Phenotypic and molecular characterisation of *Pseudomonas aeruginosa* infections from companion animals and potential reservoirs of antibacterial resistance in humans.

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

Andrea Catherine Scott

May 2018

Acknowledgements

I would like to thank my primary supervisors Dr Joanne Fothergill and Dr Alan Radford.

I also thank my secondary supervisors Dr Dorina Timofte, Dr Vanessa Schmidt, Dr Gina Pinschbeck and Dr Neil McEwan and the Institute of Infection and Global Health, University of Liverpool and Professor Craig Winstanley, along with the Small Animal Teaching Hospital (SATH), Leahurst, University of Liverpool and the Veterinary Diagnostic Laboratory (VDL), Leahurst.

Matthew Moore (PhD student at University of Liverpool) performed Bioinformatics work for this project.

The sequencing in this project was performed as part of the International Pseudomonas Consortium.

The Masters thesis of this work was supported through a University of Liverpool/Wellcome Trust Research taster fellowship and internal funding.

Abbreviations

AMR – Antimicrobial resistance

BSAVA – British Small Animal Veterinary Association

BVA – British Veterinary Association

CDC - Centre for Disease Control and Prevention

CF – Cystic fibrosis

COPD – Chronic obstructive pulmonary disorder

CRE – Carbepenam-resistant enterobacteriacea

ECDPC - European Centre Disease Prevention and Control

FDA – US Food and Drug Administration

HAIs – Hospital acquired infections

ICEs – Integrative and conjugative elements

ICUs – Intensive care units

IDSA - Infectious Diseases Society of America

LPS – Lipopolysaccharide

LES –Liverpool epidemic strain

MDR - Multi drug resistant

MMR – Mis-match repair genes

MRSA - Methicillin-resistant *Staphylococcus aureus*

OM - Outer membrane

PCR – Polymerase chain reaction

PFGE – Pulse-field gel electrophoresis

PHE – Public Health England

VAP – Ventilator associated pneumonia

VMTH – Veterinary Medicine Teaching Hospital

WHO - World Health Organisation

WGS – Whole genome sequencing

Phenotypic and molecular characterisation of *Pseudomonas aeruginosa* infections from companion animals and potential reservoirs of antibacterial resistance in humans.

Andrea Catherine Scott - May 2018

Abstract

Antimicrobial resistance (AMR) is a multifactorial and complex issue, affecting healthcare worldwide. This is an escalating concern, highlighted by European and International bodies. AMR is not only a concern in human health but also animal health. The close interface between people, pets, food production animals and the environment exemplifies the importance that these two areas of health should not be considered as independent factions. *Pseudomonas aeruginosa* is a formidable pathogen due to its innate resistance making it naturally insensitive to many classes of antimicrobials, alongside its ability to acquire further resistance mechanisms. *P. aeruginosa* in the veterinary setting is a clinically relevant pathogen often associated with disease.

In this project, I determined the susceptibility of a set of veterinary clinical *P. aeruginosa* isolates (n=24) to polymyxin antibiotics, colistin (polymyxin E) and polymyxin B, by broth micro dilution and showed that the MICs for polymyxin B to be significantly higher than in a panel of human *P. aeruginosa* isolates (n= 37). Resistance to other antibiotics used in human medicine was low but higher levels of resistance were detected to ticarcillin (21%), and also to marbofloxacin (21%) and enrofloxacin (33%), these being two widely used veterinary antibiotics. Using the array tube typing method, the *P. aeruginosa* veterinary isolates were found to be distributed throughout the *P. aeruginosa* population, with shared array types from human infections such as keratitis and respiratory infections. Using whole genome sequencing on a limited subset of isolates, the veterinary isolates were generally clustered within the main *P. aeruginosa* population; however one isolate, did not

cluster with any other previously sequenced isolate. The genome sequencing also revealed that isolates had mutations in genes associated with polymyxin resistance and other antimicrobial resistance-related genes. These findings may suggest that treating animal infections with antimicrobials could lead to resistance in isolates capable of causing human infections.

The prevalence of resistance to beta-lactams, aminoglycosides and fluoroquinolones in isolates from companion animals particularly in *P. aeruginosa* is a relatively understudied area. Here, the resistance characteristics of a set of *P. aeruginosa* isolates from companion animals (n=106), collected, at the Veterinary Diagnostic laboratory (VDL) at the Small Animal Teaching Hospital, Leahurst (University of Liverpool, UK) was investigated. Several resistance genes including those relating to ESBLs (bla_{TEM}, bla_{SHV} and bla_{OXA}) and those relating to fluroquinolone resistance (*qnr*) were identified. In particular the identification of *qnrA* and *qnrB* genes in *P.aeruginosa* isolates of companion animal origin is a novel finding with no known reports to current date in the UK. Of the 8 confirmed sequenced positive amplicons they comprised 7 different isolates all canine in origin. Multidrug resistance was also demonstrated among some isolates.
These findings further support the likelihood that companion animals are an understudied source of antimicrobial resistant *P. aeruginosa* isolates, and merit sustained surveillance of the veterinary niche as a potential reservoir for resistant, clinically-relevant bacteria.

Chapter 1 : Introduction

1.1 *Pseudomonas aeruginosa*

P. aeruginosa features and habitats

Pseudomonas aeruginosa is a Gram negative rod shaped aerobic bacterium that is motile with a single polar flagella and can act as an opportunistic pathogen. Key features of the bacterium are listed in Table 1.1 *P. aeruginosa* is metabolically versatile and can grow both aerobically and as a facultative anaerobe, in addition to utilising other metabolic systems (such as those using nitrate or benzoate) thus contributing to its ability to be both ubiquitous and opportunistic in nature. These factors also explain the organisms' presence in numerous environments, including within natural water sources such as lakes and marshes, in soil and also within both the hospital and community environments (Khan & Cerniglia, 1994). *P. aeruginosa* may be isolated from hospital respiratory ventilator equipment, antiseptics, sinks and cleaning equipment (Trautmann et al, 2008; Trautmann et al, 2005). In the non-hospital environment reservoirs have been reported in recreational water venues such as poorly maintained swimming pools, hot tubs, whirlpools, spas (Ratnam, 1986) and also in contact lens cleaning solution, (Dutta et al, 2012; Kaye et al., 2017) soil and vegetables (Correa et al, 1991; Deredjian et al., 2014; Green et al, 1974; Khan & Cerniglia, 1994; Mena et al., 2008; Youenou et al, 2014).

Table 1.1 Key features of *P. aeruginosa* features

Description	Gram negative rod shaped aerobic bacterium that is motile with a single polar flagella and can act as an opportunistic pathogen
Diversity	Found in diverse environments including water (Cabral, 2010), soil, sewage (Slekovec et al., 2012) and also humans, animals and plants
Form	Found in planktonic forms or biofilms (O'Tool et al, 2000)
Colony types	Variable, smooth or mucoid in appearance;

	Wild-type isolates found naturally from soil or water frequently produce a small, rough colony. Clinical isolates are usually either a large, smooth colony type with flat edges and elevated appearance. Or, they may have a mucoid appearance, this colony type being associated with the over production of the polysaccharide alginate and are typically found in those samples from the respiratory and urinary tract (Cole et al, 2014; Distefano, 2015).
Pigment production	*P. aeruginosa* can produce a variety of pigments; Pyocyanin a blue coloured phenzine pigment; Pyoverdine a siderophore, involved in iron metabolism, it is a yellow-green brightly fluroscent pigment; Pyorubin a red brown pigment; Pyomelanin a brown pigment, melanin like compound synthesized from tyrosine. (Lau et al 2004)

P. aeruginosa genome

P. aeruginosa has a comparatively large genome of approximately 6.3 million base pairs (Mbp) (referred to in Figure 1.1). It is known to consist of a main conserved core genome and variable accessory segment (Stover C-K., et al 2000). It has been estimated that approximately 10% of its genome varies from strain to strain (Ozer et al 2014) and is thus referred to as the accessory genome. Genetic elements within the accessory genome of *P. aeruginosa* have been associated with differences in virulence and antibiotic resistance (Kung et al , 2010; Ozer et al., 2014). Virulence factors associated with *P. aeruginosa* include flagella, adhesion and extracellular proteins, or secondary metabolites that have proteolytic/cytotoxic activity (such as exotoxin A, elastase, proteases, pyocyanin, hemolysins) (Driscoll et al, 2007). The known virulence factors of *P. aeruginosa* are reported in Table 1.2.

P. aeruginosa infection process and virulence

The *P. aeruginosa* infection process consists of three stages; bacterial adhesion and colonization; local invasion; and disseminated systemic disease (Strateva & Mitov, 2011). The colonization phase, primarily involves the cell-associated virulence factors (Table 1.2). The infectious stage (which may then develop to either an acute infection or a chronic infection) is characterized by the production of extracellular

virulence factors. In acute infections high production of these factors are typical, whereas inchronic infections lower amounts of these determinants are produced (Strateva & Mitov, 2011).

P. aeruginosa virulence factors and quorum sensing

The virulence of *P. aerurginosa* depends on a varied number of both cell-associated and extracellular factors (Table 1.2). The majority of *P. aeruginosa* infections are both invasive and toxinogenic. Many of the extracellular virulence factors (proteases, exotoxin A, pyocyanin, siderophores, haemolysins) are controlled by quorum sensing (QS). This is a cell-to-cell signaling system that enables the bacteria to produce these factors in a coordinated, cell-density-dependent manner and overwhelm the host defense mechanisms during acute infection. The QS system can also contribute to biofilm formation and so may participate in the pathogenesis of chronic infection. Two-component sensor kinases such as RetS, LadS and GacS are also recognized as controlling the production of virulence factors as well as the switch from acute to chronic infection (Jimenez et al., 2012).

As mentioned, the production of many of the virulence factors is regulated by quorum sensing (QS), a bacterial cell-to-cell communication mechanism. There exists two well defined QS systems in *P. aeruginosa*, the las and rhl systems (Pearson et al 1997; Pesci et al., 1997) although two other system have also been identified (*pqs* and *iqs*). These rely on auto-inducer signal molecules (n N-acyl homoserine lactone molecules, or other molecules whose production depends on *S*-adenosylmethionine as a substrate). Auto-inducers are produced in the cell and freely diffuse across the inner and outer membranes. These molecules accumulate in the environment as the bacterial population density increases, and bacteria track this information and collectively alter gene expression. QS controls genes that direct activities that are beneficial when performed by groups of bacteria acting in synchrony. The term “fitness” is used to describe this ability to adjust metabolism to suit the environmental conditions, in order to survive and grow (Beceiro et al., 2013; Millan et al., 2015). Processes controlled by QS include bioluminescence, sporulation, competence, antibiotic production, biofilm formation, and virulence factor secretion (Ruparell et al., 2016; Karatuna & Yagci, 2010)

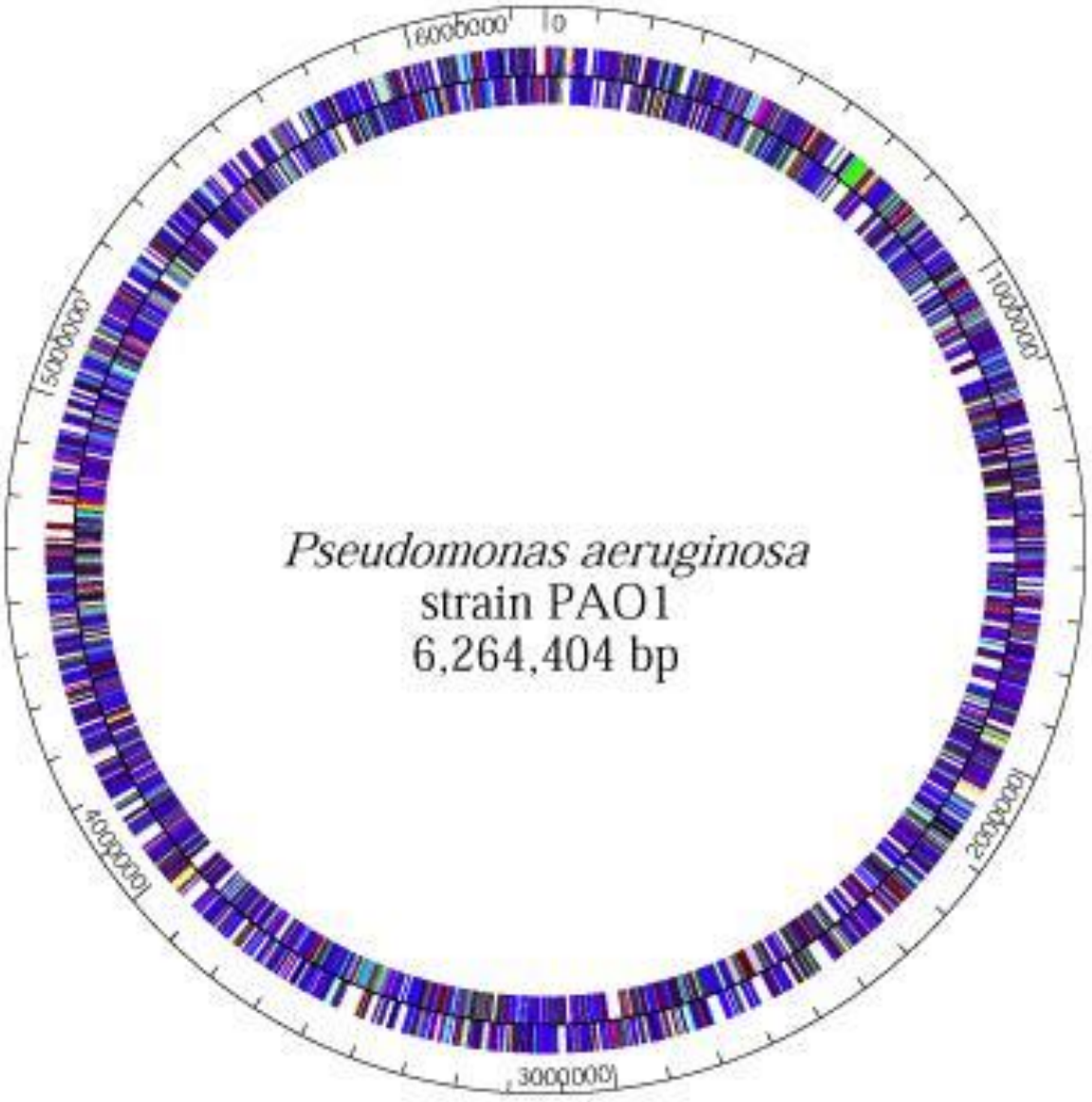


Figure 1.1 *P.aeruginosa* (PA01) genome sequence (Pseudomonas Genome Database

Table 1.2 Summary of the Virulence Determinants of Pathogenic *Pseudomonas aeruginosa* Table adapted from (Strateva & Mitov, 2011)

Virulence Factors	Cell associated factors	Extra cellular factors
Adhesins		
Type IV pili (N-methyl-phenylalanine pili)	✓	
Polysaccharide capsule (glycocalyx)	✓	
Alginate slime (biofilm)	✓	
Carbohydrate-binding	✓	
Proteins (lectins)	✓	
Adhesion Facilitation		
Neuraminidase (sialidase)		✓
Invasins		
Elastases (LasB and LasA)		✓
Alkaline protease		✓
Haemolysins (phospholipase and rhamnolipid)		✓
Cytotoxin (leukocidin)		✓
Siderophores and siderophore uptake systems		✓
Pyocyanin diffusible pigment		✓
		✓
Motility/chemotaxis		
Flagella (swimming motility)	✓	
Retractile pili (twitching motility)	✓	
Toxinogenesis		
Exotoxin A		✓
Lipopolysaccharide (endotoxin)	✓	
LecA and LecB lectins	✓	
Type III effector cytotoxins (ExoS, ExoU, ExoT, ExoY)		✓
Enterotoxin		✓
Antiphagocytic surface properties		
Capsules, slime layers		✓
Lipopolysaccharide	✓	
Biofilm	✓	
Defense against serum bactericidal reaction		
Slime layers, capsules, biofilm	✓	
Lipopolysaccharide	✓	

Protease enzymes		✓
Defense against immune responses Capsules, slime layers, biofilm Protease enzymes	✓	✓
Genetic attributes Genetic exchange by transduction and conjugation Inherent (natural) drug resistance R factors and drug resistance plasmids	**N/A**	**N/A**
Ecological criteria Adaptability to minimal nutritional requirements Metabolic diversity Widespread occurrence in a variety of habitats	**N/A**	**N/A**

1.2 *P. aeruginosa* infections in humans

Introduction

P. aeruginosa is rarely the primary cause of infections in healthy individuals. However, as an opportunistic pathogen it is a known cause of disease in the immune-compromised host (for example in severe burns, cystic fibrosis, diabetes mellitus, cancer patients). *P. aeruginosa* is a serious pathogen in hospitals (Shorr 2009), accounting for approximately 10% of all such infections in most European Union hospitals. According to the Centre for Disease Control and Prevention (CDC) of the United States, *P. aeruginosa* is the fourth most commonly-isolated nosocomial pathogen, responsible for 10.1% of all hospital acquired infections (HAIs) (Centre for Disease Control and Prevention, 2013). This is similar to that reported in European hospitals (De Bentzmann & Plésiat, 2011). In the CDC Antibiotic Resistant Threats in US Report of 2013, approximately 13% of hospital acquired *P. aeruginosa* infections were multidrug resistant (Centre for Disease Control and Prevention, 2013). *P. aeruginosa* is a cause of severe hospital-acquired (nosocomial) infections such as pneumonia (ventilator associated, VAP) and is

involved in the etiology of several diseases including bronchopneumonia, ocular, burn and wound infections, bacteremia, endocarditis and meningitis. The organism most commonly affects the lower respiratory system in humans and is an important respiratory pathogen in patients with cystic fibrosis (CF) and also other chronic lung diseases such as non-CF bronchiectasis and severe chronic obstructive pulmonary disease (COPD) (Govan et al, 2007).

P. aeruginosa is a known cause of bacteraemia and this is associated with significant mortality and morbidity. Mortality rates within the first 3-5 days of the onset of bacteremia are high and fifty percent of isolates are resistant to the standard antibiotics used in empirical treatment. Poorer clinical outcomes have also been demonstrated with pneumonia caused by infections due to *P. aeruginosa* due to associated multidrug resistance of the organism (Tam et al., 2010). *P. aeruginosa* is reported as the most common Gram-negative pathogen causing nosocomial pneumonia in the United States, and it is frequently implicated in hospital-acquired urinary tract and bloodstream infections (Peleg et al, 2010; Mehrad et al, 2015)

Specific *P. aeruginosa* human infections including some epidemiology and prevalences

Bacteremia

A UK government (UKGov, 2017) Public Health England (PHE) Infection Surveillance report looking at bloodstream infections showed that the overall rate of *Pseudomonas* as 6.2 per 100,000 population (July 2015) which was an 11% decrease from 2007 (the analyses of this report were based on data relating to diagnoses of *Pseudomonas* spp. and *Stenotrophomonas* spp. bacteramia 2007 – 2014 in England, Wales and Northern Ireland. The most frequently identified *Pseudomonas* species in blood isolates in 2014, according to the same surveillance report, was *P. aeruginosa* at 81%. The proportion of which were reported as resistant to one of the key antimicrobials in 2014 remained steady or increased slightly compared to

2013. A steady increase in reported resistance of *Pseudomonas* isolates to piperacillin/tazobactam was observed reported 2010-2014 from 7%-10%. In England, dual resistance to ciprofloxacin and ceftazidime was reported in 2% of *Pseudomonas* spp. bacteraemia. Imipenem resistance increased the most from 13% of isolates in 2013 to 16% isolates in 2014. In 2014 the proportion of resistant *Pseudomonas* spp. bacteraemia isolates reported was 10% piperacillin/tazobactam, 11% ciprofloxacin, 7% ceftazidime, 10% meropenem, 16% imipenem and 4% gentamicin.

Comparatively, a US study (Larru et al., 2016) evaluated the epidemiology and antimicrobial resistance of bloodstream infections occurring during an 11-year period in a large, tertiary care children's hospital in the United States and found 20% of these infections to be ESKAPE (includes *P. aeruginosa*) pathogen in origin. No clinically significant increases in ceftazidime-resistant *P. aeruginosa* were observed during the study, although MRSA rates did increase over time (Larru et al., 2016).

Keratitis

In humans *P. aeruginosa* is a known problem in contact lens wearers, were it can be a common cause of ulcerative keratitis (Stapleton et al, 1995). Ulcerative keratitis is a rare, but potentially serious, complication of cosmetic contact lens wear. It has the potential to affect vision and cause blindness (Austin et al, 2017; Zegans et al., 2016). There are several different types of microbes that colonize lenses and can lead to infection and inflammation, but one of the most common causes of microbial keratitis remains *P. aeruginosa*. Its ability to produce proteases, to either invade or kill corneal cells, and to coordinate expression of virulence factors via quorum-sensing have been shown to be important during microbial keratitis. Another important factor that contributes to the destruction of the cornea during microbial keratitis is excessive activation of the host defense system. *P. aeruginosa* can activate several pathways of the immune system and activation often involves

receptors on the corneal epithelial cells called toll-like receptors (Hazlett, 2004; Willcox, 2007). *P. aeruginosa* has a tendency to adhere to the contact lens surface and is transferred over scratched corneal epithelium penetrating the cornea's deeper layers and leading corneal ulcers. Permanent blindness can be caused by a severe infection. The lens, ocular environment, and storage case may offer an appropriate survival niche for the organism to adhere to and colonize lens materials during wear and storage (Hedayati, et al 2015).

A 2015 retrospective case control study (Vazirani et al, 2015) looked at ninety episodes of *P. aeruginosa* keratitis from a tertiary care eye institution from 2007 - 2014 (23 multi-drug resistant as cases and 67 episodes drug-sensitive as controls). Antimicrobial resistance in the MDR *P.aeruginosa* keratitis isolates lowest for both colistin and imipenem at 56.52% each. Complications (such as corneal perforation, cyanoacrylate glue application and keratoplasty) were more common in when infections involved MDR isolate. Studies report that various factors, such as the use of ocular lubricant, a compromised ocular surface, and bandage contact lens are associated with MDR (multi-drug resistant) *P. aeruginosa* keratitis. An addition, preservative-free lubricant ointment may act as a source or reservoir of infection in this condition (Vazirani et al., 2015). A 2015 study by (Hedayati et al., 2015) looked at contact lens wearers in Iran with 26 patients at a hospital from 2012-2013. With a low number (8 samples) of the scraped ulcers being reported as sterile, and of the positive cultures, 80% isolated *P. aeruginosa*. In this particular study 84% of the microorganism cases were sensitive to ciprofloxacin, while imipenem, meropenem, and ceftazidime were the second most effective antibiotics (susceptibilities tested by disc diffusion method). The percentage of isolates sensitive to imipenem, were somewhat higher in this study. Colistin susceptibility here was not tested however. Fernandes et al (Fernandes et al 2016) specifically looked at extensively and pan drug resistant *P. aeruginosa* keratitis. In their study (of 15 eyes from 13 different patients, at an Eye Institute in India 2009-2013) 40% were sensitive only to imipenem, 20% to colistin, 13.3% to neomycin and 6.7% each to imipenem and colistin, impienem and ceftazidime and azithromycin respectively (susceptibilities tested by disc diffusion method). The imipenem resistance in this study was higher

but it specifically looked at those isolates that were MDR from the Opthalmology Microbiology Unit. By comparison a larger UK study (in the area of Kent) over a ten year period to 2008 (Shalchi et al, 2011) looked at corneal scrapings isolate sensitivity to chloramphenicol, cefuroxime, gentamicin and ciprofloxacin. This was determined by microdilution (using Microscan System). The study assessed 476 scrapes from 440 patients. Bacterial keratitis accounted for 162 isolates (94.2%), of which 99 (61.1%) were Gram-negative. There was a general increase in the number of Gram-negative isolates with time. Testing showed widespread Gram-negative resistance to chloramphenicol (74.1%), with reducing sensitivity over the time frame of the study. There was 97.3% sensitivity to combination gentamicin and cefuroxime, and 94.4% sensitivity to ciprofloxacin.

Cystic Fibrosis

In patients with CF, *P. aeruginosa* is the major pathogen and is the major cause of morbidity and mortality (Hirsch & Tam, 2011; Tam et al., 2010). It has a high prevalence and once established, a chronic infection with mucoid strains almost inevitably occurs. Treatment strategies may involve controlling infection or attempting to eradicate the organism in the early stages of infection. Inhaled antibiotics are a mainstay of maintenance therapy while intravenous antibiotics are usually required if pulmonary exacerbations develop.

Good hygiene measures are important in prevention and to limit cross-infection in patients. A large variety of *P. aeruginosa* strains are seen to infect CF patients and it is common for the same clone type to persist in the airways of CF patients (Marvig et al 2014) (Johansen et al, 2008). *P. aeruginosa* populations within the lung in infected CF patients are highly diverse and dynamic and extreme diversification within a specific strain type during a chronic infection has been observed (Ashish et al, 2012; Hogardt & Heesemann, 2010). There may be impaired mucociliary clearance or dysfunction of antibacterial peptides, increased availability of bacterial receptors, reduced ingestion of pathogens by CF cells and impaired defences (related to low levels of molecules such as nitric oxide or glutathione). Conversion to mucoid colony types and the formation of biofilms are successful mechanisms by which the organism then enhances its survival and avoids host defences in the

established infection.

1.3 Circulating *P. aeruginosa* strain types in humans and diversity of the population structure

The genome of *P. aeruginosa* is large and complex and its size ranges from 5.2-7Mbp. It is known to consist of a main core genome and a variable accessory segment. The core genome demonstrates low variability of nucleotide divergence (0.5%) and multiple alleles at a few loci that are subject to diversifying selection (Wiehlmann et al., 2007; Smith et al., 2005). The accessory portion of the genome constitutes genomic islands and islets that are highly variable (Kung et al., 2010). Typing of particular traits allows identification of bacterial isolates to the strain level. MLST (multilocus sequence typing) is a typing scheme that uses sequence data of DNA fragments of seven housekeeping genes (Wiehlmann et al., 2007). MLST represents the genetic diversity of the 7 genes, which are all confined to the core genome. MLST databases are published for multiple species of bacteria including *P. aeruginosa.* The *P.aeruginosa* isolate that was first sequenced was PA01, a reference strain widely used in laboratories and research studies (Curran at al., 2004). This strain is 6.3Mbp and contains 5570 predicted open reading frames. The *P. aeruginosa* genome contains a large number of genes predicted to encode outer membrane proteins involved in adhesion, motility, antibiotic efflux, virulence factors, export and environmental sensing by two-component systems. There are also a large number of genes encoding transport systems and enzymes involved in nutrient uptake and metabolism (Stover et al., 2000). The large genome allows *P. aeruginosa* to be highly adaptable to many different environments and can utilize a variety of carbon sources.

A great number of other *P.aeruginosa* isolates have also subsequently been genome sequenced allowing important comparative genomic studies (Mathee et al., 2008; Roy et al., 2010; Valot et al., 2015; Winstanley et al., 2009). *P. aeruginosa* has a non-clonal structure but a few sequence types are widely distributed and frequently encountered (Woodford et al., 2011) . These are referred to as clones. Some are

thought to be significant in the spread of resistance (Oliver et al., 2015). Importantly the accessory genome is a source of genes encoding virulence factors and resistance to multiple classes of antibiotics (Ballarini et al., 2012; Kos et al., 2015; Kung et al., 2010; Talbot et al., 2006; Wiehlmann et al., 2007).

A panmictic population has been suggested for the population structure of *P. aeruginosa* from a number of studies (Denamur, 1993; Picard et al., 1994). Panmictic populations are characterised by extreme genetic diversity, whereby each clinical isolate can be genetically distinct from another. Wiehlmann et al (Wiehlmann et al., 2007) assessed 240 *P. aeruginosa* strains using DNA array tube assay and reported that the majority of strains belonged to a few dominant clones widespread in disease and environmental habitats. Overall the *P. aeruginosa* population structure is considered non-clonal, that clinical isolates are indistinguishable from environmental isolates, and that there are no specific clones with a specific habitat (disease) selection (Pirnay et al., 2009). However, the study reported the emergence, spread and persistence of multidrug resistant clones in hospitals, predominantly in those wards such as intensive care units, whereby a high antibiotic selection pressure may exist. Two serotypes, O11 and O12, are highly associated with these epidemic strains (Farmer et al, 1982). Typing of these strains supported a heterogeneous population in serotype O11 but those of serotype O12 often appeared to lack significant diversity. There is also the emergence of a number of 'transmissible' CF clones in *P. aeruginosa*, these have been reported across the world (Scott & Pitt, 2004; Syrmis et al., 2004) and may indicate the development of specific clones that have adapted to the CF airway environment and have the ability to spread within CF patient populations (Fothergill, et al., 2012). A study by Pirnay et al in 2009 (Pirnay et al., 2009) carried out work to provide a reference evolutionary framework and to position the emergent *P. aeruginosa* clones in the global population structure. The collection consisted of 328 unrelated isolates (collected over the last 125 years from 69 localities in 30 countries on 5 continents). They included isolates from diverse clinical (both human and animal) and environmental habitats. Pirnay et al (Pirnay et al., 2009) confirmed from their data the non-clonal epidemic population structure

of *P. aeruginosa*. Their findings also indicated that there are no widespread CF epidemic clones. They suggested that 'CF strains are part of a successful and ubiquitous core lineage that have infected CF patients from the natural environment and spread through short to medium range transmission between patients in CF clinics and holiday and rehabilitation camps'. They also reported of the worldwide spread and persistence of the MDR clone O12. Inferring that the excessive use of antibiotics has caused a worldwide preferential selection for multiple resistant or pan-resistant *P. aeruginosa* strains (Pirnay et al., 2009; Fothergill, et al., 2012).

It had generally been thought that individual CF patients acquired their infections separately and harboured their own individual strain that were non transferable between patients. However, Cheng et al (Cheng et al., 1996) reported the development of a drug resistant strain in a CF children's ward in Liverpool centre and spread among patients, termed the Liverpool Epidemic Strain (Al-Aloul et al., 2004; Ashish et al., 2012; Fothergill et al., 2012). Other epidemic strains have since been reported in Manchester (A. M. Jones et al., 2001) and Midlands (Scott & Pitt, 2004) CF centers and worldwide (Aaron et al., 2014; Leão et al, 2010; Parkins et al., 2014).

The LES has a number of characteristic features which include transmissibility and superinfection, that is it has the ability to superinfect patients previously chronically infected with a different strain of *P. aeruginosa* (McCallum et al., 2001). It has also been reported to cause respiratory infections of both non-CF parents of the same CF patient (McCallum et al, 2002). In addition to this a report in 2008 indicated that the LES has also been associated with transmission to a pet cat (Mohan et al., 2008). It has also been documented that LES isolates are significantly more antibiotic resistant than other *P. aeruginosa* CF isolates and that resistance in LES isolates is more likely to develop over time (Ashish et al., 2012; Salunkhe et al., 2005).

LES is known to be associated with increased morbidity and mortality (Al-Aloul et al., 2004), increased resistance to antibiotics (Ashish et al., 2012) ,the ability to

superinfect patients already harbouring their unique strains of *P. aeruginosa* (McCallum et al., 2001) and overproduction of important virulence factors (Fothergill et al, 2012; Fothergill et al., 2007)

Due to the high transmissibility of the LES, Midlands and Manchester strains a policy of microbiological surveillance for monitoring *P. aeruginosa* cross-infection of these particular strains is in place. Along with the introduction of new infection control measures in CF centres. Although much study and surveillance continues regarding these genotypes in human population little is known of their dispersion among the animal *P. aeruginosa* population.

Table 1.3 Summary of the antibacterial classes and the route of administration commonly used in management of CF P. aeruginosa lung infections. Table adapted from (Kanj & Kanafani, 2011).

Antibacterial Classes	**Antibacterials and Route of administration commonly used in management of CF**
Antipseudomonal penicillins	Ticarcillin administered iv.
Beta-*Lactam/*Beta-Lactamase Inhibitor Combinations	Ticarcillin-clavulanate and piperacillin- tazobactam administered iv. Inhaled antibiotics may be used in the treatment of *P. aeruginosa* pneumonia in particular CF patients, those used include colistin, tobramycin and beta lactams,
Cephalosporins	Cephalosporins considered good treatment of choice because of their good activity and narrow spectrum of activity as compared to carbapenems so Useful if isolate is susceptible ie Ceftazidime and cefepime administered iv.

Monobactams	Monobactams such as Aztreonam dosed iv. *P. aeruginosa* isolates that produce metallo-beta lactamases may be susceptible to aztreonam.
Carbapenems	Carbapenems such as meropenem, imipenem, doripenem administered iv. The various carbapenems have different levels of activity against *Pseudomonas* isolates. In vitro studies have, doripenem, like other carbapenems, has minimal activity against metallo-β-lactamase–producing *Pseudomonas* isolates compared with other carbapenems. Whether these in vitro differences among carbapenems translate into clinical out- come differences has not yet been determined. Carbapenems are usually used in the empirical treatment of suspected *Pseudomonas* species infections or when a polymicrobial infection is considered a possibility. In view of their broad spectrum of activity and the inherent risk of selecting for MDR organisms including *P aeruginosa* and *Acinetobacter* species, antibiotic therapy should be de-escalated when possible based on culture results.
Fluroquinolones	Fluroquinolones such as ciprofloxacin and levofloxacin dosed iv.
Polymyxins	Colistin (polymyxinE) The increasing rates of MDR *Pseudomonas* isolates have prompted clinicians to turn to agents such as the polymyxins that had previously fallen out of favour as a result of their adverse effects (namely nephrotoxicity). The polymyxins are often used as a salvage therapy when therapeutic choices are seriously limited. Inhaled antibiotics may be used in the treatment of *P. aeruginosa* pneumonia in particular CF patients, those used include colistin.
Aminoglycosides	Inhaled antibiotics may be used in the treatment of *P. aeruginosa* pneumonia in particular CF patients, those used include tobramycin.
Other Antibacterials (Combination therapy)	Other antibacterials are generally not recommended for monotherapy due to their high propensity to induce drug resistance. They may be considered used usually in combination therapy with other anti pseudomonal agents such as aminoglycosides, amikacin, gentamycin, tobramycin or rifampicin.

(Kanj & Kanafani, 2011)

1.4 Limited antimicrobial treatment options in treatment of *P. aeruginosa* infections in humans

P. aeruginosa is one of the so-named ESKAPE pathogens by the IDSA (Infectious Diseases Society of America) and is categorized by the CDC as a serious level of threat. These are significant antibiotic resistant threats for varying reasons including a reduced availability of therapeutic options. *P. aeruginosa* is now detailed on the WHO urgent list for priority pathogens for reasearch and development of new antibiotics (WHO Feb 2017). ESKAPE pathogens are highlighted as being of ever-growing relevance to antimicrobial chemotherapy in future years (Boucher et al., 2009; Santajit & Indrawattana, 2016; Zilahi et al, 2016). *P. aeruginosa* presents a serious therapeutic challenge for treatment of both community-acquired and nosocomial infections, and selection of the appropriate antibiotic to initiate therapy is essential to improve the clinical outcome. Unfortunately, selection of the most appropriate antibiotic is complicated by the ability of *P. aeruginosa* to develop resistance to multiple classes of antibacterial agents, even during the course of treating an infection. Epidemiological outcome studies have shown that infections caused by drug-resistant *P. aeruginosa* are associated with significant increases in morbidity, mortality, need for surgical intervention, length of hospital stay and chronic care, and overall cost of treating the infection. Even more problematic is the development of resistance during the course of therapy, a complication which has been shown to double the length of hospitalization and overall cost of patient care. *P. aeruginosa* can develop resistance to antibacterials either through the acquisition of resistance genes on mobile genetic elements (i.e., plasmids) or through mutational processes that alter the expression and/or function of chromosomally encoded mechanisms. Both strategies for developing drug resistance can severely limit the therapeutic options for treatment of serious infections (Cosgrove, 2006).

Antimicrobial resistance

Treatment of infectious diseases becomes more challenging particularly with the ability of bacteria to rapidly mutate and adapt. This is apparent in *P. aeruginosa,* in particular with its ability to develop a MDR (defined as non susceptibility to at least one agent in three or more antimicrobial categories) (Magiorakos et al., 2012) phenotype and also the potential passage of resistance mechanisms on mobile genetic elements. Most notably with *P. aeruginosa* its ability to rapidly develop resistance to multiple classes of antibiotics during the course of treating a patient further complicates the challenge (Lister et al., 2009).

P. aeruginosa is a bacterium that possess a great and varied armory in its defences. Among others; mediator activation via endotoxin release, exotoxins and enzymatic products, designed to evade host defences, and chromosomal and plasmid-mediated antibiotic resistance factors (El Solh & Alhajhusain, 2009). A multidrug-resistant phenotype can arise in *P. aeruginosa* through the acquisition of multiple imported resistance mechanisms on mobile genetic elements, a combination of imported and chromosomally encoded resistance mechanisms, accumulation of multiple chromosomal changes over time, and/or a single mutational event leading to the overexpression of a multidrug resistance mechanism (such as an efflux pump) (Lister et al 2009). Examples of specific gene mutations include ciprofloxacin resistance being associated with a mutation in *gyrA* (Cambau et al., 1995; Lomholt & Kilian, 2003) and mutations to topoisomerases II and IV that confer fluoroquinolone resistance. The up-regulation of MexAB-OprM is known to compromise the fluoroquinolones, penicillins, cephalosporins, and, to some degree, meropenem (not imipenem). It also enhances resistance to many other drugs that lack useful anti-pseudomonal activity. Up-regulation of other efflux systems, such as MexCD-OprJ and MexEF-OprN, confers resistance to fluoroquinolones and some β-lactams. The up-regulation of MexXY-OprM also affects aminoglycosides (Hocquet et al., 2006; Morita et al, 2013). There are mutations in genes encoding key regulators for example, *mexT* and *ampR*. Together, the traits affected by these mutations have been termed ‘pathoadaptive’ traits (Winstanley et al, 2016).

As observed in chronic CF patients *P. aeruginosa* exhibits phenotypic diversity, often characterized by the appearance of different colony morphology types. These may include mucoid (hyperalginate producers), and small colony variants (Sousa & Pereira, 2014). The small colony variants are typically slow growing and may exhibit higher levels of resistance. They may adhere well to surfaces and be involved in biofilm formation, which further contributes to treatment challenges. The phenotypic variation of *P. aeruginosa* isolates of the same genotype can also be seen in variation of antibiotic susceptibility profiles amongst isolates within a single patient sample.

Hypermutators are known to occur within *P. aeruginosa* populatons. These are bacteria with mutations in their DNA repair or mutation avoidance genes. Hypermutators are found at a relatively higher prevalence in CF patients chronically infected with *P. aeruginosa* (Ferroni et al., 2009; Lutz et al., 2013; Maciá et al, 2005). They are also found in other chronic lung disease such as severe COPD and non-CF bronchiectasis (Parameswaran & Sethi, 2012; Sahuquillo-Arce et al, 2016).

Adaptation during chronic infections

P. aeruginosa evolves from a state of early, recurrent intermittent colonization of the airways of patients with CF to a chronic infection state (Folkesson et al., 2012). The genetic adaptation of *P. aeruginosa* in the chronic CF lung involves specific gene mutations that can produce beneficial phenotypic changes to enable its persistence in the lung. The mutations most commonly observed are those inactivating the regulators *mucA* (alginate biosynthesis), *lasR* (quorum sensing) and *mexZ* (regulator multidrug-efflux pump MexXY). Along with those inactivating the DNA mismatch repair system (MRS) leading to hyper-mutator genotypes (Feliziani et al., 2010). CF infections involving *P. aeruginosa* are dynamic and see a gradual development from an acute early infection to a host-adapted pathogen of chronic infection (Sousa et al., 2014). However, virulence has shown to be reduced in these isolates that have become adapted to their niche environment. Lore, N.I. et al (Lorè et al., 2012) showed that niche-specific selection in *P. aeruginosa* reduced its ability to

cause acute infections across a range of hosts while maintaining the capacity for chronic infection in the CF host (Lorè et al., 2012).

Populations of *P. aeruginosa* in chronic CF lung infections typically exhibit high phenotypic diversity. As this population diversity is dynamic over time, it makes accurate diagnosis and treatment challenging. This phenotypic diversity includes clinically important traits such as antibiotic resistance and toxin production. In such highly diverse populations it is possible for false diagnoses (for example based upon antimicrobial susceptibility testing using single/pairs of isolates). Given the already limited efficacy of current antibiotics in these chronically infected CF patients, research in this area is ongoing to help improve understanding of the evolution of bacterial populations during chronic infections especially with the use of affordable high throughput genome sequencing (Winstanley et al., 2016).

Chronic *P.aeruginosa* lung infection is an endobronchiolitis characterized by the micro-colony mode of growth (biofilm). The most commonly used anti-pseudomonal agents (Table 1.3) used are the extended-spectrum penicillins, beta-lactams, aminoglycosides, cephalosporins, fluoroquinolones and polymxins (Høiby, 2011). However, the treatment of infections caused by multi-drug resistant organisms is problematic as antibacterial options and the development of new drugs for clinical use are limited. The drug development process is a long process with numerous restrictions along the pipeline of antibiotic development. In addition, no new novel agents with efficacy against the Gram negative pathogens in particular, have been developed in the last 30 years (Briers & Lavigne., 2015; Coates et al., 2011).

1.5 *P.aeruginosa* infections in animals

In the veterinary setting *P.aeruginosa* can be a cause of disease in a variety of different species. It may be involved in ear, skin, respiratory, urinary, reproductive, ocular and wound infections. Similar to its nature in humans *P. aeruginosa* is also

less common as a primary cause of disease in the healthy animal subject. In the dog, *P.aeruginosa* is a frequently isolated pathogen in chronic otitis externa and otitis media (Morris, 2004; Nuttall & Cole, 2007; Rubin et al., 2008; Steen & Paterson, 2012) and may be found in chronic deep pyodermas (Hillier et al, 2006; Rubin et al., 2008). In the canine patient *P.aeruginosa* related otitis can be notoriously difficult to treat and is a painful condition. In these patients it is often that the dog will have another underlying skin issue such as that of allergic skin disease, termed atopy (Hillier et al., 2006) whereby the effective skin-barrier function has become compromised. The recommended management of canine otitis externa consists of identifying and treating the predisposing factors and primary disease. Ear cleaning, flushing, appropriate topical therapy and if indicated, systemic antimicrobial medications (Morris, 2004; Nuttall & Cole., 2007).

A number of other infections involving *P.aeruginosa* in animals are known and reported in the literature, including that of contagious equine metritis (Atherton & Pitt., 1982; Ensink et al., 1993) and in chronic equine wounds were the ability of *P.aeruginosa* to form and survive within protective biofilms has been studied (Percival et al, 2015; Westgate et al, 2011). Westgate et al (Westgate et al, 2011) reported the first identification of biofilms in chronic wounds of horses. They looked at 13 tissues samples from 18 chronic wounds and identified the most common bacteria isolated from each wound, one of the most common from skin and wound samples was *P.aeruginosa* (Westgate et al 2008).

There are reports of *P. aeruginosa* infections affecting chinchillas and the organism has been isolated in high numbers in laboratory animals of this species (Wideman, 2006). In mink, *P.aeruginosa* is also a known cause of haemorrhagic pneumonia. In particular, it is an acute and fatal disease in farmed mink (Salomonsen et al., 2013; Shimizu et al., 1974; Wilson et al., 2015). A 2009 study (Pedersen et al., 2009) reporting the useage of antimicrobials and occurrence of AMR among bacteria from mink found that all the 39 *P.aeruginosa* samples in their study were sensitive gentamicin and colistin (Pedersen et al., 2009).

In the veterinary environment *P.aeruginosa* in also encountered in various eye conditions including the non-healing (so called 'melting') corneal ulcers and bacterial ulcerative keratitis in horses and occasionally dogs (Delgado & Subtil, 2015; Hindley et al, 2016). A study by Hindley et al, 2016, looked at bacterial isolates, from dogs with keratitis attending two Australian referral practices (2012-2014). Positive cultures were obtained from 42 of the 71 ulcers sampled (59%), with 45 bacteria isolated. The most commonly isolated bacteria were β-hemolytic *Streptococcus* (14/45, 31%), Pseudomonas (14/45, 31%), and *Staphylococcus* species (8/45, 18%). *P. aeruginosa* isolates were resistant to chloramphenicol, cephalexin, and fusidic acid; however, >90% were susceptible to ciprofloxacin, polymyxin B, and gentamicin. In comparison, a study of 97 dogs with bacterial keratitis identified from teaching hospital medical records at the Universities of Tennessee and Florida (1993 to 2003) were reviewed. The most commonly isolated bacteria were *Staphylococcus intermedius* (29%), beta-hemolytic *Streptococcus spp* (17%), and *P. aeruginosa* (21%). Here isolates of *P. aeruginosa* were susceptible to tobramycin and gentamicin and had limited resistance to ciprofloxacin and enrofloxacin. Among bacterial species isolated, there was no evidence of development of increased AMR over time.

In horses, *P. aeruginosa* is one of the most commonly identified causative organisms in ulcerative keratitis. Keller et al (Keller & Hendrix, 2005) documented bacterial isolates and their antimicrobial susceptibilities from horses with ulcerative keratitis treated at the University of Tennessee (1993 -2004). Of the 43 horses, 51 bacterial isolates were isolated. *S. equi* ssp. zooepidemicus and *P. aeruginosa* were the most frequently isolated bacterial organisms in equine ulcerative keratitis. No significant trends in aminoglycoside or fluoroquinolone resistance were noted among these organisms. A retrospective study by Sauer et al (Sauer et al, 2003) looked at all cases of equine bacterial ulcerative keratitis seen at the University of Florida's VMTH (1991-2000). The 65 identified bacterial isolates were subjected to sensitivity testing via disc diffusion and of these isolates, *P. aeruginosa* accounted for 14 of the bacterial isolates (22%). A statistically significant increase in resistance of *P. aeruginosa* isolates to the antibiotics gentamicin and tobramycin was found

between the isolates from 1992 to 1998 and those from 1999 to 2000 (Sauer et al., 2003).

P. aeruginosa is reported as a pathogen in mastitis in goats. A recent study looking at this condition in goats (Scaccabarozzi et al., 2015) showed caprine *P. aeruginosa* isolates to be phenotypically diverse, able to cause both transient and chronic infections and with both clinical and subclinical manifestations. *P. aeruginosa* also initiated a more pronounced inflammatory response compared to other pathogens. The study showed that the *P. aeruginosa* was able to persist during the dry period through the subsequent lactation, demonstrating the difficult clearance of this pathogen from the mammary gland (Scaccabarozzi et al., 2015). The multidrug resistance patterns demonstrated by all of the isolates tested, contributes to the difficulty to effectively cure the intra mammary infections. In the study the highest levels of resistance were found to be against the most commonly used beta-lactam antibiotics. There was also the suggestion of unique patterns of virulence-related phenotypes with certain isolates possibly being better adapted to the udder environment. For example an association between hemolysin production and swimming motility may infer that some virulence factors would be capable of making an isolate more adapted to the goat udder (Scaccabarozzi et al., 2015). *P. aeruginosa* infections have also been reported in sheep causing mastitis (Wright et al., 2015). This study reported two clonal *P. aeruginosa* isolates (PSE305 and PSE306) from a mastitis infection outbreak, representing distinct colony morphology variants with differences in phenotypic characteristics between the two clones.

Examples of infections in animals involving *P.aeruginosa* are summarized in Table 1.4.

Table 1.4. Examples of infections in animals involving *P. aeruginosa*

Species	**Examples of infections involving *P. aeruginosa* in animals**
Horses	- Contagious equine metritis - Non healing lower limb wounds - Bacterial ulcerative keratitis - Non healing corneal ulcers
Goats	- Mastitis
Sheep	- Mastitis
Mink	- Haemorrhagic pneumonia
Dogs	- Otitis - Deep pyodermas - Bacterial keratitis
Chinchillas	-Pneumonia - Septicaemia - Enteritis - Otitis media and interna

1.6 Antimicrobial treatments used for *P.aeruginosa* infections in animals

Sulfonamides were the first antimicrobial to be introduced into food animal medicine in the 1940s. The subsequent discoveries and availabilities of newer antibiotics in the 1950's has progressed to the widespread therapeutic usage of antibacterials for management of infectious diseases in the majority of food animal species. Antibiotics have also been given to food production animals for growth promotion (in animal feeds) and prophylactic medication (Landers et al., 2012).

Non-therapeutic use of antibacterials in animals may include use as growth promoters (added in low doses to the feed of farm animals, they improve their growth performance) or prophylactic/metaphylactic purposes. For instance, an animal may be treated with antibiotics post surgery or trauma. In companion animal veterinary medicine, antibiotics are commonly used to control secondary bacterial infection (for example during surgical procedures) and managing infection-promoting disease conditions (such as in urolithiasis). Also, in herd/flock management, whereby they may be given antibiotics if they are at risk of suffering an outbreak of infectious disease due to exposure to disease agents or extremely unfavorable host or environmental conditions. In poultry and livestock, mass administration of antibiotics is often practiced when transporting or moving young animals, during dry-cow therapy in dairy cows, and in preventing respiratory and intestinal disease when animals have been subjected to severely stressful conditions. With the emergence of antibiotic resistance, the use of growth promoters has been banned on all/or particular classes of antibiotics, with different countries having different lists of approved and banned growth promoter antibiotics. In 2005, the EU banned antibiotics in animal feed as growth promoters (Maron et al, 2013). A number of veterinary bodies currently implement guidelines on antibacterial usage and prescribing promoting judicious use and antimicrobial stewardship. For example, those guidelines by the British Small Animal Veterinary Society (BSAVA 2017a; BSAVA 2017b) and the British Veterinary Association (BVA 2017).

In most countries, there are no reliable consumption figures on antimicrobial agents administered to pet animals. Most national monitoring programs focus on food animals and do not include data on antimicrobial resistance in companion animals. Some long-term studies in Europe have described trends in antimicrobial resistance. Several countries have national agencies for the purpose of monitoring antimicrobial usage and rates of AMR in food animals, food and of people. For example; in the USA National Antimicrobial Resistance Monitoring System (NARMS). This is a collaborative project of state and local public health departments (FDA,

CDC and USDA). The Canadian Integrated Program for Antimicrobial Resistance (CIPARS) in Canada; Observatoire National de Epidémiologie de la Résistance Bactérienne aux Antibiotiques (ONERBA) in France and The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) in Denmark. DANMAP monitors sales for humans and animals annually, along with rates of AMR in bacteria from people, food animals, and food products. The component that monitors antibiotic usage in veterinary practice is VetStat, which collects data from pharmacies, veterinary surgeons and feed mills (Bywater., 2004; Chantziaras et al., 2014; Cogliani et al., 2011; Garcia-Migura et al., 2014; DANMAP, 2014; Bywater, 2004).

Polymyxin useage in veterinary species

It is difficult to identify usage of polymyxins within both companion and food producing veterinary species as the small animal usage is not well documented. In a report of antibiotics most commonly prescribed in animals in Europe (De Briyne et al., 2013) a survey of 3004 practitioners from 25 European countries was completed. It showed polymyxins were cited relatively infrequently by practitioners for use in cats and dogs, however this was higher in cattle pigs and horses. However, polymyxins were reported most in the treatment of diarrhoea in cattle and calves (40% frequency of citation of this class of antibiotic for this indication in this species). Polymyxins were also cited frequently as prescribed for diarrhoea (including colibacillosis and dysentery) in pigs at 30%. Colistin is often used in control of colibacillosis in poultry and pig production administered in the oral form. Resistant isolates are sometimes received from pathogenic cases especially in piglets. Colistin resistance was reported in *E.coli* and *Salmonella enterica* of poultry and swine (De Briyne et al., 2013). However, in Europe the percentage resistance to colistin in *E.coli* strains isolated from digestive tract microbiota of healthy animals remains very low at <1%.

A surveillance study of carbapenem-resistant *Enterobacteriacea* (CRE) isolates from a University hospital in Shanghai, China, reported the independent emergence of colistin resistance in KPC (*Klebsiella pneumonia* carbapenemase) producing CRE isolates without treatment with colistin (S. Chen et al., 2011). Wang et al reported the IMP-45 (Imipenemase metallo-β-lactamase) producing MDR *P.aeruginosa* that was of canine origin (Y. Wang et al., 2014). This is an important MBL (metallo beta lactamase) gene, which can be disseminated horizontally among Gram-negative bacteria including major pathogens of humans and animals. IMP type carbapenemases represent one of the most clinically relevant MBL groups in terms of epidemiological dissemination.

The main indications for polymyxin useage in the veterinary sector are infections caused by Enterobacteriaceae in rabbits, pigs, poultry, cattle, sheep and goats (EMA 2017a; EMA 2017b). In addition, colistin is utilised in laying hens and cattle, sheep and goats producing milk for human consumption. Most often, colistin products are administered orally, as a drench, in feed, in drinking water or through milk replacer diets. Combinations of colistin with other antimicrobials are available for group treatments of food-producing animals in some European countries. Products for parenteral and intra-mammary administration are also available, and Gram-negative infections in ruminants including endotoxinaemia are claimed indications. Polymyxin B is used for systemic treatment of endotoxaemia associated with severe colic and other gastrointestinal diseases. Several proprietary forms of colistin and polymyxin B are available for topical use in companion animals; prescription eye and eardrops are available as colistin alone, or in combination with other antimicrobials. Colistin tablets are available for calves for the prevention and treatment of neonatal colibacillosis. In some EU Member States, veterinary medicinal products containing colistin are not on the market and not used at all (ESVAC, 2012). The 2010 and 2011 FDA (FDA, 2017) reports on sales of veterinary antimicrobials show no sales of polymyxins, but there are reports of off label use of polymyxin B in horses in cases of endotoxemia (Werners et al, 2005).

1.7 AMR in *P. aeruginosa*

Antimicrobial resistance is a multifactorial and complex issue, affecting healthcare worldwide and is an escalating concern as a developing public health crisis. European and International bodies have highlighted AMR as a global public health issue of imperative importance. The World Health Organisation (WHO), the European Centre Disease Prevention and Control (ECDPC) and Infectious Diseases Society of America (IDSA) have included within their concerns and priorities, emerging antimicrobial resistance mechanisms, surveillance and antibiotic stewardship, considering within this, roles of both the veterinary and medical communities. AMR is not only a concern in human health but also animal health. The close interface between people, pets, food production animals and the environment exemplifies the importance that these two areas of health should not be considered as entirely independent with regards to AMR. Adopting a 'one-health' approach in combatting this global problem is of imperative importance in understanding and potentially controlling the advances of resistance among the microbial population. *P. aeruginosa* is one of the so-named ESKAPE pathogens by the IDSA and is categorized by the CDC (Centre for Disease Control and Prevention) as a serious level of threat. These are significant antibiotic resistant threats for varying reasons including a reduced availability of therapeutic options (Santajit & Indrawattana, 2016).

Relevant β-lactams and aminoglycosides remain active against 70%–98% of *P. aeruginosa* isolates in the United States and the United Kingdom (Lister et al., 2009; Livermore, 2002). Nevertheless, resistance is more frequent in specific areas, most notably, in hospital units for the management of patients with burns or CF, or in ICUs. The high levels of aminoglycoside resistance in isolates from patients with CF is apparent in both the United Kingdom and the United States (Poole, 2005a), and also imipenem resistance among isolates from patients in ICUs. More generally, there are higher levels of resistance among isolates from hospitalized patients, compared with those from outpatients (da Silva Filho et al, 2001; Mesaros et al., 2007; Pitt, 2002; Vicente et al., 2013; Winstanley et al 2016). Snapshot surveys

performed in 1982, 1993, and 1999 show little increase in resistance among *P. aeruginosa* strains in the United Kingdom (Livermore, 2002); however, resistance to ciprofloxacin in particular but, also to piperacillin and gentamicin, has recently risen among such strains in the United States.

1.8 Potential for cross-species transmission between humans and animals

The occurrence and risks of *P. aeruginosa* transmission between animals and humans is an area of limited study. There are a small number of reports in the literature of cross transmission between humans and animals. In 2008 Mohan *et al* (Mohan et al., 2008) documented a case report of a cat being infected by a transmissible strain of *P.aeruginosa* from a CF owner. The pet cat presented with a 6wk history of serous nasal discharge and sneezing and required an extensive antibiotic treatment course of 6wks marbofloxacin and was found to be harbouring the LES of *P.aeruginosa*. The owner of the cat was an adult CF patient infected with the LES, a known highly transmissible strain of *P. aeruginosa* and transmission was deemed to be from human to animal in this instance.

Conversely, Maeda & Tazumi *et al* (Tazumi et al., 2009) looked at the potential for transmission of bacteria between reptiles and a CF patient but concluded from their case study there was no evidence of the CF patient's reptile collection acting as a source of any of his respiratory flora or vice versa. However, it did highlight increased risk of transmission of pathogens to the reptiles in the direct care of CF patients.

Animals being a potential source of *P.aeruginosa* infection to humans is highlighted again in the work by Hirakawa et al (Hirakawa et al., 2010). This study reported that *P. aeruginosa* was isolated at high rates from pet and laboratory chinchillas, with typing analysis showing the isolates to be diversified however low antimicrobial susceptibility was identified in several isolates. The isolates in the study were found to contain the gene *pilL* that was previously identified only in highly virulent strains of *P. aeruginosa* (Battle et al, 2008). Thus, it is necessary to take into account the

risk of infection from pets or laboratory chinchillas to humans. This risk can be even more relevant in situations whereby pets are within the household of medical patients. A report (Broughton et al, 2010) looking at zoonotic causes of infectious peritonitis as complications in peritoneal dialysis patients, raises the importance of considering companion animals (or the patients working with animals) as sources of these infections. *P. aeruginosa* being one of the common causative agents in infectious peritonitis of peritoneal dialysis patients.

Other species have also displayed human-bacterial transmission. A report in 2012 on interspecies transmission between a CF patient and their pet cat revealed *Bordetella bronchiseptica* (BB) being transferred from a kitten to a CF patient (Register et al, 2012). BB was isolated from routine sputum samples of an 11yr old stable CF patient. There had been the recent acquisition of a kitten displaying acute respiratory disease. Genetic characterization of the isolate and comparison with other isolates of human and feline origin strongly suggested the kitten was the source of infection. Genetic traits of the isolate were consistent with it being of feline origin (Register et al., 2012). There has also been a report of a transplant patient developing BB pneumonia from pet dogs (two cases out of 60 patients with BB pneumonia contracted from pet dogs) in CF patient lung transplant recipients (Ner et al., 2003). For these patients, the infection was fatal. Due to a lack of research in this area, it is not known whether such occurences are a rare phenomenom or an emerging risk as a route of infection. Gisel et al (Gisel et al, 2010) also documented a case of a kidney-pancreas transplant patient who suffered postoperative complications requiring further surgery for adhesions and two weeks postoperatively the patient developed pneumonia (failing to respond to repeated courses of antibiotics). BB was isolated following diagnostics and it was discovered that the patient's dogs had recently received a live-attenuated BB intranasal vaccination. The patient recovered after 21 days of therapy with doxycycline based upon susceptibility testing. However, in this study as polymerase chain reaction testing was not performed, comparing the isolated strain to the vaccination strain, the association here is presumptive and further confirmation would need to be achieved.

There are reports of interspecies transmission, whereby studies suggest that pets such as cats and dogs may be potential sources for methicillin-resistant *Staphylococcus aureus* (MRSA) infection (Ferreira et al., 2011; Morris et l.,, 2010; Morrow et al., 2014; Wang et al., 2008). A report of a case of MRSA infection in a family that involved the pet cat reported the transmission of PVL-positive (Panton Valentine leukocidin) MRSA increased virulence bacteria between a symptomatic woman and both her asymptomatic family and their healthy pet cat (Wang et al., 2008). However, it is unclear if the cat was source of patient's infection or vice versa. A further study reported PVL positive MRSA strains with identical resistance patterns cultured from recurrent infections in a 51yr old patient, her healthy husband, son and pet dog. Pulsed field gel electrophoresis showed all strains to be indistinguishable (Weese et al., 2006). Furthermore a study in 2009 (Murphy et al., 2009) documented the high prevalence of concurrent MRSA colonization and identification of indistinguishable strains in humans and pet dogs and cats in the same household. Investigations of six situations where MRSA was identified in one or more animals in a household or veterinary facility were performed. MRSA was isolated from 8 animals (5 dogs and 3 cats) with clinical infections, 1 cat that was in contact with 2 infected cats and 14/88 (16%) of household contacts or veterinary personnel (Murphy et al., 2009). An indistinguishable MRSA isolate was recovered from at least one human that was in contact with each animal case. All isolates were classified as Canadian epidemic MRSA-2, the predominant community-associated MRSA clone in humans in Canada. They concluded that transmission of MRSA between humans and animals, in both directions, was suspected (Murphy et al., 2009; Weese et al., 2006). A recent study looking at the carriage of methicillin-resistant staphylococci between humans and animals on a small farm by Loncaric, et al (Loncaric et al., 2016) also reported of carriage of both MRSA and MRCoNS (methicillin-resistant coagulase negative *staphylococci*) among humans and various animals within a shared environment. The detection of strains with indistinguishable molecular characteristics strongly suggested transmission of these MRSA between the various animal species and humans. These studies highlight that interspecies transmission of MRSA is possible. It is therefore likely that pets may be

considered as possible household reservoirs that can cause infection and reinfection of humans.

