance to amoxicillin-clavulanic acid were ESBL/AmpC-producers. A limitation of this project was that not all the clinical Enterobacteriaceae isolated by the diagnostic laboratories were screened for ESBL/AmpC production due to operational constraints. Hence, the results of the study presented in Chapter 2, which analysed isolates provided by diagnostic laboratories, do not allow to draw conclusions about the prevalence of ESBL/AmpC-producers in all clinical specimens from companion animals. Future studies should address this limitation.

Consistent with the literature, ESBL/AmpC producing isolates were more likely to be resistant to other antimicrobial families, and be MDR than non-ESBL/AmpC-producers (Jacoby 2005), regardless of clinical/ faecal carriage status. The most common antimicrobial resistance profile observed among *E. coli* isolates was resistance to cephalothin and sulphamethoxazole-trimethoprim. Interestingly, the rate of susceptibility to gentamicin was high, consistent with a report indicating low level of usage of gentamicin for clinical treatment by veterinarians in New Zealand (Pleydell *et al.* 2012). Conversely, the level or resistance to enrofloxacin among ESBL-E and PAmpC-E was high (ESBL: 80%; PAmpC-E: ~50%), reflecting the increasing resistance observed to this compound in New Zealand and overseas (Matsumura *et al.* 2013; Woerther *et al.* 2013; Heffernan *et al.* 2014b). The ESBL-E, in

particular carrying *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$, were categorised as susceptible to ceftazidime by the disk diffusion test. This result adds to the body of data suggesting the CLSI zone diameter interpretive criteria for ceftazidime disk diffusion test may not be adequate for the diagnosis of ESBL-E in regions where the *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$ are prevalent (Williamson *et al.* 2012). Co-resistance to other antimicrobial compounds, such as fluoroquinolones, was also observed at high frequency in ESBL-E and PAmpC-E. In the absence of adequate *in vitro* testing, ESBL-E and PAmpC-E can be underdiagnosed, and likely to pose therapeutic challenges.

In order to identify ESBL/AmpC-producing Enterobacteriaceae, veterinary diagnostic laboratories in New Zealand must reassess their testing protocols and introduce the double synergy test for ESBL production and/or boronic acid confirmatory test for AmpC production (Dierikx *et al.* 2012). Treatment according to the results of the current susceptibility panel that includes testing for amoxicillin-clavulanic acid can lead to therapeutic failure in some cases, for instance when an inducible AmpC producer is not detected by the disk diffusion test (Jacoby 2009).

The MLST results of the ESBL/AmpC-E indicated that most isolates infecting or carried in the faeces of companion animals in New Zealand belong to leading human clinical STs. Of note, three of the five major globally distributed human-associated extra-intestinal *E. coli* STs (ST131, ST69 and ST73), and four other dominant clones (ST9, ST12, ST10 and ST127 (Riley 2014) were found also in this PhD project in the studies presented in Chapter

2 and

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Chapter 3. In the last decade the drug-resistant ST131 attracted much attention and became the most prevalent extra-intestinal pathogenic *E. coli* strain identified worldwide, and a high proportion of the isolates are ESBL-producers, carrying in particular *bla*$_{CTX-M14}$ and *bla*$_{CTX-M15}$ (Rogers *et al.* 2011). The ST131 also contributed to more than half of the ESBL-E isolates belonging to the main clonal complex identified from human clinical infections in New Zealand (Heffernan *et al.* 2014b) and overseas (Rogers *et al.* 2011). One recently published study about AmpC-E (including PAmpC-E and *ampC*-E with chromosomal mutations) from clinical infections in humans in New Zealand indicated that the prevalent phylogenetic group was D, of which 2% (n=4/200) were ST131 PAmpC-E (Drinkovic *et al.* 2015). In general, *E. coli* STs appear to lack host specificity, and ST131, ST69 and ST73 have been reported globally in human and animal infections (Corvec *et al.* 2010; Ewers *et al.* 2012; Hasan *et al.* 2012; Adams-Sapper *et al.* 2013; Hansen *et al.* 2013; Heffernan *et al.* 2014b; Riley 2014; Bogaerts *et al.* 2015; Day *et al.* 2016b). The presence of the same STs at different time intervals from different host species during the year 2012-2013 underlines the success of these STs in disseminating the ESBL/AmpC genes in the community. The limitation of MLST is that it only provides information about the core genome. It would be interestingly in future studies to also analyze the accessory genomes by means of whole genome sequencing (Clermont *et al.* 2015).

Another interesting finding of this project was the variation in the antimicrobial susceptibility test results of clinical and faecal ESBL-E and AmpC-E isolates within each ST, evidence of horizontal gene transfer of resistome (Alghoribi *et al.* 2015). For instance, ST131 was identified from clinical isolates harbouring and non-harbouring ESBL genes, and were all MDR. This suggests that ST131 is prone to acquisition of resistome. In addition, ST131 was isolated from UTI and non-UTI infections, concordant with the literature on animal and human infections (Ewers *et al.* 2010; Platell *et al.* 2010; Riley 2014). Another multidrug resistant ST was ST648, that has been identified from animal infections in Australia, with similar resistance patterns (Guo *et al.* 2015). Interestingly, ST12 was found in 12 isolates, was mostly isolated from UTI cases and was the least resistant ST identified in

this study (all ST12 isolates in this study were susceptible to all the antimicrobial families). This is concordant with the results previous studies performed overseas that described *E. coli* ST12 among the non-multidrug resistant STs causing infections in humans (Alghoribi *et al.* 2015; Day *et al.* 2016a) and animals (Hansen *et al.* 2016). We also identified 11 STs not previously reported in the Warwick database (ST4390-4395, 4166, 4167, 4200-4202, and 4406).

In the study presented in Chapter 2, rarefaction curves were generated to compare ST richness of *E. coli* isolates that include ESBL-E and/or AmpC-E and non-ESBL/AmpC-E from UTI and non-UTI sources. The shapes of the curves indicated a lower ST richness among UTI isolates (24 ST from 50 isolates) than non-UTI isolates (28 ST from 39 isolates). This is probably due to the selection of specific clones that accumulate virulence factors and antimicrobial resistance genes that confer them advantage, such as the case of uropathogenic STs such as ST131 (Croxall *et al.* 2011; Chung *et al.* 2012). In fact, in animals, it is estimated that 10-20% of UTI cases are caused by a small group of highly adapted *E. coli* STs (Manges *et al.* 2008; Banerjee *et al.* 2013; Nebbia *et al.* 2014).