The Figure 1.2 (starts p38) details consequences of antimicrobial use in small animal veterinary practice and human medicine on exchange of resistant bacteria and transfer of resistance genes between pet animals and humans with impact on human health and general factors contributing to antimicrobial use and resistance. Figure is adapted from (Guardabassi et al.,2002)

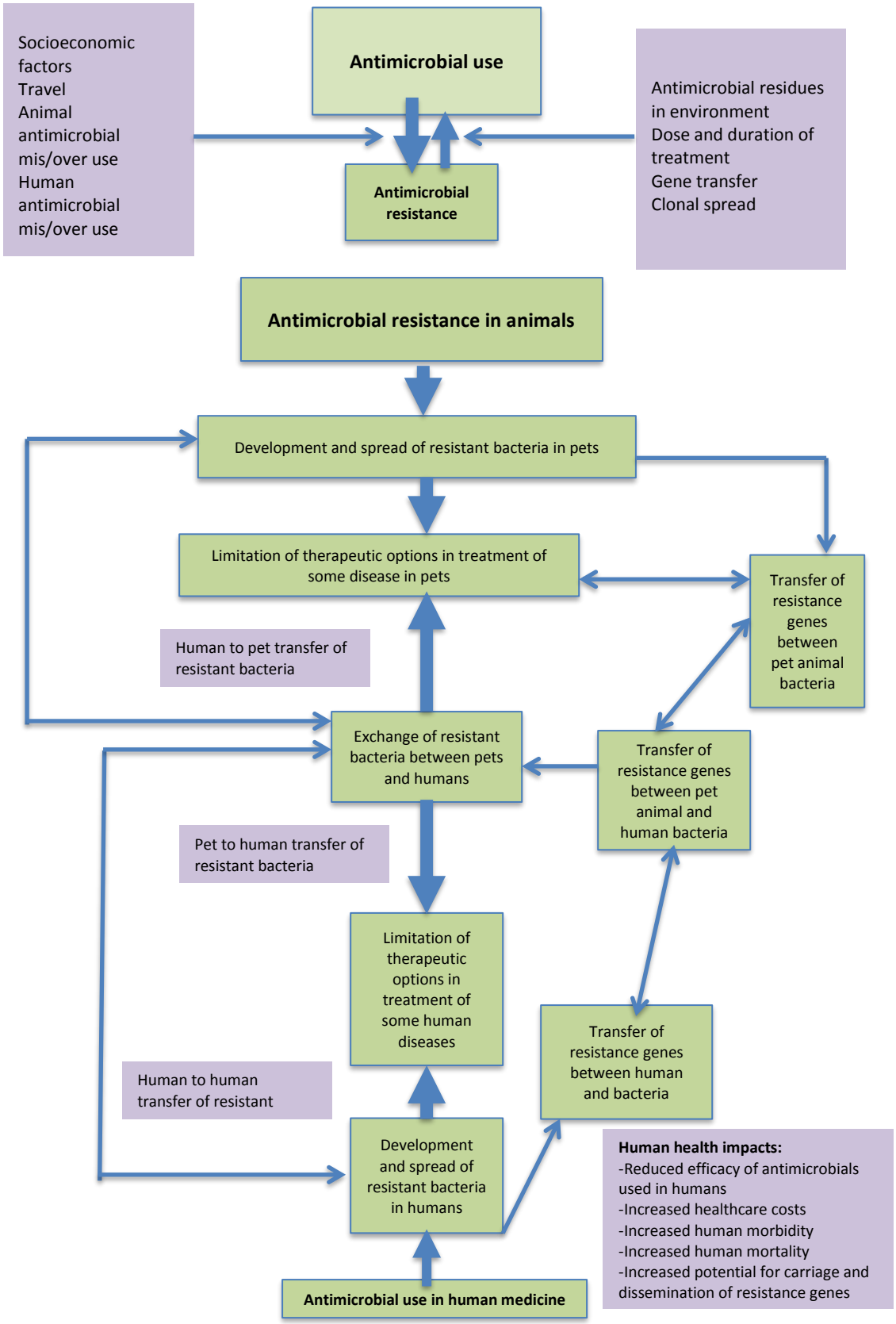


Figure 1.2 Flow chart to show consequences of antimicrobial use in small animal veterinary practice and human medicine on exchange of resistant bacteria and transfer of resistance genes between pet animals and humans with impact on human health and general factors contributing to antimicrobial use and resistance.

Figure 1.3 details some examples of the reservoirs of antibacterial resistance involving both humans and animals.

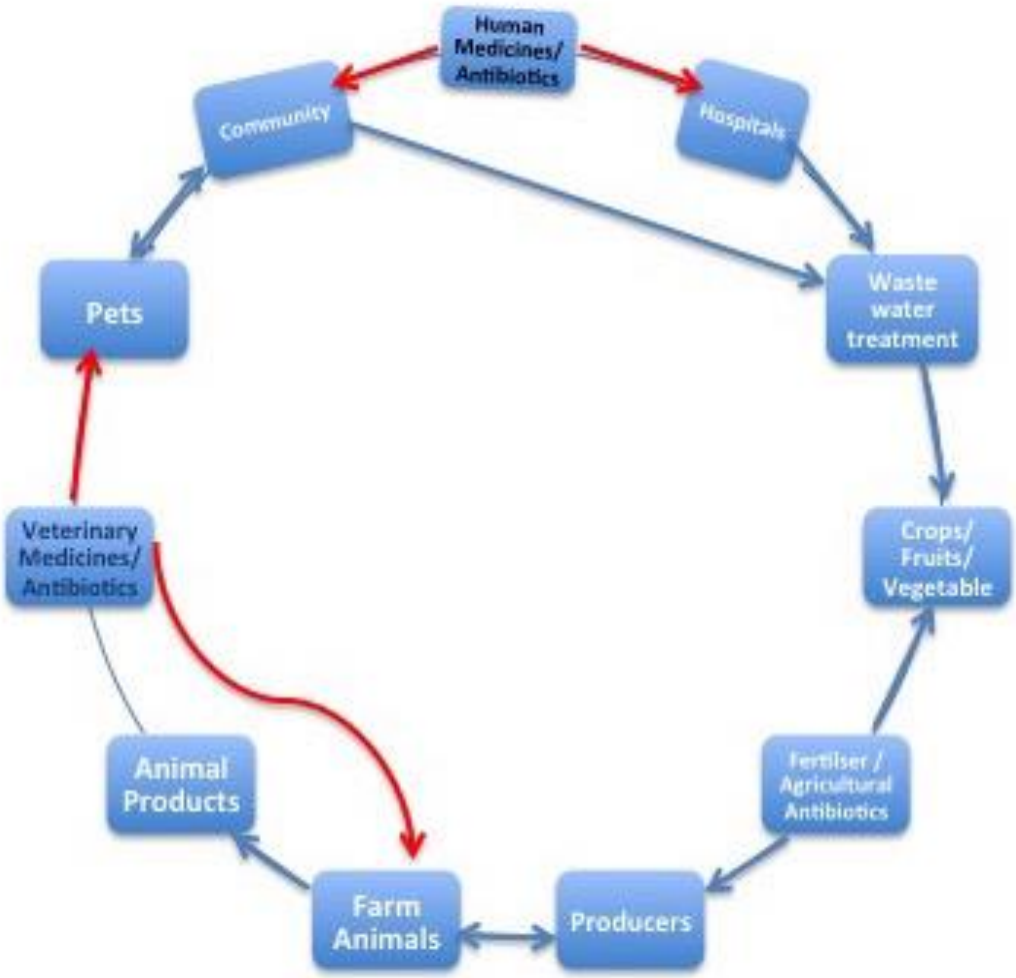


Figure 1.3 Diagram to demonstrate the potential reservoirs of antibacterial resistance (blue arrows) involving humans and animals and the selection pressures (red arrows).

Materials and Methods for Results Chapter 3 - Reservoirs of resistance: polymyxin resistance in veterinary-associated isolates of *Pseudomonas aeruginosa*:

2.1 Bacterial Isolates

24 *P. aeruginosa* isolates from 22 companion animal attending Leahurst Veterinary Teaching Hospital (UK) in 2012 were collected for this study (Table 2.1). No prior clinical details or antibiotic use for the cases were available. Human-associated *P. aeruginosa* isolates (Table 2.1). from a previously published panel were used as a comparison (De Soyza et al., 2013). Those isolates from this published panel that were of non-human origin (eg. environmental origin) were excluded. All isolates were stored in LB supplemented with glycerol and stored at -80 ^{o}C. When needed, bacteria were grown on Columbia agar for 24hrs at 37^{o}C.

Table 2.1 Veterinary and human bacterial isolates used in this study

Strain or Isolate	Description (Origin/Source)	Animal/Human Isolate	Reference
1115	*P.aeruginosa (*Canine testicle/ scrotum swab)	Animal	This study
1114	*P.aeruginosa* (Canine neck wound swab)	Animal	This study
1107	*P.aeruginosa* (Canine Bile sample)	Animal	This study
1098	*P.aeruginosa* (Canine Vaginal swab)	Animal	This study
1095	*P.aeruginosa* (Canine Buccal swab)	Animal	This study
1090	*P.aeruginosa* (Canine Skin lesion swab)	Animal	This study
1070	*P.aeruginosa* (Canine neck swab)	Animal	This study
1055	*P.aeruginosa* (Canine Lip fold swab)	Animal	This study
994	*P.aeruginosa* (Canine Right elbow swab)	Animal	This study
984	*P.aeruginosa* (Canine Right ear swab)	Animal	This study
982	*P.aeruginosa* (Canine Urine sample)	Animal	This study

978	*P.aeruginosa* (Canine Non-Healing Wound swab)	Animal	This study
970	*P.aeruginosa* (Canine Wound swab)	Animal	This study
969	*P.aeruginosa* (Canine Oropharyngeal tube swab)	Animal	This study
897	*P.aeruginosa* (Canine ear swab)	Animal	This study
886-1 &-2	*P.aeruginosa* (Canine nail pus swab)	Animal	This study
992	*P.aeruginosa* (Feline Oropharyngeal tube stoma swab)	Animal	This study
856	*P.aeruginosa* (Equine Abdominal incision swab)	Animal	This study
903	*P.aeruginosa* (Equine Urine sample)	Animal	This study
823	*P.aeruginosa* (Canine Left ear swab)	Animal	This study
811	*P.aeruginosa* (Equine nasal swab)	Animal	This study
467-L	*P.aeruginosa* (Canine Left Ear swab)	Animal	This study
467-R	*P.aeruginosa* (Canine Right Ear swab)	Animal	This study
AES-1R (AUST01)	Paediatric CF (1992), Melbourne Australia	Human	De Soyza et al., 2013
AUS23 (AUST02)	Adult CF (2007), Brisbane Australia	Human	De Soyza et al., 2013
AUS52 (AUST03)	Adult CF (2008), Hobart Australia	Human	De Soyza et al., 2013
2192	Chronic CF patient, Boston MA	Human	De Soyza et al., 2013
AMT 0023-30 (AMT2330)	Paediatric CF, Seattle WA	Human	De Soyza et al., 2013
AMT 0060-3 (AMT60-3)	Paediatric CF, Seattle WA	Human	De Soyza et al., 2013
AMT 0060-2 (AMT60-2)	Paediatric CF, Seattle WA	Human	De Soyza et al., 2013
AMT 0023-34 (AMT2334)	Paediatric CF, Seattle WA	Human	De Soyza et al., 2013
U018a	CF patient, Hobart Australia	Human	De Soyza et al., 2013
57P31PA	Chronic obstructive pulmonary disease, USA	Human	De Soyza et al., 2013
IST27N	CF patient, spontaneous non mucoid variant, Lisbon Portugal	Human	De Soyza et al., 2013
IST27mucoid (IST27muc)	CF patient, Lisbon Portugal	Human	De Soyza et al., 2013
TB CF10839 (TB)	CF, Germany	Human	De Soyza et al., 2013
UCBPP-PA14 (PA14)	Human burn isolate	Human	De Soyza et al., 2013
NH57388A	Denmark, CF	Human	De Soyza et al., 2013
A5803	Community acquired pneumonia	Human	De Soyza et al., 2013
39016	ICU (acute infection), Spain	Human	De Soyza et al., 2013
AMT 0060-1 (AMT60-1)	Paediatric CF, Seattle WA	Human	De Soyza et al., 2013
15108/1 (5801)	ICU (acute infection), Spain	Human	De Soyza et al., 2013
13121/1 (13121)	ICU (acute infection), France	Human	De Soyza et al., 2013
LES400	CF, UK	Human	De Soyza et al., 2013

39177	Keratitis, Manchester UK	Human	De Soyza et al., 2013
CHA	CF	Human	De Soyza et al., 2013
PAK	Clinical non CF	Human	De Soyza et al., 2013
1709.12	Leuven Belgium, Non clinical CF (2004)	Human	De Soyza et al., 2013
LES 431	Non CF parent of CF patient, UK	Human	De Soyza et al., 2013
AA43	CF (late), Germany	Human	De Soyza et al., 2013
DK2	CF Denmark (earliest isolates 1973-2007)	Human	De Soyza et al., 2013
C3719	CF, Manchester UK	Human	De Soyza et al., 2013
RP1	CF, Germany	Human	De Soyza et al., 2013
Mi162	Non CF burn, Ann Arbour, MI , 1997	Human	De Soyza et al., 2013
AA2	CF (early), Germany	Human	De Soyza et al., 2013
KK1	CF, Germany	Human	De Soyza et al., 2013
679	Non CF urine sample, male, Wroclaw Poland, 2011	Human	De Soyza et al., 2013
AA44	CF (late), Germany	Human	De Soyza et al., 2013
PA01 (ATCC 15692) (Pa01esh)	Genome sequenced isolate	Human	De Soyza et al., 2013
NN2	CF, Germany	Human	De Soyza et al., 2013
LES B58 (B58)	CF, UK (1988)	Human	De Soyza et al., 2013

2.2 Susceptibility Testing

Minimum Inhibitory Concentration (MIC) Assays

For all isolates, minimum inhibitory concentration (MIC) for polymyxin B and colistin from overnight broths of LB were performed using BSAC standardised methods (BSAC) (Andrews, 2001). In brief, broth dilutions were performed in 96-well plates with antibiotic concentrations from 128 ug/ml to 0.25 ug/ml. Positive and negative controls were used throughout and all assays were performed in triplicate. When isolates displayed high levels of resistance, additional assays using higher concentrations up to 1024 ug/ml were performed. The plates were incubated at 37^{o}C and assessed after 24 and 48 hrs of growth. Median values of the triplicate MIC results were used to calculate resistance levels.

The susceptibility of each isolate was determined by the last concentration of polymyxin B at which each isolate was unable to grow. For colistin, BSAC standard

breakpoints were used (4 ug/ml). This is consistent with the guidelines most commonly used by human clinical microbiology diagnostic laboratories. However, breakpoints for polymyxin B are not clearly defined. MIC values were interpreted according to CLSI guidelines as BSAC and EUCAST do not provide breakpoint guidelines for polymyxin B. CLSI recommended using breakpoints of S≤ 2 mg/L, I= 4 mg/L, R≥ 8 mg/L. It was considered that these breakpoints were justified for use in this study as no alternatives were provided by BSAC/EUCAST. (BSAC, 2015).

MIC for Companion Animal Veterinary Antibiotic panel

Subcultures were made of the human and companion animal samples by passage on blood agar or Columbia agar, cultured at 37^{o}C overnight. Following incubation, a few colonies were suspended in 5 mL sterile water solution to produce a uniform turbidity of 0.5 McFarland. A 10 µL aliquot of this solution was then transferred to 11 mL Mueller–Hinton broth, and this broth then transferred to the Sensititre COMPAN1F 96 well microtitre plate via the Trek Sensititre® AIM™ (TREK Diagnostics, Cleveland, OH) automated inoculation delivery system. The plates were incubated at 37 ^{o}C for 24 hrs and the plates read using Sensititre OptiRead™ Automated Fluorometric Plate Reading SystemTrek Sensititre® (TREK Diagnostic Systems) to measure the MIC for inhibition of growth.

2.3 Clondiag Tube Array Genotyping for *P. aeruginosa*

Clondiag Tube Arrays (Alere Technologies) were used to type the isolates according to the protocol. In brief, a sweep of each isolate was emulsified in 1 ml of sterile diluted water (SDW) and centrifuged at 13000 rpm for 2 mins. The supernatant was removed and resuspended in 200 uL of SDW, boiled for 5 mins and centrifuged at 13000 rpm for 2 mins. A master mix of Labelling buffer B1 and Labelling enzyme B2 was prepared. 5 ul of the supernatant was added to 5 ul of master mix. PCR was performed (5 mins at 96 for 1 cycle, 50 cycles of 20 secs at 62 ^{o}C, 40 secs at 72 ^{o}C and 60 secs at 96 ^{o}C. Following hybridization and washing, reagent C3 (containing Horse Radish Peroxidase conjugate) was used to label the chip (the process is depicted in Figure 3). Detection was performed using the Iconoscan, Iconoclust Software (Alere Technologies). Tube Array images were transformed into array

types as previously described (Wiehlmann et al., 2007). In order to study the veterinary isolates in the context of the wider population, an array type database of >900 recorded *P. aeruginosa* strains was used (Cramer et al., 2012). For displaying the wider *P. aeruginosa* population, the eBURST algorithm was applied (Feil et al, 2004). The number of isolates = 981, number of STs = 256, number of loci per isolate =16 and number of resampling for bootstrapping = 1000 (for statistical confidences).

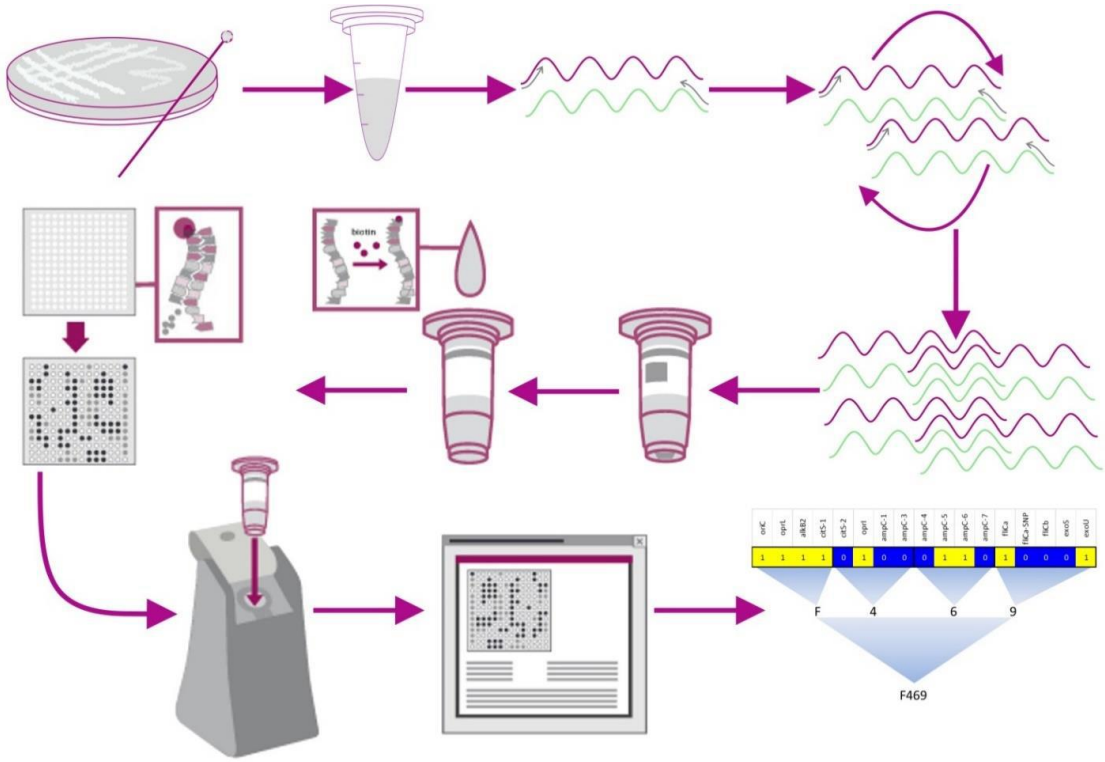


Figure 2.1: Stages of the Clondiag Array Tube system adapted from Alere Technologies.

(Alere, 2017a & Alere 2017b)

Layout of the AT chip

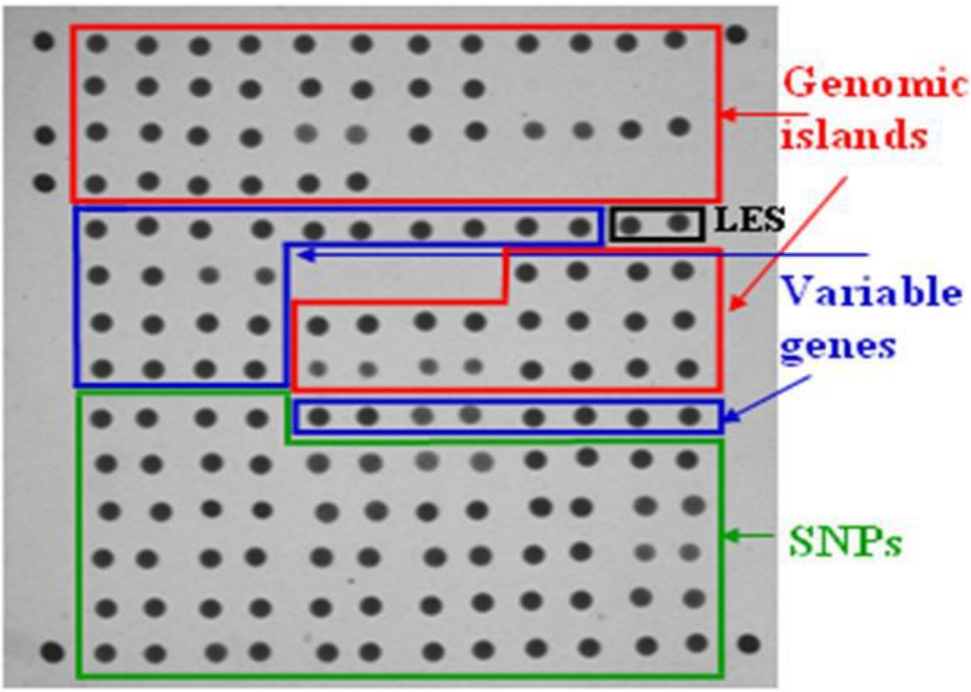
The AT consists of 77 oligonucleotides immobilised and embedded in a microchip in the base of a tube (Wiehlmann et al., 2007). The layout is divided in to 3 sections, as shown in Figure 2.2. These are; genomic islands, variable genes, and SNPs. The chip contains 29 markers for a range of genomic islands and islets, 15 markers for variable genes, a LES PS21 marker, and 16 markers relation to SNP loci. The markers for the genomic islands, variable genes, and the LES PS21 marker are represented by two spots on the chip, and the SNPs by four spots.

Interpretation of the AT chip

Analysis of the 13 SNP patterns at 7 conserved loci (*ampC, citS, alkB2, fliCa, oprI, oprL,* and *oriC*) and the presence or absences of 3 variable genes encoding the type III secretion virulence proteins ExoU and ExoS and the flagellin protein FliC provides a genetic profile, which is initially read as a 16 digit binary code, and then translated into a 4 digit hexidecimal code. The genomic islands and variable genes are represented by two oligonucleotides and the presence of the spots on the chip following hybridisation indicates whether the target is present. If the gene is present the isolate is assigned a "1" for that locus. If the gene is not present it is assigned a "0". Each SNP is represented by four oligonucleotides: two on the left which have sequences matching with PAO1 and two on the right with sequences that do not match with PAO1 (these are considered a "mutant strain"). If the hybridisation spots are stronger on the left (PAO1 sequence) the isolate is assigned a "0" for that locus. If the hybridisation spots are stronger on the right ("mutant" sequence) the isolate is assigned a "1" for that locus. Figure 5 demonstrates examples of binding for both wild type (PAO1) and non-PAO1 (mutant) variants of the hybridisation spots which were utilized to act as a guide when reading the AT image.

Hexadecimal code conversion

The resulting hexadecimal binary code can then be converted to a 4 digit code as shown in figure 6 and a genetic fingerprint is produced and assigned to the strain. This code can then be compared to isolate information from several large, previously described databases (Ballarini et al., 2012; De Soyza et al., 2013; Hall et al., 2014; Wiehlmann et al., 2007). A combined dataset was created including information from the isolates of the previous databases. The comparison allows for further information to be acquired about the strain in question, in particular if it has previously been assigned a clone type and environments from which isolates of the same strain type have been previously collected.



C-45	**C-46**	**C-47**	**PAGI-3-1**	**PAGI-3-8**	**PAGI-2-1**
PAGI-2/3-1	**PAGI-2/3-4**		**PAGI-2/3-5**	**PAGI-2/3-6**	
pKL-1	**pKL-3**	**TB-C47-1**	**TB-C47-2**	**PAPI-1-Pili ch**	**PAPI-1-LuBiPr.**
pKLC-unknown		**pKLC-adhesion**		**pKLC-metabol**	
Pyov. Rec. I	**Pyov. Rec. IIa**	**Pyov. Rec. IIb**	**Pyov. Rec. III**	**Pyov. Rec. B**	**LES**
PA0636	**PA0722**		**PAGI-1**	**PA0980**	
PA0728	**PA2185**	**Fla-island-1**	**Fla-2-orfA**	**47D7-1**	**PAPI-2-Actr**
PA2221	**PA3835**	**Fla-2orfI**	**Fla-2orfj**	**47D7-2**	**PAPI-2-xF1753**
ampC-7	**fliCa**	**fliCb**	**exoS**	**exoU**	
ampC-4		**ampC-5**		**ampC-6**	
oprI		**ampC-1**		**ampC-3**	
citS-1		**citS-2**		**oprI**	
fliCa		**fliCa**		**alkB2**	
oriC		**oprL a**		**oprL**	

Figure 2.2. Positions of SNPs, genomic islands, and variable genes on the AT chip.
C45, C46, C47 – gene island in Clone C , **PAGI** – Pathogenicity Island, **pKLC** – pKLC102 plasmid in Clone C, **pyov.rec 1,2a,2b,3** – pyoverdine type receptors I,II and III fpvA, **pyov.rec B** – pyoverdine type I receptor fpvB, **LES PS21** marker, **PA0636, PA0722, PA0728, PA2185, PA2221, PA3835** – PAO1 sequences, **TB –C47-1, TB- C47-2** - TB, pKLC102 related gene island integrated in tRNA(Lys), **fla-islands 1,2 orfA, orfI** and **orfJ** – flagellin glycosylation islands. , **fliCa, fliCb** – Flagellin proteins, **ExoU/ExoS** – Type III secreted virulence factors, **ampC** – βlactamase, **oprL** – outer membrane lipoprotein, **citS** – citrate synthase, **oprI** – outer membrane lipoprotein, **alkB2** – alkaline hydroxylase alkB2

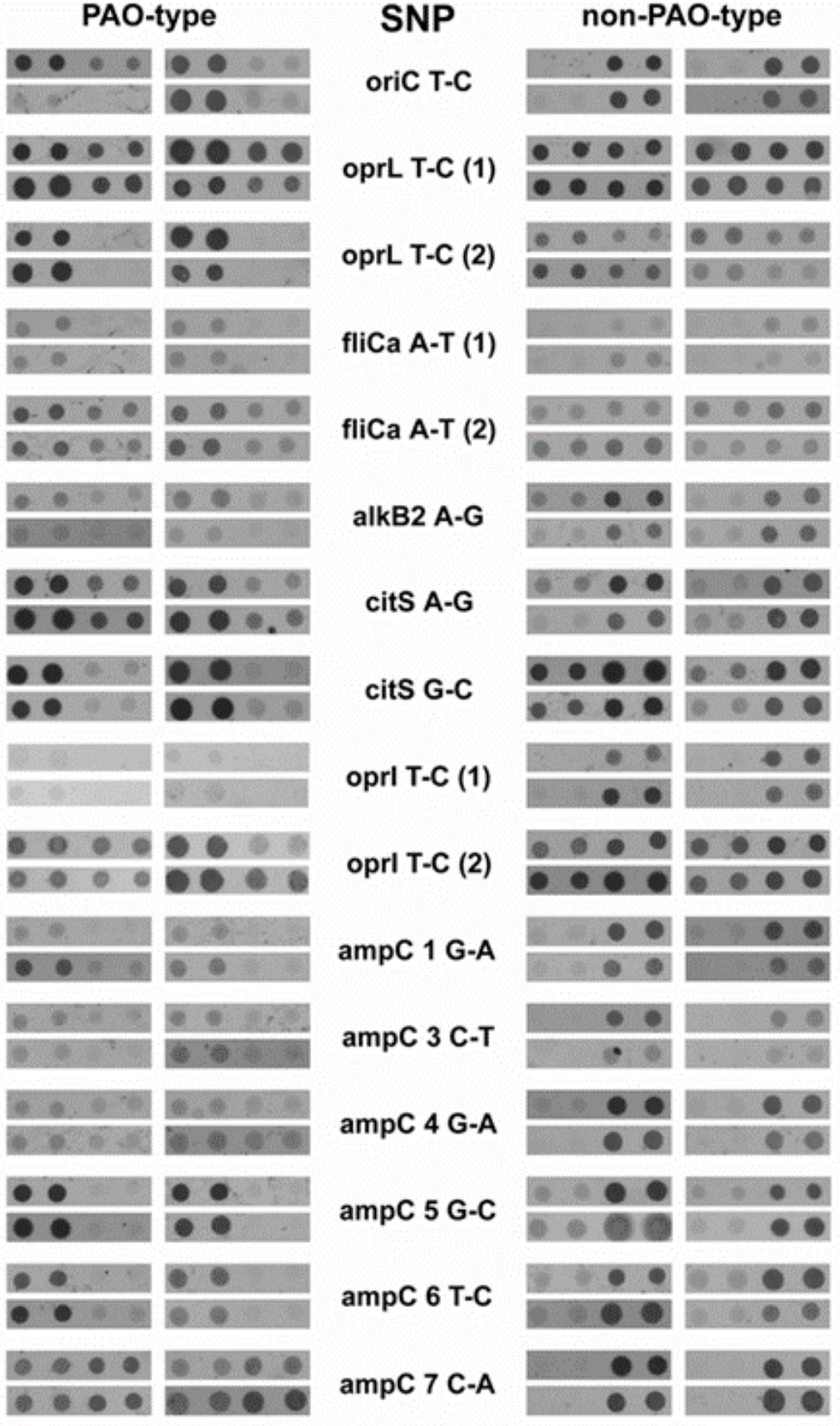


Figure 2.3: Predetermined table for comparison of hybridisation patterns for the seven conserved loci used for AT chip interpretation.

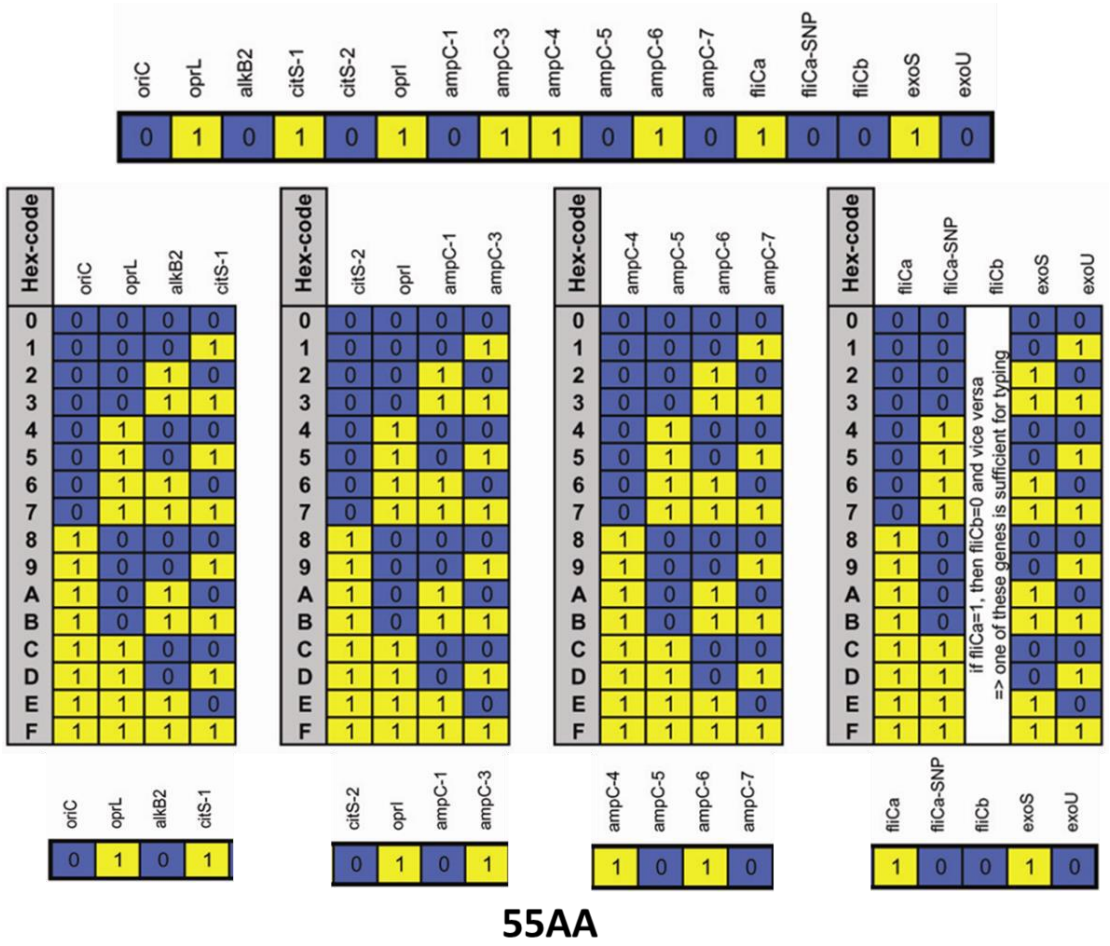


Figure 2.4: Conversion of the hexadecimal binary code. Adapted from (Wiehlmann et al., 2007)

2.4 Genomic DNA extraction for Illumina sequencing

A selection of 7 isolates were selected to undergo whole genome sequencing (some already carried out as part of preliminary unpublished work by Pottenger S,.& Fothergill,J. 2014). Genomic DNA (gDNA) was extracted from these isolates using a Promega Wizard Genomic DNA Purification Kit. The basic protocol was followed, with some minor adjustments to improve yield and purity. Asterisks indicate where the method used deviates from that provided by the manufacturer (experience of

lab has found these alterations gave better yields of DNA).

A single colony was picked and grown overnight in 5 ml LB broth in a shaking incubator at 37°C and 180 rpm. To pellet the cells, 1 ml overnight culture was centrifuged for 2 min at 14 000 rpm and the supernatant discarded. Cells were lysed by adding 600 µl Nuclei Lysis Solution and mixing gently. The tubes were then incubated at 80°C for 5 min and then cooled on ice for a further 5 min*. Once cool 3 µl RNase A Solution (Promega) was added and gently mixed. The tubes were then incubated at 37°C for 1 h and then cooled on ice for 5 min*. Protein was precipitated by adding 400 µl Protein Precipitation Solution* and vortexing immediately. The tubes were kept on ice for 5 min and then centrifuged at 14 000 rpm for 5 min*. The supernatant was then transferred to a tube containing 600 µl room temperature isopropanol and mixed by inverting the tube several times. Following centrifugation at 14 000 rpm for 15 min* the supernatant was removed. Once dry, 600 µl room temperature 70% ethanol (V/V) was added to each tube and the samples were centrifuged at 14 000 rpm for 15 min*. The supernatant was then removed and the tubes left to air dry completely. The DNA pellet was then rehydrated by adding 100 µl DEPC-treated water and storing overnight at 4°C.

Quantification of genomic DNA by Qubit fluorometer

This process was carried out by colleague at University of Liverpool, Yasmin Hilliam. Quantification of gDNA in samples was performed using a Qubit 3.0 fluorometer and Qubit dsDNA broad range assay kit (Life Technologies). A working solution was made using concentrated assay reagent and dilution buffer in a 1:200 dilution. 190 µl working solution was added to 10 µl of each of the two pre-diluted standards for a total reaction volume of 200 µl. 2 µl sample was used per reaction, and made up to a final volume of 200 µl with working solution. The tubes were vortexed for 2 – 3 seconds and then incubated at room temperature for 2 min before being inserted into the Qubit fluorometer to measure fluorescence. Samples were required to contain >20 ng µl-1 gDNA in order to be sent for sequencing.

Quantification and purity testing of genomic DNA by NanoDrop spectrophotometer

This process was performed by colleague at University of Liverpool, Yasmin Hilliam. Further quantification and purity testing of gDNA in samples was carried out using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Extracted DNA had previously been eluted in DEPC-treated water and so the same was used as a blank for NanoDrop measurements. 2 µl DEPC-treated water was carefully pipetted on to the lower measurement pedestal of the spectrophotometer and the sampling arm then closed to form a liquid column to allow the machine to make a blank measurement. 2 µl sample was pipetted in the same manner on to the lower measurement pedestal of the machine and measurements made. Both the upper and lower measurement pedestals were wiped clean with a soft clean tissue between sample measurements (Nanodrop, 2017a).

NanoDrop measurements provide a wide range of information on the absorbance of the sample but the measurements most highly relating to the purity of the sample are the ratios of sample absorbance at 260/280 nm and 260/230 nm. 260/280 ratio of absorbance assesses purity of DNA present in the sample. A ratio of ~1.8 is generally accepted as "pure" for DNA samples. If the ratio is much lower than 1.8 it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. 260/230 ratio of absorbance is a secondary measure of DNA purity and 260/230 ratios for "pure" samples are often higher than the respective 260/280 ratios, often in the range of 1.8 – 2.2. If the ratio is lower than expected, it may indicate the presence of contaminants which absorb at 230nm (Nanodrop, 2017b).

2.5 Whole Genome Sequencing (Illumina) of Bacterial Isolates

Genomic DNA from the seven polymyxin resistant isolates was extracted from overnight cultures using the DNeasy Blood and Tissue Kit (QIAGEN). Genomic DNA (500 ng) was mechanically fragmented for 40 sec using a Covaris M220 (Covaris, Woburn MA, USA) with default settings. Fragmented DNA was transferred to PCR tubes and library synthesis was performed with the Kapa Hyperprep kit (Kapa

biosystems, Wilmington MA, USA) according to manufacturer's instructions. TruSeq HT adapters (Illumina, SanDiego, CA, USA) were used to barcode the samples and libraries were sequenced along with 41 other bacterial genomes in an Illumina MiSeq 300 bp paired-end run at the Plateforme d'Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (Laval University, Quebec, Canada) (Freschi et al., 2015). Resistant isolate genomes were assembled using the A5 assembler version (Coil et al., 2015) and annotated using prokka version 1.5 (Seemann, 2014).

2.6 Genomics

Genome assembly work was performed by Matthew Moore (University of Liverpool). in collaboration with the Pseudomonas Genomics Consortium (PGC) (Levesque RC, Kukavica-Ibrulj I, Jeukens J, Freschi L; L'Institut de biologie intégrative et des systèmes (IBIS), Pavillon Charles-Eugène-Marchand 1030, avenue de la Médecine, Labo 4142 Université Laval, Québec (QC), G1V 0A6 Canada).

The 7 polymyxin resistant isolate genomes were assembled using the A5 assembler version (Coil et al, 2015) and annotated using prokka version 1.5 (Seemann, 2014).

All 7 polymyxin resistant genomes were aligned to reference genome PA01 and separately, PA14 using bwa mem version 0.7.5a (McKenna et al, 2010). Resulting .sam alignment files were sorted, converted to .bam format and duplicates marked and removed using picardtools version 1.85. The Genome Analysis Toolkit (GATK) version 3.3 was used to create indel targets, realign them and call variants using the Unified Genotyper (UG) module. All variants were filtered using vcffilter version GitHub (GitHub 2015) 'DP >9' and 'QUAL >10' to produce the final .vcf files.

Reference genomes PA01, PA14, PA7 and LESB58 and 338 deemed to represent the wider population (Kos et al., 2015) were downloaded from the Pseudomonas Database (Winsor et al., 2011). The core genome was defined and extracted by Panseq (Laing et al., 2010) as 500bp fragments matching in all 342 genomes with >=85% similarity. MEGA6 (Tamura et al, 2013) was used for all phylogenetic

analyses. Phylogeny was estimated using the maximum likelihood method (ML) and tamura-nei substitution model from concatenated polymorphic sites within the defined core genome. Inner node support was based on 100 bootstrap replicates. The tree was drawn using figtree (Figtree, 2015).

Ortholog sequences for 31 polymyxin resistance associated genes were downloaded from the Pseudomonas Database (Winsor et al., 2011). A custom blast database was curated consisting of the 7 polymyxin resistant isolate genomes using the BLAST+(55) makeblastdb software. Each polymyxin resistance associated gene was aligned against the custom database using BLAST+ blastall. A python script was used to extract matching regions and convert to amino acid sequences from the 7 polymyxin resistant isolate genomes.

2.7 Comprehensive Antibiotic Resistance Database (CARD) sequence data analysis

The CARD integrates disparate molecular and sequence data, provides a unique organizing principle in the form of the Antibiotic Resistance Ontology (ARO), and can quickly identify putative antibiotic resistance genes in new unannotated genome sequences (CARD 2015). In addition to extensive browsing tools, integrated within the CARD and available as tools on the website are BLAST databases for all genes stored in the CARD. BLAST searches can include all genes or subsets of CARD reflecting antibiotic resistance genes, antibiotic targets, and antibiotic biosynthesis genes. The Resistance Gene Identifier (RGI) is a novel analytical tool that provides a preliminary annotation of the submitted DNA sequence(s) based upon the data available in the CARD. RGI accepts GenBank accession or GI numbers, pasted sequences, or uploaded nucleotide sequence files in FASTA format. Data with two or more FASTA sequences, such as whole-genome-sequencing (WGS) assembly contigs, can be accepted (maximum size, 20 Mb). The RGI analyzes the submitted sequences and provides a detailed output of predicted antibiotic resistance genes and targeted drug classes. This includes resistance to antibiotics via mutations in their targets or via dedicated antibiotic resistance gene

products (enzymes, protective proteins, and efflux systems). RGI results are then summarized using a "resistance wheel," with overall antibiotic resistance in the center, antibiotic resistance classes in the middle, and individual antibiotic resistance genes on the outer ring (McArthur et al., 2015; McArthur et al., 2013). Seven of the sequenced isolates were uploaded in FASTA format and analysed using this particular tool.

2.8 eBURST algorithm

The eBURST algorithm (eBurst, 2015) was devised as a way of displaying the relationships between closely-related isolates of a bacterial species or population. Using a model of bacterial evolution in which a founding genotype increases in frequency in the population, and while doing so, begins to diversify to produce a cluster of closely-related genotypes that are all descended from the founding genotype. This cluster of related genotypes is referred to as a "clonal complex". The algorithm predicts descent of genotypes from the ancestor and displays the output as a radial diagram. eBURST was originally devised and developed by Ed Feil from the University of Bath (Feil et al, 2004). An enhanced version (eBurstv3) has been developed with funding from the Wellcome Trust, and is hosted by department of Infectious Disease Epidemiology Imperial College London. The eBURST algorithm has mainly been applied to MLST data, although in this study it has been used to display the relationships between genotypes identified through AT genotyping. For use with the AT genotypes, the profiles were converted into a tab delineated text file and uploaded to the eBURST site. For analysis, the programme was set to estimate the relatedness based on 16 loci, with a minimum of one identical loci for group definition, a single locus variant (SLV) count of 0 for subgroup definition, and number of re-samplings for bootstrap support set to 1000. The data is divided into groups of sequence types (STs) that have a level of similarity in their SNP profiles. Within a single group all STs must be an SLV of at least one other ST in the group. The primary founder of any group is defined as the ST that differs from the largest number of other STs at only a single locus. In the case of two STs having the same number of SLVs, the one with the greater number of double

locus variants (DLVs) is selected as the founding member. More than one group can be displayed, appearing as a cluster, in a single eBURST diagram along with any unlinked STs. The eBURST diagram shows the patterns of descent within a group in a radial fashion with lines connecting the founder to each of its SLVs, and lines connecting these STs to other STs varying from them at only one locus. The size of the node representing an ST indicates the relative abundance of that ST within the population.

2.9 Statistical Analysis:

SigmaPlot (version12.3) was used to make comparisions between the human and veterinary polymyxin susceptibilities, using Fisher's exact test. Non parametric test were used on the basis that the results were not normally distributed.

Materials and Methods for Results Chapter 4 - PCR characterisation of antibiotic resistant determinants in *P. aeruginosa* from companion animals:

2.10 Bacterial Isolates

Clinical *P. aeruginosa* (n=106) isolates from companion animal diagnostic samples sent to Veterinary Diagnostic Laboratory at the Small Animal Teaching Hospital, Leahurst (University of Liverpool, UK) between 2010-2015 were selected for this study (Table 2.2). Selection of isolates for this study was made on the basis of species (ie. any isolate that was from a companion animal species) and bacterial species identification (all *P. aeruginosa*). Any duplicates were excluded. No clinical data or prior knowledge of use of antibiotics in these cases was known. Overall, 106 isolates from companion animals (canine n=93, equine n=11, feline n=2) were selected. The collection of isolates were stored at -80°C (Microbank, Pro-Lab Diagnostics, Cheshire, UK). When required isolates were then transferred to Mueller-Hinton nutrient agar (MHA, Oxoid, Basingstoke, UK) and incubated overnight at 37^{o}C in aerobic conditions. *P. aeruginosa* ATCC27853 was used as a control strain.

Table 2.2 Clinical veterinary bacterial isolates used in this study from companion animals

Isolate Ref number	Description	Species
1	Chest drain fluid	Canine
2	Right ear swab	Canine
3	Skin swab	Canine
4	Abdominal fluid	Canine
5	Ear swab	Canine
6	Vulval swab	Canine
7	Urine	Canine
8	Ear swab	Canine
9	Ventral abdomen tissue	Canine
10	Chin furuncle and foot swab	Canine
11	Left stifle fluid	Canine
12	Ear swab	Canine
13	Ear swab	Canine
14	Right ear swab	Canine
15	Wound swab Right hind limb	Canine

16	Left ear swab	Canine
17	Nasal swab	Equine
18	Left ear swab	Canine
19	Abdominal incision swab	Equine
20	Ear swab	Canine
21	Urine	Equine
22	Wound swab	Canine
23	Urine	Canine
24	Wound swab	Canine
25	Swab right mandible (Non healing wound)	Canine
26	Neck swab	Canine
27	Urine	Canine
28	Right ear swab	Canine
29	Oesophagostomy tube stoma swab	Feline
30	Right elbow wound swab	Canine
31	Bile	Canine
32	Vaginal swab	Canine
33	Right ear swab	Canine
34	Lip fold	Canine
35	Right ear swab	Canine
36	Buccal swab	Canine
37	Skin lesion swab	Canine
38	Left neck swab	Canine
39	Testicle/scrotum swab	Canine
40	Tissue	Canine
41	Vaginal swab	Canine
42	Wound swab	Canine
43	Right ear swab	Canine
44	Tracheal	Canine
45	Left ear swab	Canine
46	Right ear swab	Canine
47	Tissue	Canine
48	Right ear swab	Canine
49	Right ear swab	Canine
50	Bronchial aspirate	Canine
51	Skin	Canine
52	Urine	Canine
53	Urinary catheter	Canine
54	Right ear swab	Canine
55	PEG tube stoma wound	Canine
56	Left ear swab	Canine
57	Bronchial aspirate	Canine
58	Tissue	Canine
59	Skin swab	Canine
60	Wound swab	Canine
61	Tracheal	Canine

62	Wound swab	Canine
63	Wound swab	Canine
64	Right anal sac swab	Canine
65	Catheter	Equine
66	Tracheal wash	Equine
67	Guttural pouch washes	Equine
68	Tissue	Canine
69	Right ear swab	Canine
70	Wound serous discharge and fat tissue	Canine
71	Urine	Canine
72	Tissue	Canine
73	Wound swab	Canine
74	Right and Left BAL	Feline
75	Left ear swab	Canine
76	Urine catheter	Canine
77	Maxillary sinus	Equine
78	Colic incision swab	Equine
79	Urine	Canine
80	Urine	Canine
81	Tissue	Canine
82	Vulval fluid swab	Canine
83	Urine	Canine
84	Skin	Canine
85	Left ear swab	Canine
86	Right ear swab	Canine
87	Ear swab	Canine
88	Right ear swab	Canine
89	Ear swab	Canine
90	Left ear swab	Canine
91	Right ear swab	Canine
92	Urine	Equine
93	Urine	Canine
94	Midline swab	Equine
95	Ear swab	Canine
96	Ear swab	Canine
97	Vulval swab	Canine
98	TrWash ETTmu	Canine
99	Ear swab	Canine
100	Vaginal swab	Canine
101	Urine	Canine
102	Wound swab	Canine
103	Urine	Canine
104	Wound swab	Canine
105	Right Ear Swab	Canine
106	Urine	Equine

Table 2.3 Clinical veterinary bacterial isolates used in this study from companion animals and identification of the sources of samples used.

Source of isolate	Total No	No from Equine	No from Canine	No from Feline
Ear	32		32	
Skin (includes lip fold and neck swabs)	8		8	
Wound/Nonhealing wound	14			
Urine	14	2	12	
Urine catheter	2		2	
Catheter	1	1		
Testicle/Scrotum swab	1		1	
Vulval/Vaginal swab	6		6	
Surgical wound/incision (including midline swab and colic incision)	4	1	3	
Abdominal fluid	1		1	
Foot swab	1		1	
Stifle fluid	1		1	
Tracheal/Tracheal wash	4	1	3	
Guttural pouch	1	1		
Maxillary sinus	1		1	
Bronchial aspirate	2		2	
Tissue swab (including abdominal tissue)	7		7	
Anal sac swab	1		1	
Buccal swab	1		1	
Bile	1		1	
Oesophogostomy tube stoma swab	1		1	
Chest drain fluid	1		1	
Bronchoalveolar lavage	1			1

2.11 Susceptibility Testing

Determination of Minimal Inhibitory Concentration (MIC) to a selection of veterinary used antibacterials

Minimal Inhibitory Concentration (MIC) testing was performed using commercially available Trek Sensititre COMPAN1F microtitre 96 well plates (Trek Diagnostics Systems, West Sussex, UK), in accordance with manufacturer's guidelines and as per the method detailed earlier in Chapter 2 'MIC for Companion Animal Veterinary

Antibiotic panel's – refer to page 31**)**. *P. aeruginosa* ATCC27853 was used as the quality control strain for MIC evaluations.

The MIC ranges were tested to a series of twenty two veterinary-used antimicrobials (concentration ranges tested in brackets in mg/mL); ampicillin (0.25-16), amoxicillin/clavulanic acid (4/2-32/16), trimethoprim/sulphamethoxazole (0.5/9.5-2/38), penicillin (0.06-8), cefovecin (0.25-8), cefpodoxime (2-16), erythromycin (0.5-4), oxacillin+2%NaCl (0.25-4), cefoxitin (2-16), clindamycin (0.5-4), doxycycline (2-8), cefazolin (4-16), chloramphenicol (4-16), rifampicin (1-2), amikacin (4-32), ceftiofur (2-16), enrofloxacin (0.25-2), gentamicin (1-8), imipenem (1-8), marbofloxacin (0.25-2), ticarcillin (8-64), ticarcillin/clavulanic acid (8/2-64/2). Three positive control wells were included on the plate. Results were interpreted using standard EUCAST or CLSI breakpoint guidelines as appropriate.

Phenotypic detection of extended spectrum beta-lactamses (ESBL)

Detection of ESBL production was performed by double disc synergy test (DDST) (S.-P. Lin, Liu, Lin, & Shi, 2012). Pairs of discs (MastDiscs, MAST Diagnostics Group Ltd, Bootle, Merseyside, UK) containing the extended spectrum cephalosporins cefotaxime, ceftazidime and cefpodoxime alone and in combination with clavulanic acid (cefotaxime/clavulanic acid 30 mg/10 µg, ceftazidime/clavulanic acid 30 mg/10 µg and cefpodoxime/ clavulanic acid 30 mg/10 µg) were placed on the plates using a multi-dispenser. Results were read after 24 hrs incubation at 37 °C. The test isolate was regarded as an ESBL producer if the zone of inhibition around the combination disc was at least 5mm larger (≥5mm larger) than that of the cephalosporin alone (MAST group 2017).

Cloxacillin or boronic acid (250 µg/mL; both from Sigma-Aldrich Company Ltd. Dorset, UK) was added to MHA to inhibit chromosomal cephalosporinase activity. Following overnight culture, test isolates were suspended to 0.5 turbidity using McFarland standards, and used to inoculate a Mueller-Hinton agar plate containing the 250µg/mL cloxacillin or boronic acid (Lin et al., 2012) (Aghazadeh et al., 2014). After plate drying, the discs were placed as described earlier.

Disc diffusion susceptibility testing against a human antibacterial panel

Antimicrobial susceptibility testing was performed by disc diffusion method to a panel of clinically relevant commonly used human antibacterials containing aztreonam (30 µg), meropenem (10 µg), cefepime (30µg), piperacillin (30 µg), piperacillin (30 µg)/tazobactam (36 µg), and cefoxitin (30 µg) (all from Oxoid, Oxoid Ltd, Thermo Fisher Scientific group, UK). Preparation of a standard bacterial suspension equal to 0.5 McFarland was inoculated on MHA and the antibiotic discs were placed on the plate using a multi-dispenser. Results were read after 24 hrs incubation at 37 °C. The diameter of zone of growth inhibition were measured interpretation performed by using the European Committee on Antimicrobial Susceptibility Testing breakpoints (EUCAST 2015). Disc diffusion susceptibility testing is a published, acceptable method for determining susceptibility and was chosen in this instance as it enables large scale screening that wouldn't be possible in the time frame of this project if MICs were used for all antibiotics.

2.12 DNA Extraction

For DNA extraction a standard boil preparation method was followed. Subcultures were made of the isolates by passage on MHA and incubation for 24 hrs at 37 °C. A few bacterial colonies (two 5 µl sterile loops) were added to 1ml of sterile water in 1.5ml Eppendorf tube and mixed. The solution was then heated for 10 mins at 95 °C on a thermoplate, followed by centrifuging at 10,000 rpm for 2 mins. The supernatant was removed to a fresh sterile Eppendorf tube for DNA use and labeled appropriately for each isolate. This was stored at 4 °C for direct usage.

DNA Extraction – Wizard® Genomic DNA Purification Kit Method

Bacterial DNA was extracted from a selection of the *P. aeruginosa* isolates using a commercially available Genomic DNA Purification kit (Wizard® Genomic DNA

Purification Kit, Promega Corporation, Madison, WI 53711-5399 USA) according to the manufacturers guidelines.

1 ml of an overnight culture was added to a 1.5 ml microcentrifuge tube and centrifuged at 13,000–16,000 *× g* for 2 minutes to pellet the cells. The supernatant was removed. 3µl of RNase Solution was added to the cell lysate and the tube inverted 2–5 times to mix. The sample was incubated at 37°C for 15–60 minutes and then cooled to room temperature. A 200µl aliquot of Protein Precipitation Solution was added to the RNase-treated cell lysate. This was then vortexed vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate. The sample was then incubated the on ice for 5 minutes. Then centrifuged at 13,000–16,000 *× g* for 3 minutes. The supernatant (containing the DNA) was transfered to a clean 1.5ml microcentrifuge tube containing 600 µl of room temperature isopropanol. This was gently mixed by inversion until the thread-like strands of DNA form a visible mass. Then centrifuged at 13,000–16,000 *× g* for 2 minutes. The supernatant was poured off and drained the tube. 600 µl of room temperature 70% ethanol was added and the tube gently inverted several times to wash the DNA pellet. After centrifuging at 13,000–16,000 *× g* for 2 minutes, aspirate the ethanol. The tube was drained on clean absorbent paper and the pellet allowed to air dry for 10–15 minutes. Then adding 100 µl of DNA Rehydration Solution to the tube and rehydrate the DNA by incubating the solution overnight at room temperature or at 4 °C then stored the DNA at 2–8 °C.

2.13 Polymerase Chain Reaction (PCR)

DNA extraction was performed as previously described by boil preparation method. PCR amplification (for both simplex and multiplex) was performed in a final volume of 25 µl, using the primers as presented in Table 2.5.
The composition of PCR mixture was: 5 µl of a Master Mix, FIREPol® (5 x FIREPol® Master Mix Ready to Load, Solis BioDyne, Estonia, a premixed ready-to-use solution containing all reagents required for PCR - except template, primers and water).

In addition for each genes tested the forward and reverse primers (final primer concentrations were 10pcm/µl PCR reaction), water and template DNA were added in the quantities as listed in Table 2.3 for multiplex and Table 2.4 for simplex.

The PCR products were electrophoresed in 1% agarose gels containing PeqGREEN® dye (VWR International Ltd, Leicestershire, England). The DNA fragments were imaged with UV transilluminator and UViProMW software documentation system. The DNA loading dye DNA/BsuRI (HaeIII) Marker 9, 50 mg (0.5 µg/µl)(ThermoScientific Molecular Biology), was placed on each gel run.

Table 2.4 Multiplex PCRs conditions

Genes Tested	PCR Mixture	PCR Conditions (using Applied BioSystems, Thermocycler 2720)	References
TEM, SHV, OXA	5 µl of FIREPol® Mastermix, 1µl of each forward and reverse primers, 12 µl water and 2 µl of each template DNA.	The amplification program for all of the aforementioned genes consisted of initial denaturation at 94 °C for 10 mins, annealing at 94 °C for 40 secs, 60 °C for 40 secs, extension at 72 °C for 1 min (for 30 cycles) and a final extension at 72 °C for 7 mins in a thermocycler.	(Dallenne et al., 2010)
GES, VEB, PER	5 µl of FIREPol® Mastermix, 0.75 µl of each forward and reverse primers, 13.5 µl water and 2 µl of each template DNA.		(Dallenne et al., 2010)
VIM, IMP, KPC	5 µl of FIREPol® Mastermix, 1.25 µl of each forward and reverse primers, 10.5 µl water and 2 µl of each template DNA.		(Dallenne et al., 2010)
qnrA, qnrB, qnrS	5 µl of FIREPol® Mastermix, 1 µl of each forward and reverse primers, 12 µl water and 2 µl of each template DNA.	initial denaturation at 94 °C for 10 mins, annealing at 94 °C for 1 min, 53 °C for 1 min, extension at 72 °C for 1 min (for 35 cycles) and a final extension at 72 °C for 10 mins in a thermocycler	(Robicsek et al., 2006)

The multiplexPCRs were not continued throughout due to misleading results (refer to detail in Results Chapter 4) therefore it was decided to do all PCRs as simplex method (details in Table 2.5).