In general, the rapid and extensive transmission of ESBL/AmpC producing Enterobacteriaceae in companion animals in the community, particularly the ESBL/AmpC-E pandemic clonal lineages, is an intriguing epidemiological phenomenon (Pitout *et al.* 2005; Can *et al.* 2016; Karkaba *et al.* 2017). These STs have a high ability to colonize the intestines and urinary epithelium (Niki *et al.* 2011; Can *et al.* 2016). In this project, a total of 13 STs

were shared by UTI-clinical isolates (Chapter 2) and faecal isolates (

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Chapter 3). Most of the common STs corresponded to leading uropathogenic strains such as ST405 and ST38, that were also identified from a range of extra intestinal infections from humans and companion animals overseas (Alghoribi *et al.* 2015; Schaufler *et al.* 2015; Nakamura *et al.* 2016). Although the faecal and the UTI isolates were not identified from the same animals, the occurrence of the same STs across carriage and disease support an ascending faecal-urethral pathogenesis of ESBL/AmpC-E UTI in dogs and cats, as is in humans (Yamamoto *et al.* 1997; Niki *et al.* 2011; Ruppe *et al.* 2013; Schaufler *et al.* 2015), or exogenous infections from other carriers. Some authors have postulated that infections with faecal ESBL/AmpC-E may occur in animals following faecal contamination of extra intestinal sites (Vo *et al.* 2007; Maddox *et al.* 2012).

This PhD project made extensive use of faecal culture for the isolation of MDR-E, ESBL/AmpC-E, in particular in the prevalence study described in

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Chapter 3. A preliminary analysis of the published literature revealed several studies that analysed the occurrence of MDR-E and ESBL/AmpC-E in the faeces of humans (Huang *et al.* 2010; Grohs *et al.* 2013; Willems *et al.* 2013; Sturod *et al.* 2014; Blane *et al.* 2016), and cats and dogs (Damborg *et al.* 2011; Wedley *et al.* 2011; Leonard *et al.* 2012; Decristophoris *et al.* 2013a; Hamilton *et al.* 2013; Hordijk *et al.* 2013; Szmolka and Nagy 2013). These studies used a variety of culture methods, and no gold standard was defined or agreed upon by the different authors. Hence, we perform a pilot study in order to select a robust culture method for the isolation of ESBL/AmpC-E from the faeces of cats and dogs. In terms of sensitivity, the culture method selected for this project outperformed all the other five methods used in parallel (Appendix 1).

Interestingly, in the pilot study, ESBL/AmpC-E were isolated from 4/10 (40%) specimens from hospitalised, as opposed to only 3/31 (9.6%) from non-hospitalised animals (Fisher's two-tailed $P<0.05$). The proportion of ESBL/AmpC-E positive specimens in the hospitalised animals was also greater than that found in the prevalence study reported in Chapter 3 ($P<0.01$). This is consistent with the idea that hospitalisation and/or recent antimicrobial treatment could increase the risk of faecal carriage of ESBL/AmpC-E in pets. This is in agreement with the commonly held view that hospitals represent hotspots of selective pressure for antimicrobial resistance (Guo *et al.* 2013; Johard *et al.* 2015). It needs to be noted that this study assessed the intestinal carriage of MDR-E during the hospitalisation, and the increase in the rate of carriage may be a temporary and reversible effect, as it has been observed that in the long term the intestinal flora of hospitalised animals reverses to the resistance levels seen in the community (Johns *et al.* 2012). The MLST and β-lactamase profiles of the ESBL/AmpC-E isolated in the pilot study have been widely reported internationally in human infections (Lau *et al.* 2008; Stoesser *et al.* 2012; Riley 2014), and some have been also identified in animals. The isolation of the same clone ST744 from three hospitalised animals suggested that nosocomial transmission may have occurred.

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Chapter 3, the prevalence of faecal carriage of MDR-E and ESBL/AmpC-E in cats and dogs presenting to veterinarians in Auckland city was 2%, 0.3%, and 6.1% (5.8% PAmpC and 0.5% *ampC* chromosomal mutations), respectively. These prevalence's are comparable with a few similar studies in cats and dogs (Wedley *et al.* 2011; Schmidt *et al.* 2015), but the ESBL-E prevalence was lower than the 5-7% prevalence found in a normal healthy human population (Stromdahl *et al.* 2011; Ben Sallem *et al.* 2012; Geser *et al.* 2012). The difference in the carriage frequencies can be explained by different risk factors for the acquisition of MDR organisms, the different bacteriological isolation methods, and the inconsistent classification of intermediate resistant as resistant isolates. The clinical significance of the MDR-E and ESBL/AmpC-E isolates found in this study could not be determined as the questionnaire did not include information on the clinical condition of the sampled animals. However, human studies have shown that faecal carriage of ESBL-E is linked to UTI with the same organism (endogenous infections), even in patients that do not receive antimicrobials (Pena *et al.* 1997; Ruppe *et al.* 2013).

The study in Chapter 4 provides insight of some of the risk factors of MDR-E and ESBL/AmpC-E carriage, and some factors identified in that study have been previously reported in humans. For instance, in our study, dogs and cats which owners reported overseas travel had increased odds of carriage of ESBL/AmpC genes, as well as MDR-E, which seems to be consistent with previously described risk factor of travelling for the faecal carriage of these bacteria in humans (Meyer *et al.* 2012; Valverde *et al.* 2015). Dogs and cats belonging to veterinary personnel had also increased odds of carriage. This is consistent with previous observations that human healthcare workers have an increased rate of carriage of these bacteria (Bassyouni *et al.* 2015). The selection pressures on the gut microbiota within veterinary clinics could be similar to the pressures exerted in human healthcare centres. Lastly, the use of antimicrobial treatment has been described by several studies as a risk factor for faecal carriage of MDR-E and ESBL/AmpC-E in cats and dogs for (Gibson *et al.* 2011; Belas *et al.* 2014; Schmidt *et al.* 2015), and the same result was found in our study.

In this thesis, 25 non-ESBL/AmpC-E from clinical infections and four faecal isolates displayed phenotypic resistance or intermediate resistance to the 3rd generation cephalosporin cefovecin, according to the manufacturer's interpretive criteria. Cefovecin is a relatively new long acting injectable drug. All these isolates were negative by PCR for any of the β-lactamase genes tested. It is important to note that the inhibition-zone diameter cut off defining cefovecin-resistance was provided by the manufacturer and not endorsed by CLSI. However, to the best of the candidate's knowledge these interpretive criteria are routinely used by some diagnostic laboratories in New Zealand. The clinical impact of such reporting by diagnostic laboratories is hard to assess. The resistance to cefovecin warrants further investigation due to the widespread use of this compound in veterinary practice and the possibility that its use may select for co-resistance against cephalosporins used in human medicine,

Chapter 6 deals with MRSA. The isolates identified in this study belonged to five epidemic clones, three of which contributed to more than 68% of the human MRSA infections in New Zealand (AK3, MRSA-15, WSPP) (Heffernan *et al.* 2013), which suggest spillover of human MRSA strains to animal populations. The isolation of the MRSA strains from the North and South Island shows that these strains are not restricted to one geographical area. All the inferred strain types found in this study were previously isolated from animals also overseas (Walther *et al.* 2009; Fall *et al.* 2012; Ishihara *et al.* 2014; Loncaric *et al.* 2014). Interestingly, the temporal change in the MRSA strains found in humans was reflected in animals. On the contrary, no MRSA was detected in the carriage study in dogs (nasal) and cats (perianal) in the Auckland region. The low level of MRSA carriage in dogs and cats reflects the comparatively low level of carriage in humans, although some bias may have been introduced by the sampling of animals treated with antibiotics. Sampling of additional anatomical sites may also have increased the probability of detection of MRSA.

In summary, companion animals in New Zealand were found to carry ESBL/AmpC-E, and be infected with ESBL/AmpC Enterobacteriaceae and MRSA. The implications of these infections on clinical practice are unknown

and require further investigation. Future studies should assess the degree of cross transmission between animal and humans, and also assess in detail the virulence factors and resistome of the isolates, and make use of whole genome sequencing to compare human and animal populations using higher resolution.

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Appendix 1: Comparison between different culture methods for the isolation of extended spectrum β-lactamase and AmpC β-lactamase producing *Escherichia coli* from faeces of cats and dogs

This appendix presents data to support our choice of culture method in

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Ali Karkaba

Name/Title of Principal Supervisor: Alex Grinberg

Name of Published Research Output and full reference:

Karkaba, A. Grinberg, J. Benschop, E. Pleydell (2016). Characterisation of extended spectrum β-lactamase and AmpC β-lactamase-producing Enterobacteriaceae isolated from companion animals in New Zealand. New Zealand Veterinary Journal

In which Chapter is the Published Work: Chapter 2

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  The candidate designed the study, collected the samples and processed them at Massey University. He wrote the first draft of the paper and responded to the co-authors' feedback and qualified as first and correspondent author.

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Chapter 3. A preliminary analysis of the published literature revealed a large number of studies that analysed the occurrence of MDR-E and ESBL/AmpC-E in the faeces of humans (Huang *et al.* 2010; Grohs *et al.* 2013; Willems *et al.* 2013; Sturod *et al.* 2014; Blane *et al.* 2016), and several studies in cats and dogs (Damborg *et al.* 2011; Wedley *et al.* 2011; Leonard *et al.* 2012; Decristophoris *et al.* 2013a; Hamilton *et al.* 2013; Hordijk *et al.* 2013; Szmolka and Nagy 2013). The studies used a variety of culture methods, and no gold standard was defined or agreed upon by the different authors. Therefore, it was necessary to select one method for the prevalence study

presented in

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Ali Karkaba

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With the aim of selecting a culture method with an optimal balance between its ability to detect antimicrobial resistant MDR-E and ESBL/AmpC-E, ease of use, and cost, this preliminary study was performed using faecal specimens collected from hospitalised and non-hospitalised cats and dogs in Palmerston North.

Materials and Methods

Collection of faecal samples

Forty-one, fresh voided faecal specimens were obtained from cats and dogs in Palmerston North, New Zealand in August 2012. The samples originated from animals hospitalised at the Veterinary Teaching Hospital, Massey University (MU) (n=10; 4 cats and 6 dogs), and from animals belonging to residents of the Palmerston North area (n=31; 7 cats and 24 dogs). Inclusion criteria for the hospitalised animals included that both hospital admission and commencing antibiotic treatment occurred at least two days before sample collection. The reasons for hospitalisation were not available. Ten faecal specimens from hospitalised animals were collected by hospital staff into a plastic pottle from the cages' floor. No information on the time of voiding was available.

Thirty-one community sourced animals were healthy cats and dogs with no history of hospitalisation or antimicrobial treatment in the 2 months prior to the sampling. These animals were owned by MU staff members, or by acquaintances of the PhD candidate and supervisory team, of which none worked at the animal hospital nor was in clinical veterinary practice. The owners were instructed to collect faeces from the ground in a provided plastic bottle within 2-3 hours of voiding, and deliver them personally to MU. Additional faecal specimens were taken from two dogs and one cat within the same household of a community animal that resulted positive for ESBL-*E. coli.* No animal ethics approval was necessary for this study as no animal manipulations were performed. The samples were processed at the Microbiology Laboratory, IVABS on the same day of arrival to MU.

Laboratory methods

Five primary culture methods were used in parallel for the initial detection of multi-resistant bacterial growth in each specimen, followed by the isolation of antimicrobial resistant *E. coli* from the growth. These methods were retrieved from the literature, or were modifications of the same. For each method, a cotton swab previously immersed in faeces was used to inoculate the primary culture media. The following methods were used:

Stage One: Detection of multi-resistant bacterial growth

Method 1 (M1) (modified from (Bartoloni *et al.* 2006). The swab was immersed in buffered peptone water (BPW) (primary culture medium) and incubated overnight at 37°C in aerobic conditions. The next day, the broth was diluted to a turbidity equivalent to the 0.5 McFarland standard by adding normal saline, and a new swab was used to inoculate the suspension onto the surfaces of two MacConkey agar plates (Fort Richards, Auckland, New Zealand) (Figure A.1). Within 15 minutes of inoculation, eight antimicrobial-impregnated discs were deposited on the surface of plates (four discs on two plates) using a replicator, and the plates were incubated as above. The eight antimicrobial-impregnated discs were selected as they were commonly used in veterinary panels by diagnostic laboratories in New Zealand. These were cephalothin (30µg); amoxicillin-clavulanic acid (30µg); cefotaxime (30µg); cefovecin (30µg); tetracycline (30µg); gentamicin (10µg); trimethoprim/sulphamethoxazole (25µg); and enrofloxacin (5µg) (Oxoid, Wade Road, Basingstoke, UK).

Method 2 (M2) (Upton *et al.* 2011): the swab was immersed in MacConkey broth (Fort Richard, Auckland) (primary culture medium) and incubated overnight at 37°C in aerobic conditions. After incubation, the broth was diluted to a turbidity equivalent to the 0.5 McFarland standard by adding normal saline, and a new swab was used to inoculate the suspension onto the surfaces of two Mueller Hinton agar plates (Fort Richards, Auckland, New Zealand). Within 15 minutes of inoculation, the same eight antimicrobial-impregnated discs used in M1 were deposited on the surface of each plate using a replicator, and the plates were incubated as above.

Method 3 (M3) (Vieira *et al.* 2008): the swab was immersed in normal saline and the suspension adjusted to a turbidity equivalent to the 0.5 McFarland standards by adding normal saline. A new swab was used to inoculate the suspension onto the surfaces of two MacConkey agar plates, which were incubated overnight at 37°C in aerobic conditions. The same eight antimicrobial-impregnated discs used in M1 and M2 were deposited on the plates incubated as above.

Method 4 (M4) (Damborg *et al.* 2012): the faecal swab was directly plated onto two MacConkey agar plates, the antimicrobial discs deposited and plates were incubated as for M1.