Table 2.5 Simplex PCR conditions

Genes Tested	PCR Mixture	PCR Conditions (using Applied BioSystems, Thermocycler 2720)	References
bla$_{TEM}$, bla$_{SHV}$, bla$_{OXA}$	5 µl of FIREPol® Mastermix, 1µl of each forward and reverse primers, 16 µl water and 2 µl of template DNA.	The amplification program for all of the aforementioned genes consisted of initial denaturation at 94 °C for 10 mins, annealing at 94 °C for 40 secs, 60 °C for 40 secs, extension at 72 °C for 1 min (for 30 cycles) and a final extension at 72 °C for 7 mins in a thermocycler.	(Dallenne et al., 2010)
bla$_{GES}$, bla$_{VEB}$, bla$_{PER}$	5 µl of FIREPol® Mastermix, 0.75 µl of each forward and reverse primers, 16.5 µl water and 2 µl of template DNA.		(Dallenne et al., 2010)
bla$_{VIM}$, bla$_{IMP}$, bla$_{KPC}$	5 µl of FIREPol® Mastermix, 1.75 µl of each forward and reverse primers, 15.5 µl water and 2 µl of template DNA.		(Dallenne et al., 2010)
group *OXAs; OXA-1, OXA-2, OXA-10*	5 µl of FIREPol® Mastermix, 0.5 µl of each forward and reverse primers, 18 µl water and 1µl of template DNA.	initial denaturation at 95 °C for 5 mins, annealing at 95 °C for 1 min, 55 °C for 40 secs, extension at 72 °C for 1 min (for 25 cycles) and a final extension at 72 °C for 10 mins in a thermocycler	(Poirel, et al, 2010)
qnrA, qnrB, qnrS	5 µl of FIREPol® Mastermix, 0.5 µl of each forward and reverse primers, 18 µl water and 1 µl of template DNA.	initial denaturation at 94 °C for 10 mins, annealing at 94 °C for 1 min, 55 °C for 40 secs, extension at 72 °C for 1 min (for 25 cycles) and a final extension at 72 °C for 10 mins in a thermocycler	(Robicsek et al., 2006)
AmpC	5 µl of FIREPol® Mastermix, 0.5 µl of each forward and reverse primers, 18 µl water and 1 µl of template DNA.	initial denaturation at 94 °C for 3 mins, annealing at 94 °C for 1 min, 60 °C for 40 secs, extension at 72 °C for 1 min (for 25 cycles) and a final extension at 72 °C for 5 mins in a thermocycler	(Rodríguez-Martínez et al, 2009)
CTX-MU, *BEL-1*, *CS(integrons)*	5 µl of FIREPol® Mastermix, 0.5 µl of each forward and reverse primers, 17 µl water and 2 µl of template DNA.	initial denaturation at 94 °C for 3 mins, annealing at 94 °C for 1min, 56.9 °C for 40 secs, extension at 72 °C for 1 min (for 25 cycles) and a final extension at 72 °C for 5 mins in a thermocycler	(Wedley et al., 2011) (Bogaerts, et al, 2007) (Zhao et al., 2003)
AAC(lb) cr variant	5 µl of FIREPol® Mastermix, 1 µl of each forward and reverse primers, 16 µl water and 2 µl of template DNA.	initial denaturation at 94 °C for 3 mins, annealing at 94 °C for 45 secs, 55 °C for 45 secs, extension at 72 °C for 45 secs (for 34 cycles) and a final extension at 72 °C for 5 mins in a thermocycler	(Hong et al., 2009; Park et al, 2006)

2.14 Primers

Table 2.6 DNA sequencing primers used in this study.
Primers purchased from Eurofins Genomics (Eurofins, Ebersberg, Germany)

Primer name	Sequence	Product size (bp)	Reference
TEM Fw	CATTTCCGTGTCGCCCTTATTC	800	(Dallenne et al., 2010)
TEM Rev	CGTTCATCCATAGTTGCCTGAC		
SHV Fw	AGCCGCTTGAGCAAATTAAAC	713	(Dallenne et al., 2010)
SHV Rev	ATCCCGCAGATAAATCACCAC		
OXA Fw	GGCACCAGATTCAACTTTCAAG	564	(Dallenne et al., 2010)
OXA Rev	GACCCCAAGTTTCCTGTAAGTG		
GES Fw	AGTCGGCTAGACCGGAAAG	399	(Dallenne et al., 2010)
GES Rev	TTTGTCCGTGCTCAGGAT		
VEB Fw	CATTTCCCGATGCAAAGCGT	648	(Dallenne et al., 2010)
VEB Rev	CGAAGTTTCTTTGGACTCTG		
PER Fw	GCTCCGATAATGAAAGCGT	520	(Dallenne et al., 2010)
PER Rev	TTCGGCTTGACTCGGCTGA		
VIM Fw	GATGGTGTTTGGTCGCATA	390	(Dallenne et al., 2010)
VIM Rev	CGAATGCGCAGCACCAG		
IMP Fw	TTGACACTCCATTTACDG	139	(Dallenne et al., 2010)
IMP Rev	GATYGAGAATTAAGCCACYCT		
KPC Fw	CATTCAAGGGCTTTCTTGCTGC	538	(Dallenne et al., 2010)
KPC Rev	ACGACGGCATAGTCATTTGC		
qnrA Fw	ATTTCTCACGCCAGGATTTG	516	(Robicsek et al., 2006)
qnrA Rev	GATCGGCAAAGGTTAGGTCA		(Robicsek et al., 2006)
qnrB Fw	GATCGTGAAAGCCAGAAAGG	469	(Robicsek et al., 2006)
qnrB Rev	ACGATGCCTGGTAGTTGTCC		(Poirel et al., 2010)
qnrS Fw	ACGACATTCGTCAACTGCAA	417	(Poirel et al 2010)
qnrS Rev	TAAATTGGCACCCTGTAGGC		(Poirel et al., 2010)
OXA-1 Fw	AGCCGTTAAAATTAAGCCS	908	(Wedley et al., 2011)
OXA-1 Rev	CTTGATTGAAGGGTTGGGCG		
OXA-2 Fw	GCCAAAGGCACGATAGTTGT	700	(Bogaerts et al., 2007)
OXA-2 Rev	GTCCGAGTTGACTGCCGG		
OXA-10 Fw	TCTTTCGAGTTACGGCATTAGC	760	(Zhao et al., 2003)
OXA-10 Rev	CCAATGATGCCCTCACTTTCC		
CTX-MU Fw	TGGGTRAARTARGTSACCAGAAYCAGCGG	593	(Hong et al., 2009)
CTX-MU Rev	TGGGTRAARTARGTSACCAGAAYCAGCGG		
BEL-1 Fw	CGACAATGCCGCAGCTAACC		(Rodríguez-Martínez et al., 2009)
BEL-1 Rev	CAGAAGCAATTAATAACGCCC		
CS-1 Fw	GGCATCCAAGCACAAGC		
CS-1 Rev	AAGCAGACTTGACTGAT		
AAC-61b Fw	TTGCGATGCTCTATGAGTGGCTA	482	
AAC-61b Rev	CTCGAATGCCTGGCGTGTTT		
AmpC Fw	ATGCAGCCAACGACAAAGG	1243	
AmpC Rev	CGCCCTCGCGAGCGCGCTTC		

2.15 Quantification and purity testing of genomic DNA by NanoDrop spectrophotometer

Further quantification and purity testing of gDNA in samples was carried out using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Extracted DNA had previously been eluted in DEPC-treated water and so the same was used as a blank for NanoDrop measurements. 1 µl DEPC-treated water was carefully pipetted on to

the lower measurement pedestal of the spectrophotometer and the sampling arm then closed to form a liquid column to allow the machine to make a blank measurement. 1 µl sample was pipetted in the same manner on to the lower measurement pedestal of the machine and measurements made. Both the upper and lower measurement pedestals were wiped clean with a soft clean tissue between sample measurements.

PCR products of adequate quantity were sent for sequencing to Source BioScience (Source BioScience). The concentration of DNA required was dependent upon the length of the sample sent. A concentration of 1 ng/ µl per 100 base pairs is required for successful sequencing from putified PCR samples. Primers forwards and reverse were also sent. These were required to be sent at 3.2 pmol/µl. For sequencing 5 µl per reaction of both primer and DNA at the above specified concentrations was required thus 15 µl of each was prepared.

2.16 DNA Sequencing

Genomic DNA from positive simplex PCR *P. aeruginosa* isolates was extracted using a commercially available PCR clean up Gel extraction kit (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel GmbH & Co, Duren, Germany) according to the manufacturers guidelines.

The PCR amplicons were purified and sequenced on both strands. The DNA sequences were analyzed with Staden software and compared with published DNA sequences in the GenBank database https://blast.ncbi.nlm.nih.gov/Blast.cgi

2.17 Statistical Analysis

The 95% CIs were performed on proportions using the Wilson method (http://epitools.ausvet.com.au)

3. Reservoirs of resistance: polymyxin resistance in veterinary-associated isolates of *Pseudomonas aeruginosa:*

3.1 Introduction

Polymyxins are produced naturally by *Bacillus polymyxa*. They demonstrate bactericidal activity against a wide range of species of Gram-negative bacilli including *Escherichia coli*, *Salmonella* spp and *P. aeruginosa*. Polymyxins that are utilised in clinical practice include polymyxin B and polymyxin E (colistin). These are cationic peptides and the main difference between the two molecules is that polymyxin B contains phenylalanine in position 6 (from the N-terminal), while colistin contains D-leucine (Tran et al., 2016; Velkov et al., 2014). The outer membrane normally serves as a protective barrier for Gram-negative bacteria against antimicrobial peptides and disinfectants. The mechanism of action by which polymyxins exert their effect is through electrostatic interaction with divalent cations of the outer bacterial membrane. The polymyxin antibacterial peptides can selectively bind to the lipopolysaccharide (LPS) of the outer cell membrane, the membrane permeability is disturbed and this destabilization results in lysis of the bacteria and ultimately leads to cell death (Chen et al., 2011; Yu., et al, 2015).

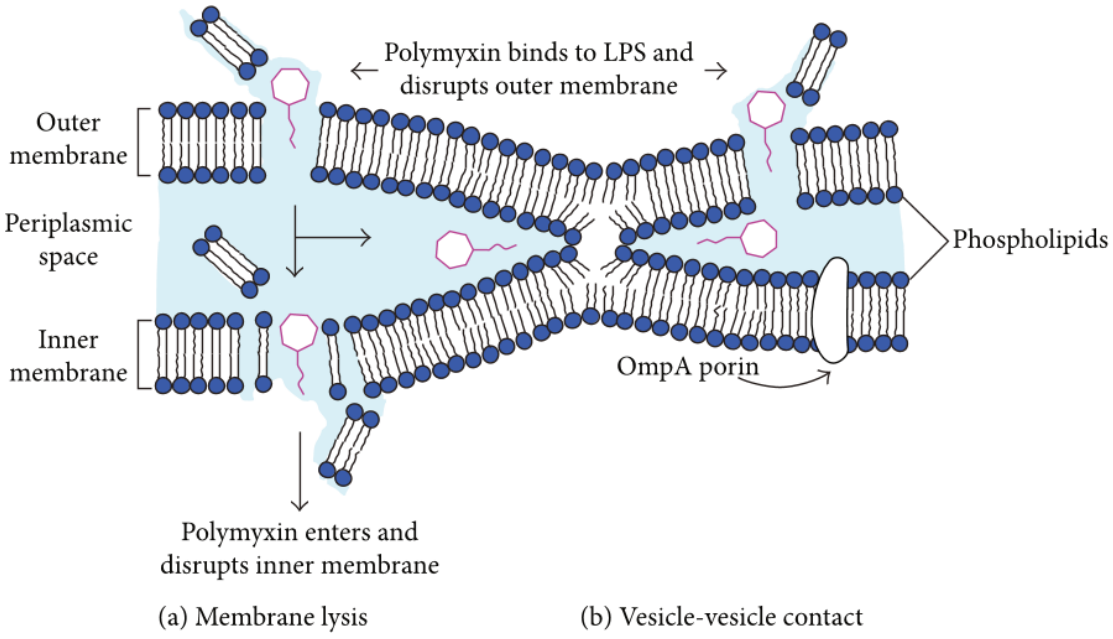


Figure 3.1 Antibacterial mechanisms of polymyxin: (a) classic mechanism of membrane lysis; (b) alternative mechanism of vesicle-vesicle contact. Polymyxin is shown as red. LPS-lipopolysaccharide Referenced from (Chen et al., 2011; Yu et al, 2015)

Mechanisms of resistance in *P. aeruginosa* to polymyxins include adaptive resistance (where various cell surface alterations are reported) (Fernández et al., 2010; Moore et al, 1984) and genetic mutations.

Adaptive resistance describes a reversible refractoriness to the bactericidal effect of an antibacterial agent. Adaptive resistance is a phenomenon by which certain environmental cues, including sub-inhibitory concentrations of antibiotics, can transiently induce resistance to otherwise lethal doses of antimicrobial agents (often refered to as tolerance). The cause is not deemed a genetic mutational change but rather a protective phenotypic alteration in bacterial characteristics (Darmon & Leach, 2014). This can include the reversible down-regulation of the active transport of aminoglycosides into Gram-negative bacteria (Barclay & Begg, 2001; Poole, 2005a). In *P. aeruginosa*, adaptive resistance to polymyxins and cationic antimicrobial peptides occurs in response to limiting extracellular concentrations of divalent Mg^{2+} and Ca^{2+} cations. This adaptation is controlled by

the two-component regulators PhoP-PhoQ and PmrA-PmrB which up-regulate the expression of the *arnBCADTEF* lipopolysaccharide (LPS) modification operon (Fernández et al., 2010; Olaitan et al., 2014). The products of these *arn* genes participate in the addition of 4-aminoarabinose to lipid A of LPS, thereby reducing the net negative charge of LPS and limiting the interaction and self-promoted uptake of polycationic antibiotics, such as polymyxins, host defense peptides, and aminoglycosides, with the outer membrane (Fernández et al., 2010). However, this adaptation to low divalent cations is unlikely to be clinically meaningful. Sub-inhibitory concentrations of peptides can induce the expression of the *pmrAB* and *arnBCADTEF* operons in *P. aeruginosa*. However, neither PhoP-PhoQ nor PmrA-PmrB seems to be essential for this upregulation. Thus, an unidentified regulatory system has been proposed to respond to peptides and promote the activation of the *arnBCADTEF* and *pmrAB* operons independently of PhoP-PhoQ and PmrA-PmrB, consequently increasing resistance to antimicrobial peptides (Fernández et al., 2012; Gooderham et al., 2009; Macfarlane et al, 1999; McPhee et al, 2003). That is the peptides themselves are able to induce adaptive resistance via LPS modification (Fernández et al., 2010).

Genetic mutation is more stable and is inheritable. It is characterized by alterations in the outer membrane. A study by Fernandez *et* al (Fernández et al., 2013) reported a relatively small polymyxin B resistome involving 17 susceptibility/intrinsic resistance determinants. Their findings demonstrated that the changes in susceptibility to polymyxin B and to colistin suggested evidence of cross resistance. They showed that many of the same genes influence the susceptibility of *P. aeruginosa* to different antibiotic classes and suggest that certain genes may coordinate multidrug resistance possibly by affecting regulatory pathways. In their study they determined the MICs of these strains to other cationic antimicrobial peptides, including colistin. All of the tested transposon mutants had altered susceptibility to colistin. The fold change in susceptibility of the mutant was determined compared to that of wild-type PA14 by broth microdilution for the polymyxins. In particular, it was of note that a 2 fold change in

MIC for polymyxin B also produced an identical 2 fold change in the MIC for colistin (Fernández et al., 2013). This would suggest that the resistance genes of *P.aeruginosa* appear to have the same effect on colistin as they do on polymyxin B and may infer cross-resistance in the two polymyxins. This raises concern regarding the use of polymyxins within the veterinary community whereby their use may help drive development of polymyxin B/colistin resistant *P. aeruginosa* strains. These could act as a potential source of resistant *P.aeruginosa*.

Several indications for polymyxin usage in veterinary medicine exist (as referenced earlier in more detail in Chapter 1.1.5). Infections caused by Enterobacteriaceae in rabbits, pigs, poultry, cattle, sheep and goats are a main indication for their use (Bassetti et al 2015). In Europe, colistin and polymyxin B are used for treating infections caused by *Enterobacteriaceae* in rabbits, broilers, veal, beef cattle, dairy cattle and primarily pigs. In other areas of the world, polymyxins are still utilised as growth promoters, however in Europe this usage has been banned since 2006 (Castanon, 2007; Millet et al., 2011). Polymyxin B is an antibiotic utilised in veterinary medicine as a common first line topical therapy for treatment of otitis externa in the form of proprietary ear drops product and may be used for systemic treatment of endotoxaemia associated with severe colic and other gastrointestinal diseases (Bassetti et al 2015). A 2013 European Medicines Agency report on colistin usage in animals within the EU stated that across 19 EU/EEA European countries for which sales data are available for 2010, polymyxins were the 5th most sold group of antimicrobials (4.5%), after tetracyclines (39%), penicillins (23%), sulphonamides (11%), and macrolides (9%) (Bassetti et al., 2015). However, this was based on food producing animals (including horses) only and would not include companion animals such as cats and dogs. The use of polymyxins specifically in companion animals is not reported. However, several studies looking at the usage of antimicrobial prescribing in small animal practice exist (Bassetti & Righi, 2015). More recently there has been the development of a number of real time data surveillance schemes that monitor illness and disease in pet animals using veterinary practice and laboratory data SAVSNET (SAVSNET, 2017). Such surveillance systems may prove useful for future analysis and development of

studies of antibiotic resistance data from companion animal across the UK (Jones et al., 2014; Radford et al., 2011; Singleton et al., 2017).

Mechanisms of resistance in *P. aeruginosa* to polymyxins include adaptive resistance and genetic mutations and generally characterized by alterations in the outer membrane (Moore et al., 1984). A study by Fernandez *et al* (Fernández et al., 2013) reported a relatively small polymyxin B resistome involving 17 susceptibility/intrinsic resistance determinants including evidence of cross resistance between colistin and polymyxin B (Fernandez, 2013). This raises concern regarding use of polymyxins within the veterinary community whereby their use may help drive development of polymyxin B/colistin resistant *P. aeruginosa* strains. Potentially selecting in veterinary medicine polymyxin-resistant isolates that may subsequently be transferred to humans. These could then act as a source of resistant *P. aeruginosa* populations (Moore et al., 2012).

Table 3.1. Polymyxin Resistome Genes and Gene Function

Gene	Gene Function
pyrC	*Cell metabolism-associated*
pyrB	*Cell metabolism-associated*
phoQ	*Sensor kinase, mediates virulence*
pdxB	*Cell metabolism-associated*
sucC	*Cell metabolism-associated*
parR	*Acquired/adaptive resistance through LPS modification*
galU	*Biosynthetic processes/pathogenesis*
pyrD	*Biosynthetic/metabolic processes*
Mpl	*Recycling of cell wall components*
rmlD	*Biosynthetic processes*
ampR	*Transcriptional regulator-associated with resistance to antibiotics*
lptC	*LPS binding*
tpiA	*Cell metabolism-associated*
pmrA	*Activation of LPS-modifying operon by mutations in TCSs*
wapR	*LPS biosynthesis-related functions*
Ssg	*LPS biosynthetic processes*
aroB	*Biosynthetic/metabolic processes*

http://www.pseudomonas.com

Polymyxin Resistance Mechanisms

PhoP-PhoQ Two-Component System:

The OM usually serves as a permeability barrier to protect Gram-negative bacteria from various antibacterial peptides and chemicals. The electrostatic interaction between positively charged Dab residues on polymyxin and negatively charged phosphate groups on lipid A of LPS are critical in its bactericidal activity. The bacterial cell is able to reduce the initial electrostatic attraction by reducing net negative charge of OM via lipid A modification, thereby increasing resistance to polymyxins. The most common polymyxin-resistance mechanism in bacteria is attributed to the 'shielding'of phosphates on lipid A with positively charged groups. This is mediated by PhoP-PhoQ regulatory system encoded by *phoP* locus (Yu., et al, 2015). Activated by PhoP-PhoQ, the PmrA-PmrB encoded by *pmrCAB* operon is the major regulator to mediate the LPS modification in Gram-negative bacteria. PmrA-dependent modification can occur on each of the three distinct LPS domains, namely, lipid A, core polysaccharide, and O-antigen chain (McPhee et al., 2006; Gellatly et al, 2012; McPhee et al., 2003).

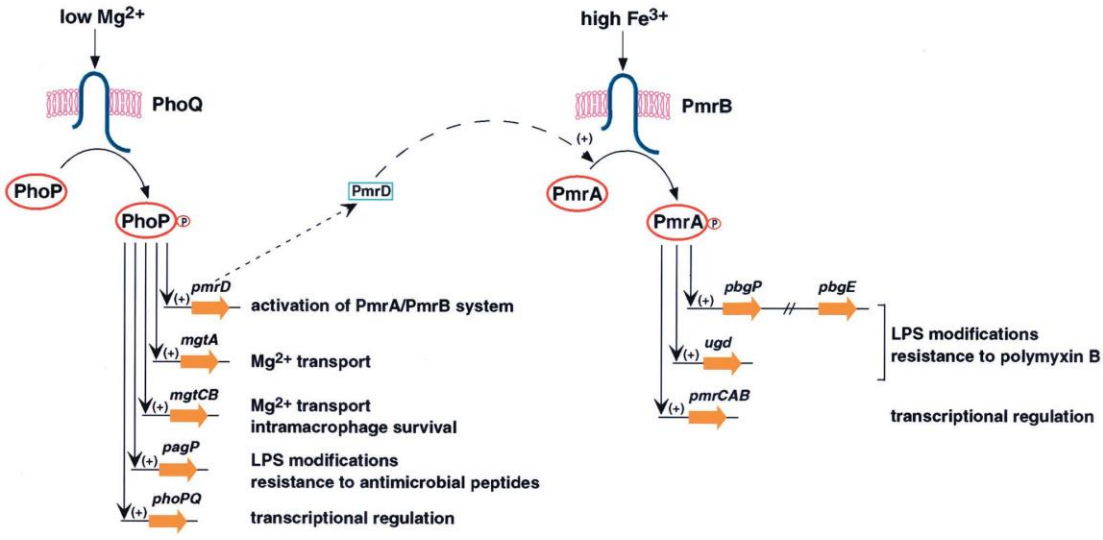


Figure 3.2 Model describing the signals controlling expression of PhoP-PhoQ-regulated determinants and the interaction between the PhoP-PhoQ and PmrA-PmrB two-component systems, as well as some of the genes and phenotypes governed by the PhoP-PhoQ system. Referenced from (Groisman, 2001).

MexAB-OprM efflux pump:

The MexA-MexB-OprM efflux system (Li et al., 1995) contributes to both intrinsic and acquired resistance in *P. aeruginosa*. Reported studies with mutants that overproduce or lack MexAB-OprM demonstrated that this efflux system extrudes quinolones, macrolides, tetracycline, chloramphenicol, novobiocin, and most β-lactams but not imipenem (Li et al., 1995; Masuda & Ohya, 1992; Srikumar et al, 1997). The MexAB-OprM efflux pump in *P. aeruginosa* has been proposed to confer tolerance towards polymyxin E, due to the increase of mexAB-oprM expression in *P. aeruginosa* upon polymyxin E exposure (Henrichfreise et al., 2007; Hirakata et al., 2009; Li et al., 2000).

OM Protein:

Polymyxin resistance is reported to be associated with the expression of OM proteins in bacteria. It has been suggested that the OM protein OprH, a membrane stabilization protein, can promote resistance to polymyxin B in *P. aeruginosa* (Macfarlane et al., 1999; Olaitan et al., 2014; Yu et al., 2015).

Plasmid mediated colistin resistance:

Liu et al 2015 (Liu et al., 2016) first reported the emergence of plasmid mediated resistance gene, *mcr-1* in *E.coli* and *Klebsiella pneumonia* isolates from pigs, meat and human patients in China. In their study, the mcr-1 gene was present in 20.6% of *E. coli* isolates from pigs, 14.9% of *E. coli* isolates from retail meat (pig and poultry) and 1.4% of patients sampled (Liu et al., 2016). Occurrence of the *mcr-1* gene has been reported in *E. coli*, *Salmonella* and *Klebsiella* from Laos, Thailand, Tunisia, Nigeria, South America and several European countries from people, pigs, poultry and foodstuffs and in the UK (Anjum et al., 2016; Castanheira et al., 2016; Falgenhauer et al., 2016; Kluytmans-van den bergh et al., 2016; Malhotra-Kumar et al., 2016).

3.2 Aims

The significance of the role of companion animals as reservoirs of antimicrobial resistance is an understudied area and merits further investigation. There is a risk of transfer of resistant bacteria and/or resistance genes from animals to humans.
The overall aim of this study is to evaluate susceptibility of *P. aeruginosa* veterinary isolates to polymyxin antibacterials and to determine whether the veterinary niche represents a potential reservoir of resistant isolates.

Specific aims are to:

- Measure the susceptibility of a set of clinical *P. aeruginosa* isolates to polymyxin antibiotics colistin (polymyxin E) and polymyxin B by broth micro dilution.
- Compare these susceptibilities to a panel of human *P. aeruginosa* isolates.
- Determine the susceptibility of the *P. aeruginosa* isolates in the study to a selection of antibacterials used in human clinical medicine by disc diffusion.
- Determine susceptibility of the *P. aeruginosa* isolates in the study to a selection of commonly used veterinary antibacterials using an automated microdilution system.
- Perform genotyping of the veterinary *P. aeruginosa* isolates using the Clondiag Array Tube.
- Perform whole genome sequencing on a selection of resistant isolates to determine genetic mutations in a defined subset of genes associated with resistance to polymyxin antibacterials.

3.3 Results

Resistance to polymyxin antibiotics

Resistance of all *P. aeruginosa* isolates to polymyxin B and colistin were tested using broth dilution MICs. For the veterinary isolates, 92 % displayed resistance to polymyxin B and the remaining isolates were classed as displaying intermediate resistance (Table 3.2), whilst 68% displayed resistance to colistin. For the human isolates 33% displayed resistance to polymyxin B and just 3% (one isolate) to colistin. Using the Fisher's exact test the levels of resistance were signicantly higher (ie. $p<0.05$) as compared to that of the human isolates ($p = 0.033$) for polymyxin B and also significantly higher for colistin ($p = 0.002$).

Table 3.2 Minimum inhibitory concentration tests to show antimicrobial resistance of *P. aeruginosa* in companion animal (n=24) and human (n=37) isolates to polymyxin antimicrobials (calculated using median of triplicate MICs).

Isolate	Antimicrobial	Resistance Breakpoint (ug/mL)	Resistant Strains (n)	Resistant Strains (%)
Animal	Polymyxin B	≥8	22	92
Animal	Colistin	>4	8	33
Human	Polymyxin B	≥8	25	68
Human	Colistin	>4	1	3

Fisher's exact test (significant = $p<0.05$):
p value for polymyxin B MIC human v animal **p=0.033**
p value for colistin MIC human v animal **p=0.002**

The highest resistance was observed in isolate 970, from a canine wound, which had an MIC of >100 mg/L (Figure 3.3). The level of resistance to colistin amongst the veterinary isolates was 33% compared to only 3% in the human-associated panel (Table 3.2). The isolates with the highest resistance to colistin were 856 and 1098

(Figure 3.3). Isolate 856 was of equine origin, from an abdominal incision and 1098 from a canine vaginal swab (refer to Chapter 2, Table 2.1).

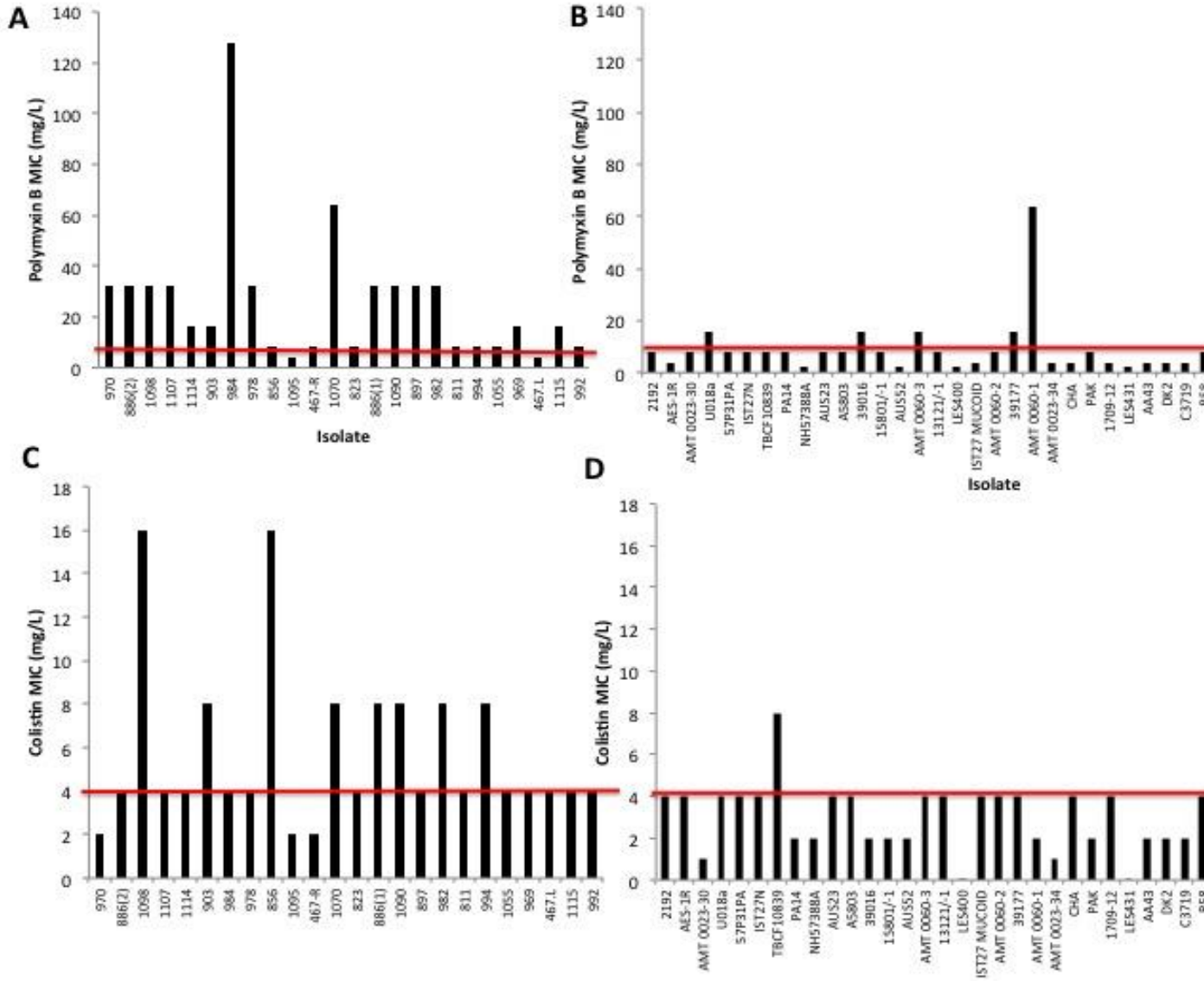


Figure 3.3. Levels of resistance of *P. aeruginosa* to polymyxins in isolates from companion animals and humans (calculated using median of triplicate MICs). A). Polymyxin B resistance in veterinary isolates B). Polymyxin B resistance in human isolates C) Colistin resistance in veterinary isolates D). Colistin resistance in human isolates.

There was some evidence for cross-resistance between the two polymyxins. All the eight veterinary-associated isolates that were resistant to colistin were also

resistant to polymyxin B. The single human associated *P. aeruginosa* isolate, that was resistant to colistin was also resistant to polymyxin B (Figure 3.3).

Resistance of veterinary-associated *P. aeruginosa* to other antibiotics

Disk diffusion susceptibility assays were performed using 5 commonly used antibiotics in humans; ciprofloxacin, ceftazidime, tobramycin, meropenem and tazobactam/piperacillin. No resistance was detected to ceftazidime, meropenem or tobramycin. Two isolates were resistant to ciprofloxacin and a further three displayed intermediate resistance. The two resistant isolates (1055 and 823) were both of canine origin. Isolate 823 was completely resistant to ciprofloxacin with no zone of inhibition (Figure 3.4). The isolates with intermediate resistance were 1095 (canine buccal swab), 467_L and 467_R (from the left and right ear of the same dog). One isolate was resistant to TZP, isolate 823, from a canine ear. Isolate 823 had the lowest susceptibility of all the isolates tested. It was resistant to only ciprofloxacin and tazobactam/piperacillin antibacterials (and intermediate to meropenem).

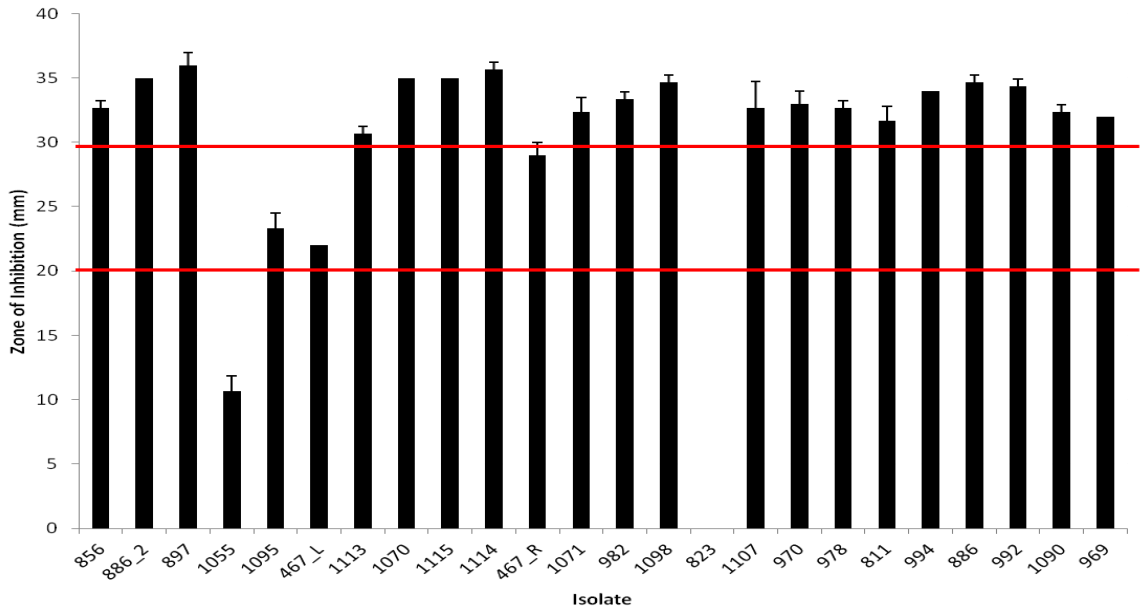


Figure 3.4 Ciprofloxacin resistance in veterinary *P. aeruginosa* isolates (n=24). The breakpoints are indicated by the red lines.

Using the Sensititre COMPAN1F mircotitre susceptibility assay, a panel of antibiotics commonly used for veterinary clinical diagnostics was tested. Table 3.3 shows the antibiotics with interpretation guidelines for *P. aeruginosa*.

Table 3.3 Antimicrobial resistance of *P. aeruginosa* isolated from companion animals (n=24)

Antimicrobial	Breakpoint mg/L	Resistant strains (*n*)	Intermediate strains (*n*)	Sensitive strains (*n*)	Resistant strains (%)
Amikacin	S=8, I=16), R>16	0	0	24	0
Ceftiofur	S=2, I=4, R>4	22	2	0	92
Enrofloxacin	S=0.5, I=2, R>2	8	13	3	33
Gentamicin	S=4, I=8. R>8	1	0	23	4
Imipenem	S=4, I=8. R>8	0	2	22	0
Marbofloxacin	S=1, I=2. R>2	5	2	17	21
Ticarcillin	S=64, R>64	5	0	19	21
Ticarc/clavulanic acid	S=64, R>64	1	0	23	4

No resistance to amikacin was detected in the companion animal isolates and only intermediate resistance was detected to imipenem. Four percent of isolates were resistant to gentamicin and ticarcillin/clavulanic acid. Higher levels of resistance were detected to ticarcillin (21%), marbofloxacin (21%) and enrofloxacin (33%). The majority of isolates were resistant to ceftiofur (92%). The three most resistant companion animal isolate to this panel of antibiotics were 823 (canine ear swab), 984 (canine ear swab) and 1055 (canine lip fold swab). Each of these isolates was resistant to four of the antibiotics panel tested including enrofloxacin, marbofloxacin and ticarcillin. These three isolates could be considered as multi drug resistant (MDR) being non susceptible to 3 antimicrobial categories; ie. anti-pseudomonal penicillin/beta lactamase inhibitor, fluroquinolones and a 3rd

generation cephalosporin. The companion animal isolates 467R and 467L (samples from the same dog but Right and Left ear) were the least resistant.

The human isolates were also tested using the Sensititre COMPAN1F mircotitre susceptibility assay. A similar trend in resistance was observed with the highest resistance to ceftiofur and the lowest to amikacin. The human isolates showed generally higher levels of resistance to the antibiotics tested (Table 3.4). The most resistant human isolate to this panel was AUST03.

Table 3.4. Antimicrobial resistance of a human panel of *P. aeruginosa* strains (n=37)

Antimicrobial	BreakPoints mg/L	Resistant strains (*n*)	Intermediate strains (*n*)	Sensitive strains (*n*)	Resistant strains (%)
Amikacin	S=8, I=16), R>16	2	3	32	5.4
Ceftiofur	S=2, I=4, R>4	35	2	0	95
Enrofloxacin	S=0.5, I=2, R>2	13	21	3	35
Gentamicin	S=4, I=8. R>8	10	0	27	27
Impinenem	S=4, I=8. R>8	8	7	22	22
Marbofloxacin	S=1, I=2. R>2	13	0	24	35
Ticarcacillin	S=64, R>64	8	0	29	22
Ticarc/clavulanic acid	S=64, R>64	7	0	30	19

Circulating strain types of *P. aeruginosa*

The Clondiag Tube Array has been used to determine the population structure of *P. aeruginosa* and a database of 955 isolates can be used to infer the wider population (Wiehlmann et al., 2007). This typing technique was used to classify the companion animal isolates. From the 24 isolates, 20 different array types were identified (Table 3.5).

Table 3.5. Clondiag Array Tube Hexidecimal codes of veterinary *P. aeruginosa* isolates. SNP=single nucleotide polymorphism.

ANIMAL ISOLATE NUMBER	SNP CODE	DETAILS OF ISOLATES SHARING THE SAME CLONE TYPE AS THE ANIMAL ISOLATES
1114	**2C12**	Water, Germany
467.L	**0002**	Same group as PA01 CF Germany. Pneumonia, Switzerland.
1115	**AF92**	COPD, USA.
467.R	**0002**	CF Germany. Pneumonia, Switzerland.
1098	**B429**	Keratitis, Great Britain. River, Germany.
1095	**AA0A**	Novel
978	**6C12**	CF, Italy. COPD, USA
984	**241A**	Acute infection, source unknown. Catheter, Germany.
823	**241A**	Acute infection, source unknown. Catheter, Germany.
982	**2FAA**	CF throat swab, Water, Italy and Germany
1070	**2D92**	Novel
811	**AA01**	Novel
1107	**2422**	Novel
992	**AF92**	COPD, USA.
856	**B420**	Sputum, nose and water, Germany.
969	**840A**	Keratitis, Bristol.
903	**B429**	Keratitis, Great Britain and River Germany.

994	**0C12**	CF sputum Germany.
897	**848A**	Novel
970	**0B9A**	Novel
886-1	**2B92**	Novel
886-2	**2B92**	Novel
1090	**2O22**	Novel
1055	**0812**	Found in CF patients, bacteraemia and ICU, France, Switzerland and Austria.

Eight of these were novel array types not previously identified in the database. One array type had previously been associated only with the environment from water). However, the remaining array types had been previously associated with human infections such as keratitis, cystic fibrosis and pneumonia lung infections and catheter associated infections (Table 3.5). Using the database of array types to generate a *P. aeruginosa* population structure, the isolates were mostly distributed amongst the main *P. aeruginosa* clades. Five isolates were located as outliers, isolates 811, 1107, 856 and the two isolates from the same dog, 467L and 467R (Figure 3.5). The latter two isolates share an array type with the widely used laboratory strain of *P. aeruginosa*, PAO1.

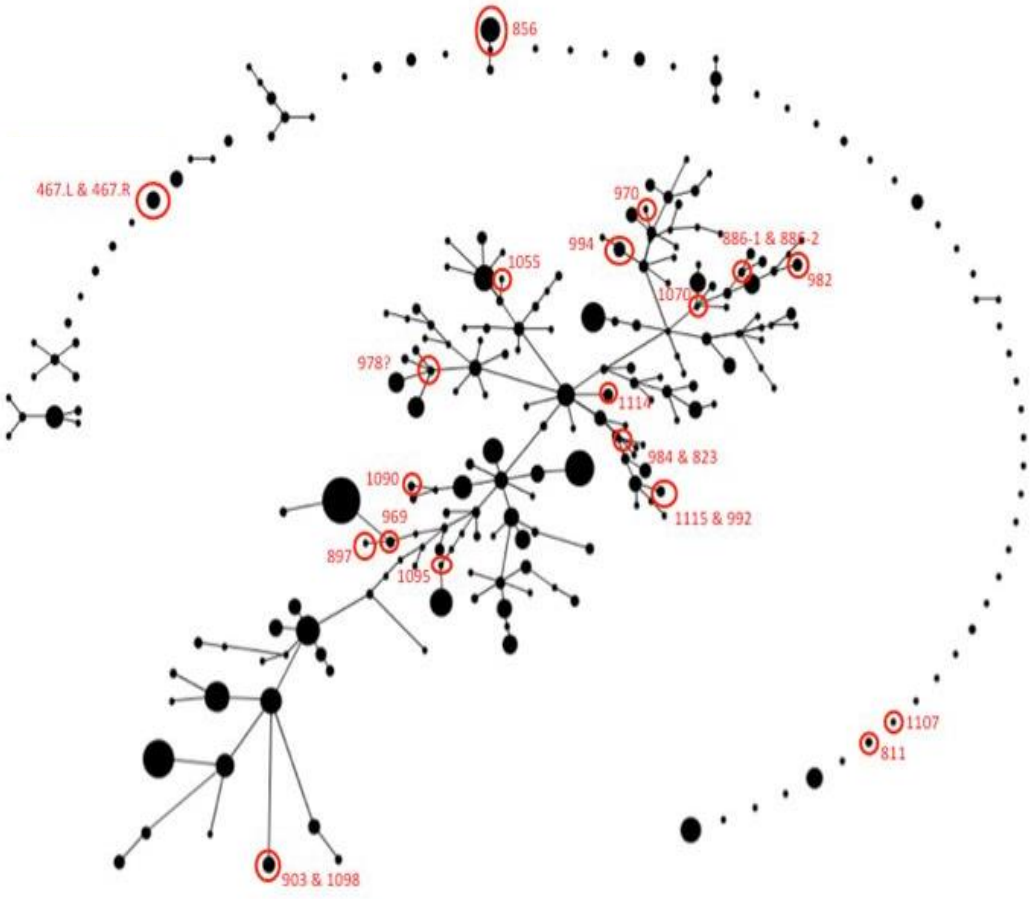


Figure 3.5. Distribution of the array tubes codes of the veterinary *P. aeruginosa* isolates amongst a larger panel of *P. aeruginosa* isolates (Cramer *et al.,* 2012) using Eburst analysis. Veterinary isolates highlighted in red.

Genome sequencing and analysis of the polymyxin resistome

Seven isolates that displayed resistance to colistin and polymyxin B were selected for whole genome sequencing. A phylogenetic tree of the veterinary isolates and 342 *P. aeruginosa* available genomes (Figure 3.6) shows that four of the isolates are located in clade I and another two in clade II of the main *P. aeruginosa* population. Although none of the isolates were found to be PA7-like, isolate 856 was diverse from the main population and was located on a new arm of the phylogenetic tree (Figure 3.6).

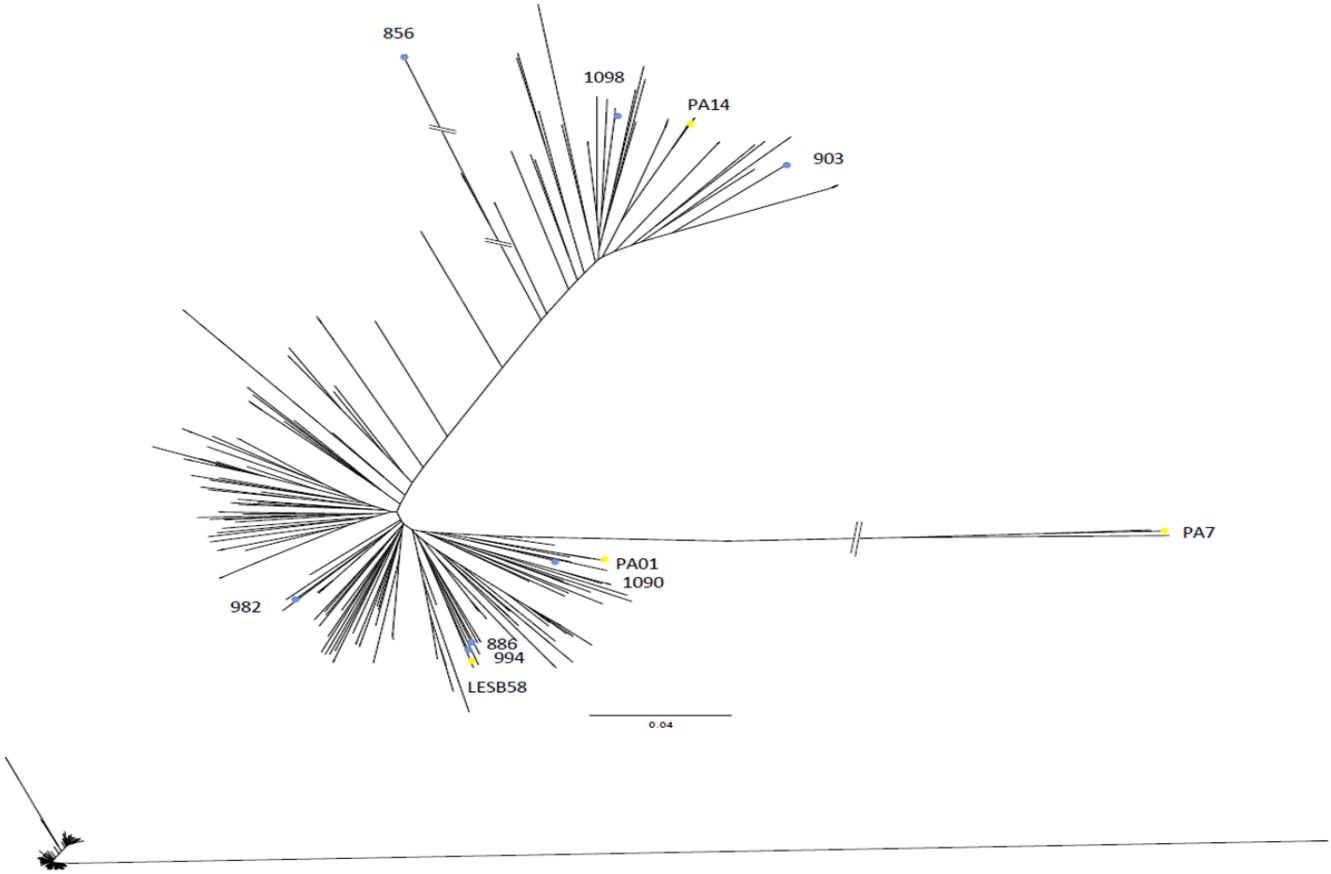


Figure 3.6. **Maximum likelihood tree using the Tamura-Nei substitution model of *P. aeruginosa* core genome SNPs.** The core genome is defined as polymorphic sites within 500bp fragments matching => 85% similarity in 342 genomes. The tree is based on 1336 phylogenetically informative polymorphic sites after gaps and N's were removed. Three clear groups are distinguishable: that containing PA14, PAO1 and PA7 (indicated by yellow coloured circles) respectively. Branches to PA7 and 856 (veterinary sequenced isolated indicated by blue coloured circles) have been truncated for clarity, so an impression of the real distances in the same tree has also been provided (the smaller diagram depicted below).

This genome had 125303 SNPs and 1884 indels compared to PAO1 and 130312 SNPs and 1985 indels in comparison to PA14 (Table 3.6). This was double the number of SNPs and indels than any other isolate sequenced.

Table 3.6. Counts of overall variable sites, SNPs and Indels between each polymyxin resistant isolate genome compared with PA01 and PA14 respectively, by short read mapping and variant calling. Ts = transition and Tv = Transversion.

Strain	SNP count	Indel count	Total count	Ts	Tv	Ts/Tv
vs PAO1 reference genome						
903	56361	887	57248	43128	13233	3.26
856	125303	1884	127187	92796	32507	2.85
1098	57288	927	58215	43734	13554	3.23
982	29399	498	29897	21979	7420	2.96
1090	28373	538	28911	21404	6969	3.07
994	28454	507	28961	21415	7039	3.04
886_1	29138	532	29670	22016	7122	3.09
vs PA14 reference genome						
903	35750	613	36363	27611	8139	3.39
856	130312	1985	132297	96630	33682	2.87
1098	42853	768	43621	32050	10803	2.97
982	59452	953	60405	45147	14305	3.16
1090	61403	1050	62453	46431	14972	3.1
994	60900	1004	61904	46129	14771	3.12
886_1	59378	950	60328	45211	14167	3.19

31 genes associated with resistance to polymyxins were analysed (Table 3.7 and Table 3.8).

Multiple stop codons were identified in isolate 856, these causing potential loss of function mutations. A stop codon in pyrC was identified in isolate 1098. Additional amino acid modifications resulting in a change in hydrophobicity were identified in all 7 genomes (Table 3.7).

Table 3.7. Amino acid changes (presence of stop codons and changes in hydrophobicity) identified in the 7 genome sequenced veterinary *P. aeruginosa* isolates, for a selection of 31 genes analysed through whole genome sequencing compared with PA01 and PA14 respectively, by short read mapping and variant calling.

Function	**Amino Acid/ Protein**	**856**	**886**	**903**	**994**	**982**	**1090**	**1098**
Cell metabolism	*pyrC*	G423STOP						G423STOP
associated	*pdxB*			V109K		D324A		V109K
	aroB	A200E	A200E	A200E	A200E	A200E	A200E	A200E
Acquired/adaptive	*parR*	L153R		L153R	L153R	L153R		L153R
resistance through								
LPS modification	*parS*	A115E						
Recycling of cell	*mpl*	A404P						
wall components		E451STOP						
Transcriptional	*ampR*	E114A	E114A	E114A				E114A
regulator associated		M228R	E172V	M228R				M228R
with resistance to		R291STOP						
antibiotics								
Activation of LPS-	*pmrA*	L71R	L71R		L71R		L71R	L71R
modifying operon by								
mutations in TCSs								
LPS biosynthesis-	*wapR*	W114R						
related functions		295STOP						
Modification of lipid	*arnC*	P23A						
A or Kdo with	*arnD*	A284D						
aminoarabinose	*arnF*	P137L						

This showed the presence of resistance determinants including beta-lactamase (PDC-1-7, amr), efflux (smeB, mex), fluoroquinolone resistance and elfamycin. Isolate 856 had the least additional resistance genes however this was the most divergent isolate through sequencing. In addition, the genome sequences were analysed using the CARD database (refer to Figure 3.7).

Table 3.8. All amino acid changes to the selected 31 genes, identified in the 7 genome sequenced veterinary *P. aeruginosa* isolates for a selection of 31 genes

analysed through whole genome sequencing compared with PA01 and PA14 respectively, by short read mapping and variant calling.

Gene	856	886	903	994	982	1090	1098
pyrC	D16G T184S Q247R N297S S355A R368S V402I G423STOP	D16G Q383E	D16G	D16G	D16G	D16G G130S R363H	D16G G423STOP
pyrB	P272S						
phoQ	D90E E267Q V369L						
pdxB	Q369L	A307V T337N Q365R	V109K H183Y P192T	A105V L310M A361T	E191G D324A Q365R	R179H R247H	V109K H183Y P192T
parR	T135A L153R		L153R S170N	P128S L153R S170N	L153R S170N		L153R S170N
Mpl	S221T I226S Q347R A404P E451STOP	V358I	V297M A415V	V297M			
rmlD	V98I						
ampR	E114A G283E M228R R291STOP	E114A E172V A208T	E114A G283E M228R				E114A I251V G283E M228R
tpiA	S47G T78I						
pmrA	L71R	L71R		L71R		L71R	L71R
wapR	A83T T85A W114R 295STOP	T85A	R58H R78K T85A	T85A	R78K T85A		R58H R78K T85A
Ssg	S35G						A109V
aroB	L54F Q58E V85A E89G A200E	V85A A200E	V85A A200E	V85A A200E S235L I297T	V85A A200E	V85A A200E I297T	V85A A200E
amgS	Q100R		I260V			I260V	I260V
galU					N338S		
pyrD							
sucC							
lptC							
arnA	T42I L50F H199R V250I T297A C312S S313G	C312S S313G S509N I551V	H59Y F80Y C312S S313G	C312S S313G I551V	C312S S313G	C312S S313G	F80Y C312S S313G

	V564I						
arnB	G120S T143A V153I I231V K286E V302A S321T H332R R363G E376D	A316V V302A	K286E V302A E376D	V302A A316V	V302A A316V	V302A	V302A K286E E376D
arnC	P23A E35G A265S T316A						
arnD	E25D F58L V123T G208S A284D		F58L				
arnE	A32T D33S T26A L56V A84V F87L S114N						R28H
arnF	A129T P137L				V14M	T106I	
arnT	C7W L93F L163F A267S P290A L337Q D440G T443A H447Y R502Q I509V	R502Q I509V	C7W H151Y L337Q I509V T16S E386D	R502Q	A267S R448H R502Q I509V	V266I A282S	C7W A214V T443A I509V
colS	Q60K V425I						
cprR	V27A I59V E183D						
cprS	V22I A78E D89G V159I V165I T170A E386D						T16S
parS	E90K A115E V304I H398R	H398R	H398R R243H	H398R	H398R	H398R	H398R
pmrB							
colR							

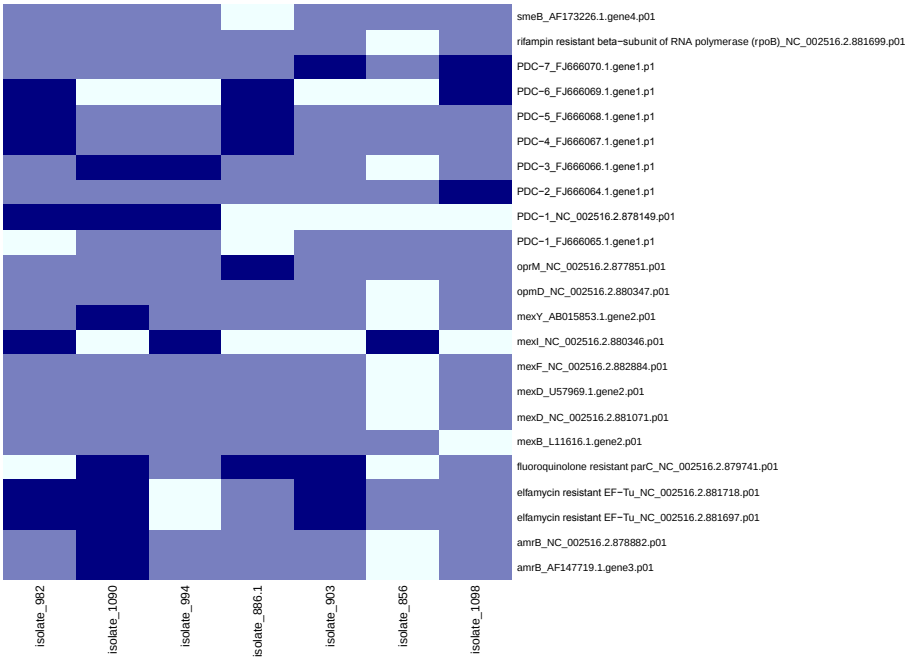


Figure 3.7. Heat map of resistance genes present in 7 veterinary *P. aeruginosa* isolates identified through whole genome sequencing. The shading of colour refers to the percentage similarity between the resistance genes in the 7 isolates (the darker colour representing highest % value (perfect match); mid-blue representing a variant of the known AMR gene sequence and white for no sequence present. Similar values are displayed as similar areas of colour.)

3.4 Discussion

In the veterinary setting *P. aeruginosa* can cause disease in a number of species. As previously discussed in earlier Chapter 1 these include ears, skin, respiratory tract, urinary tract, reproductive, ocular and wound infections. Similar to its nature in humans, *P. aeruginosa* is also less common as a primary cause of disease in the healthy animal. In canines, it is a frequently isolated pathogen in chronic otitis externa, otitis media and chronic deep pyodermas (Morris, 2004; Nuttall & Cole,

2007). One of the commonly used first line therapies for canine otitis externa is the effective commercial product Surolan® which contains polymyxin B as one of its active ingredients. It is licenced for use in cats and dogs for several ear conditions and skin infections.

A high proportion of the isolates within this companion animal sample set are of canine origin and from skin (including wounds) or ear, sample sites. A recent report looking at antimicrobial susceptibility profiles of bacterial isolates in the canine ear in Australia identified *P. aeruginosa* as one of the 5 most commonly isolated bacterial pathogen (Bugden, 2013). Of 3541 canine ear swabs, 35.5% isolated *P. aeruginosa*. However, although they raised concerns of resistance levels in the other Gram negative bacterial isolates (*Escherichia coli* and *Proteus sp.*) to polymyxin B, the resistance in *P. aeruginosa* isolates was comparatively low to polymyxin B (7%) and gentamicin (5%), although enrofloxacin was reasonably high (36%) (Bugden, 2013). This is in contrast to the findings presented in our study whereby polymyxin resistance levels of veterinary isolates was high. However, it is of note that the samples are from a referral centre and as such the cases seen here may be particularly extreme and difficult to treat having been sent from first opinion general practice veterinary surgeons.

A number of other diseases involving *P. aeruginosa* in animals are known, including that of contagious equine metritis (Allen et al., 2011) and in chronic equine wounds where the ability of *P. aeruginosa* to form and survive within protective biofilms has been reported (Freeman, 2009; Westgate 2011). Three of the samples within this study were from horses; a urine sample, a nasal swab and an abdominal incision wound and isolate 856 (equine abdominal incision swab) was shown to be the most diverse from the main *P. aeruginosa* population. These findings add further evidence that the veterinary niche is underrepresented in many population-wide studies (Guardabassi et al., 2004).

Polymyxin B is used as a common treatment in veterinary medicine and it is feasible that this longterm use in veterinary medicine has contributed to the increased

resistance. Therapeutics often use polymyxin B sulphate in the form of a topical preparation (eg. Surolan®). A number of other formulations that include polymyxin B for topical ocular solutions/ointments and ear preparations are available in other countries (eg. Australia, Netherlands) but these are not marketed in the UK. Polymyxin B in the form of polymyxin B sulphate administered intravenously has previously been reported as a use in equine colics as a treatment of endotoxaemia. Several studies document its efficacy (Barton et al., 2004; Werners et al., 2005). This would be an off license use. Polymyxin B is on the list of substances essential for the treatment of equidae for systemic treatment for endotoxaemia (antitoxigenic effect, not antibacterial as such) associated with severe colic and other gastrointestinal diseases (EU Agency, 2013; Barton et al., 2004; Richez & Burch, 2016). In the EU/EEA, colistin has been used in veterinary medicine since the 1950s (Koyama et al., 1950), primarily for pigs including group treatments and prevention of diarrhoea caused by *E. coli* and *Salmonella* spp., as first choice treatments for neonatal diarrhoea in piglets (Timmerman et al., 2006) and veal calves (Pardon et al., 2012) caused by *E. coli* as well as for the therapy of mild colibacillosis in poultry. Colistin is not a therapeutic in use for companion animals in the UK although it has previously been available as a licensed product for administration in various food production animals for the indication of diarrhoea. However, in 2015 the organisation RUMA (Responsible Use of Medicines in Agriculture Alliance) announced that they would voluntarily restrict the use of colistin in UK livestock (RUMA, 2015). This was following the reports of a new gene (*MRC-1*) that makes common bacteria resistant to colistin, reports have been in *E. coli* (Castanheira et al., 2016; Liu et al., 2016; Zhi et al., 2016a). There are now recent reports of *Salmonella* and *E. coli* colistin resistance reported in the UK (Munja et al.,2016). It is possible that the use of the polymyxins among veterinary species has driven resistance within this niche of *Pseudomonas* isolates. Hence we have seen higher levels of resistance in companion animal isolates as compared to that of the comparative human panel.

Within the EU Member States, colistin and polymyxin B are authorised nationally. The main indication for colistin in veterinary medicine is for preventing infection of

the gastrointestinal tract caused by non-invasive *E. coli* in pigs, poultry, cattle, sheep, goats and rabbits. Colistin is also used in laying hens and cattle, sheep and goats producing milk for human consumption. Colistin is also active against endotoxins produced by some *E. coli* strains in the gastrointestinal tract. Typically, colistin products are administered orally, in feed, in drinking water, as a drench, or through milk replacer diets. Combinations of colistin with other antimicrobials are available for group treatments of food-producing animals in some EU countries (EMA, 2016b). Products for parenteral and intra-mammary administration are also available, and infections due to Gram-negative bacteria in ruminants including endotoxaemia are claimed indications.

As in human medicine, colistin and polymyxin B are registered for topical administration to individual veterinary patients, except for food-producing animals in the case of polymyxin B, in the absence of Meat Residue Limits. In companion animals, prescription eye and eardrops are available with colistin alone, or in combination with other antimicrobials. Colistin tablets are available for calves for the prevention and treatment of neonatal colibacillosis. In some EU Member States, veterinary medicinal products (VMPs) containing colistin are not on the market, i.e. not commercialised (EMA/ESVAC, 2015).

Colistin products (polymyxin E) have never been marketed for use in animals in the United States (US Food and Drug Administration, 2016). Sources from the FDA have indicated that there is only one polymyxin B product (ophthalmic ointment, combination of polymyxin B and oxytetracycline) approved for use in food-producing species. In recent years, this product has been marketed in 2009 and 2012-2015, although it has been marketed in small quantities. Polymyxin B is also available in the US as a component of approved ophthalmic products (for use in dogs and cats) and otic products (for use in dogs). There is documented legal off-label use in other non-food-producing species, such as horses. Sources from the Public Health Agency of Canada have indicated that there are no approved colistin products (polymyxin E) for use in animals in Canada (Public Health Agency of Canada, 2016).

In 2013, the total sales of polymyxins in the 26 EU/EEA countries reporting data to the ESVAC project (EMA/ESVAC, 2015), including tablets (but excluding topical forms), polymyxins were the 5th most sold group of antimicrobials (6.1%), after tetracyclines (36.7%), penicillins (24.5%), sulphonamides (9.6%), and macrolides (7.4%). Total sales in weight summed up 495 tonnes. Of those 99.7% were for oral forms as follows: 43.3% were oral solution (powder and liquid for use in drinking water), 42.5% were premix (premixes for medicated feeding stuff) and 14.0% were oral powder (powder to be administered with the feed or milk). Small amounts were sold as: injectables (0.2%), tablets (0.1%) and intramammaries, intrauterines and oral paste (less than 0.0% for each of the three forms). Of the group of polymyxins, colistin represented more than 99.9% of the sales. In addition combinations of colistin with other antimicrobials are authorised in some Member States. The sales of those combination products represented less than 10% of the overall sales of colistin (EMA/ESVAC, 2015). Colistin is used in aquaculture for the prevention of Gram-negative infections (Xu et al., 2012), consumption data are not available separately for this food production sector. In the Danish monitoring programme (DANMAP), details on consumption do not refer to the use of colistin in fish (DANMAP, 2012; EMA 2016).

Resistance to polymyxins has been attributed to modifications to the outer membrane include modifications to lipid A and LPS as well as two-component regulators.

The outer membrane normally serves as a protective barrier for Gram-negative bacteria against the cationic antimicrobial peptides. The main difference between the two molecules (polymyxin B and polymyxin E) being that polymyxin B contains phenylalanine in position 6 (from the N-terminal), while colistin contains D-leucine (Falagas & Kasiakou, 2005; Yu et al., 2015). Fernandez et al (Fernández et al., 2013) reported a relatively small polymyxin B resistome involving 17 susceptibility/intrinsic resistance determinants. This study demonstrated considerable cross-resistance in susceptibility to polymyxin B and to colistin. This would suggest that the resistance genes of *P.aeruginosa* appear to have the same

effect on colistin as they do on polymyxin B and suggests some cross-resistance in the two polymyxins. Seven of the veterinary isolates were genome sequenced and mutations, including stop codons, were detected in genes previously implicated in polymyxin resistance. Many of the sequences studied showed modifications in genes leading to an altered amino acid, many of which had altered hydrophobic/hydrophilic properties. Here, it is possible that a combination of mutations leads to a change in phenotype through membrane remodelling.

A study by Manivannan et al (Manivannan et al, 2016) looking at a draft genome of a MDR *P. aeruginosa* clinical isolate (from a female patient's urine sample having had ureteric reimplant) documented the presence of genes responsible for virulence factors, quorum sensing and biofilm formation. The isolate was extensively drug resistant and found to be resistant to penicillins, second and third generation cephalosporins, aminoglycosides, carbapenems, tetracyclines, quinolones, and trimethoprim-sulfamethoxazole and sensitive to only colistin, as tested with the automated antibiotic susceptibility testing system Vitek®-2 (Manivannan et al., 2016). In their study, analysis indicated that the draft genome has genes encoding for resistance to aminoglycosides, fosfomycin, beta-lactams, sulfonamides, and trimethoprim (Manivannan et al., 2016).

A study by Lee et al (Lee et al., 2014), investigated genetic variations involved in the acquisition and loss of colistin resistance in three clinical isogenic *P. aeruginosa* isolates taken from a single patient and assessed their impacts on colistin resistance.
Through the use of whole genome sequencing to identify single nucleotide polymorphisms and insertions or deletions in two colistin-resistant isolates, which, were then compared with a susceptible isolate. Their results showed 37 non-synonymous mutations in 33 coding sequences detected in the colistin-resistant isolates. One gene was significantly down-regulated in both colistin-resistant isolates (a gene encoding erythronate-4-phosphate dehydrogenase). Eight genes were up-regulated in the colistin-resistant isolates. Of these three encoded hypothetical proteins and five were predicted to be involved in core biological

functions, encoding a cell wall-associated hydrolase, a response regulator EraR, a sensor/response regulator hybrid, a glycosyltransferase and an arabinose efflux permease (Lee et al., 2014). None of theses genes were identified in our polymyxin study. They may be novel determinants that may be associated with the acquisition of colistin resistance (Li., et al, 2014).

Using both the tube array typing method and genome sequencing on a limited subset of isolates, the *P. aeruginosa* veterinary isolates were found to be distributed thoughout the *P. aeruginosa* population. Using tube array, 9 of the isolates had no previous description and were classified as novel. 15 isolates shared array types with isolates previously found to cause human infections such as pneumonia, cystic fibrosis and chronic obstructive pulmonary disease lung infection, keratitis and bacteraemia and therefore highlight the ability of these bacteria to cause human infections. Using whole genome sequencing, the veterinary isolates were generally clustered within the main *P. aeruginosa* population however one isolate, 856, did not cluster with any other previously sequenced isolate. Through mapping to both PAO1 and PA14, the isolates had double the number of SNPs and indels than any of the other isolates sequenced. The isolate was of equine origin and this highlights the importance of studying alternative and underrepresented niches in order to fully characterise the *P. aeruginosa* pan genome.

In this study it is likely that polymyxin resistance involved chromosomal mutations and there was no evidence of mcr-1 plasmid mediated colistin resistance. However, a recent study discussed the emergence of plasmid-mediated colistin resistance mechanism MCR-1 resistance in humans and animals (Liu et al., 2016). This study was based in China and during a routine surveillance project on antimicrobial resistance in commensal *E.coli* from food animals in China, a major increase of colistin resistance was observed. When an *E. coli* strain, SHP45, possessing colistin resistance that could be transferred to another strain, was isolated from a pig, further analysis of possible plasmid-mediated polymyxin resistance was carried out. This particular study demonstrated the emergence of the first plasmid-mediated

polymyxin resistance mechanism, MCR-1, in *Enterobacteriaceae*. The plasmid-borne mcr-1 gene encodes a phosphoethanolamine transferase that mediates addition of phosphoethanolamine to the lipid A moiety of the lipopolysaccharide and thus transferring resistance to polymyxins (Liu et al., 2016). This is a gene that to date has predominantly been identified in *E. coli* and also *Klebsiella spp* and *Salmonella spp*. Since the first report by Liu et al 2016, further identifications worldwide have emerged with reports in countries including in Algeria, Denmark, France, Germany, Laos, the Netherlands, Malaysia, Nigeria, Switzerland and Thailand (Castanheira et al., 2016; Doumith et al., 2016; Falgenhauer et al., 2016; Kluytmans-van den bergh et al., 2016; Liu et al., 2016; Malhotra-Kumar et al., 2016; Rolain et al., 2016; Liu et al., 2016; Zhi et al., 2016). The gene was also identified from imported food products in Denmark – meat products (Hasman & Hammerum, 2015) and Switzerland, vegetables (Zurfluh, 2016). Plasmid-encoded carbapenem and colistin resistance may be co-associated in *E. coli* (Falgenhauer et al., 2016). Positive Salmonella *enterica* isolates of different serotypes were identified from food samples in Portugal in 2011, and France in 2012 and 2016 (Figueiredo et al., 2016; Lin et al., 2012; Zhi et al., 2016a). An epidemiological survey conducted in France on a collection of ESBL-producing *E. coli* isolates recovered from the faeces of diarrhoeic veal calves on farms from 2005 to 2014 showed a very high rate of MCR-1-positive isolates (20.5%) (Haenni et al., 2015). Following these new reports it was felt prudent to carry out further genomic assessment (courtesy of Matthew Moore, University of Liverpool) of the seven *P. aeruginosa* isolates that had already undergone WGS in this study. This additional work was performed by Matthew Moore and Dr Jo Fothergill (University of Liverpool) after the main body of this thesis work was completed (as this was only a one year funded Fellowship). The seven isolates were screened for the presence of the *mcr-1* determinant but none were identified in this instance. As yet there does not appear to be reports of plasmid encoded polymyxin resistance mechanism *mcr-1* in *P. aeruginosa* isolates (Baron et al., 2016).

In conclusion, all of these findings raise concerns regarding the use of polymyxins within the veterinary community whereby their use may help drive development of

polymyxin B/colistin resistant *P. aeruginosa* strains. These could then potential act as a source of resistant genetic material for the human associated *P. aeruginosa* population. The findings suggest that future surveillance of polymyxin resistance in animal associated *P. aeruginosa* strains should be recommended and highlights the importance of improved correspondence between veterinary and medical professions with regards to use of polymyxins. The veterinary setting, particular in companion animals is an, often ignored, niche that provides close proximity between humans and companion animals and therefore cross infection of resistant organisms would be possible. The preservation of colistin as a "last resort" successful anti-pseudomonal drug is of great concern (Kempf et al, 2016).

3.5 Conclusions

In this study, the increased resistance to polymyxin antibiotics, polymyxin B and colistin in a panel of veterinary isolates from companion animals is described. To the author's knowledge, this is the first description of increased resistance to colistin in this environmental niche. Resistance to other commonly used human antibiotics was low.

Companion animals are an understudied source of antimicrobial resistant *P. aeruginosa* isolates however, resistance to polymyxin amongst these isolates is high. These findings merit sustained surveillance of the veterinary niche as a potential reservoir for resistant, clinically-relevant bacteria.

4. **PCR characterisation of antibiotic resistant determinants in *P. aeruginosa* from companion animals**

4.1 Introduction

AMR mechanisms in *P. aeruginosa*

P. aeruginosa can develop resistance to antibacterials either through the acquisition of resistance genes on mobile genetic elements (i.e.integrative and conjugative elements (ICEs)) or through mutational processes that alter the expression and/or function of chromosomally encoded mechanisms. The main mechanisms associated with antibiotic resistance are shown in Figure 4.1

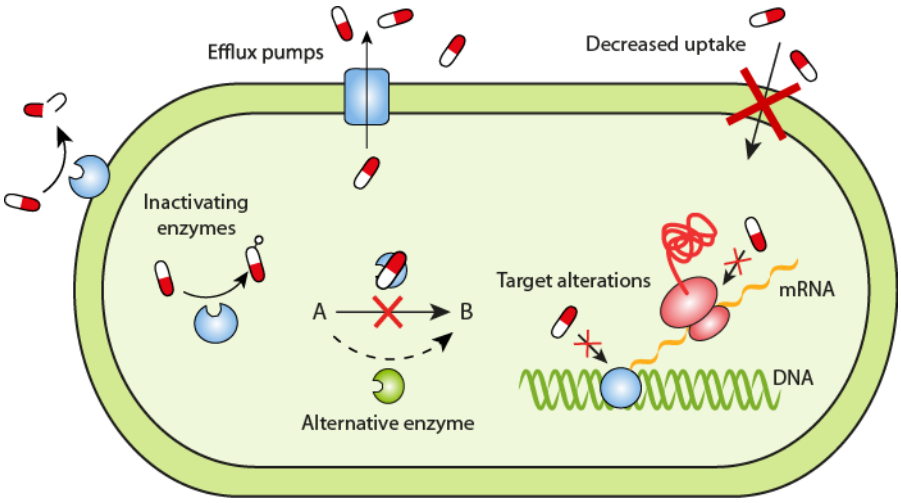


Figure 4.1 Mechanisms of resistance in *P. aeruginosa*. Image from (Gullberg et al., 2011)

Transmissible resistance refers to the ability to spread resistance genes via horizontal gene transfer. ICEs (including plasmids) are found in particular in Enterobacteriaceae where they may play a role as important carriers of antimicrobial resistance genes. Like plasmids, ICEs can exist in *P. aeruginosa* as circular extrachromosomal elements and can impact on resistance to the beta-lactams and aminoglycoside antibiotics. This is in contrast to resistance mechanisms to fluoroquinolone among *P. aeruginosa* isolates which has mainly been associated with chromosomal genes mutations such as *gyrA, gyrB*, *parC* and *parE* and/or overexpression of multidrug efflux pumps (Fàbrega et al, 2009). Although the plasmid-mediated *qnr* and the fluoroquinolone-modifying enzyme *aac* 6Ib-cr can contribute to fluoroquinolone resistance among strains of *Enterobacteriaceae* (Jacoby, 2005; Robicsek et al., 2006; Strahilevitz et al, 2009b), these two plasmid-encoded mechanisms have not been found in clinical isolates of *P. aeruginosa.*

Resistance to the beta-lactams involves the production of inactivating beta-lactamases and several families of these have been identified among clinical isolates of *P. aeruginosa* (Lister et al., 2009). The most common imported beta-lactamases found among *P. aeruginosa* isolates are penicillinases. The therapeutic impact of these penicillinases is relatively limited since they do not impact the clinical efficacy of extended-spectrum cephalosporins, monobactams, or carbapenems.

P. aeruginosa shows high intrinsic resistances to antibiotics and this can, in part, be attributed to its' low outer membrane permeability. The low permeability can further contribute to other resistance mechanisms such as inducible cephalosporinase or antibiotic efflux pumps therefore leading to highly resistant bacteria. *P. aeruginosa*, in addition to being intrinsically resistant to several antimicrobial agents, can acquire resistance to conventional anti-pseudomonal antibiotics including anti-pseudomonal penicillins, ceftazidime, carbapenems, aminoglycosides and ciprofloxacin.

Table 4.1 summarises the resistance mechanisms of *P. aeruginosa*.

Table 4.1. Examples of resistance mechanisms in *Pseudomonas aeruginosa.*

(Referenced from Gellatly et al., 2012).

Mechanism	Example(s)
Efflux pumps	MexAB–OprM, MexCD–OprJ, MexEF–OprN, MexXY–OprM (cephalosporins, carbapenems, aminoglycosides, quinolones, ureidopenicillins)
Outer membrane impermeability	OprF, OprD, OprB (carbapenems, aminoglycosides, quinolones)
β-lactamases	AmpC (penicillins)
Targeted mutation	DNA gyrase, DNA topoisomerase (quinolones) MexZ (quinolones, cefepimes, aminoglycosides)
Horizontal transfer	Metallo-β-lactamases, ESBLs (penicillins, cephalosporins, carbapenems)
Membrane changes	Lipid A modification (aminoglycosides, polymyxins) AmpC upregulation (penicillins)

ESBL, extended spectrum β-lactamase

Inherent and aquirred resistance can severely limit the therapeutic options for treatment of serious infections (Lister et al., 2009).

Resistance types

Changes can be made to a bacteria's genetic inheritance in two ways; through mutations that alter the pre-existing DNA of the cell—these alterations, base changes and DNA deletions, insertions and inversions (Avison, 2005), change genes already possessed but do not add new genes, to the cell genome. Alternatively, resistance can also be obtained through the acquisition of new genetic material (Bennett 2008).

1. Acquisition of additional genetic material

In bacteria, new genetic material can be acquired through mobile genetic elements (ICEs) and can include plasmids, phages, transposons and integrons. The acquisition of new genetic material is a naturally occurring process in bacteria and is facilitated

by the lack of a nucleus membrane protecting the genome. A study by Johnston et al (Johnston., et al 2014) stated a total of 82 species having been shown to be naturally transformable showing that acquisition through transformation is common amongst many different species of bacteria. Gene transfer through plasmids is one of the major routes for spreading antibiotic resistance in bacteria. Many of the ICEs carrying antibiotic-resistant genes can be transferred between different species of bacteria and therefore widespread resistance to a specific antibiotic can develop rapidly. One example is a plasmid carrying a gene that encodes the enzyme beta-lactamase. Beta-lactamases alters the structure of the beta-lactam antibiotics (such as penicillin), rendering them inactive (Araujo et al., 2016; Drawz & Bonomo, 2010). Outbreaks of life-threatening multi-drug resistant superbugs such as New Delhi metallo-β-lactamase-1 (NDM-1) bacteria are often due to the transfer of antibiotic resistance genes in ICEs (Abraham et al, 2014; Ji et al, 2007; Liu et al., 2016; Poirel et al., 2010; Schmidt & Beutin, 1995).

Integrons are genetic elements first described at the end of the 1980s (Stokes & Hall, 1989). Although most integrons were initially described in human clinical isolates, they have now been identified in many non-clinical environments, such as water and soil (Domingues et al, 2012; Nardelli et al., 2012). Integrons consist of two conserved segments of DNA separated by a segment of variable length and a sequence often carrying resistance determinants. Class 1, 2 and 3 integrons are recognized (Domingues et al., 2012). Most resistance integrons belong to class 1, which is the most frequent among isolates of human origin and has been found in *P. aeruginosa* isolates from dogs (Lin et al., 2012; Rubin et al., 2008).
The class 1 integrons are important in the dissemination of resistance in Gram-negative bacteria. Class 1 integrons being important in the worldwide problem of antibiotic resistance, as they can capture and express diverse resistance genes and may often be embedded in plasmids and transposons, facilitating their lateral transfer into a wide range of pathogens (Gillings et al., 2008).

As described above, the acquisition of new genetic material requires gene transfer from some outside source. Bacteria have three methods by which DNA may be transferred from one cell to another; transformation, transduction and conjugation (Bennett, 2009).

Resistance acquisition mechanisms:

i) Conjugation –

Conjugation is the process by which one bacterium transfers genetic material to another through direct contact. During conjugation, one bacterium serves as the donor of the genetic material, and the other serves as the recipient. Conjugative plasmids, commonly present in bacterial cells (Sherley et al, 2003; Shintani et al, 2015) can be transferred to other cells by conjugation. This process often requires sex pili to establish a mating-pair by promoting cell aggregation (Cabezón et al, 2015). Conjugation usually imposes a fitness cost on plasmid hosts as it requires resources, alters cell physiology and determines viral susceptibility because sex pili can serve as receptors for viruses (Cabezón et al., 2015).

This requires the participation of either a conjugative plasmid or a conjugative transposon, each of which encodes a DNA transfer system that has evolved specifically to mediate horizontal transfer of itself. Conjugation is responsible for much of the horizontal gene transfer, particularly of resistance genes, seen among prokaryotic cells, both Gram-negative and Gram-positive (Van Hoek et al., 2011).

ii) Transduction –

This involves gene transfer by bacterial 'viruses', called bacteriophages ('phages'). Phages tend to have very narrow host ranges. Transduction is the transfer of DNA from one cell to another, mediated by a bacteriophage and occurs as a

consequence of rare errors in phage reproduction, when a small percentage of the phage particles produced contain DNA sequences from the host cell, in place of or in addition to the normal phage genome. Transduction is responsible for some of the transfer of bacterial drug resistance genes (Munita et al, 2016). Fragments of bacterial genomes or small bacterial plasmids can be moved from one bacterial cell to another in this way. Survival of the DNA in the new cell requires either recombination, prophage integration or the transferred DNA must be able to replicate independently in its new host i.e. act as a plasmid (Van Hoek et al., 2011).

iii) Transformation –

Transformation requires no connection between the donor and recipient cells, other than the DNA itself. The bacterial cell uptakes the DNA from its environment and incorporates it into its own genome (Van Hoek et al., 2011).

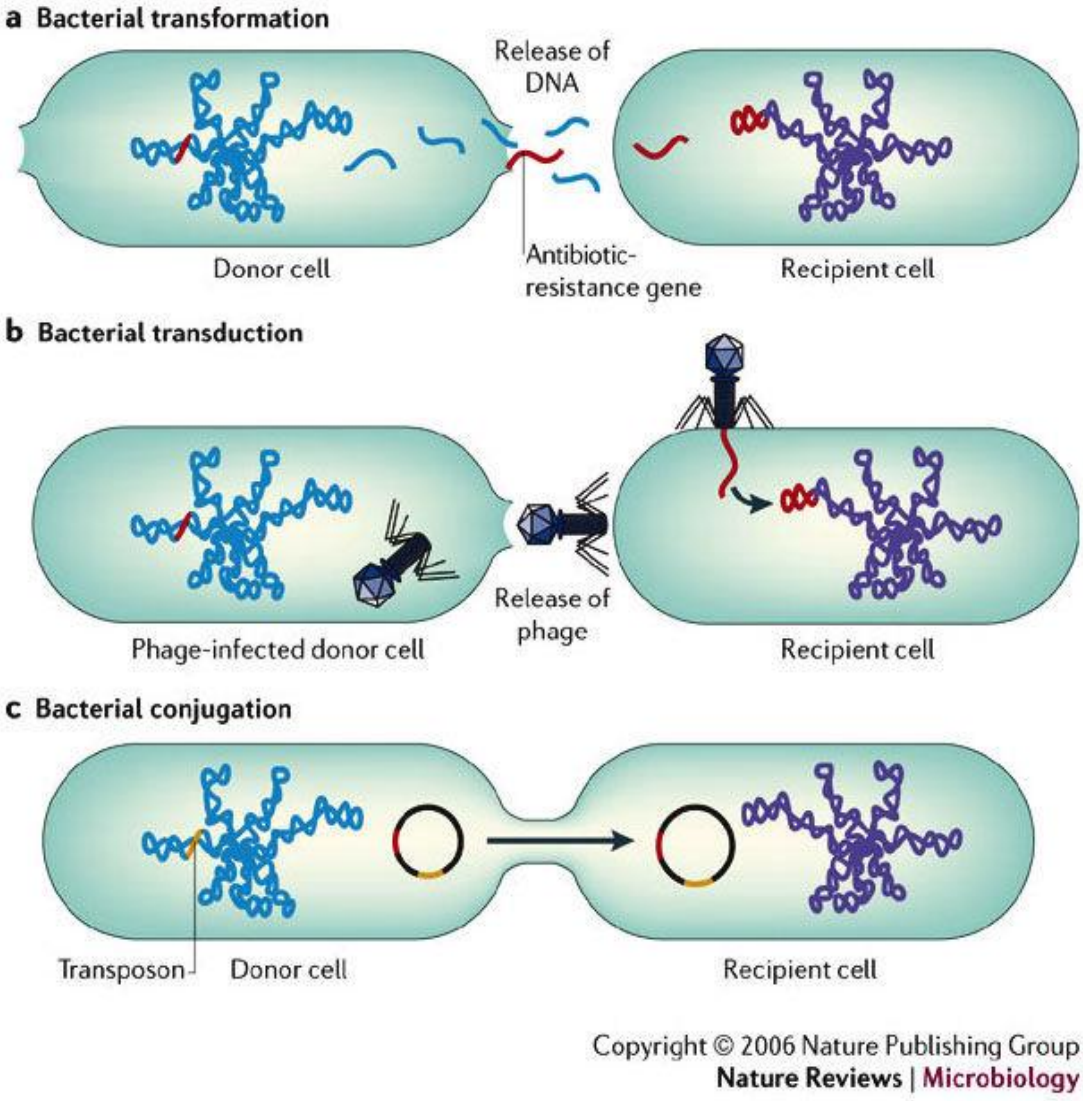


Figure 4.2 The processes of bacterial, transformation(a), transduction(b) and conjugation(c). (Furuya & Lowy, 2006)

a. Transformation occurs when DNA is released on lysis of an organism and is taken up by another organism. The antibiotic-resistance gene can be integrated into the chromosome or plasmid of the recipient cell.

b. In transduction, antibiotic-resistance genes are transferred from one bacterium to another by means of bacteriophages and can be integrated into the chromosome of the recipient cell.

c. Conjugation occurs by direct contact between two bacteria: plasmids form a mating bridge across the bacteria and DNA is exchanged, which can result in acquisition of antibiotic-resistance genes by the recipient cell. Transposons are sequences of DNA that carry their own recombination enzymes that allow for transposition from one location to another; transposons can carry antibiotic-resistance genes. (Furuya & Lowy, 2006)

2 Intrinsic Resistance:

Intrinsic resistance is the ability of a bacterial species to resist activity of a particular antibacterial agent through its inherent structural or functional features, which allow tolerance of a particular drug or class of antibacterials (Munita et al., 2016). This characteristic may be due to a lack of affinity of the drug for the bacterial target. An example of such would be *P. aeruginosa* and the sulphonamides/trimethoprim, tetracyclines and chloramphenicol whereby there is a lack of uptake. Intrinsic resistance may also be due to inaccessibility of the drug into the bacterial cell. This is the case for vancomycin and Gram-negative bacteria, whereby the lack of uptake results from inability of vancomycin to penetrate the outer membrane. Intrinsic resistance may be due to the extrusion of the drug by chromosomally encoded active exporters or innate production of enzymes that inactivate the drug. For example the production of beta lactamase enzymes by that destroy beta lactam antibiotic such as ampicillin and destroy it before it can reach the PBP (penicillin binding protein) target (Kong et al, 2010; Zeng & Lin, 2013) or AmpC type beta lactamases which confer resistance to beta lactams. *P. aeruginosa* also has constitutive expression of AmpC beta-lactamase (cephalasporinases) and efflux pumps, combined with a low permeability of the outer membrane (Cabot et al., 2011; Jacoby, 2009). The natural resistance of the species relates to the beta-lactams: penicillin G; aminopenicillins, including those combined with beta-lactamase inhibitors; first and second generation cephalosporins. Strains of a particular bacterial species will exhibit the same mechanisms of innate resistance and this is unaffected by prior antibiotic exposure (Beceiro et al., 2013; Schroeder et al, 2017).

P. aeruginosa as discussed is intrinsically resistant to several antibiotics because of the low permeability of its outer-membrane, the constitutive expression of various efflux pumps, and the production of antibiotic-inactivating enzymes (e.g. constitutive expression of AmpC beta-lactamase - cephalosporinases). Furthermore, it also has a remarkable ability to develop or acquire further mechanisms of resistance to multiple groups of antimicrobial agents, including

beta-lactams, aminoglycosides and fluoroquinolones (Mesaros et al., 2007a; Mesaros et al., 2007b). *P. aeruginosa* exhibits many of the known mechanisms of antimicrobial resistance; derepression of chromosomal AmpC cephalosporinase; acquisition of integron-mediated beta-lactamases from different molecular classes (carbenicillinases and extended-spectrum beta-lactamases belonging to class A, class D oxacillinases and class B carbapenem-hydrolysing enzymes); diminished outer membrane permeability (loss of OprD proteins); overexpression of active efflux systems with wide substrate profiles; synthesis of aminoglycoside-modifying enzymes (phosphoryltransferases, acetyltransferases and adenylyltransferases); and structural alterations of topoisomerases II and IV determining quinolone resistance. Concerningly, these mechanisms are often present simultaneously, thereby conferring multi-resistant phenotypes (Strateva & Yordanov, 2009).

Membrane Permeability

P. aeruginosa, has a relatively impermeable outer membrane (OM) and expression of numerous MDR efflux pumps that effectively reduce the intracellular concentration of the given drug (Li et al., 1995). The OM is a semi-permeable barrier to the uptake of antibiotics or nutrient molecules. Small hydrophilic molecules (such as beta-lactam antibiotics) (refer to Figure 4.3. Blair et al, 2015) have uptake restricted to a small portion of the OM (namely the water filled channels of porin proteins) and as a result the entry of these molecules into the cell is limited (Hancock, 1998). This low overall OM permeability is especially true of *P. aeruginosa,* which is considered to be lower than some other Gram negatives. It is estimated that the permeability of *P. aeruginosa* and *Acinetobacter baumannii* outer membranes are only 1-8% of that *E. coli* and that non specific slow porins OprF and OmpA-AB, respectively, restrict access of molecules larger than approx. 200Da, which is the size of a typical monosaccharide (Zgurskaya et al, 2015). The major porin OprF is responsible for the large exclusion limit of the *P. aeruginosa* OM. The porin OprD only mediates passage of zwitterionic carbapenem beta lactams (eg. imipenem, meropenem) see Figure 4.5), as this porin contains a binding site specifically for these dipeptides. Uptake across the OM of polycationic antibiotics (eg. gentamicin, tobramycin and colistin) are mediated by a system

termed self-promoted uptake. This system involves the interactions of the polycation with divalent cation binding site that are on the LPS cell surface and that normally stabilise the OM. Since the polycationic antibiotic molecules are much larger than the native divalent cations, they cause a disruption to the OM that makes it become more permeable. – see Figures 4.4 and 4.5 (Lister et al., 2009).

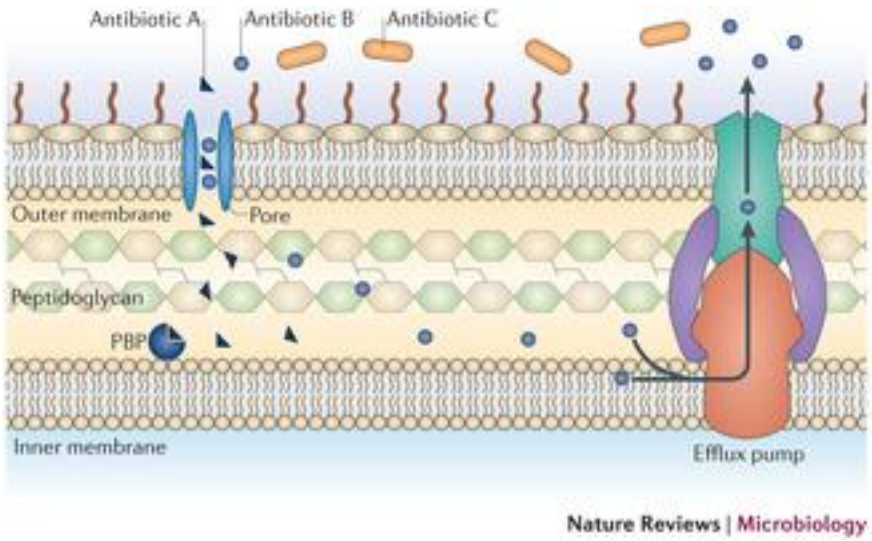


Figure 4.3 Overview of intrinsic resistance mechanisms (Blair et al, 2015).

Depicting β-lactam antibiotics targeting a penicillin-binding protein (PBP). Antibiotic A can enter the cell via a membrane-spanning porin protein, reach its target and inhibit peptidoglycan synthesis. Antibiotic B can also enter the cell via a porin, but unlike Antibiotic A, it is efficiently removed by efflux. Antibiotic C cannot cross the outer membrane and so is unable to access the target PBP (Blair et al, 2015).

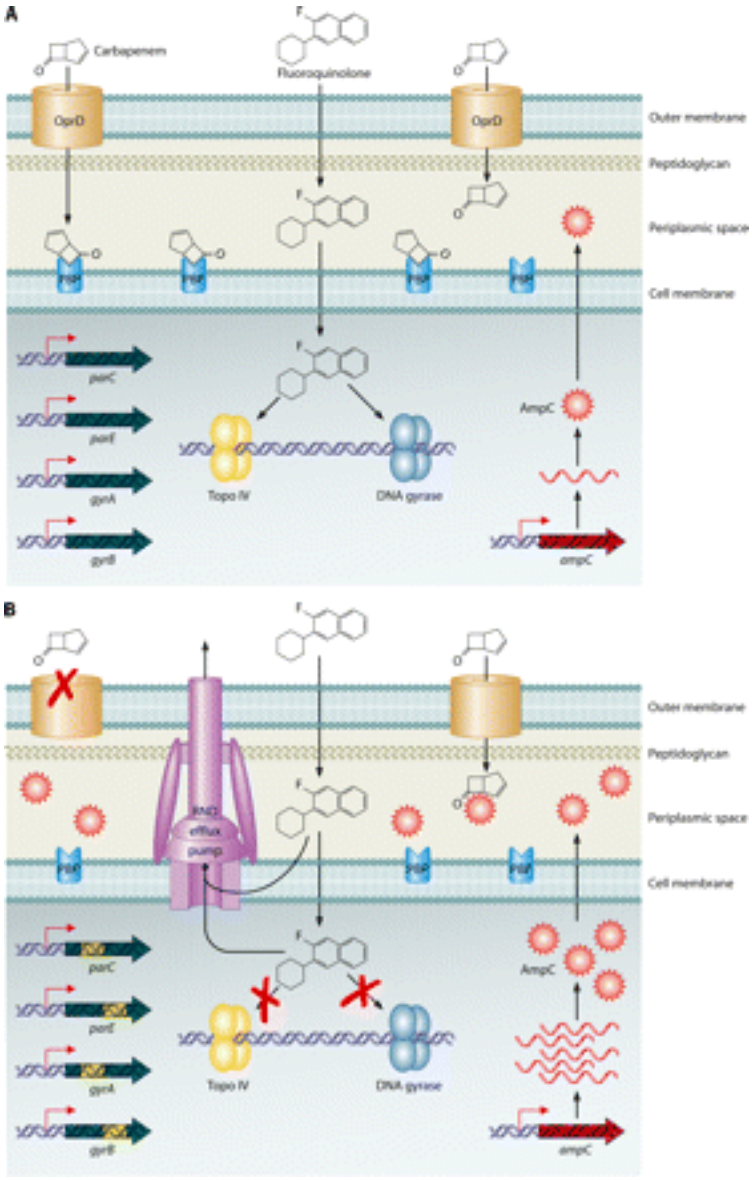


Figure 4.4 Mutational resistance to fluoroquinolones and carbapenems involving chromosomally encoded mechanisms expressed by *P. aeruginosa* (Lister et al., 2009).

Image (A) Depicts interactions of fluoroquinolones and carbapenems with susceptible *P. aeruginosa* expressing basal levels of AmpC, OprD, and non-mutated fluoroquinolone target genes (gyrA, gyrB, parC, and parE). Fluoroquinolone molecules pass through the outer membrane, peptidoglycan, periplasmic space, and cytoplasmic membrane and interact with DNA gyrase and topoisomerase IV (Topo IV) targets in the cytoplasm when these enzymes are complexed with DNA. The carbapenem molecules pass through the outer membrane-specific porin OprD and interact with their target PBPs (Penecillin-binding proteins), located on the outside of the cytoplasmic membrane.
Image (B) Depicts chromosomally encoded mechanisms of resistance to fluoroquinolones and carbapenems. Fluoroquinolone resistance is mediated by (i) overexpression of RND efflux pumps extruding the drug molecules from the periplasmic and cytoplasmic spaces and/or (ii) mutational changes within the target genes. Locations of the QRDRs within target genes are highlighted in yellow. Carbapenem resistance is mediated primarily by (i) decreased production or loss of functional OprD in the outer membrane and/or (ii) overproduction of RND efflux pumps (with the exception of imipenem). Minor changes in susceptibility can be observed due to overexpression of AmpC, adding to the resistance potential (Lister et al., 2009)

Efflux Pumps

Efflux pumps are transport proteins involved in the extrusion of toxic substrates from within cells into the external environment (Figure 4.5). These are known to be important for intrinsic resistance of *P. aeruginosa* to antibiotics such as some fluroquinolones, beta-lactams (except imipenem), tetracyclines and chloramphenicol antibiotics. The active efflux systems of *P. aeruginosa* involve a number of efflux systems including MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM (Webber & Piddock, 2003).

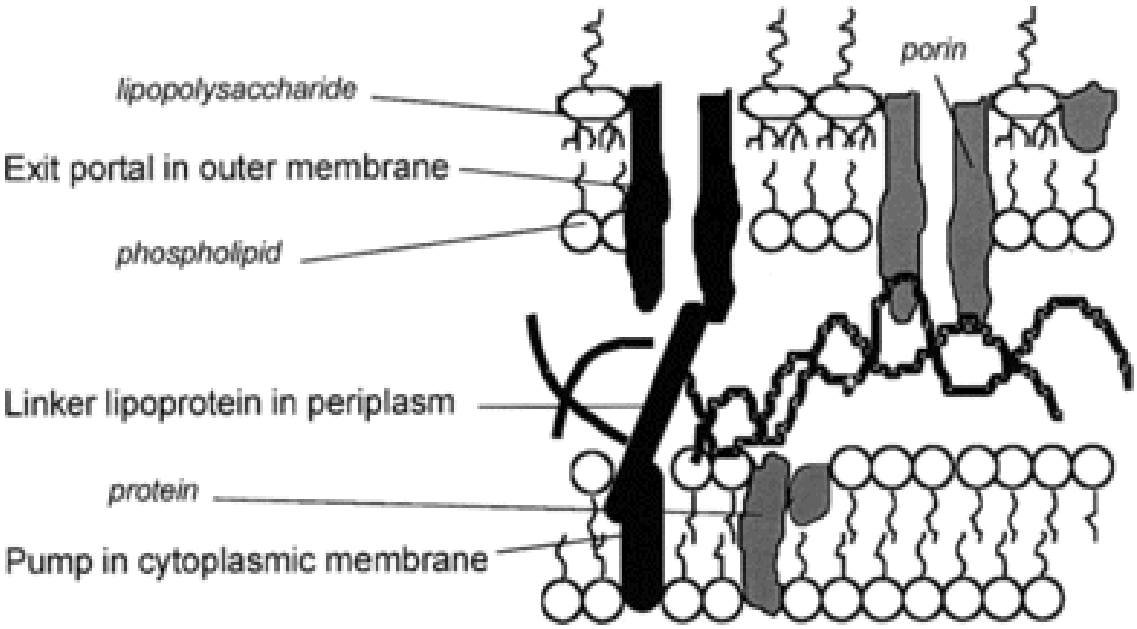


Figure 4.5. Three-component efflux pump (Livermore, 2002).

The pump itself (MexB, MexD, or MexF, according to the system) lies in the cytoplasmic membrane and is attached via a linker lipoprotein (MexA, MexC, or MexE) to the exit portal (OprM, OprJ, or OprN). Efflux system components appear in large roman type; other membrane components appear in small italic type (Livermore, 2002).

3. Mutation of existing DNA:

This refers to the change by mutation of an existing gene product or control mechanism. Changes in the bacterial genome through mutation may consequently lead to a change in the nature of proteins expressed by the organism and this can result in changes in antibacterial resistance. There are many mechanisms of resistance through the accumulation of mutations. These are often through gene 'loss of function' mutations resulting from small nucleotide polymorphisms (SNPs) or insertions/deletions of DNA. In *P. aeruginosa*, these mutations are very common and contribute to the high resistance in many *P. aeruginosa* isolates. The accumulation of mutations is also very common during chronic infections and repeated exposure to antibiotics can help to select for mutated isolates with higher resistance. These mutations can results in altered target sites and altered activity of existing mechanisms such as efflux pumps. The target site may be altered by mutation such that it evades the action of or no longer binds the antimicrobial agent (e.g. ciprofloxacin). Mutations that result in alterations of an antibiotic's target can also confer resistance, for example where a mutation in DNA gyrase reduces the binding affinity of the enzyme for fluoroquinolones leading to resistance (Breidenstein et al, 2011; Gellatly et al., 2012; Schweizer, 2003). Mutations in bacterial DNA may change the target enzyme in a metabolic pathway to therefore bypass the primary target (e.g. trimethoprim) (Munita et al., 2016; Van Hoek et al., 2011). Bacteria may develop higher activity of efflux pumps that expel the antimicrobial agent before it reaches an effective concentration (e.g tetracyclines). Examples of these include, natural inducers, such as aminoglycosides and other antibiotics targeting ribosomes, a mutation in *mexZ*, which normally suppresses expression of *mexXY* , leads to the overexpression of the MexXY efflux pump (Matsuo et al, 2004).

Tolerance and resistance of bacteria growing in biofilms:

Bacteria can grow as multicellular communities called biofilms, distinct from planktonic growth. Biofilm-associated bacteria typically cause subacute (subacute being not yet chronic >3mths, but has passed the acute phase <1mth. It may also indicate that the condition is not as severe as the acute stage) and chronic infections. They are clinically significant due to their persistence despite sustained antimicrobial treatments and adequate host defenses. Biofilm bacteria are highly resistant to a wide range of antimicrobial compounds and disinfectants, and the mechanism underlying this resistance is multifactorial. Biofilms can be difficult to eradicate when they cause biofilm-related diseases, e.g., implant infections, cystic fibrosis, urinary tract infections, and periodontal diseases. A number of phenotypic features of the biofilm can be involved in biofilm-specific tolerance and resistance. Little is known about the molecular mechanisms involved (Olsen, 2015).

Antibiotic resistance typically refers to an increase in the minimum inhibitory concentration (MIC) value of an antibiotic due to a permanent change in the bacteria, e.g. mutation or resistance acquired through horizontal gene transfer. However, antibiotic tolerance is the ability of cells to survive the effect of an antibiotic due to a reversible phenotypic state. In biofilms, antimicrobial tolerance is related to the mode of growth of the biofilm. This is in contrast to bacteria growing in planktonic culture. A number of mechanisms have been reported to operate together to produce a high total biofilm tolerance or resistance (Cao et al., 2015; Sousa & Pereira, 2014; Strateva & Mitov, 2011). Biofilms are not regarded as uniform cultures of physiologically identical cells and different mechanisms can protect subpopulations of cells in the biofilm (O'Toole et al., 2000). Antibiotic tolerance mechanisms in biofilms include failure of antibiotics to penetrate biofilms, slow growth rate, altered metabolism, persister cells, oxygen gradients, and extracellular biofilm matrix (Olsen, 2015).

Biofilms play an increasing role within the medical and veterinary community. Due to the increased resistance of a biofilm, they can complicate chronic non-healing wounds, implant/prosthesis infection and mastitis (Pye et al., 2013; Scaccabarozzi et al., 2015; Westgate et al., 2011). With maturity, biofilms may become more resistant to the effects of antimicrobials, which make the infection harder to treat (Olsen, 2015; Sousa & Pereira, 2014). Many antibiotic therapies currently used to treat bacterial infections are aimed at planktonic bacterial cells as opposed to cells encased in a biofilm; this makes their treatment increasingly problematic. Without adequate diagnostic and treatment protocols to treat veterinary biofilms, their impact will remain a significant challenge.

Resistance to Beta-lactam Antibiotics

Beta-lactamases and Classification

β-lactam antibiotics are one of the most effective and commonly used antibacterials with their useage approximating 60% of the global antibiotic use (Kong et al., 2010). The Infectious Diseases Society of America (IDSA) reported that three of the top six listed dangerous pathogens are β -lactam resistant bacteria (IDSA, 2017). Resistance to β -lactam antibiotics may be through production of β -lactamase enzymes that hydrolyse the β -lactam ring of the antibiotic or, changes of the penicillin binding proteins that maintain the peptidoglycan structure in bacterial cell wall. Also via alteration of porin channels and by initiation of efflux exporter proteins (Öztürk et al, 2015).

The most widely used classification of the β-lactamases is the Ambler classification. This divides β-lactamases into four classes (A, B, C and D) based upon their homology of amino acid sequences (Table 4.2). Ambler class A is the active-site serine β-lactamases; Ambler class B, is the metallo-β-lactamases that require a bivalent metal ion (usually Zn^{2+}) for activity. Ambler class C refers to the serine β-

lactamases that were found to have dissimilar sequence to those already identified as the class A enzymes. Within the designated class C, includes the 'AmpC' β-lactamases. Ambler class D is a further classification of serine β-lactamases, referred to as the OXA β-lactamases (Bush & Jacoby, 2010; Hall & Barlow, 2005; Weldhagen et al., 2003).

The metallo-β-lactamases, are a group of enzymes that hydrolyse β-lactams through an enzymatic process that is distinctly different from that of the serine β-lactamases, and they are structurally unrelated to the serine β-lactamases. All metallo-β-lactamases have been defined as class B β-lactamases. These are sub-dived into three subgroups, B1, B2 and B3.6 (Hall & Barlow, 2005).

IMP and VIM are metallo—β-lactamases that rapidly hydrolyze penicillins, cephalosporins, and carbapenems (not aztreonam) (Hall & Barlow, 2005). Resistance to penicillins and cephalosporins usually accompanies production, but carbapenem resistance may also require the loss of OprD. Various IMP enzymes have been reported in Japan, Canada, Italy, France, Greece, and South Korea,Taiwan (Deshpande et al., 2013; Docquier et al., 2003; Hayakawa et al., 2014; Jeannot et al., 2012; Tada et al., 2016). VIM enzymes resemble IMP types with regard to their hydrolytic properties, they but share only 30%–40% amino acid identity (Livermore, 2002).

The genes for VIM and IMP enzymes, like those for OXA-ESBLs, are often carried as cassettes within integrons, which are natural recombination systems that assemble series of acquired genes behind a single promoter. This organization facilitates gene recombination. The β-lactamase genes are often adjacent to aminoglycoside 6'-*N* acetyltransferase [*aac(6')-1b*] determinants (Araujo et al., 2016; Galimand et al, 1993). A *P. aeruginosa* strain with this combination of cassettes is susceptible only to polymyxins, ciprofloxacin, and, perhaps, aztreonam. If MexAB-OprM is up-regulated by mutation, or if the isolate has topoisomerase and *ampD* mutations, then only susceptibility to polymyxins remains (Livermore, 2002).

Table 4.2 Ambler Classification of beta-lactamases (Bush & Jacoby, 2010; B. G. Hall & Barlow, 2005).

Class	Active site	Enzyme type
A	Serine	Narrow spectrum
A	Serine	Extended spectrum
A	Serine	Carbapenemases
B	Zinc	Metallo beta lactamases (carbapenemases)
C	Serine	Cephalasporinases
D	Serine	OXA type enzymes (ESBLs, carbapenemases)

There currently exists a second classification scheme for the beta-lactamases which classifies the beta lactamases on their hydrolytic properties. The functional classification scheme is based on the 1995 proposal by Bush et al (Bush et al., 1995). It takes into account substrate and inhibitor profiles to group the enzymes in ways that can be correlated with their phenotype in clinical isolates. Major groupings generally correlate with the more broadly based molecular classification. The updated system includes group 1 (class C) cephalosporinases; group 2 (classes A and D) broad-spectrum, inhibitor-resistant, and extended-spectrum beta-lactamases and serine carbapenemases; and group 3 metallo-beta-lactamases. Several new subgroups of each of the major groups are described, based on specific attributes of individual enzymes. A list of attributes is also suggested for the description of a new beta-lactamase, including the requisite microbiological properties, substrate and inhibitor profiles, and molecular sequence data that provide an adequate characterization for a new beta-lactam-hydrolyzing enzyme (Bush & Jacoby, 2010).

Table 4.3 shows the selection of genes used in this study and their relevant classifications.

Table 4.3. A selection of genes used in this study known to be associated with ESBLs and involved in transmissible resistance (Strateva & Yordanov, 2009)

Gene	Description/Function	Reference
bla$_{TEM}$	Ambler Class A beta lactamases and extended spectrum beta lactamases	(Bush & Jacoby, 2010; Weldhagen et al., 2003)
bla$_{SHV}$	Ambler Class A beta lactamases and extended spectrum beta lactamases	(Bush & Jacoby, 2010; Weldhagen et al., 2003)
bla$_{OXA}$	Ambler Class A beta lactamases and extended spectrum beta lactamases	(Bush & Jacoby, 2010)
bla$_{GES}$	Ambler Class A extended spectrum beta lactamases	(Bush & Jacoby, 2010; Weldhagen et al., 2003)
bla$_{VEB}$	Ambler Class A extended spectrum beta lactamases	(Bush & Jacoby, 2010; Weldhagen et al., 2003)
bla$_{PER}$	Ambler Class A extended spectrum beta lactamases	(Weldhagen et al., 2003)
bla$_{KPC}$	Mechanism of carbapenemase resistance, plasmid transferable in Enterobacteracea	(Chen et al, 2012; Poirel et al , 2010; Xu et al., 2015)
bla$_{VIM}$	Ambler class B (metallo beta lactamase) beta lactamases, plasmid transferable	(Bush & Jacoby, 2010; Rossolini et al, 2007)

bla$_{IMP}$	Ambler class B (metallo beta lactamase) beta lactamases, plasmid transferable	Rossolini et al., 2007 and Bush & Jacoby 2010
bla$_{OXA-1}$	Extended spectrum oxacillinases from Ambler Class D	(Bush & Jacoby, 2010)
bla$_{OXA-2}$	Extended spectrum oxacillinases from Ambler Class D	(Bush & Jacoby, 2010)
bla$_{OXA-10}$	Extended spectrum oxacillinases from Ambler Class D	(Bush & Jacoby, 2010)
BEL-1	Clavulanic acid inhibited Ambler Class A specific extended beta lactamase, chromosome encoded, embedded in class1 integrons. Activity inhibited by clav acid, tazobactam, cefoxitin, moxobactam, imipenem. Hydrolyses most extended spectrum cephalosprins and aztreonam.	(Bush & Jacoby, 2010; Weldhagen et al., 2003)
CTX-M	Ambler Class A , Extended spectrum beta-lactamase	(Bush & Jacoby, 2010; Weldhagen et al., 2003)
Amp-C	Overexpression of naturally occurring Amp C is associated wit a reduced susceptibility or resistance to extended spectrum cephalosporins such as ceftazidime. AmpC beta-lactamases (AmpC) are enzymes which convey resistance to penicillins, second and third generation cephalosporins and cephamycins. They also result in resistance to combinations of these antibiotics and substances which are actually intended to inhibit the effect of beta-lactamases. They do not convey resistance to fourth generation cephalosporins. The genes for these enzymes occur naturally in some bacteria species as so-called chromosomal AmpC (e.g. in *E. coli*, but not in Salmonella). The enzymes are only actually formed and only become effective under certain conditions. The important thing is the increasing number of AmpC genes localised outside the chromosome on so-called plasmids, which is why they are often referred to as ”plasmidic AmpC” (pAmpC). They ensure the constant formation of the enzyme and lie on transmissible gene	(Rodríguez-Martínez et al., 2009)

	sections. These can be exchanged between bacteria of the same type or of different types (horizontal gene transfer).	
CS integrons	Integrons are naturally occurring genetic elements found as part of the Tn21 transposon family or located on various broad host-range plasmids. Principle importance in clinical isolates as they mediate resistance.	(Bass et al., 1999; Lévesque et al, 1995)

(Lister et al., 2009)

Many acquired beta-lactamases have been noted in *P. aeruginosa* (Cuzon et al., 2011; Godfrey & Bryan, 1984; Poole, 2005b; Sacha et al., 2008; Williams, 1991). Clinical *P. aeruginosa* isolates producing class A extended-spectrum beta-lactamases (ESBLs) such as TEM, SHV, PER, GES, VEB, BEL and CTX-M are occasionally described (Weldhagen et al., 2003). The Ambler class B enzymes (metallo-beta-lactamases, such as IMP – Imipenemase and VIM - Verona imipenemase) are also spread among clinical *P. aeruginosa* isolates, especially in Asia and Europe (Rossolini et al., 2007). Metallo-β-lactamases (MBL) are a set of enzymes that catalyze the hydrolysis of a broad range of β-lactam drugs including carbapenems. The enzyme mechanisms differ based on whether one or two zincs are bound in the active site, which in turn, is dependent on the subclass of β-lactamase. The dissemination of genes encoding these enzymes is widely reported in *P. aeruginosa* making them an important cause of resistance (Palzkill, 2014). The first plasmid-mediated MBL was reported in *P. aeruginosa* in Japan in 1991 (Butt et al, 2005). Since then many reports from a number of countries have documented the prevalence of MBLs (Akya et al.,2015; Marra et al., 2006; Pitout et al., 2005; Pitout et al., 2008; Varaiya et al, 2008).

P. aeruginosa strains producing extended-spectrum oxacillinases from the Ambler class D (i.e. OXA-10, OXA-2 and OXA-1 derivatives) have sporadically been described but knowledge of their spread in the clinical setting remains poor (Bert et al 2002; Farshadzadeh et al, 2014; Strateva & Yordanov, 2009).

Extended spectrum beta lactamases (ESBLs)

Extended spectrum beta-lactamase (ESBLs)-producing *P. aeruginosa* have spread rapidly worldwide and pose a serious threat as a healthcare-associated infections (Goossens & Grabein, 2005; Marcel et al., 2008; Microbiology et al., 2004). Since the discovery of ESBLs in 1983, their prevalence has been reported in many regions of the world and now comprises over three hundred variants (Rossolini et al., 2007).

Inactivating enzymes are prominent facilitators of resistance. Extended-spectrum beta-lactamases (ESBLs) refer to enzymes that have activity against a broad spectrum of beta-lactam antibiotics, thus making them ineffective. Bacteria which produce these enzymes become resistant to important active substances such as aminopenicillins (e.g. ampicillin), cephalosporins (including those of the third and fourth generation) and monobactams (Chen et al., 2015; Shaikh et al., 2015). The genes for these enzymes are found on transmissible gene sections that can be exchanged between bacteria of the same type or of different types (horizontal gene transfer). ESBLs are plasmid-mediated bacterial enzymes typically found in enteric Gram-negative bacteria. Plasmids encode several beta lactamases (eg TEM-1, OXA-1, OXA-2). This resistance can be detected in various bacteria species, in particular enterobacteria (eg. *Salmonella, Klebsiella* and *E.coli*). Pathogens that produce ESBLs are difficult to treat and are increasingly responsible for serious healthcare-associated infections (HAIs) (Peleg & Hooper, 2011; Santajit & Indrawattana, 2016).

The prevalence of different ESBLs genotypes in *Enterobacteriaceae* varies in different countries and regions (Bradford, 2001). The PER, VEB, GES TEM, SHV and CTX genotypes are prevalent in Asian countries and regions. They have been extensively reported in members of the family *Enterobacteriaceae* since the early 1980s In *P. aeruginosa* ESBL producers were described only more recently (Chen et al., 2015; Crespo et al., 2004; Empel et al., 2007; Glupczynski et al., 2010; Kouda et al., 2009; Laudy et al., 2017; Libisch et al., 2008; Patzer et al., 2009; Woodford et al., 2008) These enzymes are either of the TEM and SHV types, which are also well

known in the *Enterobacteriaceae*; with the PER type, mostly originating from Turkish isolates; the VEB type from Southeast Asia; and recently, the GES and IBC types, which have been reported in France, Greece and South Africa (Chen et al., 2015; Tawfik et al, 2012; Woodford et al., 2008)

In *P. aeruginosa* specifically, many acquired β-lactamases have been noted (Bert et al., 2002; Z. Chen et al., 2015; Crespo et al., 2004; Empel et al., 2007; Glupczynski et al., 2010; Kouda et al., 2009; Laudy et al., 2017; Libisch et al., 2008; Patzer et al., 2009). Some of these are widely prevalent among isolates from southern Europe, Turkey, and Southeast Asia, although they are not known to be widely prevalent in the United Kingdom. β-lactamases that give wider resistance are emerging in *P. aeruginosa* (Paterson & Bonomo, 2005; Shaikh et al., 2015), and include PER-1 β-lactamase and the extended-spectrum OXA types. PER-1, a class A β-lactamase, confers high-level resistance to ceftazidime, with susceptibility restored by the addition of clavulanate, but it has little *in vitro* effect on piperacillin; carbapenems are stable to PER-1 producers. PER-1 is frequently noted in *P. aeruginosa* from Turkey and has also occasionally been reported in Europe (Empel et al., 2007; Kolayli et al., 2005; Libisch et al., 2008; Llanes et al., 2006; Vahaboglu et al., 1997). The OXA-ESBLs, are also, mainly are reported in *P. aeruginosa* from Turkey. A few *P. aeruginosa* isolates from Turkey produce PER ESBLs, often together with potent aminoglycoside-modifying enzymes (discussed in later section of this Chapter).

Since the first description of ESBL producing *Enterobacteriaceae* isolated from hospitalized humans, many nosocomial outbreaks have been reported and furthermore an increase in the detection of ESBL producing strains in the community. Reports have also noted the dissemination of ESBL producing *E. coli* in healthy food producing animals in several countries in Europe, and Asia, and USA in food products including meat (Geser et al, 2012) fish and raw milk. There are reports of ESBL producing *P. aeruginosa* in animal related isolates. There has been the emergence of livestock associated ESBL-producing *P. aeruginosa* in cows, poultry and pigs (Odumosu et al, 2016).

Plasmid mediated AmpC

AmpC β-lactamases are clinically important cephalosporinases encoded on the chromosomes of many of the Enterobacteriaceae and a few other organisms, where they mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and β-lactamase inhibitor-β-lactam combinations. In many bacteria, AmpC enzymes are inducible and can be expressed at high levels by mutation. Overexpression confers resistance to broad-spectrum cephalosporins including cefotaxime, ceftazidime, and ceftriaxone.

P. aeruginosa can be susceptible to carboxypenicillins, ceftazidime and aztreonam; however, it can develop resistance to third generation cephalosporins. The most frequent mechanism by which this occurs is through the constitutive hyper-production of AmpC beta-lactamase (stable derepression) (Bagge et al, 2000). *P. aeruginosa* produces an inducible chromosome-encoded AmpC beta-lactamase (cephalosporinase). Usually the enzyme is produced in low quantities ('low-level' expression) and determines resistance to aminopenicillins and most of the early cephalosporins (Langaee et al, 2000). However, chromosomal cephalosporinase production in *P. aeruginosa* may increase from 100 to 1000 times in the presence of inducing beta-lactams (especially imipenem) (Bagge et al, 2000). This resistance is usually mediated by derepression of chromosomal beta-lactamase, caused by the *bla1* mutation. However, derepression of the chromosomal *ampC* beta lactamase reduces susceptibility to penecillins and cephalosporins, the levels of resistance will depend upon the degree of derepression.

Transmissible plasmids have acquired genes for AmpC enzymes, which consequently can then appear in bacteria lacking or poorly expressing a chromosomal *blaAmpC* gene. Resistance due to plasmid-mediated AmpC enzymes is less common than extended-spectrum β-lactamase production in most parts of the world but may be both harder to detect and broader in spectrum (Jacoby, 2009). Carbapenems can usually be used to treat infections due to AmpC-producing bacteria, but carbapenem resistance can arise in some organisms by mutations that

reduce influx (outer membrane porin loss) or enhance efflux (efflux pump activation) (Jacoby, 2009).