Method 5 (M5) (Bartoloni *et al.* 2006): the faecal swab was directly plated onto two MacConkey Number 3 agar plates (Thermo Fisher Scientific, New Zealand) as previously described. The above antimicrobial discs were added and the plates incubated overnight as for the previous methods.

Stage Two: Isolation of antimicrobial resistant E. coli The inhibition zone diameter interpretive criteria established by the Clinical Laboratory Standard Institute (CLSI 2013) for resistant Enterobacteriaceae isolates were used to identify antimicrobial resistant *E. coli*. The interpretive criterion for cefovecin was not available in the CLSI and was provided by the drug manufacturer (Zoetis, USA). When a well-separated colony was seen within the inhibition zone defining a resistant isolate for that antimicrobial, the colony was selected and streaked onto a selective-differential medium for *E. coli* (ECC CHROMagar®; Fort Richard Laboratories, Auckland). If no isolated colonies were seen within the growth inhibition zone, a loopful of bacteria was taken from the edge of the growth. Because eight antimicrobials were tested, this procedure could result in 0 to 8 subculture events per specimen. These plates were incubated overnight at 37°C in aerobic conditions. A single presumptive *E. coli* colony growing on ECC CHROMagar® was subcultured onto nutrient agar overnight at 37°C in aerobic conditions.

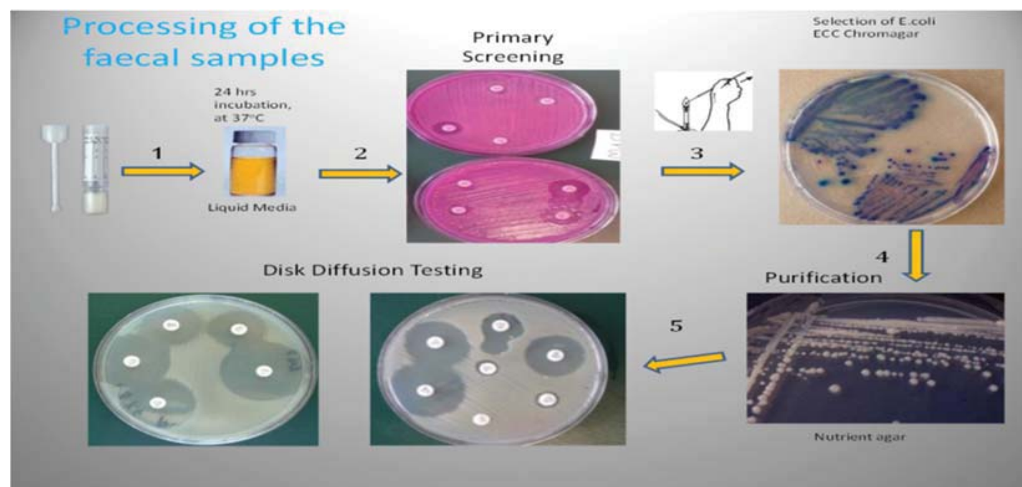

Figure A.1: Flow chart of the microbiologic analysis of faecal samples as per (M1)

Isolates were defined as *E. coli* based on the results of indole and urease production tests, MRVP (Methyl Red and Voges-Proskauer) reactions, growth patterns in triple sugar iron and Kligler agar, and citrate utilisation (Winn and Koneman 2006). After the isolation of an *E. coli*, antimicrobial susceptibility testing was performed according to CLSI guidelines (CLSI 2013). The following commercially available antimicrobials were used: cephalothin (30µg); amoxicillin-clavulanic acid (30µg); cefoxitin (30µg);

cefotaxime (30µg); ceftazidime (30µg); cefovecin (30µg); imipenem (10µg); tetracycline (30µg); gentamicin (10µg); trimethoprim/sulphamethoxazole (25µg), enrofloxacin (5µg) and aztreonam (30µg) (Oxoid, Basingstoke, UK). *E. coli* isolates that had acquired resistance to at least one antimicrobial drug from three or more families were considered multidrug-resistant, as proposed by (Magiorakos *et al.* 2012). For each batch of isolates tested for antimicrobial susceptibility, quality control was performed using *E. coli* ATCC25922 and *K. pneumoniae* ATCC700603 (supplied by Institute of Environmental Science and Research Ltd, Porirua, New Zealand).

Phenotypic identification of ESBL/AmpC-E.

Isolates showing a presumed ESBL- and/or AmpC-E phenotype based on a resistance to cefoxitin and/or cefotaxime and/or ceftazidime, were subjected to phenotypic and genotypic confirmatory tests. The phenotypic identification of an ESBL-E was based on synergy testing, seen as a distortion of the zone of no growth, with a ≥5mm increase in the zone diameters for cefotaxime or ceftazidime in proximity to amoxicillin-clavulanic acid (CLSI 2013). The phenotypic identification of AmpC-E was based on the observation of ≥5mm increase in the growth inhibition zone for ceftazidime, cefotaxime, and cefoxitin, in the presence of 3-aminophenylboronic acid (APB) (Yagi *et al.* 2005). Briefly, a solution was prepared by mixing 1ml dimethyl-sulphoxide (DMSO) with 100 mg APB (Acros Organics, New Jersey, USA) and 3 µL were deposited on the ceftazidime, cefotaxime and cefoxitin discs.

All isolates showing resistance to ≥2 antimicrobials, and all the ESBL-E and AmpC-E were suspended in glycerol broth and stored at -80°C for future testing.

Molecular identification of ESBL/AmpC-E

PCR-sequencing was used to confirm and characterise the phenotypically positive ESBL-E and AmpC-E. DNA was extracted using a precipitation method (Mullner et al., 2010). Briefly, frozen isolates were thawed and subcultured on 5% sheep blood agar plates. Two or three colonies of each isolate were suspended into a 2ml conical tube containing 100 µl of 2% Chelex® chelant (Thermo Fisher Scientific, New Zealand) with the addition of H_2SO_4 to a final pH of 7.4. The samples were vortexed and heated to 95°C in

a heat block for 5 min. This was followed by centrifugation at 13,000 rpm for 3 minutes. The supernatant was transferred to a 2ml tube containing 200µl of DNA/RNA free H_2O and the DNA quantified using spectrophotometry (NanoDrop, Thermo Fisher Scientific, Wilmington, DE). The primers used for the PCR and sequencing of ESBL (bla_{TEM}, bla_{SHV}, bla_{CTX-M}) and AmpC variants (bla_{AmpC}, chromosomal *ampC* gene) are described in Table A.1. These sets of primers cover Class A and Class C β-lactamase enzymes, which are the predominant enzymes reported internationally (Bush 2013a). The amplification conditions have been previously described (Caroff *et al.* 1999; Pérez-Pérez and Hanson 2002; Rayamajhi *et al.* 2008; Woodford 2010a). For the bla_{CTX-M} groups 2, 8 and 25 genes, the following sets of primers were used in series: 1, the consensus primer bla_{CTX-M} (Saladin *et al.* 2002) for detection of bla_{CTX-M} – positive isolates; and 2, a multiplex PCR (Woodford 2010a) protocol for detection and differentiation of CTX-M groups. Finally, PCR was used to amplify and sequence bla_{CTX-M} groups 1 and 9 genes (Jeong *et al.* 2005; Costa *et al.* 2006).