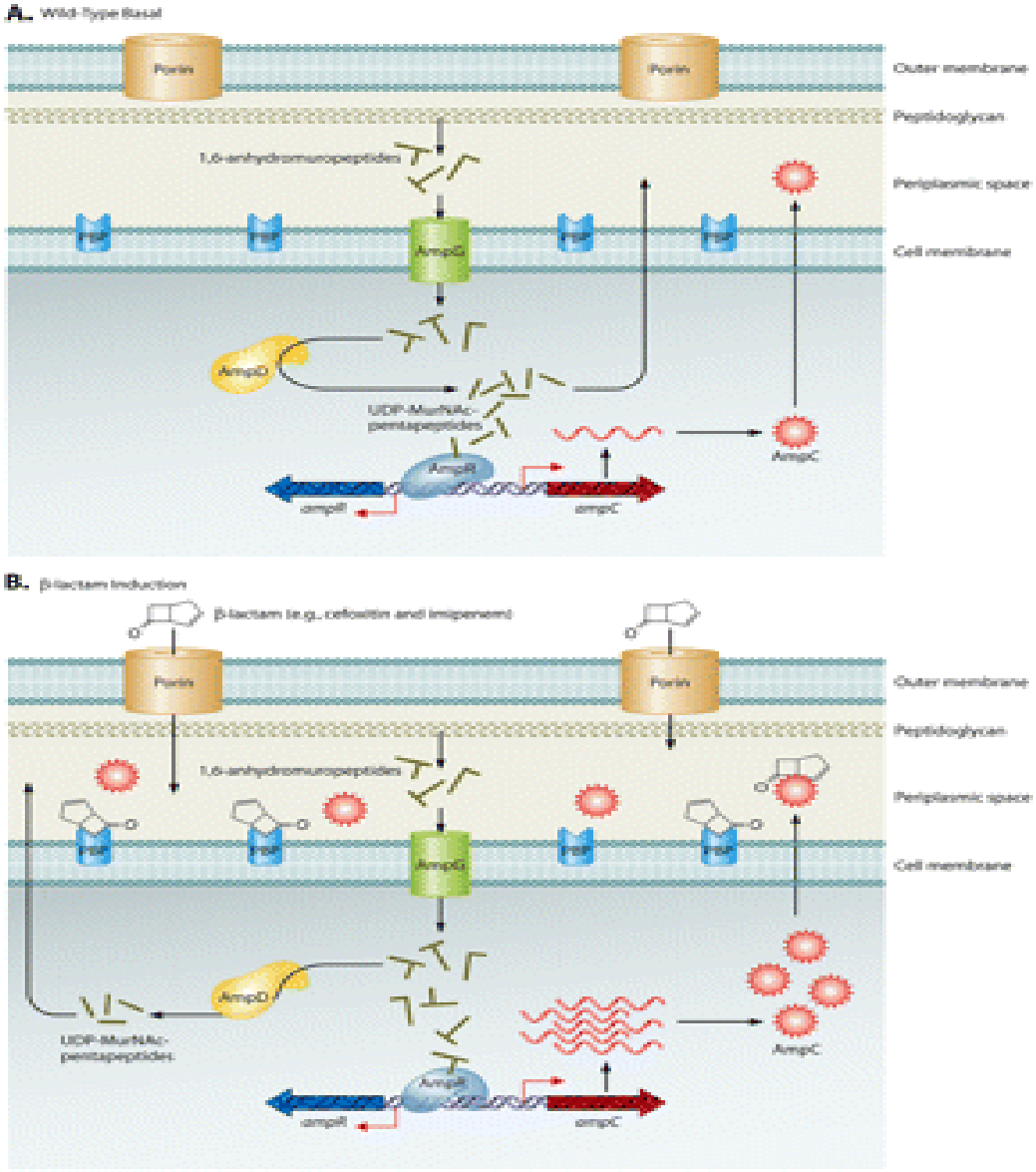


Figure 4.6. Mechanisms involved in regulation of *ampC* expression (Lister et al., 2009)**.**

These figures represent the current knowledge obtained from studies with members of the Enterobacteriaceae and appear to parallel events in P. aeruginosa. (A) Wild-type basal expression of ampC. During normal cell wall recycling, 1,6-anhydromuropeptides are removed from the cell wall and transported into the cytoplasm via the AmpG permease. The 1,6-anhydromuropeptides are cleaved by AmpD to generate free tripeptides, which are later converted into UDP-MurNAc-pentapeptides. UDP-MurNAc-pentapeptide interacts with AmpR bound to the ampR-ampC intergenic region, creating a conformation that represses transcription of ampC. Low basal levels of AmpC are produced, and the enzyme is localized to the periplasmic space. (B) β-Lactam induction of ampC expression. Inducing β-lactams, such as cefoxitin and imipenem, cross the outer membrane through porins, enter the periplasmic space, and interact with target PBPs. An increase in pools of 1,6-anhydromuropeptides is observed, and AmpD is unable to efficiently process the higher levels of cell wall fragments. The anhydro-MurNAc-peptides (inducing peptides) replace UDP-MurNAc-pentapeptides (suppressing peptides) bound to AmpR, causing a conformational change in the protein. AmpR is converted into a transcriptional activator, ampC is expressed at higher levels, and levels of AmpC increase in the periplasmic space. When the amount of β-lactam decreases below "inducing levels," the cytoplasmic pool of anhydro-MurNAc-peptides also decreases, and AmpD is able to efficiently cleave these peptides, restoring wild-type ampC expression, as shown in panel A. (C) AmpD-associated derepression of ampC expression. Mutations leading to the inactivation of AmpD or decreased expression of ampD impair the processing of cell wall recycled products and lead to increased levels of anhydro-MurNAc-peptides (inducing peptides) in the cytoplasm. As a result, the binding of inducing peptides to AmpR is favored, AmpR is "locked" in a conformation for transcriptional activation of ampC expression, and high-level constitutive expression of ampC is observed (Lister et al., 2009).

Polymyxins

The polymyxins, colistin (polymyxin E) and polymyxin B, are cationic polypeptide antibiotics that disrupt the outer bacterial cell membrane by binding to the anionic outer membrane and thereby causing bacterial cell death. This has been reviewed extensively in Chapter 3.

Resistance to Fluroquinolones

Fluoroquinolones exhibit concentration-dependent bactericidal activity by inhibiting the activity of DNA gyrase and topoisomerase enzymes essential for bacterial DNA replication. After oral and parenteral administration, fluoroquinolones are widely distributed in most extracellular and intracellular fluids and are concentrated in the prostate, lungs, and bile. Most fluoroquinolones are metabolized in the liver and excreted in urine, reaching high levels in urine. The fluoroquinolones are a group of antibiotics with considerable application for use in veterinary dermatology. They are most useful in the management of recurrent pyoderma and in chronic, deep pyoderma with extensive scar tissue. In addition, fluouroquinolones frequently are the drugs of choice for canine ear infections caused by *P. aeruginosa.* For many years the only veterinary-licensed fluoroquinolone available was enrofloxacin. The selection of a fluoroquinolone has now become wider with the availability of orbifloxacin, difloxacin,marbofloxacin and pradofloxacin. Resistance to fluroquinolones in *P. aeruginosa,* namely enrofloxacin is widely reported in worldwide studies in dogs/cats predominantly in cases of canine otitis, from Canada (Rubin et al., 2008), Denmark (Pedersen et al., 2007), USA (McKay et al., 2007), Japan (Harada et al, 2012) and Brazil.

In veterinary species, fluroquinolones are frequently used in farm animal species and avian and 'exotic' species along with small animals (cats and dogs) for various respiratory conditions, mastitis, and skin or urinary tract infections. However, the

BVA (British Veterinary Association) advocates that the use of fluoroquinolones (along with third and fourth generation cephalosporins and macrolides) should always be carefully considered. It is strongly discouraged for use in groups or flocks of animals except in very specific situations, and special attention should be given to the risk of antimicrobial resistance. Use of these antimicrobials should be reserved for the treatment of clinical conditions that have responded poorly, or are expected to respond poorly, to other classes of antimicrobials. Off label use is strongly discouraged (BVA, 2017).

Quinolone resistance can be both chromosome or plasmid mediated. Two major mechanisms are described in *P.aeruginosa* strains: active efflux pump and structural changes in target enzymes due to point mutations in chromosomal DNA gyrase (*gyrA, gyrB*) and topoiso- merase IV (*parC* and *parE*). Mutations in the *gyrA* and *gyrB* genes occur in a quinolone resistance determinant region (QRDR) near the active enzyme site (Hooper, 2000). Resistance may also be plasmid-mediated (*qnrA, qnrB, qnrS* and *aac(6)Ib* and the *cr* variant) whilst efflux systems have also been reported as alternate mechanisms of resistance. The acquisition of certain plasmids can lead to the production of enzymes that can also modify the aminoglycosides by variously acetylating, adenylating or phosphorylating the antibiotic molecule (Galimand et al., 1993; Park et al., 2006; Strateva & Yordanov, 2009). Plasmid mediated quinolone resistance genes have been found in a variety of *Enterobacteriaceae*, especially *E. coli* and species of *Enterobacter, Klebsiella,* and *Salmonella*. They have occasionally been reported in P*. aeruginosa,* other *Pseudomonas* spp. (Cattoir et al., 2007; Hong et al., 2009; Ogbolu et al, 2011; Strahilevitz et al., 2009a). Although most prevalence studies have surveyed hospital isolates, animals have not been neglected. Plasmid mediated quinolone resistance genes have been found in samples of Enterobacter from domestic or wild birds, chicken, cattle, horses, cats, dogs, reptiles and zoo animals (Ma et al., 2009; Mcmahon et al, 2015; Schink et al., 2013; Schink et al, 2012; Verner-jeffreys et al., 2009; Yang et al., 2015).

Table 4.4. A selection of genes used in this study known to be associated with fluroquinolones and involved in transmissible resistance

Gene	Description/Function	Ref
aac(6')-Ib-cr	aminoglycoside acetyltransferase enzyme capable of also modifying ciprofloxacin plasmid transferable	(Galimand et al., 1993)
qnrA	Fluroquinolone resistance, protects DNA gyrase and topoisomerase IV from quinolone inhibition plasmid transferable	(G. Jacoby et al., 2008; Strahilevitz et al., 2009a)
qnrB	Fluroquinolone resistance, protects DNA gyrase and topoisomerase IV from quinolone inhibition, plasmid transferable	(G. Jacoby et al., 2008; Strahilevitz et al., 2009a)
qnrS	Fluroquinolone resistance, protect DNA gyrase and topoisomerase IV from quinolone inhibition, plasmid transferable	(G. Jacoby et al., 2008; Strahilevitz et al., 2009a)

(Lister et al., 2009)

Resistance to Aminoglycosides

Aminoglycosides are important antibacterial agents for the treatment of various infections in humans and animals, although they are seldom the sole treatment option. In veterinary medicine in the European Union (EU), aminoglycosides account for 3.5% of the total sales of antimicrobials (EMA, 2017).

In *P. aeruginosa* specifically, many aminoglycoside-modifying enzymes have been noted. Some of these are widely prevalent among isolates from southern Europe, Turkey, and Southeast Asia, although they are not known to be widely prevalent in the United Kingdom (Fàbrega et al., 2009; Hong et al., 2009). As discussed

previously, *P. aeruginosa* isolates from Turkey have been shown to produce both PER enzymes and ESBLs, often together with potent aminoglycoside-modifying enzymes (Nordmann & Naas, 1994). Acquired resistance occurs through several mechanisms, but enzymatic inactivation of aminoglycosides is the most common one. Resistance mechanisms differ between the aminoglycoside molecules and between bacterial species. Cross-resistance to several aminoglycosides by a single mechanism/plasmid CIEs does occur, but generally there is no complete cross-resistance to all aminoglycosides by one mechanism. Mechanisms conferring resistance to streptomycin and spectinomycin usually differ from those of the other aminoglycosides (Ramirez et al., 2010). Aminoglycosides resistance has been found in many different bacterial species, including those with zoonotic potential (Garneau-tsodikova & Labby, 2016).

Resistance genes are often located on mobile elements facilitating their spread between different bacterial species and between animals and humans. The same resistance genes have been found in isolates from humans and animals (Ho et al., 2010). In human medicine, gentamicin, tobramycin and amikacin are of greater importance than the other aminoglycosides. Resistance to gentamicin, tobramycin and amikacin is generally still considered scarce in veterinary organisms and use of these aminoglycosides in animals is more often through local administration (topical eye or ear medications) or by injection. Aminoglycosides are important in human medicine for the treatment of MDR tuberculosis, Gram-negative infections and enterococcal/streptococcal endocarditis and have been categorized by WHO as critically important for human medicine (WHO CIA, 2017). Aminoglycosides are, however, rarely the sole treatment option in either veterinary or human medicine. Veterinary-authorised aminoglycosides useage should be considered on the basis of (i) their importance in human medicine and (ii) the high potential for transmission of resistance determinants between animals and humans and the potential for co-selection of resistance.

Acquired resistance to aminoglycosides can be due to the production of aminoglycoside-modifying enzymes encoded by horizontally acquired resistance

determinants, or by mutations that reduce aminoglycoside accumulation in the bacterial cell. The most prevalent aminoglycoside-modifying enzymes found in *P. aeruginosa* are the acetyl-transferases (AAC). Reduced aminoglycoside uptake could be due to mutations causing lipopolysaccharide changes or up-regulation of efflux systems based on the MexX–MexY linked-pump module. Unlike resistance mediated by modifying enzymes, the spectrum of which can be variable depending on the nature of the enzyme, resistance mediated by efflux systems tends to be more broad spectrum (Galimand et al., 1993; Hocquet et al., 2006; Morita et al., 2012).

Table 4.5 A selection of genes used in this study known to be associated with aminoglycoside resistance and involved in transmissible resistance

Gene	Description/Function	Ref
aac(6')-Ib-cr	Aminoglycoside resistance, plasmid transferable	(Park et al., 2006)

4.2 Aims

Resistance to beta-lactams, aminoglycosides and fluoroquinolones have been widely reported through various mechanisms in human clinical isolates and in some isolates of animal origin, predominantly the Enterobacteriaceae. However, prevalence in isolates from companion animals particularly in *P. aeruginosa* is relatively understudied. Here, the aim was to study the resistance characteristics of a set of 106 *P. aeruginosa* isolates from companion animals, collected between 2010- 2015, at the Veterinary Diagnostic laboratory (VDL) at Leahurst Veterinary Teaching Hospital (University of Liverpool, UK).

More specifically, the aim of the work presented in this chapter was to:

- Characterize the resistance of the isolates to beta-lactam antibiotics using disk diffusion, MICs and double-disc synergy testing (DDST). Isolates were also screened by PCR for a subset of genetic markers associated with beta-lactam resistance.

-Screen the isolates to study phenotypic resistance to aminoglycosides and the presence of genes associated with the presence of aminoglycoside resistance (*aac(6')-Ib-cr*).

- Screening for genes (*qnr A*, *qnr B*, *qnrS* and *aac(6')-Ib-cr*) associated with the presence fluroquinolone resistance.

4.3 Results

The panel of isolates used were sourced from companion animals comprising 11 equine, 2 feline, and 93 canine samples (refer to Chaper 2 Methods, Table 2.2). Samples originated from mostly ear infections, urine, wounds/non-healing wounds including skin, vaginal/vulval, scrotal/testicle swabs and also limited respiratory samples (tracheal washes, maxillary sinus, bronchoalveolar lavage) (refer to Chaper 2 Methods, Table 2.3). The inclusion criteria for isolates in this study were performed on the basis of species (ie. any isolate that was from a companion animal species) and bacterial species identification (all *P. aeruginosa*). Only a single isolate from each sample was included in the analysis. It was accepted that in being a Diagnostic Laboratory closely associated with the Small Animal Teaching Hospital, Leahurst and so receiving a high number of samples from the referral centers (equine and small animal Veterinary Teaching Hospitals) that the isolates from these are more likely to be associated with disease more difficult to treat (hence the patients being referred to a specialist center from a first opinion veterinary practitioner).

Resistance to Beta-Lactam Antibiotics

There was expected high occurrence of resistance to third generation cephalosporin ceftiofur (98.1% resistant). The prevelance of fourth generation cephalosporin, cefepime (Figure 4.9) was found to be 9.4%. Resistance to piperacillin, piperacillin/tazobactam, ticarcillin and ticarcillin/clavulanate were 17%, 17.9%, 9.4% and 5.7% respectively. The resistance to beta-lactam antibiotics is detailed in Table 4.6 and Table 4.7. Of the carbapenems; meropenam resistance (Figure 4.8) was particularly low (3.7%) and also low for imipenam (8.5%) (Table 4.7), although slightly higher in aztreonam (Figure 4.7) at 11%.

Table 4.6 Susceptibility testing of *Pseudomonas aeruginosa* isolates from companion animal (n=106) isolates against beta-lactam antibiotics using disk diffusion methodology (EUCAST 2015)

Antimicrobial	Antibiotic Class	Resistance Breakpoint S≥/<R (mm)	Resistant Strains (n)	Intermediate Strains (n)	Sensitive Strains (n)	Resistant Strains (%)
Aztreonam	Monobactam	50/16	12	94	0	11.3 [6.6, 18.8]
Meropenem	Carbapenem	24/18	4	11	91	3.7 [1.5, 9.3]
Cefepime	4th Generation Cephalosporin	19/19	10	-	96	9.4 [5.2,16.5]
Piperacillin	Ureidopenicillin (4th Generation B-lactam)	18/18	18	-	88	17.0 [1.1, 25.3]
Piperacillin/tazobactam	Ureidopenicillin with B-lactamase inhibitor	18/18	19	-	87	17.9 [11.8,26.3]

Table 4.7 Minimum inhibitory concentration tests to show antimicrobial resistance of *P. aeruginosa* in companion animal (n=106) isolates to a panel of beta-lactam antibiotics using the TREK COMPAN1F diagnostic plates assay.

Antimicrobial	Antibiotic class	Breakpoint mg/L	MIC mg/mL										MIC $_{50}$	MI C_{90}	Resistant strains (%)
			0.25	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**			
Ceftiofur	Caphalosporin (veterinary)	S≤2, I=4, R≥8	1				1	104					8	8	98.1 [93, 99]
Imipenem	Carbapenem	S≤4, I=8, R>8			42	42	13	6	3				2	4	8.5 [5, 15]
Ticarcacillin	Carboxypenecillin	S≤64, R>64						5	28	57	6	10	32	32	9.4 [5, 17]
Ticarc/clavul acid	Carboxypenecillin and B-lactamase inhibitor	S≤64/2, R>64/2						3	28	63	6	6	32	32	5.6 [3, 12]

Confidence intervals are 95% {Lower, Upper) Wilson method

Other antibacterials (amoxicillin/clavulanic acid, ampicillin, cefazolin, cefovecin, cefoxitine, cefpodoxime, chloramphenicol, clindamycin, doxycycline, erythromycin, oxacillin, +2%NaCl, penicillin, rifampicin, trimethoprim sulfamethoxazole) included in the Trek COMPAN1F plate were also tested but are not reported due to intrinsic *P.aeruginosa* resistance.

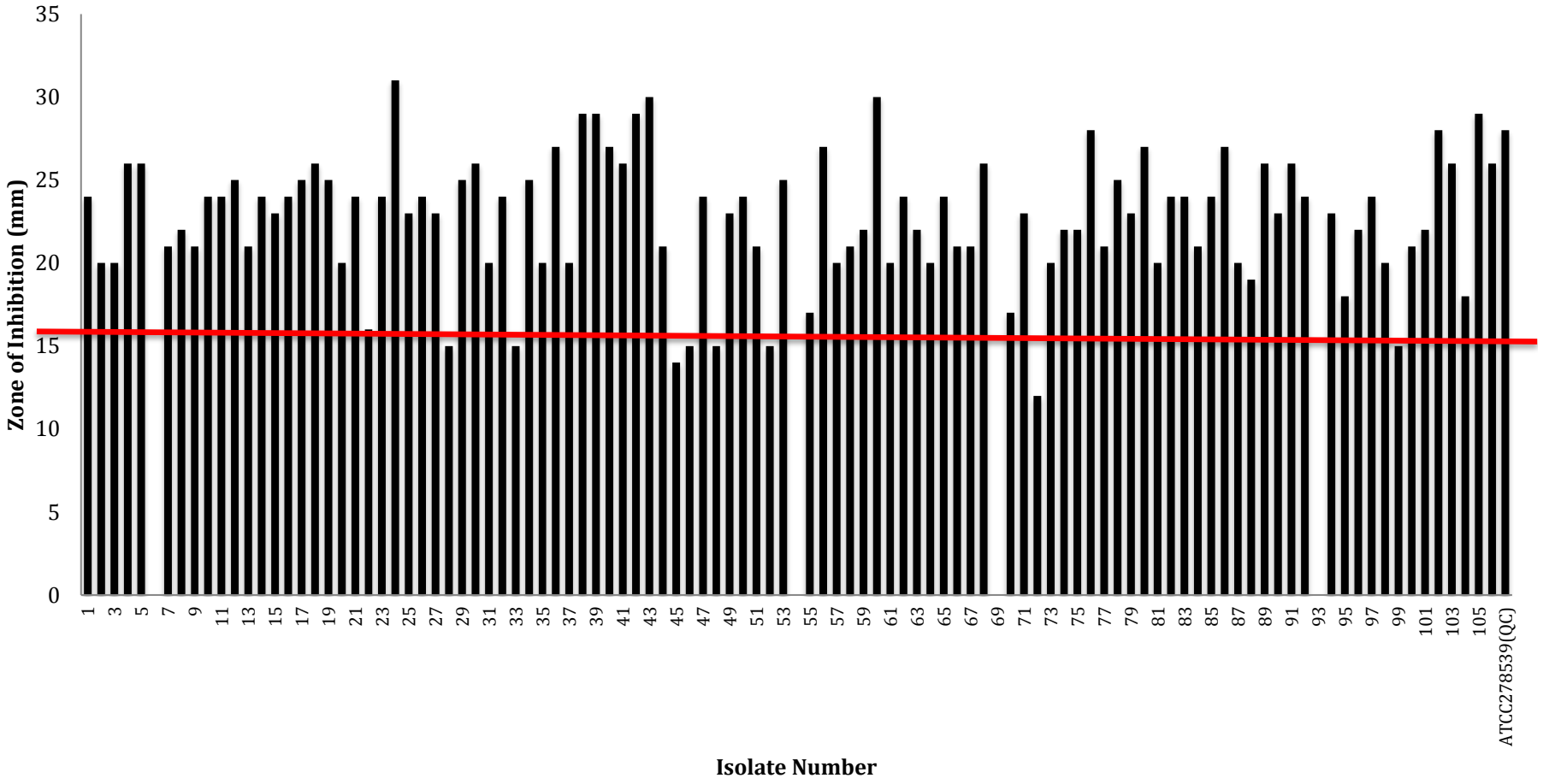


Figure 4.7 Susceptibility testing results of the *P.aeruginosa* isolates from companion animals (n=106) against Aztreonam 30mcg (DDST) (red line indicates 'cut off' value. Quality control used ATCC278539.

Figure 4.8 Susceptibility testing results of the *P.aeruginosa* isolates from companion animals (n=106) against Meropenem 10mcg (DDST) (red line indicates 'cut off' value. Quality control used ATCC278539.

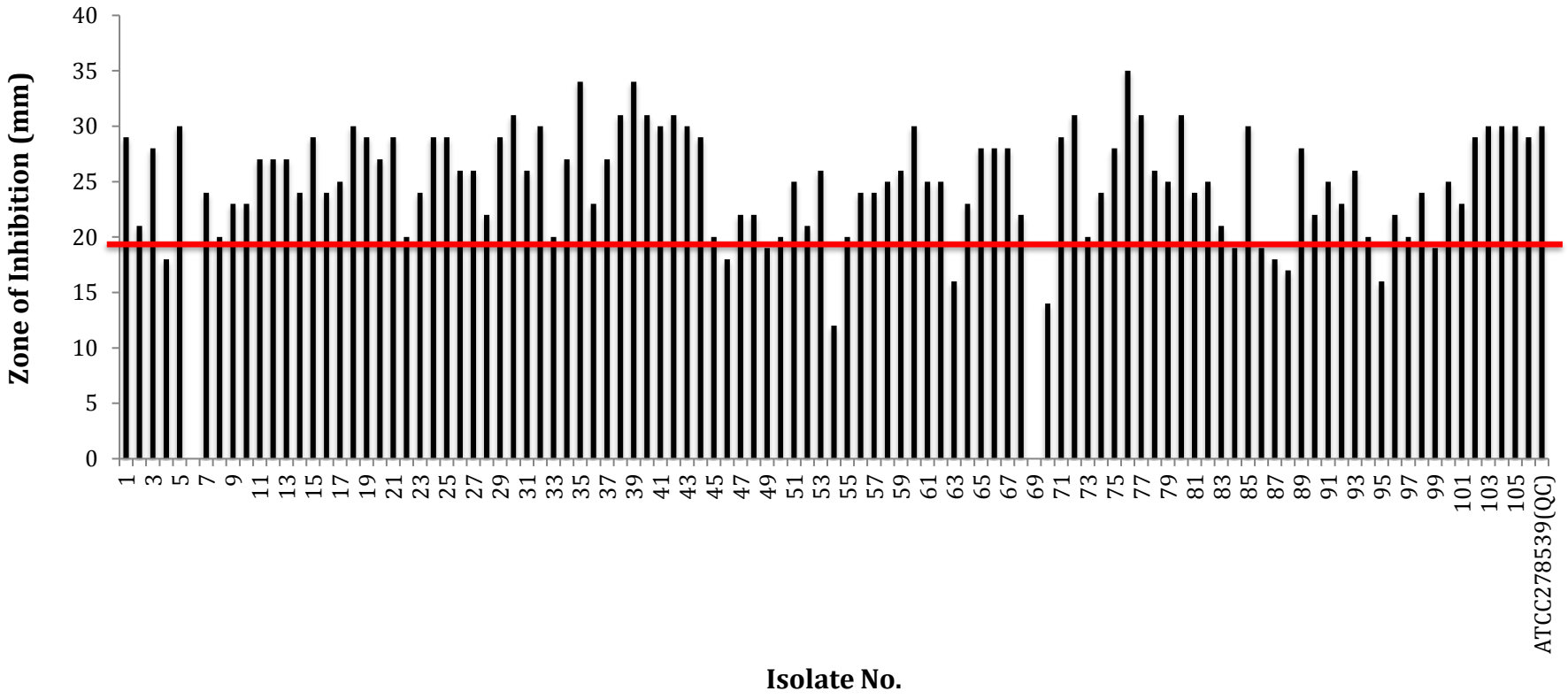


Figure 4.9 Susceptibility testing results of the *P.aeruginosa* isolates from companion animals (n=106) against Cefepime 30mcg (DDST) (red line indicates 'cut off' value. Quality control used ATCC278539.

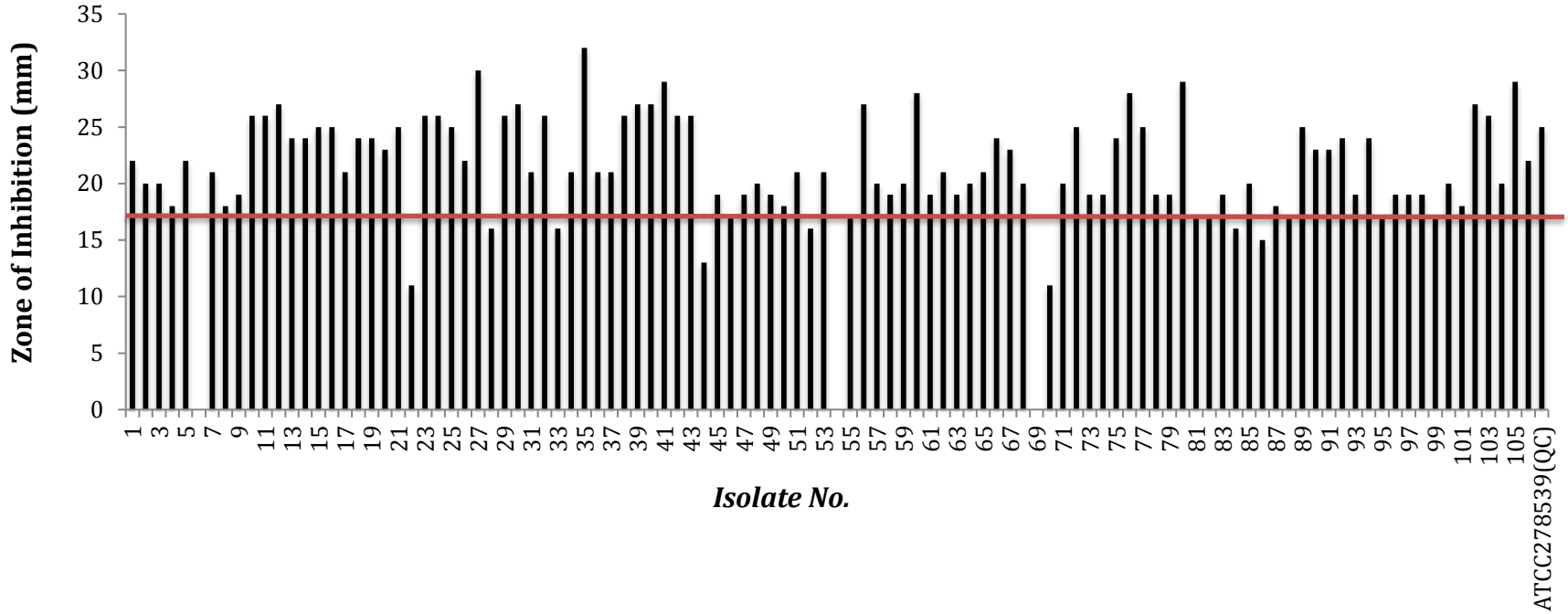


Figure 4.10 Susceptibility testing results of the *P.aeruginosa* isolates from companion animals (n=106) against Piperacillin 30mcg (DDST) (red line indicates 'cut off' value. Quality control used ATCC278539.

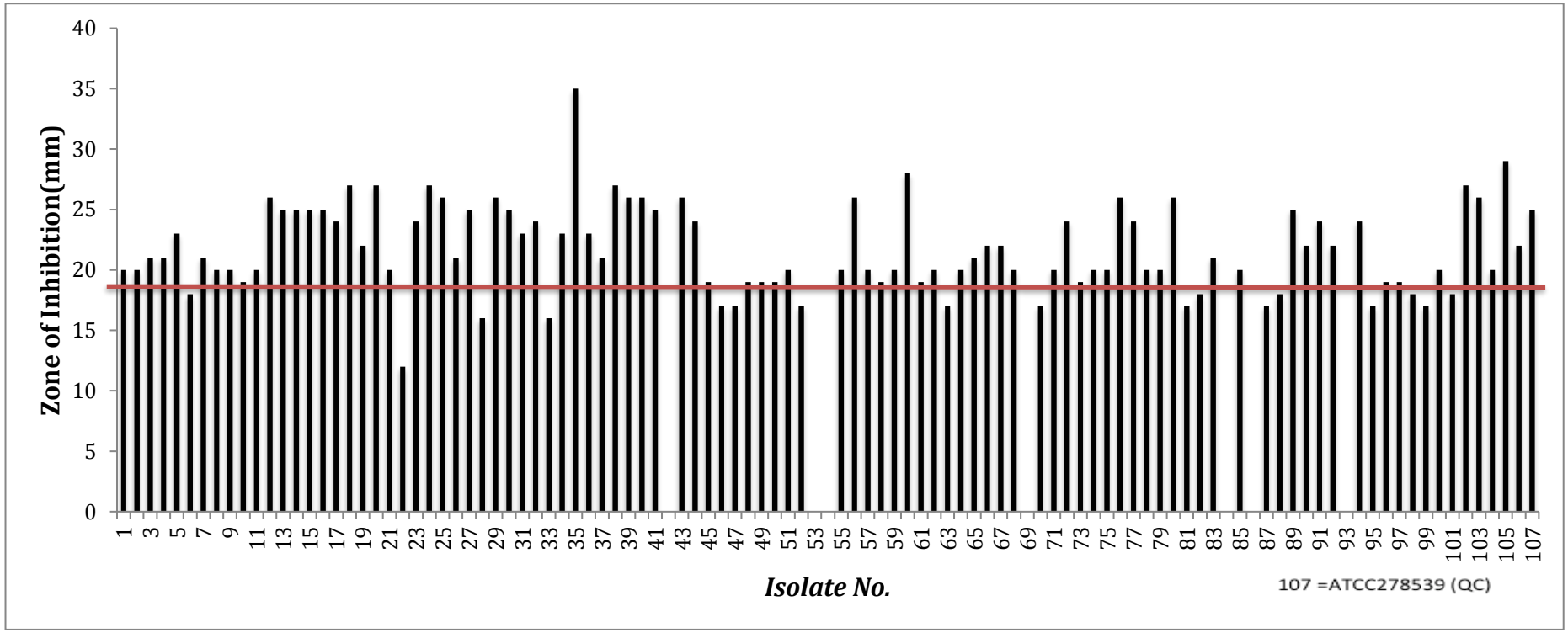


Figure 4.11 Susceptibility testing resultsof the *P.aeruginosa* isolates from companion animals (n=106) against Piperacillin/Tazobactam 36mcg (DDST) (red line indicates 'cut off' value. Quality control used ATCC278539 (labelled as isolate 107).

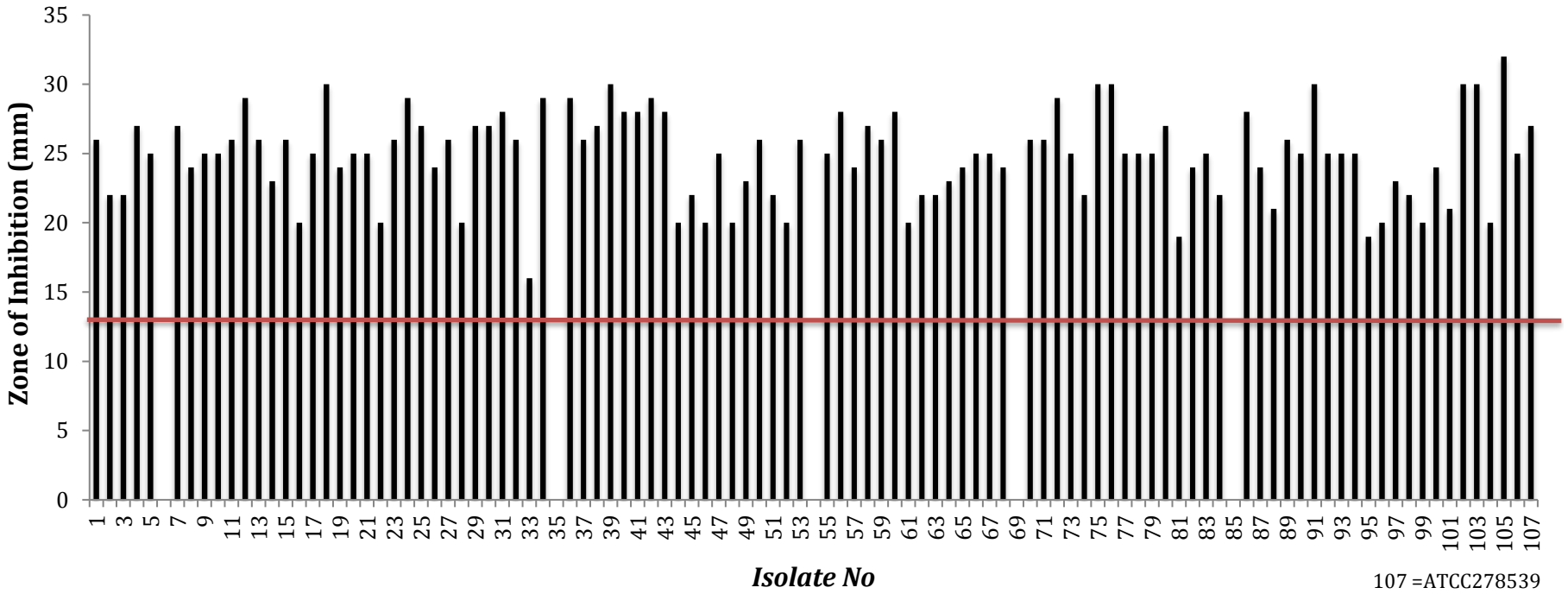


Figure 4.12 Susceptibility testing results of the *P.aeruginosa* isolates from companion animals (n=106) against Ceftazidime 30mcg (DDST) (red line indicates 'cut off' value. Quality control used ATCC278539 (labelled as isolate 107)

ESBL phenotyping results

Results are detailed for both methods in Table 4.8. The two methods did not produce consistent results. Of the 106 isolates tested 3 were positive ESBL producers by cloxacilliin method and 7 were positive by boronic acid method. (begins page p138)

Table 4.8. ESBL testing results performed via two methods of the 106 *P. aeruginosa* veterinary isolates

Isolate	ESBL Results (Cloxacillin method)	ESBL results (Boronic acid method)	Isolate	ESBL Results (Cloxacillin method)	ESBL results (Boronic acid method)	Isolate	ESBL Results (Cloxacillin method)	ESBL results (Boronic acid method)
1	-	-	37	-	-	73	-	-
2	-	-	38	-	-	74	+	-
3	-	-	39	-	-	75	-	-
4	-	-	40	-	-	76	-	-
5	-	-	41	-	-	77	-	-
6	-	-	42	-	-	78	-	-
7	-	-	43	-	+	79	-	-
8	-	-	44	-	+	80	-	-
9	-	-	45	-	+	81	-	-
10	-	-	46	-	+	82	-	-
11	-	-	47	-	-	83	-	-
12	-	-	48	-	-	84	-	-
13	-	-	49	-	-	85	-	-
14	-	-	50	-	-	86	-	+
15	-	-	51	-	-	87	-	-
16	-	-	52	-	-	88	-	-
17	-	-	53	-	-	89	-	-
18	-	-	54	.	-	90	-	-
19	-	-	55	+	-	91	-	-
20	-	-	56	-	-	92	-	-
21	-	-	57	-	-	93	-	-
22	-	-	58	-	-	94	-	-
23	-	-	59	-	-	95	-	+
24	-	-	60	-	-	96	-	-
25	-	-	61	-	-	97	-	-
26	-	-	62	-	-	98	-	-
27	-	-	63	-	-	99	-	-
28	-	-	64	-	-	100	+	-
29	-	-	65	-	-	101	-	-
30	-	-	66	-	-	102	-	-
31	-	-	67	-	-	103	-	-
32	-	-	68	-	-	104	-	-
33	-	-	69	-	-	105	-	-
34	-	-	70	-	-	106	-	-
35	-	+	71	-	-			
36	-	-	72	-	-			

Tables 4.9 -4.12 show details of PCR screening results for the ESBL-associated resistance genes within the veterinary *P. aeruginosa* isoaltes for the genes *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$; *bla*$_{GES}$, *bla*$_{VEB}$, *bla*$_{PER}$; *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{KPC}$ and *bla*$_{OXA-1}$, *bla*$_{OXA-2}$, *bla*$_{OXA-10}$ respectively. Of the 106 isolates, 11 isolates (highlighted with grey shading in Table 4.9) showed positive PCR product for ESBL-associated resistance gene *bla*$_{TEM}$. While eight isolates identified positive for *bla*$_{SHV}$ (Table 4.9) and three isolates positive for *bla*$_{OXA}$ (Table 4.9). The three isolates that were identified as positive resistance determinants for *bla*$_{OXA}$ (isolates 71, 72 and 73) – refer to Figure 4.12, were also positive for the other ESBL-associated resistance genes *bla*$_{TEM}$ and *bla*$_{SHV}$.

(begins page 140)

Table 4.9. Results of the PCR screening of ESBL-associated resistance genes within the veterinary *P. aeruginosa* isolates (n=106) - shows results for the genes *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$ genes.

Isolate	*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{OXA}$	Isolate	*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{OXA}$
1	-	-	-	54	+	+	-
2	-	-	-	55	-	-	-
3	-	-	-	56	-	-	-
4	-	-	-	57	-	-	-
5	-	-	-	58	+	+	-
6	-	-	-	59	-	-	-
7	-	-	-	60	-	-	-
8	-	-	-	61	-	-	-
9	-	-	-	62	-	-	-
10	-	-	-	63	-	-	-
11	-	-	-	64	-	-	-
12	-	-	-	65	-	-	-
13	+	-	-	66	-	-	-
14	-	-	-	67	-	-	-
15	-	-	-	68	-	-	-
16	-	-	-	69	-	-	-
17	-	-	-	70	+	+	+
18	-	-	-	71	+	+	+
19	-	-	-	72	+	+	+
20	-	-	-	73	-	-	-
21	-	-	-	74	-	-	-
22	-	-	-	75	-	-	-
23	-	-	-	76	-	-	-
24	-	-	-	77	-	-	-
25	-	-	-	78	-	-	-
26	-	-	-	79	-	-	-
27	-	-	-	80	-	-	-
28	-	-	-	81	-	-	-
29	-	-	-	82	-	-	-
30	-	-	-	83	-	-	-
31	-	-	-	84	-	-	-
32	-	-	-	85	+	-	-
33	-	-	-	86	-	-	-
34	-	-	-	87	-	-	-
35	-	-	-	88	-	-	-
36	-	-	-	89	-	-	-
37	-	-	-	90	-	-	-
38	-	-	-	91	-	-	-
39	-	-	-	92	-	-	-
40	-	-	-	93	-	-	-
41	-	-	-	94	-	-	-
42	+	+	-	95	-	-	-
43	+	+	-	96	-	-	-
44	+	+	-	97	-	-	-
45	+	-	-	98	-	-	-
46	-	-	-	99	-	-	-
47	-	-	-	100	-	-	-
48	-	-	-	101	-	-	-
49	-	-	-	102	-	-	-
50	-	-	-	103	-	-	-
51	-	-	-	104	-	-	-
52	-	-	-	105	-	-	-
53	-	-	-	106	-	-	-

The Figure 4.13 shows the PCR gel results for the *bla*$_{OXA}$ genes as reported.

Figure 4.13 Simplex PCR assay using primers to detect bla*OXA* gene performed on isolates from isolates from the VDL, Leahurst. Isolates 70, 71, 72 appear to show strong amplification of *bla*$_{OXA-1}$. The DNA loading dye DNA/BsuRI (HaeIII) Marker 9, 50 mg (0.5 µg/µl)(ThermoScientific Molecular Biology), was placed on each gel run.

Table 4.10 details the results of the PCR screening of ESBL-associated resistance genes within the veterinary *P. aeruginosa isolates* for the genes *bla*$_{GES}$, *bla*$_{VEB}$, *bla*$_{PER}$. Of the 106 isolates, five isolates (highlighted with grey shading in Table 4.10) showed positive PCR product for ESBL-associated resistance gene *bla*$_{GES}$. While four isolates identified positive for *bla*$_{PER}$ (Table 4.10) there were no positives identified for *bla*$_{VEB}$. It is noted that although there was amplification of the multiple ESBL-associated resistance genes as detailed here, some were feint and only weakly positive but are still noted above in the table.

Table 4.10. Results of the PCR screening of ESBL-associated resistance genes within veterinary *P. aeruginosa* isolates (n=106) – shows the detection of *bla*$_{GES}$, *bla*$_{VEB}$, *bla*$_{PER}$ genes.

Isolate	*bla*$_{GES}$	*bla*$_{VEB}$	*bla*$_{PER}$	Isolate	*bla*$_{GES}$	*bla*$_{VEB}$	*bla*$_{PER}$
1	-	-	-	54	+	-	-
2	-	-	-	55	-	-	-
3	-	-	-	56	-	-	-
4	-	-	-	57	-	-	-
5	+	-	-	58	-	-	-
6	-	-	-	59	-	-	-
7	-	-	-	60	-	-	-
8	-	-	+	61	-	-	-
9	-	-	-	62	-	-	-
10	-	-	-	63	-	-	-
11	-	-	-	64	-	-	-
12	-	-	-	65	-	-	-
13	-	-	-	66	-	-	-
14	-	-	+	67	-	-	-
15	-	-	-	68	-	-	-
16	-	-	-	69	-	-	-
17	-	-	-	70	-	-	-
18	-	-	-	71	-	-	-
19	-	-	-	72	-	-	-
20	-	-	-	73	-	-	-
21	-	-	-	74	-	-	-
22	-	-	-	75	-	-	-
23	-	-	-	76	-	-	-
24	-	-	-	77	-	-	-
25	-	-	-	78	-	-	-
26	-	-	-	79	-	-	-
27	-	-	-	80	-	-	-
28	-	-	-	81	-	-	-
29	-	-	-	82	-	-	-
30	-	-	-	83	-	-	-
31	-	-	-	84	-	-	-
32	-	-	+	85	-	-	-
33	-	-	-	86	-	-	-
34	-	-	-	87	-	-	-
35	-	-	-	88	-	-	-
36	-	-	-	89	-	-	-
37	-	-	-	90	-	-	-
38	-	-	-	91	-	-	-
39	-	-	-	92	-	-	-
40	-	-	-	93	-	-	-
41	-	-	-	94	-	-	-
42	-	-	+	95	-	-	-
43	-	-	-	96	-	-	-
44	-	-	-	97	-	-	-
45	-	-	-	98	-	-	-
46	-	-	-	99	-	-	-
47	+	-	-	100	-	-	-
48	+	-	-	101	-	-	-
49	-	-	-	102	-	-	-
50	-	-	-	103	-	-	-
51	-	-	-	104	-	-	-
52	-	-	-	105	-	-	-
53	+	-	-	106	-	-	-

***Although there was amplification of the multiple ESBL-associated resistance genes, some were feint and only weakly positive but are still noted above in table.**

In the table 4.11 results of the PCR screening of ESBL-associated resistance genes of the veterinary *P. aeruginosa isolates* for the bla_{VIM} , bla_{IMP}, bla_{KPC} genes are shown. Of the 106 isolates, 26 isolates (highlighted with grey shading in Table 4.11) showed positive PCR product for ESBL-associated resistance gene bla_{GES}, while six isolates identified positive for bla_{KPC} (Table 4.11). There were no positives identified for bla_{IMP}. It is noted that although there was amplification of the multiple ESBL-associated resistance genes as detailed here, some were feint and only weakly positive but are still noted above in the table. There was also the presence of multiple unspcific bands produced on the gels making the resultant images difficult to interpret. An example gel is depicted in Figure 4.13 and this issue is discussed later in this Chapter. (Table 4.11 begins p144)

Table 4.11. Results of the PCR screening of ESBL-associated resistance genes of the veterinary panel of *P. aeruginosa* isolates (n=106) - shows the genes *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{KPC}$.

Isolate	*bla*$_{VIM}$	*bla*$_{IMP}$,	*bla*$_{KPC}$	Isolate	*bla*$_{VIM}$	*bla*$_{IMP}$,	*bla*$_{KPC}$
1	-	-	-	54	-	-	-
2	-	-	-	55	-	-	-
3	-	-	-	56	-	-	-
4	-	-	-	57	-	-	-
5	-	-	-	58	+	-	-
6	+	-	-	59	-	-	-
7	-	-	-	60	+	-	-
8	-	-	-	61	-	-	-
9	-	-	-	62	-	-	-
10	-	-	-	63	+	-	-
11	-	-	-	64	+	-	-
12	-	-	-	65	-	-	-
13	-	-	-	66	-	-	-
14	+	-	-	67	-	-	-
15	+	-	-	68	+	-	+
16	+	-	-	69	-	-	-
17	-	-	-	70	+	-	+
18	-	-	-	71	-	-	-
19	+	-	-	72	-	-	-
20	+	-	-	73	+	-	-
21	-	-	-	74	-	-	-
22	-	-	-	75	-	-	-
23	+	-	-	76	-	-	-
24	-	-	-	77	-	-	-
25	-	-	-	78	-	-	-
26	-	-	-	79	-	-	-
27	-	-	-	80	+	-	-
28	-	-	-	81	-	-	-
29	-	-	-	82	-	-	-
30	-	-	-	83	-	-	-
31	-	-	-	84	-	-	-
32	-	-	-	85	-	-	-
33	+	-	+	86	+	-	-
34	+	-	-	87	+	-	-
35	-	-	-	88	-	-	-
36	-	-	-	89	+	-	-
37	+	-	-	90	-	-	+
38	+	-	-	91	-	-	+
39	-	-	-	92	+	-	-
40	-	-	-	93	-	-	-
41	-	-	-	94	+	-	-
42	-	-	-	95	+	-	+
43	-	-	-	96	-	-	-
44	-	-	-	97	-	-	-
45	+	-	-	98	-	-	-
46	-	-	-	99	-	-	-
47	-	-	-	100	-	-	-
48	-	-	-	101	-	-	-
49	-	-	-	102	-	-	-
50	-	-	-	103	-	-	-
51	-	-	-	104	-	-	-
52	-	-	-	105	-	-	-
53	-	-	-	106	-	-	-

*Although there was amplification of the multiple ESBL-associated resistance genes, some were feint and only weakly positive but are still noted above in table. There was also multiple banding produced on the gels making the resultant images difficult to interpret.

Table 4.12 shows details the results of the PCR screening of ESBL-associated resistance genes of the veterinary *P. aeruginosa isolates* for the genes bla_{OXA-1}, bla_{OXA-2}, bla_{OXA-10}. Of the 106 isolates, three isolates (highlighted with grey shading in Table 4.12) showed positive PCR product for ESBL-associated resistance gene bla_{OXA-2}. There were no positives identified however for bla_{OXA-1} and bla_{OXA-10}. It is noted that although there was amplification of the multiple ESBL-associated resistance genes as detailed here, some were feint and only weakly positive but are still noted above in the table. (Table 4.12 begins page 146)

Table 4.12. Results of the PCR screening of ESBL-associated resistance genes within the larger veterinary panel of *P. aeruginosa* isolates (n-106) - shows the genes *bla*$_{OXA-1}$, *bla*$_{OXA-2}$, *bla*$_{OXA-10}$.

Isolate	*bla*$_{OXA-1}$	*bla*$_{OXA-2}$	*bla*$_{OXA-10}$	Isolate	*bla*$_{OXA-1}$	*bla*$_{OXA-2}$	*bla*$_{OXA-10}$
1	-	-	-	54	-	-	-
2	-	-	-	55	-	-	-
3	-	-	-	56	-	-	-
4	-	-	-	57	-	-	-
5	-	-	-	58	-	-	-
6	-	-	-	59	-	-	-
7	-	+	-	60	-	-	-
8	-	+	-	61	-	-	-
9	-	-	-	62	-	-	-
10	-	-	-	63	-	-	-
11	-	-	-	64	-	-	-
12	-	-	-	65	-	-	-
13	-	-	-	66	-	-	-
14	-	-	-	67	-	-	-
15	-	-	-	68	-	-	-
16	-	-	-	69	-	-	-
17	-	-	-	70	-	-	-
18	-	-	-	71	-	-	-
19	-	-	-	72	-	-	-
20	-	-	-	73	-	-	-
21	-	-	-	74	-	-	-
22	-	-	-	75	-	-	-
23	-	-	-	76	-	-	-
24	-	-	-	77	-	-	-
25	-	-	-	78	-	-	-
26	-	-	-	79	-	-	-
27	-	-	-	80	-	-	-
28	-	-	-	81	-	-	-
29	-	-	-	82	-	-	-
30	-	-	-	83	-	-	-
31	-	-	-	84	-	-	-
32	-	-	-	85	-	-	-
33	-	-	-	86	-	-	-
34	-	+	-	87	-	-	-
35	-	-	-	88	-	-	-
36	-	-	-	89	-	-	-
37	-	-	-	90	-	-	-
38	-	-	-	91	-	-	-
39	-	-	-	92	-	-	-
40	-	-	-	93	-	-	-
41	-	-	-	94	-	-	-
42	-	-	-	95	-	-	-
43	-	-	-	96	-	-	-
44	-	-	-	97	-	-	-
45	-	-	-	98	-	-	-
46	-	-	-	99	-	-	-
47	-	-	-	100	-	-	-
48	-	-	-	101	-	-	-
49	-	-	-	102	-	-	-
50	-	-	-	103	-	-	-
51	-	-	-	104	-	-	-
52	-	-	-	105	-	-	-
53	-	-	-	106	-	-	-

*Although there was amplification of the multiple ESBL-associated resistance genes, some were feint and only weakly positive but are still noted above in table.

The genes *BEL-1 CTX-MU, AmpC* and *CS Integrons* are not presented as all isolates were PCR negative for these markers.

Multiplex Polymerase Chain Reaction (PCR) Assays

Following the initial multiplex PCR (refer to Chapter 2 for Methods and Results as detailed above) on all 106 isolates for the previously described genes it was concluded that the results of the multiplex PCR were not as clear to interpret as had been expected. Many multiple faint positive bands or unexplained banding were present in some isolates interfering with the reading of results. The primers chosen were mostly previously used for Enterobacteriacea isolates by the VDL and experience of their use with *P.aeruginosa* isolates was more limited. As there was insufficient time and further funding on this project for carrying out repeated PCRs with alternative gene primers or for performing a multitude of gradient PCRs to refine PCR conditions, it was decided to repeat all as simplex PCRs but for a smaller subset of isolates which had the potential to carry resistance genes based on their susceptibility phenotype. The selection of this smaller subset of 32 isolates is detailed below and listed in Table 4.13 (the results of these simplex PCRs on this subset of isolates are presented under the section of work entitled: **Findings from Simplex Polymerase Chain Reaction (PCR) Assays)**.

Although the initial screening of 106 isolates via multiplex PCR for the TEM, SHV and OXA set of genes did show some fairly clear positive/weak positive amplicons, for the remainder of genes screened, the results were not easily interpretable due to multiple non-specific banding (Figure 4.14).

An example of a gel that yielded potential unconfirmed positive amplicons is shown in Figure 4.14.

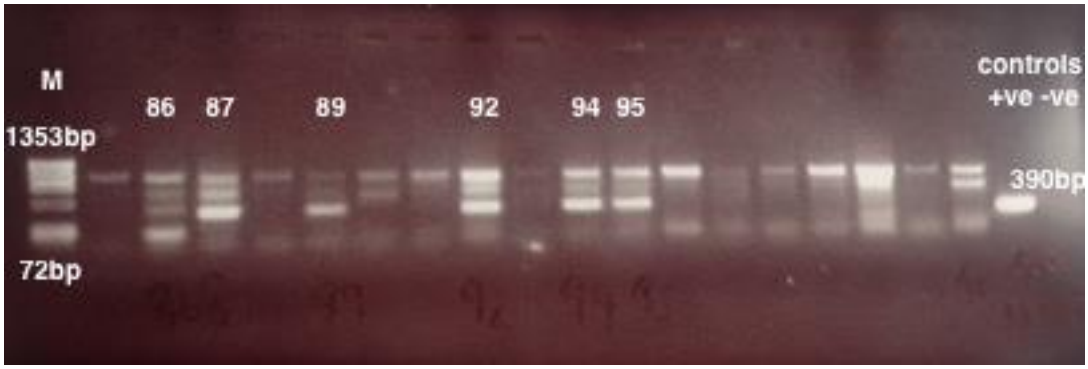


Figure 4.14 PCR assay using primers to detect *bla*$_{VIM}$ gene performed on isolates from VDL, Leahurst. Multiple isolates (86, 87, 89, 92, 94, 95) appear to show strong amplification of *bla*$_{VIM}$ shown with the positive control and negative controls and multiple bands. The DNA loading dye DNA/BsuRI (HaeIII) Marker 9, 50 mg (0.5 µg/µl)(ThermoScientific Molecular Biology), was placed on each gel run.

This was thought to be due to multiplex PCR and so it was repeated but as simplex presuming that this would improve the clarity of results. However, the resultant gel images on the simplex PCRs that were repeated on the same 106 isolates the results were generally overall difficult to interpret. The screening of all of the described genes were then repeated but only on the narrowed down sample size of 32 isolates (as described earlier, along with how they were selected) and their DNA extraction method was improved (refer to Materials and Methods; DNA Extraction), due to time and cost constraints for this study this had to be limited. Improved results were achieved following gradient PCRs to adjust the thermocycler conditions for optimisation (conditions used as detailed in Table 4.13).

It is suggested that multiple or non specific PCR products can occur due to the following potential reasons

Table 4.13 Potential causes of non specific PCR product

Possible causes of non specific product	**Possible adjustments**	**Any method adjustments made after gradient PCRs for optimisation**
Premature replication	Use a hot start polymerase, such as One*Taq* Hot Start DNA Polymerase Set up reactions on ice using chilled components and add samples to thermocycler preheated to the denaturation temperature	N/A Already done in method
Primer annealing temperature too low	Increase annealing temperature	Annealing temperature increased
Incorrect Mg^{++} concentration	Adjust Mg^{++} in 0.2–1 mM increments	N/A as within a compound polymerase mastermix solution Firepol® so Mg non adjustable
Poor primer	Check specific product literature for recommended primer design Verify that primers are non-complementary, both internally and to each other Increase length of primer Avoid GC-rich 3′ ends	N/A Insufficient time/funding to reorder alternative primers to trial
Excess primer	Primer concentration can range from 0.05–1 µM in the reaction. Please see specific product literature for ideal conditions.	Primer concentrations were reduced
Contamination with exogenous DNA	Use positive displacement pipettes or non-aerosol tips Set-up dedicated work area and pipettor for reaction setup Wear gloves during reaction setup	All of this was already in place
Template conc too high	For low complexity templates (i.e. plasAmid, lambda, BAC DNA), use 1 pg–10 ng of Dper 50 µl re	A lower conc dna used

Findings from Simplex Polymerase Chain Reaction (PCR) Assays

This further narrowed down subset of 32 isolates (Table 4.14) from the 106 were selected on the basis of the criteria of being either ESBL positive phenotypes and/or those that were resistant to ceftazidime (a third generation cephalosporin) plus any of the antibiotics; aztreonam, meropenam (carbapenems), cefepime (fourth generation cephalosporin), piperacillin or piperacllin/tazobactam (ESBLs). These isolates underwent PCR following repeat subculture and DNA extraction using an alternative extraction method (as referenced in Materials and Methods section - **Wizard® Genomic DNA Purification Kit Method**). Resultant PCR products were electrophoresed (in 1% agarose gels containing PeqGREEN® dye).

Table 4.14 A further narrowed down selection of isolates (n=32) from the 106 that were used for the characterisation study.

Isolate	Source
4	Abdominal fluid
6	Vulval swab
22	Wound swab
28	Right ear swab
33	Right ear swab
42	Wound
43	Right ear swab
44	Tracheal
45	Left ear
46	Right ear swab
47	Tissue
48	Right ear swab
52	Urine
53	Urine catheter
54	Right ear swab
55	PEG tube Stoma wound
63	Wound
64	Right anal sac swab
69	Right ear swab
70	Wound serous discharge and fat tissue
74	Right and Left BAL
72	Tissue
81	Tissue
82	Vulval fluid swab

84	Skin
86	Right ear swab
87	Ear swab
88	Right ear swab
93	Urine
95	Ear swab
99	Ear swab
100	Vaginal swab

Of the positive amplicons obtained for each resistant determinant presented in the Tables 4.9-4.12) only a small selection were subsequently sequenced due to time and cost constraints (sequenced amplicons and sequencing results are listed in Table 4.15)

Table 4.15. Selected isolates and results of the simplex PCR sequenced isolates of ESBL-associated resistance genes from veterinary panel of *P. aeruginosa*

Isolate sent for sequencing	**Positive amplicon**	**Results of sequencing**
13	TEM	Confirmed as TEM1/TEM 188/
71	TEM	Confirmed as TEM1
54	SHV	Confirmed as SHV 12
58	SHV	Confirmed as SHV 1/11/148/149/150/33
70	OXA	Confirmed as OXA 1 - 100%
42	PER	Non specific amplification
6	VIM	Non specific amplification
80	VIM	Non specific amplification
33	KPC	Non specific amplification
70	KPC	Non specific amplification
91	KPC	Non specific amplification

The ESBL-associated resistance genes which generated a weak amplicon were not included for sequencing.

The table below (Table 4.16) summarises the findings of isolates within the smaller subset selected that were confirmed PCR positive and sequenced along with the results of phenotypic antibacterial resistance testing for these isolates.

Table 4.16. Summary of the isolates and resistance genes confirmed by simplex PCR and sequencing for selected veterinary *P. aeruginosa* isolates associated with ESBL resistance (n=32)

Gene	Simplex PCR positive isolates	Isolates confirmed by sequencing	Results of additional phenotypic antibacterial resistance to testing
bla_{TEM}	6 isolates (43, 44, 45, 54, 55, 93)	Isolate 71 (canine urine) =blaTEM-1, Isolate 13 (canine ear swab)=blaTEM1/188	71 = Intermediate Resistance to aztreonam. Intermediate Resistant to enrofloxacin. * 13= Intermediate Resistance to aztreonam. Intermediate Resistance to enrofloxacin. * Susceptible to others.
bla_{SHV}	4 isolates (43, 44, 54, 55)	Isolate 58 (canine tissue swab) = SHV 1/148/149/150/33 Isolate 54 (canine ear swab) =SHV 12	Intermediate Resistant to aztreonam. Intermediate Resistant to enrofloxacin. * Resistant to aztreonam, cefepime, piperacillin, pip/taz. Intermediate Resistant to Meropenam. Intermediate Resistant to Gentamicin. Resistant to enrofloxacin and Marbofloxacin. *
bla_{OXA}	3 isolates (70, 71, 72)	Isolate 70 (canine wound serous discharge and fat tissue) = OXA-1	Intermediate Resistant to aztreonam. Resistant to cefepime, piperacillin, pip/taz. Intermediate Resistant to enrofloxacin. *

Resistance to aminoglycoside antibiotics

In order to determine the resistance to aminoglycosides, MICs to two clinically used aminoglycoside antibiotics; amikacin and gentamicin were performed. From the panel of 106 isolates, seven were found to be resistant to amikacin with an MIC of ≥16 mcg/ml. Nine isolates displayed intermediate resistance and 90 were found to be susceptible. Of the seven amikacin resistant isolates, six were of canine origin with one from equine origin. Of the 11 gentamicin resistant isolates, 10 were from canine infections and one from equine (Table 4.17 and Table 4.18).

MIC resistance levels for amikacin and gentamicin were found to be at similar levels, at 6.6% and 10.4% respectively (Table 4.17).

Table 4.17. Minimum inhibitory concentration testing of *P. aeruginosa* from companion animal (n=106) isolates to aminogycosides

Antimicrobial	Breakpoint	MIC mcg/mL										MIC_{50}	MIC_{90}	Resistant strains (%)
	mcg/L	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**			
Amikacin	S≤4, I=8, R≥16					90	9	6			1	4	4	6.6 [3, 13]
Gentamicin	S≤2, I=4, R≥8			25	54	16	6	5				2	4	10.4 [0.176, 0.059]

Confidence intervals are 95% {Lower, Upper) Wilson method

Other antibacterials (amoxicillin/clavulanic acid, ampicillin, cefazolin, cefovecin, cefoxtine, cefpodoxime, chloramphenicol, clindamycin, doxycycline, erythromycin, oxacillin, +2%NaCl, penicillin, rifampicin, trimethoprim sulfamethoxazole) included in the Trek COMPAN1F plate were also tested but are not reported due to intrinsic *P.aeruginosa* resistance.

There were seven isolates that were resistant to amikacin and 11 were resistant to gentamicin. One of these isolates (isolate 43, from canine ear swab) was confirmed by sequencing as carrying the *qnrB* resistance gene (Table 4.22). Of the seven amikacin resistant isolates six were similarly resistant to gentamicin. These consisted of five isolates from canine infections and one from an equine sample.

The majority were sourced from ear swabs (10), with remainder being from wound swab (1) and from tissue swab (1) (Table 4.18).

Table 4.18. Clinical veterinary bacterial isolates from companion animals resistant to both aminoglycosides, amikacin and gentamicin

Isolate Ref number	Description	Species
16	Left ear swab	Canine
43	Right ear swab	Canine
48	Right ear swab	Canine
49	Right ear swab	Canine
72	Tissue	Canine
75	Left ear swab	Canine
86	Right ear swab	Canine
87	Ear swab	Canine
89	Ear swab	Canine
94	Midline swab	Equine
95	Ear swab	Canine
105	Right Ear Swab	Canine

Resistance to Fluoroquinolones

In order to determine the resistance to fluoroquinolones, MICs to two widely used fluroquinolone antibiotics; enrofloxacin and marbofloxacin were performed. From the panel of 106 isolates, 38 were found to be resistant to enrofloxacin with an MIC of 4 mcg/ml. 55 isolates displayed intermediate resistance and 13 were found to be susceptible. Of the 38 enrofloxacin resistant isolates, all were of canine origin. For marbofloxacin 29 were found to be resistant with a MIC of 4mcg/ml. 21 isolates displayed intermediate resistance and 55 were found to be susceptible. Of the 29 resistant isolates, all were of canine origin (Table 4.19).

Table 4.19. Minimum inhibitory concentration tests to show antimicrobial resistance of *P. aeruginosa* in companion animal (n=106) isolates to fluroquinolone veterinary antimicrobials

Antimicrobial	Breakpoint	MIC mg/mL										MIC_{50}	MIC_{90}	Resistant strains (%)
	mg/L	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**			
Enrofloxacin	S<1, I ≥1 ≤2, R>4	3	10	43	12	38						0.5	4	35.8 [27.4, 45.3]
Marbofloxacin	S<1, I ≥1 ≤2, R>4	16	39	14	7	29						1	4	27.4 [19.8, 36.5]

Confidence intervals are 95% {Lower, Upper) Wilson method

Other antibacterials (amoxicillin/clavulanic acid, ampicillin, cefazolin, cefovecin, cefoxtine, cefpodoxime, chloramphenicol, clindamycin, doxycycline, erythromycin, oxacillin, +2%NaCl, penicillin, rifampicin, trimethoprim sulfamethoxazole) included in the Trek COMPAN1F plate were also tested but are not reported due to intrinsic *P.aeruginosa* resistance.

MIC resistance levels for enrofloxacin were notably slightly higher at a level of 35.8% compared to 27.3% in marbofloxacin. There were 38 isolates that were resistant to enrofloxacin and 29 were resistant to marbofloxacin. All 29 isolates that were resistant to marbofloxacin were also resistant to enrofloxacin. These were isolates all of canine origin and the majority were sourced from ear swabs (17), with remainder being from wound swabs (3), from tissue swabs (2), from vulva/vaginal swab (2), from other skin sites (neck/lip folds) (2), from urine (1) and abdominal fluid (1) (Table 4.20).

Table 4.20. Clinical veterinary bacterial isolates from companion animals resistant to both fluroquinolones, enrofloxacin and marbofloxacin.

Isolate Ref number	Description	Species
3	Skin swab	Canine
4	Abdominal fluid	Canine
14	Right ear swab	Canine
18	Left ear swab	Canine
26	Neck swab	Canine
28	Right ear swab	Canine
32	Vaginal swab	Canine
33	Right ear swab	Canine
34	Lip fold	Canine
35	Right ear swab	Canine
42	Wound	Canine
43	Right ear swab	Canine
45	Left ear swab	Canine
46	Right ear swab	Canine
52	Urine	Canine
54	Right ear swab	Canine
56	Left ear swab	Canine
63	Wound swab	Canine
68	Tissue	Canine
72	Tissue	Canine
85	Left ear swab	Canine
86	Right ear swab	Canine
87	Ear swab	Canine
89	Ear swab	Canine
95	Ear swab	Canine
97	Vulval swab	Canine
99	Ear swab	Canine
104	Wound swab	Canine
105	Right Ear Swab	Canine

In order to determine whether the resistance phenotypes observed via MIC assays were associated with the presence of fluroquinolone resistance determinants, PCR for the genes *qnrA, qnrB, qnrS* and *aac 6'Ib-cr* were performed. Six isolates showed positive amplifications for resistance determinants *qnrA* (one – isolate 57) and qnrB (five – isolates 42, 43, 44, 57, 70, 72) – refer to Figures 4.14 and 4.15. Of these isolates three (isolates 42, 43, 72) were resistant to both fluroquinolones

enrofloxacin and marbofloxacin, one (isolate 57) was resistant to just enrofloxacin alone and two demonstrated intermediate resistance to enrofloxacin (but were susceptible to marbofloxacin). 16 isolates showed positive amplicons for resistance determinants *qnrS* (Figure 4.21) and seven positive to *aac(6')-Ib* -cr (Figure 4.22).

However, of these amplicons several were feint and considered only weakly potential positives (detailed in Table 4.21). (begins on p158)

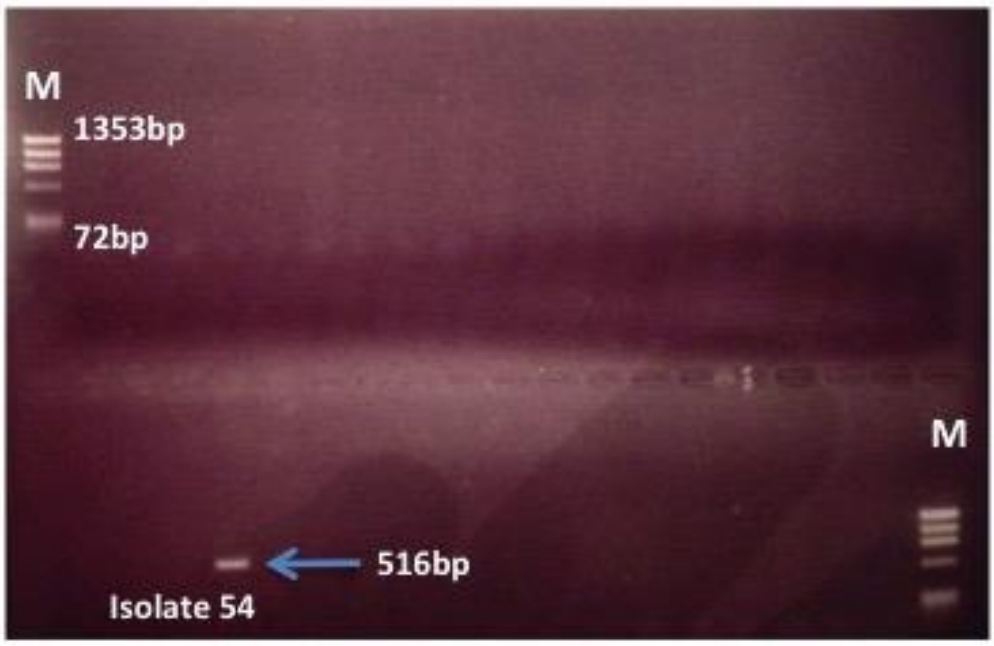
Table 4.21 Results of the PCR screening of fluroquinolone-associated resistance genes within the larger veterinary panel of *P. aeruginos*a

Isolate	*qnrA*	*qnrB*	*qnrS*	*aac(6')-Ib-cr*	Isolate	*qnrA*	*qnrB*	*qnrS*	*aac(6')-Ib-cr*
1	-	-	-	-	54	+	-	-	-
2	-	-	-	-	55	-	-	+	-
3	-	-	-	-	56	-	-	-	-
4	-	-	-	-	57	+	-	-	-
5	-	-	-	-	58	-	-	+	-
6	-	-	-	-	59	-	-	-	-
7	-	-	-	-	60	-	-	+	-
8	-	-	-	-	61	-	-	-	-
9	-	-	+	-	62	-	-	-	-
10	-	-	-	-	63	-	-	+	-
11	-	-	+	-	64	-	-	-	-
12	-	-	-	-	65	-	-	-	-
13	-	-	-	-	66	-	-	-	-
14	-	-	-	-	67	-	-	-	-
15	-	-	-	-	68	-	-	-	-
16	-	-	-	-	69	-	-	-	-
17	-	-	+	-	70	-	+	-	+
18	-	-	-	-	71	-	-	-	+
19	-	-	-	-	72	-	+	-	+
20	-	-	-	-	73	-	-	-	-
21	-	-	-	-	74	-	-	+	-
22	-	-	-	-	75	-	-	-	-
23	-	-	-	-	76	-	-	-	-
24	-	-	+	-	77	-	-	-	-
25	-	-	-	-	78	-	-	-	-
26	-	-	-	-	79	-	-	+	-
27	-	-	-	-	80	-	-	-	-
28	-	-	-	-	81	-	-	-	+
29	-	-	+	-	82	-	-	-	-
30	-	-	+	-	83	-	-	-	-
31	-	-	-	-	84	-	-	-	-
32	-	-	-	-	85	-	-	-	-
33	-	-	-	-	86	-	-	-	-
34	-	-	-	-	87	-	-	-	-
35	-	-	-	-	88	-	-	-	-
36	-	-	-	-	89	-	-	-	-
37	-	-	-	+	90	-	-	-	-
38	-	-	-	-	91	-	-	+	-
39	-	-	-	-	92	-	-	-	-
40	-	-	+	-	93	-	-	-	-
41	-	-	-	-	94	-	-	-	-
42	-	+	-	+	95	-	-	-	-
43	-	+	-	+	96	-	-	-	-
44	-	+	-	+	97	-	-	-	-
45	-	-	-	-	98	-	-	-	-
46	-	-	-	-	99	-	-	+	-
47	-	-	-	-	100	-	-	-	-
48	-	-	-	-	101	-	-	-	-
49	-	-	-	-	102	-	-	+	-
50	-	-	-	-	103	-	-	-	-
51	-	-	-	-	104	-	-	-	-
52	-	-	+	-	105	-	-	-	-
53	-	-	-	-	106	-	-	-	-

*Although there was amplification of the multiple fluroquinolone-associated resistance genes, some were feint and only weakly positive but are still noted above in table.

The figures below depict some of the PCR gel results for the *qnrA* gene, *qnrB*, *qnrS*, *aac(6')-Ib-cr* genes as reported.

(a)



(b)

Figure 4.15 Simplex PCR assay using primers to detect *qnrA* gene performed on isolates from VDL, Leahurst. An isolate (54) appears to show strong amplification of *qnrA* shown on the first gel image (a), the second gel image (b) shows the positive control with positive result. The DNA loading dye DNA/BsuRI (HaeIII) Marker 9, 50 mg (0.5 µg/µl)(ThermoScientific Molecular Biology), was placed on each gel run.

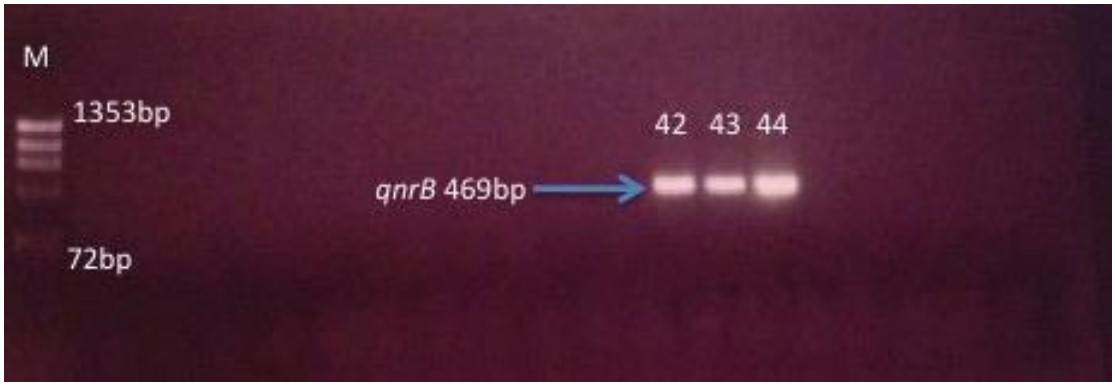


Figure 4.16 Simplex PCR assay using primers to detect *qnrB* gene performed on isolates from VDL, Leahurst. Here isolates 42,43 and 44 appear to show strong amplification. The PCR products were electrophoresed in 1% agarose gels containing PeqGREEN® dye (VWR International Ltd, Leicestershire, England). The DNA fragments were imaged with UV transilluminator and UViProMW software documentation system. The DNA loading dye DNA/BsuRI (HaeIII) Marker 9, 50 mg (0.5 µg/µl)(ThermoScientific Molecular Biology), was placed on each gel run.

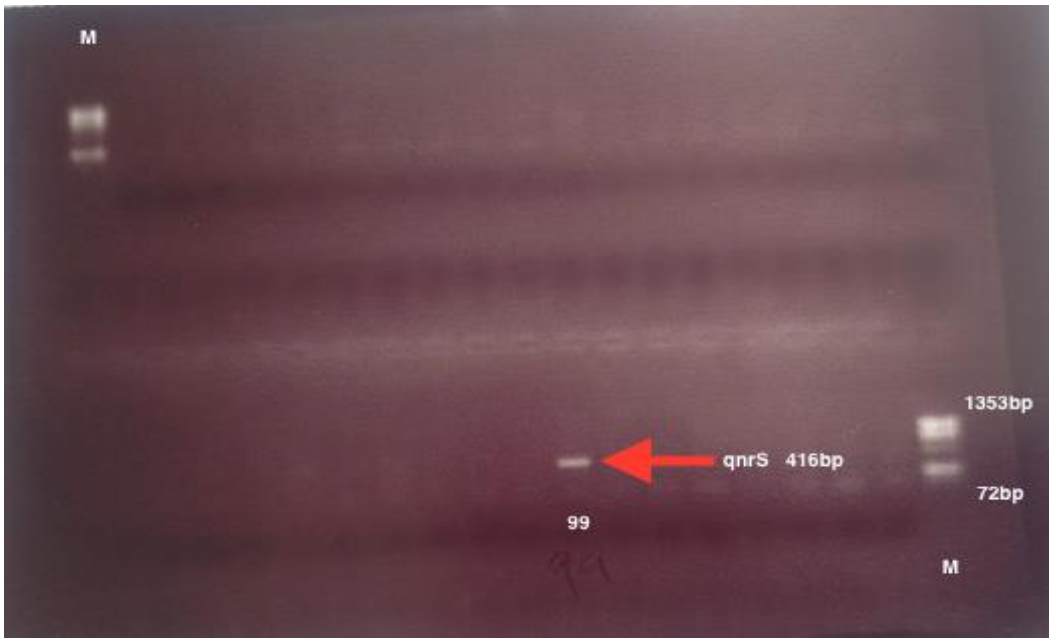
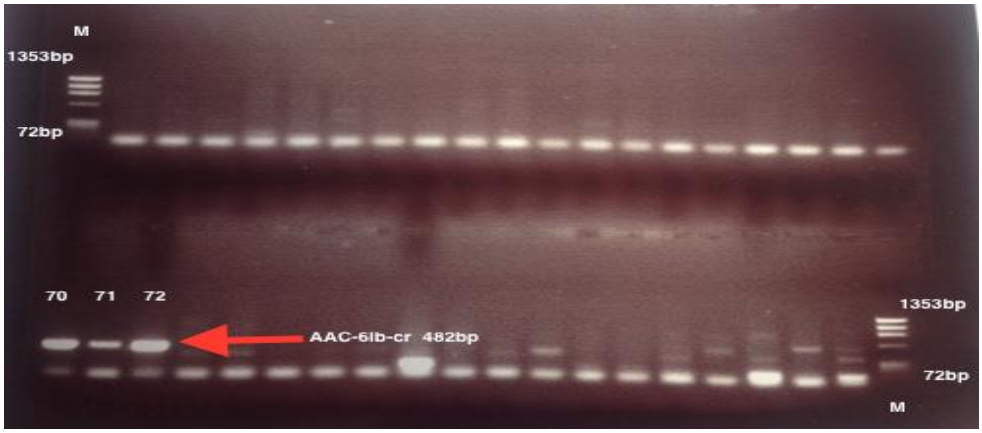
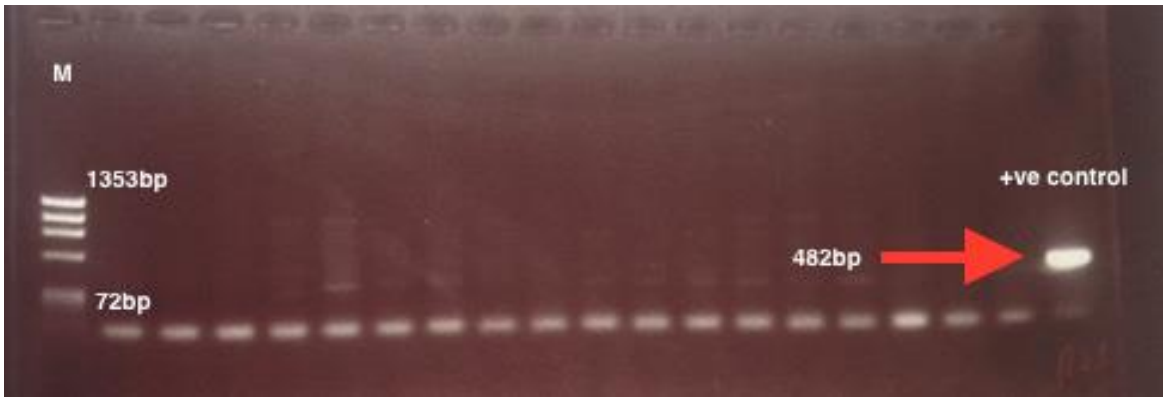


Figure 4.17 Simplex PCR assay using primers to detect *qnrS* gene performed on isolates from VDL, Leahurst. An isolate (99) appears to show strong amplification of *qnrS* shown on the gel image. The DNA loading dye DNA/BsuRI (HaeIII) Marker 9, 50 mg (0.5 µg/µl)(ThermoScientific Molecular Biology), was placed on each gel run.

(a)



(b)



(c)

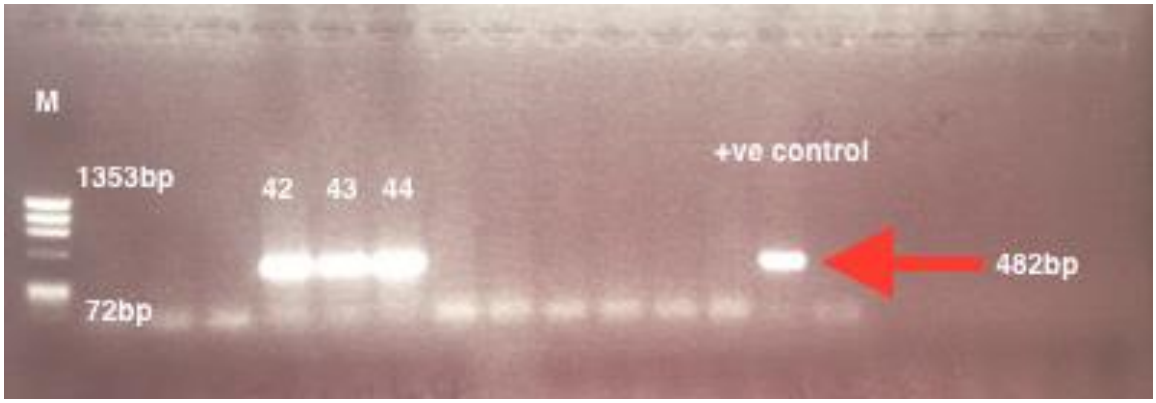


Figure 4.18 Simplex PCR assay using primers to detect *aac(6')-Ib -cr* gene performed on isolates from VDL, Leahurst. Three isolates (70, 71, 72) appear to show strong amplification of *aac(6')-Ib* -cr shown on the first gel image (a), the second gel image (b) shows the positive control, the third gel image (c) shows 3 isolates (42, 43,44 showing strong amplification of the *aac(6')-Ib* -cr gene – the positive control is also identified. The DNA loading dye DNA/BsuRI (HaeIII) Marker 9, 50 mg (0.5 µg/µl)(ThermoScientific Molecular Biology), was placed on each gel run.

To verify the sequence identity a small selection (due to time and cost limitations) of the amplified bands were sent for sequencing for each fluroquinolone-associated resistance determinant, these are detailed in (Table 4.22) along with the result of sequencing.

Table 4.22 Selected isolates and results of the PCR sequenced isolates of fluroquinolone-associated resistance genes from veterinary panel of *P. aeruginosa*

Isolate sent for sequencing	**Positive amplicon**	**Results of sequencing**
54	*qnr A*	Confirmed as qnrA1
43	*qnrB*	Confirmd as qnrB2
70	*qnrB*	Non specific amplification
99	*qnrS*	Didn't reproduce a strong positive amplicon therefore not sent
72	*aac -6'lb-cr*	Confirmed as acetyltransferase AAC(6')-Ib-cr (aac(6')-Ib-cr) *However this result was excluded because the initial Simplex PCR showed positive amplicon but the subsequent simplex PCR on the smaller selection of isolates did not produce positive PCR.
81	*aac -6'lb -cr*	Non specific amplification

Detailed in Table 4.23 are the molecular and phenotypic characteristics of the specific isolates with fluroquinolone associated resistance determinants as confirmed via sequencing along with the number of positive amplicons identified on PCR from the smaller subset (n 32) of veterinary *P.aeruginosa* isolates.

Table 4.23. Summary of the confirmed PCR positive and sequenced isolates associated with fluroquinolone resistance within the smaller (n 32) subset of *P.aeruginosa* veterinary isolates combined with the phenotypic characteristics

Gene	Number of positive PCR isolates	Sequencing confirmed positive isolates	Results of additional phenotypic antibacterial resistance to testing
qnrA	2 isolates (54 and 55)	Isolate 54 (canine ear swab) = *qnrA1*	Intermediate Resistant to aztreonam. Resistant to cefepime, piperacillin, pip/taz. Intermediate Resistant to enrofloxacin. *
qnrB	2 isolates (43 and 44)	Isolate 43 (canine ear swab) = *qnrB*	Intermediate Resistant to aztreonam. Intermediate Resistant to Gentamicin. Resistant to enrofloxacin and marbofloxacin.
aac(6')-Ib-cr	7 isolates (4 stronger positive amplicons 43, 44, 45,100 and 3 weakly positive amplicons 42, 48, 54)	Isolate 72 (canine tissue swab) – *aac 6'Ib -cr*	Resistant to aztreonam. Resistant to ticarcallin and ticacillin/clavulanate Resistant to enrofloxacin and marbofloxacin, Resistant to gentamicin. *

***** Other antibacterials (amoxicillin/clavulanic acid, ampicillin, cefazolin, cefovecin, cefoxtine, cefpodoxime, chloramphenicol, clindamycin, doxycycline, erythromycin, oxacillin, +2%NaCl, penicillin, rifampicin, trimethoprim sulfamethoxazole) included in the Trek COMPAN1F plate were also tested but are not reported due to intrinsic *P.aeruginosa* resistance.
(Nikaido 1994)

Multidrug resistance (MDR) in isolates from companion animals.

The PCR amplicons confirmed by DNA sequencing to encode for the screened genes, originated from 8 different Pseudomonas isolates, with 5 of these isolates harbouring more than one of the investigated resistance genes.

Isolates 43 (canine ear swab) and 44 (canine tracheal swab) were found to carry the *bla*$_{TEM}$, *bla*$_{SHV}$ and *qnrB* genes. Isolate 45 (canine ear swab) was found to carry both the *bla*$_{TEM}$ and *bla*$_{OXA}$ genes. Isolate 54 (canine ear swab) and 55 (canine PEG tube stoma wound) was found to carry the *bla*$_{TEM}$, *bla*$_{SHV}$ and *qnr*A genes. Isolate 93 (canine urine) was found to carry just the *bla*$_{TEM}$ gene and isolates 84 (canine skin swab) and 99 (canine ear swab) were both found to carry only the *bla*$_{OXA}$ gene.

Isolate 71 (canine urine sample) was found to be carrying *TEM-1* gene with intermediate resistance to aztreonam and enrofloxacin. Isolate 13 (canine ear swab) was found to be carrying *TEM-1/188* gene and similarly with intermediate resistance to aztreonam and enrofloxacin. Isolate 58 (canine tissue sample) was found to be carrying *SHV-1/148/149/150/33* gene also with intermediate resistance to aztreonam and enrofloxacin. Isolate 70 (canine wound sample) was found to be carrying *OXA-1* gene with resistance to cefepime, piperacillin and pipercillin/tazobactam, along with intermediate resistance to aztreonam and enrofloxacin. One particularly interesting isolate was isolate 54, from a canine ear swabs and showing multidrug resistance characteristics. Isolate 54 was found to be carrying *SHV-12* gene and *qnrA1* with resistance to aztreonam, enrofloxacin, to both piperacillin and piperacillin/tazobactam and also to the 4th generation cephalosporin cefepime, as such making it multidrug resistant (MDR). It also demonstrated intermediate resistance to meropenam and gentamicin. Isolate 43 (canine ear swab) was found to harbour *qnr*B gene with resistance to enrofloxacin and marbofloxacin. It also demonstrated intermediate resistance to aztreonam and gentamicin.

Isolate 72 (canine tissue swab) was found to be carrying the *aac(6')-Ib* -cr variant resistant determinant by sequencing and was resistant to aztreonam, ticarcacillin, ticacillin/clavulanate, enrofloxacin, marbofloxacin and gentamicin and therefore was multidrug resistant. However, although this sequencing result is reported (in Table 4.22) there was disparity in the PCR results for the isolate. Isolate 72 showed a positive band on the PCR in the original simplex PCR (Table 4.21) but was then negative in repeated simplex PCR for the smaller subset of isolates. As such this isolate is being considered as negative for this gene in this isolate. There is the potential possibility contamination may have resulted in misleading results in this case.

4.4 Discussion

The true extent and importance of AMR in companion animals is not entirely understood and limited surveillance contributes to this (P. H. Jones et al., 2014; Radford et al., 2011; Scott Weese, 2008). As discussed previously in Chapter 1, potential for interspecies human-animal transmission exists. Studies have demonstrated that AMR is problematic in a number of important pathogens and commensals, such as *Staphylococci, Enterococci, E. coli* and *Salmonella*, although in *P. aeruginosa* this appears less well reported in animals as compared to humans. Much of the literature has focussed on AMR in food producing animals. This area may be a reservoir and source of AMR genes that could be transmitted to the human population (Arnold et al., 2016; Weese, 2008). Studies have shown that antimicrobial use in food animals contributes to the selection of antimicrobial resistance and poses risks to humans due to transmission of resistant zoonotic bacteria via the food chain and indirect transfer of resistance genes from animals to humans (Aarestrup et al., 1998; Smith et al., 2002; Tang et al,. 2017; van Den Bogaard & Stobberingh, 2000).

Companion animals are another potential reservoir source for AMR to develop and is less well studied. The pet population is ever growing (PFMA, 2017) and in 2017 it

is estimated that 12m (44%) of households have pets giving a pet population of around 54 million. 24% of households owning dogs and 17% of households owning cats (PFMA, 2017). Several reports have highlighted the presence of resistance genes in both farm (Timofte et al., 2014) and companion animals (Schmidt et al., 2015; Timofte et al., 2011) including equines (Ahmed et al., 2012; Maddox et al., 2011). Cats and dogs in particular, represent a potential source of spread of antimicrobial resistance due to the extensive use of antimicrobial agents in these animals and their close contact with humans. There are several studies looking at AMR in companion animals (mainly in that of dogs and horses), predominantly involving *E.coli* (Schmidt et al., 2015; Wedley et al., 2011). However, fewer studies of AMR and *P. aeruginosa* isolates exist.

A number of antimicrobial classes are frequently used in small animal veterinary medicine including penicillins, cephalosporins, macrolides, lincosamides, fusidic acid, tetracyclines, chloramphenicol, potentiated sulphonamides, aminoglycosides and fluoroquinolones. The most frequent causes of antimicrobial treatment in dogs and cats are skin and wound infections, otitis externa, respiratory infections, and urinary tract infections (UTI) (Radford et al., 2011; Singleton et al., 2017).

Due to the presence of several drug efflux systems and porins, *P. aeruginosa* is intrinsically resistant to a wide range of antimicrobials. The results here confirmed the intrinsic resistance to antibacterials amoxicillin/clavulanic acid, ampicillin, cefazolin, cefovecin, cefoxitine, cefpodoxime, chloramphenicol, clindamycin, doxycycline, erythromycin, oxacillin, +2%NaCl, penicillin, rifampicin, trimethoprim sulfamethoxazole (these were included in the Trek COMPAN1F plate and were also tested but results not reported).

There was high occurrence of resistance to the third generation cephalosporin ceftiofur (98.1% resistant). Although the prevelance of resistance to fourth generation cephalosporin, cefepime was found to be 9.4%. Beta-latam resistance to piperacillin, piperacillin/tazobactam, ticarcillin and ticacillin/clavulanate were 17%, 17.9%, 9.6% and 5.6% respectively. Of the carbapenems; meropenam resistance was particularly low (3.7%) and also low for imipenem (8.5%), although

slightly higher in aztreonam (11%). Resistance to aminoglycosides was low (6.6% for amikacin and 10.4% for gentamicin).

The fluoroquinolones are some of the most commonly prescribed effective antimicrobials against *P. aeruginosa* infections, in recent years bacterial resistance to fluroquinolones has been promoted with overuse of these antibacterials (Jacoby, 2005) and as such, the monitoring of antimicrobial susceptibility is important for selecting effective antimicrobial agents in the treatment of this disease. Quinolones enter bacteria through porins or directly through the lipid and cytoplasmic membrane and target DNA topoisomerases (Jacoby et al., 2008) achieving their bactericidal effects through the inhibition of DNA *gyrase* and topoisomerase IV. There are three plasmid-mediated quinolone resistance determinants discussed in the literature. The *qnr* genes (*qnrA, qnrB, qnrS), aac(6')-Ib -cr,* and *qepA*. Qnr proteins are known to be a part of the pentapeptide repeat family and protect DNA gyrase and topoisomerase IV from quinolone inhibition (Ma et al., 2009). The *aac(6')-Ib*-cr is a variant of *aac(6')-Ib*. *AAC (6')-Ib* is an enzyme that causes aminoglycoside resistance through acetylation, and a variant of this enzyme, *aac (6')-Ib*-cr, is deemed responsible for plasmid-mediated quinolone resistance (Ma et al., 2009).

Studies have largely shown that resistance to the fluroquinolones is mainly due to: (i) the point mutations in the DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) genes, (ii) the presence of transferable plasmid-mediated quinolone resistance (PMQR) determinants, and (iii) mutations in genes regulating the expression of efflux pumps and decreased expression of outer membrane porins (D. Lin et al., 2012). Plasmid-associated resistance to fluroquinolones can be mediated by the production of qnr proteins, which preserve DNA gyrase and topoisomerase IV from inhibition by quinolones (Strahilevitz et al., 2009a). The fluoroquinolones are approved for indications such as respiratory tract, urinary tract infections and soft tissue infections and in dogs and cats along with colibacillosis in poultry (chickens and turkeys). Fluoroquinolone use is licenced for conditions such as canine otitis externa and in rabbits, small mammals and reptiles for alimentary and respiratory tract infections. The fluroquinolones are also licensed for veterinary use

in treatment of acute mastitis and respiratory infections in bovines and neonatal *E. coli* gastroenteritis in calves. Other indications for which the fluoroquinolones have been used in animal health include deep-seated infections, prostatitis, and other bacterial infections resistant to standard antimicrobial therapy (Brown, 1996). In this study, *P. aeruginosa* isolates showed 36% resistance to enrofloxacin and 27% to marbofloxacin. With increasing utilization of fluoroquinolones in both human and veterinary medicine, emerging resistance has become a significant concern (Cattoir et al., 2007; Hong et al., 2009; Strahilevitz et al., 2009a; Sumrall et al, 2014).

A study of a University Hospital in North eastern Poland looked at a relatively small number of *P.aeruginosa* human isolates (from 2002-2009) and reported 20% (5/25) isolates had detectable *qnrB* by PCR (Michalska et al., 2014). The same study did also identify *aac(6')-Ib* in 28% (7/25) of isolates. Compared to none found in the results of this work. Among *Enterobacteriaceae* screened for production of plasmid-mediated fluoroquinolone resistance determinants, *qnrB* was reported as the most prevalent gene (Kim et al 2009). However there is not a similar longitudinal study to date to compare prevalence in *P.aeruginosa* or in veterinary isolates (for comparison to human isolates). However, plasmid mediated quinolone resisatnce (PMQR) genes (*qnrA*, *qnrB*, *qnrS*, *qnrD* and *qepA*) have been reported in veterinary clinical isolates in China (Yang et al., 2015; Zhao et al., 2010). The *qnrA1* and *qnrB* determinants identified in *P. aeruginosa* isolates of this study are novel findings in UK in companion animal isolates. Plasmid mediated quinolone resistance (PMQR) determinants have been demonstrated among a variety of animal sources in the *Enterobactereaceae* bacteria. A number of studies have reported presence of the *qnr* genes in faeces from healthy animals or clinical isolates, including in chicken faeces from poultry production systems, cattle faeces, pig farms, other food production animals and companion animals (Ajayi et al, 2012; Huang et al., 2009; Kuo et al., 2009; Ma et al., 2009) and appears to be distributed throughout the world. Fluroquinolone resistance genes *qnrA* and *qnrB* are reported in *P. putida* in isolates from imported shrimp (Tran et al, 2011) and in *P. fluoroscens* although none currently reported in animal *P. aeruginosa* isolates. Cayci et al (Cayci et al., 2014),

performed screening for plasmid mediated quinolone resistance genes in *P. aeruginosa* clinical (human) isolates, although no *qnr* genes were identified.
The *qnrB1* has been reported present in an ESBL producing *E.coli* isolates from Tunisia and in *E.coli* isolates from human and poultry origin in Ecuador based study (Armas-Freire et al., 2015). In a study of AMR resistance risk factors in *E.coli* in healthy Labrador retrievers (Schmidt et al., 2015) the *qnr* genes were not detected in the sample set investigated in that study.
Bacterial resistance to fluroquinolones results from mutations in the quinolone resistance determining regions of the drug targets, over expression of efflux pumps and/or the more recently identified plasmid mediated low level resistance mechanism (Cayci, Y.T 2014; Strahilevitz et al., 2009a).

In this study, whilst *qnr* determinants where confirmed, the *aac(6')-Ib-cr* determinants were not. The *qnr* and *aac(6')-Ib-cr* determinants are widely reported in human clinical isolates of *Enterobacteriaceae* with a worldwide distribution (Hong et al., 2009; Ma et al., 2009; Park et al., 2006; Robicsek et al., 2006; Rodríguez-Martínez et al., 2011; Sumrall et al., 2014; Xiao et al., 2012). There is suggestion of transferability of resistant bacteria or mobile resistance determinants between animals and humans potentially via the food chain or direct contact (Fey et al., 2000; Manian, 2003). The quinolones are an antibacterial with widespread use in livestock veterinary care and in treatment of companion animals in some countries, including the UK. However, few reports on the occurrence of plasmid-mediated quinolone resistant (PMQR) determinants among bacteria from companion animals and food-producing animals have been published. Quinolones and β-lactams are among the most commonly used antimicrobials in both human and veterinary clinical medicine.

4.5 Conclusions

The findings in this work have identified several resistance genes including those relating to ESBLs production (*bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$) and those relating to fluroquinolone resistance (*qnr*). In particular, the identification of *qnrA* and *qnrB* genes in *P.aeruginosa* isolates of companion animal origin is a novel finding with no known reports to current date in the UK. Whilst reports demonstate their prevelance worldwide and in Enterobacteriaceae, reports on *P aeruginosa* of companion animal origin remain non reported in literature. It would be advantageous to have further time and funds on this study to be able to expand upon the study sample size and also investigate the transmissibility of these identified genes through conjugation studies and to further define the mechanisms of resistance. In addition, genotyping of the resistant isolates to determine their relative distribution within the wider *P.aeruginosa* population would be beneficial.

This study further confirms the likelihood that companion animals are an understudied source of antimicrobial resistant *P. aeruginosa* isolates and merit sustained surveillance of the veterinary niche as a potential reservoir for resistant, clinically-relevant bacteria.

Chapter 5: General Discussion and Future Studies

Antimicrobial resistance is a multifactorial and complex issue affecting healthcare worldwide. It is an escalating concern as a developing public health crisis and European and International bodies have highlighted AMR as a global public health issue of imperative importance. The World Health Organisation (WHO), the European Centre Disease Prevention and Control (ECDPC) and Infectious Diseases Society of America (IDSA) have included within their concerns and priorities, emerging antimicrobial resistance mechanisms, surveillance and antibiotic stewardship, applicable to both the veterinary and medical communities.
AMR is not only a concern in human health but also animal health. The close interface between people, pets, food production animals and the environment exemplifies the importance that these two areas of health should not be considered as entirely independent factions, particularly in relation to AMR. Adopting a 'one-health' approach in combatting this global problem is of great importance in understanding and potentially controlling the advances of resistance among the microbial population.

P. aeruginosa is one of the so-named ESKAPE pathogens by the IDSA and is categorized by the CDC as a serious level of threat (CDC, 2013). It was also named recently by the WHO as in the top three bacteria for which there is an urgent need for new antibiotics for therapy (WHO, Feb 2017). The WHO specified in particular carbapenem resistant *P. aeruginosa* in the WHO priority pathogens list for R&D of new antibiotics as Priority 1 - Critical (WHO 2017; Important & Priority, 2017). Increased resistance has resulted in limited therapeutic options. ESKAPE pathogens are highlighted as being of ever-growing relevance to antimicrobial chemotherapy in future years (Boucher et al., 2009; Santajit & Indrawattana, 2016). *P. aeruginosa* is a formidable pathogen due to its innate resistant making it naturally insensitive to many classes of antimicrobials, alongside its ability to acquire further resistance mechanisms (Henrichfreise et al., 2007; Lambert, 2002). In addition to these factors, it is known to form and survive in biofilms and is ubiquitous and opportunistic nature.

This thesis reports the levels of antibacterial resistance in a panel of *P. aeruginosa* isolates from companion animals including a high number of resistant isolates from canine ear infections. It was shown that resistance to polymyxins (a class of antimicrobials of particular importance in the management of *P. aeruginosa* lung infections in patients with CF), particularly polymyxin B was significantly higher in the panel of *P. aeruginosa* isolates taken from companion animals as compared to that of a human panel. This study highlighted the importance of sustained surveillance of the veterinary niche as source of antibacterial resistance.

Whole genome sequencing of a selection of polymyxin B resistant isolates from animal sources was performed. These were found to be a unique and an interesting source of genomic data. The sequencing revealed some of these resistant isolates to be incredibly diverse. Notably isolate 856 (sample from an equine abdominal incision) was diverse from the main population and was located on a distinct arm of the phylogenetic tree (refer to Chapter 3, Figure 3.6). This genome had 125303 SNPs and 1884 indels compared to PAO1 and 130312 SNPs and 1985 indels in comparison to PA14 (refer to Chapter 3, Figure 3.8). This was double the number of SNPs and indels than any other isolate sequenced.

An abundance of *P. aeruginosa* genome sequencing data information already exists, compiled predominantly from human isolates and environmental sources. *P. aeruginosa* isolates derived from animal infections are currently under-represented and this study highlights the importance of including such isolates. Increased sequencing and analysis of veterinary-associated *P. aeruginosa* isolates may add important additional data to the *P. aeruginosa* pan genome and population level analyses. Using the Clondiag Tube Array, a commercially available strain typing system, it was demonstrated that strain types identified from these companion animals had also been associated with human infections and therefore there may be a potential for transmission of infection through close contact. Indeed, there have been case studies reporting this between human and animal (Deora et al, 2013; Ferreira et al., 2011; Mohan et al., 2008b; Register et al., 2012; Wannet et al,

2005) however this area is understudied and therefore the true risk is unknown. By characterising and WGS a sample set of veterinary isolates in future studies it would be potentially possible to offer novel findings in this area contributing to knowledge of the *P. aeruginosa* population diversity in an understudied, under represented field and potentially identifying it as a reservoir of antimicrobial resistance.

The results from this study suggest that a larger study is warranted. In such a study, samples could be obtained on a random basis from a commercial UK veterinary reference diagnostic laboratory (for example Idexx Veterinary Diagnostic Laboratories – a commercial laboratory utilised by many veterinary practices) and would therefore be from a variety of UK geographic areas, different species and sites of origin. Characterisation through genomics and phenoptypic studies (including virulence factor production, biofilm formation and antimicrobial susceptibility testing) would provide a wealth of information which could both contribute to knowledge of *P. aeruginosa* population biology as well as providing a full characterisation of the features of isolates capable of causing infections in particular species of animals. This information could contribute to appropriate therapeutic choices and veterinary management of animals therefore ultimately providing a rationale and basis for treatment for veterinary practitioners.

It is acknowledged that there are limitations in the interpreting the results of this thesis. For instance, whilst the results of Chapter 3 showed the polymyxin resistance to be higher in the veterinary clinical isolates as compared to the human isolates and this was statistically significant ($p < 0.05$ – refer to Chapter 3 Results) however these results being from a relatively small sample set may not necessarily extrapolate to the wider population. It is also acknowledged that the two sample sets used (animal versus human) were not of equal size. In further studies it would be advantageous to acquire additional 'background' information such as that of the clinical history from the cases isolates were sampled from. This may help to better understand and postulate mechanisms of resistance development in the isolates. In this work, the samples were those sent in from a referral university practice/external veterinary practices directly to a Diagnostic Laboratory for

Microbiological assessment and then stored there, hence no clinical history would be available. To know if any of the patients from which the isolates originated had been previously treated with antibiotics would add value to understanding the full significance of the levels of antibiotic resistance reported from this work. To collate this prior clinical history in future study would be of benefit.

Limitations of the methods used, in particular that of the multiplex versus simplex pcr where identified during the study. However, it is understood that part of these issues may have been due to using some primers that were designed for use in *Enterobactereaccea* and thermocycler protocols published for these, it is acknowledged that to carry out prior optimisation PCRs to refine the conditions and to have *Pseudomonas* specific designed primers for the regions being targeted would have been an improvement that may have overcome these issues encountered. Further improvements would have been to use WGS to identify resistance detrminants, if funding would allow.

Whilst the larger sample set of 106 (Results reported in Chapter 4) is a statistically better sample size, ultimately there was a reduced sample size of 32 used for refining the results. These were chosen on basis of being 'interesting' isolates from the initial screening ie they had identified some resistance in specific antibitics or ESBL positive, however it is understood some bias would result in doing this. It is also noted that the inconsistencies of utilising human clinical breakpoints ion isolates of animla origin would not be ideal. However, some antibiotics especially those not routinely used in veterinary medicine do not have established veterinary breakpoints to use. VetCAST (a subcommittee of EUCAST) has been formed in 2015 and provides some guidance on this area of using available breakpoints.

Other findings as a result of the work performed within this thesis, has identified several resistance genes including those relating to ESBLs production (blaTEM, blaSHV and blaOXA) and those relating to fluroquinolone resistance (qnr) from *P. aeruginosa* isolates of veterinary origin. In particular the identification of *qnrA* and *qnrB* genes in *P.aeruginosa* isolates of companion animal origin is a novel finding with no known reports to current date in the UK. Whilst reports demonstate their prevelance worldwide and in *Enterobacteriaceae*, reports in *P. aeruginosa* of

companion animal origin remain non reported in literature, to the best of my knowledge. These findings would suggest that further exploration of isolates of animal origin could provide sources of resistant bacteria. That may have implications as a niche reservoir of resistant bacteria to provide a source of cross-resistance to human bacteria and also have implications in animal welfare and disease treatment as well as human health. In addition further genotyping of resistant animal isolates may provide novel information regarding their relative distribution within the wider *P.aeruginosa* population.

There are studies in the literature, for example that by Haenni et al., (Haenni et al., 2015), looking at population structure and antimicrobial susceptibility of *P. aeruginosa* from animal infections in France. They found resistance phenotypes were much more frequent in dogs (as compared to cows and horses), and multidrug resistant *P. aeruginosa* appeared to emerge mainly in those suffering from otitis. However, they noted such multidrug phenotypes are comparatively rare in other animal species. It would be of interest to further expand this study to include a greater number of *P.aeruginosa* isolates from a greater variety of animal species.

P. aeruginosa in the veterinary setting is a clinically relevant pathogen and a known to be associated with disease. In companion animals, *P. aeruginosa* can cause pyoderma, chronic otitis, ulcerative keratitis, wound infections, respiratory tract infections and urinary tract infections in a number of species. A number of other diseases involving *P. aeruginosa* in animals are known, including that of equine metritis and in chronic equine wounds where the ability of *P. aeruginosa* to form and survive within protective biofilms has been reported (Percival et al., 2015; Westgate et al., 2011). Canine chronic otitis is a common presentation in small animal veterinary medicine and a challenging infection to treat. Much is already known regarding *P. aeruginosa* in human disease, in particular that of cystic fibrosis (CF). From the extensive research carried out in the area of CF whereby these patients suffer from long-term chronic lung infections caused by *P. aeruginosa*. It is known that these are complicated and difficult to treat with routine antimicrobials. This is, in part, attributed to the complex behaviour of *P. aeruginosa* in chronic

infections. During these chronic infections the bacteria diversify, leading to populations with mixed levels of resistance or susceptibility to drugs. Further complicated by the ability of *P. aeruginosa* to form complex biofilm communities and coexist with many other bacterial species. The use of some antimicrobials may even drive diversification and increased resistance to drugs. It is unknown if these diverse populations occur in chronic infection of other species. Therefore, it would be insightful to study the population biology and dynamics in chronic *P. aeruginosa* infections of the canine ear by analysis of ear swab samples taken during therapy. This could improve current knowledge of microbial population dynamics within microbial communities in such infections and may inform diagnostic practice such as testing "sweeps" of *P. aeruginosa* from a samples rather than a single isolate when testing for antimicrobial susceptibility. By studying populations of *P. aeruginosa* from animal infections it would be possible to assess the impact of treatment strategies, fluctuations in the bacterial population biology and diversity, and potential drivers of antimicrobial resistance. Cross resistance of antimicrobial classes and transmission of resistant isolates between human and animal *P. aeruginosa* are major issues and therefore merit continued study.

Little is known about *P. aeruginosa* carriage in seemingly healthy companion animals and their owners. Suggested future avenues of study would be to determine the levels of *P. aeruginosa* carriage in the human owners/contact animal handlers of companion animals, particularly those canine patients with otitis. This would allow the study of potential cross transmission routes between species. A US study by Ferreira et al (Ferreira et al., 2011) looked at Methicillin-resistant *Staphylococcus aureus* (MRSA) transmission between companion animals and infected human patients presenting to outpatient medical care facilities. MRSA was isolated from at least one companion animal in 4/49 (8.2%) households of MRSA-infected outpatients compared to none of the pets of the 50 uninfected human controls. Using PFGE, patient-pets MRSA isolates were identical for three pairs and discordant for one pair. The results of this study suggest that companion animals of MRSA-infected patients can be culture-positive for MRSA, thus representing a potential source of infection or re-infection for humans. However, there is the

possibility that both parts became infected from different sources and direction of transmission also cannot be determined. Weese *et al* (Weese et al., 2006) studied the transmission of MRSA in veterinary clinics and in the households, after the identification of a MRSA positive animal. The authors described six cases. MRSA was isolated from 16% (14/88) of household contacts or veterinary personnel and in all of the 6 cases it was possible to find at least one human isolate identical to the animal (initial) one (Weese et al., 2006). Faires *et al* (Faires et al, 2009) evaluated both the rate of MRSA transmission from infected animals to humans and vice-versa. When the MRSA-infected animal was initially identified, at least one MRSA-colonized person was identified in over one-quarter (6/22; 27.3%) of the study households. By contrast, only one of the 8 (12.5%) study households of MRSA-infected humans contained an MRSA-colonised pet (Faires et al, 2009). The high prevalence of concurrent MRSA colonization as well as identification of indistinguishable strains in humans and pet dogs and cats in the same household suggested that interspecies transmission of MRSA is possible. Longitudinal studies are required to identify factors associated with interspecies transmission. Similar studies for investigation of such occurrence and factors involved for *P.aeruginosa* would be similarly useful.

The close nature of contact of animals and humans in both the home and working environments can allow for an opportunity of transmission of infection from human to animal and vice versa. This is an area where relatively few studies exist (Deora et al, 2013; Ferreira et al., 2011; Mohan et al., 2008b; Register et al., 2012; Wannet et al, 2005). There is potential to expand this study by collating samples from owners/handlers in contact with the corresponding pets for which canine chronic ear swab samples are collected from a Small Animal Teaching Hospital could provide a further interesting body of work. Potential results from such data could help to identify risk factors and interventions with the aim of improving the health of both humans and animals.

Antimicrobials are highly valuable in the prevention and treatment of infectious diseases in dogs and cats, and the occurrence of antimicrobial resistance threatens

their efficacy and utility. Overuse of antimicrobials is an important contributor to the occurrence of antimicrobial resistance. Few studies describing antimicrobial use in small animal practice have been published, in particular the off license use of antimicrobials. From the available evidence, antimicrobial use is common in small animal practice, although the overall quantity prescribed to small animals is often much smaller than in humans or food animal species (DANMAP 2015). In one veterinary teaching hospital, the rate of antimicrobial prescriptions ranged from 168 to 235 prescriptions per 1,000 admissions to the hospital (Weese et al., 2006). There is no specific recording scheme of the usage of antimicrobials for veterinary use per country but the European Medicines Agency reports (EMA, 2016) sales of antimicrobial agents in 29 European countries. Several epidemiological studies in dogs have demonstrated that prior antimicrobial exposure was associated with antimicrobial resistance in a number of bacterial species including *E. coli* isolates from faeces (Murphy et al., 2009; Ogeer-Gyles et al., 2006; Schmidt et al., 2015; Wedley et al., 2011) and opportunistic pathogens. Antimicrobial use needs to be appropriate and prudent, considering factors such as when to use antimicrobials, length of therapy and antimicrobial selection. Several veterinary professional organizations have published guidelines on the prudent use of antimicrobials (BSAVA 2017a). The PROTECT message developed out of an initiative of Small Animal Medicine Society (SAMSoc) to review and promote responsible antibacterial prescribing. This led to the PROTECT poster (BSAVA 2016b), produced by BSAVAs (British Small Animal Veterinary Association) and SAMSoc. A 2017 study (Singleton et al., 2017) describes antimicrobial agent prescription patterns over a 2 year period in small animal veterinary practices involved in a real time surveillance network scheme. The importance of guidelines or policies promoting responsible antibiotic use was identified as being important in guiding practitioner prescribing (Singleton et al., 2017).

There are documented areas within prescribing for small animal veterinary practice that antimicrobial use by veterinary surgeons could be improved (Coyne et al., 2016; Hughes et al., 2012; Mateus et al, 2014). These include eliminating the common use of antimicrobials in elective, routine neutering procedures (eg.

ovariohysterectomy, castration) and reducing antimicrobial use in other clean surgical procedures However, a study by Pratesi et al (Pratesi et al, 2015) looked at the efficacy of postoperative antimicrobial use for clean orthopedic implant surgery in dogs (a prospective randomized study in 100 consecutive cases) and showed that overall postoperative infection rate was 12.9%. Infection occurred in 2 cases (4.3%) administered postoperative oral antimicrobials and in 10 cases (21.3%) not administered postoperative antimicrobials. Use of postoperative antimicrobials was associated with a significant reduction in the risk of infection by 84% and risk of infection was increased by 2% for each minute increase in anesthesia time. The authors of the study concluded that administration of oral postoperative antimicrobials had a protective effect. The BSAVA provides guidance in the use of prophylaxis use of antibiotics and selection based on classification of wounds and surgical wounds (BSAVA, 2017). Antimicrobial use could likely be reduced for diseases such as feline lower urinary tract disease (FLUTD), feline upper respiratory tract disease (URT) and canine infectious tracheobronchitis. These are three particular conditions fairly commonly presented in veterinary first opinion practice and, although in some instances may involve primary or secondary bacterial pathogens, they are typically not primarily bacterial in origin. Traditional empirical practice in the afore mentioned conditions has frequently been to 'cover' these presentations with antibiotics.

Certain regulatory bodies already offer advice and protocols/prescribing pipeline for antimicrobial use in veterinary practice, notably the BVA (BVA 2017) and the BSAVA. The BSAVA provides guidelines to practicing veterinary surgeons (BSAVA 2017). PROTECT encourages a practice policy for empirical prescribing (whilst awaiting cultures) that can optimize therapy, and minimize inappropriate use of antibacterials. There are very strong arguments that antibaterials with restricted use in human medicine (eg. imipenem, vancomycin) should not be used in animals. These include amikacin, 3rd and 4th generation cephalosporins (except cefovecin) and fluroquinolones. These antibacterials should only be used when other agents are inappropriate and or ineffective and culture and sensitivity testing indicates that they will be effective. Following the veterinary prescribing Cascade is advocated.

Using antimicrobials only when appropriate is important, as is antimicrobial selection and application. A better understanding of the links between antibiotic resistance in animals and humans and its transmission and relevant selection pressures and drivers would also help guide the use of antibiotics within the veterinary industry.

Overall it is apparent that resistance of many antibacterials and limited new therapeutic options create a challenge in therapeutics of conditions in both humans and animals. The potential for animals as a reservoir for the development of these resistance antimicrobial genes has been eluded to earlier in this discussion but there is also the capability and potential for rapid spread of these resistance genes within this niche reservoir and furthermore the significant potential for transmissibility including between humans and animals. Antimicrobial resistance in animal *P. aeruginosa* should be closely monitored in the future, in line with possible animal-to-human transfers between pets and owners. Among other conditions this may be especially important for patients with cystic fibrosis, which often results from unique *P. aeruginosa* strains acquired in the environment of the patient. Animal welfare is of prime importance and whilst we need to preserve the availability of antibiotics for treating animal infections, this needs to be done responsibly to ensure that we do not promote further antimicrobial resistance.

In summary the findings of this work suggest that *P. aeruginosa* veterinary isolates maybe an understudied reservoir of antibacterial resistance. The identification of some novel findings using these two sets veterinary isolates studied in this body of work demonstrate this. Whilst the study using the larger sample set has not been exhaustive in its methods and further work (as discussed earlier in Chapter 3.2) could have expanded upon these findings if time constraints had not limited this. A further conclusion from this thesis has been that that any results should be verified via multiple methods for clarity and confirmation purposes. In that whilst PCR technique was used as a screening tool for identification of resistance determinants present or absent in the sample sets, whole genome sequencing on all positive amplicons (detailed in Results Chapter 4) would have been the'gold standard'

approach. As WGS becomes more widely available and affordable, it is hoped that further funding will be secured in order to continue this work.

References/Bibliography

Aarestrup, F. M., Bager, F., Jensen, N. E., Madsen, M., Meyling, A., & Wegener, H. C. (1998a). Resistance to antimicrobial agents used for animal therapy in pathogenic-, zoonotic- and indicator bacteria isolated from different food animals in Denmark: a baseline study for the Danish Integrated Antimicrobial Resistance Monitoring Programme (DANMAP). *Apmis*, *106*(8), 745–770. http://doi.org/10.1111/j.1699-0463.1998.tb00222.x

Aarestrup, F. M., Bager, F., Jensen, N. E., Madsen, M., Meyling, A., & Wegener, H. C. (1998b). Surveillance of antimicrobial resistance in bacteria isolated from food animals to antimicrobial growth promoters and related therapeutic agents in Denmark. *Apmis*, *106*(6), 606–622. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9725794

Aaron, S. D., Vandemheen, K. L., Giesbrecht-lewis, T., Tullis, E., Freitag, A., Paterson, N., … Doucette, S. (2014). With Cystic Fibrosis, *304*(19), 2145–2153.

Abraham, S., Wong, H. S., Turnidge, J., Johnson, J. R., & Trott, D. J. (2014). Carbapenemase-producing bacteria in companion animals: A public health concern on the horizon. *Journal of Antimicrobial Chemotherapy*, *69*(5), 1155–1157. http://doi.org/10.1093/jac/dkt518

Agency, E. M. (2013). Antimicrobial resistance - European Medicines Agency provides advice on use of colistin and tigecycline in animals, *44*(July), 0–2.

Aghazadeh, M., Hojabri, Z., Mahdian, R., Nahaei, M. R., Rahmati, M., Hojabri, T., … Pajand, O. (2014). Role of efflux pumps: MexAB-OprM and MexXY(-OprA), AmpC cephalosporinase and OprD porin in non-metallo-β-lactamase producing Pseudomonas aeruginosa isolated from cystic fibrosis and burn patients. *Infection, Genetics and Evolution*, *24*, 187–192. http://doi.org/10.1016/j.meegid.2014.03.018

Ahmed, M. O., Williams, N. J., Clegg, P. D., van Velkinburgh, J. C., Baptiste, K. E., & Bennett, M. (2012). Analysis of Risk Factors Associated with Antibiotic-Resistant *Escherichia coli*. *Microbial Drug Resistance*, *18*(2), 161–168.

http://doi.org/10.1089/mdr.2011.0213

Ajayi, A. O., Oluduro, A. O., Olowe, O. A., Odeyemi, A. T., & Famurewa, O. (2012). Plasmid-mediated Fluoroquinolone-resistance Qnr A and Qnr B Genes among Escherichia coli from Cattle in Ado-Ekiti , Nigeria Genes Q nr A y Qnr B de la Resistencia a la Fluoroquinolona Mediada por Plásmidos entre las Bacterias Escherichia coli del Ganado e. *West Indian Medicine Journal*, *61*(8), 784–788.