Positive control strains used are listed in Table A.1 (positive controls for blaCTX-M groups 2, 8 and 25 were not available in New Zealand and were not used). PCR amplicons were verified by 2% agarose gel electrophoresis and amplicons showing positive bands of the appropriate size were purified using PureLink® PCR Purification Kit (Thermo Fisher Scientific, New Zealand) according to the manufacturer's instructions. The Massey University Genome Service Unit (Palmerston North, New Zealand) performed bidirectional sequencing of PCR products using an ABI 377 instrument. Forward and reverse sequences were aligned and edited manually to produce consensus sequences, using Geneious Pro 5.6.7® (Biomatters Ltd, Auckland, New Zealand). Consensus sequences were mapped to sequences downloaded from a publicly available online database for β-lactamases (maintained by Prof. George Jacoby at Lahey Clinic Indiana, USA; http://www.lahey.org/Studies/) using the Geneious program. Data were stored in a predesigned database (www.epimanager.com/Epicentre/en-nz).

A multi Locus Sequence Typing (MLST) scheme was used to determine the genetic relatedness of the ESBL/AmpC-E isolates (http://www2.warwick.ac.uk/mlst). PCR amplification of the seven housekeeping genes for the MLST, DNA purification and sequencing was performed by a commercial provider (Institute of Environmental Science and Research Ltd., Porirua, New Zealand) as previously described (Wirth *et al.* 2006). MLST results were compared with sequence types (ST) published in a publically available database (http://www2.warwick.ac.uk/mlst).

The two-tailed Fisher's exact test was used to compare proportions of interest (R version 3.0.3, Copyright (C) 2014 The R Foundation for Statistical Computing), and $P<0.05$ threshold was considered statistically significant.

Table A.1: Primers used for ESBL/AmpC detection by PCR in *E. coli* isolates

Gene name	Primer name	Primer sequence	PCR product size (bp)	Reference	Positive Control
*bla*TEM	TEM-F	5'-TCGGGGAAATGTGCG-3'	1074	(Rayamajhi *et al.* 2008)	ESR; NIL05/26
	TEM-R	5'TGCTTAATCAGTGAGGCACC- '3			
	TEM-F	5'-GTATCCGCTCATGAGACAATA-3'	966	(Dubois *et al.* 2002)	
	TEM-R	5'-TCTAAAGTATATATGAGTAAAC-3			
*bla*SHV	SHV-F	5'-GCCGGGTTATTCTTATTTGTCGC3'	1016	(Rayamajhi *et al.* 2008)	ESR; NIL05/26
	SHV-R	5'-ATGCCGCCGCCAGTCA-3'			
	SHV-R	5'-TCTTTCCGATGCCGCCGCCAGTCA-3'	1017	(Tenover and Rasheed 2004)	
*bla*CTX-M	CTX-F	5'-SCSATGTGCAGYACCAGTAA-3'	543	(Saladin *et al.* 2002)	ESR; NIL05/26
	CTX-R	5'-CCGCRATATCRTTGGTGGTG-3			
*bla*CTX-M group 1	CTX1-F	5'-AAAAATCACTGCGCCAGTTC-3'	415		ESR; NIL05/26
	CTX1-R	5'-AGCTTATTCATCGCCACGTT-3'		(Woodford 2010a)	
*bla*CTX-M group 2	CTX2-F	5'-CGACGCTACCCCTGCTATT-3'	552		Not available in New Zealand
	CTX2-R	5'-CCAGCGTCAGATTTTTCAGG -3'			
*bla*CTX-M group 8	CTX8-F	5'-TCGCGTTAAGCGGATGATGC-3'	666		Not available in New Zealand
	CTX8-R	5'-AACCCACGATGTGGGTAGC-3'			
*bla*CTX-M group 9	CTX9-F	5'- CAAAGAGAGTGCAACGGATG-3'	205		ESR; ARL03/641
	CTX9-R	5'- ATTGGAAAGCGTTCATCACC-3'			
*bla*CTX-M group 25	CTX25-F	5'- GCACGATGACATTCGGG-3'	327		Not available in New Zealand
	CTX25-R	5'-AACCCACGATGTGGGTAGC3'			
*bla*CTX-M group 1	CTX1-F	5'-CCC TGGTTAAAAAATCACTG-3'	891	(Jeong *et al.* 2005)	ESR; NIL05/26
	CTX1-R	5'-CCGTTTCCGCTATTACAAAC-3'			
*bla*CTX-M group 9	CTX9-F	5'-GTGACAAAGAGAGTGCAACGG-3'	857	(Costa *et al.* 2006)	ARL03/641
	CTX9-R	5'-ATGATTCTCGCCGCTGAAGCC-3'			
MOX-, MOX2, C-1MY, CMY-8 to AMY-11	MOXM-F	5'-GCTGCTCAAGGAGCACAGGAT-3'	520	(Pérez-Pérez and Hanson 2002)	' Nancy Hanson'
	MOXM-R	5'-CACATTGACATAGGTGTGGTG C-3'			
LAT-1 to LAT4 CMY-2 to CMY-7, BIL-1	CITM-F	5'-TGGCCAGAACTGACAGGCAAA-3'	462		ARS06/841
	CITM-R	5'-TTTCTCCTGAACGTGGCTGGC-3'			
DHA-1 DHA-2	DHAM-F	5'-CCGTACGCATACTGGCTTTGC-3'	405		ARL06/624
	DHAM-R	5'-AACAGCCTCAGCAGCCGGTTA-3'			
ACC	ACCM-F	5'-AACAGCCTCAGCAGCCGGTTA-3'	346		ERL10/3021
	ACCM-R	5'-TTCGCCGCAATCATCCCTAGC-3'			
MIR-1T ACT-1	EBCM-F	5'-TCGGTAAAGCCGATGTTGCGG-3'	302		ARL11/565
	EBCM-R	5'-CTTCCACTGCGGCTGCCAGTT-3'			
FOX-1 to FOX-5b	FOXM-F	5'-AACATGGGGTATCAGGGAGATG-3'	190		ARL11/62
	FOXM-R	5'-CAAAGCGCGTAACCGGATTGG-3'			
CIT (sequencing)	CMY-2-F	5'-AACACACTGATTGCGTCTGAC-3'	1200		ARS06/841
	CMY-2-R	5'-CTGGGCCTCATCGTCAGTTA-3'			
ampC promoter	AmpC1	$_{-71}$5'-AATGGG-TTTTCTACGGTCTG-3'$_{-52}$		(Caroff *et al.* 1999)	
	AmpC2	$_{120}$5'-GGGCAGCAAATGTGGAGCAA-3'$_{101}$			

Results

Comparison of isolation of AMR-E between methods

The number of faecal specimens that showed bacterial growth within the Enterobacteriaceae resistance zone for each antimicrobial, and the number of *E. coli* isolated from these specimens are summarized in Table A.2. Method 1 (BPW) as primary culture was more likely to detect bacterial growth within the resistance zone of seven out of the eight antimicrobials tested, although statistical comparisons between proportions of resistant specimens for the single antimicrobials were not significant (Fisher's exact test P=0.58). M1 was also more likely to detect MDR and ESBL/AmpC-E isolates than the other methods (P>0.7) and was more likely to detect resistant *E. coli* isolates against 7/8 antimicrobials than the other methods. With all five methods, the highest prevalence of resistance in *E. coli* was observed against cephalothin, sulphamethoxazole-trimethoprim, and tetracycline. No resistance to imipenem was observed.

Comparison of isolation of AMR-E between hospitalised and non-hospitalised animals

If the results of the five culture methods are merged, for 7/8 antimicrobials tested the proportion of faecal specimens that showed growth within the resistance zone was greater in hospitalised, than non-hospitalised animals (Fisher's exact two-tailed P<0.01). When only M1 was considered, a greater proportion of faecal specimens from hospitalised (9/10; 90%) compared with non-hospitalised (17/31; 55%) animals (P=0.063) showed growth within the Enterobacteriaceae resistance zones, requiring subculture. Using M1, AMR *E. coli* were identified in 15/41 (37%) specimens. The rate of isolation of AMR *E. coli* in hospitalised was greater than non-hospitalised animals (hospitalised: *E. coli* found in 9/10 subcultured specimens; non-hospitalised: *E. coli* found in 6/17 subcultured specimens; P<0.01).

The susceptibilities of the *E. coli* isolates obtained using M1 to the individual antimicrobials, and the resistance profiles of the isolates are reported in Table A.3Table A.4. The most commonly observed resistance in *E. coli* isolates was to cephalothin and sulphamethoxazole-trimethoprim (10/41;

24.3%). A total of seven (17%; 95% CI= 7.1%-32%) of the *E. coli* isolates were defined as multi-drug resistant.

Detection of ESBL/AmpC-E by the different culture methods

Over all, ESBL/AmpC-E phenotypes were isolated from 3/10 and 1/10 specimens from hospitalised and 1/31 and 2/31 non-hospitalised animals, respectively. The number of ESBL/AmpC-E isolates obtained from faecal specimens varied between the five methods. In particular, the use of culture method M1 was more likely to result in the detection of ESBL-E and AmpC-E isolates in more specimens than any other method, and in two specimens, such isolates were only detected by M1 (Isolates 2 and 6; Table A.5). ESBL-E and AmpC-E phenotypes from five specimens were isolated also by other methods. There was inadequate statistical power to assess the significance of ESBL/AmpC detection between methods.

Table A.2: The antimicrobial susceptibility test results of faecal specimens in stage 1 with results of the confirmed *Escherichia coli* isolates in stage 2, stratified by microbiological method used

Antimicrobial agent	Buffered Peptone Water (M1)		MacConkey Broth (M2)		Saline (M3)		MacConkey Agar (M4)		MacConkey No3 (M5)	
	No of faecal specimens resistant (%)	No of resistant *E. coli*	No of faecal specimens resistant (%)	No of resistant *E. coli*	No of faecal specimens resistant (%)	No of resistant *E. coli*	No of faecal specimens resistant (%)	No of resistant *E. coli*	No of faecal specimens resistant (%)	No of resistant *E. coli*
Cephalothin[a]	20 (48)	10 (24)	12 (29)	7 (17)	7 (17)	6 (15)	12 (29)	7 (17)	13 (32)	6 (15)
AMC[a]	11 (27)	9 (22)	12 (29)	6 (15)	5 (12)	4 (10)	9 (22)	7 (17)	8 (20)	6 (15)
Cefoxitin	-	3 (7)	-	2 (5)	-	1 (2)	-	1 (2)	-	1 (2)
Cefovecin[a]	10 (24)	8 (20)	8 (20)	6 (15)	6 (15)	4 (10)	9 (22)	5 (12)	8 (20)	5 (12)
Cefotaxime[a]	8 (20)	6 (15)	7 (17)	4 (10)	4 (10)	4 (10)	7 (17)	5 (12)	6 (15)	4 (10)
Ceftazidime[a]	-	2 (5)	-	2 (5)	-	1 (2)	-	1 (2.4)	-	1 (2)
Imipenem	-	0	-	0	-	0	-	0	-	0
Gentamycin	10 (24)	4 (10)	6 (15)	2 (5)	5 (12)	3 (7)	7 (17)	3 (7)	8 (20)	3 (7)
Enrofloxacin	8 (20)	4 (10)	7.3 (3)	1 (2)	2 (5)	2 (5)	4 (10)	3 (7)	6 (15)	4 (10)
SXT	17 (41)	10 (24)	26.8 (11)	6 (15)	10 (24)	7 (17)	12 (29.2)	7 (17)	12 (29)	9 (22)
Tetracycline	20 (49)	9 (22)	13 (32)	4 (10)	8 (20)	4 (10)	14 (34)	7 (17)	13 (32)	6 (15)
Aztreonam[a]	-	5 (12)	-	4 (10)	-	3 (7)	-	4 (10)	-	4 (10)

AMC: Amoxicillin Clavulanic Acid; SXT: sulphamethoxazole-trimethoprim
No (%) is describing the number of samples (from 41) and their percentage of the total number of samples that are resistant to the antibiotic used for susceptibility testing at stage 1
Confirmed resistant *E. coli*: describes the number of *E. coli* isolates and percentage retrieved from the preceded sample at stage1. One isolate per sample was used for the analysis of this table.
(-) antibiotics that were not used at stage 1
[a] Isolates that are intermediate and resistant are included

Table A.3: Antimicrobial resistance patterns of the 15 *Escherichia coli* isolates detected by method M1

AMR Patterns	Number of isolates (%)
SXT	3 (7.3)
CEP-AMC-CTX-CVN-CN-ENR-SXT-TET-ATM	4 (9.8)
TET	1 (2.4)
CEP-SXT-TET	2 (4.9)
CEP-AMC-CVN	1 (2.4)
CEP-AMC-FOX-CTX-CAZ-CVN-SXT-ATM	1 (2.4)
AMC-ENR-SXT-TET	1 (2.4)
CEP-AMC-FOX-CTX-CAZ-CVN-TET	1 (2.4)
CEP-AMC-FOX-CVN	1 (2.4)
Total multi-drug resistant	7 (17)

Table A.4: The antimicrobial susceptibility test results of the faecal specimens and *Escherichia coli* isolates from hospitalised and non-hospitalised animals using M1 method

Antimicrobial agent	Hospitalised		Non-Hospitalised		Total Number of Isolates	
	Sample (n=10)	Confirmed *E. coli* (%)	Sample (n=31)	Confirmed *E. coli*	Number *(%)	95%CI
Cephalothin[a]	8 (80)	5 (50)	13 (42)	5 (16.1)	10 (24.3)	16.8-46.5
AMC[a]	7 (70)	6 (60)	7 (22.5)	3 (9.6)	9 (21.9)	8.7-35.1
Cefoxitin	-	1 (10)		2 (6.4)	3 (7.3)	0.1-15.6
Cefovecin[a]	5 (50)	5 (50)	6 (19.3)	3 (9.6)	8 (19.5)	6.8-32.1
Cefotaxime[a]	5 (50)	4 (40)	5 (16.1)	2 (6.4)	6 (14.6)	3.3-25.9
Ceftazidime[a]	-	1 (10)	-	1 (3.2)	2 (4.8)	0.0-11.7
Imipenem	-	0	-	0	0 (0)	0.0
Gentamycin	4 (40)	3 (30)	6 (19.3)	1 (3.2)	4 (9.8)	0.27-19.2
Enrofloxacin	1 (10)	3 (30)	3 (9.6)	1 (3.2)	4 (9.8)	0.27-19.2
SXT	6 (60)	6 (60)	10 (32.2)	4 (12.9)	10 (24.3)	16.8-46.5
Tetracycline	4 (40)	4 (40)	16 (51.2)	8 (25.8)	9 (21.9)	8.7-35.1
Aztreonam[a]	-	4 (40)	-	1 (3.2)	5 (12.2)	1.7-22.6
MDR		4 (40)		3 (9.6)	7 (17)	5-29.1

AMC: amoxicillin clavulanic acid, SXT: sulphamethoxazole-trimethoprim

[a] Isolates that are intermediate and resistant are included

One isolate was taken per sample for this table analysis

MDR: Multi-Drug resistant: resistant to three or more antimicrobial classes

Molecular characterisation of ESBL/AmpC-positive-E

The ESBL/AmpC-positive-E isolates were genotypically characterised using the β-lactamase genes (Table A.5). In total, the *bla*$_{CTX-M}$ gene was detected in seven ESBL-E isolates, three independent (isolates 1, 2, 3), and four from the same household (4.1, 4.2, 4.3, 4.4), and were all identified as *bla*$_{CTX-M-14}$. The *bla*$_{AmpC}$ gene was detected in 2/3 AmpC-E isolates (isolates 6 and 7), and these were *bla*$_{CMY-2}$-positive. The third AmpC-E isolate (isolate 8) was negative for the *bla*$_{AmpC}$ by PCR and was therefore tested by another PCR designed for detection of chromosomal mutations within the 191 bp region of the promoter and attenuator of the AmpC gene (Caroff et al., 1999). The amplicon was sequenced and the sequence compared with the *E. coli* K-12 reference strain's sequence (GenBank accession U00096). A number of mutations were observed in the promoter region. There were substitutions at positions -18 (G to A), -9 (A to T), and outside the promoter and attenuator regions at positions -1 (C to T) and +58 (C to T), and a TA insertion at position +1.

The four *bla*$_{CTX-M-14}$ *E. coli* were also carrying the *bla*$_{TEM-1.}$ One of the *bla*$_{CMY-2}$ positive isolates (isolate 6) was also positive for the *bla*$_{TEM-1.}$ None of the isolates were *bla*$_{SHV}$ positive. Isolates originating from the same specimens always showed the same ESBL/AmpC genetic profiles, even if the AMR profiles differed.

MLST was performed to investigate the clonal relatedness of the seven (ESBL/AmpC) *E. coli* isolates. There were five sequence types (ST) among the seven isolates. Three ST did not belong to any known clonal complex (CCs), and these were ST744 (international ST, Stoesser *et al*., 2012), ST4394 (new ST), and ST2175 (found abroad, Matsumura *et al*., 2013). Two ST belonged to known pandemic CCs and were the ST48 (CC10) and the ST155 (CC155) (Table A.5). One isolate was a novel strain, carrying a new *pur*-336 gene. This new MLST was submitted to the University of Warwick MLST database (http://www2.warwick.ac.uk/mlst) and was assigned a new ST4394 (clonal complex, none). Finally, one isolate belonged to ST2175.

Table A.5: Antimicrobial resistance profiles of ESBL/AmpC-producing *E. coli* isolates and β-lactamase genes carried on plasmids of these isolates

Isolate number	H/C	Species	Antimicrobial profile	ESBL/AmpC genes	MLST ST	MLST CC	First Isolated/Country/ Pathogenicity/Year
1	H	Canine	CEP-AMC-CTX-CVN-CN-ENR-SXT-TET-ATM	$bla_{CTX-M-14}$+ bla_{TEM-1}	744		Human; Paris, France; None; 2006
2	H	Feline	CEP-AMC-CTX-CVN-CN-ENR-SXT-TET-ATM	$bla_{CTX-M-14}$+ bla_{TEM-1}	744		Human; Paris, France; None; 2006
3	H	Feline	CEP-AMC-CTX-CVN-CN-ENR-SXT-TET-ATM	$bla_{CTX-M-14}$+ bla_{TEM-1}	744		Human; Paris, France; None; 2006
4.1*	C	Feline	CEP-AMC-CTX-CVN-CN-SXT-TET-ATM	$bla_{CTX-M-14}$+ bla_{TEM-1}	155	155	Human; Ipetu-Modu, Nigeria; EAEC; 1995
4.2*	C	Canine	CEP-AMC-CTX-CVN-CN-SXT-TET-ATM	$bla_{CTX-M-14}$+ bla_{TEM-1}	155	155	Human; Ipetu-Modu, Nigeria; EAEC; 1995
4.3*	C	Feline	CEP-AMC-CTX-CVN-CN-ENR-SXT-TET-ATM	$bla_{CTX-M-14}$+ bla_{TEM-1}	48	10	Human; Ghana; None;1994
4.4*	C	Canine	CEP-AMC-CTX-CVN-CN-ENR-SXT-TET-ATM	$bla_{CTX-M-14}$+ bla_{TEM-1}	744		Human; Paris, France; None; 2006
5	H	Feline	CEP-AMC-CVN		73	73	Human; Freiburg, Germany; non-pathogenic;1917
6	H	Canine	CEP-AMC-FOX-CTX-CAZ-CVN-SXT	bla_{CMY-2}+ bla_{TEM-1}	4394/New		Canine; Palmerston North, New Zealand; 2012
7	C	Canine	CEP-AMC-FOX-CTX-CAZ-CVN-TET	bla_{CMY-2}	155	155	Human; Ipetu-Modu, Nigeria;EAEC; 1995
8	C	Canine	CEP-AMC-FOX-CTX-CAZ-CVN-ATM	*ampC* gene mutation	2175		Human; Nagoya, Japan; bla_{CMY-2} ;2009

CEP: Cephalothin; AMC: Amoxicillin-clavulanic acid; FOX Cefoxitin; CTX Cefotaxime; CAZ: Ceftazidime; CVN: Cefovecin; TE: Tetracycline; CN Gentamicin; SXT: Sulphamethoxazole-Trimethoprim; ENR: Enrofloxacin; ATM: Aztreonam.
The naming of an antimicrobial drug indicates resistance or intermediate resistance to the antimicrobial, as observed by disk diffusion test.
H: hospitalised animal; C: community animal
* Isolates originating from different animals on the same household.

Four ESBL-positive isolates were obtained from two dogs and two cats belonging to the same household, and belonged to three different sequence types (isolates 4.1 - 4.4, Table A.5).

In addition, one isolate (isolate 5) showing resistance to the 3rd generation cephalosporin (cefovecin) and belonging to ST73 (pandemic) was negative for ESBL/AmpC by PCR. For confirmation, the phenotypic and PCR tests were repeated, with identical results.

Comparison of phenotypes and genotypes of ESBL/AmpC-E

There were five antimicrobial resistance profiles among the seven ESBL/AmpC-E isolates. A number of ESBL/AmpC-E carrying the same ESBL/AmpC genes displayed different phenotypes (Table A.5). In particular, the four *bla*$_{CTX-M}$ ESBL-carrying *E. coli* exhibited two antimicrobial resistance profiles. Three *bla*$_{CTX-M}$ positive isolates from hospitalised animals (isolates 1, 2, and 3) shared the same antimicrobial and genotypic profiles, and one isolate from the community displayed a different antimicrobial resistance profile, differing from the previous three by its susceptibility to enrofloxacin.

The four ESBL-positive *E. coli* isolated from the same household displayed two antimicrobial and genetic profiles and each pair of isolates displaying the same genetic profile shared the same antimicrobial resistance profile.

The three AmpC-E isolates exhibited three different antimicrobial resistance profiles. One isolate originated from a hospitalised dog and carried the *bla*$_{AmpC.}$ This isolate differed from the other two AmpC-positive *E. coli* by its sulphamethoxazole-trimethoprim resistance. One isolate originated from a non-hospitalised dog, and harboured the *bla*$_{AmpC}$ gene and was resistant to tetracycline, while the third isolate originated from a non-hospitalised dog, had a chromosomal *ampC* mutation, and was resistant only to the β-lactams.

One isolate (isolate 5, Table A.5) originated from a hospitalised cat and displayed a distinctive antibiogram and was resistant only to some β-lactams (cephalothin, amoxicillin-clavulanic acid and intermediate resistance to cefovecin). However, this isolate was cefotaxime and ceftazidime susceptible (with zone diameters > 28 mm). Thus, according to CLSI guidelines (2013), it

did not display the ESBL phenotype. Furthermore, the isolate was PCR-negative for *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ (the susceptibility tests and PCRs were repeated (using re-extracted DNA), with similar results).

Cost and utility considerations

The costs of media and antimicrobial discs in New Zealand dollars for the processing of each faecal specimen and *E. coli* isolate, per culture method are reported in Table A.6. In general, in regards to the ease of use and availability of reagents in New Zealand, the M1 procedure was the easiest to manage and use. Processing of a specimen using M1 and M2 required one additional day than the other methods. The cost for processing each faecal specimen varied according to the number of antimicrobials around which there was growth within the resistance zone, with the lowest cost associated with specimens which did not require subculture (showing no growth within the resistance zones). The costs of processing increased as the number of antimicrobials around which there was growth in the resistance zone increased.

Table A.6: Maximum and minimum costs/specimen and duration of processing the specimens according to the protocol chosen

Methods		M1	M2	M3	M4	M5
Cost (NZ	Min	2.4-9.1	3.14-9.7	2.26	2.23	3
currency $)	Max	9.1	9.7	8.86	8.83	9.6
Duration	Min	48	48	12-14	12-14	12-14
(hrs.)	Max	120	120	96	96	96

Discussion and conclusions

This pilot study aimed to compare five culture methods for the isolation of AMR *E. coli* from faeces cats and dogs in New Zealand, in order to choose a

suitable method to be used in the prevalence study presented in

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We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Ali Karkaba

Name/Title of Principal Supervisor: Alex Grinberg

Name of Published Research Output and full reference:

Karkaba, A. Grinberg, J. Benschop, E. Pleydell (2016). Characterisation of extended spectrum β-lactamase and AmpC β-lactamase-producing Enterobacteriaceae isolated from companion animals in New Zealand. New Zealand Veterinary Journal

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Candidate's Signature

02/03/2017

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Alex Grinberg Digitally signed by Alex Grinberg Date: 2017.03.03 10:55:04 +13'00'

Principal Supervisor's signature

03/03/2017

Date

Chapter 3. The sampling of hospitalised animals was performed in order to enhance the isolation of AMR *E. coli,* given that the literature indicated a higher rate of isolation of AMR in hospitalised humans and pets (Nseir *et al.* 2011; Damborg *et al.* 2012).

A number of studies have compared different methods for the isolation of antimicrobial resistant *E. coli* from human (Finney *et al.* 2003; Bartoloni *et al.* 2006; Liss *et al.* 2013) and animal (Vieira *et al.* 2008; Leonard *et al.* 2012) faeces, but there is no agreed gold standard method. The results of the present study indicated method M1 was more likely to result in the detection of AMR-E, MDR-E and ESBL/AmpC-E from faeces of hospitalised and non-hospitalised cats and dogs than the other methods. Resistance to at least one antimicrobial was detected in 36% of the specimens, including 90% of specimens from hospitalised animals. Multidrug resistant *E. coli* were detected in 17% of the specimens, of which four isolates were ESBL/AmpC producers.

M1 yielded a greater proportion of faecal specimens with growth within the resistance zone for 7 out of the 8 antimicrobials used, than the other methods (Table A.2). Likewise, M1 yielded more AMR *E. coli* isolates to these antimicrobials than the other methods and was the second most favorable method in terms of costs (Table A.5). In addition, M1 was also more likely to detect ESBL/AmpC-E in comparison with the other methods, and all the *E. coli* exhibiting the ESBL/AmpC phenotype were confirmed by genotyping.

In summary, the comparison between five different culture methods for the isolation of ESBL/AmpC-E from faecal matrices, and suggested that the most

suitable method for use in the study presented in

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Candidate's Signature

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Chapter 3 is M1.