Akya, A., Salimi, A., Nomanpour, B., & Ahmadi, K. (2015). Prevalence and Clonal Dissemination of Metallo-Beta-Lactamase-Producing Pseudomonas aeruginosa in Kermanshah. *Jundishapur Journal of Microbiology*, *8*(6), 0–4. http://doi.org/10.5812/jjm.20980v2

Al-Aloul, M., Crawley, J., Winstanley, C., Hart, C. a, Ledson, M. J., & Walshaw, M. J. (2004). Increased morbidity associated with chronic infection by an epidemic Pseudomonas aeruginosa strain in CF patients. *Thorax*, *59*(4), 334–336. http://doi.org/10.1136/thx.2003.014258

Allen, J. L., Begg, a. P., & Browning, G. F. (2011). Outbreak of equine endometritis caused by a genotypically identical strain of Pseudomonas aeruginosa. *Journal of Veterinary Diagnostic Investigation*, *23*(6), 1236–1239. http://doi.org/10.1177/1040638711425589

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *The Journal of Antimicrobial Chemotherapy*, *48 Suppl 1*, 5–16. http://doi.org/10.1093/jac/48.suppl_1.5

Anjum, M. F., Duggett, N. A., AbuOun, M., Randall, L., Nunez-Garcia, J., Ellis, R. J., … Teale, C. (2016). Colistin resistance in Salmonella and Escherichia coli isolates from a pig farm in Great Britain. *Journal of Antimicrobial Chemotherapy*, *71*(8), 2306–2313. http://doi.org/10.1093/jac/dkw149

Anton Y. Peleg, M.B., B.S., M.P.H. and David C. Hooper, M. . (2010). Hospital-Acquired Infections Due to Gram-Negative Bacteria. *The New England Journal of Medicine*, *362*(19), 1804–1813. http://doi.org/10.1056/NEJMra0904124.Hospital-Acquired

Araujo, B. F., Ferreira, M. L., De Campos, P. A., Royer, S., Batistão, D. W. D. F., Dantas, R. C. C., … Ribas, R. M. (2016). Clinical and molecular epidemiology of multidrug-resistant p. aeruginosa carrying aac(6')-ib-cr, qnrs1 and blaspm

genes in Brazil. *PLoS ONE*, *11*(5), 1–15. http://doi.org/10.1371/journal.pone.0155914

Armas-Freire, P. I., Trueba, G., Proaño-bolaños, C., Levy, K., Marrs, C. F., Cevallos, W., & Eisenberg, J. N. S. (2015). Unexpected distribution of qnrB gene in E. coli isolates from different origins in Ecuador. *Int. Microbiol*, *18*(2), 85–90. http://doi.org/10.2436/20.1501.01.237.Unexpected

Arnold, K. E., Williams, N. J., & Bennett, M. (2016). "Disperse abroad in the land": the role of wildlife in the dissemination of antimicrobial resistance. *Biology Letters*, *12*(8), 20160137. http://doi.org/10.1098/rsbl.2016.0137

Ashish, A., Shaw, M., Winstanley, C., Ledson, M. J., & Walshaw, M. J. (2012). Increasing resistance of the Liverpool Epidemic Strain (LES) of Pseudomonas aeruginosa (Psa) to antibiotics in cystic fibrosis (CF)-A cause for concern? *Journal of Cystic Fibrosis*, *11*(3), 173–179. http://doi.org/10.1016/j.jcf.2011.11.004

Atherton, J. G., & Pitt, T. L. (1982). Types of Pseudomonas aeruginosa isolated from horses. *Equine Veterinary Journal*, *14*(4), 329–332. http://doi.org/10.1111/j.2042-3306.1982.tb02446.x

Austin, A., Lietman, T., & Rose-Nussbaumer, J. (2017). Update on the Management of Infectious Keratitis. *Ophthalmology*. http://doi.org/10.1016/j.ophtha.2017.05.012

Avison, M. B. (2005). New approaches to combating antimicrobial drug resistance. *Genome Biology*, *6*(13), 243. http://doi.org/10.1186/gb-2005-6-13-243

Bagge, N., Ciofu, O., Skovgaard, L. T., & Høiby, N. (2000). Rapid development in vitro and in vivo of resistance to ceftazidime in biofilm-growing Pseudomonas aeruginosa due to chromosomal beta-lactamase. *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, *108*(9), 589–600. http://doi.org/10.1034/j.1600-0463.2000.d01-102.x

Ballarini, A., Scalet, G., Kos, M., Cramer, N., Wiehlmann, L., & Jousson, O. (2012). Molecular typing and epidemiological investigation of clinical populations of Pseudomonas aeruginosa using an oligonucleotide-microarray. *BMC Microbiology*, *12*(1), 152. http://doi.org/10.1186/1471-2180-12-152

Barclay, M. L., & Begg, E. J. (2001). Aminoglycoside adaptive resistance: importance

for effective dosage regimens. *Drugs*, *61*(6), 713–21. http://doi.org/10.2165/00003495-200161060-00001

Baron, S., Hadjadj, L., Rolain, J. M., & Olaitan, A. O. (2016). Molecular mechanisms of polymyxin resistance: knowns and unknowns. *International Journal of Antimicrobial Agents*, *48*(6), 583–591. http://doi.org/10.1016/j.ijantimicag.2016.06.023

Barton, M. H., Parviainen, a, & Norton, N. (2004). Polymyxin B protects horses against induced endotoxaemia in vivo. *Equine Veterinary Journal*, *36*(5), 397–401. http://doi.org/10.2746/0425164044868350

Bass, L., Liebert, C. A., Lee, M. D., Summers, A. O., White, D. G., Thayer, S. G., & Maurer, J. J. (1999). Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian Escherichia coli. *Antimicrobial Agents and Chemotherapy*, *43*(12), 2925–2929.

Bassetti, M., & Righi, E. (2015). Development of novel antibacterial drugs to combat multiple resistant organisms. *Langenbeck's Archives of Surgery*, 153–165. http://doi.org/10.1007/s00423-015-1280-4

Battle, S. E., Meyer, F., Rello, J., Kung, V. L., & Hauser, A. R. (2008). Hybrid pathogenicity island PAGI-5 contributes to the highly virulent phenotype of a Pseudomonas aeruginosa isolate in mammals. *Journal of Bacteriology*, *190*(21), 7130–7140. http://doi.org/10.1128/JB.00785-08

Beceiro, A., Tomás, M., & Bou, G. (2013). Antimicrobial resistance and virulence: A successful or deleterious association in the bacterial world? *Clinical Microbiology Reviews*, *26*(2), 185–230. http://doi.org/10.1128/CMR.00059-12

Bennett, P. M. (2009). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology*, *153*(S1), S347–S357. http://doi.org/10.1038/sj.bjp.0707607

Bergen, P., Landersdorfer, C., & Zhang, J. (2012). Pharmacokinetics and pharmacodynamics of "old"polymyxins: what is new? *… and Infectious Disease*, *74*(3), 213–223. http://doi.org/10.1016/j.diagmicrobio.2012.07.010.Pharmacokinetics

Bert, F., Branger, C., & Lambert-Zechovsky, N. (2002). Identification of PSE and OXA beta-lactamase genes in Pseudomonas aeruginosa using PCR-restriction

fragment length polymorphism. *The Journal of Antimicrobial Chemotherapy*, *50*(1), 11–8. http://doi.org/10.1093/jac/dkf069

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O., & Piddock, L. J. V. (2015). Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology*, *13*(1), 42–51. http://doi.org/10.1038/nrmicro3380

Bogaerts, P., Bauraing, C., Deplano, A., & Glupczynski, Y. (2007). Emergence and dissemination of BEL-1-producing Pseudomonas aeruginosa isolates in Belgium [3]. *Antimicrobial Agents and Chemotherapy*, *51*(4), 1584–1585. http://doi.org/10.1128/AAC.01603-06

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., … Bartlett, J. (2009). Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. *Clinical Infectious Diseases*, *48*(1), 1–12. http://doi.org/10.1086/595011

Bradford, P. (2001). Extended spectrum betalactamase in the 21 century: characterization, epidemiology, and detection of this important resistant threat. *Clinical Microbiol Rev*, *14*(4), 933–951. http://doi.org/10.1128/CMR.14.4.933

Breidenstein, E. B. M., de la Fuente-Núñez, C., & Hancock, R. E. W. (2011). Pseudomonas aeruginosa: All roads lead to resistance. *Trends in Microbiology*, *19*(8), 419–426. http://doi.org/10.1016/j.tim.2011.04.005

Briers, Y., & Lavigne, R. (2015). Breaking barriers: expansion of the use of endolysins as novel antibacterials against Gram-negative bacteria. *Future Microbiology*, *10*(3), 377–390. http://doi.org/10.2217/fmb.15.8

Broughton, A., Verger, C., & Goffin, E. (2010). Pets-related peritonitis in peritoneal dialysis: Companion animals or trojan horses? *Seminars in Dialysis*, *23*(3), 306–316. http://doi.org/10.1111/j.1525-139X.2010.00726.x

Brown, S. A. (1996). Fluoroquinolones in animal health. *Journal of Veterinary Pharmacology and Therapeutics*, *19*(1), 1–14. http://doi.org/10.1111/j.1365-2885.1996.tb00001.x

Bugden, D. L. (2013). Identification and antibiotic susceptibility of bacterial isolates from dogs with otitis externa in Australia. *Australian Veterinary Journal*, *91*(1-2), 43–46. http://doi.org/10.1111/avj.12007

Bush, K., & Jacoby, G. A. (2010). Updated functional classification of ??-lactamases. *Antimicrobial Agents and Chemotherapy*, *54*(3), 969–976. http://doi.org/10.1128/AAC.01009-09

Bush, K., Jacoby, G. A., & Medeiros, A. A. (1995). MINIREVIEW A Functional Classification Scheme for NL -Lactamases and Its Correlation with Molecular Structure, *39*(6), 1211–1233.

Butt, T., Usman, M., Ahmad, R. N., & Saif, I. (2005). Emergence of metallo-beta-lactamase producing Pseudomonas aeruginosa in Pakistan. *Journal of the Pakistan Medical Association*, *55*(7), 302–304.

Bywater, R. J. (2004). Veterinary use of antimicrobials and emergence of resistance in zoonotic and sentinel bacteria in the EU. *Journal of Veterinary Medicine Series B: Infectious Diseases and Veterinary Public Health*, *51*(8-9), 361–363. http://doi.org/10.1111/j.1439-0450.2004.00791.x

Cabezón, E., Ripoll-Rozada, J., Peña, A., de la Cruz, F., & Arechaga, I. (2015). Towards an integrated model of bacterial conjugation. *FEMS Microbiology Reviews*, *39*(1), 81–95. http://doi.org/10.1111/1574-6976.12085

Cabot, G., Ocampo-Sosa, A. A., Tubau, F., Macia, M. D., Rodríguez, C., Moya, B., … Oliver, A. (2011). Overexpression of AmpC and efflux pumps in Pseudomonas aeruginosa isolates from bloodstream infections: Prevalence and impact on resistance in a Spanish multicenter study. *Antimicrobial Agents and Chemotherapy*, *55*(5), 1906–1911. http://doi.org/10.1128/AAC.01645-10

Cabral, J. P. S. (2010). Water microbiology. Bacterial pathogens and water. *International Journal of Environmental Research and Public Health*, *7*(10), 3657–3703. http://doi.org/10.3390/ijerph7103657

Cambau, E., Perani, E., Dib, C., Petinon, C., Trias, J., & Jarlier, V. (1995). Role of mutations in DNA gyrase genes in ciprofloxacin resistance of Pseudomonas aeruginosa susceptible or resistant to imipenem. *Antimicrob Agents Chemother*, *39*(10), 2248–2252. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8619577

Cao, B., Christophersen, L., Thomsen, K., Sønderholm, M., Bjarnsholt, T., Jensen, P. Ø., … Moser, C. (2015). Antibiotic penetration and bacterial killing in a

Pseudomonas aeruginosa biofilm model. *Journal of Antimicrobial Chemotherapy*, *70*(7), 2057–2063. http://doi.org/10.1093/jac/dkv058

Castanheira, M., Griffin, M. A., Deshpande, L. M., Mendes, R. E., Jones, R. N., & Flamm, R. K. (2016). Detection of mcr-1 among Escherichia coli clinical isolates collected worldwide as part of the SENTRY Antimicrobial Surveillance Program during 2014-2015. *Antimicrobial Agents and Chemotherapy*, *316*(8), 806–807. http://doi.org/10.1128/AAC.01267-16

Castanon, J. I. R. (2007). History of the Use of Antibiotic as Growth Promoters in European Poultry Feeds. *Poultry Science*, *86*(11), 2466–2471. http://doi.org/10.3382/ps.2007-00249

Cattoir, V., Poirel, L., Rotimi, V., Soussy, C. J., & Nordmann, P. (2007). Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. *Journal of Antimicrobial Chemotherapy*, *60*(2), 394–397. http://doi.org/10.1093/jac/dkm204

Chantziaras, I., Boyen, F., Callens, B., & Dewulf, J. (2014). Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: A report on seven countries. *Journal of Antimicrobial Chemotherapy*, *69*(3), 827–834. http://doi.org/10.1093/jac/dkt443

Chen, L. F., Anderson, D. J., & Paterson, D. L. (2012). Overview of the epidemiology and the threat of *Klebsiella pneumoniae* carbapenemases (KPC) resistance. *Infection and Drug Resistance*, *5*, 133–141. http://doi.org/10.2147/IDR.S26613

Chen, S., Hu, F., Zhang, X., Xu, X., Liu, Y., Zhu, D., & Wang, H. (2011). Independent emergence of colistin-resistant Enterobacteriaceae clinical isolates without colistin treatment. *Journal of Clinical Microbiology*, *49*(11), 4022–4023. http://doi.org/10.1128/JCM.01233-11

Chen, Z., Niu, H., Chen, G., Li, M., Li, M., & Zhou, Y. (2015). Prevalence of ESBLs-producing pseudomonas aeruginosa isolates from different wards in a Chinese teaching hospital. *International Journal of Clinical and Experimental Medicine*, *8*(10), 19400–19405.

Cheng, K., Smyth, R. L., Govan, J. R., Doherty, C., Winstanley, C., Denning, N., … Hart, C. a. (1996). Spread of beta-lactam-resistant Pseudomonas aeruginosa in a cystic fibrosis clinic. *Lancet*, *348*(9028), 639–642.

http://doi.org/10.1016/S0140-6736(96)05169-0

Coates, A. R., Halls, G., & Hu, Y. (2011). Novel classes of antibiotics or more of the same? *British Journal of Pharmacology*, *163*(1), 184–194. http://doi.org/10.1111/j.1476-5381.2011.01250.x

Cogliani, C., Goossens, H., & Greko, C. (2011). Restricting Antimicrobial Use in Food Animals: Lessons from Europe. *Microbe*, *6*(6), 274–279. http://doi.org/10.1128/microbe.6.274.1

Coil, D., Jospin, G., & Darling, A. E. (2015). A5-miseq: An updated pipeline to assemble microbial genomes from Illumina MiSeq data. *Bioinformatics*, *31*(4), 587–589. http://doi.org/10.1093/bioinformatics/btu661

Cole, S. J., Records, A. R., Orr, M. W., Linden, S. B., & Lee, V. T. (2014). Catheter-associated urinary tract infection by Pseudomonas aeruginosa is mediated by exopolysaccharide-independent biofilms. *Infection and Immunity*, *82*(5), 2048–2058. http://doi.org/10.1128/IAI.01652-14

Correa, C. M. C., Tibana, A., & Gontijo Filho, P. P. (1991). Vegetables as a Source of Infection with Pseudomonas-Aeruginosa in a University and Oncology Hospital of Rio De Janeiro Brazil. *Journal of Hospital Infection*, *18*(4), 301–306.

Cosgrove, S. E. (2006). The Relationship between Antimicrobial Resistance and Patient Outcomes : Mortality , Length of Hospital Stay , and Health Care Costs, *21287*(Suppl 2), 82–89.

Coyne, L. A., Latham, S. M., Williams, N. J., Dawson, S., Donald, I. J., Pearson, R. B., … Pinchbeck, G. L. (2016). Understanding the culture of antimicrobial prescribing in agriculture: A qualitative study of UK pig veterinary surgeons. *Journal of Antimicrobial Chemotherapy*, *71*(11), 3300–3312. http://doi.org/10.1093/jac/dkw300

Cramer, N., Wiehlmann, L., Ciofu, O., Tamm, S., Høiby, N., & Tümmler, B. (2012). Molecular Epidemiology of Chronic Pseudomonas aeruginosa Airway Infections in Cystic Fibrosis. *PLoS ONE*, *7*(11). http://doi.org/10.1371/journal.pone.0050731

Crespo, M. P., Woodford, N., Sinclair, A., Kaufmann, M. E., Glover, J., Velez, J. D., … Castan, C. R. (2004). Outbreak of Carbapenem-Resistant Pseudomonas aeruginosa Producing VIM-8 , a Novel Metallo- β -Lactamase, in a Tertiary Care

Center in Cali , Colombia Outbreak of Carbapenem-Resistant Pseudomonas aeruginosa Producing VIM-8, a Novel Metallo-Lactamase, *42*(11), 5094–5101. http://doi.org/10.1128/JCM.42.11.5094

Curran, B., Jonas, D., Grundmann, H., Pitt, T., & Dowson, C. G. (2004). Development of a Multilocus Sequence Typing Scheme for the Opportunistic Pathogen Pseudomonas aeruginosa Development of a Multilocus Sequence Typing Scheme for the Opportunistic Pathogen Pseudomonas aeruginosa. *Journal of Clinical Microbiology*, *42*(12), 5644–5649. http://doi.org/10.1128/JCM.42.12.5644

Cuzon, G., Naas, T., Villegas, M. V., Correa, A., Quinn, J. P., & Nordmann, P. (2011). Wide dissemination of Pseudomonas aeruginosa producing β-lactamase bla KPC-2 gene in Colombia. *Antimicrobial Agents and Chemotherapy*, *55*(11), 5350–5353. http://doi.org/10.1128/AAC.00297-11

da Silva Filho, L. V, Levi, J. E., Bento, C. N., Rodrigues, J. C., & da Silvo Ramos, S. R. (2001). Molecular epidemiology of Pseudomonas aeruginosa infections in a cystic fibrosis outpatient clinic. *J Med Microbiol*, *50*(3), 261–267. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11232773

Dallenne, C., da Costa, A., Decré, D., Favier, C., & Arlet, G. (2010). Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy*, *65*(3), 490–495. http://doi.org/10.1093/jac/dkp498

Darmon, E., & Leach, D. R. F. (2014). Bacterial Genome Instability. *Microbiology and Molecular Biology Reviews*, *78*(1), 1–39. http://doi.org/10.1128/MMBR.00035-13

De Bentzmann, S., & Plésiat, P. (2011). The Pseudomonas aeruginosa opportunistic pathogen and human infections. *Environmental Microbiology*, *13*(7), 1655–1665. http://doi.org/10.1111/j.1462-2920.2011.02469.x

De Briyne, N., Atkinson, J., Borriello, S. P., & Pokludová, L. (2014). Antibiotics used most commonly to treat animals in Europe. *Veterinary Record*, *175*(13), 325–325. http://doi.org/10.1136/vr.102462

De Briyne, N., Atkinson, J., Pokludová, L., Borriello, S. P., & Price, S. (2013). Factors influencing antibiotic prescribing habits and use of sensitivity testing amongst veterinarians in Europe. *Veterinary Record*, *173*(19), 475–475. http://doi.org/10.1136/vr.101454

De Soyza, A., Hall, A. J., Mahenthiralingam, E., Drevinek, P., Kaca, W., Drulis-Kawa, Z., … Winstanley, C. (2013). Developing an international Pseudomonas aeruginosa reference panel. *MicrobiologyOpen*, *2*(6), 1010–1023. http://doi.org/10.1002/mbo3.141

Delgado, E., & Subtil, S. (2015). Identification of multi-resistant strains in bacteria associated with canine external ocular diseases, 930–937.

Denamur, E. (1993). The absence of correlation between allozyme and rrn RFLP analysis indicates a high gene flow rate within human clinical Pseudomonas aeruginosa isolates, *110*, 275–280.

Deora, R., Carolina, N., & Street, E. M. (2013). Possible Transmission from a Household Cat, *59*(4), 246–250. http://doi.org/10.1111/j.1863-2378.2011.01446.x.Bordetella

Deredjian, A., Colinon, C., Hien, E., Brothier, E., Youenou, B., Cournoyer, B., … Nazaret, S. (2014). Low occurrence of Pseudomonas aeruginosa in agricultural soils with and without organic amendment. *Frontiers in Cellular and Infection Microbiology*, *4*(April), 53. http://doi.org/10.3389/fcimb.2014.00053

Deshpande, L. M., Davies, T. A., Blandino, G., Nicoletti, G., Jones, R. N., & Castanheira, M. (2013). IMP-33, a new IMP variant detected in Pseudomonas aeruginosa from sicily. *Antimicrobial Agents and Chemotherapy*, *57*(12), 6401–6403. http://doi.org/10.1128/AAC.02532-12

Distefano, M. D. (2015). NIH Public Access, *154*(5), 213–223. http://doi.org/10.1007/978-1-62703-673-3

Docquier, J.-D., Riccio, M. L., Mugnaioli, C., Luzzaro, F., Endimiani, A., Toniolo, A., … Rossolini, G. M. (2003). IMP-12, a new plasmid-encoded metallo-beta-lactamase from a Pseudomonas putida clinical isolate. *Antimicrobial Agents and Chemotherapy*, *47*(5), 1522–8. http://doi.org/10.1128/aac.47.5.1522-1528.2003

Domingues, S., da Silva, G. J., & Nielsen, K. M. (2012). Integrons: Vehicles and

pathways for horizontal dissemination in bacteria. *Mobile Genetic Elements*, *2*(5), 211–223. http://doi.org/10.4161/mge.22967

Doumith, M., Godbole, G., Ashton, P., Larkin, L., Dallman, T., Day, M., … Woodford, N. (2016). Detection of the plasmid-mediated mcr-1 gene conferring colistin resistance in human and food isolates of Salmonella enterica and Escherichia coli in England and Wales. *Journal of Antimicrobial Chemotherapy*, *71*(8), 2300–2305. http://doi.org/10.1093/jac/dkw093

Drawz, S. M., & Bonomo, R. A. (2010). Three decades of beta-lactamase inhibitors. *Clinical Microbiology Reviews*, *23*(1), 160–201. http://doi.org/10.1128/CMR.00037-09

Driscoll, J. A., Brody, S. L., & Kollef, M. H. (2007). The epidemiology, pathogenesis and treatment of Pseudomonas aeruginosa infections. *Drugs*, *67*(0012-6667 (Print)), 351–368. http://doi.org/10.2165/00003495-200767030-00003

Dutta, D., Cole, N., & Willcox, M. (2012). Factors influencing bacterial adhesion to contact lenses. *Molecular Vision*, *18*(January), 14–21.

El Solh, A. A., & Alhajhusain, A. (2009). Update on the treatment of Pseudomonas aeruginosa pneumonia. *Journal of Antimicrobial Chemotherapy*, *64*(2), 229–238. http://doi.org/10.1093/jac/dkp201

Empel, J., Filczak, K., Mrówka, A., Hryniewicz, W., Livermore, D. M., & Gniadkowski, M. (2007). Outbreak of Pseudomonas aeruginosa infections with PER-1 extended-spectrum β-lactamase in Warsaw, Poland: Further evidence for an international clonal complex. *Journal of Clinical Microbiology*, *45*(9), 2829–2834. http://doi.org/10.1128/JCM.00997-07

Ensink, J. M., Van Klingeren, B., Houwers, D. J., Klein, W. R., & Vulto, A. G. (1993). In-vitro susceptibility to antimicrobial drugs of bacterial isolates from horses in The Netherlands. *Equine Veterinary Journal*, *25*(4), 309–313. http://doi.org/10.1111/j.2042-3306.1993.tb02969.x

Fàbrega, A., Madurga, S., Giralt, E., & Vila, J. (2009). Mechanism of action of and resistance to quinolones. *Microbial Biotechnology*, *2*(1), 40–61. http://doi.org/10.1111/j.1751-7915.2008.00063.x

Falagas, M. E., & Kasiakou, S. K. (2005). Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clinical*

Infectious Diseases : An Official Publication of the Infectious Diseases Society of America, *40*(9), 1333–1341. http://doi.org/10.1097/01.inf.0000174577.97635.7b

Falgenhauer, L., Waezsada, S. E., Yao, Y., Imirzalioglu, C., Käsbohrer, A., Roesler, U., … Chakraborty, T. (2016). Colistin resistance gene mcr-1 in extended-spectrum β-lactamase-producing and carbapenemase-producing Gram-negative bacteria in Germany. *The Lancet Infectious Diseases*, *16*(3), 282–283. http://doi.org/10.1016/S1473-3099(16)00009-8

Farmer, J. J., Weinstein, R. A., Zierdt, C. H., & Brokopp, C. D. (1982). Hospital outbreaks caused by Pseudomonas aeruginosa: Importance of serogroup O11. *Journal of Clinical Microbiology*, *16*(2), 266–270.

Farshadzadeh, Z., Khosravi, A. D., Alavi, S. M., Parhizgari, N., & Hoveizavi, H. (2014). Spread of extended-spectrum β-lactamase genes of blaOXA-10, blaPER-1 and blaCTX-M in Pseudomonas aeruginosa strains isolated from burn patients. *Burns : Journal of the International Society for Burn Injuries*, *40*(8), 6–11. http://doi.org/10.1016/j.burns.2014.02.008

Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P., & Spratt, B. G. (2004). eBURST: Inferring Patterns of Evolutionary Descent among Clusters of Related Bacterial Genotypes from Multilocus Sequence Typing Data. *Journal of Bacteriology*, *186*(5), 1518–1530. http://doi.org/10.1128/JB.186.5.1518-1530.2004

Feliziani, S., Luján, A. M., Moyano, A. J., Sola, C., Bocco, J. L., Montanaro, P., … Smania, A. M. (2010). Mucoidy, quorum sensing, mismatch repair and antibiotic resistance in pseudomonas aeruginosa from cystic fibrosis chronic airways infections. *PLoS ONE*, *5*(9), 1–12. http://doi.org/10.1371/journal.pone.0012669

Fernandes, M., Vira, D., Medikonda, R., & Kumar, N. (2016). Extensively and pan-drug resistant Pseudomonas aeruginosa keratitis: clinical features, risk factors, and outcome. *Graefe's Archive for Clinical and Experimental Ophthalmology*, *254*(2), 315–322. http://doi.org/10.1007/s00417-015-3208-7

Fernández, L., Álvarez-Ortega, C., Wiegand, I., Olivares, J., Kocíncová, D., Lam, J. S., … Hancock, R. E. W. (2013). Characterization of the polymyxin B resistome of Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*, *57*(1),

110–119. http://doi.org/10.1128/AAC.01583-12

Fernández, L., Gooderham, W. J., Bains, M., McPhee, J. B., Wiegand, I., & Hancock, R. E. W. (2010). Adaptive resistance to the “last hope” antibiotics polymyxin B and colistin in Pseudomonas aeruginosa is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrobial Agents and Chemotherapy*, *54*(8), 3372–3382. http://doi.org/10.1128/AAC.00242-10

Fernández, L., Jenssen, H., Bains, M., Wiegand, I., Gooderham, W. J., & Hancock, R. E. W. (2012). The two-component system CprRS senses cationic peptides and triggers adaptive resistance in Pseudomonas aeruginosa independently of ParRS. *Antimicrobial Agents and Chemotherapy*, *56*(12), 6212–6222. http://doi.org/10.1128/AAC.01530-12

Ferreira, J. P., Anderson, K. L., Correa, M. T., Lyman, R., Ruffin, F., Reller, L. B., & Fowler, V. G. (2011). Transmission of mrsa between companion animals and infected human patients presenting to outpatient medical care facilities. *PLoS ONE*, *6*(11), 1–6. http://doi.org/10.1371/journal.pone.0026978

Ferroni, A., Guillemot, D., Moumile, K., Bernede, C., Le Bourgeois, M., Waernessyckle, S., … Taddei, F. (2009). Effect of mutator P. aeruginosa on antibiotic resistance acquisition and respiratory function in cystic fibrosis. *Pediatric Pulmonology*, *44*(8), 820–825. http://doi.org/10.1002/ppul.21076

Fey, P. D., Safranek, T. J., Rupp, M. E., Dunne, E. F., Ribot, E., Iwen, P. C., … Hinrichs, S. H. (2000). Ceftriaxone-Resistant Salmonella Infection Acquired by a Child from Cattle. *New England Journal of Medicine*, *342*(17), 1242–1249. http://doi.org/10.1056/NEJM200004273421703

Figueiredo, R., Card, R. M., Nunez, J., Pomba, C., Mendonça, N., Anjum, M. F., & Da Silva, G. J. (2016). Detection of an mcr-1-encoding plasmid mediating colistin resistance in Salmonella enterica from retail meat in Portugal. *Journal of Antimicrobial Chemotherapy*, *71*(8), 2338–2340. http://doi.org/10.1093/jac/dkw240

Folkesson, A., Jelsbak, L., Yang, L., Johansen, H. K., Ciofu, O., Høiby, N., & Molin, S. (2012). Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective. *Nature Reviews Microbiology*, *10*(12), 841–851. http://doi.org/10.1038/nrmicro2907

Fothergill, J. L., Panagea, S., Hart, C. a, Walshaw, M. J., Pitt, T. L., & Winstanley, C. (2007). Widespread pyocyanin over-production among isolates of a cystic fibrosis epidemic strain. *BMC Microbiology*, *7*, 45. http://doi.org/10.1186/1471-2180-7-45

Fothergill, J. L., Walshaw, M. J., & Winstanley, C. (2012). Transmissible strains of Pseudomonas aeruginosa in cystic fibrosis lung infections. *European Respiratory Journal*, *40*(1), 227–238. http://doi.org/10.1183/09031936.00204411

Freschi, L., Jeukens, J., Kukavica-Ibrulj, I., Boyle, B., Dupont, M. J., Laroche, J., … Levesque, R. C. (2015). Clinical utilization of genomics data produced by the international Pseudomonas aeruginosa consortium. *Frontiers in Microbiology*, *6*(SEP), 1–8. http://doi.org/10.3389/fmicb.2015.01036

Furuya, E. Y., & Lowy, F. D. (2006). Antimicrobial-resistant bacteria in the community setting. *Nature Reviews Microbiology*, *4*(1), 36–45. http://doi.org/10.1038/nrmicro1325

Galimand, M., Lambert, T., Gerbaud, G. U. Y., & Courvalin, P. (1993). Characterization of the aac (6 ’) -Ib Gene Encoding an Aminoglycoside 6 ' -N-Acetyltransferase in Pseudomonas aeruginosa BM2656, *37*(7), 1456–1462. http://doi.org/10.1128/AAC.37.7.1456.Updated

Garcia-Migura, L., Hendriksen, R. S., Fraile, L., & Aarestrup, F. M. (2014). Antimicrobial resistance of zoonotic and commensal bacteria in Europe: The missing link between consumption and resistance in veterinary medicine. *Veterinary Microbiology*, *170*(1-2), 1–9. http://doi.org/10.1016/j.vetmic.2014.01.013

Garneau-tsodikova, S., & Labby, K. J. (2016). Mechanisms of Resistance to Aminoglycoside Antibiotics: Overview and Perspectives, *7*(1), 11–27. http://doi.org/10.1039/C5MD00344J.Mechanisms

Gellatly, S. L., Needham, B., Madera, L., Trent, M. S., & Hancock, R. E. W. (2012). The Pseudomonas aeruginosa phoP-phoQ two-component regulatory system is induced upon interaction with epithelial cells and controls cytotoxicity and inflammation. *Infection and Immunity*, *80*(9), 3122–3131. http://doi.org/10.1128/IAI.00382-12

Geser, N., Stephan, R., & Hächler, H. (2012). Occurrence and characteristics of extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae in food producing animals, minced meat and raw milk. *BMC Veterinary Research*, *8*(1), 21. http://doi.org/10.1186/1746-6148-8-21

Giedraitienė, A., Vitkauskienė, A., Naginienė, R., & Pavilonis, A. (2011). Antibiotic resistance mechanisms of clinically important bacteria. *Medicina (Kaunas, Lithuania)*, *47*(3), 137–46. http://doi.org/1103-01e [pii]

Gillings, M., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Holley, M., & Stokes, H. W. (2008). The evolution of class 1 integrons and the rise of antibiotic resistance. *Journal of Bacteriology*, *190*(14), 5095–5100. http://doi.org/10.1128/JB.00152-08

Gisel, J. J., Brumble, L. M., & Johnson, M. M. (2010). Bordetella bronchiseptica pneumonia in a kidney-pancreas transplant patient after exposure to recently vaccinated dogs: Case report. *Transplant Infectious Disease*, *12*(1), 73–76. http://doi.org/10.1111/j.1399-3062.2009.00451.x

Glupczynski, Y., Bogaerts, P., Deplano, A., Berhin, C., Huang, T. D., van Eldere, J., & Rodriguez-villalobos, H. (2010). Detection and characterization of class a extended-spectrum-β-lactamase-producing Pseudomonas aeruginosa isolates in Belgian hospitals. *Journal of Antimicrobial Chemotherapy*, *65*(5), 866–871. http://doi.org/10.1093/jac/dkq048

Godfrey, a J., & Bryan, L. E. (1984). Resistance of Pseudomonas aeruginosa to new beta-lactamase-resistant beta-lactams. *Antimicrobial Agents and Chemotherapy*, *26*(4), 485–488. http://doi.org/10.1128/AAC.26.4.485

Gooderham, W. J., & Hancock, R. E. W. (2009). Regulation of virulence and antibiotic resistance by two-component regulatory systems in Pseudomonas aeruginosa. *FEMS Microbiology Reviews*, *33*(2), 279–294. http://doi.org/10.1111/j.1574-6976.2008.00135.x

Goossens, H., & Grabein, B. (2005). Prevalence and antimicrobial susceptibility data for extended-spectrum ??-lactamase- and AmpC-producing Enterobacteriaceae from the MYSTIC Program in Europe and the United States (1997-2004). *Diagnostic Microbiology and Infectious Disease*, *53*(4), 257–264. http://doi.org/10.1016/j.diagmicrobio.2005.10.001

Govan, J. R., Brown, A. R., & Jones, A. M. (2007). Evolving epidemiology of Pseudomonas aeruginosa and the Burkholderia cepacia complex in cystic fibrosis lung infection. *Future Microbiology*, *2*(2), 153–164. http://doi.org/10.2217/17460913.2.2.153

Green, S. K., Schroth, M. N., Cho, J. J., Kominos, S. K., & Vitanza-jack, V. B. (1974). Agricultural plants and soil as a reservoir for Pseudomonas aeruginosa. *Applied Microbiology*, *28*(6), 987–91. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4217591\nhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC186868\nhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=186868&tool=pmcentrez&rendertype=abstract

Groisman, E. A. (2001). The Pleiotropic Two-Component Regulatory System PhoP-PhoQ MINIREVIEW The Pleiotropic Two-Component Regulatory System PhoP-PhoQ. *Journal of Bacteriology*, *183*(6), 1835–1842. http://doi.org/10.1128/JB.183.6.1835

Guardabassi, L., Schwarz, S., & Lloyd, D. H. (2004). Pet animals as reservoirs of antimicrobial-resistant bacteria. *Journal of Antimicrobial Chemotherapy*, *54*(2), 321–332. http://doi.org/10.1093/jac/dkh332

Gullberg, E., Cao, S., Berg, O. G., Ilbäck, C., Sandegren, L., Hughes, D., & Andersson, D. I. (2011). Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathogens*, *7*(7), 1–9. http://doi.org/10.1371/journal.ppat.1002158

Gutu, A. D., Sgambati, N., Strasbourger, P., Brannon, M. K., Jacobs, M. A., Haugen, E., … Moskowitz, S. M. (2013). Polymyxin resistance of Pseudomonas aeruginosa phoQ mutants is dependent on additional two-component regulatory systems. *Antimicrobial Agents and Chemotherapy*, *57*(5), 2204–2215. http://doi.org/10.1128/AAC.02353-12

Haenni, M., Hocquet, D., Ponsin, C., Cholley, P., Guyeux, C., Madec, J.-Y., & Bertrand, X. (2015). Population structure and antimicrobial susceptibility of Pseudomonas aeruginosa from animal infections in France. *BMC Veterinary Research*, *11*(1), 1–5. http://doi.org/10.1186/s12917-015-0324-x

Hall, A. J., Fothergill, J. L., McNamara, P. S., Southern, K. W., & Winstanley, C. (2014). Turnover of strains and intraclonal variation amongst Pseudomonas

aeruginosa isolates from paediatric CF patients. *Diagnostic Microbiology and Infectious Disease*, *80*(4), 324–326. http://doi.org/10.1016/j.diagmicrobio.2014.09.007

Hall, B. G., & Barlow, M. (2005). Revised Ambler classification of β-lactamases [1]. *Journal of Antimicrobial Chemotherapy*, *55*(6), 1050–1051. http://doi.org/10.1093/jac/dki130

Hancock, R. E. W. (1998). Resistance Mechanisms in Pseudomonas aeruginosa and Other Nonfermentative Gram-Negative Bacteria. *Clin Infect Dis*, *27*, S93–S99. http://doi.org/10.1086/514909

Harada, K., Arima, S., Niina, A., Kataoka, Y., & Takahashi, T. (2012). Characterization of Pseudomonas aeruginosa isolates from dogs and cats in Japan: Current status of antimicrobial resistance and prevailing resistance mechanisms. *Microbiology and Immunology*, *56*(2), 123–127. http://doi.org/10.1111/j.1348-0421.2011.00416.x

Hasman, H., & Hammerum, A. M. (2015). Hansen Fet al. Detection of mcr-1 encoding plasmid-mediated colistin-resistant Escherichia coli isolates from human bloodstream infection and imported …. *Euro Surveill*, 1–5.

Hayakawa, K., Miyoshi-Akiyama, T., Kirikae, T., Nagamatsu, M., Shimada, K., Mezaki, K., … Ohmagari, N. (2014). Molecular and epidemiological characterization of IMP-Type metallo-β-lactamase-producing enterobacter cloacae in a large tertiary care hospital in Japan. *Antimicrobial Agents and Chemotherapy*, *58*(6), 3441–3450. http://doi.org/10.1128/AAC.02652-13

Hazlett, L. D. (2004). Corneal response to Pseudomonas aeruginosa infection. *Progress in Retinal and Eye Research*, *23*(1), 1–30. http://doi.org/10.1016/j.preteyeres.2003.10.002

Hedayati, H., Ghaderpanah, M., Rasoulinejad, S. A., & Montazeri, M. (2015). Clinical Presentation and Antibiotic Susceptibility of Contact Lens Associated Microbial Keratitis. *Journal of Pathogens*, *2015*, 1–5. http://doi.org/10.1155/2015/152767

Henrichfreise, B., Wiegand, I., Pfister, W., & Wiedemann, B. (2007). Resistance mechanisms of multiresistant Pseudomonas aeruginosa strains from Germany and correlation with hypermutation. *Antimicrobial Agents and Chemotherapy*,

51(11), 4062–4070. http://doi.org/10.1128/AAC.00148-07

Hillier, A., Alcorn, J. R., Cole, L. K., & Kowalski, J. J. (2006). Pyoderma caused by Pseudomonas aeruginosa infection in dogs: 20 Cases. *Veterinary Dermatology*, *17*(6), 432–439. http://doi.org/10.1111/j.1365-3164.2006.00550.x

Hindley, K. E., Groth, A. D., King, M., Graham, K., & Billson, F. M. (2016). Bacterial isolates, antimicrobial susceptibility, and clinical characteristics of bacterial keratitis in dogs presenting to referral practice in Australia. *Veterinary Ophthalmology*, *19*(5), 418–426. http://doi.org/10.1111/vop.12325

Hirakata, Y., Kondo, A., Hoshino, K., Yano, H., Arai, K., Hirotani, A., … Kaku, M. (2009). Efflux pump inhibitors reduce the invasiveness of Pseudomonas aeruginosa. *International Journal of Antimicrobial Agents*, *34*(4), 343–346. http://doi.org/10.1016/j.ijantimicag.2009.06.007

Hirakawa, Y., Sasaki, H., Kawamoto, E., Ishikawa, H., Matsumoto, T., Aoyama, N., … Amao, H. (2010). Prevalence and analysis of Pseudomonas aeruginosa in chinchillas. *BMC Veterinary Research*, *6*(1), 52. http://doi.org/10.1186/1746-6148-6-52

Hirsch, E. B., & Tam, V. H. (2011). Impact of multidrug-resistant Pseudomonas aeruginosa infection on patient outcomes. *Expert Review of Pharmacoeconomics & Outcomes Research*, *10*, 1–18. http://doi.org/10.1586/erp.10.49.Impact

Ho, P. L., Wong, R. C., Lo, S. W., Chow, K. H., Wong, S. S., & Que, T. L. (2010). Genetic identity of aminoglycoside-resistance genes in Escherichia coli isolates from human and animal sources. *Journal of Medical Microbiology*, *59*(6), 702–707. http://doi.org/10.1099/jmm.0.015032-0

Hocquet, D., Nordmann, P., Garch, F. El, Plésiat, P., Cabanne, L., & Ple, P. (2006). Involvement of the MexXY-OprM Efflux System in Emergence of Cefepime Resistance in Clinical Strains of Pseudomonas aeruginosa Involvement of the MexXY-OprM Efflux System in Emergence of Cefepime Resistance in Clinical Strains of Pseudomonas aeruginosa. *Society*, *50*(4), 1347–1351. http://doi.org/10.1128/AAC.50.4.1347

Hogardt, M., & Heesemann, J. (2010). Adaptation of Pseudomonas aeruginosa during persistence in the cystic fibrosis lung. *International Journal of Medical*

Microbiology, *300*(8), 557–562. http://doi.org/10.1016/j.ijmm.2010.08.008

Høiby, N. (2011). Recent advances in the treatment of Pseudomonas aeruginosainfections in cystic fibrosis. *BMC Medicine*, *9*(1), 32. http://doi.org/10.1186/1741-7015-9-32

Hong, B. K., Chi, H. P., Chung, J. K., Kim, E. C., Jacoby, G. A., & Hooper, D. C. (2009). Prevalence of plasmid-mediated quinolone resistance determinants over a 9-year period. *Antimicrobial Agents and Chemotherapy*, *53*(2), 639–645. http://doi.org/10.1128/AAC.01051-08

Hooper, D. C. (2000). Mechanisms of Action and Resistance of Older and Newer Fluoroquinolones. *Clinical Infectious Diseases*, *31*(Supplement_2), S24–S28. http://doi.org/10.1086/314056

Huang, S.-Y., Dai, L., Xia, L.-N., Du, X.-D., Qi, Y.-H., Liu, H.-B., … Shen, J.-Z. (2009). Increased prevalence of plasmid-mediated quinolone resistance determinants in chicken escherichia coli isolates from 2001 to 2007. *Foodborne Pathogens and Disease*, *6*(10), 1203–1209. http://doi.org/10.1089/fpd.2009.0348

Hughes, L. A., Williams, N., Clegg, P., Callaby, R., Nuttall, T., Coyne, K., … Dawson, S. (2012). Cross-sectional survey of antimicrobial prescribing patterns in UK small animal veterinary practice. *Preventive Veterinary Medicine*, *104*(3-4), 309–316. http://doi.org/10.1016/j.prevetmed.2011.12.003

Important, C., Important, H., Priority, H., & Priority, H. (2005). for Human Medicine (WHO CIA list).

Jacoby, G. A. (2005). Mechanisms of resistance to quinolones. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *41 Suppl 2*(Supplement_2), S120–6. http://doi.org/10.1086/428052

Jacoby, G. A. (2009). AmpC ??-Lactamases. *Clinical Microbiology Reviews*, *22*(1), 161–182. http://doi.org/10.1128/CMR.00036-08

Jacoby, G., Cattoir, V., Hooper, D., Martínez-Martínez, L., Nordmann, P., Pascual, A., … Wang, M. (2008). Qnr Gene Nomenclature. *Antimicrobial Agents and Chemotherapy*, *52*(7), 2297–2299. http://doi.org/10.1128/AAC.00147-08

Jeannot, K., Poirel, L., Robert-Nicoud, M., Cholley, P., Nordmann, P., & Plésiat, P. (2012). IMP-29, a novel IMP-Type metallo-β-lactamase in pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*, *56*(4), 2187–2190.

http://doi.org/10.1128/AAC.05838-11

Ji, Y. L., Sheng, H., Keun, S. S., Yong, H. P., & Hovde, C. J. (2007). Characterization of an Escherichia coli O157:H7 plasmid O157 deletion mutant and its survival and persistence in cattle. *Applied and Environmental Microbiology*, *73*(7), 2037–2047. http://doi.org/10.1128/AEM.02643-06

Johansen, H. K., Moskowitz, S. M., Ciofu, O., Pressler, T., & Høiby, N. (2008). Spread of colistin resistant non-mucoid Pseudomonas aeruginosa among chronically infected Danish cystic fibrosis patients. *Journal of Cystic Fibrosis*, *7*(5), 391–397. http://doi.org/10.1016/j.jcf.2008.02.003

Jones, A. M., Govan, J. R. W., Doherty, C. J., Dodd, M. E., Isalska, B. J., Stanbridge, T. N., & Webb, A. K. (2001). Spread of a multiresistant strain of Pseudomonas aeruginosa in an adult cystic fibrosis clinic Superinfection with a transmissible strain of Pseudomonas aeruginosa in adults with cystic fibrosis chronically colonised by P aeruginosa For personal use . Onl. *The Lancet*, *358*, 557–558.

Jones, P. H., Dawson, S., Gaskell, R. M., Coyne, K. P., Tierney, Setzkorn, C., … Noble, P. J. M. (2014). Surveillance of diarrhoea in small animal practice through the Small Animal Veterinary Surveillance Network (SAVSNET). *Veterinary Journal*, *201*(3), 412–418. http://doi.org/10.1016/j.tvjl.2014.05.044

Kanj, S. S., & Kanafani, Z. A. (2011). Current concepts in antimicrobial therapy against resistant gram-negative organisms: extended-spectrum beta-lactamase-producing Enterobacteriaceae, carbapenem-resistant Enterobacteriaceae, and multidrug-resistant Pseudomonas aeruginosa. *Mayo Clinic Proceedings*, *86*(3), 250–9. http://doi.org/10.4065/mcp.2010.0674

Karatuna, O., & Yagci, A. (2010). Analysis of quorum sensing-dependent virulence factor production and its relationship with antimicrobial susceptibility in Pseudomonas aeruginosa respiratory isolates. *Clinical Microbiology and Infection*, *16*(12), 1770–1775. http://doi.org/10.1111/j.1469-0691.2010.03177.x

Kaye, R., Kaye, A., Sueke, H., Neal, T., Winstanley, C., Horsburgh, M., & Kaye, S. (2017). Recurrent Bacterial Keratitis, 4136–4139. http://doi.org/10.1167/iovs.13-12130

Keller, R. L., & Hendrix, D. V. H. (2005). Bacterial isolates and antimicrobial

susceptibilities in equine bacterial ulcerative keratitis (1993--2004). *Equine Veterinary Journal*, *37*(3), 207–211. http://doi.org/10.2746/0425164054530731

Kempf, I., Jouy, E., & Chauvin, C. (2016). Colistin use and colistin resistance in bacteria from animals. *International Journal of Antimicrobial Agents*, *48*(6), 598–606. http://doi.org/10.1016/j.ijantimicag.2016.09.016

Khan, A. A., & Cerniglia, C. E. (1994). Detection of Pseudomonas aeruginosa from clinical and environmental samples by amplification of the exotoxin A gene using PCR. *Appl. Environ. Microbiol.*, *60*(10), 3739–3745. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7986047

Kluytmans-van den bergh, M., Huizinga, P., Bonten, M. J., Bos, M., De Bruyne, K., Friedrich, A. W., … Kluytmans, J. A. (2016). Presence of mcr-1-positive Enterobacteriaceae in retail chicken meat but not in humans in the Netherlands since 2009. *Eurosurveillance*, *21*(9), 1–7. http://doi.org/10.2807/1560-7917.ES.2016.21.9.30149

Kolayli, F., Gacar, G., Karadenizli, A., Sanic, A., & Vahaboglu, H. (2005). PER-1 is still widespread in Turkish hospitals among Pseudomonas aeruginosa and Acinetobacter spp. *FEMS Microbiology Letters*, *249*(2), 241–245. http://doi.org/10.1016/j.femsle.2005.06.012

Kong, K.-F., Schneper, L., & Mathee, K. (2010). NIH Public Access. *Apmis*, *118*(1), 1–36. http://doi.org/10.1111/j.1600-0463.2009.02563.x.Beta-lactam

Kos, V. N., Draspe, M., McLaughlin, R. E., Whiteaker, J. D., Roy, P. H., Alm, R. A., … Gardner, H. (2015). The resistome of Pseudomonas seudomonas aeruginosa in relationship to phenotypic susceptibility. *Antimicrobial Agents and Chemotherapy*, *59*(1), 427–436. http://doi.org/10.1128/AAC.03954-14

Kouda, S., Ohara, M., Onodera, M., Fujiue, Y., Sasaki, M., Kohara, T., … Sugai, M. (2009). Increased prevalence and clonal dissemination of multidrug-resistant Pseudomonas aeruginosa with the blaIMP-1 gene cassette in Hiroshima. *Journal of Antimicrobial Chemotherapy*, *64*(1), 46–51. http://doi.org/10.1093/jac/dkp142

Kung, V. L., Ozer, E. A., & Hauser, A. R. (2010). The Accessory Genome of

Pseudomonas aeruginosa. *Microbiology and Molecular Biology Reviews*, *74*(4), 621–641. http://doi.org/10.1128/MMBR.00027-10

Kuo, H.-C., Chou, C.-C., Tu, C., Gong, S.-R., Han, C.-L., Liao, J.-W., … Lee, C.-N. (2009). Characterization of plasmid-mediated quinolone resistance by the qnrS gene in Escherichia coli isolated from healthy chickens and pigs. *Veterinarni Medicina*, *54*(10), 473–482.

Laing, C., Buchanan, C., Taboada, E. N., Zhang, Y., Kropinski, A., Villegas, A., … Gannon, V. P. (2010). Pan-genome sequence analysis using Panseq: an online tool for the rapid analysis of core and accessory genomic regions. *BMC Bioinformatics*, *11*(1), 461. http://doi.org/10.1186/1471-2105-11-461

Lambert, P. a. (2002). Mechanisms of antibiotic resistance in Pseudomonas aeruginosa. *Journal of the Royal Society of Medicine*, *95 Suppl 4*(Figure 1), 22–26.

Landers, T. F., Cohen, B., Wittum, T. E., & Larson, E. L. (2012). A Review of Antibiotic Use in Food Animals: Perspective, Policy, and Potential. *Public Health Reports*, *127*(1), 4–22. http://doi.org/10.1177/003335491212700103

Langaee, T. Y., Gagnon, L., & Huletsky, A. (2000). Inactivation of the ampD gene in Pseudomonas aeruginosa leads to moderate-basal-level and hyperinducible AmpC β-lactamase expression. *Antimicrobial Agents and Chemotherapy*, *44*(3), 583–589. http://doi.org/10.1128/AAC.44.3.583-589.2000

Larru, B., Sulieman, S. E., Localio, R., Ross, R. K., Sharland, M., Zaoutis, T. E., & Gerber, J. S. (2016). Frontline clinician knowledge of antimicrobial prescribing in an academic tertiary children's hospital: A point prevalence study. *Journal of the Pediatric Infectious Diseases Society*, *5*(4), 462–464. http://doi.org/10.1093/jpids/piv026

Lau, G. W., Hassett, D. J., Ran, H., & Kong, F. (2004). The role of pyocyanin in Pseudomonas aeruginosa infection. *Trends in Molecular Medicine*, *10*(12), 599–606. http://doi.org/10.1016/j.molmed.2004.10.002

Laudy, A. E., Róg, P., Smolinska-Król, K., Ćmiel, M., Søoczynska, A., Patzer, J., … Tyski, S. (2017). Prevalence of ESBL-producing Pseudomonas aeruginosa isolates in Warsaw, Poland, detected by various phenotypic and genotypic methods. *PLoS ONE*, *12*(6), 1–15. http://doi.org/10.1371/journal.pone.0180121

Leão, R. S., Carvalho-assef, A. P. D., Ferreira, A. G., Folescu, T. W., & Barth, A. L. (2010). Comparison of the worldwide transmissible, 1079–1081.

Lee, J. Y., Na, I. Y., Park, Y. K., & Ko, K. S. (2014). Genomic variations between colistin-susceptible and-resistant pseudomonas aeruginosa clinical isolates and their effects on colistin resistance. *Journal of Antimicrobial Chemotherapy*, *69*(5), 1248–1256. http://doi.org/10.1093/jac/dkt531

Lévesque, C., Piché, L., Larose, C., Roy, P. H., & Roy, P. H. (1995). PCR mapping of integrons reveals several novel combinations of resistance genes . These include : PCR Mapping of Integrons Reveals Several Novel Combinations of Resistance Genes, *39*(1), 185–191. http://doi.org/10.1128/AAC.39.1.185.Updated

Levy, S. B. (2002). Factors impacting on the problem of antibiotic resistance. *Levy, Stuart B*, *49*(1), 25–30. http://doi.org/10.1093/jac/49.1.25

Li, X. Z., Barré, N., & Poole, K. (2000). Influence of the MexA-MexB-oprM multidrug efflux system on expression of the MexC-MexD-oprJ and MexE-MexF-oprN multidrug efflux systems in Pseudomonas aeruginosa. *The Journal of Antimicrobial Chemotherapy*, *46*(6), 885–893.

Li, X. Z., Nikaido, H., & Poole, K. (1995). Role of MexA-MexB-OprM in antibiotic efflux in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*, *39*(9), 1948–1953. http://doi.org/10.1128/AAC.39.9.1948

Libisch, B., Poirel, L., Lepsanovic, Z., Mirovic, V., Balogh, B., Pászti, J., … Nordmann, P. (2008). Identification of PER-1 extended-spectrum β-lactamase producing Pseudomonas aeruginosa clinical isolates of the international clonal complex CC11 from Hungary and Serbia. *FEMS Immunology and Medical Microbiology*, *54*(3), 330–338. http://doi.org/10.1111/j.1574-695X.2008.00483.x

Lin, D., Foley, S. L., Qi, Y., Han, J., Ji, C., Li, R., Wang, Y. (2012). Characterization of antimicrobial resistance of Pseudomonas aeruginosa isolated from canine infections. *Journal of Applied Microbiology*, *113*(1), 16–23. http://doi.org/10.1111/j.1365-2672.2012.05304.x

Lin, S.-P., Liu, M.-F., Lin, C.-F., & Shi, Z.-Y. (2012). Phenotypic detection and polymerase chain reaction screening of extended-spectrum β-lactamases produced by Pseudomonas aeruginosa isolates. *Journal of Microbiology,*

Immunology, and Infection = Wei Mian Yu Gan Ran Za Zhi, *45*(3), 200–7. http://doi.org/10.1016/j.jmii.2011.11.015

Lister, P. D., Wolter, D. J., & Hanson, N. D. (2009). Antibacterial-resistant Pseudomonas aeruginosa: Clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clinical Microbiology Reviews*, *22*(4), 582–610. http://doi.org/10.1128/CMR.00040-09

Liu, Y. Y., Wang, Y., Walsh, T. R., Yi, L. X., Zhang, R., Spencer, J., … Shen, J. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. *The Lancet Infectious Diseases*, *16*(2), 161–168. http://doi.org/10.1016/S1473-3099(15)00424-7

Livermore, D. M. (2002). Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: our worst nightmare? *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *34*(5), 634–640. http://doi.org/10.1086/338782

Llanes, C., Neuwirth, C., Garch, F. El, Hocquet, D., Ple, P., & Bacte, L. De. (2006). Genetic analysis of a multiresistant strain of Pseudomonas aeruginosa. *Microbiology*.

Lomholt, J. a, & Kilian, M. (2003). Ciprofloxacin susceptibility of Pseudomonas aeruginosa isolates from keratitis. *The British Journal of Ophthalmology*, *87*, 1238–40. http://doi.org/10.1136/bjo.87.10.1238

Loncaric, I., Künzel, F., Klang, A., Wagner, R., Licka, T., Grunert, T., … Monecke, S. (2016). Carriage of meticillin-resistant staphylococci between humans and animals on a small farm. *Veterinary Dermatology*, *27*(3), 191–e48. http://doi.org/10.1111/vde.12321

Lorè, N. I., Cigana, C., De Fino, I., Riva, C., Juhas, M., Schwager, S., … Bragonzi, A. (2012). Cystic Fibrosis-Niche Adaptation of Pseudomonas aeruginosa Reduces Virulence in Multiple Infection Hosts. *PLoS ONE*, *7*(4), e35648. http://doi.org/10.1371/journal.pone.0035648

Lutz, L., Leao, R. S., Ferreira, A. G., Pereira, D. C., Raupp, C., Pitt, T., … Barth, A. L. (2013). Hypermutable Pseudomonas aeruginosa in cystic fibrosis patients from two Brazilian cities. *Journal of Clinical Microbiology*, *51*(3), 927–930.

http://doi.org/10.1128/JCM.02638-12

Ma, J., Zeng, Z., Chen, Z., Xu, X., Wang, X., Deng, Y., … Wang, M. (2009). High prevalence of plasmid-mediated quinolone resistance determinants qnr, aac(6')-Ib-cr, and qepA among ceftiofur-resistant Enterobacteriaceae isolates from companion and food-producing animals. *Antimicrobial Agents and Chemotherapy*, *53*(2), 519–524. http://doi.org/10.1128/AAC.00886-08

Macfarlane, E. L. a, Kwasnicka, A., Ochs, M. M., & Hancock, R. E. W. (1999). PhoP-PhoQ homologues in Pseudomonas aeruginosa regulate expression of the outer-membrane protein OprH and polymyxin B resistance. *Molecular Microbiology*, *34*(2), 305–316. http://doi.org/10.1046/j.1365-2958.1999.01600.x

Maciá, M. D., Blanquer, D., Togores, B., Pérez, J. L., & Oliver, A. (2005). Hypermutation Is a Key Factor in Development of Multiple-Antimicrobial Resistance in Pseudomonas aeruginosa Strains Causing Chronic Lung Infections Hypermutation Is a Key Factor in Development of Multiple-Antimicrobial Resistance in Pseudomonas aeruginosa. *Society*, *49*(8), 3382–3386. http://doi.org/10.1128/AAC.49.8.3382

Maddox, T. W., Williams, N. J., Clegg, P. D., O'Donnell, A. J., Dawson, S., & Pinchbeck, G. L. (2011). Longitudinal study of antimicrobial-resistant commensal Escherichia coli in the faeces of horses in an equine hospital. *Preventive Veterinary Medicine*, *100*(2), 134–145. http://doi.org/10.1016/j.prevetmed.2011.02.006

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Monnet, D. L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*, *18*(3), 268–281. http://doi.org/10.1111/j.1469-0691.2011.03570.x

Malhotra-Kumar, S., Xavier, B. B., Das, A. J., Lammens, C., Butaye, P., & Goossens, H. (2016). Colistin resistance gene mcr-1 harboured on a multidrug resistant plasmid. *The Lancet Infectious Diseases*, *16*(3), 283–284. http://doi.org/10.1016/S1473-3099(16)00012-8

Manian, F. a. (2003). Asymptomatic nasal carriage of mupirocin-resistant,

methicillin-resistant Staphylococcus aureus (MRSA) in a pet dog associated with MRSA infection in household contacts. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *36*(2), e26–e28. http://doi.org/10.1086/344772

Manivannan, B., Mahalingam, N., Jadhao, S., Mishra, A., & Nilawe, P. (2016). Draft Genome Sequence of a Clinically Isolated Extensively Drug- Resistant Pseudomonas aeruginosa Strain, *4*(2), 2015–2016. http://doi.org/10.1128/genomeA.00162-16.Copyright

Marcel, J. P., Alfa, M., Baquero, F., Etienne, J., Goossens, H., Harbarth, S., … Jarlier, V. (2008). Healthcare-associated infections: Think globally, act locally. *Clinical Microbiology and Infection*, *14*(10), 895–907. http://doi.org/10.1111/j.1469-0691.2008.02074.x

Maron, D., Smith, T. J., & Nachman, K. E. (2013). Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. *Globalization and Health*, *9*(1), 48. http://doi.org/10.1186/1744-8603-9-48

Marra, A. R., Pereira, C. A. P., Gales, A. C., Menezes, L. C., Cal, R. G. R., Souza, M. A. De, … Einstein, A. (2006). Bloodstream Infections with Metallo- NL -Lactamase-Producing Pseudomonas aeruginosa : Epidemiology , Microbiology , and Clinical Outcomes. *Antimicrobial Agents and Chemotherapy*, *50*(1), 388–390. http://doi.org/10.1128/AAC.50.1.388

Marvig, R. L., Sommer, L. M., Molin, S., & Johansen, H. K. (2014). Convergent evolution and adaptation of Pseudomonas aeruginosa within patients with cystic fibrosis. *Nature Genetics*, *47*(1), 57–64. http://doi.org/10.1038/ng.3148

Masuda, N., & Ohya, S. (1992). Cross-resistance to meropenem, cephems, and quinolones in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*, *36*(9), 1847–1851. http://doi.org/10.1128/aac.

Mateus, A. L. P., Brodbelt, D. C., Barber, N., & Stärk, K. D. C. (2014). Qualitative study of factors associated with antimicrobial usage in seven small animal veterinary practices in the UK. *Preventive Veterinary Medicine*, *117*(1), 68–78. http://doi.org/10.1016/j.prevetmed.2014.05.007

Mathee, K., Narasimhan, G., Valdes, C., Qiu, X., Matewish, J. M., Koehrsen, M., …

Lory, S. (2008). Dynamics of Pseudomonas aeruginosa genome evolution. *Proceedings of the National Academy of Sciences*, *105*(8), 3100–3105. http://doi.org/10.1073/pnas.0711982105

Matsuo, Y., Eda, S., Gotoh, N., Yoshihara, E., & Nakae, T. (2004). MexZ-mediated regulation of mexXY multidrug efflux pump expression in Pseudomonas aeruginosa by binding on the mexZ-mexX intergenic DNA. *FEMS Microbiology Letters*, *238*(1), 23–28. http://doi.org/10.1016/j.femsle.2004.07.010

McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., … Wright, G. D. (2013). The comprehensive antibiotic resistance database. *Antimicrobial Agents and Chemotherapy*, *57*(7), 3348–3357. http://doi.org/10.1128/AAC.00419-13

McArthur, A. G., & Wright, G. D. (2015). Bioinformatics of antimicrobial resistance in the age of molecular epidemiology. *Current Opinion in Microbiology*, *27*, 45–50. http://doi.org/10.1016/j.mib.2015.07.004

McCallum, S. J., Corkill, J., Gallagher, M., Ledson, M. J., Anthony Hart, C., & Walshaw, M. J. (2001). Superinfection with a transmissible strain of *Pseudomonas aeruginosa* in adults with cystic fibrosis chronically colonised by *P. aeruginosa*. *The Lancet*, *358*(9281), 558–560. http://doi.org/10.1016/S0140-6736(01)05715-4

McKay, L., Rose, C. D. S., Matousek, J. L., Schmeitzel, L. S., Gibson, N. M., & Gaskin, J. M. (2007). Antimicrobial testing of selected fluoroquinolones against Pseudomonas aeruginosa isolated from canine otitis. *Journal of the American Animal Hospital Association*, *43*(6), 307–312.

Mcmahon, T., Zijl, P. C. M. Van, & Gilad, A. A. (2015). NIH Public Access, *27*(3), 320–331. http://doi.org/10.1002/nbm.3066.Non-invasive

McPhee, J. B., Bains, M., Winsor, G., Lewenza, S., Kwasnicka, A., Brazas, M. D., … Hancock, R. E. W. (2006). Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg2+-induced gene regulation in Pseudomonas aeruginosa. *Journal of Bacteriology*, *188*(11), 3995–4006. http://doi.org/10.1128/JB.00053-06

McPhee, J. B., Lewenza, S., & Hancock, R. E. W. (2003). Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that

regulates resistance to polymyxin B and cationic antimicrobial peptides in Pseudomonas aeruginosa. *Molecular Microbiology*, *50*(1), 205–217. http://doi.org/10.1046/j.1365-2958.2003.03673.x

Mehrad, B., Clark, N. M., Zhanel, G. G., & Lynch, J. P. (2015). Antimicrobial resistance in hospital-acquired gram-negative bacterial infections. *Chest*, *147*(5), 1413–1421. http://doi.org/10.1378/chest.14-2171

Mena, A., Smith, E. E., Burns, J. L., Speert, D. P., Moskowitz, S. M., Perez, J. L., & Oliver, A. (2008). Genetic adaptation of Pseudomonas aeruginosa to the airways of cystic fibrosis patients is catalyzed by hypermutation. *Journal of Bacteriology*, *190*(24), 7910–7917. http://doi.org/10.1128/JB.01147-08

Mesaros, N., Glupczynski, Y., Avrain, L., Caceres, N. E., Tulkens, P. M., & Van Bambeke, F. (2007). A combined phenotypic and genotypic method for the detection of Mex efflux pumps in Pseudomonas aeruginosa. *Journal of Antimicrobial Chemotherapy*, *59*(3), 378–386. http://doi.org/10.1093/jac/dkl504

Mesaros, N., Nordmann, P., Plesiat, P., Roussel-Delvallez, M., Van Eldere, J., Glupczynski, Y.,Van Bambeke, F. (2007). Pseudomonas aeruginosa: Resistance and therapeutic options at the dawn of the 2d millenium. *Antibiotiques*, *9*(3), 189–198. Retrieved from http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L47518817\nhttp://dx.doi.org/10.1016/S1294-5501(07)91378-3\nhttp://sfx.library.uu.nl/utrecht?sid=EMBASE&issn=12945501&id=doi:10.1016/S1294-5501(07)91378-3&atitle=Pseudomonas+aerugino

Mesaros, N., Nordmann, P., Plésiat, P., Roussel-Delvallez, M., Van Eldere, J., Glupczynski, Y., … Van Bambeke, F. (2007). Pseudomonas aeruginosa: Resistance and therapeutic options at the turn of the new millennium. *Clinical Microbiology and Infection*, *13*(6), 560–578. http://doi.org/10.1111/j.1469-0691.2007.01681.x

Michalska, A. D., Sacha, P. T., Ojdana, D., Wieczorek, A., & Tryniszewska, E. (2014). Prevalence of resistance to aminoglycosides and fluoroquinolones among Pseudomonas aeruginosa strains in a University Hospital in Northeastern Poland. *Brazilian Journal of Microbiology*, *45*(4), 1455–1458.

http://doi.org/10.1590/S1517-83822014000400041

Microbiology, C., Goossens, H., Eraksoy, H., Unal, S., Grabein, B., Masterton, R., … Information, T. (2004). Research note, *10*(8), 1290–1292.

Millan, A. S., Santos-Lopez, A., Ortega-Huedo, R., Bernabe-Balas, C., Kennedy, S. P., & Gonzalez-Zorn, B. (2015). Small-plasmid-mediated antibiotic resistance is enhanced by increases in plasmid copy number and bacterial fitness. *Antimicrobial Agents and Chemotherapy*, *59*(6), 3335–3341. http://doi.org/10.1128/AAC.00235-15

Millet, S., & Maertens, L. (2011). The European ban on antibiotic growth promoters in animal feed: From challenges to opportunities. *Veterinary Journal*, *187*(2), 143–144. http://doi.org/10.1016/j.tvjl.2010.05.001

Mohan, K., Fothergill, J. L., Storrar, J., Ledson, M. J., Winstanley, C., & Walshaw, M. J. (2008a). Transmission of *Pseudomonas aeruginosa* epidemic strain from a patient with cystic fibrosis to a pet cat. *Thorax*, *63*(9), 839–840. http://doi.org/10.1136/thx.2007.092486

Mohan, K., Fothergill, J. L., Storrar, J., Ledson, M. J., Winstanley, C., & Walshaw, M. J. (2008b). Transmission of Pseudomonas aeruginosa epidemic strain from a patient with cystic fibrosis to a pet cat. *Thorax*, *63*(9), 839–840. http://doi.org/10.1136/thx.2007.092486

Moore, J. E., & Elborn, J. S. (2012). Implications for colistin use in patients with cystic fibrosis (CF). Letter in response to " Prophylactic and metaphylactic antimicrobial use in Belgian fattening pig herds" by Callens et al. [Prev. Vet. Med. 106 (2012) 53-62]. *Preventive Veterinary Medicine*, *107*(3-4), 286–287. http://doi.org/10.1016/j.prevetmed.2012.09.006

Moore, R. a, Chan, L., & Hancock, R. E. (1984). Evidence for two distinct mechanisms of resistance to polymyxin B in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*, *26*(4), 539–545. http://doi.org/10.1128/AAC.26.4.539

Morita, Y., Tomida, J., & Kawamura, Y. (2012). Mexxy multidrug efflux system of Pseudomonas aeruginosa. *Frontiers in Microbiology*, *3*(NOV), 1–13. http://doi.org/10.3389/fmicb.2012.00408

Morita, Y., Tomida, J., & Kawamura, Y. (2013). Responses of Pseudomonas aeruginosa to antimicrobials. *Frontiers in Microbiology*, *4*(JAN), 1–8.

http://doi.org/10.3389/fmicb.2013.00422

Morris, D. O. (2004). Medical therapy of otitis externa and otitis media. *Veterinary Clinics of North America - Small Animal Practice*, *34*(2), 541–555. http://doi.org/10.1016/j.cvsm.2003.10.009

Morris, D. O., Boston, R. C., O'Shea, K., & Rankin, S. C. (2010). The prevalence of carriage of meticillin-resistant staphylococci by veterinary dermatology practice staff and their respective pets. *Veterinary Dermatology*, *21*(4), 400–407. http://doi.org/10.1111/j.1365-3164.2010.00866.x

Morrow, C. B., Raraigh, K. S., Green, D. M., Blackman, S. M., Cutting, G. R., & Collaco, J. M. (2014). Cat and Dog Exposure and Respiratory Morbidities in Cystic Fibrosis. *The Journal of Pediatrics*, *165*(4), 830–835.e2. http://doi.org/10.1016/j.jpeds.2014.05.046

Muller, C., Plésiat, P., & Jeannot, K. (2011). A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and β-lactams in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*, *55*(3), 1211–1221. http://doi.org/10.1128/AAC.01252-10

Munita, J. M., Arias, C. A., Unit, A. R., & Santiago, A. De. (2016). HHS Public Access. *Mechanisms of Antibiotic Resistance*, *4*(2), 1–37. http://doi.org/10.1128/microbiolspec.VMBF-0016-2015.Mechanisms

Murphy, C., Reid-Smith, R. J., Prescott, J. F., Bonnett, B. N., Poppe, C., Boerlin, P., … McEwen, S. A. (2009). Occurrence of antimicrobial resistant bacteria in healthy dogs and cats presented to private veterinary hospitals in southern Ontario: A preliminary study. *The Canadian Veterinary Journal. La Revue Vétérinaire Canadienne*, *50*(10), 1047–1053.

Nadal Jimenez, P., Koch, G., Thompson, J. A., Xavier, K. B., Cool, R. H., & Quax, W. J. (2012). The Multiple Signaling Systems Regulating Virulence in Pseudomonas aeruginosa. *Microbiology and Molecular Biology Reviews*, *76*(1), 46–65. http://doi.org/10.1128/MMBR.05007-11

Nardelli, M., Scalzo, P. M., Ramírez, M. S., Quiroga, M. P., Cassini, M. H., & Centrón, D. (2012). Class 1 integrons in environments with different degrees of urbanization. *PLoS ONE*, *7*(6). http://doi.org/10.1371/journal.pone.0039223

Ner, Z., Ross, L. a, Horn, M. V, Keens, T. G., MacLaughlin, E. F., Starnes, V. a, & Woo,

M. S. (2003). Bordetella bronchiseptica infection in pediatric lung transplant recipients. *Pediatric Transplantation*, *7*(5), 413–417. http://doi.org/10.1034/j.1399-3046.2003.00074.x

Nordmann, P., & Naas, T. (1994). Sequence analysis of PER-1 extended-spectrum beta-lactamase from Pseudomonas aeruginosa and comparison with class A beta-lactamases. *Antimicrobial Agents and Chemotherapy*, *38*(1), 104–14. http://doi.org/10.1128/AAC.38.1.104

Nuttall, T., & Cole, L. K. (2007). Evidence-based veterinary dermatology: A systematic review of interventions for treatment of Pseudomonas otitis in dogs. *Veterinary Dermatology*, *18*(2), 69–77. http://doi.org/10.1111/j.1365-3164.2007.00575.x

O'Toole, G., Kaplan, H. B., & Kolter, R. (2000). Biofilm Formation as Microbial Development. *Annual Review of Microbiology*, *54*(1), 49–79. http://doi.org/10.1146/annurev.micro.54.1.49

Odumosu, B. T., Ajetunmobi, O., Dada-Adegbola, H., & Odutayo, I. (2016). Antibiotic susceptibility pattern and analysis of plasmid profiles of Pseudomonas aeruginosa from human, animal and plant sources. *SpringerPlus*, *5*(1), 1381. http://doi.org/10.1186/s40064-016-3073-9

Ogbolu, D. O., Daini, O. A., Ogunledun, A., Alli, A. O., & Webber, M. A. (2011). High levels of multidrug resistance in clinical isolates of Gram-negative pathogens from Nigeria. *International Journal of Antimicrobial Agents*, *37*(1), 62–66. http://doi.org/10.1016/j.ijantimicag.2010.08.019

Ogeer-Gyles, J., Mathews, K., Weese, J. S., Prescott, J. F., & Boerlin, P. (2006). Evaluation of catheter-associated urinary tract infections and multi-drug-resistant Escherichia coli isolates from the urine of dogs with indwelling urinary catheters. *Journal of the American Veterinary Medical Association*, *229*(10), 1584–1590. http://doi.org/10.2460/javma.229.10.1584

Olaitan, A. O., Morand, S., & Rolain, J.-M. (2014). Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Frontiers in Microbiology*, *5*(November), 1–18. http://doi.org/10.3389/fmicb.2014.00643

Oliver, A., Mulet, X., López-Causapé, C., & Juan, C. (2015). The increasing threat of Pseudomonas aeruginosa high-risk clones. *Drug Resistance Updates*, *21-22*,

41–59. http://doi.org/10.1016/j.drup.2015.08.002

Olsen, I. (2015). Biofilm-specific antibiotic tolerance and resistance. *European Journal of Clinical Microbiology and Infectious Diseases*, *34*(5), 877–886. http://doi.org/10.1007/s10096-015-2323-z

Ozer, E. A., Allen, J. P., & Hauser, A. R. (2014). Characterization of the core and accessory genomes of Pseudomonas aeruginosa using bioinformatic tools Spine and AGEnt. *BMC Genomics*, *15*(1), 737. http://doi.org/10.1186/1471-2164-15-737

Öztürk, H., Ozkirimli, E., & Özgür, A. (2015). Classification of beta-lactamases and Penicillin Binding Proteins using ligand-centric network models. *PLoS ONE*, *10*(2), 1–23. http://doi.org/10.1371/journal.pone.0117874

Palzkill, T. (2014). NIH Public Access, 91–104. http://doi.org/10.1111/j.1749-6632.2012.06796.x.Metallo-

Parameswaran, G. I., & Sethi, S. (2012). Pseudomonas infection in chronic obstructive pulmonary disease. *Future Microbiology*, *7*(10), 1129–1132. http://doi.org/10.2217/fmb.12.88

Park, C. H., Robicsek, A., Jacoby, G. A., Sahm, D., & Hooper, D. C. (2006). Prevalence in the United States of aac(6???)-Ib-cr encoding a ciprofloxacin-modifying enzyme. *Antimicrobial Agents and Chemotherapy*, *50*(11), 3953–3955. http://doi.org/10.1128/AAC.00915-06

Parkins, M. D., Glezerson, B. A., Sibley, C. D., Sibley, K. A., Duong, J., Purighalla, S., … Rabin, H. R. (2014). Twenty-five-year outbreak of Pseudomonas aeruginosa infecting individuals with cystic fibrosis: Identification of the prairie epidemic strain. *Journal of Clinical Microbiology*, *52*(4), 1127–1135. http://doi.org/10.1128/JCM.03218-13

Paterson, D. L., & Bonomo, R. a. (2005). Extended-Spectrum NL -Lactamases : a Clinical Update. *Society*, *18*(4), 657–686. http://doi.org/10.1128/CMR.18.4.657

Patzer, J. A., Walsh, T. R., Weeks, J., Dzierzanowska, D., & Toleman, M. A. (2009). Emergence and persistence of integron structures harbouring VIM genes in the Children's Memorial Health Institute, Warsaw, Poland, 1998-2006. *Journal of Antimicrobial Chemotherapy*, *63*(2), 269–273.

http://doi.org/10.1093/jac/dkn512

Pearson, J. P., Pesci, E. C., & Iglewski, B. H. (1997). Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes . Roles of Pseudomonas aeruginosa las and rhl Quorum-Sensing Systems in Control of Elastase and Rhamnolipid Biosynthesis Genes Dow. *Journal of Bacteriology*, *179*(18), 5756–5767. http://doi.org/10.1128/JB.179.18.5756-5767.1997

Pedersen, K., Hammer, A. S., Sørensen, C. M., & Heuer, O. E. (2009). Usage of antimicrobials and occurrence of antimicrobial resistance among bacteria from mink. *Veterinary Microbiology*, *133*(1-2), 115–122. http://doi.org/10.1016/j.vetmic.2008.06.005

Pedersen, K., Pedersen, K., Jensen, H., Finster, K., Jensen, V. F., & Heuer, O. E. (2007). Occurrence of antimicrobial resistance in bacteria from diagnostic samples from dogs. *Journal of Antimicrobial Chemotherapy*, *60*(4), 775–781. http://doi.org/10.1093/jac/dkm269

Peleg, A. Y., & Hooper, D. C. (2011). Hospital-Acquired Infections Due to Gram-Negative Bacteria. *New England Journal of Medicine*, *362*(19), 1804–1813. http://doi.org/10.1056/NEJMra0904124.Hospital-Acquired

Percival, S. L., McCarty, S. M., & Lipsky, B. (2015). Biofilms and Wounds: An Overview of the Evidence. *Advances in Wound Care*, *4*(7), 373–381. http://doi.org/10.1089/wound.2014.0557

Pesci, E. C., Pearson, J. P., Seed, P. C., Pesci, E. C., Pearson, J. P., Seed, P. C., & Iglewski, B. H. (1997). Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa Regulation of las and rhl Quorum Sensing in Pseudomonas aeruginosa. *Strain*, *179*(10), 3127–3132. http://doi.org/10 3127-3132

Picard, B., Denamur, E., Barakat, A., Elion, J., Goullett, P., Microbiologie, L. De, … Huchard, D. H. (2017). G en et ic heterogeneity of Pseudomonas aeruginosa c I i n ica I isolates revealed by esterase electrophoretic polymorphism and restriction fragment length polymorphism of the ribosomal RNA gene region, *40*(1994), 313–322.

Pirnay, J. P., Bilocq, F., Pot, B., Cornelis, P., Zizi, M., Van Eldere, J., … De Vos, D.

(2009). Pseudomonas aeruginosa population structure revisited. *PLoS ONE*, *4*(11). http://doi.org/10.1371/journal.pone.0007740

Pitout, J. D. D., Gregson, D. B., Poirel, L., Mcclure, J., & Le, P. (2005). Detection of. *Society*, *43*(7), 3129–3135. http://doi.org/10.1128/JCM.43.7.3129

Pitout, J. D. D., Revathi, G., Chow, B. L., Kabera, B., Kariuki, S., Nordmann, P., & Poirel, L. (2008). Metallo-β-lactamase-producing Pseudomonas aeruginosa isolated from a large tertiary centre in Kenya. *Clinical Microbiology and Infection*, *14*(8), 755–759. http://doi.org/10.1111/j.1469-0691.2008.02030.x

Pitt, T. L. (2002). Cross infection of cystic fibrosis patients with Pseudomonas aeruginosa. *Thorax*, *57*(11), 921. http://doi.org/10.1136/thorax.57.11.921

Poirel, L., Lagrutta, E., Taylor, P., Pham, J., & Nordmann, P. (2010). Emergence of metallo-??-lactamase NDM-1-producing multidrug-resistant Escherichia coli in Australia. *Antimicrobial Agents and Chemotherapy*, *54*(11), 4914–4916. http://doi.org/10.1128/AAC.00878-10

Poirel, L., Nordmann, P., Lagrutta, E., Cleary, T., & Munoz-Price, L. S. (2010). Emergence of KPC-producing Pseudomonas aeruginosa in the United States. *Antimicrobial Agents and Chemotherapy*, *54*(7), 3072. http://doi.org/10.1128/AAC.00513-10

Poole, K. (2005a). Aminoglycoside Resistance in Pseudomonas aeruginosa. *Antimicrob Agents Chemother*, *49*(2), 479–487. http://doi.org/10.1128/AAC.49.2.479

Poole, K. (2005b). *Efflux-mediated antimicrobial resistance*. *Journal of Antimicrobial Chemotherapy* (Vol. 56). http://doi.org/10.1093/jac/dki171

Pratesi, A., Moores, A. P., Downes, C., Grierson, J., & Maddox, T. W. (2015). Efficacy of Postoperative Antimicrobial Use for Clean Orthopedic Implant Surgery in Dogs: A Prospective Randomized Study in 100 Consecutive Cases. *Veterinary Surgery*, *44*(5), 653–660. http://doi.org/10.1111/vsu.12326

Pye, C. C., Yu, A. a., & Weese, J. S. (2013). Evaluation of biofilm production by Pseudomonas aeruginosa from canine ears and the impact of biofilm on antimicrobial susceptibility in vitro. *Veterinary Dermatology*, *24*(4). http://doi.org/10.1111/vde.12040

Radford, a. D., Noble, P. J., Coyne, K. P., Gaskell, R. M., Jones, P. H., Bryan, J. G. E., …

Dawson, S. (2011). Antibacterial prescribing patterns in small animal veterinary practice identified via SAVSNET: the small animal veterinary surveillance network. *The Veterinary Record*, *169*(12), 310. http://doi.org/10.1136/vr.d5062

Ratnam, S., Hogan, K., March, S. B., & Butler, R. W. (1986). Whirlpool-associated follicilitis caused by Pseudomonas aeruginosa: Report of an outbreak and review. *J Clin Microbiol*, *23*(3), 655–659.

Register, K. B., Sukumar, N., Palavecino, E. L., Rubin, B. K., & Deora, R. (2012). Bordetella bronchiseptica in a Paediatric Cystic Fibrosis Patient: Possible Transmission from a Household Cat. *Zoonoses and Public Health*, *59*(4), 246–250. http://doi.org/10.1111/j.1863-2378.2011.01446.x

Report, C. (2002). from a patient with cystic fibrosis (CF) to non-CF relatives, (February 2000), 559–561.

Richez, P., & Burch, D. G. S. (2016). Colistin in animals: a high risk for resistance selection in Europe? *Veterinary Record*, *178*(4), 101.2–102. http://doi.org/10.1136/vr.i381

Robicsek, A., Jacoby, G. A., & Hooper, D. C. (2006). Review The worldwide emergence of plasmid-mediated quinolone resistance. *October*, 629–640. http://doi.org/doi:10.1016/S1473-3099(06)70599-0. PMID 17008172.%0A

Robicsek, A., Strahilevitz, J., Sahm, D. F., Jacoby, G. A., & Hooper, D. C. (2006). qnr prevalence in ceftazidime-resistant Enterobacteriaceae isolates from the United States. *Antimicrobial Agents and Chemotherapy*, *50*(8), 2872–2874. http://doi.org/10.1128/AAC.01647-05

Rodríguez-Martínez, J. M., Cano, M. E., Velasco, C., Martínez-Martínez, L., & Pascual, Á. (2011). Plasmid-mediated quinolone resistance: An update. *Journal of Infection and Chemotherapy*, *17*(2), 149–182. http://doi.org/10.1007/s10156-010-0120-2

Rodríguez-Martínez, J. M., Poirel, L., & Nordmann, P. (2009). Extended-spectrum cephalosporinases in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*, *53*(5), 1766–1771. http://doi.org/10.1128/AAC.01410-08

Rolain, J.-M., Kempf, M., Leangapichart, T., Chabou, S., Olaitan, A. O., Le Page, S., … Raoult, D. (2016). Plasmid-mediated *mcr-1* gene in colistin-resistant clinical

isolates of *Klebsiella pneumoniae* in France and Laos. *Antimicrobial Agents and Chemotherapy*, *60*(11), AAC.00960–16. http://doi.org/10.1128/AAC.00960-16

Rossolini, G. M., Mantengoli, E., Docquier, J. D., Musmanno, R. A., & Coratza, G. (2007). Epidemiology of infections caused by multiresistant Gram-negatives: ESBLs, MBLs, panresistant strains. *New Microbiologica*, *30*(3), 332–339.

Roy, P. H., Tetu, S. G., Larouche, A., Elbourne, L., Tremblay, S., Ren, Q., … Paulsen, I. T. (2010). Complete genome sequence of the multiresistant taxonomic outlier Pseudomonas aeruginosa PA7. *PLoS ONE*, *5*(1), 1–10. http://doi.org/10.1371/journal.pone.0008842

Rubin, J., Walker, R. D., Blickenstaff, K., Bodeis-Jones, S., & Zhao, S. (2008). Antimicrobial resistance and genetic characterization of fluoroquinolone resistance of Pseudomonas aeruginosa isolated from canine infections. *Veterinary Microbiology*, *131*(1-2), 164–172. http://doi.org/10.1016/j.vetmic.2008.02.018

Ruparell, A., Dubern, J. F., Ortori, C. A., Harrison, F., Halliday, N. M., Emtage, A., … Hardie, K. R. (2016). The fitness burden imposed by synthesising quorum sensing signals. *Scientific Reports*, *6*(1), 33101. http://doi.org/10.1038/srep33101

Sacha, P., Wieczorek, P., Hauschild, T., Zórawski, M., Olszańska, D., & Tryniszewska, E. (2008). Metallo-beta-lactamases of Pseudomonas aeruginosa--a novel mechanism resistance to beta-lactam antibiotics. *Folia Histochemica et Cytobiologica*, *46*(2), 137–142. http://doi.org/10.2478/v10042-008-0020-9

Sahuquillo-Arce, J., Méndez, R., Hernández-Cabezas, A., & Menéndez, R. (2016). Non-cystic fibrosis bronchiectasis: The long road to multidrug resistant bacteria. *Community Acquired Infection*, *3*(4), 110. http://doi.org/10.4103/2225-6482.198491

Salomonsen, C. M., Boye, M., Høiby, N., Jensen, T. H., & Hammer, A. S. (2013). Comparison of histological lesions in mink with acute hemorrhagic Pneumonia associated with pseudomonas aeruginosa or Escherichia coli. *Canadian Journal of Veterinary Research*, *77*(3), 199–204.

Salunkhe, P., Smart, C. H. M., Morgan, A. W., Panagea, S., Walshaw, M. J., Hart, A., … Winstanley, C. (2005). A Cystic Fibrosis Epidemic Strain of. *Journal of*

Bacteriology, *187*(14), 4908–4920. http://doi.org/10.1128/JB.187.14.4908

Santajit, S., & Indrawattana, N. (2016). Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. *BioMed Research International*, *2016*. http://doi.org/10.1155/2016/2475067

Sauer, P., Andrew, S. E., Lassaline, M., Gelatt, K. N., & Denis, H. M. (2003). Changes in antibiotic resistance in equine bacterial ulcerative keratitis (1991-2000): 65 Horses. *Veterinary Ophthalmology*, *6*(4), 309–313. http://doi.org/10.1111/j.1463-5224.2003.00312.x

Scaccabarozzi, L., Leoni, L., Ballarini, A., Barberio, A., Locatelli, C., Casula, A., … Moroni, P. (2015). Pseudomonas aeruginosa in dairy goats: Genotypic and phenotypic comparison of intramammary and environmental isolates. *PLoS ONE*, *10*(11). http://doi.org/10.1371/journal.pone.0142973

Schink, A. K., Kadlec, K., Hauschild, T., Brenner Michael, G., Dörner, J. C., Ludwig, C., … Schwarz, S. (2013). Susceptibility of canine and feline bacterial pathogens to pradofloxacin and comparison with other fluoroquinolones approved for companion animals. *Veterinary Microbiology*, *162*(1), 119–126. http://doi.org/10.1016/j.vetmic.2012.08.001

Schink, A. K., Kadlec, K., & Schwarz, S. (2012). Detection of qnr genes among Escherichia coli isolates of animal origin and complete sequence of the conjugative qnrB19-carrying plasmid pQNR2078. *Journal of Antimicrobial Chemotherapy*, *67*(5), 1099–1102. http://doi.org/10.1093/jac/dks024

Schmidt, H., & Beutin, L. (1995). Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157 : H7 strain EDL 933. *Infection and Immunity*, *63*(3), 1055–1061.

Schmidt, S. (2009). Measuring absorptive capacity. *Proceedings of the International Conference on Intellectual Capital, Knowledge Management & Organizational Learning*, *20*, 254–260. http://doi.org/10.1101/gr.107524.110.20

Schmidt, V. M., Pinchbeck, G. L., Nuttall, T., McEwan, N., Dawson, S., & Williams, N. J. (2015). Antimicrobial resistance risk factors and characterisation of faecal E. coli isolated from healthy Labrador retrievers in the United Kingdom. *Preventive Veterinary Medicine*, *119*(1-2), 31–40. http://doi.org/10.1016/j.prevetmed.2015.01.013

Schroeder, M., Brooks, B. D., & Brooks, A. E. (2017). The complex relationship between virulence and antibiotic resistance. *Genes*, *8*(1). http://doi.org/10.3390/genes8010039

Schweizer, H. P. (2003). Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. *Genet Mol Res*, *2*(1), 48–62. http://doi.org/S01 [pii]

Scott, F. W., & Pitt, T. L. (2004). Identification and characterization of transmissible Pseudomonas aeruginosa strains in cystic fibrosis patients in England and Wales. *Journal of Medical Microbiology*, *53*(7), 609–615. http://doi.org/10.1099/jmm.0.45620-0

Scott Weese, J. (2008). Antimicrobial resistance in companion animals. *Animal Health Research Reviews*, *9 (2)*(2), 169–176. http://doi.org/10.1017/S1466252308001485

Seemann, T. (2014). Prokka: Rapid prokaryotic genome annotation. *Bioinformatics*, *30*(14), 2068–2069. http://doi.org/10.1093/bioinformatics/btu153

Shaikh, S., Fatima, J., Shakil, S., Rizvi, S. M. D., & Kamal, M. A. (2015). Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. *Saudi Journal of Biological Sciences*, *22*(1), 90–101. http://doi.org/10.1016/j.sjbs.2014.08.002

Shalchi, Z., Gurbaxani, A., Baker, M., & Nash, J. (2011). Antibiotic resistance in microbial keratitis: Ten-year experience of corneal scrapes in the United Kingdom. *Ophthalmology*, *118*(11), 2161–2165. http://doi.org/10.1016/j.ophtha.2011.04.021

Sherley, M., Gordon, D. M., & Collignon, P. J. (2003). Species differences in plasmid carriage in the Enterobacteriaceae. *Plasmid*, *49*(1), 79–85. http://doi.org/10.1016/S0147-619X(02)00107-5

Shimizu, T., Homma, J. Y., Aoyama, T., Onodera, T., & Noda, H. (1974). Virulence of Pseudomonas aeruginosa and spontaneous spread of Pseudomonas pneumonia in a mink ranch. *Infection and Immunity*, *10*(1), 16–20.

Shintani, M., Sanchez, Z. K., & Kimbara, K. (2015). Genomics of microbial plasmids: Classification and identification based on replication and transfer systems and host taxonomy. *Frontiers in Microbiology*, *6*(MAR), 1–16.

http://doi.org/10.3389/fmicb.2015.00242

Singleton, D. A., Sánchez-Vizcaíno, F., Dawson, S., Jones, P. H., Noble, P. J. M., Pinchbeck, G. L., … Radford, A. D. (2017). Patterns of antimicrobial agent prescription in a sentinel population of canine and feline veterinary practices in the United Kingdom. *Veterinary Journal*, *224*, 18–24. http://doi.org/10.1016/j.tvjl.2017.03.010

Slekovec, C., Plantin, J., Cholley, P., Thouverez, M., Talon, D., Bertrand, X., & Hocquet, D. (2012). Tracking Down Antibiotic-Resistant Pseudomonas aeruginosa Isolates in a Wastewater Network. *PLoS ONE*, *7*(12). http://doi.org/10.1371/journal.pone.0049300

Smith, D. L., Harris, A. D., Johnson, J. a, Silbergeld, E. K., & Morris, J. G. (2002). Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *99*, 6434–6439. http://doi.org/10.1073/pnas.082188899

Smith, E. E., Sims, E. H., Spencer, D. H., Kaul, R., & Olson, M. V. (2005). Evidence for diversifying selection at the pyoverdine locus of Pseudomonas aeruginosa. *Journal of Bacteriology*, *187*(6), 2138–2147. http://doi.org/10.1128/JB.187.6.2138-2147.2005

Sousa, A., & Pereira, M. (2014). Pseudomonas aeruginosa Diversification during Infection Development in Cystic Fibrosis Lungs—A Review. *Pathogens*, *3*(3), 680–703. http://doi.org/10.3390/pathogens3030680

Srikumar, R., Li, X. Z., & Poole, K. (1997). Inner membrane efflux components are responsible for beta-lactam specificity of multidrug efflux pumps in Pseudomonas aeruginosa. *Journal of Bacteriology*, *179*(24), 7875–81. http://doi.org/10.1128/AAC.49.5.1782-1786.2005

Stapleton, F., Dart, J. K., Seal, D. V, & Matheson, M. (1995). Epidemiology of Pseudomonas aeruginosa keratitis in contact lens wearers. *Epidemiology and Infection*, *114*(3), 395–402. http://doi.org/10.1017/S0950268800052109

Steen, S. I., & Paterson, S. (2012). The susceptibility of Pseudomonas spp. isolated from dogs with otitis to topical ear cleaners. *Journal of Small Animal Practice*, *53*(10), 599–603. http://doi.org/10.1111/j.1748-5827.2012.01262.x

Stokes, H. W., & Hall, R. M. (1989). A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Molecular Microbiology*, *3*(12), 1669–1683. http://doi.org/10.1111/j.1365-2958.1989.tb00153.x

Stover C-K., Pham X-Q., Erwin A-L., Mizoguchi S-D., Warrener P., H. M.-J., & Brinkman F-S., Hufnagle W-O., Kowalik D-J., Lagrou M. Garber R-L., Goltry L., Tolentino E., Westbrock-Wadman S., Yuan Y., Brody L-L., Coulter S-N., Folger K-R., Kas A., Larbig K., Lim R., Smith K., Spencer D., Wong G-K., Wu Z., Paulsen I-T., Reizer J., Sa, O. M.-V. (2000). Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature*, *406*(6799), 959–964. http://doi.org/10.1038/35023079

Strahilevitz, J., Jacoby, G. A., Hooper, D. C., & Robicsek, A. (2009a). Plasmid-mediated quinolone resistance: A multifaceted threat. *Clinical Microbiology Reviews*, *22*(4), 664–689. http://doi.org/10.1128/CMR.00016-09

Strahilevitz, J., Jacoby, G. A., Hooper, D. C., & Robicsek, A. (2009b). Plasmid-mediated quinolone resistance: A multifaceted threat. *Clinical Microbiology Reviews*, *22*(4), 664–689. http://doi.org/10.1128/CMR.00016-09

Strateva, T., & Mitov, I. (2011). Contribution of an arsenal of virulence factors to pathogenesis of Pseudomonas aeruginosa infections. *Annals of Microbiology*, *61*(4), 717–732. http://doi.org/10.1007/s13213-011-0273-y

Strateva, T., & Yordanov, D. (2009). Pseudomonas aeruginosa - A phenomenon of bacterial resistance. *Journal of Medical Microbiology*, *58*(9), 1133–1148. http://doi.org/10.1099/jmm.0.009142-0

Sumrall, E. T., Gallo, E. B., Aboderin, A. O., Lamikanra, A., & Okeke, I. N. (2014). Dissemination of the transmissible quinolone-resistance gene qnrS1 by IncX plasmids in Nigeria. *PLoS ONE*, *9*(10), 1–10. http://doi.org/10.1371/journal.pone.0110279

Syrmis, M. W., O'Carroll, M. R., Sloots, T. P., Coulter, C., Wainwright, C. E., Bell, S. C., & Nissen, M. D. (2004). Rapid genotyping of Pseudomonas aeruginosa isolates harboured by adult and paediatric patients with cystic fibrosis using repetitive-element-based PCR assays. *Journal of Medical Microbiology*, *53*(11), 1089–1096. http://doi.org/10.1099/jmm.0.45611-0

Tada, T., Nhung, P. H., Miyoshi-Akiyama, T., Shimada, K., Tsuchiya, M., Phuong, D. M., … Kirikae, T. (2016). Multidrug-resistant sequence type 235 Pseudomonas aeruginosa clinical isolates producing IMP-26 with increased carbapenem-hydrolyzing activities in Vietnam. *Antimicrobial Agents and Chemotherapy*, *60*(11), 6853–6858. http://doi.org/10.1128/AAC.01177-16

Talbot, G. H., Bradley, J., Edwards, J. E., Gilbert, D., Scheld, M., & Bartlett, J. G. (2006). Bad Bugs Need Drugs: An Update on the Development Pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. *Clinical Infectious Diseases*, *42*(5), 657–668. http://doi.org/10.1086/499819

Tam, V. H., Rogers, C. A., Chang, K. T., Weston, J. S., Caeiro, J. P., & Garey, K. W. (2010). Impact of multidrug-resistant Pseudomonas aeruginosa bacteremia on patient outcomes. *Antimicrobial Agents and Chemotherapy*, *54*(9), 3717–3722. http://doi.org/10.1128/AAC.00207-10

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725–2729. http://doi.org/10.1093/molbev/mst197

Tang, Y., Fang, L., Xu, C., & Zhang, Q. (2017). Antibiotic resistance trends and mechanisms in the foodborne pathogen, Campylobacter. *Animal Health Research Reviews*, 1–12. http://doi.org/10.1017/S1466252317000135

Tawfik, A. F., Shibl, A. M., Aljohi, M. A., Altammami, M. A., & Al-Agamy, M. H. (2012). Distribution of Ambler class A, B and D ??-lactamases among Pseudomonas aeruginosa isolates. *Burns*, *38*(6), 855–860. http://doi.org/10.1016/j.burns.2012.01.005

Tazumi, a, Maeda, Y., Buckley, T., Millar, B., Goldsmith, C., Dooley, J., … Moore, J. (2009). Molecular epidemiology of clinical isolates of Pseudomonas aeruginosa isolated from horses in Ireland. *Irish Veterinary Journal*, *62*(7), 456–459. http://doi.org/10.1186/2046-0481-62-7-456

Timofte, D., Dandrieux, J., Wattret, A., Fick, J., & Williams, N. J. (2011). Detection of extended-spectrum-β-lactamase-positive Escherichia coli in bile isolates from two dogs with bacterial cholangiohepatitis. *Journal of Clinical Microbiology*, *49*(9), 3411–3414. http://doi.org/10.1128/JCM.01045-11

Timofte, D., Maciuc, I. E., Evans, N. J., Williams, H., Wattret, A., Fick, J. C., & Williams, N. J. (2014). Detection and molecular characterization of Escherichia coli CTX-M-15 and Klebsiella pneumoniae SHV-12 ??-lactamases from bovine mastitis isolates in the United Kingdom. *Antimicrobial Agents and Chemotherapy*, *58*(2), 789–794. http://doi.org/10.1128/AAC.00752-13

Toleman, M. A., Bennett, P. M., Walsh, T. R., Nemec, A., Dolzani, L., Brisse, S., … Tolmasky, M. E. (2011). NIH Public Access. *Sciences-New York*, *13*(6), 151–171. http://doi.org/10.1016/j.drup.2010.08.003.Aminoglycoside

Tomás, M., Doumith, M., Warner, M., Turton, J. F., Beceiro, A., Bou, G., … Woodford, N. (2010). Efflux pumps, OprD porin, AmpC beta-lactamase, and multiresistance in Pseudomonas aeruginosa isolates from cystic fibrosis patients. *Antimicrobial Agents and Chemotherapy*, *54*(5), 2219–2224. http://doi.org/10.1128/AAC.00816-09

Tran, Q. T., Nawaz, M. S., Deck, J., Nguyen, K. T., & Cerniglia, C. E. (2011). Plasmid-mediated quinolone resistance in Pseudomonas putida isolates from imported shrimp. *Applied and Environmental Microbiology*, *77*(5), 1885–1887. http://doi.org/10.1128/AEM.01176-10

Tran, T. B., Velkov, T., Nation, R. L., Forrest, A., Tsuji, B. T., Bergen, P. J., & Li, J. (2016). Pharmacokinetics/pharmacodynamics of colistin and polymyxin B: are we there yet? *International Journal of Antimicrobial Agents*, *48*(6), 592–597. http://doi.org/10.1016/j.ijantimicag.2016.09.010

Trautmann, M., Halder, S., Hoegel, J., Royer, H., & Haller, M. (2008). Point-of-use water filtration reduces endemic Pseudomonas aeruginosa infections on a surgical intensive care unit. *American Journal of Infection Control*, *36*(6), 421–429. http://doi.org/10.1016/j.ajic.2007.09.012

Trautmann, M., Lepper, P. M., & Haller, M. (2005). Ecology of Pseudomonas aeruginosa in the intensive care unit and the evolving role of water outlets as a reservoir of the organism. *American Journal of Infection Control*, *33*(5 SUPPL. 1), 41–49. http://doi.org/10.1016/j.ajic.2005.03.006

Vahaboglu, H., Öztürk, R., Aygün, G., Coşkunkan, F., Yaman, A., Kaygusuz, A., … Otkun, M. (1997). Widespread detection of per-1-type extended-spectrum β-lactamases among nosocomial Acinetobacter and Pseudomonas aeruginosa

isolates in Turkey: A nationwide multicenter study. *Antimicrobial Agents and Chemotherapy*, *41*(10), 2265–2269.

Valot, B., Guyeux, C., Rolland, J. Y., Mazouzi, K., Bertrand, X., & Hocquet, D. (2015). What it takes to be a Pseudomonas aeruginosa? The core genome of the opportunistic pathogen updated. *PLoS ONE*, *10*(5), 1–15. http://doi.org/10.1371/journal.pone.0126468

van Den Bogaard, A., & Stobberingh, E. E. (2000). Epidemiology of resistance to antibiotics. Link between animals and humans. *International Journal of Antimicrobial Agents*, *14*, 327–335.

Van Hoek, A. H. A. M., Mevius, D., Guerra, B., Mullany, P., Roberts, A. P., & Aarts, H. J. M. (2011). Acquired antibiotic resistance genes: An overview. *Frontiers in Microbiology*, *2*(SEP), 1–27. http://doi.org/10.3389/fmicb.2011.00203

Varaiya, A., Kulkarni, N., Kulkarni, M., Bhalekar, P., & Dogra, J. (2008). Incidence of metallo beta lactamase producing Pseudomonas aeruginosa in ICU patients. *Indian Journal of Medical Research*, *127*(4), 398–402.

Vazirani, J., Wurity, S., & Ali, M. H. (2015). Multidrug-resistant Pseudomonas aeruginosa keratitis: Risk factors, clinical characteristics, and outcomes. *Ophthalmology*, *122*(10), 2110–2114. http://doi.org/10.1016/j.ophtha.2015.06.007

Velkov, T., Roberts, K. D., Nation, R. L., Wang, J., Thompson, P. E., & Li, J. (2014). Teaching “old” polymyxins new tricks: New-generation lipopeptides targeting gram-negative “superbugs.” *ACS Chemical Biology*, *9*(5), 1172–1177. http://doi.org/10.1021/cb500080r

Verner-jeffreys, D. W., Welch, T. J., Schwarz, T., Pond, M. J., Woodward, M. J., Haig, S. J., … Baker-Austin, C. (2009). High prevalence of multidrug-tolerant bacteria and associated antimicrobial resistance genes isolated from ornamental fish and their carriage water. *PLoS ONE*, *4*(12). http://doi.org/10.1371/journal.pone.0008388

Vicente, L., Ferreira, R., Ferreira, F. D. A., José, F., Reis, C., Carlos, M., … Ribeiro, J. D. (2013). Review Article, *39*(April), 495–512.

Wang, R., Lagakos, S. W., & Drazen, J. M. (2008). in a Family and Its Pet Cat, 4–5.

Wang, Y., Wang, X., Schwarz, S., Zhang, R., Lei, L., Liu, X., … Shen, J. (2014). IMP-45-

producing multidrug-resistant Pseudomonas aeruginosa of canine origin. *Journal of Antimicrobial Chemotherapy*, *69*(9), 2579–2581. http://doi.org/10.1093/jac/dku133

Wannet, W. J. B., Duijkeren, E. Van, Wolfhagen, M. J. H. M., & Heck, M. E. O. C. (2005). Transmission of a Panton-Valentine Staphylococcus aureus Strain between Humans and a Dog Transmission of a Panton-Valentine Leucocidin-Positive , Methicillin-Resistant Staphylococcus aureus Strain between Humans and a Dog. *Journal of Clinical Microbiology*, *43*(12), 8–11. http://doi.org/10.1128/JCM.43.12.6209

Warne, R. T. (2011). An Investigation of, *235*(5), 1–4. http://doi.org/10.1177/1932202X11414821

Wayne, A., Mccarthy, R., & Lindenmayer, J. (2011). Therapeutic antibiotic use patterns in dogs: Observations from a veterinary teaching hospital. *Journal of Small Animal Practice*, *52*(6), 310–318. http://doi.org/10.1111/j.1748-5827.2011.01072.x

Webber, M. A., & Piddock, L. J. V. (2003). The importance of efflux pumps in bacterial antibiotic resistance. *Journal of Antimicrobial Chemotherapy*, *51*(1), 9–11. http://doi.org/10.1093/jac/dkg050

Wedley, a L., Maddox, T. W., Westgarth, C., Coyne, K. P., Pinchbeck, G. L., Williams, N. J., & Dawson, S. (2011). Prevalence of antimicrobial-resistant Escherichia coli in dogs in a cross-sectional, community-based study. *The Veterinary Record*, *168*(13), 354. http://doi.org/10.1136/vr.d1540

Weese, J. S., Dick, H., Willey, B. M., McGeer, A., Kreiswirth, B. N., Innis, B., & Low, D. E. (2006). Suspected transmission of methicillin-resistant Staphylococcus aureus between domestic pets and humans in veterinary clinics and in the household. *Veterinary Microbiology*, *115*(1-3), 148–155. http://doi.org/10.1016/j.vetmic.2006.01.004

Weldhagen, G. F., Poirel, L., & Nordmann, P. (2003). Ambler Class A Extended-Spectrum β -Lactamases in Pseudomonas aeruginosa : Novel Developments and Clinical Impact MINIREVIEW Ambler Class A Extended-Spectrum ɴʟ -Lactamases in Pseudomonas aeruginosa : Novel Developments and Clinical

Impact. *Antimicrobial Agents and Chemotherapy*, *47*(8), 2385–92. http://doi.org/10.1128/AAC.47.8.2385

Werners, a H., Bull, S., & Fink-Gremmels, J. (2005). Endotoxaemia: a review with implications for the horse. *Equine Veterinary Journal*, *37*(4), 371–383. http://doi.org/10.2746/0425164054529418

Westgate, S. J., Percival, S. L., Knottenbelt, D. C., Clegg, P. D., & Cochrane, C. a. (2011). Microbiology of equine wounds and evidence of bacterial biofilms. *Veterinary Microbiology*, *150*(1-2), 152–159. http://doi.org/10.1016/j.vetmic.2011.01.003

Wideman, W. L. (2006). Pseudomonas aeruginosa otitis media and interna in a chinchilla ranch. *Canadian Veterinary Journal*, *47*(8), 799–800.

Wiehlmann, L., Wagner, G., Cramer, N., Siebert, B., Gudowius, P., Morales, G., … Tümmler, B. (2007). Population structure of Pseudomonas aeruginosa. *Pnas*, *104*(19), 8101–6. http://doi.org/10.1073/pnas.0609213104

Willcox, M. D. P. (2007). Pseudomonas aeruginosa infection and inflammation during contact lens wear: a review. *Optometry and Vision Science*, *84*(4), 273–278. http://doi.org/10.1097/OPX.0b013e3180439c3e

Williams, J. D. (1991). Mechanisms of beta-lactam resistance i n british isolates of. *Journal of Antimicrobial Chemotherapy*, (May 1982).

Wilson, D. J., Baldwin, T. J., Whitehouse, C. H., & Hullinger, G. (2015). Causes of mortality in farmed mink in the Intermountain West, North America. *Journal of Veterinary Diagnostic Investigation*, *27*(4), 470–475. http://doi.org/10.1177/1040638715586438

Winsor, G. L., Lam, D. K. W., Fleming, L., Lo, R., Whiteside, M. D., Yu, N. Y., … Brinkman, F. S. L. (2011). Pseudomonas Genome Database: Improved comparative analysis and population genomics capability for Pseudomonas genomes. *Nucleic Acids Research*, *39*(SUPPL. 1), 596–600. http://doi.org/10.1093/nar/gkq869

Winstanley, C., Brien, S. O., & Brockhurst, M. A. (2016). Pseudomonas aeruginosa Evolutionary Adaptation and Diversification in Cystic Fibrosis Chronic Lung Infections. *Trends in Microbiology*, *xx*(5), 1–11. http://doi.org/10.1016/j.tim.2016.01.008

Winstanley, C., Langille, M. G. I., Fothergill, J. L., Kukavica-Ibrulj, I., Paradis-Bleau, C., Sanschagrin, F., … Levesque, R. C. (2009). Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the liverpool epidemic strain of pseudomonas aeruginosa. *Genome Research*, *19*(1), 12–23. http://doi.org/10.1101/gr.086082.108

Winstanley, C., O'Brien, S., & Brockhurst, M. A. (2016). Pseudomonas aeruginosa evolutionary adaptation and diversification in cystic fibrosis chronic lung infections. *Trends in Microbiology*, *24*(5), 327–337. http://doi.org/10.1016/j.tim.2016.01.008

Woodford, N., Turton, J. F., & Livermore, D. M. (2011). Multiresistant Gram-negative bacteria: The role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiology Reviews*, *35*(5), 736–755. http://doi.org/10.1111/j.1574-6976.2011.00268.x

Woodford, N., Zhang, J., Kaufmann, M. E., Yarde, S., Tomas, M. del M., Faris, C., … Livermore, D. M. (2008). Detection of Pseudomonas aeruginosa isolates producing VEB-type extended-spectrum β-lactamases in the United Kingdom. *Journal of Antimicrobial Chemotherapy*, *62*(6), 1265–1268. http://doi.org/10.1093/jac/dkn400

Wright, E. A., Di Lorenzo, V., Trappetti, C., Liciardi, M., Orru, G., Viti, C., … Winstanley, C. (2015). Divergence of a strain of Pseudomonas aeruginosa during an outbreak of ovine mastitis. *Veterinary Microbiology*, *175*(1), 105–113. http://doi.org/10.1016/j.vetmic.2014.11.011

Xiao, M., Wang, Y., Yang, Q. W., Fan, X., Brown, M., Kong, F., & Xu, Y. C. (2012). Antimicrobial susceptibility of Pseudomonas aeruginosa in China: A review of two multicentre surveillance programmes, and application of revised CLSI susceptibility breakpoints. *International Journal of Antimicrobial Agents*, *40*(5), 445–449. http://doi.org/10.1016/j.ijantimicag.2012.07.002

Xu, Y., Gu, B., Huang, M., Liu, H., Xu, T., Xia, W., & Wang, T. (2015). Epidemiology of carbapenem resistant Enterobacteriaceae (CRE) during 2000-2012 in Asia. *Journal of Thoracic Disease*, *7*(3), 376–385. http://doi.org/10.3978/j.issn.2072-1439.2014.12.33

Yang, X., Xing, B., Liang, C., Ye, Z., & Zhang, Y. (2015). Prevalence and

fluoroquinolone resistance of pseudomonas aeruginosa in a hospital of South China. *Int J Clin Exp Med*, *8*(1), 1386–1390. Retrieved from www.ijcem.com

Youenou, B., Brothier, E., & Nazaret, S. (2014). Diversity among strains of Pseudomonas aeruginosa from manure and soil, evaluated by multiple locus variable number tandem repeat analysis and antibiotic resistance profiles. *Research in Microbiology*, *165*(1), 2–13. http://doi.org/10.1016/j.resmic.2013.10.004

Yu, Z., Qin, W., Lin, J., Fang, S., & Qiu, J. (2015). Antibacterial mechanisms of polymyxin and bacterial resistance. *BioMed Research International*, *2015*. http://doi.org/10.1155/2015/679109

Zegans, M. E., DiGiandomenico, A., Ray, K., Naimie, A., Keller, A. E., Stover, C. K., … Lietman, T. M. (2016). Association of Biofilm Formation, Psl Exopolysaccharide Expression, and Clinical Outcomes in *Pseudomonas aeruginosa* Keratitis. *JAMA Ophthalmology*, *134*(4), 383. http://doi.org/10.1001/jamaophthalmol.2015.5956

Zeng, X., & Lin, J. (2013). Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. *Frontiers in Microbiology*, *4*(MAY), 1–9. http://doi.org/10.3389/fmicb.2013.00128

Zgurskaya, H. I., Weeks, J. W., Ntreh, A. T., Nickels, L. M., & Wolloscheck, D. (2015). Mechanism of coupling drug transport reactions located in two different membranes. *Frontiers in Microbiology*, *6*(FEB), 1–13. http://doi.org/10.3389/fmicb.2015.00100

Zhao, J., Chen, Z., Chen, S., Deng, Y., Liu, Y., Tian, W., … Liu, J. H. (2010). Prevalence and dissemination of oqxAB in Escherichia coli isolates from animals, farmworkers, and the environment. *Antimicrobial Agents and Chemotherapy*, *54*(10), 4219–4224. http://doi.org/10.1128/AAC.00139-10

Zhao, S., Qaiyumi, S., Friedman, S., Singh, R., Foley, S. L., White, G., … White, D. G. (2003). Characterization of Salmonella enterica Serotype Newport Isolated from Humans and Food Animals Characterization of Salmonella enterica Serotype Newport Isolated from Humans and Food Animals. *Jornal of Clinical Microbiology*, *41*(12), 5366–5371. http://doi.org/10.1128/JCM.41.12.5366

Zhi, C., Lv, L., Yu, L. F., Doi, Y., & Liu, J. H. (2016a). Dissemination of the mcr-1

colistin resistance gene. *The Lancet Infectious Diseases*, *16*(3), 292–293. http://doi.org/10.1016/S1473-3099(16)00063-3

Zhi, C., Lv, L., Yu, L. F., Doi, Y., & Liu, J. H. (2016b). Dissemination of the mcr-1 colistin resistance gene. *The Lancet Infectious Diseases*, *16*(3), 292–293. http://doi.org/10.1016/S1473-3099(16)00063-3

Zilahi, G., Artigas, A., & Martin-Loeches, I. (2016). What's new in multidrug-resistant pathogens in the ICU? *Annals of Intensive Care*, *6*(1), 96. http://doi.org/10.1186/s13613-016-0199-4

Zurfluh, K. (2016). Escherichia coli Isolates of Different Origins, *60*(9), 5589–5591. http://doi.org/10.1128/AAC.00935-16.Address

Website References

Alere 2017a, Alere Technologies. *Accessed 20 November 2016*. https://alere-technologies.com/products/lab-solutions/platforms/arraytube-at.html

Alere 2017b, Alere Technologies. *Accessed 20 November 2016*. https://alere-technologies.com/en/products/lab-solutions/assay-principles/isothermal-amplification-labelling.html

BSAC 2015, British Society for Antimicrobial Chemotherapy. Methods for Antimicrobial Susceptibility Testing. *Accessed 20 November 2017. http://bsacorguk/wp-content/uploads/2012/02/BSAC-disc-susceptibility-testing-method-Jan-2015pdf*

BSAVA 2017a, British Small Animal Veterinary Association Resources, Medicine Guide, Prescribing cascade. 2017. *accessed 20 November 2017.* https://www.bsava.com/Resources/Veterinary-resources/Medicines-Guide/Prescribing-cascade

BSAVA 2017b, The BSAVA British Small Animal Veterinary Association, Resources, PROTECT. 2017. *accessed 20 November 2017*

https://www.bsava.com/Resources/Veterinary-resources/PROTECT

BVA 2017, British Veterinary Association, Medicine Guide, Good Practice Guide. 2017. *accessed 20 November 2017.* https://www.bva.co.uk/Workplace-guidance/Medicines/veterinary-medicines-good-practice-guide/

CARD 2017, CARD Comprehensive Antibiotic Resistance Database. *Accessed July 2015*. http://arpcard.mcmaster.ca

CDC 2013, Centre for Disease Control and Prevention, 2013, Antibiotic Resistance Threats in the United States 2013, US Department of Health and Human Services, *accessed 16 November 2017*. http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf#page=69

DANMAP 2015, Danish Integrated Antimicrobial Resistance Monitoring and Research Programme. July 2015. *accessed 20 November 2017.* http://www.danmap.org/About%20Danmap.aspx

eBurst 2015, eBURST Algorithm. *Accessed July 2015*. http://eburst.mlst.net

EMA 2016a, European Medicines Agency, Sales of veterinary antimicrobial agents in 29 European Countries in 2014. *accessed 20 November 2017.* http://www.ema.europa.eu/docs/en_GB/document_library/Report/2016/10/WC500214217.pdf

EMA 2016b, European Medicines Agency, Updated advice on the use of colistin products in animals within the European Union: development of resistance and possible impact on human and animal health July 2016. *accessed 20 November 2017.* http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/07/WC500211080.pdf

EUCAST 2015, European Committee for Antimicrobial Sensitivity Testing Guidelines 2015. *accessed 20 November 2017* http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_5.0_Breakpoint_Table_01.pdf

FDA 2017, US Food and Drug Administration. 2010 Summary Report. Antimicrobials Sold or Distributed for Use in Food Producing Animals. 28 October 2011. *Accessed 26 November 2017*. https://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM277657.pdf

Figtree 2015, Molecularevolution.org. *Accessed 23 July 2015*. http://www.molecularevolution.org/software/phylogenetics/figtree

GitHub 2015, GitHub. *Accessed 23 July 2015*. https://github.com/ekg/vcflib

GOV 2014 Government UK; UK Veterinary Antimicrobial Resistance and Sales Surveillance Report 2014. *accessed 12 January 2018.* https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/477788/Optimised_version_-_VARSS_Report_2014__Sales___Resistance_.pdf

MAST group 2017, The Importance and Detection of AmpC and ESBL Enzymes. *accessed 20 November 2017.* http://www.mastgrp.com/%5CIdentification%20strips%20discs%20and%20rings%5CReferences%5CLivermore%20poster%20v2.0.pdf

Nanodrop 2017a, Product User Guide. Thermofisher Scientific. *Accessed 20 November 2016*. http://www.nanodrop.com/library/nd-1000-v3.7-users-manual-8.5x11.pdf

Nanodrop 2017b, Nanodrop Nucleic Acid Purity. Thermofisher Scientific. *Accessed 20 November 2016*. http://www.nanodrop.com/Library/T009-NanoDrop%201000-&-NanoDrop%208000-Nucleic-Acid-Purity-Ratios.pdf

PFMA 2017a, Pet Food Manufacturers Association. Pet Population 2017 . *accessed 26 November 2017.* https://www.pfma.org.uk/pet-population-2017

Pseudomonas Genome Database 2017, *P.aeruginosa* (PA01) genome sequence. *accessed 20 November 2016.* http://beta.pseudomonas.com

RUMA 2015 Responsible Use of Medicines in Agriculture Alliance (RUMA); RUMA announces voluntary restrictions on colistin use in UK livestock. *accessed 12 January 2018.* http://www.ruma.org.uk/ruma-announces-voluntary-restrictions-on-colistin-use-in-uk-livestock/

SAVSNET Small Animal Veterinary Surveillance Network, British Small Animal Veterinary Association (BSAVA) and the University of Liverpool. *accessed 19 November 2017.* http://www.savsnet.co.uk

Source BioScience Sanger Sequencing, 2017. Source BioScience Nottingham UK. 2017 *accessed 19 November 2015.* http://www.lifesciences.sourcebioscience.com/genomic-services/sanger-sequencing-service/

UKGov 2017, GOV.UK – Guidance – Gram-Negative bacteria: prevention, surveillance and epidemiology. 01 August 2017. *accessed 20 February 2016*. https://www.gov.uk/guidance/gram-negative-bacteria-prevention-surveillance-and-epidemiology

WHO 2017, Publication; Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. The World Health Organisation, February 2017. *accessed 20 November 2017.* http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/

WHO CIA 2017, Publication; Critically Important Antibiotics for Human Medicine.

5th edition. The World Health Organisation, February 2017. *accessed 21 December 2017*
http://www.who.int/foodsafety/publications/antimicrobials-fifth/en/

WHO Feb 2017, Publication: Global Priority list of antibiotic-resistant bacteria to guide research, discovery and development of new antibiotics. The World Health Organisation, February 2017. *accessed 22 December 2017*
http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf