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Antimicrobial resistance in clinical isolates of
Pseudomonas aeruginosa

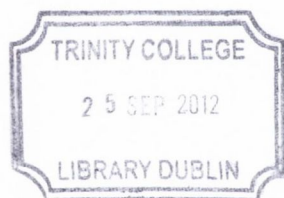
A dissertation presented for degree of Doctor of Philosophy, in the Faculty of Health
Sciences, Trinity College Dublin

By

Sarah Ellen Molan

2011

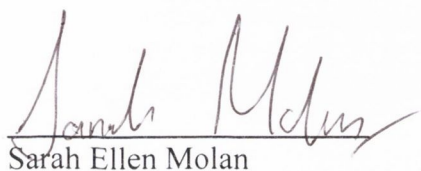
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Declaration

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A handwritten signature in dark ink, appearing to read 'Sarah Molan', is written over a horizontal line.

Sarah Ellen Molan

Summary

Pseudomonas aeruginosa is one of the most feared Gram-negative bacterial pathogens. It is not only among the most frequently isolated Gram-negative organisms in bloodstream and wound infections, pneumonia, intra-abdominal-sepsis and urogenital-sepsis but it is also a major cause of bacterial complication leading to chronic colonisation and acute respiratory distress in cystic fibrosis patients. The increasing prevalence and spread of multidrug resistant strains threatens the efficacy of currently available antimicrobials.

Agar dilution or disc diffusion test methods were employed to determine the susceptibilities of 194 non-cystic fibrosis (NCF) and 99 cystic fibrosis (CF) *P. aeruginosa* isolates, according to Clinical and Laboratory Standard Institute (CLSI) and The British Society for Antimicrobial Chemotherapy (BSAC) guidelines. Subsequently, 27, 102 and 65 NCF and 0, twelve and 8y seven CF isolates were categorised as antimicrobial susceptible, resistant and MDR, respectively. Higher rates of resistance were detected in the CF group compared to the NCF group for all antimicrobials tested. In contrast to emerging worldwide trends, analysis of the molecular mechanisms of resistance, by PCR amplification and sequencing, revealed a low prevalence of acquired resistance genes, aminoglycoside modifying enzymes: $n=3$, bla_{VIM-2} $n=6$.

Quantitative real-time PCR revealed the over expression of efflux pump genes in 60% of NCF and 78% of CF isolates examined. There was also elevated expression of the chromosomal β -lactamase gene, *ampC* in 50% of NCF and 30% of CF isolates examined. Reduced expression of the outer member protein gene *oprD*, indicating loss of OprD, was seen in 53% NCF and 56% of CF isolates examined. A total of six clonal PFGE clusters were detected among the NCF and CF isolates, three separate cluster groups in each.

NCF and CF isolates which were categorised as very multi-drug resistant (vMDR) were investigated to determine their mutation rate. Twelve CF isolates were categorised as hypermutators, these results compare favourably with other studies. Data presented in this thesis found an equal proportion of hypermutators were mucoid or non-mucoid which suggests that both are capable of developing a hypermutator phenotype in the CF lung. A total of 67% of the hypermutator isolates had at least one mutation in the mismatch repair genes examined.

Colistin is the last line drug available for the treatment of MDR *P. aeruginosa* and has been increasingly used to treat serious infection in CF patients. Not surprisingly, a greater proportion of CF isolates were resistant (28%) to colistin compared to NCF isolates (12%). A total of 9% of the colistin resistant NCF isolates were vMDR, compared to the CF collection, where 21% of the isolates were vMDR and 7% were hypermutators. Susceptibility testing methods which are frequently used in clinical laboratories were compared to agar dilution. Etest and disc diffusion were comparable to agar dilution results in the NCF *P. aeruginosa* isolates compared to CF isolates. However, there were many NCF isolates which were defined as susceptible according to the disc diffusion methods which were resistant according to the agar dilution method. Since many busy clinical laboratories rely on disc or Etest methods to detect colistin susceptibility levels in *P. aeruginosa* isolates, there is potential for colistin resistance to go undetected in Irish hospitals.

In conclusion, the prevalence of antimicrobial resistance in *P. aeruginosa* as documented in this thesis highlights the need for continued surveillance, hospital infection control and antimicrobial stewardship in order to minimise the consequences of resistance for public health. This is the first study which examines the antimicrobial susceptibility profiles of *P. aeruginosa* isolates from two distinct groups in Ireland.

Presentations

ORAL PRESENTATIONS

Molan S.E. (2009) Antimicrobial Resistance Mechanisms in Clinical Isolates of *Pseudomonas aeruginosa*. School of Medicine Post graduate research Day 2009.

Molan S.E. (2008) Resistance Mechanisms of multiresistant *Pseudomonas aeruginosa* isolates. RAMI Biomedical Sciences Section Annual Meeting 2008

POSTERS PRESENTED

Molan S.E. (2008) Antimicrobial Resistance Mechanisms in Clinical Isolates of *Pseudomonas aeruginosa*. ECCMID Conference Barcelona 2008.

Molan S.E. (2007) Elucidation of Antibiotic Resistance Levels in *Pseudomonas aeruginosa*. SGM Conference Manchester 2007.

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List of Abbreviations

Abbreviation	Full Name
API	analytical profile index
ATCC	American type culture collection
bp	base-pair
CFU/ml	colony forming units per ml
CF	cystic fibrosis
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HPSC	high purity salt free
kbp	kilo base-pair
kDa	kilodalton
LPS	lipopolysaccharide
MBP	maltose-binding protein
M β L	Metallo- β -lactamase
MOPS	3-(N-Morpholino) propanesulfonic acid
MMR	Mismatch repair
NCTC	national collection of type cultures
NCF	non-cystic fibrosis
OD	optical density
PAI	pathogenicity-associated island
PBS	phosphate-buffered saline
PBPs	penicillin-binding proteins
PFU/ml	plaque forming units per ml
psi	pound-force per square inch
SJH	St. James's Hospital
SVH	St. Vincent's Hospital
SSC	saline-sodium citrate
SSPE	saline-sodium phosphate-ethylenediaminetetraacetic acid
TBE	tris-borate-EDTA buffer
QS	Quorum sensing
QRDR	quinolone resistance determining regions
qRT-PCR	Quantitative real time polymerase chain reaction
vMDR	very multi-drug resistant

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Chapter 1 General Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic rod belonging to the bacterial family *Pseudomonadaceae*. Since the revisionist taxonomy based on conserved macromolecules (e.g. 16S ribosomal RNA), the family consists only of members of the genus *Pseudomonas*, which are cleaved into 8 groups. Those species associated with human infections include; *P. aeruginosa*, *P. fluorescens*, *P. putida* and *P. stutzeri*. Because of its close association with serious human infection *P. aeruginosa* is considered the most important of the pseudomonads ^{1 623}, *Pseudomonas aeruginosa* and *P. maltophilia* account for approximately 80% of the pseudomonads recovered from clinical specimens. It can cause a variety of diseases as outlined in Table 1.1.

P. aeruginosa is an opportunistic pathogen capable of causing both local and systemic infection, the difference in the severity of infection is ultimately due to the altered host defences. It is not generally part of human microbial flora, rates of colonisation range from 0-6% for the throat, 0-3.3% for nasal mucosa ². However, rates of colonisation during hospitalisation can exceed 50%, especially among patients with an impaired immune system or patients where antimicrobial therapy has disrupted normal microbial flora ². *Pseudomonas aeruginosa* is capable of infecting virtually any tissue ³; however, infection is often facilitated by the presence of underlying disease (e.g. cystic fibrosis) or the breakdown in non-specific host defences. Localised infections can occur following surgery or in burns, often resulting in serious bacteraemia. A review of surveillance data from by the CDC found that *P. aeruginosa* was responsible for 9% of all hospital-acquired infections in the US ⁴. A study of European intensive care units found that *P. aeruginosa* was responsible for the highest burden of healthcare-associated infections ⁵. Data from the National Nosocomial Infections Surveillance System found that *P. aeruginosa* was responsible for 3% of bloodstream infections, 10% of urinary tract infections, 2% of pneumonias in the US ⁶. A similar European study found that *P. aeruginosa* was responsible for 10% of bloodstream infections, 19% of urinary tract infections and 30% of pneumonias ⁷.

Table 1.1 Diverse sites of *P. aeruginosa* infection

Site	Infection
Brain	CNS infection
Ear	Localised infection
Eye	Localised infection
Blood	Bacteremia
Heart	Endocarditis
Kidney/bladder	Urinary tract infection
Lung	Respiratory tract chronic infection Acute pneumonia
Skin/musculoskeletal tissues	Burn wounds, surgical wounds

Most *P. aeruginosa* strains are known to produce two types of soluble pigments, pyoverdinin and pyocyanin. Pyoverdinin is a siderophore, a small iron chelator scavenging iron from the environment to be used in *P. aeruginosa* metabolism. Pyocyanin is a blue pigment metabolite of *P. aeruginosa*, it has been shown to have numerous pathogenic effects such as increasing IL-8, depressing host responses and induced apoptosis in cells⁸. In animal models, pyocyanin was shown to be essential to *P. aeruginosa* virulence⁹. Pyoverdine has been shown to play a role in regulation of virulence factors¹⁰.

Three colony types exist for *P. aeruginosa*; environmental strains isolated from water and soil usually have small, rough colony morphology. Clinical strains usually display one of two colony types; the first has a smooth, fried egg appearance, the colonies are large and smooth with flat edges and an elevated appearance. The second is a mucoid colony type, which is attributed to alginate production, Fig 1.1.¹¹

Pseudomonas aeruginosa is an ubiquitous organism found in many diverse settings. It can be isolated from different living sources including plants, animals and humans². *P. aeruginosa* can survive on a variety of carbon sources for energy, when O₂ is not available it can utilise nitrogen as a terminal electron acceptor under anaerobic conditions¹². This ability to survive on minimal nutritional requirements allows *P. aeruginosa* to survive and persist in the community and the hospital setting². In the community, reservoirs of this organisms include water-based sources such as swimming pools, whirlpools and hot tubs. It can also be found in soil, plants and vegetables^{2,13}. In the hospital setting *P. aeruginosa* is found on respiratory equipment, antiseptics, sinks, mops, medicines and medical equipment¹³ and can be spread from patient –to- patient

on the hands of hospital personnel, by direct patient contact and through contaminated reservoirs and ingested of contaminated water and food ¹.

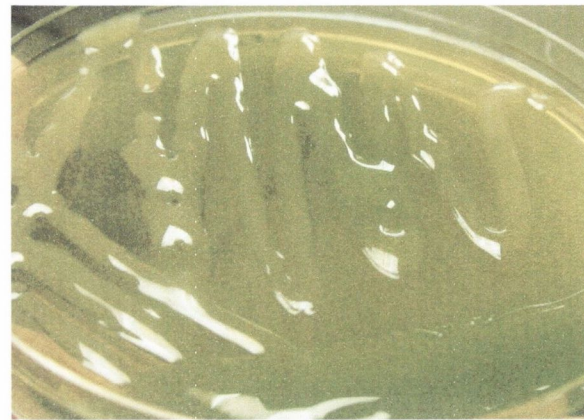
Pseudomonas aeruginosa's ability to survive in a variety of environments is due to its metabolic versatility. Its genome is large and complex. Sequencing studies from the first *P. aeruginosa* isolate sequenced, (PAO1: a strain widely used in research and originally isolated from a wound), in 2000 indicated that this isolate has a genome size of 6.3 Mbp containing 5,570 predicted open reading frames (ORFs) ¹⁴. The large size of *P. aeruginosa* genome reflects the numerous and distinct gene families that it contains. This differs from other large bacterial genomes, whose size reflects gene duplication events rather than greater genetic and functional diversity ¹⁵. Because *P. aeruginosa* is a soil dwelling organism it is likely that it acquired many genes through horizontal transfer, based on the high GC content of many of its genes. The *P. aeruginosa* genome contains a disproportionately large number of genes which are predicted to encode outer membrane proteins involved in adhesion, motility, antimicrobial efflux, virulence factor export and environmental sensing by two component systems. Not surprising the genome contains a large number of genes (8.4% of total) predicted to be involved in gene regulation ¹⁴. Data from comparative genomic analysis of a number of fully sequenced *P. aeruginosa* clinical isolates, led to the conclusion that *P. aeruginosa* genome is composed of two components: a core genome and an accessory genome ¹⁵. The core genome contributes to 90% of the total genome; it encodes a set of metabolic and pathogenic factors shared by all strains, irrespective of origin. In contrast, the accessory genome encompasses genes that are found in some but not all *P. aeruginosa* isolates. These segments tend to cluster in certain loci, termed regions of genomic plasticity ¹⁶. Genetic sequences occupying many regions of genomic plasticity are often referred to as genomic islands (>10 Kb) or islets (<10 Kb), which can possess genetic elements that encode properties that contribute to niche-based adaptation or antimicrobial resistance. The *P. aeruginosa* genome is characterised by a high G +C content (66.6%). Genes acquired from other species (much of the accessory genome) generally have a lower G + C content than that of the core genome. Apart from deletions, rearrangements and mutations within the core genome, the horizontal transfer of components of the accessory genome is primarily contributing to *P. aeruginosa* diversity and evolution. The accessory genome is a source of genes encoding antimicrobial resistance determinates ¹⁷ and virulence factors ¹⁸.

A



Small rough *P. aeruginosa*
colonies

B



Mucoid *P. aeruginosa*

Fig 1.1 Clonal appearance of *Pseudomonas aeruginosa*. **(A)** Environmental *P. aeruginosa*, © Kenneth Taylor www.textbookofbacteriology.net. **(B)** Clinical mucoid *P. aeruginosa* isolate.

As previously discussed *P. aeruginosa* is an opportunistic pathogen, after being acquired from the environment, it colonizes the respiratory epithelium in patients with impaired immune systems or predisposing conditions. The first step towards the establishment of infection is the binding of *P. aeruginosa* to the host epithelial cell. *P. aeruginosa* has numerous virulence factors which aid in its dissemination. Flagella and pili (which are responsible for bacterial motility) act as tethers in the initial binding of *P. aeruginosa* to epithelial cell surface glycolipids. Additionally, lipopolysaccharide plays a similar role in bacterial adhesion through asialo-GMI binding. After contact is made with the host cell, the type III secretion system (TTSS) is activated. This system allows *P. aeruginosa* to inject secreted toxins through a syringe-like apparatus directly into the hosts cytoplasm. Four effector proteins (ExoY, ExoS, ExoT and ExoU) are known to participate, to varying degrees, in the cytotoxicity of *P. aeruginosa* resulting in invasion and dissemination of *P. aeruginosa*. TTSS also secrete other virulence factors into the extracellular space, such as elastase, alkaline phosphatase, exotoxin A and phospholipase C. These virulence factors aid in the invasion process by destroying the protective glycocalyx of the respiratory epithelium and exposing epithelial ligands to *P. aeruginosa*. In acute infections, invasion dissemination and extensive tissue damage predominate⁸. However, in chronic infections *P. aeruginosa* adapts to the host environment by losing its most immunogenic feature such as flagella and pili to avoid clearance. Whether in acute or chronic infections *P. aeruginosa* has a multitude of regulating systems at its disposal which allows it to adapt to its environment and most importantly to the host defences. Quorum sensing (QS) is an example of one of these systems and demonstrates the adaptability of *P. aeruginosa*. QS systems allow *P. aeruginosa* isolates growing in biofilm to communicate and coordinate the production and release of virulence factors⁸.

1.2 Cystic fibrosis

Cystic fibrosis (CF) is the most common inherited lethal single gene genetic disorder of Caucasian populations. Birth prevalence varies from country-to-country, and with ethnic background. For example, the disease occurs in roughly 1 in 3000 white Americans, 1 in 4000-10,000 Latin Americans and 1 in 15,000-20,000 African Americans¹⁹. Cystic fibrosis is uncommon in Africa and Asia, with reported frequency of 1 in 350,000 in Japan¹⁹. Ireland has one of the high incidences of CF in the world with an estimated incidence 3 in every 10,000 with the disease and 1 in 19 individuals

classed as carriers ²⁰. The incidence of CF in Ireland is almost four times the average rate in other EU countries and the United States ²⁰. The outlook for people diagnosed with CF has substantially improved in the last 10-20 years. The life expectancy of a CF patient has increased from thirty one to thirty seven years over the past decade ¹⁹. A study by Dodge *et al* ²¹ predicts that an individual born with CF today will typically live to 50 years of age, thanks to early diagnosis and improved therapies.

Cystic fibrosis is caused by a mutation in a gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein. This protein mainly functions as a chloride channel in epithelial cells. It also has other regulatory functions including inhibition of sodium transport through the epithelial sodium channel, regulation of the outwardly rectifying chloride channel, regulation of ATP channels, regulation of intracellular vesicle transport, acidification of intracellular organelles and inhibition of endogenous calcium-activated chloride channels ²²⁻²⁶.

Over 1500 CFTR mutations have been identified ¹⁹, but only a small portion of the mutant *cfr* genes have been functionally examined, Table 1.2. The absence of phenylalanine at position 508 accounts for two-thirds of mutated alleles in northern Europe and North American populations ¹⁹.

Table 1.2 Mutations found in CFTR^a

Sample mutations	Effect on CFTR
Stop codons, splicing defects with no protein production	Lack of protein production
Phe508del, Asn1303Lys, Gly85Glu, Leu1065Pro, Asp1507, Ser549Arg	Protein trafficking defect with ubiquitination and degradation in endoplasmic, results in substantially reduced CFTR function
Gly551Asp, Ser492Phe, Val520Phe, Arg553Gly, Arg560Thr, Arg560Ser	Defective regulation; CFTR not activated by ATP or cyclic AMP. CFTR not functioning
Ala455Glu, Arg117Cys, Asp1152His, Leu227Arg, Arg334Trp, Arg117His	Reduced chloride transport through CFTR at the apical membrane. Functional CFTR present.
3849+10kb C→T, 1811+1.6kb A→G, IVS8-5T G→A	Splicing defect with reduced production of normal CFTR. Functional CFTR present.

^a Table adapted from ¹⁹

The *cftr* gene is located on the short arm of chromosome 7 and encodes a transmembrane protein termed the cystic fibrosis transmembrane regulator (CFTR). This membrane-bound glycoprotein is a member of the ABC transporter family, the CFTR protein consists of two transmembrane domains, TMD1 and TMD2²⁷. Each of these domains is composed of six membrane spanning alpha helices that make up the core structure of the pore²⁸. As with many proteins belonging to the ABC transporter family, the transmembrane domains are each followed by a nucleotide binding domain. These domains allow for the ATP-dependant gating of the channel²⁹. When the two nucleotide binding domains bind ATP, they form a heterodimer which causes the CFTR channel to open and allow the flow of Cl⁻ down the electrochemical gradient³⁰.

The CFTR channel is believed to be involved in the regulation of many membrane permeability pathways³⁰. The majority of the salt and water transport in the airways involves the down regulation of the epithelial sodium channel (ENaC), which is activated by ATP. ATP release is actively facilitated by the cAMP-dependent Cl⁻ channel³¹⁻³³. ENaC is expressed in the airways and the alveolar epithelium, its channel is responsible for sodium reabsorption which helps maintain a constant depth of the airway surface fluid^{30,34}. The CFTR channel is also involved in the positive regulation of the outward rectifying chloride channel (ORCC)³⁵⁻³⁹. In the absence of the CFTR channel, ENaC activity increases which leads to excessive absorption of sodium, water transport follows the gradient which in combination with the decrease in Cl⁻ secretion from the CFTR and ORCC channels, leads to depletion in the CF airway³⁰. Therefore, as well as affecting its own chloride channel activity, defects in the *cftr* gene lead to an altered cascade of regulation of various ion transporters that causes an overall imbalance in the water content of the airways, Fig 1.2.³⁰

Patients who are homozygous for mutant alleles of the *cftr* gene have major defects in chloride ion transport. This causes a build up sticky dehydrated mucus in male sex ducts, ducts of the pancreas and the airways in the lungs⁴⁰. In a functioning lung, CFTR can act as a receptor for *P. aeruginosa* and normally initiates a co-ordinated and rapid inflammatory response that quickly eliminates the bacteria and returns the tissue to its baseline state⁴¹. The mucus layer traps inhaled foreign particles including bacteria which are then propelled upwards towards the pharynx by cilia (mucociliary escalator) and then either expectorated or swallowed. Inflammation is resolved by induction of apoptosis to restore homeostasis⁴².

In the CF lung, mucous clearance is impaired and over time bacteria become permanent residents of the normally sterile lower airways ⁴³. In response to infection the hosts immune systems excretes substances into the airways, including lysozyme (which is able to hydrolyse the peptidoglycan backbone of the bacterial cell wall), lactoferrin (an iron chelator), phospholipase A₂, proteases, complement and secretory IgA ⁴⁴. It has also been observed that epithelial cells of the airways (and elsewhere) can synthesise and excrete antimicrobial peptides ⁴⁴⁻⁴⁷.

A large number of pro-inflammatory factors travel to the CF bronchial tree including neutrophils, macrophages and inflammatory mediators such as tumour necrosis factor- α (TNF), interleukin-1 (IL-1) and IL-8, ⁴⁴⁻⁴⁷. Recent studies have suggested that one of the major causes of bacterial infection in CF patients is an imbalance between proinflammatory and anti-inflammatory cytokines in the airways ⁴⁸. It has been found that cells which lack a functional CFTR secrete larger amounts of IL-1, IL-8/ keratinocyte chemoattractant (KC), TNF- α and macrophage inflammatory protein MIP-2 after infection with *P. aeruginosa* compared to control cells ⁴⁸⁻⁵³. Currently the mechanisms by which a mutant *cfr* gene effects the enhanced release of proinflammatory cytokines while decreasing the formation of anti-inflammatory cytokines are unknown ⁴⁸. Nevertheless, it does appear that prolonged microbial colonisation and infection, which is characteristic of the CF lung, results from defeats in the innate/ non-specific immune system, Fig 1.3. ⁴⁴.

1.2.1 Clinical manifestations

The severity of symptoms in CF patients varies; Table 1.3 illustrates the approximate age of onset and some of the major clinical complications.

Established understanding of CF infection is that CF patients are generally colonized by pathogens in a sequential manner beginning with *Haemophilus influenzae* and *Staphylococcus aureus* and subsequently followed by *P. aeruginosa* and organisms of the *Burkholderia cenocepacia* complex ^{40,44}, Table 1.4. Other pathogens which have also been found in CF patients who are chronically infected with *P. aeruginosa* include *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Mycobacterium* spp, *Aspergillus fumigatus*, *Pandora* spp, *Ralstonia* spp and a variety of uncommon organisms ⁴¹.

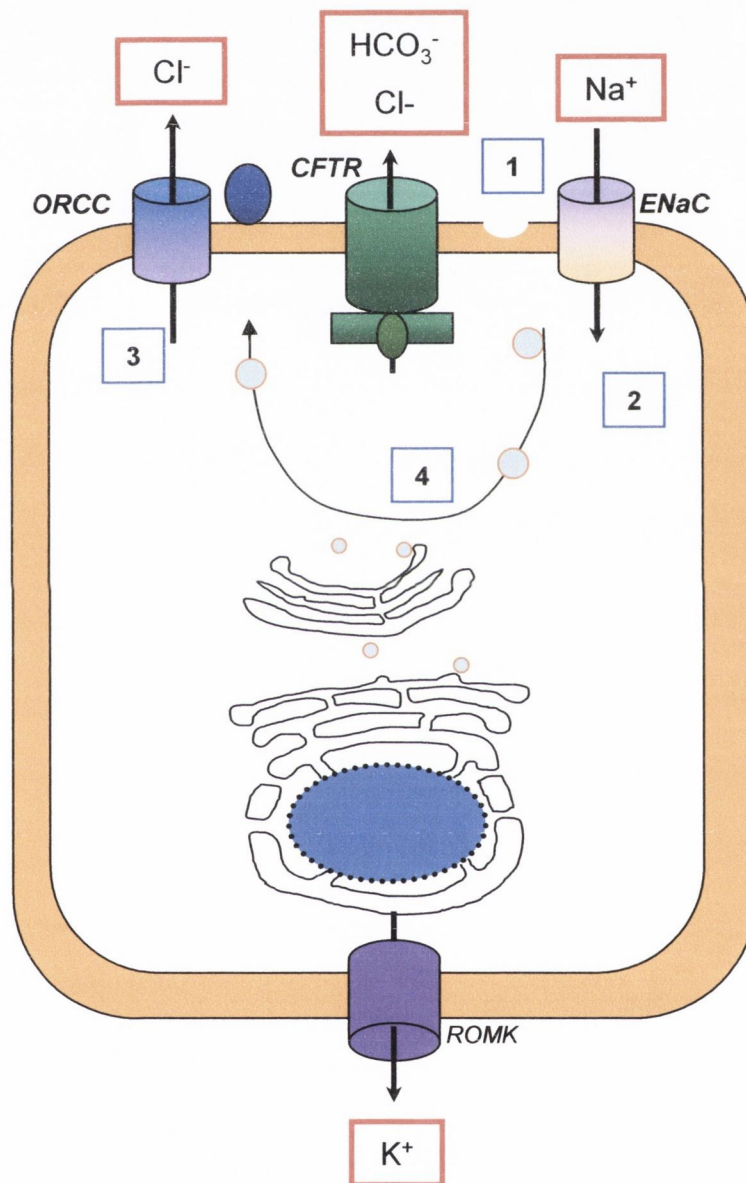


Fig 1.2 Functions and interactions of CFTR. (1) The channel itself acts as a cAMP-regulated Cl^- channel, which allows Cl^- and HCO_3^- to be released and in the process ATP is also released. (2) This in turn down-regulates ENaC activity and positively regulates ORCC activity (3). CFTR is also implicated in the regulation of vesicle trafficking (4), intracellular compartment acidification and protein processing and the modulation of the renal outer medullary potassium channel's (ROMK) sensitivity to sulfonylureas, ³⁰. Diagram adapted from ³⁰.

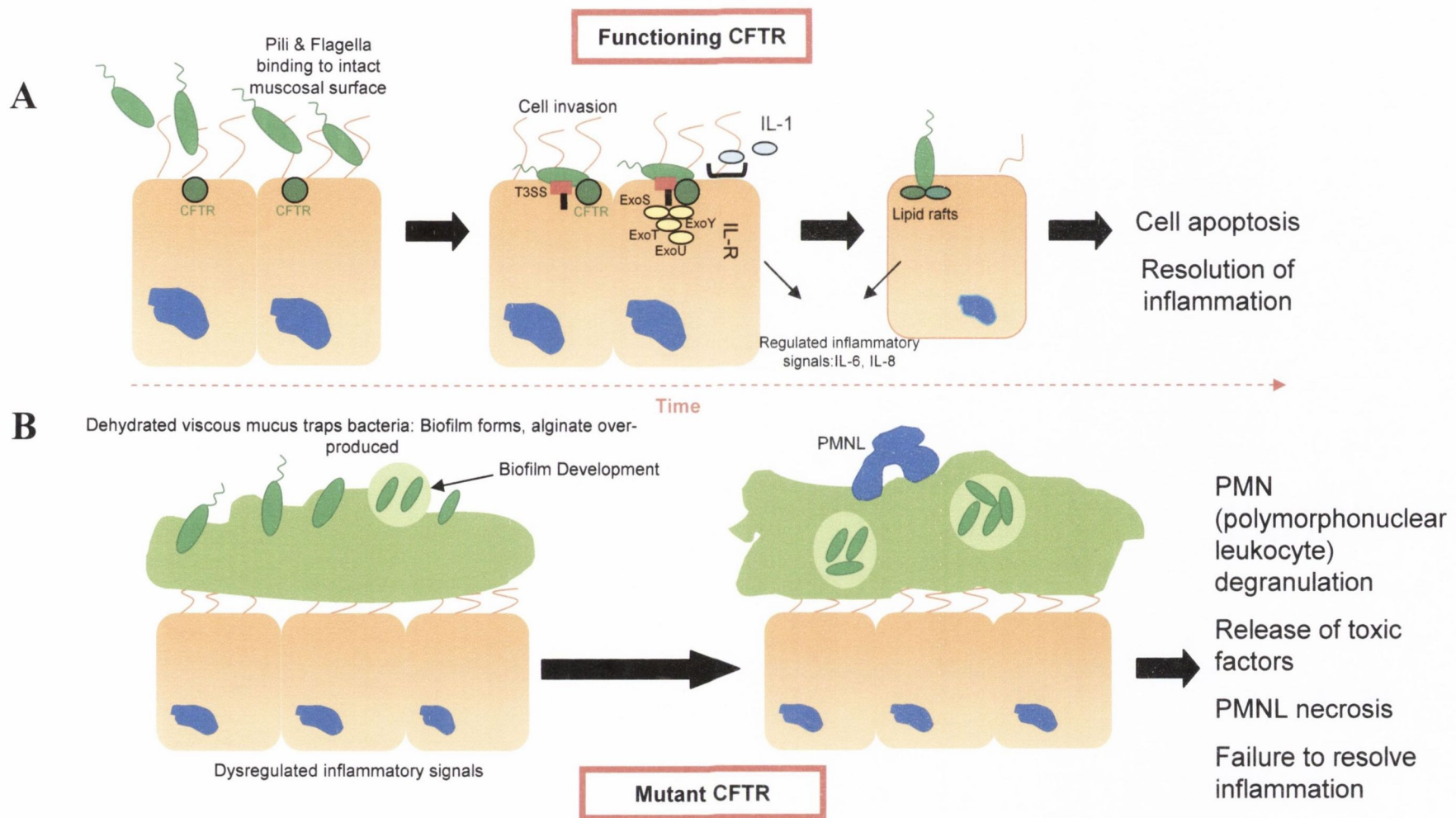


Fig 1.3 Overview of *P. aeruginosa* invasion and epithelial response. **(A)** Wild type CFTR, *P. aeruginosa* binds to epithelial cell via flagella and pili to various structures including asialoGM1, *P. aeruginosa* has a range of virulence factors including flagella, pili and a type 3 secretion systems (T3SS) which allows *P. aeruginosa* to inject effector proteins directly into the host cell cytoplasm (at least four effector proteins have been described ExoS, ExoT, ExoU and ExoY). In the host cell with a functional CFTR a regulated inflammatory response occurs to eliminate the infecting *P. aeruginosa* and to restore tissue homeostasis. **(B)** In the mutant CFTR containing cell, *P. aeruginosa* infects an already inflamed surface with a defective mucociliary elevator. The infecting organisms may or may not be piliated and may already exist in a 'biofilm' state if acquired from another patient. The infection occurs strictly in the mucous layer where the organisms bind to cell debris rather than epithelial surface. The microcolonies elicit a strong immune response but are resistant to the actions of the neutrophils, which may actually damage surrounding tissue in their efforts to eradicate *Pseudomonas*. Diagram adapted from ⁴³ and ⁴¹.

Table 1.3 Symptoms of cystic fibrosis in relation to age^a

Any age	Neonatal	Infancy	Childhood	Adolescence and adulthood
Cough with sputum production	<i>Meconium ileus</i> (thickening of ileum, often first sign of CF)	Failure to thrive	Chronic pansinusitis	Allergic bronchopulmonary aspergillosis
Mucoid <i>P. aeruginosa</i> isolated from the airway	Jaundice	Anasarca or hypoproteinaemia (widespread swelling of the skin due to effusion of fluid into extracellular space)	Steatorrhoea	Chronic pansinusitis or nasal polyposis
Hypochloraemic metabolic alkalosis	Abdominal or scrotal calcifications	Chronic diarrhoea	Rectal prolapse	Bronchiectasis
	Intestinal atresia (malformation of the intestine)	Abdominal distension	Distal intestinal obstruction syndrome	Haemoptysis
		Cholestasis (condition where bile can not flow from liver to duodenum)	Idiopathic recurrent or chronic pancreatitis	Idiopathic recurrent pancreatitis
		<i>S. aureus</i> Pneumonia	Liver disease	Portal hypertension
		Vitamin A deficiency		Delayed puberty
		Vitamin E deficiency causes anaemia		Azoospermia secondary to congenital bilateral absence of vas deferens

^a Adapted from ¹⁹.

Table 1.4 Medically important pseudomonads in CF infection.

rRNA homology group	Species	Infection in CF
I <i>Pseudomonas</i>	<i>P. aeruginosa</i>	Major pathogen
IIa <i>Burkholderia</i>	<i>B. cepacia</i> (genomovars I, III, VI)	Major pathogen
	<i>B. multivorans</i> (formally genomovar II)	Common
	<i>B. stabilis</i> (formally genomovar IV)	Common
	<i>B. vietnamiensis</i> (formally genomovar V)	Common

Before the advent of antimicrobial therapy, most CF patients died in infancy from Staphylococcal infections⁴⁰. Today with improved understanding of the pathogenesis of CF and improved strategies for treating patients the prognosis for newly diagnosed individuals has improved. However, most CF patients will eventually succumb to concurrent pulmonary exacerbations caused by persistent *P. aeruginosa* colonisation, typically over a period of 10-20 years⁴⁰. During the course of life the average CF patient will be exposed to a number of antimicrobials. These antimicrobials can also potentially effect the commensal flora. Indeed it has been suggested that commensal flora may act as a natural reservoir for bacterial resistance, as a higher number of bacteria and species are present. Thereby allowing multiple horizontal gene transfers to pathogens as well as direct infection by commensal bacteria⁵⁴⁻⁵⁵.

1.2.2 Mucoid *P. aeruginosa* and CF

Mucoid forms of *P. aeruginosa* were first associated with chronic pulmonary infection in CF patients in the 1960's⁵⁶⁻⁵⁷. A study by Doggett *et al*⁵⁶⁻⁵⁷ found that 90% of CF patients examined were colonized by mucoid *P. aeruginosa*. Although mucoid *P. aeruginosa* isolates are associated with CF patients, it appears that the mechanisms underlying the emergence and establishment of mucoid isolates are not specific to CF but rather instead reflect the molecular regulation and biological properties of alginate and microbial adaptation to the host environment⁴⁰. Mucoid *P. aeruginosa* isolates arise in up to 40% of non-CF patients with chronic bronchitis⁵⁸. The emergence of mucoid *P. aeruginosa* often correlates with poor patient outcome⁵⁹⁻⁶².

The appearance of mucoid isolates is a result of the excessive production of alginate, which is an oxygen-radical scavenger. It protects *P. aeruginosa* against phagocytosis and clearance from the lungs, the mucoid phenotype is therefore better protected compared to the non-mucoid phenotype against the inflammatory defence mechanisms of the host ⁶³, Table 1.5.

Table 1.5 Features of mucoid and non-mucoid *P. aeruginosa* isolates from the CF lung^a

Features	Phenotype	
	Mucoid	Non-mucoid
Location	Lung, respiratory zone ^b , Conductive zone ^c in sputum	Lung, Conductive zone in sputum
Biofilm <i>in vivo</i>	Yes	Yes
Motility	No	Yes
Multiple antimicrobial resistance	Seldom	Frequent
Responsible for lung tissue damage	Yes	No
Induces antibody response	Yes	Yes

^{a63}, ^b 3000ml: 95% of lung volume, includes bronchioles, alveolar ducts, alveolar sacs, no cilia. ^c 150ml: 5% of lung volume, includes trachea, bronchi, terminal bronchioles, has cilia. Mucous is produced in this zone

Mucoid *P. aeruginosa* biofilms are located in the respiratory zone (3000ml: 95% of lung volume which includes respiratory bronchioles, alveolar ducts and alveolar sacs). This section of the lung has no cilia. Development of bacterial biofilms over time has been intensively studied *in vitro* by confocal scanning laser microscopy ⁶³. The formation of biofilm is believed to develop in a number of stages; initial attachment, formation of microcolonies and biofilm maturation ⁶³, Fig 1.4. Bacteria in biofilm communicate by means of synthesising and reacting to secreted molecules ⁶⁴⁻⁶⁶. The term quorum sensing (QS) refers to the system which allows bacteria to sense when a critical number of bacteria are present within the biofilm and respond by activating certain genes. In *P. aeruginosa*, QS regulates the production of virulence factors such as extracellular enzymes and cellular lysins, which are important for pathogenesis of

infections by functioning as a protective shield against phagocytes ⁶⁷⁻⁶⁹. QS may also influence the development of the biofilm ⁷⁰. It has been shown to determine the tolerance of *P. aeruginosa* biofilms to antimicrobial therapy and to the innate inflammatory response ⁷¹. The connection between QS and biofilms has been named sociomicrobiology ⁷².

Although *in vitro* studies have indicated that alginate may not be essential for biofilm formation ⁷³⁻⁷⁴, laboratory studies of clinical strains have shown that biofilm produced by mucoid alginate producing strains is generally more robust and thicker than that produced by isogenic non-mucoid strains ⁷⁵⁻⁷⁶.

Pseudomonas aeruginosa colonizes the CF lung after a succession of other bacteria, as mentioned in the previous section. Intermittent colonisation by *P. aeruginosa* can also occur early in the life of a CF patient. Infections start with a predominant environmental non-mucoid phenotype, which is speculated to be involved in initial colonisation ⁷⁷. Indeed no natural ecological niche for overtly mucoid forms of *P. aeruginosa* has been found.

Inside the CF lung non-mucoid strains undergo alterations that can give rise to alginate overproducing mucoid variants, frequently due to mutations in the regulatory genes, mainly *mucA* ⁷⁸. This gene encodes the anti-sigma factor MucA ⁷⁹.

It has been observed that during chronic infection both mucoid and non-mucoid phenotypes have been isolated simultaneously from the CF lung ⁸⁰⁻⁸¹, and often these mucoid and non-mucoid strains differ in their antimicrobial susceptibility patterns ⁸². One explanation for this which has been suggested previously, is that during chronic *P. aeruginosa* infection both phenotypes may exist within a biofilm, ⁸²⁻⁸³ where the mucoid alginate overproducing isolates ensure the survival of the biofilm and the non-mucoid isolates may play a role in antimicrobial resistance. The heterogeneity of *P. aeruginosa* phenotypes within the CF lung presents a difficult therapeutic challenge.

1.3 Antimicrobial resistance and *P. aeruginosa*

The early success of antimicrobial therapies in the 1960s, led many to believe that infectious diseases, caused by bacterial pathogens were a thing of the past ⁸⁴. Unfortunately, this is not the case and today infectious diseases remain the second leading cause of death world-wide ⁸⁵. In fact since the earliest use of antimicrobials, resistant bacteria have been observed. A report in 1940 ⁸⁶ described a penicillin-

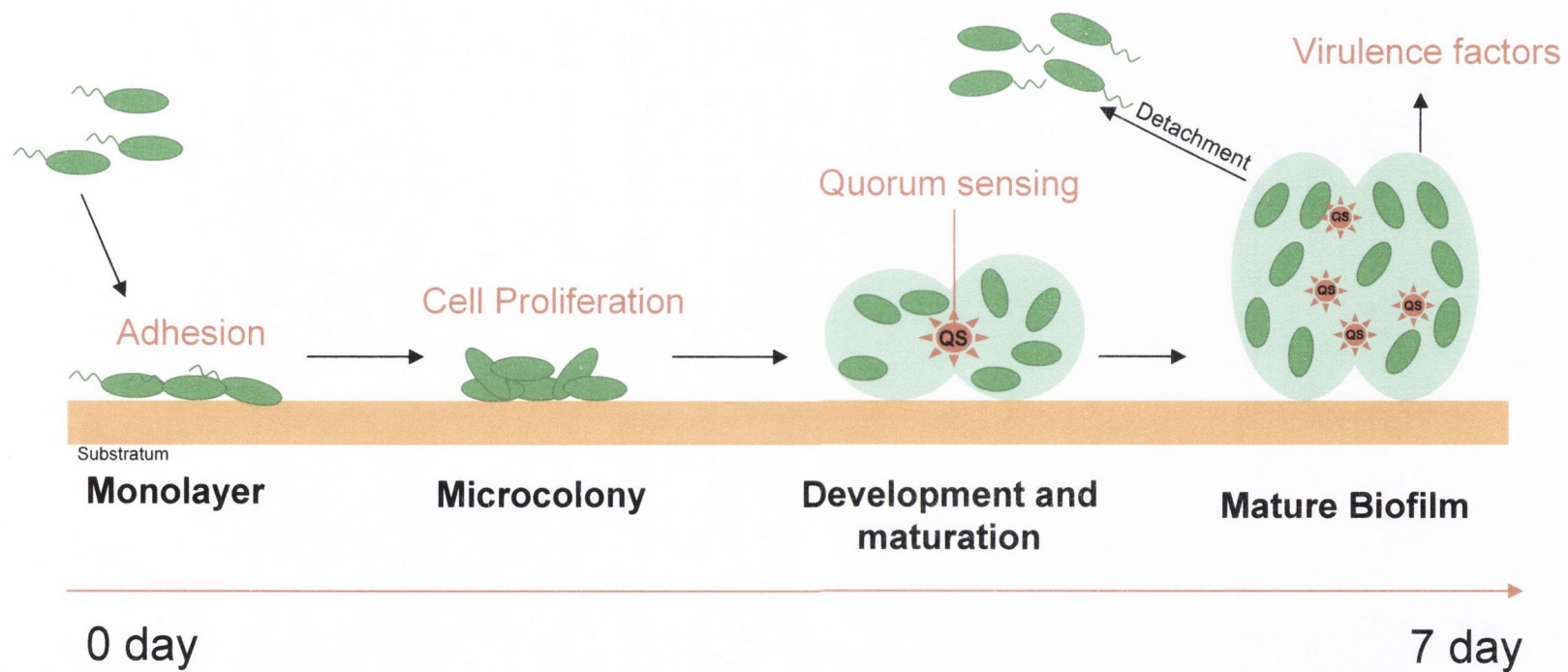


Fig 1.4 Biofilm formation in *P. aeruginosa*. Biofilm development *in vitro* 5-7days. Diagram adapted from ⁶¹.

inactivating enzyme produced by *E. coli*. By the late 1940s around 50% of *S. aureus* isolates were found to produce penicillinase⁸⁷. The emergence of antimicrobial resistant bacteria has been closely linked to the use of antimicrobials⁸⁷ and this is especially true in the hospital environment⁸⁸⁻⁹⁰ where the increased use of antimicrobials acts as a selective pressure on bacteria which drives antimicrobial resistance.

Infections caused by antimicrobial resistant *P. aeruginosa* present a major public health threat and challenge for the treatment of both community-acquired and nosocomial infection^{2,91}. The selection of appropriate antimicrobials to treat such infections is essential in order to optimise the clinical outcome⁹²⁻⁹³. Studies have shown that infections caused by antimicrobial resistant bacteria result in higher mortality, prolonged hospital stays, higher healthcare costs compared to antimicrobial susceptible bacteria^{91,94-95}. The emergence of resistant bacteria during the course of antimicrobial therapy is also a major concern and has been shown to double the length of hospitalisation and overall cost of patient care².

Antimicrobial resistance in *P. aeruginosa* can arise, for example through the acquisition of resistance genes on plasmids, or through mutational changes which alters the expression of chromosomally encoded genes. Mechanisms which increase *P. aeruginosa* resistance severely limit the treatment options available for treatment of serious infection.

1.3.1 Efflux mediated resistance

Multidrug efflux systems have been extensively investigated in *P. aeruginosa* since their discovery in the 1990s⁹⁶⁻⁹⁸. The major role efflux pumps play in multi-drug resistance (MDR) in clinical isolates has been well documented⁹⁹⁻¹⁰⁴. The reduction in the accumulation of antimicrobials in the bacterial cell can be achieved by active export by membrane associated pumps². Efflux pumps are categorised into different families based on their amino acid sequence identity, the energy source required to drive the active export and the substrate specificity of the different pumps¹⁰⁵⁻¹⁰⁶. There are five efflux pumps families which are associated with MDR in *P. aeruginosa*¹⁰⁷, i) the ATP-binding cassette (ABC) family ii) the small multidrug resistance family (a subgroup of the drug metabolite transporter family) iii) the major facilitator super family iv) the resistance-nodulation-division (RND) family and v) the multidrug and toxic compound family². The most clinically important of these families in *P. aeruginosa* is the RND family, 12 RND systems have been identified in *P. aeruginosa*¹⁴. RND efflux systems

are widespread among Gram-negative bacteria and catalyse the active efflux of a wide variety of antimicrobial substrates including many antibiotics and chemotherapeutic agents, Table 1.6.

Table 1.6 RND efflux pumps found in *P. aeruginosa*

Operon ^a	Gene Product	Function ^b	Regulator ^c	Substrate/s	
				Primary	Antimicrobials ^d Additional compounds
<i>mexAB-oprM</i>	MexA	MFP	MexR	FQ, β -L/ β -LI, Tetra, Chlora, Macro, Sulf	Biocides, detergents, dyes, aromatic hydrocarbons
	MexB	RND	NalD		
	OprM	OMF			
<i>mexCD-oprJ</i>	MexC	MFP	NfxB	FQ, β -L, Tetra, Chlora, Macro,	Biocides, detergents, aromatic hydrocarbons
	MexD	RND			
	OprJ	OMF			
<i>mexEF-oprN</i>	MexE	MFP	MexT	FQ, Chlora	Biocides, aromatic hydrocarbons
	MexF	RND			
	OprN	OMF			
<i>mexXY</i>	MexX	MFP	MexZ	FQ, β -L, tetra, Amino, Macro, Chlora	
	MexY	RND			
	OprM/Opm ^e	OMF			
<i>mexJK</i>	MexJ	MFP	MexL	Tetra, erythromycin	Biocides
	MexK	RND			
	OprM/OpmH	OMF			
<i>mexGHI-opmD</i>	MexG		SoxR	FQ	Vanadium
	MexH	MFP			
	MexI	RND			
<i>mexVW</i>	OprmD	OMF			
	MexV	MFP	-	FQ, Tetra, Chlora,	
	MexW	RND			
<i>mexPQ-opmE</i>	OpmE	OMF			
	MexP	MFP	-	FQ, Tetra, Chlora, Macro	
	MexQ	RND			
<i>mexMN</i>	OpmE	OMF			
	MexM	MFP	-	Chlora	
	MexN	RND			
<i>triABC</i>	OprM	OMF			
	TriA	MFP	-		Biocide (Triclosan)
	TriB	MFP			
	TriC	RND			
	OpmH	OMF			

^a Mex is an acronym for multiple efflux, Tri refers to triclosan efflux. ^bMFP: membrane, RND: resistance-nodulation-division transporter, OMF: outer membrane factor. ^c Regulatory proteins that directly control expression of efflux operons, -: not identified. ^d MexXY may also use OpmB, OpmG, OpmH and/ or OmpI as outer membrane factor. ^d FQ: fluoroquinolones, Amino: aminoglycosides, Tetra: tetracyclines, Chlora: chloramphenicol, Macro: macrolides, Sulf: sulfonamides, β -L: β -lactams, β -LI: β -lactam inhibitors. ².

The RND family of efflux pumps are secondary active transporter pumps that gain the energy required for active export of compounds by proton motive force ². A disruption in the proton gradient increases the accumulation of substrates which are normally exported ¹⁰⁸⁻¹⁰⁹. RND transporters exist as a three part system i) periplasmic membrane fusion protein (MFP) ii) an outer membrane factor (OMF) and iii) cytoplasmic membrane (RND) transporter, Fig 1.5. These parts form a tripartite complex channel which spans the entire membrane, thus allowing the export of lipophilic and amphiphilic antimicrobials from the periplasmic and cytoplasm to the extracellular environment ². The genes which encode the efflux pumps are organized into operons in *P. aeruginosa*, Table 1.6.

The ten RND efflux pumps (excluding the metal cation transporters) are listed in Table 1.6. Substrates of these pumps include antimicrobials, biocides, dyes, detergents, organic solvents, aromatic hydrocarbons and homoserine lactones ¹¹⁰⁻¹¹¹.

The role of some of these efflux pumps in pathogenicity has been examined in by Piddock *et al* ¹⁰⁷. The fact that some bacterial efflux pumps export not only antimicrobials but also naturally occurring substances such as dyes, detergents and host derived antimicrobial agents has led to the suggestion that efflux pumps may play a role in the export of naturally occurring substances thereby allowing the bacterium to survive in a particular ecological niche ¹⁰⁷. Indeed it has been shown by a number of studies that the MexAB-OprM efflux pump was essential for the invasion of an epithelial cell ¹¹² and *P. aeruginosa*-induced pneumonia ¹¹³. Recent evidence has suggested that RND efflux pumps do not participate in biofilm resistance to frequently used antipseudomonal agents ¹¹⁴ but may be involved with azithromycin resistance in biofilms ¹¹⁵.

1.3.1.1 MexAB-OprM efflux pump

The MexAB-OprM efflux pump was discovered by Poole *et al* in 1993¹¹¹ and is capable of exporting several different classes of antimicrobials including; fluoroquinolones, tetracyclines, chloramphenicol, β -lactams, β -lactamase inhibitors, macrolides, novobiocin, trimethoprim and sulfonamides^{97,116-119}. MexAB-OprM has the broadest substrate profile for the β -lactam class². It can export carboxypenicillins, aztreonam, extended spectrum cephalosporins (e.g. ceftazidime, cefepime), penicillins (e.g. piperacillin) and carbapenems (meropenem and panipenem but not imipenem or biapenem). The MexAB-OprM efflux pump is constitutively expressed and participates in the intrinsic resistance of *P. aeruginosa*¹²⁰. Knock out studies have confirmed the role of this pump in intrinsic resistance^{97,121-122}. A number of loci influence the expression of the *mexAB-oprM* operon. The *mexR* gene (which is located upstream from *mexAB-oprM*) encodes a repressor protein MexR which binds as a stable homodimer to two different sites within the *mexR-mexA* intergenic region¹²³⁻¹²⁴. NalD is another repressor protein which binds to a sequence upstream of *mexAB-oprM* but downstream of *mexR* binding site¹²⁵. Binding of these repressor proteins represses transcription of the *mexAB-oprM* operon. Mutations within the genes known to regulate *mexAB-oprM* may not be the only mechanism responsible for increased expression of *mexAB-oprM*. For example, it has been shown that during oxidative stress, two residues within MexR are oxidised, which promotes the formation of an intermonomer disulfide bond which alters the formation of MexR¹²⁶⁻¹²⁷, thereby allowing transcription of the *mexAB-oprM* operon.

1.3.1.2 MexCD-OprJ efflux pump

The MexCD-OprJ efflux pump was sequenced and cloned by Poole *et al* in 1996¹²³. This pump can export many different antimicrobial classes including, fluoroquinolones, chloramphenicol, tetracycline, novobiocin, trimethoprim and macrolides^{117,119,122,128}. Unlike MexAB-OprM, the MexCD-OprJ efflux pump does not have a wide β -lactam substrate profile², it exports fourth generation cephalosporins (e.g. cefepime, cefpirome and ceftazopran)¹²²⁻¹²³. This efflux pump is not thought to contribute to intrinsic resistance^{116,121}. The expression of *mexCD-oprJ* is controlled by the gene product of *nfxB*, which is located upstream of *mexCD-oprJ*¹²³. NfxB binds to a site within the *nfxB-mexC* intergenic region thereby negatively regulating expression of

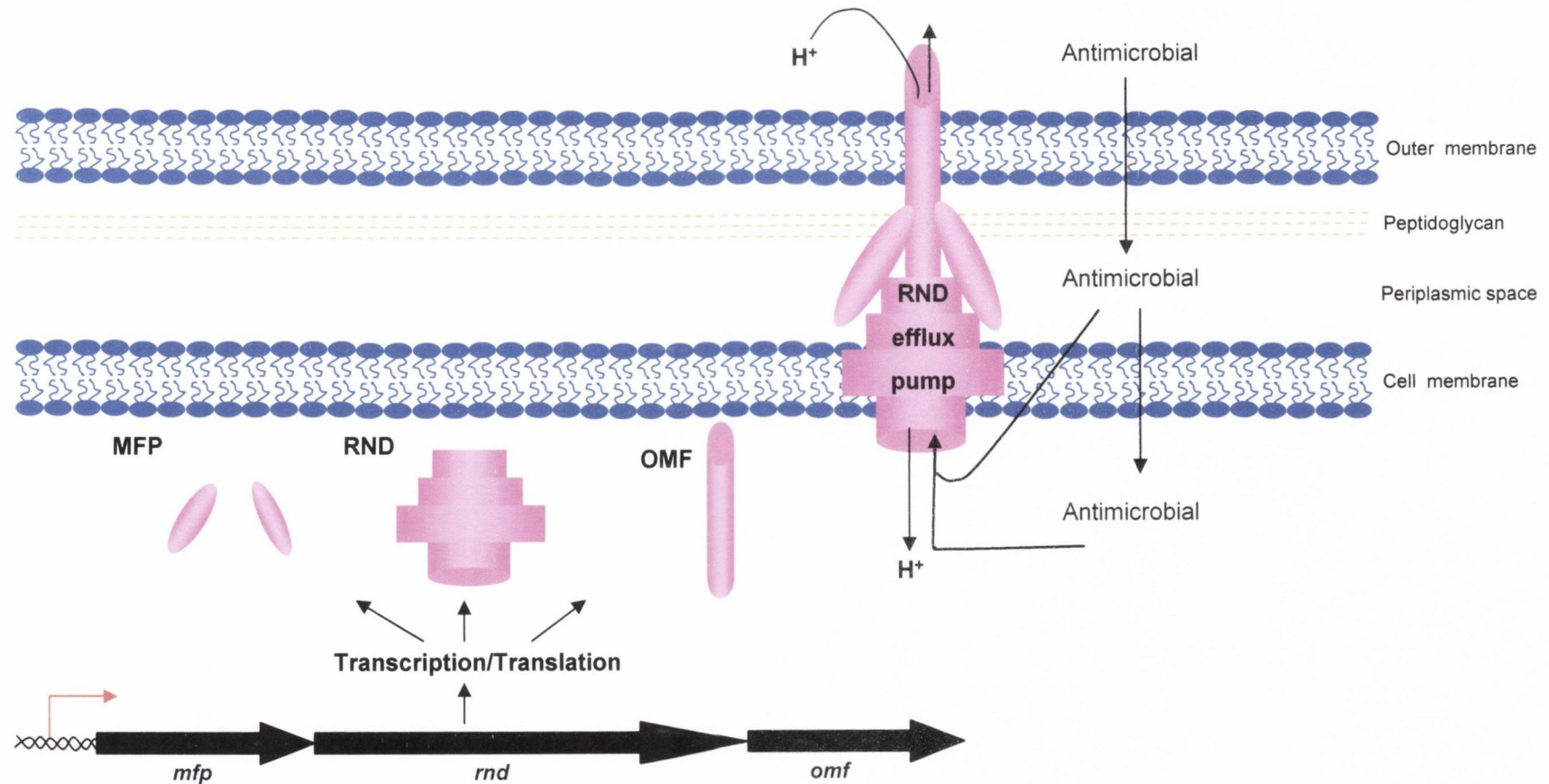


Fig 1.5 Structure of RND efflux pumps in *P. aeruginosa*. RND efflux pumps are typically made up of three parts, the periplasmic fusion protein (MFP), the cytoplasmic membrane (RND) transporter and the outer membrane factor (OMF). These parts form a complex which spans the entire membrane, allowing for proton-driven transport of lipophilic and amphiphilic antimicrobials from the cytoplasm of the cell across the peptidoglycan and across the outer membrane. RND efflux pumps can also extrude antimicrobial from the periplasmic space before they cross the cytoplasmic membrane, adapted from ¹⁷⁰.

mexCD-oprJ as well as its own expression^{123,129}. Mutations within *nfxB* may alter the repressor activity of NfxB leading to the overexpression of *mexCD-oprJ*^{122-123,129}. Regulation of MexCD-OprJ may not be limited to altered functioning of NFXB, in PAO1 deletion of *mexAB-oprM* and *oprM* caused an increase in *mexCD-oprJ* expression which may indicate that MexAB-OprM is somehow involved in the expression of *mexCD-oprJ*¹³⁰, this mechanism has not been fully elucidated.

1.3.1.3 MexEF-OprN efflux pump

MexEF-OprN was characterised by Kohler *et al* in 1997¹³¹. It can export fluoroquinolones, chloramphenicol and trimethoprim unlike MexAB-OprM or MexCD-OprJ it can not export currently available β -lactams¹³¹. The *mexEF-oprN* is expressed at low levels in wild-type *P. aeruginosa*^{2,130}. Like MexEF-OprN, MexCD-OprJ is not involved in intrinsic resistance¹³¹. Multiple factor control the expression of the *mexEF-oprN* operon, in contrast to the previously discussed efflux pumps, it does not have a negative regulator¹³¹.

The *mexT* gene which is located upstream of *mexEF-oprN* is thought to positively regulate *mexEF-oprN* expression¹³²⁻¹³³. However, as the sequence of *mexT* varies in the wild-type *P. aeruginosa* isolates, the activation of MexT is thought to occur through different pathways¹³⁴. Another factor controlling *mexEF-oprN* expression is MvaT which is a member of the histone-like nucleoid structuring protein family which acts as a global regulator of genes involved in virulence, housekeeping and biofilm formation¹³⁵⁻¹³⁷. The regulation of *mexCD-oprJ* appears to involve many complex pathways.

1.3.1.4 MexXY efflux pump

The MexXY efflux pump of *P. aeruginosa* was characterised by Mine *et al* in 1999¹³⁸. MexXY does not have a gene which encodes an outer membrane protein; instead MexXY is able to recruit the outer membrane protein OprM¹³⁸⁻¹⁴⁰ and possibly other outer membrane components such as OmpB, OmpG, OpmH and OpmI to form a functional tripartite system^{139,141}. MexXY is able to export fluoroquinolones, certain, β -lactams (i.e. cefepime), aminoglycosides, tetracycline, chloramphenicol and erythromycin^{110,142}. MexXY is also necessary for adaptive resistance to aminoglycosides¹⁴³. The expression of MexXY can be induced when cells are grown in

the presence of tetracycline, erythromycin and gentamicin ¹⁴⁴. It is believed that MexXY is involved in intrinsic resistance to tetracycline, erythromycin and gentamicin ^{121,144}. However, MexXY does not contribute to intrinsic resistance to fluoroquinolones as these antimicrobials do not induce MexXY expression ¹⁴⁴. The expression of *mexXY* is negatively regulated by the gene product of *mexZ*, which is located upstream of *mexXY* ^{140,145}. Mutations in *mexZ* and *mexZ-mexX* intergenic region have been associated with overexpression of *mexXY* ¹⁴⁶. However, overexpression of *mexXY* has been reported in isolates which do not have any mutations within *mexZ* or the *mexZ-mexX* intergenic region ¹⁴⁷⁻¹⁴⁹.

1.3.1.5 Additional RND efflux pumps

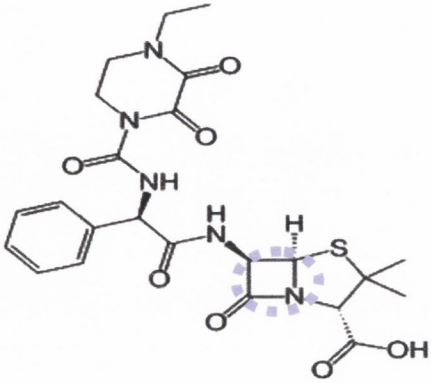
The role of the remaining RND efflux pumps in resistance and the factors which govern their expression are beginning to be elucidated. The MexJK efflux pump was discovered in a *P. aeruginosa* isolate following selection with broad-spectrum biocide triclosan ¹⁵⁰. Like MexXY, MexJK recruits OprM to form a tripartite system ^{139,150}. MexJK is regulated by the gene product *mexL* which is located upstream of *mexJK* ¹⁵⁰.

The contribution of this and other (MexGHI-opmD, MexVW-MexMN, MexPQ-OpmE, TriABC) efflux pumps mentioned in this section to clinical resistance has not yet been fully understood.

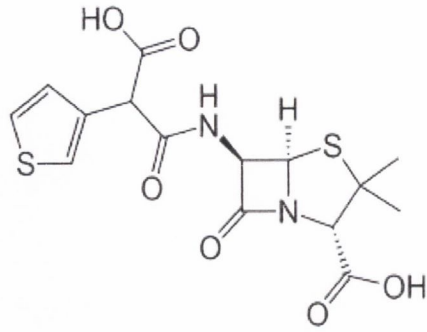
1.3.2 β -lactams : mechanism of action and mode of resistance

β -lactams are a broad-spectrum antimicrobial class. Since the first β -lactam was introduced in 1940, a large number of different β -lactams have been developed, including: penicillins, cepheems (cephalosporins and cephamycins), monobactams, carbapenems and β -lactamase inhibitors. Members of this class of antimicrobial all share a common four-atom structure known as the β -lactam ring, which consists of a lactam (a cyclic amide) with a heteroatomic ring structure consisting of three carbon atoms and a nitrogen atom, Fig 1.6. The β -lactams exert their antimicrobial effect by acting on transpeptidase and carboxypeptidase (so called penicillin binding proteins, PBPs), thereby inhibiting formation of peptidoglycan ¹⁵¹. Peptidoglycan is an essential component of the bacterial cell wall, which maintains the cell shape and structural

A

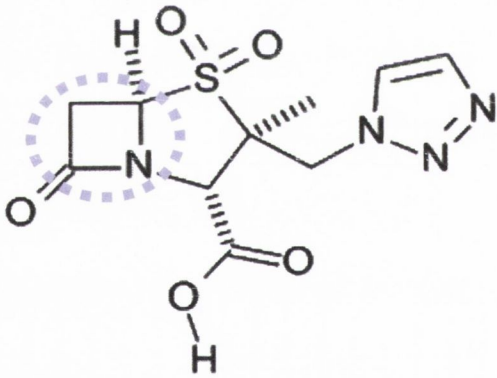


Piperacillin

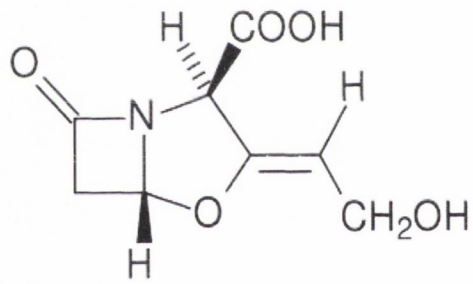


Ticarcillin

B

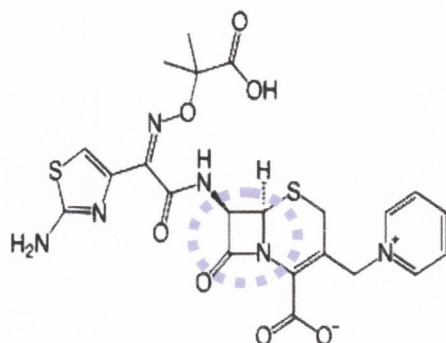


Tazobactam

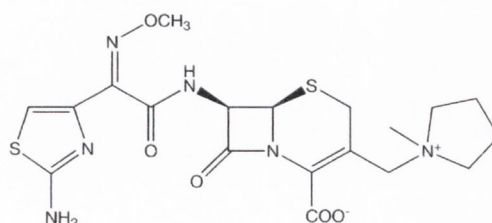


Clavulanic acid

C

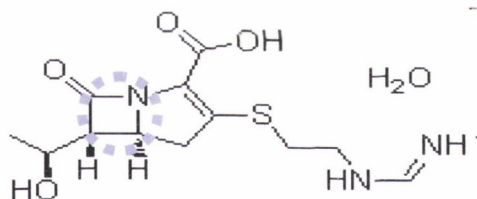


Ceftazidime

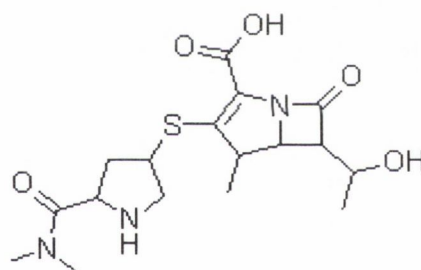


Cefepime

D



Imipenem



Meropenem

Fig 1.6 Chemical structure of antimicrobials from the β -lactam class. **(A)** Members from the penicillin family **(B)** Members from the β -lactamase inhibitor family **(C)** Members from the extended-spectrum and broad spectrum β -lactam family. **(D)** Ceftazidime belongs the third generation cephalosporins while cefepime belongs to the fourth generation cephalosporins. Imipenem and meropenem belong to the carbapenems. Dashed purple circles indicate the characteristic β -lactam ring structure.

integrity. Alterations in the formation of peptidoglycan ultimately leads to cell wall damage. β -lactams are the most widely used antimicrobials in the clinical setting, they account for 60% of all antimicrobial use by weight¹⁵². As a result of their extensive use, resistance to β -lactams is now endemic.

1.3.2.1 Classification of β -lactamases

β -lactamases are enzymes which destroy β -lactams by catalyzing the hydrolysis of the β -lactam ring, thereby inactivating the antimicrobial ability to bind PBPs¹⁵¹. Two classification schemes which are based on molecular and functional properties of the β -lactamases are standardly used. Molecular classification is based on the amino acid sequences. β -lactamases are divided into four classes (A-D)¹⁵³. Classes A, C and D are serine type enzymes, which have a serine moiety at the active site. Class B are enzymes which require divalent cations (usually zinc) as metal co-factors and are therefore called metallo- β -lactamases (M β L). Functional classification is based on substrate and inhibitor profiles of β -lactamases¹⁵³⁻¹⁵⁵. Under this type of classification β -lactamases are divided into a number of groups: Group 1: cephalosporinases (class C), Group 2: broad spectrum, inhibitor resistant and β -lactamases and serine carbapenemases (class A and D), Group 3: metallo- β -lactamases (class B), several subgroups of the major groups have also been described.

Over 800 different β -lactamases belonging to over 30 groups have been described in Gram-negative bacteria¹⁵¹. The genes which encode β -lactamases are generally located either on the chromosome or on a plasmid. At present the spread of β -lactamases in Gram-negative bacteria is mediated by integrons¹⁵¹. Integrons are genetic structures which are capable of capturing and mobilising antibiotic resistance. The majority of β -lactamase genes are carried on class 1 integrons¹⁵⁶⁻¹⁵⁷. Over 120 different β -lactamases belonging to 18 groups have been found in clinical isolates of *P. aeruginosa*¹⁵¹.

The first plasmid-mediated β -lactamase discovered was found in *E. coli* in 1963¹⁵⁸. The majority of TEMs (167 known) are found in *Enterobacteriaceae*. By 1979, TEM-1 and TEM-2 were found in *P. aeruginosa*¹⁵⁹. Subsequently, TEM-4, TEM-21, TEM-24, TEM-42 and TEM-116 were also found in *P. aeruginosa*¹⁵⁹⁻¹⁶³.

SHV is another common β -lactamase, the first SHV was identified in *E. coli*¹⁶⁴. SHV-1, SHV-2, SHV-2a, SHV-5 and SHV-12 have been found in *P. aeruginosa*^{163,165-168}.

Oxacillin-hydrolyzing (OXA-type) β -lactamases are widely distributed among *Acinetobacter* spp and *P. aeruginosa*. To date at least 36 of the known 158 OXA's have been detected in *P. aeruginosa*¹⁵¹. Among the β -lactamases so far identified in *P. aeruginosa* AmpC, ESBLs and M β LS are the most clinically significant.

1.3.2.2 AmpC

AmpC β -lactamases are cephalosporinases (belonging to class C). They contribute to intrinsic resistance to most penicillins, narrow spectrum cephalosporins, β -lactams/ β -lactamase inhibitors^{2,169}. Overproduction of chromosomally-encoded AmpC enzyme in *P. aeruginosa* may be induced by exposure to specific β -lactams and β -lactamase inhibitors (e.g. ceftiofur, imipenem, clavulanate)¹⁷⁰⁻¹⁷⁴. In contrast, AmpC derepression occurs when proteins involved in the induction pathway are compromised through mutational changes¹⁷⁵⁻¹⁷⁹. There is no doubt that AmpC overproduction has a major clinical effect. A study by Tan *et al* reported that patients were 67.5 times more likely to be given inappropriate antimicrobials when the infection was caused by *P. aeruginosa* overproducing AmpC compared to infections caused by *P. aeruginosa* which did not overproduce AmpC¹⁸⁰.

1.3.2.3 Carbapenemases

Carbapenemases are enzymes which hydrolyse imipenem and/ or meropenem. Naturally occurring carbapenemases are chromosomally-encoded, while acquired carbapenemases are integron and plasmid-mediated¹⁸¹. Class B β -lactamases (metallo- β -lactamases, MBLs) have a number of characteristics, i) able to hydrolyse virtually all carbapenems and other β -lactams (aztreonam being the exception), ii) resistant to commercially available β -lactamase inhibitors, iii) susceptible to inhibition by metal ion chelators such as EDTA. Acquired MBLs are generally encoded by transferable genes and the majority of the acquired MBLs have been discovered in *P. aeruginosa*.

IMP-type MBL is one of the acquired MBLs which have been detected in *P. aeruginosa*. The first, IMP-1 was discovered in a *P. aeruginosa* isolate from Japan in

1988¹⁸². To date *bla*_{IMP-1} has been detected in more than 18 species of Gram-negative bacteria¹⁸³, suggesting horizontal transfer of *bla*_{IMP-1} gene has occurred¹⁸⁴. As *bla*_{IMP-1} gene cassette, the IMP-1 gene was inserted in a class 1 integron and mostly co-existed with an aminoglycoside resistance gene¹⁸⁵. The IMP-1 gene has a world-wide distribution¹⁸³. Currently 26 types of IMPs (IMP-1-26) have been assigned and at least 18 types have been identified in *P. aeruginosa*.

Verona integron-encoded metallo-β-lactamase or VIM was first discovered in a strain of *P. aeruginosa* in Verona, Italy in 1997¹⁸⁶. VIM type MβLs are the second most prevalent group of acquired MβL¹⁵¹. Similar to *bla*_{IMP} most of the *bla*_{VIM} genes are located on class 1 integrons. VIM-1 is distantly related to IMP-1 (31.4% identity)¹⁵¹.

SPM-1 (Sao Paulo MβL) was found in a bloodstream isolate of *P. aeruginosa* from Brazil in 2001, as part of the SENTRY Antimicrobial Surveillance Programme in Latin America. Unlike *bla*_{IMP} and *bla*_{VIM}, *bla*_{SPM-1} is not harboured by an integron but is associated with a region containing a novel type of transposable structure with potential recombinase and promoter sequences¹⁵¹. The *bla*_{SPM-1} is located on a plasmid¹⁸⁷.

GIM-1 (German imipenemase) was found in a number of isolates of *P. aeruginosa* in Germany during the SENTRY Antimicrobial Surveillance Study Programme in 2002¹⁸⁸. The *bla*_{GIM-1} gene is harboured on a class 1 integron on a non-transferable plasmid along with three resistance genes, *aacA4*, *aadA1*, *bla*_{OXA-2}¹⁸⁸.

1.3.2.4 Extended-spectrum β-lactamase

Extended-spectrum β-lactamases (ESBLs) are a rapidly growing group of β-lactamases that can hydrolyse broad and narrow spectrum cephalosporins and penicillins¹⁸⁹, they do not hydrolyse cephamycins or carbapenems. Over 200 different ESBLs have been identified in Gram-negative bacteria¹⁹⁰. Of these 32 have been detected in *P. aeruginosa* including, TEM, SHV, CTX-M, PER, VEB, GES, BEL and OXA-type β-lactamases¹⁵¹.

1.3.2.5 Outer membrane pump OprD

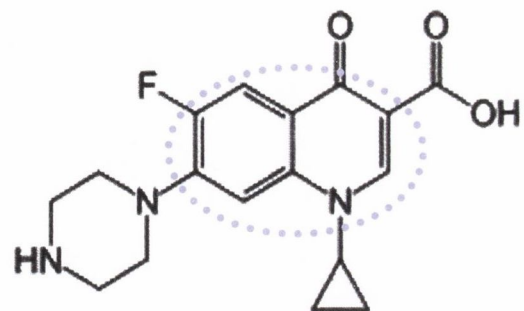
OprD is one of the many outer membrane proteins found in *P. aeruginosa*¹⁹¹. Porins play an important physiological role in the transport of sugars, amino acids, phosphates, divalent cations and siderophores¹⁹¹. Certain hydrophilic antimicrobials such as β -lactams, aminoglycosides and some fluoroquinolones have been shown to transverse the outer membrane through porin channels¹⁹². It is no surprise therefore that the loss of an outer membrane protein can correlate to reduced susceptibility in a bacterial isolate. OprD is the preferred port of entry for carbapenems, and its loss from the outer membrane decreases the susceptibility of *P. aeruginosa* to carbapenems^{133,193-196}. Resistance can involve mechanisms which decrease the transcriptional expression of *oprD* and/or mutations that disrupt the translational production of a functional porin for outer membrane¹⁹⁷⁻¹⁹⁸. Interesting mechanisms which affect the transcription of *oprD* are those that are linked to the regulation of expression of the *mexEF-oprN* efflux pump. The *mexEF-oprN* regulator gene *mexT*, in wild-type *P. aeruginosa* is inactive and this allows *mexEF-oprN* to be expressed at basal levels. In mutants MexT becomes active through a mutation within the structural gene; the activated MexT positively regulates transcription of *mexEF-oprN*. Simultaneously MexT negatively regulates *oprD* at the transcriptional and post-transcriptional level leading to decreased OprD production,^{2,132-133}. This highlights the complexity by which *P. aeruginosa* can regulate resistance mechanisms.

1.3.3 Fluoroquinolones: mechanism of action and mode of resistance

Fluoroquinolones (e.g. ciprofloxacin and levofloxacin) are members of a fully synthetic, broad-spectrum class of antimicrobials which also includes quinolones (e.g. nalidixic acid, oxolinic acid). Their basic active structure is the quinolone ring which is composed of a double ring structure consisting of 8 carbon atoms and two nitrogen atoms, Fig 1.7.

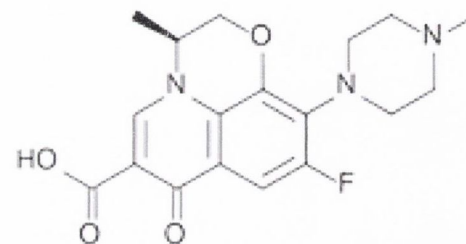
Nalidixic acid is considered the predecessor of all members of the quinolone family. It was introduced into the clinic in 1962¹⁹⁹. The addition of a fluorine at the C-6 position in the 1970s yielded norfloxacin, the first fluoroquinolone, which was first available in 1986²⁰⁰.

A



Ciprofloxacin

B



Levofloxacin

Fig 1.7 Chemical structure of antimicrobials from the fluoroquinolone class. Members of the fluoroquinolone family: **(A)** ciprofloxacin and **(B)** levofloxacin. Dashed purple circle highlights the characteristic quinolone ring structure.

Ciprofloxacin, probably the most used fluoroquinolone ²⁰¹, was introduced into the clinical market in 1987. Since then, structural revisions of the quinolone molecules have provided numerous new agents suited to treatment of a variety of bacterial infections.

Fluoroquinolones enter the bacterial cell through the lipids of the periplasmic and cytoplasmic membranes, Fig 1.8, and subsequently elicit their bactericidal effect by interfering with DNA synthesis ²⁰². Fluoroquinolones target two related but functionally distinct essential bacterial type II topoisomerases, i) DNA gyrase ii) topoisomerase IV ²⁰³. DNA gyrase is composed of two subunits: GyrA (a protein which is encoded by the *gyrA* gene), GyrB (encoded by *gyrB*), these subunits catalyse the negative supercoiling of DNA, a process that is essential in DNA replication, recombination, transcription and repair. Topoisomerase is composed of two subunits; ParC and ParE, which are primarily involved in decatenation, the unlinking of replicated daughter chromosomes. These two enzymes work together to transiently break both strands of double stranded DNA and driven by an ATP-dependant reaction, pass a second DNA double helix through the break, which is then resealed. Fluoroquinolones block the reaction and trap gyrase and topoisomerase IV as a drug-enzyme-DNA complex, with the ultimate release of lethal, double stranded breaks.

Resistance to fluoroquinolones was initially thought to be unlikely for two reasons i) Bacteria would need to spontaneously acquire multiple resistance mutations to survive at clinical concentrations (drug concentrations 1000-fold of those required to inhibit growth are routinely achieved) ii) Many resistance genes have co-evolved in nature with antimicrobials that they counteract, because fluoroquinolones are fully synthetic it seemed unlikely that resistance genes would be available for recruitment onto mobile elements ²⁰⁴. Yet over the last 20 years since the introduction of fluoroquinolones, resistance has become common and widespread and is generally not clonal ²⁰⁴. Fluoroquinolones have become one of the most frequently prescribed antimicrobials worldwide due to their high potency, broad spectrum of activity, good bioavailability and low incidence of side effects ²⁰⁵. They are frequently used to treat a diverse range of bacterial infections both in humans and animals. Due to their extensive use, resistance has inevitably emerged. Depending on the source and geographic location, current *P. aeruginosa* resistance rates to fluoroquinolones ranges from 20 to over 80% in the clinical setting and continues to increase ^{2,206}. Resistance to fluoroquinolones is generally mediated by the accumulation of point mutations in the

quinolone-resistance-determining region (QRDR) of DNA gyrase and topoisomerase IV. The highest levels of resistance are observed in isolates which have mutations in the QRDR of *gyrA* and *parC*²⁰⁷⁻²¹¹. Although mutations have been observed in the other two genes, *gyrB* and *parE*, the frequency of mutations appear to be lower²¹¹.

Four efflux pumps which actively export fluoroquinolones in *P. aeruginosa* have been described, MexAB-OprM, MexXY, MexCD-OprJ and MexEF-OprN^{101,120,123,131,144,212}.

Plasmid-encoded quinolone resistance has also emerged among Gram-negative bacteria²¹³, Table 1.7.

The presence of these genes confers low level fluoroquinolone resistance (ciprofloxacin 0.025 mg/L) but they facilitates the selection of high-level clinically significant fluoroquinolone resistance mutants (i.e. ciprofloxacin MIC 4mg/L) at more than 100 times the frequency of a plasmid-free strain in the presence of quinolones at therapeutic levels.

The geographical distribution of *qnr*-positive strains appears to be increasing world-wide, with the majority of *qnr*'s being detected in the U.S^{96,202,213-216}. As yet *qnr* genes have not been found in *P. aeruginosa*.

Table 1.7 Discovery of *qnr* genes

Date/Location	Organism	Gene	Variants	Homology	Reference
1994, U.S.A.	<i>Klebsiella pneumoniae</i>	<i>qnrA</i>	6		²¹³
2003, India	<i>K. pneumoniae</i>	<i>qnrB</i>	21	43% QnrA	²¹⁷
				44% QnrS	²¹⁸
2003, Japan	<i>Shigella flexeri</i>	<i>qnrS</i>	3	59% QnrA	²¹⁹
2006, China	<i>Proteus mirabilis</i>	<i>qnrC</i>	1	64% QnrA	²²⁰
				41% QnrB	²¹⁸
				59% QnrS	
				43% QnrD	
2009, China	<i>Salmonella enterica</i>	<i>qnrD</i>	1	48% QnrA	²²¹
				61% QnrB	
				32% QnrS	

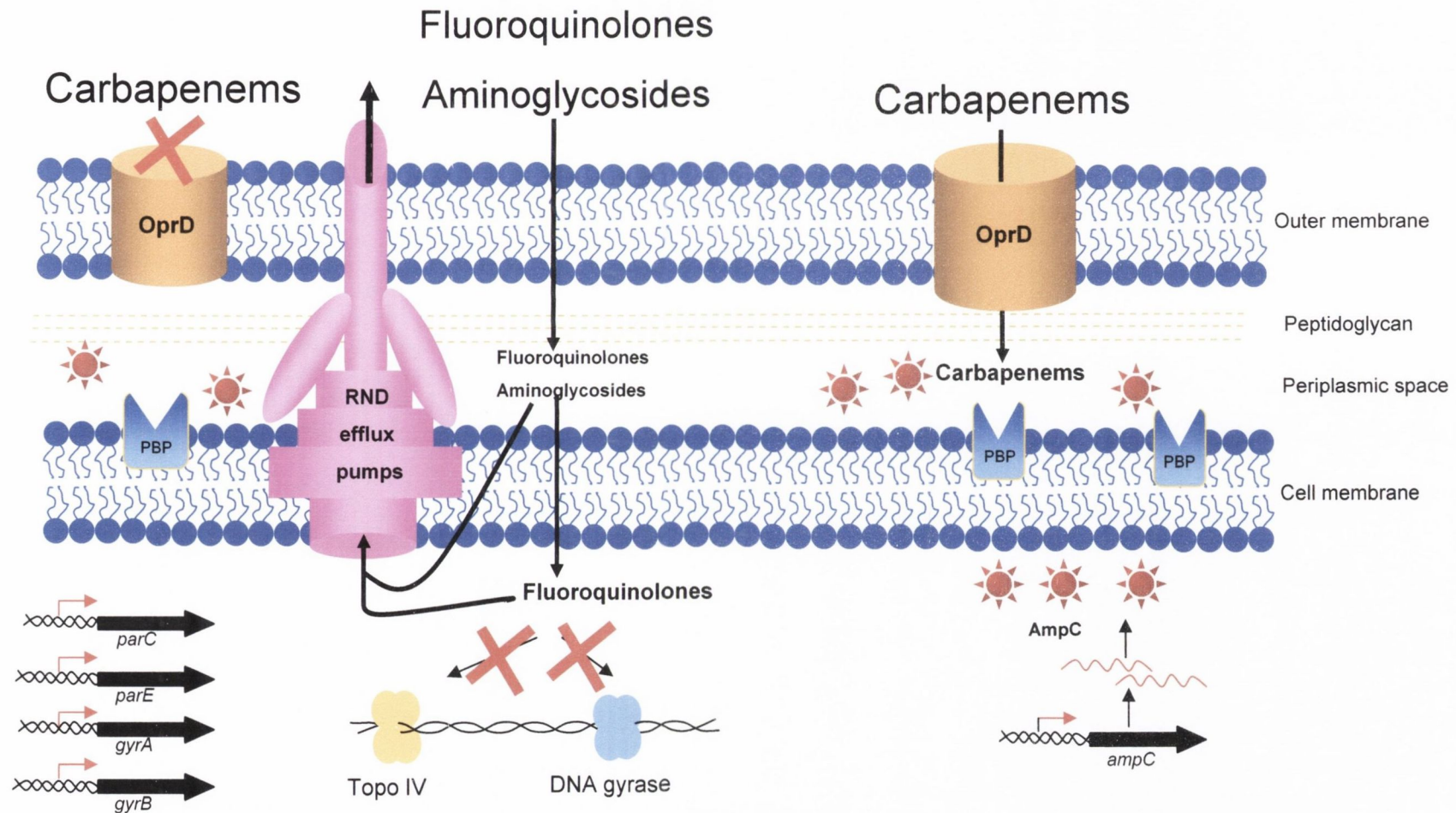


Fig 1.8 Overview of resistance mechanisms to fluoroquinolones, carbapenems and aminoglycosides in *P. aeruginosa*. Fluoroquinolone resistance is mediated by 1) overexpression of RND efflux pumps which pumps the drug from the periplasmic space and/or 2) alterations in the target genes, *gyrA*, *gyrB*, *parC* and *parE*. Aminoglycoside resistance is mediated by overexpression of RND efflux pumps. Carbapenem resistance is mediated mainly by 1) loss of OprD in the outer membrane and/or 2) overproduction of RND efflux pumps (with the exception of imipenem). Small changes in susceptibility can be observed due to the derepression of AmpC, diagram adapted from ¹⁷⁰.

1.3.4. Aminoglycosides: mechanism of action and mode of resistance

Aminoglycosides are among the most widely used broad spectrum antimicrobials used to treat a variety of infections, particularly pulmonary exacerbations in CF patients²²². The vast majority of aminoglycosides are bactericidal, they are often used in combination with other antimicrobials most notably β -lactams. The first aminoglycoside, streptomycin, was isolated from *Streptomyces griseus* in the 1940s²²³. Since then a series of aminoglycosides have been identified and characterised for clinical use, many have been altered by chemists to generate semi-synthetic derivatives with expanded activities²²⁴.

Aminoglycosides are multifunctional hydrophilic sugars that possess several amino and hydroxyl functionalities, Fig 1.9. The amine moieties are mostly protonated in biological media, and can therefore be considered polycationic for the purpose of understanding their biological interactions. Since they are considered polycationic they show a binding affinity for nucleic acids, specifically aminoglycosides have a high affinity for prokaryotic rRNA²²⁵. Aminoglycosides mechanism of action involves the following: i) Ribosomal blockage ii) Misreading in translation iii) Membrane damage iv) Irreversible uptake of the antimicrobial²²⁶. Aminoglycosides bind to the A-site of the 16S rRNA of the 30S subunit of the bacterial ribosomes²²⁷⁻²²⁸ (mammalian cells do not have a 30S subunit). This aminoglycoside-ribosome interaction impairs translational accuracy resulting in misreading of the mRNA sequence and/or premature termination of protein synthesis²²⁶. The resulting in-active proteins interact with the cell membrane causing formation of a leaky membrane and subsequently this enhances the uptake/penetration of the aminoglycosides.

Aminoglycosides are poorly lipid soluble, they pass across the outer membrane of Gram-negatives by a non-energy dependant mechanism which involves a drug induced disruption of bridges between lipopolysaccharide molecules. Transport across the inner membrane is dependant on the transmembrane electrical potential, and therefore is dependant on energy-requiring mechanisms which maintain the transmembrane potential (anaerobic environments and low pH result in a reduction in the transmembrane potential and aid in the effectiveness of aminoglycosides).

Bacterial resistance to aminoglycosides can occur by the following mechanisms; i) enzyme modification ii) low outer membrane permeability iii) active efflux and iv) rarely, target modification²²⁹. Of these resistance mechanisms, inactivation by

aminoglycoside modifying enzymes is considered the most important in terms of the level and the frequency of resistance conferred to the bacterium ²²⁴.

Aminoglycoside modifying enzymes (AME) function by attaching a phosphate, adenyl or acetyl radical to the antibiotic molecule, thereby decreasing the binding affinity of the modified antimicrobial to the target in the bacterial cell ²³⁰. AME are plasmid encoded and are divided into three families; i) Aminoglycoside nucleotidyltransferases (ANTs) ii) Aminoglycoside acetyltransferases (AACs) iii) Aminoglycoside phosphotransferases (APHs). Each family of enzymes consists of isozymic forms that differ in their substrate regiospecificity for their reactions ²²⁴. The most frequently expressed in *P. aeruginosa* are the following: AAC (6')-II (determines resistance to gentamicin, tobramycin and netilmicin), AAC (3)-I (determines resistance to gentamicin), AAC(3)-II (resistance to gentamicin, tobramycin, netilmicin), AAC(6')-I (resistance to tobramycin, netilmicin, amikacin) and ANT (2')-I (resistance to gentamicin and tobramycin) ²³¹.

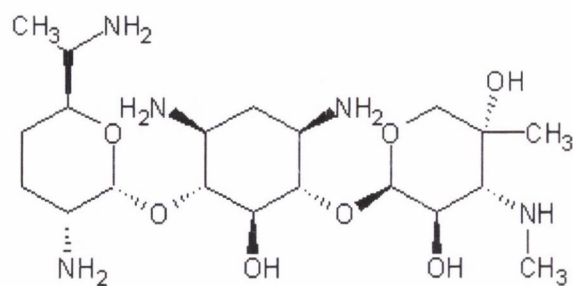
Low membrane impearability is a resistance mechanism that is independent of AME and effects all aminoglycosides ²³². This resistance mechanism is attributed to changes in the outer membrane, which make it less permeable to aminoglycosides ²²⁹. Impermeability is considered a significant cause of aminoglycoside resistance, particularly in CF *P. aeruginosa* isolates ²³³.

Aminoglycosides are also exported by the drug efflux pump MexXY ^{134,146}.

Target modification in the form of methylation of the 16s rRNA has emerged as a new mechanism of aminoglycoside resistance ²³⁴⁻²³⁵. Methylation is mediated by a newly recognised group of 16s rRNA methylases, which share some similarity to those produced by aminoglycoside producing actinomycetes. The genes responsible are located on transposons within transferable plasmids ²²⁹. The first 16s rRNA methylase, RmtA, was reported in Japan in 2003 in a clinical isolate of *P. aeruginosa* ²³⁶. The enzyme was found to confer high-level resistance to all parenterally administered aminoglycosides (including amikacin, tobramycin, isepamicin, kanamycin, gentamicin, with MICs >1024 mg/L). The second 16s rRNA methylase RmtD was identified in Brazil in 2005 in a pan-resistant *P. aeruginosa* ²³⁴.

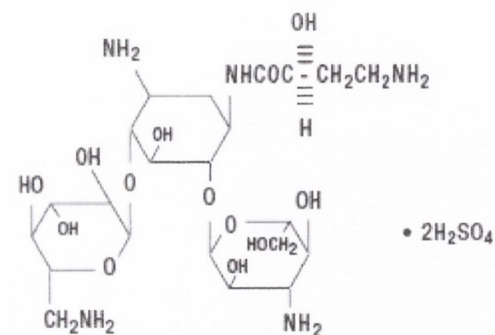
Non-enzymic aminoglycoside mechanisms were examined by El'Garch *et al* ²³⁷. In their study they showed that the accumulation of mutants leads to a gradual increase in resistance to aminoglycosides. They identified four genes in *P. aeruginosa*, *galU*,

A



Gentamicin

B



Amikacin

Fig 1.9 Chemical structure of antimicrobials from the aminoglycoside class. **(A)** gentamicin **(B)** amikacin.

nuoG, *mexZ* and *rplY* which were shown to be involved in the gradual increase in MIC to aminoglycosides²³⁷.

1.3.5 Polymyxins: mechanism of action and mode of resistance

Polymyxins are amphipathic polypeptide antimicrobials, consisting of five different compounds (polymyxin A-E). They were discovered in 1947²³⁸ but only two, polymyxin B and polymyxin E (colistin) have been used in clinical practice²³⁹. They differ by an amino acid; the D-leucine in polymyxin E is substituted by D-phenylalanine in polymyxin B²⁴⁰. Colistin, like the other member of the polymyxin group, is a multicomponent polypeptide antimicrobial, consisting of colistin A (polymyxin E1) and B (polymyxin E2), which differ only in the fatty acid side chain²⁴¹, (Fig 1.10). Colistin was used mainly in the 1950s and 1960s to treat infections caused by Gram-negative bacteria but reports of nephrotoxicity and neurotoxicity and the emergence of newer less toxic antimicrobials, meant that colistin ceased to be prescribed by many clinicians²³⁸. It was not until the late 1990s that clinicians began to re-evaluate colistin as a potential therapy option in the treatment of nosocomial infections such as pneumonia, urinary tract infections, bacteremia, surgical wound infection and meningitis. Caused by MDR bacteria such as *P. aeruginosa*, *Acinetobacter* species, *Klebsiella pneumoniae* and *Enterobacter* species^{239,242}. However, by then, a large gap existed between the years that colistin was used clinically. As a result, data regarding the pharmacokinetic and pharmacodynamics of the drug were and still are limited. The lack of information about the pharmacokinetics and pharmacodynamics of colistin in humans means that appropriate colistin dosing strategies for the different routes of administration have yet to be established. A suboptimal dose of colistin creates the risk of the development of resistance.

Colistin (polymyxin E) is a member of a group of antimicrobials known as polymyxins. Colistin has been available for clinical use since the early 1960s, but its use was curtailed because of fears of nephrotoxicity and the introduction of newer broad spectrum antimicrobials. Polymyxins interact electrostatically with the outer membrane of Gram-negative bacteria, competitively displacing the divalent cation (Mg^{2+}) from the negatively charged phosphate groups of membrane lipids²⁴³. The result is a destabilising effect on the outer membrane, leading to cell leakage and eventual cell

death ²⁴⁴. Resistance to polymyxin is mainly associated with decreased uptake into the bacterial cell which is the result of reduced capacity for initial binding ²⁴⁵ and changes in the lipid composition of the lipopolysaccharide ²⁴⁶⁻²⁴⁸.

1.4 Scope of this thesis

Bacterial resistance is on the increase worldwide, as illustrated in numerous surveillance studies ^{5,249-262}. These studies contribute to our understanding of the scale and prevalence of antimicrobial resistance. Studies like the European Antimicrobial Resistance Surveillance System (EARSS) ²⁶³ and the SENTRY Antimicrobial Surveillance Programme monitor the changing rates of resistance and the emergence and spread of new resistance mechanisms. However, data from Ireland is limited, especially in relation to *P. aeruginosa* and often only blood culture isolates are included in these studies. Very few national studies of *P. aeruginosa* resistance ²⁶⁴⁻²⁶⁷ have examined the molecular mechanisms and rates of resistance among *P. aeruginosa* isolates from CF patients.

The aim of this study was to determine the prevalence of antimicrobial resistance and the mechanisms involved in two distinct populations of *P. aeruginosa* isolates. Since many CF patients acquire *P. aeruginosa* from the environment or the hospital setting, the rate of resistance among the general population was examined, as isolates from this population could act as reservoir of resistant isolates for CF patients.

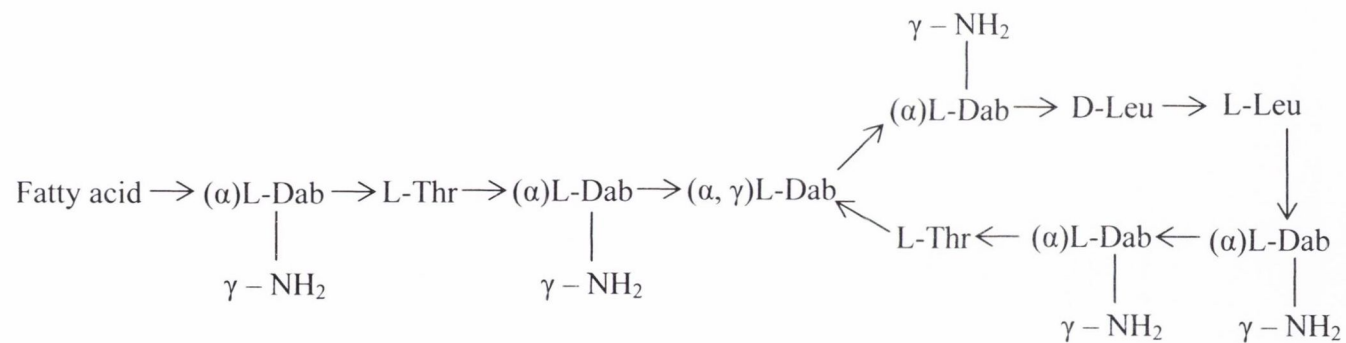


Fig 1.10 Structure of colistin A and B. Fatty acid: 6-methyloctanoic acid for colistin A and 6-methylheptanoic acid for colistin B, Thr: threonine, Leu: leucine, Dab: α , γ -diaminobutyric acid, α and γ indicate the amino group involved in the peptide linking.

Chapter 2 Materials and Methods

2.1 General Methods

2.1.1 Bacterial strains and patient data

2.1.1.1 Culture collection and storage

A total of 194 non-duplicate *Pseudomonas aeruginosa* clinical isolates were collected from the Microbiology Laboratory, St. James's Hospital (SJH) between May 2006 and August 2008 as a prospective study. 99 non-duplicate *P. aeruginosa* clinical isolates from adult cystic fibrosis patients were collected from St. Vincent's University Hospital (SVH) between June 2006 to July 2007.

All *P. aeruginosa* isolates used in this study are listed in Table 2.1. The laboratory control strain *Klebsiella pneumoniae* ATCC 700603 was also employed as a standard in antimicrobial susceptibility testing (see Section 2.2). This isolate encodes a SHV-18 extended-spectrum β -lactamase. Permanent stocks of all strains were maintained in Lennox L broth supplemented with 5% (v/v) glycerol, and stored at -80°C .

2.1.1.2 Identification of *Pseudomonas aeruginosa*

After the isolates were collected they were cultured on Mueller-Hinton (MH) agar plates, incubated at 37°C for 18 to 20 h and examined for purity. Species identification was confirmed biochemically using the API 20NE system (bioMérieux, Marcy L'Etoile, France) according to the manufacturer's instructions. Briefly, the API 20NE system uses 20 individual, miniaturised reaction wells that produce biochemical reactions to ultimately determine the genus and species of non-fastidious Gram-negative rods, not belonging to the *Enterobacteriaceae*. The dried reagent in each well was rehydrated by distributing the pure bacterial saline solution culture into the first 8 tubes, (and not the capsule). The remaining twelve tubes and capsules were filled, taking care to leave a flat or slightly convex but not concave meniscus. Mineral oil was added to three of the tubes (glucose, arginine and urea) to ensure anaerobic conditions. The strip was incubated in a small, plastic humidity chamber for 18 to 24 h at 37°C . After incubation, each well was assessed for a specific colour change indicating the presence

of a metabolic reaction. Most wells change colour due to differences in pH values e.g. amino acid decarboxylation and carbohydrate fermentation reactions. Other wells contain end products that have to be identified using additional reagents including James Reagent and VP1 (potassium hydroxide/water)/VP2 (α -naphthol/ethanol). Interpretation of the 20 reactions was converted to a seven-digit code and cross referenced in a computer database containing all know bacterial species in their database.

Table 2.1 Isolates/DNA used in this study

Strain	Relevant details/Genotype	Source
Isolates		
2006.1 to 2006.21	<i>P. aeruginosa</i> non-cystic fibrosis sputa isolates from 2006	SJH
2006.22 to 2006.99	<i>P. aeruginosa</i> non-cystic fibrosis swab isolates from 2006	SJH
2006.100 to 2006.113	<i>P. aeruginosa</i> non-cystic fibrosis MSU* isolates from 2006	SJH
2007.114 to 2007.153	<i>P. aeruginosa</i> non-cystic fibrosis sputa isolates from 2007	SJH
2007.154 to 2007.157	<i>P. aeruginosa</i> non-cystic fibrosis swab isolates from 2007	SJH
2007.158 to 2007.159	<i>P. aeruginosa</i> non-cystic fibrosis swab isolates from 2007	SJH
2008.160 to 2008.194	<i>P. aeruginosa</i> non-cystic fibrosis swab isolates from 2008	SJH
2006.195 to 2006.244	<i>P. aeruginosa</i> cystic fibrosis swab isolates from 2006	SVH
2007.245 to 2007.262	<i>P. aeruginosa</i> cystic fibrosis swab isolates from 2007	SVH
2008.263 to 2008. 293	<i>P. aeruginosa</i> cystic fibrosis swab isolates from 2008	SVH
LESB-58	<i>P. aeruginosa</i> Liverpool clone	UL
MES	<i>P. aeruginosa</i> Manchester epidemic strain	UL
Midlands-1	<i>P. aeruginosa</i> Midlands epidemic strain	UL
ATCC 27853	<i>P. aeruginosa</i> Standard in antimicrobial testing	SJH
ATCC 25922	<i>Escherichia coli</i> Standard in antimicrobial testing	SJH
ATCC 35218	<i>Escherichia coli</i> Standard in antimicrobial testing, encodes TEM-1 β -Lactamase	SJH
ATCC 700603	<i>Klebsiella pneumoniae</i> Standard in antimicrobial testing	SJH
PAO1	<i>P. aeruginosa</i> Wild Type strain	CNB
JFL30	<i>P. aeruginosa</i> , over expressing MexAB-OprM	CNB
JFL28	<i>P. aeruginosa</i> , over expressing MexCD-OprJ	CNB
JFL05	<i>P. aeruginosa</i> , over expressing MexEF-OprN	CNB
JFL10	<i>P. aeruginosa</i> , over expressing MexXY	CNB
DNA		
pUCP20	<i>Escherichia-Pseudomonas</i> shuttle vector; Ap ^r	268

pANK-M1	pUCP20 containing a 4.7-kb <i>Xba</i> I – <i>Hind</i> III digested amplicon with <i>mutS</i> from PAO1; Ap ^r	269
PW9409	lacZwp08q1A07	UWA
PW3129	lacZwp03q3D08	UWA
PW3128	lacZwp02q4F08	UWA
PW3130	Supplemental_Plate3C08	UWA
PW3131	lacZwp04q2C09	UWA
PW3132	phoAwp02q1F10	UWA
PW9677	lacZwp07q4E10	UWA
PW9676	lacZwp02q1H08	UWA
PW9679	phoAwp09q4B08	UWA
PW9678	lacZwp06q2C12	UWA
PW8507	phoAwp03q1H02	UWA
PW8508	phoAwp07q1H03	UWA
PW4183	lacZwp01q3G07	UWA
PW4184	lacZwp09q2C04	UWA
PW4181	lacZwp04q3B10	UWA
PW4182	phoAbp02q1C07	UWA
PW4180	phoAwp09q4B05	UWA
PW4179	lacZwp06q3F04	UWA
PW4178	phoAbp03q1F11	UWA
PW4177	phoAwp02q2A06	UWA
PW4176	phoAbp03q3C02	UWA

SJH: St. James's Hospital. *: Mid stream urine. SVH: St. Vincent's Hospital. UL: University of Liverpool. CNB: Centro Nacional de Biotecnologia, Madrid. UWA: University of Washington, Genome Sciences

2.1.1.3 Study setting and patient data

This study was based on isolates retrieved from two major hospitals in Dublin. St James's Hospital is a 963 bed tertiary care hospital, which serves a local community of 350,000 (in correspondence from Professor Thomas Rogers), as well as being a tertiary referral centre for hematopoietic stem cell transplantation, burns, maxilla-facial and plastic surgery, with supra-regional services for HIV medicine, cardiothoracic surgery, and oncology. It is the largest acute teaching hospital in the Republic of Ireland. In 2008, almost 300,000 patients were treated here in SJH. All available antipseudomonal antibiotics were available to be prescribed to treat *P. aeruginosa*

infections in the two patient groups. Piperacillin/tazobactam with or without an aminoglycoside, meropenem and ciprofloxacin were the antibiotics most frequently prescribed to treat *P. aeruginosa* infections in St James's Hospital. Ceftazidime or colistin was not recommended treatment options, unless the *P. aeruginosa* resistance profile indicated that it was the only suitable option.

St. Vincent's University Hospital is a major academic teaching hospital, affiliated to University College Dublin. The hospital has 554 in-patient beds, incorporating 7-day, 5-day and day care options, including intensive care, high dependency and coronary care beds as well as medical, surgical, orthopaedic, care of the elderly and psychiatry beds. St.Vincent's Hospital treats 280 to 300 adult Cystic Fibrosis patients. It has an 8 bed isolation unit. When additional beds are required beds in other wards are also used for adult cystic fibrosis patients.

2.1.2 Bacterial culture conditions

2.1.2.1 Bacterial growth medium

All media were prepared using Millipore 18 M Ω cm⁻¹ grade water generated by Medica Water purifier (ELGA LabWater, Kildare, Ireland). The chemicals were obtained from Oxoid, Fluka, Sigma and BD (Becton, Dickinson). Media were sterilised by autoclaving at 120°C and 15psi for 20 min prior to use. Additional solutions not suitable for autoclaving were sterilised by filtration through 0.2 μ m Millex filters (Millipore, Cork, Ireland). Media agar was allowed to cool to 50°C before the addition of the required supplements and antimicrobials. All quantities listed below are sufficient for 1 litre of medium.

Mueller-Hinton (MH) agar:

All antimicrobial susceptibility (with the exception of colistin) testing, i.e. agar dilution and disc diffusion test methods, were routinely performed using MH agar base. This agar powder is tested according to, and meets the acceptance limits of the current Clinical and Laboratory Standards Institute Screening Guidelines,²⁷⁰.

MH agar:

MH agar was used throughout this study for the routine culturing of all bacterial strains except where otherwise stated.

MH agar: 39 g MH agar base (BD).

After autoclaving, antimicrobials were added at the required concentrations for use in agar dilution susceptibility testing.

Iso-Sentitest agar:

Colistin disc susceptibility testing was performed using Oxoid iso-sentitest agar, as recommended by BSAC ²⁷¹.

Iso-Sentitest agar: 31.4 g Iso-Sentitest agar base (Oxoid).

After autoclaving, antimicrobials were added at the prerequisite concentrations for use in agar dilution susceptibility testing.

Mueller-Hinton (MH) broth:

MH broth was used throughout this study for routine culturing of all bacterial strains except where otherwise stated. MH broth was used for the growth of cultures prior to Miles and Misra testing.

MH broth: 23 g MH agar base (Fluka).

Cation adjusted Mueller-Hinton broth (CAMHB):

CAMHB was used for broth dilution experiments with colistin. Fresh stocks of 10 mg/ml of Ca^{2+} and 10 mg/ml Mg^{2+} were made up. Stock solutions were sterilized by membrane filtration and stored at 2°C. MH broth was per manufacturer guidelines, autoclaved and chilled overnight at 2°C, with stirring 0.1 mL of chilled Ca^{2+} and Mg^{2+} stock solution per litre of broth was added. The correct concentrations of Ca^{2+} and Mg^{2+} are 20-25 mg/L Ca^{2+} and 10-12.5 mg/L Mg^{2+} .

2.1.2.2 Antimicrobial powders and discs

Antimicrobials used in this study were supplied by the following companies: Sigma (piperacillin, gentamicin, amikacin, colistin, rifampicin), GlaxoSmithKline

(ceftazidime), Sanofi-Aventis (cefotaxime), Bayer Healthcare (ciprofloxacin), Fluka (levofloxacin, cloxacillin) and AstrqZeneca (meropenem, imipenem). All antimicrobial powders were prepared daily as stocks with molecular grade water (DNase-, RNase-free, 0.1 µl filtered) (Sigma), unless otherwise stated and used at varying working concentrations, Section 2.2.2.1. Antimicrobial discs were used when powders forms of antimicrobials were not available and were also used to test combination antimicrobials. Antimicrobial discs were supplied by Oxoid (piperacillin/tazobactam, ticarcillin/clavulanic acid and colistin) and those used to confirm ESBL producers and derepressed *ampC* were supplied by BD Diagnostics (ceftazidime, cefotaxime, ceftazidime-clavulanic acid and cefotaxime-clavulanic acid and nitrocefin sticks). All antimicrobial powders and discs were stored at 4°C, except ceftazidime-clavulanic acid discs and cefotaxime-clavulanic acid discs which were stored at -20°C.

2.1.2.3 Bacterial culture conditions

Bacterial cultures were routinely grown on MH plates and aerobically in liquid MH broth at 37°C, except where otherwise stated. Liquid cultures were inoculated by transferring single colonies from 16 to 20 h MH plate cultures into an appropriate volume of MH broth and grown overnight at 37°C with shaking at 250 rpm. Where mid-logarithmic phase cultures were required, overnight cultures were diluted 1:100 in fresh media and grown at 37°C with shaking at 250 rpm, to the appropriate optical density at 600 nm.

2.1.2.4 Hypermutation study

2.1.2.4.1 Inoculum preparation and incubation

The presence of hypermutators was investigated amongst isolates which were classified as very multiple drug resistant (high level resistant to at least one antibiotic per class, term defined in this study). Briefly, independent triplicate 10 ml overnight MH broth cultures of the *P. aeruginosa* isolates were collected by centrifugation and resuspended in 1 ml of saline solution. Serial 10-fold dilutions were plated in MH agar with and without 300 µg/ml of rifampicin. After 36 h of incubation colonies were counted and the mean fraction of the mutants estimated. Isolates were considered hypermutable if the mutation frequency was at least 20-fold higher than that obtained

for control strain *P. aeruginosa* PAO1¹⁴. The Miles and Misra Method was employed to determine viable counts.

2.1.2.4.2 Miles and Misra Method

The Miles and Misra Method is a technique for counting the number of colony forming units (CFU/ml) in a bacterial suspension, it was developed in 1938 by Miles and Misra²⁷². Serial dilutions from bacteria were prepared using phosphate buffered saline (PBS) as a diluent in a logarithmic fashion ranging from 10⁰ to 10⁻⁷ (1 µl sample in 9 µl diluent). The 7-fold dilution scheme was plated onto two MH agar plate divided up into four sections. A 10 µl aliquot of the suspension was transferred onto the appropriate section of a MH agar plate. Plates were incubated for 36 to 48 h at 37°C and observed for growth. The individual drops counts were used to yield estimates of the number of CFU/ml according to Equation 1 below. Each strain was tested in triplicate. The mutation frequency was calculated according to Equation 2 below.

Equation 1:

$$\text{CFU/ml:} \quad C \times \frac{1}{D} \times V$$

Where, C = number of colonies, D = dilution factor and V = volume factor.

Equation 2:

$$\text{Mutation frequency:} \quad \frac{\text{Rif CFU/ml} \times V}{\text{MH CFU/ml} \times V}$$

Where, Rif = rifampicin, MH= Mueller-Hinton, V= volume factor

2.1.3 Oligonucleotide primers

The sequences of all oligonucleotide primers used in this study are listed in Table 2.2. All primers were designed using Primer3 (<http://frodo.wi.mit.edu/primer3>) unless where otherwise stated, and were synthesised by Eurofins MWG Operon, Ebersberg, Germany at the synthesis scale of 0.01 µM and HPSF purification.

Table 2.2 Oligonucleotide primer pairs

Primer	Primer Sequence 5'-3'	Amplicon Size (bp)	Reference
<i>aac(6')-Ib</i>	TTGCAATGCTGAATGGAGAG	198	273
<i>(aacA4)</i>	CGTTTGGATCTTGGTGACCT		
<i>ant (2'')-Ia</i>	GAGCGAAATCTGCCGCTCTGG	299	273
<i>(aadb)</i>	CTGTTACAACGGACTGGCCGC		
<i>aac (3)-Ia</i>	ACCTACTCCCAACATCAGCC	138	273
<i>(aacc1)</i>	ATATAGATCTCACTACGCGG		
<i>gyr A</i>	TTATGCCATGAGCGAGCTGGGCAACGACT AACCGTTGACCAGCAGGTTGGGAATCTT	365	274
<i>gyr B</i>	GCGCGTGAGATGACCCGCCGT CTGGCGGTAGAAGAAGGTCAG	389	274
<i>par C</i>	ATGAGCGAACTGGGGCTGGAT ATGGCGGCGAAGGACTTGGA	209	274
<i>par E</i>	CGGCGTTCGTCTCGGGCGTGGTGAAGGA TCGAGGGCGTAGTAGATGTCCTTGCCGA	591	274
<i>qnrA</i>	AAGGAAGCCGTATGGATATT AGCTAATCCGGCAGCACTAT	670	275
<i>qnrB</i>	CGACCTGAGCGGCACTGAAT TGAGCAACGATGCCTGGTAG	515	275
<i>qnrS</i>	ACCTTCACCGCTTGCACATT CCAGTGCTTCGAGAATCAGT	571	275
<i>aac(6')Ib</i>	TGACCTTGCGATGCTCTATG TTAGGCATCACTGCGTGTTT	506	275
<i>bla_{IMP}</i>	CTACCGCAGCAGAGTCTTTG AACCAGTTTTGCCTTACCAT	587	276
<i>bla_{VIM}</i>	AGTGGTGAGTATCCGACAG ATGAAAGTGCGTGGAGAC	261	277
<i>bla_{GIM-1}</i>	ACAACCTTGACCGAACGCAG ACTCATGACTCCTCACGAGG	747	188
<i>bla_{SPM-1}</i>	CCTACAATCTAACGGCGACC	600	277

	TCGCCGTGTCCAGGTATAAC		
<i>Int (class 1 integrons)</i>	GGCATCCAAGCAGCAAG	Variable	278-2
	AAGCAGACTTGACCTGA		
<i>Int1</i>	CAGTGGACATAAGCCTGTTC	160	278-2
	CCCGAGGCATAGACTGTA		
<i>Int2</i>	TTGCGAGTATCCATAACCTG	288	278-2
	TTACCTGCACTGGATTAAGC		
<i>TEM-F</i>	ATCAGCAATAAACCAGC	516	280
<i>TEM-R</i>	CCCCGAAGAACGTTTTTC		
<i>SHV-F</i>	AGGATTGACTGCCTTTTTTG	392	280
<i>SHV-R</i>	ATTTGCTGATTTTCGCTCG		
<i>CTX-M-1-F</i>	AAAAATCACTGCGCCAGTTC	415	281
<i>CTX-M-1-R</i>	AGCTTATTCATCGCCACGTT		
<i>CTX-M-2-F</i>	GGACGCTACCCCTGCTATT	552	281
<i>CTX-M-2-R</i>	CCAGCGTCAGATTTTTTCAGG		
<i>CTX-M-9-F</i>	TCGCGTTAAGCGGATGATGC	205	281
<i>CTX-M-9-R</i>	AACCCACGATGTGGGTAGC		
<i>rpslF</i>	GCAAGCGCATGGTTCGACAAGA	201	102
<i>rpslR</i>	CGCTGTGCTCTTGCAGGTTGTGA		
<i>mexA1</i>	GCAGACGGTGACCCTGAATA	152	This s
<i>mexA2</i>	GGGTCGATCTGGTAGAGCTG		
<i>mexC3</i>	GTACCGGGCGTCATGCAGGGTTC	164	102
<i>mexC4</i>	TTACTGTTGCGGCGCAGGTGACT		
<i>mexE4</i>	CCAGGACCAGCACGAACTTCTTGC	137	102
<i>mexE5</i>	CGACAACGCCAAGGGCGAGTTCACC		
<i>oprD1</i>	ATCTACCGCACAAACGATGAAGG	156	This s
<i>oprD</i>	GCCGAAGCCGATATAATCAAACG		
<i>ampC1</i>	CGGCTCGGTGAGCAAGACCTTC	218	This s
<i>ampC2</i>	AGTCGCGGATCTGTGCCTGGTC		
<i>oprM1</i>	CTGGGAACCTCGATCTCTTCG-	206	This s
<i>oprM2</i>	TCAGGTCGAAACTCTTCTGCTAG		
PS21F (LES-PS21)	AAGCAGGCCAGCGTGTCTA	364	282
PS21R	AAAACGTAGCAAGCAGTG		
LESF9F	AACACTTGCTCCATCTGC	431	283
LESF9R	CACGATATCCAGCAAGAC		
MIDIF	TTGCGCTCCATCGTTTGA	649	284
MIDIR	CTCCAGATGCCTACGAAA		
MAI5F	GTCGGCAGATAGCCTTTGTC	308	285
MAI5R	CGACTAATACCCGTCGCTTC		

34If	CCTACGGGAGGCAGCAG	560	286
518r	CCGTCAATTCMTTGTGAGTTT		
PAOrpoB1	TCATCGATGTGCTCAAGACC	789	287
PAOrpoB2	TGACGCTGCATGTTCGAG		
PAOrpoBNEW	TCGAGGTTTCGTGACGTACAC	429	This study
	TGACGCTGCATGTTCGAG		
PAOrpoBNterminal	TGGCTTACTCATACACTGAGAAAAA	712	287
PAOrpoBCterminal	CCCTTGATGGTGGGAAGACGTT		
<i>mutS</i>	GGCGAATCGGGTTAACATT	2713	This study
	GGTGACGACGAAGGTCATTT		
<i>mutS2</i>	GGAGCTGGATATCAACCTCA		This study
<i>mutS3</i>	GCTGAACCTCGACCTGAATCG		This study
<i>mutL</i>	CTCCTGGAAAACAGCCTTGA	791	This study
	GGGTGCCATAGAGGAAGTCA		
<i>mutL2</i>	CAGCGCTATGAAGTGAGCAT	2302	This study
	AAGACATCGGAACGCTCTTG		
<i>uvrD</i>	ACGAGTTCCAGGACACCAAC	736	This study
	GGGGTGAGGTCTTCCTCTTC		
<i>uvrD2</i>	AGTGGTTTGCCTTGAAGTGG	2587	This study
	TTCGCTCTCGAAATTCACCT		
<i>pmrB</i>	GCGGTGGAGGCGGTACCGCTGG	808bp	This study
	GCTACAGATATGTGACCGCCCCG		
<i>rpoN</i>	ATGAAACCATCGCTAGTCCTCA	503bp	This study
	TTGATGCTATCGATGATGGTCA		
<i>arnB1</i>	ACGCGCGCCGCCAGGCCCTC	187bp	This study
<i>arnB2</i>	CCC AGG CCG CAG ACC TCG GC		

2.1.4 Statistical analyses

Statistical significance testing was carried out to compare proportions of given characteristics (e.g. resistant vs. susceptible) between particular populations using the Fisher’s exact test, as this was considered most accurate method, based on guidelines outlines in software programme. The lower the *P* value indicates the stronger the inference that the difference is real. *P* values of ≤ 0.05 were considered statistically significant. All tests were performed with InStat version 3 software (GraphPad).

2.2 Antimicrobial Susceptibility Testing

A phenotypic screen was performed on the *P. aeruginosa* isolate collection from CF and Non-CF patients to determine the susceptibility pattern of each isolate to thirteen antimicrobials agents from six different classes, Table 2.3. Standard agar dilution or disc diffusion methods were employed to quantitatively measure these susceptibility activities *in vitro*. *P. aeruginosa* ATCC® 27853, *Escherichia coli* ATCC® 25922 and *K. pneumoniae* ATCC® 700603 were employed as negative and positive controls, respectively, in all susceptibility tests and ESBL confirmatory tests. The accuracy and reproducibility of these test methods were maintained in accordance with current Clinical and Laboratory Standards Institute (CLSI) Guidelines, as outlined below ²⁷⁰. A “susceptible” isolate was defined as one that exhibited phenotypic susceptibility to all members of all antimicrobial families, a “resistant” isolate was defined as one that exhibited phenotypic resistance or intermediate resistance to member(s) of 1-2 antimicrobial families (as defined by CLSI), and a MDR isolate was defined as one that exhibited phenotypic resistance to member(s) of ≥ 3 antimicrobial families (as defined in this study).

Table 2.3 Antimicrobials used in this study: concentrations and solvents

Antimicrobial class	Antimicrobial	Concentration		Solvent
		MIC range	Disc content	
		(mg/L)	(µg)	
<u>β-lactams</u>				
Penicillin	Piperacillin	0.25-128	-	Water
β-lactam/β-lactamase inhibitor concentration	Piperacillin/tazobactam	-	10/1	-
	Ticarcillin-clavulanic acid	-	7.5/1	-
	Ceftazidime/clavulanic acid	-	30/10	-
	Cefotaxime/clavulanic acid	-	30/10	-

3 rd -generation Cephalosporin	Ceftazidime	0.016-128	30µg	Water
4 th -generation Cephalosporin	Cefepime	30/10	30µg	-
<u>Carbapenem</u>	Imipenem	0.016-128	-	PBS
	Meropenem	0.016-128	-	Water
<u>Aminoglycosides</u>	Gentamicin	0.016-128	-	Water
	Amikacin	0.016-128	-	PBS
<u>Fluoroquinolones</u>	Ciprofloxacin	0.016-128	-	n/a ^a
	Levofloxacin	0.016-128	-	Water
<u>Polymyxin</u>	Colistin	0.016-128	25µg	Water

^a n/a: not applicable, ciprofloxacin supplied in solution

2.2.1 Agar dilution and disc diffusion inoculum preparation

For each *P. aeruginosa* isolate, an inoculum was prepared by making a direct sterile saline suspension of 3 to 5 well-isolated colonies selected from 18 to 20 h blood agar plates. The density was adjusted to a 0.5 McFarland standard solution (approximately $1 \text{ to } 2 \times 10^8 \text{ CFU/ml}$) using a Crystalspec® Nephelometer photometric device (BD Diagnostic Systems).

2.2.2 Agar dilution procedure

2.2.2.1 Preparation of agar dilution plates

The minimal inhibitory concentration (MIC) of each isolate, to each antimicrobial, was determined by agar dilutions using serial twofold dilutions indexed to the base 1 (e.g. 1, 2, 4, 8 mg/L, etc). Dilutions of antimicrobials were prepared from standard parental stocks at concentrations of at least 1,000 mg/L or ten times the highest concentration to be tested, whichever was greater. The range of concentrations tested for each antimicrobial encompassed the interpretive MIC breakpoints defined by CLSI

guidelines. These concentration ranges and the number of dilutions tested varied for each antimicrobial, Table 2.3. Antimicrobial solutions were incorporated into 20 ml of autoclaved MH agar per plate, with quick and thorough mixing. All plates were used on same day of preparation.

2.2.2.2 Inoculation agar dilution plates

Within 15 min of inoculum preparation, the inocula were aseptically applied rapidly and simultaneously to MH agar surfaces using an inoculum-replicating apparatus that transfers 27 inocula to each plate (Biddulph & Co). The replicator pins were 1 mm in diameter and delivered approximately 0.1 to 0.2 μ l aliquots of inocula onto the agar surface. A control plate (antimicrobial-free plate) was inoculated first and then, starting with the lowest concentration, plates containing different antimicrobial concentrations were inoculated. A second control plate was inoculated last to ensure no contamination or significant antimicrobial carry-over during the inoculation procedure. Plates were allowed to stand at room temperature for a maximum of 30 min while the inoculum absorbed into the agar, and were then inverted and incubated at 37°C for 16 to 20 h.

2.2.2.3 Determination of agar dilution end points

Plates were placed on a dark, non-reflecting surface and the end points were determined visually. The MIC was defined as the lowest concentration of antimicrobial that completely inhibited bacterial growth, disregarding a single colony or a faint haze caused by the inoculum. MIC interpretive standards were defined according to CLSI breakpoints in order to classify isolates as resistant, intermediate or sensitive, Table 2.4. These MIC₅₀ and MIC₉₀ values are defined as the minimum concentration of antimicrobial required to inhibit the growth of 50% and 90% of the collection, respectively. The MIC₅₀ and MIC₉₀ values were calculated as the 50th and 90th percentile, respectively, of the MIC values of the corresponding antimicrobials.

Table 2.4 Agar dilution breakpoints and disc zone diameter interpretive criteria

Antimicrobial	Minimal inhibitory concentration			Disc diffusion zone diameter		
	(mg/L)			(mm)		
	S	I	R	R	I	S
Piperacillin	≤ 64	-	≥ 128	-	-	-
Piperacillin/Tazobactam	-	-	-	≤ 17	-	≥ 18
Ticarcillin/ clavulanic acid	-	-	-	≤ 14	-	≥ 15
Ceftazidime	≤ 8	16	≥ 32	≤ 14	15-17	≥ 18
Cefepime	-	-	-	≤ 14	15-17	≥ 18
Imipenem	≤4	8	≥16	-	-	-
Meropenem	≤ 4	8	≥ 16	-	-	-
Gentamicin	≤ 4	8	≥ 16	-	-	-
Amikacin	≤ 16	32	≥ 64	-	-	-
Levofloxacin	≤ 2	4	≥ 8	-	-	-
Ciprofloxacin	≤ 1	2	≥ 4	-	-	-
Colistin	≤2	4	≥8	≤ 13	-	≥14

2.2.3 Disc diffusion procedure

2.2.3.1 Preparation of disc diffusion test plates

Within 15 min of inoculum preparation, Section 2.2.1, a sterile cotton swab was used to aseptically inoculate an entire MH agar plate surface by streaking. This streaking procedure was repeated three times at 60° angles with respect to each other to ensure a uniformly distributed inoculum.

2.2.3.2 Application of discs to inoculated agar plates

Antimicrobial impregnated discs containing ticarcillin/clavulanic, piperacillin/tazobactam, cefepime were placed onto dried inoculated MH agar plates (one disc per plate) for no longer than 15 min after inoculation of plates. (The concentration of each antimicrobial per disc is documented in Table 2.3.) The plates

were inverted and incubated at 37°C for 16 to 20 h within 15 min of antibiotic disc application.

2.2.3.3 Interpretation of disc diffusion results

Plates were placed on a dark, non-reflecting surface to facilitate visualisation of the end points. The diameter of each zone of inhibition was measured to the nearest whole millimetre. Zone diameter interpretive criteria were defined according to CLSI breakpoints in order to classify isolates as resistant, intermediate or sensitive, Table 2.4. For most antimicrobials these criteria were developed by first comparing zone diameters to MICs of a large number of isolates, including those with known mechanisms of resistance relevant to particular drug classes, and secondly the MICs and correlated zone diameters are analysed according to the pharmacokinetics of the drug from normal dosing regimens²⁷⁰. Finally, when feasible, the tentative *in vitro* interpretive criteria are analysed according to studies of clinical efficacy and microbiologic eradication efficacy in the treatment of specific pathogens.

2.2.3.4 Double disc diffusion ESBL confirmatory test

Isolates displaying a reduced susceptibility to cefotaxime or ceftazidime were considered potential ESBL producers. These isolates underwent further screening which involved the use of the double-disc synergy test²⁸⁸. ESBL production was inferred when the zones produced by the discs with clavulante were ≥ 5 mm larger than those without inhibitor. Since ESBLs in *P. aeruginosa* are notoriously difficult to detect due to the presence of an inducible AmpC enzyme and efflux pumps, cloxacillin (an inhibitor of the AmpC enzyme) and PA β N (efflux inhibitor) were included in the media. *E. coli* ATCC® 25922 and *K. pneumoniae* ATCC® were used as negative and positive controls respectively, in antimicrobial susceptibility and confirmatory tests.

Preparation of the double-disc diffusion confirmatory test plates was carried out as in Section 2.2.3.1. The antibiotic discs were placed 25-30 mm apart per plate. The concentration of each antimicrobial per disc is documented in Table 2.3. Disc diffusion end points were determined as in Section 2.2.3.3. A positive test was recorded when the diameter of inhibition zone of cephalosporin in combination with clavulanic acid was ≥ 5 mm in comparison to that of the cephalosporin zone alone.

2.2.3.5 Cephalosporinase overproduction test

Overproduction of the chromosomal cephalosporinase AmpC was evaluated by the nitrocefin test according to the instructions in the kit (β -lactamase identification sticks, BR0066, OXOID). Development of a red colour within 5 minutes in the area of the disc where the culture is applied was considered a positive result.

2.3 Nucleic Acid Methodologies

2.3.1 Preparation of chromosomal DNA, RNA and cDNA

2.3.1.1 Purification of total genomic DNA

Chromosomal DNA was extracted by suspending a single colony in 100 μ l of ultra pure H₂O and boiling for 10 min, the supernatant was used as a DNA template. In some instances (where PCR failed) chromosomal DNA was purified from bacterial strains using the ArchivePure DNA Isolation Kit (5 PRIME). Briefly, bacteria from a 0.5 ml overnight culture were harvested by centrifugation at 10,000 $\times$ g for 5 min. The supernatant was resuspended in a proprietary cell lysis solution, containing Tris [hydroxymethyl] aminomethane, EDTA and SDS and incubated at 80°C for 5 min to lyse the bacteria. Contaminating RNA was removed by treatment with RNase for 1 h at 37°C and cellular proteins were removed by protein precipitation using ammonium acetate. The remaining DNA was precipitated with isopropanol, washed in ethanol twice to remove any contaminants and resuspended in a Tris/EDTA buffer. Stock genomic DNA solutions were stored at -80°C and working solutions (1:10 dilutions) were stored at -20°C.

2.3.1.2 Isolation of RNA

RNA is a chemically unstable molecule and is prone to digestion by ubiquitous RNases which require no cofactors to function, these enzymes can maintain active even after autoclaving or boiling. Therefore during isolation and handling of RNA, several precautions were taken to minimise the risk of contamination with exogenous RNases. These precautions included wearing gloves, working in a designated RNA handling area that had been cleaned with RNaseZAP® (Ambion) and working with designated tips, tubes, pipettes and electrophoresis tanks. Additionally, where possible, all steps in the handling of RNA were performed expediently and on ice.

Total RNA was isolated from bacterial cultures using the RiboPure™-Bacteria Kit (Ambion). Briefly, a single colony of *P.aeruginosa* PAO1 (fully sequenced) was inoculated into 3 ml MH broth and incubated overnight with shaking. The overnight bacterial culture was diluted 1:100 in 20 ml of fresh MH broth and grown to an OD_{600nm} of 0.6. Cells from a 10 ml aliquot of this suspension were harvested by centrifugation at 10,000 x g for 5 min. The cell pellet was resuspended in 1 ml PBS and centrifuged again as before. The cell pellet was then resuspended in RNAwiz (Ambion), a combination of phenolic, detergent and chaotropic denaturants that inhibit RNases and stabilise RNA. Bacterial cell walls were disrupted by vortexing this suspension in the presence of Zirconia beads (Ambion). The resultant bacterial lysate was mixed with 0.2 volumes chloroform and centrifuged to retrieve the RNA-containing upper aqueous phase. This crude RNA preparation was diluted with ethanol and bound to a silica filter. After several wash steps (with ethanol 70% w/v) to remove contaminants, the RNA was eluted in a low ionic strength solution. Contaminating genomic DNA was removed by DNaseI treatment. Total RNA samples were quantified (see Section 2.3.1.3) and stored at -80°C.

2.3.1.3 Quantitation of nucleic acids

Nucleic acid concentration (µg/ml) and purity ($A_{260nm}:A_{280nm}$ ratio) were assessed using a NanoDrop® ND-8000 spectrophotometer (NanoDrop Technologies). Pure nucleic acid (DNA or RNA) has an $A_{260nm}:A_{280nm}$ ratio equal to 1.8-2.1.

2.3.2 *In vitro* manipulation of DNA

2.3.2.1 Purification of DNA fragments

Following PCR amplification, linear DNA fragments were purified directly from solution using a QIAquick PCR Purification Kit (Qiagen) or from an agarose gel slice using a QIAquick Gel Extraction Kit (Qiagen). To purify DNA from solution, 5 volumes of Binding Buffer was added and mixed thoroughly. Binding Buffer contains isopropanol and the chaotropic salt guanidine hydrochloride, which denatures proteins. This solution was added to a spin column where the DNA fragments bind to a glass fibre matrix. Bound DNA was then purified in a series of ethanol based wash steps to remove salts, enzymes, dyes and unincorporated nucleotides. Purified DNA was eluted from the column in Elution Buffer (10 mM TrisCl, pH 8.5). Purification of DNA from an agarose gel slice was achieved by melting the gel slice at 50°C in the presence of 3 volumes of Solubilisation Buffer. Solubilisation Buffer contains the chaotropic protein denaturant guanidine thiocyanate. Once the gel slice had melted and the DNA was in solution, 1 volume of isopropanol was added and mixed thoroughly. This solution was added to a spin column where the DNA fragments bind to a glass fibre matrix. The column was washed with a further aliquot of Solubilisation Buffer to remove all traces of agarose. Bound DNA was purified in a series of ethanol-based wash steps to remove salts, enzymes, dyes and unincorporated nucleotides. Purified DNA was eluted from the column in Elution Buffer.

2.3.2.2 DNA sequencing

Nucleotide sequencing of DNA fragments of interest (both strands where necessary) was performed by The Genomics Core Facility, Belfast City Hospital. Briefly, 2 µg of purified linear DNA fragments and 10 µM of specific primers were sent for analysis. Files received back were in FASTA format. Multiple sequence alignments were performed with genes of known GenBank entries using Clustal W2 software program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) to determine nucleotide sequence homologies. Where protein sequences were needed before alignment, FASTA nucleotide sequence files were firstly translated into proteins in all reading frames using a DNA sequence translator programme (<http://searchlauncher.bcm.tmc.edu/seq-util/Options/sixframe.html>).

2.3.3 Agarose gel electrophoresis

2.3.3.1 DNA agarose gel electrophoresis

DNA samples were separated on 1.5-2.5% (w/v) agarose gels, depending on the size of the DNA. Briefly, for a 1% gel, 1 g of agarose was added to 100 ml of 0.5X TBE buffer (44.5 mM Tris borate (pH 8.3), 1 mM EDTA) and microwaved to dissolve the agarose. Ethidium bromide was added to a final concentration of 1 µg/ml and the molten gel was poured into a gel mould and allowed to set. DNA samples were prepared by adding an appropriate volume of 6X Blue/Orange Loading Dye (Promega) and these samples were subjected to electrophoresis through the gel usually at 70 V for 1 hr in 0.5X TBE buffer. The separated DNA fragments were visualised under UV light using a gel documentation system and analysed using the corresponding software program (Quantity One® UV transillumination, Bio-Rad Laboratories).

2.3.3.2 Denaturing RNA agarose gel electrophoresis

RNA samples were analysed by denaturing agarose gel electrophoresis to prevent the formation of extensive RNA secondary structures via intramolecular base pairing, which prevents it from migrating strictly according to its size. The quality and yield of RNA samples were assessed on a 1% denaturing agarose gel. Briefly, 1 g of agarose was added to 72 ml of molecular grade water and microwaved to dissolve the agarose. After cooling to 60 °C, 1X MOPS running buffer (40 mM MOPS pH 7.0, 10 mM sodium acetate, 1 mM EDTA) and 7% formaldehyde were added. The molten gel was poured into a gel mould and allowed to set. Samples containing 1 µg of total RNA in 50 µl RNA running buffer (1x MOPS running buffer, 7% (w/v) formaldehyde, 50% formamide) were prepared for electrophoresis by heating to 55°C for 5 min to denature any RNA secondary structures. Denatured RNA samples were mixed with 1 volume of formaldehyde loading dye (1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol, 60 µg/ml ethidium bromide). Samples were subjected to electrophoresis through the gel at 5 V/cm in 1X MOPS running buffer. Separation of 16S and 23S ribosomal RNA (rRNA), which infers good quality rRNA, fragments were visualised under UV light using a gel documentation system and analysed using a

corresponding software program (Quantity One® UV transillumination, Bio-Rad Laboratories).

2.3.4 Polymerase chain reaction

The polymerase chain reaction (PCR) was used for the routine amplification of specific DNA fragments responsible for antimicrobial resistance and virulence. PCR is based on the ability of certain thermostable DNA polymerases to synthesise a new DNA strand complementary to a provided single-stranded, denatured DNA template when primed with specific complementary oligonucleotides. The procedure involves successive rounds of thermal denaturation of a double-stranded DNA template, hybridisation of two complementary oligonucleotides (primers) and synthesis of the new DNA strand by the DNA polymerase. The primers are designed to be complementary to opposite strands at either end of the fragment to be amplified and orientated such that their 3' ends face each other. The new DNA strand is synthesised from provided dNTPs by the DNA polymerase in the presence of Mg^{2+} . Each new strand acts as a template in further rounds of amplification and the procedure thus results in exponential amplification of the desired DNA fragment.

2.3.4.1 Amplification of DNA by polymerase chain reaction

Two different *Taq* DNA polymerases were used for routine amplification of DNA fragments. *Taq* DNA polymerase, a recombinant purified thermostable polymerase from *Thermus aquaticus* YT-1, which has 5'→3' DNA polymerase activity and 5'→3' exonuclease activity was used in amplification of DNA fragments to detect presence/absence of genes of interest, and also in amplification of DNA fragments that were to be sequenced. *Taq* polymerase 5U/μL was used in combination with 10x buffer (Sigma, 100mMTris-HCl, 500mMKCl), dNTP mix (Promega, dATP, dCTP, dGTP and dTTP final dNTP concentration of 200μM for each dNTP) 25mM $MgCl_2$ with 0.4μM of forward and reverse primer and 10ng of template DNA. When increased sensitivity, specificity and yield of PCR product were required, a Hot Start technique was employed using AmpliTaqGold® PCR 2X Master Mix (Applied Biosystems). AmpliTaqGold is a chemically modified form of AmpliTaqGold® DNA Polymerase. When the proprietary chemical moiety is attached to the enzyme, it is inactive, allowing for flexibility in

reaction setup. The enzyme requires a heat activation step, well above optimal annealing, to activate the enzyme, therefore diminishing chances of mis-primed primer extension. AmpliTaqGold® PCR Master Mix contains 0.025 U/μl AmpliTaqGold® DNA Polymerase, GeneAmp PCR Gold Buffer (15 mM TrisHCl, 50 mM KCl), 0.2 mM of each dNTP and 2.5 mM MgCl₂. PCR amplification reactions were performed in either a PTC-100® Peltier Thermal Cycler (Bio-Rad Laboratories Inc, CA, USA) or GS1 G-Storm® Thermal cycler (Gene Technologies). As seen in Table 2.2, 15 different primer sets were designed and optimised based on the individual T_m of the primers and the optimal/recommended use of the polymerase taq, based on manufacturer guidelines.

2.3.4.2 Detection of β-lactam resistance genes

The presence of *bla*_{VIM}, *bla*_{IMP}, *bla*_{SPM} and *bla*_{GIM} genes were detected by individual PCR amplifications in isolates with a reduced susceptibility to imipenem and/or meropenem. Briefly, *bla*_{VIM}, *bla*_{IMP}, *bla*_{SPM} and *bla*_{GIM} genes were amplified with the following master mix- 0.5μl *Taq*, 3μl 25mM MgCl₂, 5μl 10X PCR buffer, 1μl dNTP mix, 0.1 μM of each forward and reverse primer, 10 ng template genomic DNA and molecular grade water to a final volume of 50 μl. The PCR reactions were transferred to the thermal cycler and incubated as follows:

	<u><i>bla</i>_{VIM}</u>	<u><i>bla</i>_{IMP}</u>	<u><i>bla</i>_{SPM}</u>	<u><i>bla</i>_{GIM}</u>
1 Initial denaturation	94°C 5min	94°C 2min	95°C 15 min	96°C 5 min
2 Denaturation	94°C 25sec	94°C 1min	95°C 1 min	96°C 1 min
3 Annealing	52°C 40s	55°C 1min	50°C 1 min	58°C 1 min
4 Extension	72°C 1min 30s	72°C 1min 30s	72°C 1 min	72°C 1 min
5 Repeat steps 2-4	30	30	additional 29 cycles	additional 34 cycles
6 Final Extension	72°C 7min	72°C 6min	72°C for 10 min	72°C for 10 min

2.3.4.3 Detection of ESBL resistance genes

Isolates which were positive for the ESBL double disc test, Section 2.2.3.4, were screened for the presence of ESBLs. PCR amplification of *bla*_{TEM} and *bla*_{SHV} were performed using 12.5µl AmpliTaq Gold® Gold 2x Master mix, 1µM of each primer, DNA for PCR amplification was prepared by making a suspension of a few colonies in molecular grade water and boiling for 10min. Following centrifugation 1µl of crude DNA was used directly in 25 µl volumes. PCR amplification of CTX-M β-lactamases were performed using 12.5µl AmpliTaq Gold® 2x Master mix, 0.4µM of each CTX-M-1F/R, CTX-M-2F/R AND CTX-M-9 F/R. The PCR reactions were transferred to the thermal cycler and incubated as follows:

	<u><i>bla</i>_{TEM}</u>	<u><i>bla</i>_{SHV}</u>	<u><i>bla</i>_{CTX-M}</u>
1 Initial denaturation	95°C for 15 min	96°C for 5 min	94°C for 5 min
2 Denaturation	95°C for 1 min	96°C for 1 min	94°C for 25s
3 Annealing	52°C for 1 min	58°C for 1 min	54°C for 40s
4 Extension	72°C for 1 min	72°C for 1 min	72°C for 50s
5 Repeat steps 2-4	30 cycles	35 cycles	29 cycles
6 Final Extension	72°C for 10 min	72°C for 10 min	72°C for 10 min

2.3.4.3 Detection of quinolone resistance genes

Point mutations in the quinolone resistance-determining regions of *gyrA*, *gyrB* and *parC*, *parE* genes were studied by specific PCR amplifications in isolates that displayed a reduced susceptibility to levofloxacin and/or ciprofloxacin antimicrobials Individual PCR amplifications of *gyrA*, *gyrB* and *parC*, *parE* genes were performed using the following master mix- 0.5µl *Taq*, 3µl 25mM MgCl₂, 5µl 10X PCR buffer, 1µl dNTP mix, 0.1 µM of each forward and reverse primer, 10 ng template genomic DNA and molecular grade water to a final volume of 50 µl., 1 µM of each *gyrA*-F, *gyrB*-F and *gyrA*-R, *gyrB*-R primer or 1 µM of each *parC*-F, *parE*-F and *parC*-R, *parE*-R primer (Table 2.2), DNA for PCR amplification was prepared by making a suspension of a few

colonies in molecular grade water and boiling for 10min. Following centrifugation 1µl of crude DNA was used directly in 25 µl volumes. The PCR reactions were transferred to the thermal cycler and incubated as follows:

	<u><i>gyrA</i></u>	<u><i>gyrB</i></u>	<u><i>parC</i></u>	<u><i>parE</i></u>
1 Initial denaturation	94°C 5min	94°C 5min	94°C 5min	94°C 5min
2 Denaturation	94°C 1min	94°C 1min	94°C 1min	94°C 1min
3 Oligonucleotide annealing	57°C 1min	65°C 1min	57°C 1min	55°C 1min
4 Extension	72°C 1min	72°C 1min	72°C 1min	72°C 1min
5 Repeat steps 2-4 for additional	35 cycles	35 cycles	35 cycles	35 cycles
6 Final Extension	72°C 10min	72°C 10min	72°C 10min	72°C 10min

2.3.4.4 Detection of *qnr* genes

The presence of plasmid-mediated quinolone resistance genes (*qnrA*, *qnrB* and *qnrS*) were analysed in all quinolone resistant isolates, along with any further isolate with a ciprofloxacin MIC of ≥ 0.25 mg/L. Amplification of these genes was performed by individual PCR amplification with the following master mix- 0.5µl *Taq*, 3µl 25mM MgCl₂, 5µl 10X PCR buffer, 1µl dNTP mix, 0.1 µM of each forward and reverse primer, DNA for PCR amplification was prepared by making a suspension of a few colonies in molecular grade water and boiling for 10min. Following centrifugation 1µl of crude DNA was used directly in 25 µl volumes containing 1 µM of each *qnrA*-F, *qnrA*-R, *qnrB*-F, *qnrB*-R, *qnrS*-F and *qnrS*-R primer, Table 2.2, DNA for PCR amplification was prepared by making a suspension of a few colonies in molecular grade water and boiling for 10min. Following centrifugation 1µl of crude DNA was used directly in 25 µl volumes. Amplification of this gene was performed by PCR with the following master mix- 0.5µl *Taq*, 3µl 25mM MgCl₂, 5µl 10X PCR buffer, 1µl dNTP mix, 0.1 µM of each forward and reverse primer, DNA for PCR amplification was prepared by making a suspension of a few colonies in molecular grade water and boiling for 10min. Following centrifugation 1µl of crude DNA was used directly in 25 µl volumes. The PCR reactions were transferred to the thermal cycler and incubated as follows:

	<u><i>qnrA</i></u>	<u><i>qnrB</i></u>	<u><i>qnrS</i></u>
1 Initial denaturation	94°C 5 min	94°C 5 min	94°C 5 min
2 Denaturation	94°C 45 s	94°C 45 s	94°C 45 s
3 Oligonucleotide annealing	58°C 45 s	56°C 45 s	55°C 45 s
4 Extension	72°C 1 min	72°C 1 min	72°C 1 min
5 Repeat steps 2-4 for additional	32 cycles	32 cycles	32 cycles
6 Final Extension	72°C 10 min	72°C 10 min	72°C 10 min

2.3.4.5 Detection of aminoglycoside modifying enzymes

The presence of aminoglycoside modifying enzymes were detected by individual PCR amplifications in isolates with a reduced susceptibility to gentamicin and/or amikacin. Briefly, *aac(6')Ib*, *ant(2'')Ia*, *aac(3)Ia* genes were amplified with the following master mix- 0.5µl *Taq*, 3µl 25mM MgCl₂, 5µl 10X PCR buffer, 1µl dNTP mix, 0.1 µM of each forward and reverse primer, DNA for PCR amplification was prepared by making a suspension of a few colonies in molecular grade water and boiling for 10min. Following centrifugation 1µl of crude DNA was used directly in 25 µl volumes. The PCR reactions were transferred to the thermal cycler and incubated as follows:

	<u><i>aac(6')Ib</i></u>	<u><i>ant(2'')Ia</i></u>	<u><i>aac(3)Ia</i></u>
1 Initial denaturation	94°C 5 min	94°C 5min	94°C 5 min
2 Denaturation	94°C 45 s	94°C 45 s	94°C 45 s
3 Oligonucleotide annealing	59°C 45 s	61°C 45s	57°C 45 s
4 Extension	72°C 1 min	72°C 45s	72°C 45 s
5 Repeat steps 2-4 for additional	33 cycles	33 cycles	33 cycles
6 Final Extension	72°C 10 min	72°C 10min	72°C for 10 min

2.3.4.6 Detection of integrons

The presence of integrons were detected by individual PCR amplifications in isolates with were positive for *bla_{VIM}*, *bla_{IMP}*, *bla_{SPM}* and *bla_{GIM}* genes. Isolates which were positive for the presence of an integron were further tested with Int1 and Int2 primers. Briefly, isolates were amplified with the following master mix- 0.5µl *Taq*, 3µl

25mM MgCl₂, 5µl 10X PCR buffer, 1µl dNTP mix, 0.1 µM of each forward and reverse primer, DNA for PCR amplification was prepared by making a suspension of a few colonies in molecular grade water and boiling for 10min. Following centrifugation 1µl of crude DNA was used directly in 25 µl volumes. The PCR reactions were transferred to the thermal cycler and incubated as follows:

	<u><i>Int</i></u>	<u><i>Int1</i></u>	<u><i>Int2</i></u>
1 Initial denaturation	94°C 5 min	94°C 5min	94°C 5 min
2 Denaturation	94°C 45 s	94°C 45 s	94°C 45 s
3 Oligonucleotide annealing	59°C 45 s	61°C 45s	57°C 45 s
4 Extension	72°C 1 min	72°C 45s	72°C 45 s
5 Repeat steps 2-4 for additional	33 cycles	33 cycles	33 cycles
6 Final Extension	72°C 10 min	72°C 10min	72°C for 10 min

2.3.4.7 Detection of Liverpool, Midlands and Manchester epidemic strains

In order to detect if the Liverpool, Midlands 1 and Manchester epidemic strains were present in the CF population, a multiplex PCR was carried out,²⁸⁹. DNA for PCR amplification was prepared by making a suspension of a few colonies in molecular grade water and boiling for 10min. Following centrifugation 1µl of crude DNA was used directly in 25 µl volumes containing, 1 x GoTaq Green Master Mix (Promega), 300 nM of each primer. 1 x Taq buffer, 1.5 mM MgCl₂ and 200µM nucleotides (dATP, dCTP, dGTP, dTTP). Amplifications were carried out as follows:

<u>Multiplex PCR</u>	
LES-PS21, LESF9, MID1, MA15, 16S rRNA	
1 Initial denaturation	95°C 5 min
2 Denaturation	95°C 1 min
3 Oligonucleotide annealing	52°C 1 min
4 Extension	72°C 2 min
5 Repeat steps 2-4 for additional	30 cycles
6 Final Extension	72°C 10 min

2.3.4.8 Detection of mutation in *rpoB* gene

Hypermutator isolates were investigated to determine if there were mutations in the *rpoB* gene which could have caused rifampicin resistance. Isolates were amplified with the following master mix- 0.5µl *Taq*, 3µl 25mM MgCl₂, 5µl 10X PCR buffer, 1µl dNTP mix, 0.1 µM of each forward and reverse primer. DNA for PCR amplification was prepared by making a suspension of a few colonies in molecular grade water and boiling for 10min. The PCR reactions were transferred to the thermal cycler and incubated as follows:

	<u>PAOrpoB</u>	<u>PAOrpoBNTerminal</u>	<u>PAOrpoBNew</u>
1 Initial denaturation	98°C 30s	98°C 30s	98°C 30s
2 Denaturation	98°C 10s	98°C 10s	98°C 10s
3 Oligonucleotide annealing	58°C 30s	58°C 30s	59°C 30s
4 Extension	72°C 21s	72°C 21s	72°C 21s
5 Repeat steps 2-4 for additional	30 cycles	30 cycles	30 cycles
6 Final Extension	72°C 10 min	72°C 10 min	72°C 10 min

2.3.4.9 Detection of mutation in genes associated with mutator phenotype

Hypermutator isolates were investigated for mutation in the *mutS*, *mutL* and *uvrD* genes as follows; isolates were amplified with the following master mix- 0.5µl *Taq*, 3µl 25mM MgCl₂, 5µl 10X PCR buffer, 1µl dNTP mix, 0.1 µM of each forward and reverse primer, DNA for PCR amplification was prepared by making a suspension of a few colonies in molecular grade water and boiling for 10min. The PCR reactions were transferred to the thermal cycler and incubated as follows:

	<u><i>mutS</i></u>	<u><i>mutL</i></u>	<u><i>uvrD</i></u>
1 Initial denaturation	98°C 30s	98°C 30s	98°C 30s
2 Denaturation	98°C 10s	98°C 10s	98°C 10s
3Oligonucleotide annealing	54°C 30s	57°C 30s	56°C 30s
4 Extension	72°C 1 min 10s	72°C 27s	72°C 27s
5 Repeat steps 2-4 for additional	30 cycles	30 cycles	30 cycles
6 Final Extension	72°C 10 min	72°C 10 min	72°C 10 min

2.3.4.10 Detection of mutation in genes associated with colistin resistance

Isolates which were resistant to colistin were investigated for mutation in the *pmrB* and *rpoN* genes as follows; isolates were amplified with the following master mix- 0.5µl *Taq*, 3µl 25mM MgCl₂, 5µl 10X PCR buffer, 1µl dNTP mix, 0.1 µM of each forward and reverse primer, DNA for PCR amplification was prepared by making a suspension of a few colonies in molecular grade water and boiling for 10min. The PCR reactions were transferred to the thermal cycler and incubated as follows:

	<u><i>pmrB</i></u>	<u><i>rpoN</i></u>
1 Initial denaturation	95°C 5 min	95°C 5 min
2 Denaturation	95°C 1 min	95°C 15 s
3 Oligonucleotide annealing	60°C 1 min	55°C 15 s
4 Extension	72°C 1 min	72°C 30 s
5 Repeat steps 2-4 for additional	30 cycles	40 cycles
6 Final Extension	72°C 10 min	72°C 10 min

2.4 Real-Time Reverse Transcriptase-Polymerase Chain Reaction

Real-time reverse transcriptase PCR (qRT-PCR) is the most sensitive and reliable method for quantification of RNA transcripts. Reverse transcriptase is a multifunctional enzyme with three distinct enzymatic activities, two of which (an RNA-dependant DNA polymerase and a ribonuclease: RNase H) are utilised in the reverse transcription of RNA to single-stranded cDNA. The quantity of cDNA produced by target genes can then be determined during the exponential phase of real-time PCR by the detection of, for example, fluorescent SYBR Green (a specific dsDNA binding dye).

2.4.1 Generation of DNA standards from each gene of interest

DNA PCR amplifications were performed for each gene of interest in order to obtain high quality and quantity DNA standards. Reactions consisted of 12.5 µl of AmpliTaq Gold® PCR 2X Master Mix mixed with 0.4 µM of each relevant forward and reverse primer, 10 ng template genomic *P. aeruginosa* DNA and molecular grade water to a final volume of 25 µl. These primers were as follows: *rpsL*: *rpsL*F and *rpsL*R, *mexA*: *mexA*1 and *mexA*2, *mexC*: *mexC*3 and *mexC*4, *mexE*: *mexE*4 and *mexE*5, *oprD*: *oprD*1 and *oprD*2, *ampC*: *ampC*1 and *ampC*2, *oprM*: *oprM*1 and *oprM*2, *arnB*: *arnB*1 and *arnB*2, Table 2.2. Each set of primers were designed to a length of 26 oligos, with a 59-60°C melting temperature, in order to generate a 150-250 bp product. The PCR reactions were transferred to the thermal cycler and incubated as follows:

- | | |
|---|--|
| 1 | Denaturation: 95°C for 3 min |
| 2 | Denaturation: 94°C for 30 s |
| 3 | Oligonucleotide annealing: 57°C for 30 s |
| 4 | Extension: 72°C for 15 s |
| 5 | Repeat steps 2-4 for an additional 29 cycles |
| 6 | Final Extension: 72°C for 10 min |

Resulting PCR products were separated by electrophoresis on a 2% (w/v) agarose gel (see Section 2.3.3.1). They were subsequently purified, see Section 2.3.2.2, and quantified, see Section 2.3.1.3, then stored at -20 °C until further use.

2.4.2 Establishment of standard DNA amplification curves

A standard DNA amplification curve is defined by the number of points in the dilution series, the number standard replicates, the starting quantity and the serial factor. Standard DNA amplification curves were set up for each gene of interest to optimise each individual reaction, using the above DNA standards as templates. This was achieved by real-time PCR amplification using the QuantiTect® SYBR® Green PCR Kit (Qiagen). Briefly, 10 µl of Qiagen QuantiTect® SYBR® Green PCR Master Mix (contains HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, SYBR Green 1 and ROX passive dye), 0.1 to 0.6 µM of each relevant reverse primer only, serial 1 in 10 dilutions of standard DNA ranging from 10 pg to 1 fg and molecular grade

water to a final volume of 20 µl. These reverse primers were as follows: *rspl*: *rspl*-R, *mexA*: *mexA*-2, *mexC*: *mexC*-4, *mexE*: *mexE*-5, *oprD*: *oprD*-2, *ampC*: *ampC*-2, *oprM*: *oprM*-2, *arnB*: *arnB*2, Table 2.2. All reactions were performed in triplicate. The reactions were transferred to an Applied Biosystems 7500 Fast System and incubated as follows using a Standard Curve Quantitation program:

1. Activation of HotStarTaq® DNA polymerase: 95°C for 15 min
2. Denaturation: 94°C for 15 s
3. Oligonucleotide annealing: 60°C for 30 s
4. Extension: 72°C for 30 s
5. Repeat steps 3-5 for an additional 30 cycles
6. Followed by Melt Curve analysis: 95°C for 15 s, 94°C for 15s, 61°C for 30 s and 72°C for 30s

Resulting standard DNA amplification curves were accessed for percentage efficiency (optimally 100%, ± 10%) and slope (optimally -3.3, ± 0.2). Furthermore, the slope and amplification efficiency of any two assays (i.e. endogenous control vs. gene of interest) must be as similar as possible to ensure accurate expression results in further testing of cDNA transcripts. The slope is the regression coefficient generated from the regression line in the standard curve, while the amplification efficiency is generated using the slope of the regression line in the standard curve. The regression line is the best-fit line from the standard curve and is calculated by the following equation:

$$C_T = m [\log (Qty)] + b,$$

Where, C_T = threshold cycle (the PCR cycle number at which the fluorescence meets the threshold in the amplification plot), m = slope, b = y-intercept and Qty = standard quantity.

2.4.3 Reverse transcription PCR for first-strand cDNA synthesis

RNA extracted from *P. aeruginosa* was reverse transcribed into single-stranded cDNA by reverse transcriptase PCR (RT-PCR). RT-PCR was performed using the Omniscript® Reverse Transcription Kit (Qiagen) for each gene of interest. Briefly, 2 µg

of template total RNA was mixed with 2 µl of 10X Qiagen Omniscript® RT buffer, 4 U of Qiagen Omniscript® RT enzyme, 0.5 mM of each dNTP, 1 µM of reverse primer (as in Section 2.3.6.2; Table 2.2), 10 U of RNase inhibitor (Promega) and RNase-free water to a final volume of 25 µl. The reactions were incubated at 37 °C for 1 h and stored at -20 °C until further use.

2.4.4 Quantification of cDNA *P. aeruginosa* targets

The quantity of cDNA expressed from *P. aeruginosa* for each gene of interest, was evaluated by real-time PCR amplification using the QuantiTect® SYBR® Green PCR Kit (Qiagen) with optimised conditions (see Section 2.3.5.3). The expression level of one gene was analysed in any one run relative to the endogenous control, *rpsL*. Briefly, 10 µl of Qiagen QuantiTect® SYBR® Green PCR Master Mix (contains HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, SYBR Green 1 and ROX passive dye), 0.1 to 0.6 µM of each forward and reverse primer (as in Section 2.3.6.1; Table 2.2), 10 ng cDNA and molecular grade water to a final volume of 20 µl. All reactions were performed with 5 replicates. The reactions were transferred to an Applied Biosystems 7500 Fast System and incubated as in Section 2.3.5.3, using a Comparative C_T (ΔΔ C_T) Quantitation program. This program measures amplification of the target (i.e. gene of interest) and of the endogenous control (*rpsL*) in the samples and in a reference sample. Measurements are normalised using the endogenous control. The program software then determines the relative quantity of target in each sample by comparing normalised target quantity in each sample to normalised target quantity in the reference sample.

2.5 Clonality

2.5.1 Pulsed field gel electrophoresis

Pulsed field gel electrophoresis is a separation technique similar to gel electrophoresis, with an exception; instead of the voltage continuously running in one direction the voltage is periodically switched in three directions. The pulse times are equal for each direction. This technique allows large pieces of DNA (30-50kb) to be

separated. The technique was first developed by Schwartz *et al*²⁹⁰ PFGE is typically used for genetic fingerprinting.

2.5.1.1 Isolate selection for pulsed field gel electrophoresis

Isolates which were classified as vMDR or colistin resistant were selected for pulsed field gel electrophoresis analysis.

2.5.1.2 Preparation of isolates for PFGE analysis

Isolates were grown on Mueller Hinton agar overnight. A single colony from each was used to make separate agar plugs, which were then suspended in 2 ml Lysis buffer (N-lauroylsarcosine 1g, EDTA Na₂ 18.6g, deionised water 80ml. Approximately 10ml of 10M NaOH was required to dissolve the EDTA Na₂. Solution was adjusted to pH 9.5, filter sterilized and stored at room temp), and 10 µl TritonX-100 (all Sigma-Aldrich unless otherwise stated) and incubated in a water bath at 55°C overnight. The plugs were then cut to size (1mm x 1mm) and placed in an eppendorf tube containing 90 µl deionised H₂O, 10µl reaction buffer, 2 µl bovine serum albumin, 2 µl dithiothreitol and 2 µl restriction enzyme (*Xba*I). The eppendorfs were then placed in a water bath at 37°C overnight. The restriction fragments were separated in 1% (w/v) agarose gel using a CHEF DR 111 (Bio-Rad). CHEF changes the direction of the electric field electronically to reorient the DNA by changing the polarity of an electrode array. The field strength was 6V/cm in recirculating 0.5X Tris-borate-EDTA buffer, pH 8.2. The running time was 16-18hr. The gel was stained with ethidium bromide (0.5 µg/ml) for 45min and analyzed using Bionumerics software. The PFGE patterns were examined, according to criteria outlined in Tenover²⁹¹.

2.6 Complementation

2.6.1 Preparing competent *Pseudomonas aeruginosa* cells

A number of colonies of the *P. aeruginosa* are inoculated into 5 ml of MH broth, grown overnight in shaking incubator at 37°C. After 18 h, 1 ml of overnight culture is inoculated into fresh MH broth (in 2 L culture flask) and grown in shaking incubator at 37°C for 2.5-3.5 h (log phase, 0.3-0.5 OD). After this time the culture is transferred into 50ml flask (which has kept on ice) and centrifugated at 7000g for 10min at 4°C. The

supernatant is drained and the cells are resuspended in 50ml of 300mM sucrose and centrifugated at 7000g for 10 min. The supernatant is drained and the cells are washed in 25ml of 300mM sucrose, the cells are centrifugated at 7000g for 10min. The supernatant is drained and the cells are resuspended in 500µl sucrose. Aliquots of 40µl are stored on ice for 10min and then stored long term at -80°C, ²⁹².

2.6.2 Preparing *Pseudomonas aeruginosa* cells for transformation

The 40 µl cell suspension is chilled on ice for 30 min, 5 µl of purified DNA (containing vector) is added to the cell suspension and transferred to a chilled 0.2 cm gap cuvette. The cuvette is placed in the Bio-RAD GenePulser®, the volts are set to 2.5, and ideally time constant is 4.3-4.8. Following delivery of the pulse, the cells are mixed with 3 ml MH broth and transferred to the shaking incubator, 37°C for 2 h. The cells are then plated onto MH plates containing 200 µg/ml carbenicillin. Each isolate to be examined is done in triplicate independent culture, component cells of the test isolate are plated out on 3 carbenicillin containing MH plates, the test isolate containing the vector are plated out onto 3 carbenicillin (200 µg/ml) containing MH plates, test isolate containing the plasmid of interest are plated out onto 3 carbenicillin (200 µg/ml) containing MH plates.

**Chapter 3 Comparative Phenotypic Analysis of
Antimicrobial Susceptibility in *Pseudomonas
aeruginosa* isolates from Non Cystic Fibrosis and
Cystic Fibrosis patients**

3.1 Introduction

The purpose of antimicrobial susceptibility testing is to assess the level of effectiveness a given antimicrobial has on a microorganism *in vitro*, thereby allowing the pathogen to be classified as susceptible, intermediate or resistant to the antimicrobial tested. This assists clinicians in the selection of appropriate antimicrobial therapy for patient care. A common way of assessing the effectiveness of an antimicrobial against bacteria is to determine the minimum inhibitory concentration (MIC). The MIC is defined as the minimum concentration of an antimicrobial agent, which is required to inhibit growth of the organism. Agar dilution and broth dilution are the most commonly used techniques to determine the MICs of antimicrobials ²⁹³. Agar dilution involves the serial dilution (usually log₂ serial dilutions) of an antimicrobial which is then incorporated into agar medium; the result is each plate contains a different concentration of the antimicrobial. A standardised bacterial suspension is then applied rapidly to the agar surfaces and incubated overnight at 37°C, after which time the MIC is read.

Phenotypic susceptibility testing methods frequently used in diagnostic and research laboratories include; disc diffusion, Etest and automated systems such as Vitek 2 (bioMérieux, Marcy-l'Etoile, France) and Phoenix (BD Diagnostics System, Sparks, MD). In general, these techniques are less time consuming and are favoured in a busy diagnostic laboratory ²⁹⁴. Vitek 2 is the automated system currently used in the diagnostic microbiology laboratory in St. James's Hospital (SJH). The Vitek 2 is an integrated system that automatically performs rapid identification using algorithms and antimicrobial susceptibility testing (based on kinetic analysis of growth data) ²⁹⁵. It has an Advanced Expert System (AES) which analyses the antimicrobial susceptibility testing data using a knowledge base of 2000 phenotypes and 2000 MIC distributions. It also offers interpretative reading of susceptibility tests which aims to analyse susceptibility patterns so that underlying resistance mechanisms may be predicted ²⁹⁵.

MICs which are determined by susceptibility test methods are interpreted by specific breakpoints, the particular MIC that differentiates resistant, assumed untreatable from susceptible which are assumed treatable. Features that determine breakpoints are MIC distributions, pharmacodynamics, pharmacokinetics and the clinical outcome of an antimicrobial. A definition of resistance (above the MIC breakpoint) is associated with a very high likelihood of therapeutic failure, whereas a definition of susceptible is associated with a greater likelihood of therapeutic success.

The therapeutic outcome of isolates from patients which are defined as intermediate is uncertain ²⁹⁶. The accepted standards for these classifications are published by a number of different organisations such as the Clinical and Laboratory Standards Institute (CLSI) ²⁷⁰ in the USA, European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the British Society for Antimicrobial Chemotherapy (BSAC) ²⁷¹.

Pseudomonas aeruginosa is a leading cause of nosocomial infections and the most prevalent bacterial pathogen in cystic fibrosis (CF), and contributes to significant levels of morbidity and mortality ^{2,7,229,297-299}. Within SJH *P. aeruginosa* is one of the top five isolates recovered from respiratory specimens, urinary isolates and eye and throat specimens (data supplied by Surveillance Scientist, SJH). At a national level Ireland has one of the highest rates of CF in the world ³⁰⁰ with incidence at 1:1353 ²⁰. With this in mind, rapid identification and determination of the susceptibility profiles of these organisms is essential for effective management of infection.

The aim of the study presented in this chapter was to determine and compare the susceptibilities of a collection of *P. aeruginosa* isolates from non-cystic fibrosis population with those from a cystic fibrosis population to a panel of antimicrobials commonly used to treat such infections. CLSI guidelines were used for all antimicrobials with the exception of colistin disc testing where BSAC guidelines were used. Agar dilution and disc testing were used as reference methods when comparing Vitek 2 system.

3.2 Results

3.2.1 Collection and identification of clinical isolates of *P. aeruginosa*

The 194 non-duplicate *P. aeruginosa* clinical isolates were collected from the microbiology laboratory, St. James's Hospital (SJH) between May 2006 and August 2008 as a prospective study. A total of 99 non-duplicate *P. aeruginosa* clinical isolates from adult cystic fibrosis patients were collected from the National Adult Referral Centre, St. Vincent's University Hospital (SVH) between June 2006 and July 2007. The isolates were confirmed as *P. aeruginosa* by standard laboratory techniques and identification using the API 20NE tests (Fig. 3.1).

3.2.2 Clinical data from patients colonised by *P. aeruginosa*

Demographic data from NCF patients (n=194) and CF patients (n=99) was gathered with the help of the Surveillance Scientist at SJH and Deirdre Keating (Department of Microbiology, SVH). In the NCF population the average patient age was 62 years: 5% were aged 16-26 years, 10% were aged 27-37 years, 14% were aged 38-48 years, 10% were aged 49-59 years, 13% were aged 60-70 years, 35% were aged 71-81 years and 13% were aged 82 + years. Among the CF collection the average patient age was 28 years: 51% were aged 16-26 years, 38% were aged 27-37 years and 11% were aged 38-48 years. In the NCF population 41% of isolates were hospital-acquired (HA), 16% were healthcare-associated (HCA) and 43% were community acquired (CA). An isolate was considered HA (SJH definition) if it was recovered from an inpatient > 48 hours in after administration to SJH. An isolate was considered HCA (SJH definition) if it was recovered from a patient with one of the following risk factors: i) inpatient in SJH in the previous 90 days, ii) outpatient in SJH in the previous 30 days, iii) referred or transferred from another hospital, iv) resident in a nursing home. Isolates were defined as CA (SJH definition) if they did not fall into HA or HCA category.

Primary sources for the NCF isolates included respiratory tract sputum (50.5%), wound swabs (41%), mid stream urine (6%), bronchoalveolar lavage (2%), bone (0.5%), (Table 3.1). The CF isolates from SVH originated from the CF reference

laboratory. All of the CF isolates (n=99) were isolated from respiratory tract, with 41% of the isolates being mucoid and 59% been non-mucoid, (Fig. 3.2).

Table 3.1 Primary sources of NCF *P. aeruginosa* isolates

Primary source	HA ^a n=80	HCA ^b n=30	CA ^c n=84
Respiratory tract sputum	52	11	35
Wound swabs	24	18	38
Mid Stream Urine	0	1	10
Bronchoalveolar Lavage	3	0	1
Bone	1	0	0

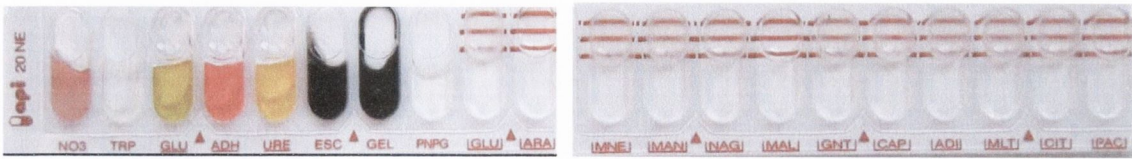
^aHA: hospital acquired, ^bHCA: healthcare-associated, ^cCA: community-acquired.

3.2.3 Determination of antimicrobial susceptibilities

A phenotypic screen was preformed to determine the susceptibility patterns of each isolate to twelve antimicrobial agents from six different antimicrobial classes. Agar dilution and double-disc test were performed in accordance with CLSI guidelines, as outlined in Section 2.2. Selection of the most appropriate antimicrobials was achieved in consultation with The Empiric Antimicrobial Guidelines of the Prescriber’s Guide, along with the CLSI recommendations for *P. aeruginosa* infections. An isolate was considered resistant if it fell into the intermediate or resistance categories as defined by CLSI or BSAC ²⁷⁰⁻²⁷¹ .

3.2.3.1 Minimum inhibitory concentrations

The MIC for each antimicrobial tested using the agar dilution method to the NCF and CF *P.aeruginosa* collection are summarised in Table 3.2 and Table 3.3 respectively. MIC₅₀ and MIC₉₀ values were calculated as outlined in chapter two. The rates of resistance to each antimicrobial are shown in Fig 3.3. The Fisher's test was chosen as it always gives the exact P value, while the chi-square test only calculates an



NO ³	TRP	GLU	ADH	URE	ESC	GEL	PNP	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OX
1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
+	-	-	+	-	+	+	-	+	-	-	+	+	-	+	+	+	+	-	-	+
1			5			5			4			5			7			4		

Fig 3.1 Identification of *P. aeruginosa* by the API 20NE system. Identification of NCF.2006.54 by the API 20NE system. The seven digit code, 1554575, corresponds to *P. aeruginosa*. Substrates: NO³ potassium nitrate, TRP : tryptophan, GLU: glucose, ADH: arginine, URE: urea, ESC: esculin, GEL: gelatine, PNP: p-nitro-phenyl-βD-galactopyranoside, GLU: glucose, ARA: arabinose, MNE: gmannose, NAG: N-acetyl-glucosamine, MAL: maltose, GNT: gluconate, CAP: caprate, ADI: adipate, MLT: malate, CIT: citrate, PAC: phenyl-acetate and OX: tetramethyl-p-phenylene diamine).

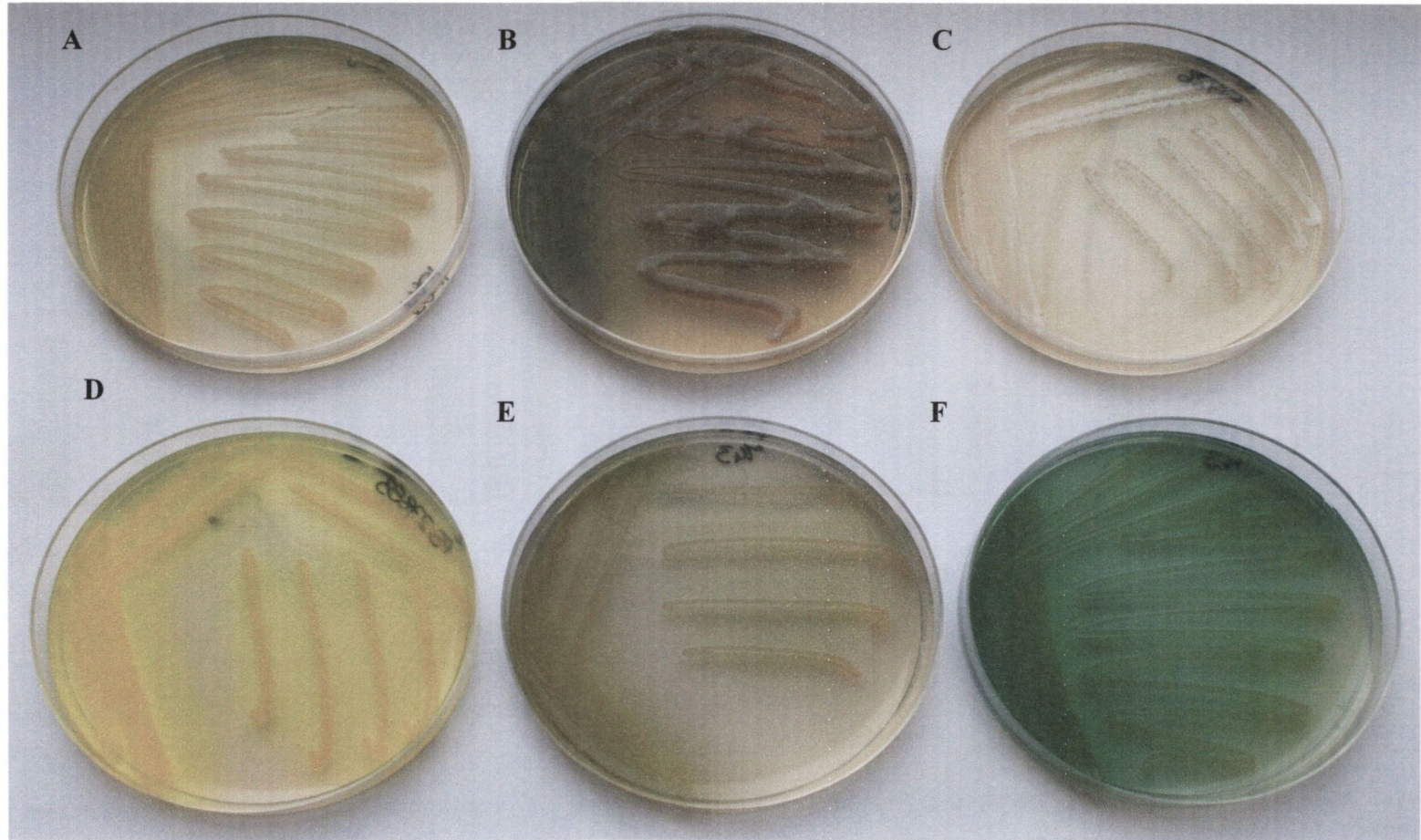


Fig 3.2 A representative collection of *P. aeruginosa* isolates. (A) PAO1, (B) Mucoid CF.2006.196, (C) Non-Mucoid CF.2008.285, (D) *P. aeruginosa* ATCC 27853, (E) NCF.2006.100, (F) NCF.2006.67.

approximate P value. In the NCF population the rate of resistance was lowest for cefepime with levels of resistance at 3%.

Table 3.2 Minimum inhibitory concentrations of antimicrobials against *P. aeruginosa* NCF collection

Antimicrobial	Range	Breakpoint			MIC ₅₀	MIC ₉₀
	mg/L	CLSI			mg/L	mg/L
		mg/L				
		R	I	S		
Piperacillin	0.06-256	≥128		≤64	16	64
Ceftazidime	0.06-128	≥32	16	≤8	4	32
Imipenem	0.06-128	≥16	8	≤4	4	32
Meropenem	0.06-128	≥16	8	≤4	2	16
Gentamicin	0.06-128	≥16	8	≤4	2	32
Amikacin	0.06-128	≥64	32	≤16	4	16
Ciprofloxacin	0.06-128	≥4	2	≤1	0.5	8
Levofloxacin	0.06-128	≥8	4	≤2	1	16
Colistin	0.06-128	≥8	4	≤2	1	2

The second most effective antimicrobial against the NCF population was piperacillin-tazobactam. Amikacin was the third most efficacious antimicrobial against the NCF population; the MIC₅₀ and MIC₉₀ were within two doubling dilutions of each other and still within the sensitive range according to CLSI guidelines. The other aminoglycoside gentamicin was not as effective against NCF isolates, with levels of resistance to the antimicrobial at 35%. The MIC₅₀ of gentamicin was in the range of sensitive, its MIC₉₀ was in the range of resistance.

The β-lactam, piperacillin had MIC₅₀ and MIC₉₀ values within the susceptible range and levels of resistance at 19%. The carbapenem meropenem was more potent against the NCF collection than imipenem which was the least effective antimicrobial against NCF population with level of susceptibility at 42%. There was a three doubling dilution difference in imipenem between the MIC₅₀ and MIC₉₀ with the MIC₉₀ values falling into the resistant category.

The cephalosporins ceftazidime and cefepime were effective against the NCF collection with resistance at 26% and 3% respectively.

The fluoroquinolones levofloxacin and ciprofloxacin were equally effective against the NCF collection. In both there was a four-fold doubling dilution difference between the MIC₅₀ and MIC₉₀, with the MIC₉₀ values for both antibiotics within the resistant range.

Table 3.3 Minimum inhibitory concentrations of antimicrobials to *P. aeruginosa* CF collection

Antimicrobial	Range	Breakpoint			MIC ⁵⁰	MIC ⁹⁰
	mg/L	CLSI			mg/L	mg/L
		mg/L				
		R	I	S		
Piperacillin	0.06-256	≥128		≤64	32	128
Ceftazidime	0.06-128	≥32	16	≤8	16	64
Imipenem	0.06-128	≥16	8	≤4	32	64
Meropenem	0.06-128	≥16	8	≤4	4	32
Gentamicin	0.06-128	≥16	8	≤4	8	64
Amikacin	0.06-128	≥64	32	≤16	16	64
Ciprofloxacin	0.06-128	≥4	2	≤1	1	4
Levofloxacin	0.06-128	≥8	4	≤2	4	16
Colistin	0.06-128	≥8	4	≤2	2	4

The MIC₅₀ and MIC₉₀ were higher in the CF population for all the antimicrobials in comparison to NCF isolates. The lowest rate of resistance in the CF population was for piperacillin-tazobactam, with levels of resistance at 22%. Colistin was the second most effective antimicrobial with levels of resistance at 30%. Of the two aminoglycosides tested, amikacin was more effective than gentamicin with levels of resistance at 55% and 83% respectively.

Between the two carbapenems tested, meropenem was more efficient than imipenem against the CF isolates (40% susceptible meropenem vs. 17% susceptible imipenem). The fluoroquinolones, ciprofloxacin and levofloxacin were equally effective against the CF isolates with levels of resistance at 63%. Of the two cephalosporins cefepime was more effective then ceftazidime against CF collection, which followed the

same trend as in the NCF population. The level of resistance to cefepime was 37% whereas with ceftazidime the level of resistance was 64% with MIC₅₀ and MIC₉₀ values of 16-64mg/L. A summary of the resistance profiles displayed by the MDR isolates is described in Table 3.4.

Table 3.4 Prevalence of MDR isolates according to resistance profile

Phenotypic characteristics	MDR isolates	
	NCF	CF
	(n=65)	(n=87)
<u>Resistant to three antimicrobial classes</u>		
FQ ^R , AMINO ^R ,CARBA ^R	7	6
FQ ^R ,AMINO ^R , POL ^R	0	2
FQ ^R , CARBA ^R , β-L/β-LI ^R	0	1
FQ ^R ,AMINO ^R ,PEN ^R	0	1
FQ ^R , AMINO ^R , CEPH ^R	1	1
FQ ^R , CARBA ^R , PEN ^R	1	0
FQ ^R , POL ^R , CARBA ^R	4	0
FQ ^R , AMINO ^R , β-L/β-LI ^R	0	1
FQ ^R , PEN ^R , β-L/β-LI ^R	0	0
AMINO ^R , POL ^R , β-L/β-LI ^R	0	1
AMINO ^R , POL ^R , CARBA ^R	1	0
AMINO ^R , CARBA ^R , PEN ^R	2	0
AMINO ^R , CARBA ^R , CEPH ^R	1	2
AMINO ^R , CARBA ^R , β-L/β-LI ^R	2	1
AMINO ^R , CEPH ^R , β-L/β-LI ^R	0	1
AMINO ^R , POL ^R , CARBA ^R	0	1
POL ^R , CARBA ^R , β-L/β-LI ^R	1	0
<u>Resistant to four antimicrobial classes</u>		
FQ ^R ,AMINO ^R ,CARBA ^R ,CEPH ^R	1	4
FQ ^R , AMINO ^R , POL ^R , PEN ^R	1	0
FQ ^R ,AMINO ^R ,CEPH ^R , PEN ^R	0	1
FQ ^R ,AMINO ^R ,POL ^R , CARBA ^R	1	2
FQ ^R , AMINO ^R , CARBA ^R ,β-L/β-LI ^R	3	0
FQ ^R , AMINO ^R , CARBA ^R ,CEPH ^R	2	0
FQ ^R , AMINO ^R , POL ^R ,β-L/β-LI ^R	1	0
FQ ^R , AMINO ^R , CEPH ^R ,β-L/β-LI ^R	0	1
FQ ^R , AMINO ^R , PEN ^R ,β-L/β-LI ^R	1	0
FQ ^R AMINO ^R CARBA ^R , PEN ^R	8	0
FQ ^R POL ^R CARBA ^R β-L/β-LI ^R	0	2

FQ ^R CARBA ^R CEPH ^R PEN ^R	0	1
FQ ^R CARBA ^R CEPH ^R β-L/β-LI ^R	1	0
AMINO ^R CARBA ^R CEPH ^R PEN ^R	1	4
AMINO ^R CARBA ^R PEN ^R β-L/β-LI ^R	1	0
AMINO ^R , CARBA ^R , CEPH ^R ,β-L/β-LI ^R	1	2
AMINO ^R , POL ^R , CARBA ^R ,β-L/β-LI ^R	1	2
AMINO ^R , POL ^R , CARBA ^R ,CEPH ^R	1	0
CARBA ^R , POL ^R , PEN ^R ,β-L/β-LI ^R	1	0
CARBA ^R , CEPH ^R , PEN ^R ,β-L/β-LI ^R	0	1
POL ^R , CARBA ^R , PEN ^R ,β-L/β-LI ^R	1	0
POL ^R , CARBA ^R ,CEPH ^R ,β-L/β-LI ^R	0	2
<u>Resistant to five antimicrobials</u>	0	0
FQ ^R ,AMINO ^R ,CARBA ^R ,CEPH ^R , β-L/β-LI ^R	1	1
FQ ^R ,AMINO ^R ,CARBA ^R ,PEN ^R , β-L/β-LI ^R	1	1
FQ ^R ,AMINO ^R ,POL ^R ,CARBA ^R , CEPH ^R	1	2
FQ ^R ,AMINO ^R ,CARBA ^R ,CEPH ^R , PEN ^R	0	5
FQ ^R ,AMINO ^R ,POL ^R ,CARBA ^R , β-L/β-LI ^R	0	1
FQ ^R ,POL ^R ,CARBA ^R ,CEPH ^R , β-L/β-LI ^R	0	3
FQ ^R ,POL ^R ,CARBA ^R ,CEPH ^R , PEN ^R	1	0
FQ ^R ,CARBA ^R ,CEPH ^R ,PEN ^R , β-L/β-LI ^R	3	3
AMINO ^R ,CARBA ^R ,CEPH ^R ,PEN ^R , β-L/β-LI ^R	0	3
AMINO ^R , POL ^R , CARBA ^R , PEN ^R , CEPH ^R	2	0
<u>Resistant to six antimicrobials</u>		
FQ ^R , AMINO ^R , CARBA ^R , CEPH ^R , PEN ^R , β-L/β-LI ^R	6	18
FQ ^R AMINO ^R , CARBA ^R , CEPH ^R , POL ^R , β-L/β-LI ^R	2	1
FQ ^N AMINO ^R , CARBA ^R , CEPH ^R , POL ^R , PEN ^R	0	1
AMINO ^R POL ^R , CARBA ^R , CEPH ^R , PEN ^R , β-L/ β-LI ^R	0	1
<u>Resistant to seven antimicrobials</u>		
FQ ^R , COL ^R , CARBA ^R , CEPH ^R , PEN ^R , AMINO ^R , β-L/β-LI ^R	2	7

FQ: fluoroquinolone (s), AMINO: aminoglycoside (s), CARBA: carbapenem (s), CEPH: cephalosporin (s), POL: Polymyxin, Colistin, PEN: penicillin and β-L/β-LI: β-lactam/β-lactamase inhibitor. R: resisitant

Extended-spectrum β-lactamase (ESBL) producing strains were identified using the double-disc confirmatory test method, Fig. 3.4. Overproduction of the chromosomal cephalosporinase AmpC was evaluated by the nitrocefin test, see Section 2.2.3.5. A total of 27 isolates (13.9%) from the NCF population were categorised as ‘susceptible’, 102 (52.6%) as ‘resistant’ and 65 (33.5%) as ‘multidrug-resistant (MDR)’ (resistant to ≥ three drug classes). All of the CF isolates (n=99) were resistant to at least one

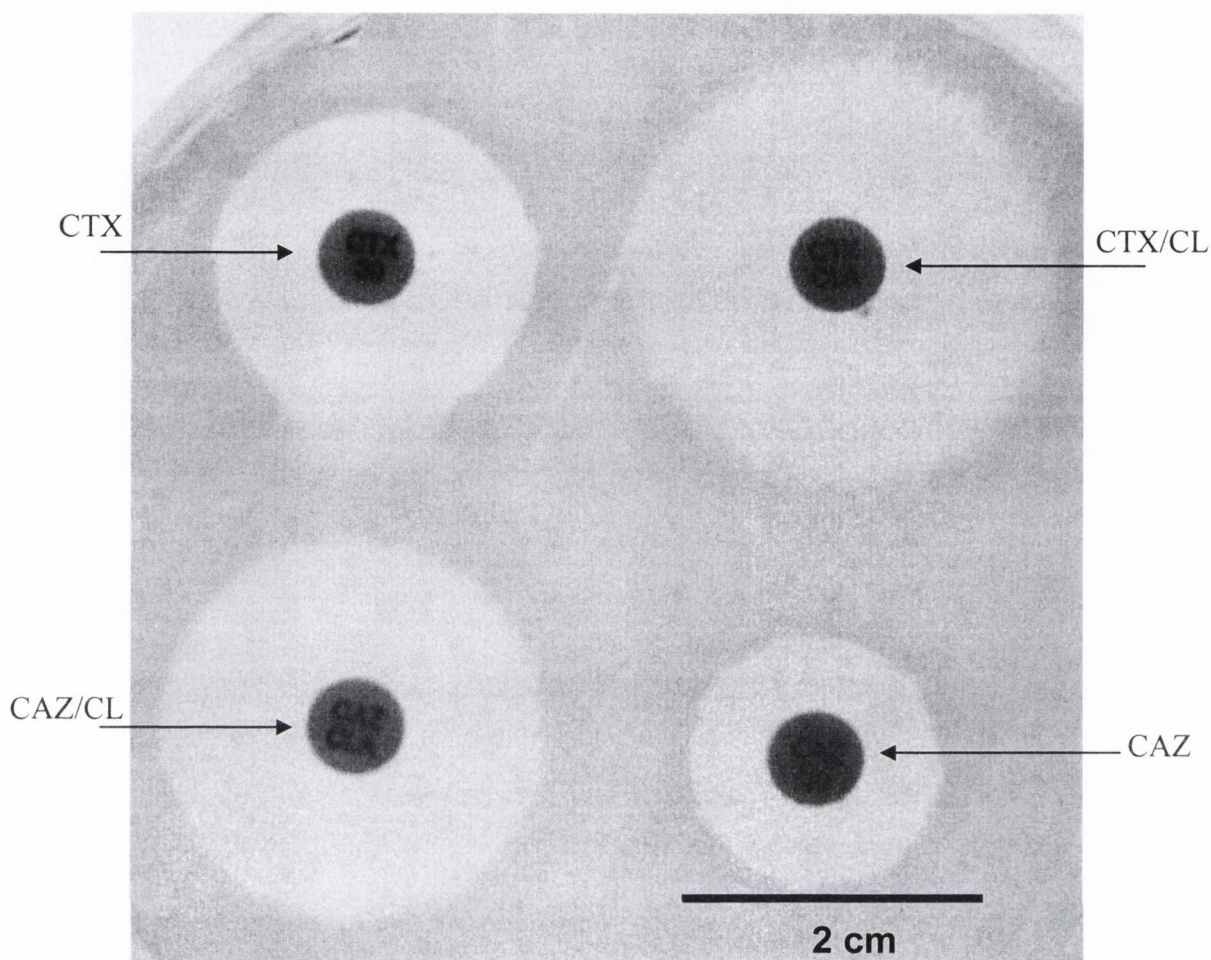


Fig 3.4 ESBL double disc testing. ESBL production was inferred in isolate NCF. 2006.110 using the double disk diffusion test. Since ESBLs in *P. aeruginosa* are notoriously difficult to detect due to the presence of an inducible AmpC enzyme and efflux pumps, cloxacillin (an inhibitor of the AmpC enzyme) and PA β N (efflux inhibitor) were included in the media. The antibiotic disks were placed 25-30 mm apart per plate. A positive test was recorded when the diameter of inhibition zone of cephalosporin in combination with clavulanic acid was ≥ 5 mm in comparison to that of the cephalosporin zone alone. CTX/CL: Cefotaxime/ Clavulanic acid. CTX: Cefotaxime. CAZ: Ceftazidime. CAZ/CL: Ceftazidime/ Clavulanic acid.

antimicrobial, a total of 12 isolates (12%) were resistant and 87 isolates (88%) were MDR.

Three isolates were confirmed as ESBL producers by the double-disc test method: NCF.2006.110, NCF.2007.142, and NCF.2008.175. All of these isolates were resistant to one or more cephalosporin. A total of thirty NCF isolates displayed derepression of chromosomal AmpC, of these isolates 73% were resistant to both imipenem and meropenem, 17% were resistant to imipenem and 10% were susceptible to both imipenem and meropenem. Forty six CF isolates displayed depression of chromosomal AmpC, 67% of these isolates were resistant to both imipenem and meropenem, 29% were resistant to imipenem while 4% were resistant to meropenem.

Isolates which were resistant to imipenem and/or meropenem were examined for metallo- β -lactamase (M β L) activity. An Etest M β L strip (AB BioDisc, Sweden) consisting of Imipenem (IP)/ Imipenem + EDTA (IPI) was used. In the NCF collection 12% of isolates and 30% of the CF isolates tested (imipenem, meropenum resistant) were shown to be positive for presence of M β Ls, (Fig 3.5).

3.2.3.2 Rates of resistance among NCF and CF isolates in relation to age

The rates of resistance among the NCF and CF population in relation to age are shown in Fig 3.6 and Fig 3.7. The levels of resistance across the age groups were higher in general in the CF collection compared to the NCF collection.

In the CF group, the highest levels of resistance to aminoglycosides, carbapenems and penicillins were seen in the 38-48 age group. The highest levels of resistance to the fluoroquinolones and cephalosporins were seen in the 27-37 age group. And the highest levels of resistance to the β -lactam/ β -lactamase inhibitor combination were seen in the 16-26 age group. Conversely in the 16-26 age group, was most susceptible to the carbapenems and cephalosporins. In the 27-37 age group the lowest levels of resistance seen were to the β -lactam/ β -lactamase inhibitor combination and the aminoglycosides. The 38-48 age group, was most susceptible to fluoroquinolones and colistin.

In the NCF population, the highest proportion of resistance was seen in the 49-59 age group, which had the highest levels of resistance to aminoglycosides, carbapenems, cephalosporins and β -lactam/ β -lactamase inhibitor combination. The

lowest level of resistance was seen in the 38-48 year group, which had the lowest level of resistance to β -lactam/ β -lactamase inhibitor combination, polymyxin and penicillins.

The highest level of resistance to fluoroquinolones was seen in the 82+ group, the lowest level of resistance to fluoroquinolones was seen in the 60-70 age group. The 60-70 age group also had the lowest level of resistance to the aminoglycosides. The lowest level of resistance to the carbapenems was seen in the 16-26 age group, however the highest level of resistance to cephalosporins was seen in this group. Similarly the lowest level of resistance to penicillins was seen in the 38-48 and 71-81 age group, but the highest level of resistance to polymyxins was seen in the 71-81 age group.

3.2.3.3 Antimicrobial susceptibility trends, comparisons in hospital acquired (HA), healthcare associated (HCA), community acquired (CA) NCF and CF *P. aeruginosa* isolates

The prevalence of antimicrobial resistance within isolates from the NCF collection, according to whether they were HA, HCA and CA compared to the CF isolates is illustrated in Fig 3.8. Overall, the levels of resistance were higher for all of the antimicrobials tested in the CF collection compared to the NCF collection.

Among the NCF isolates the highest rates of resistance for seven (ciprofloxacin, levofloxacin, meropenem, ticarcillin-clavulanic acid, piperacillin, colistin and piperacillin-tazobactam) out of the twelve antimicrobials tested were among the HA population, ranging from 6%-44%. Community acquired isolates had the highest rate of resistance for the other five antimicrobials (imipenem, gentamicin, ceftazidime, amikacin and cefepime), ranging from 5%-62%. Health-care associated and CA had a similar rate of resistance to amikacin.

The highest rates of resistance to imipenem, gentamicin and ceftazidime are in the CF and CA population. Fluoroquinolone, meropenem and ticarcillin-clavulanic acid resistance was highest in the CF and HA population. Amikacin resistance was highest in the CF followed by CA and HCA population. Piperacillin, piperacillin-tazobactam and colistin had the highest rate in CF isolates followed by HA isolates. Cefepime had the highest rate of resistance in the CF and CA population. The distribution of the multidrug resistant, resistant and susceptible phenotypes are shown in Table 3.5. There was an extremely significant difference in the rate of MDR ($P=0.0001$) between the NCF isolates and the CF isolates. Similarly there was also an extremely significant difference

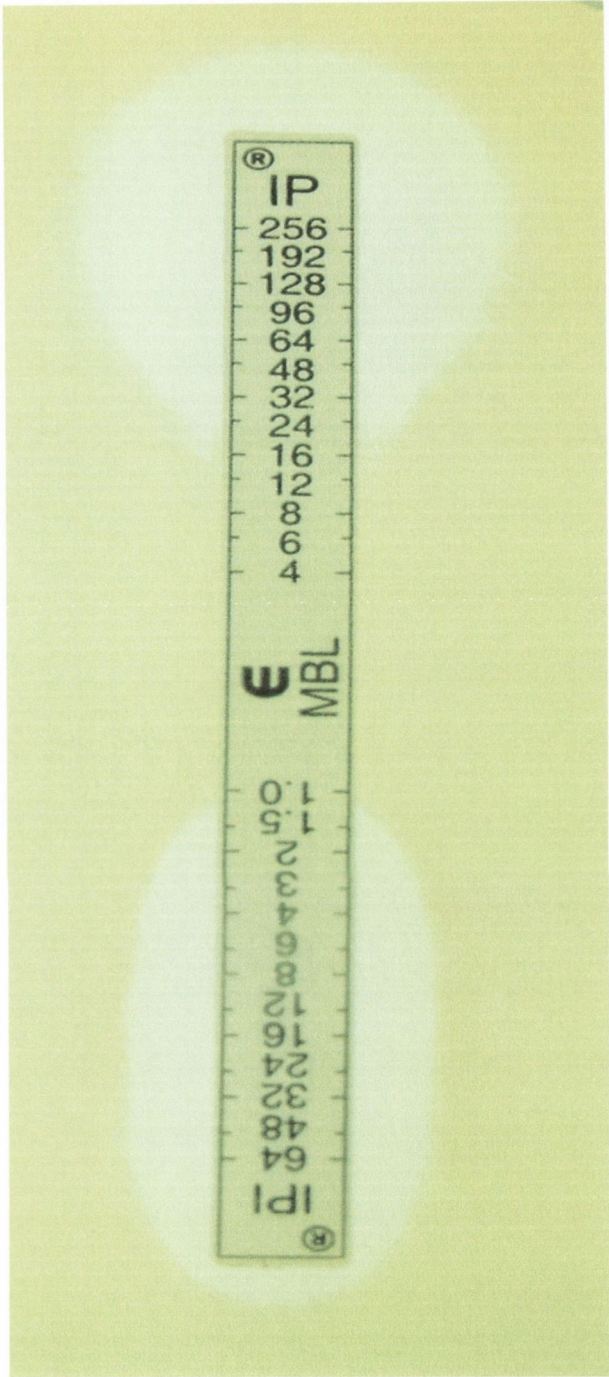


Fig 3.5 M β L production was inferred using the imipenem Etest. M β L test, containing imipenem (IP) and imipenem EDTA (IPI) isolate NCF.2006.1.

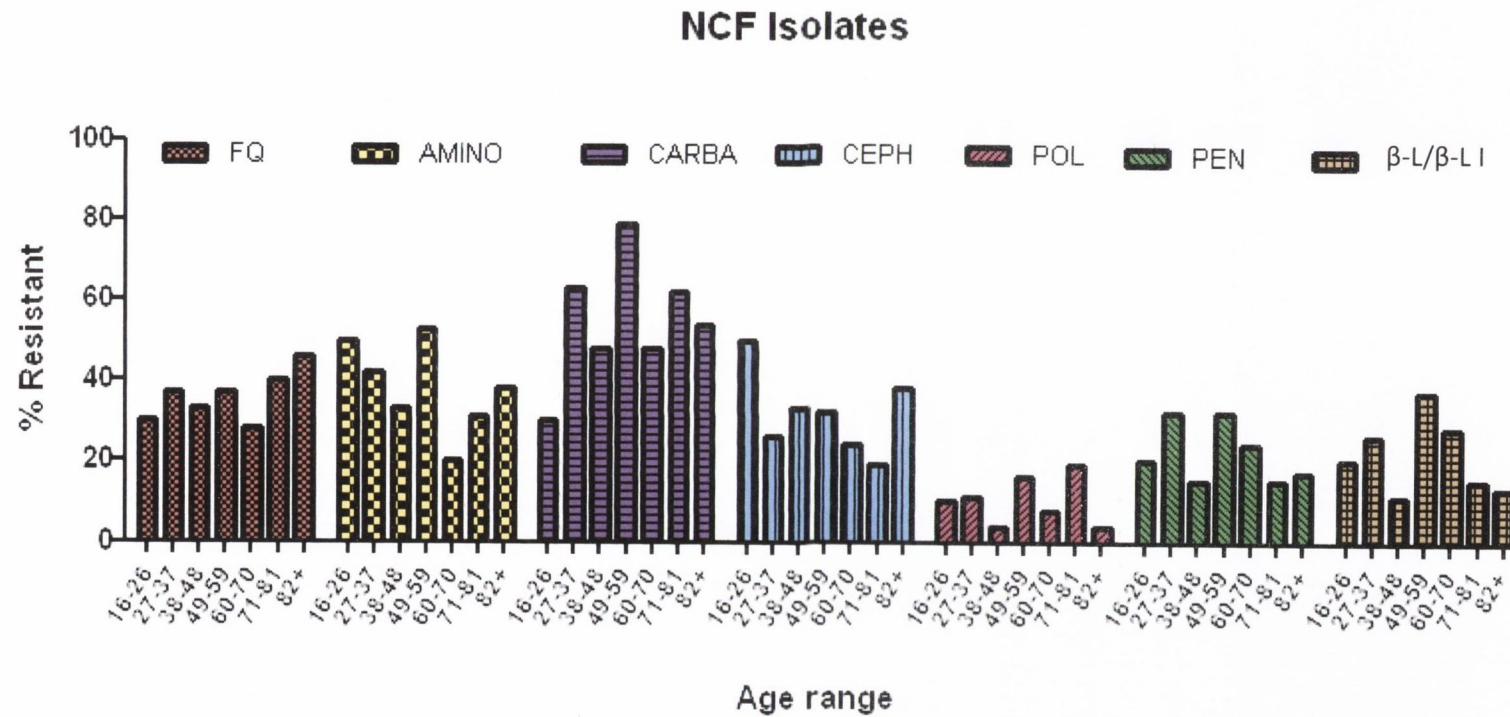


Fig 3.6 Rates of resistance in relation to age in NCF collection. FQ: Fluoroquinolones, AMINO: Aminoglycosides, CARBA: Carbapenems, CEPH: Cephalosporins, POL: Polymyxin, β-L/ β-L I: β-Lactam/ β-Lactamase Inhibitor combination.

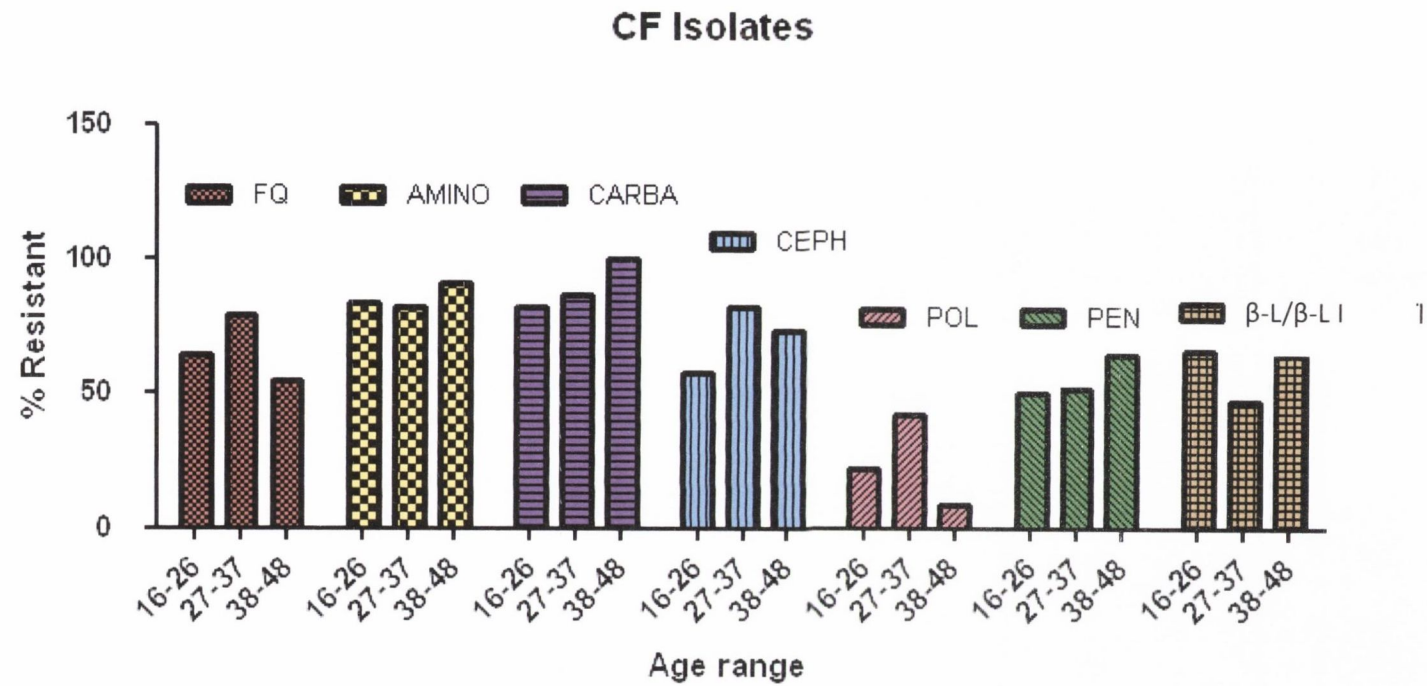


Fig 3.7 Rates of resistance in relation to age in CF collection. FQ: Fluoroquinolones, AMINO: Aminoglycosides, CARBA: Carbapenems, CEPH: Cephalosporins, POL: Polymyxin, PEN: Penicillin, β-L/ β-L I: β-Lactam/ β-Lactamase Inhibitor combination.

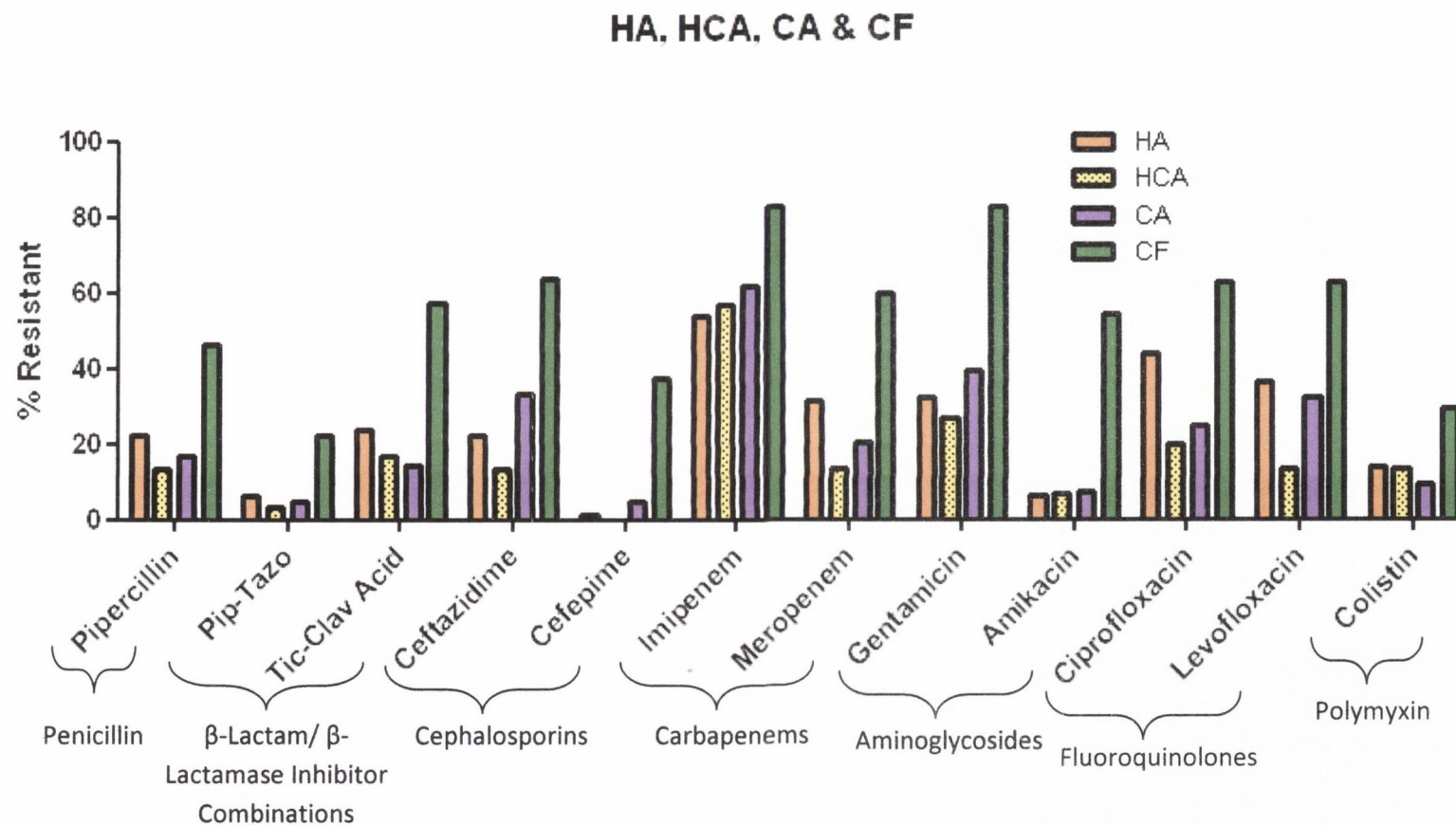


Fig 3.8 Comparison of antimicrobial resistance in hospital acquired (HA), health-care associated (HCA), community acquired (CA) and cystic fibrosis (CF) *P. aeruginosa* isolates.

in the rate of susceptible isolates in the NCF compared to CF population ($P=0.0001-0.0002$) between the NCF isolates and the CF isolates.

Table 3.5 Distribution of hospital-acquired (HA), healthcare-acquired (HCA) and community-associated (CA) NCF *P. aeruginosa* according to antimicrobial susceptibility category

Prevalence of characteristic, no (%) of isolates										
	HA n=80	HCA n=30	CA n=84	CF n=99	HA VS. HCA	HA VS. CA	HCA VS. CA	HA VS CF	HCA Vs. CF	CA Vs. CF
MDR	33	6	26	87	-	-	-	0.0001	0.0001	0.0001
R	37	18	47	12	-	-	-	-	-	-
S	10	6	11	0	-	-	-	0.0002	0.0001	0.0001

MDR: multi-drug resistant, R: resistant, S: susceptible. ^a *P* values (by Fisher’s exact test) are shown only where $P\leq 0.05$

3.2.3.4 Comparison of antimicrobial susceptibilities in CF collection based on mucoid or non-mucoid phenotype

The antimicrobial susceptibility profiles of mucoid and non-mucoid CF isolates are illustrated in Fig. 3.9. Overall, in the CF population the non-mucoid phenotype has a higher rate of resistance to all antimicrobials tested with the exception of piperacillin, where the rates of resistance are identical at 23% for both mucoid and non-mucoid morphologies. A greater number of non-mucoid isolates were MDR compared to mucoid isolates, Table 3.6. The entire CF collection originates from sputa specimens.

Table 3.6 Distribution of non-mucoid and mucoid CF isolates of *P. aeruginosa* according to antimicrobial susceptibility category

	Non Mucoid n=58	Mucoid n=41	<i>P</i> ^a comparison of NM vs. M
MDR	52	35	-
R	6	6	-
S	0	0	

MDR: multi-drug resistant, R: resistant, S: susceptible. ^a *P* values (by Fisher’s exact test) are shown only where *P*≤ 0.05

3.2.4 Comparison of antimicrobial susceptibility testing results based on testing procedures

Vitek 2 is an automated system routinely used in clinical laboratory for rapid microbial identification and antibiotic susceptibility testing. The system is used extensively in St. James’s Hospital diagnostic microbiology laboratory for *P. aeruginosa* isolates with the exception of mid-stream urine (MSU) isolates. Mid-stream urine isolates are routinely examined using disc diffusion methods. The Vitek 2 system is not used for the examination of CF isolates in St. Vincent’s hospital, disc diffusion tests are used.

3.2.4.1 Comparison of antimicrobial susceptibility testing results from Vitek 2 with the agar dilution and disc diffusion tests for NCF isolates

The antimicrobial susceptibility results determined by agar dilution and disc diffusion testing were compared to the Vitek 2 (bioMérieux, Mary-l’Etoile, France) automated system for 158 NCF isolates. MSU isolates were not included, Vitek results for the remaining NCF isolates (n=27) were not available. The results from the Vitek 2 system for seven antimicrobials were compared based on antimicrobial susceptibility categories as defined by CLSI guidelines, resistant, intermediate and susceptible, Table 3.7.

When calculating the very major (VM) and major error (M), the total number of tested isolates was used as denominator. The acceptability criteria of ≤3% for VM and ≤10% for a combination of M and minor errors (m) were applied. The correlation

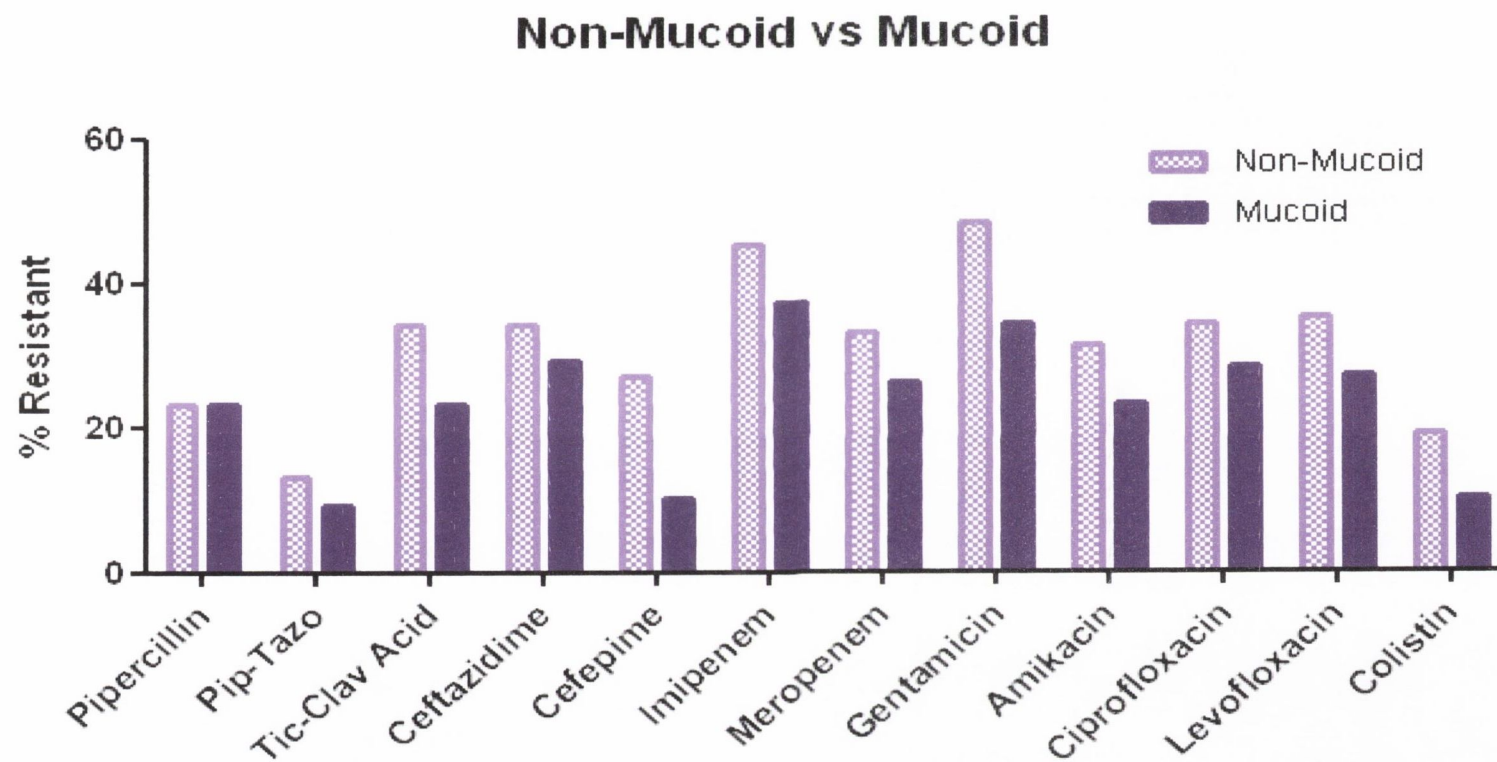


Fig 3.9 Rates of antimicrobial resistance, a comparison between mucoid and non-mucoid cystic fibrosis (CF) *P. aeruginosa* isolates n=99. The rates of resistance to cefepime were significantly different ($P = 0.0347$) and extremely significantly different for gentamicin ($P = 0.0001$) between non-mucoid and mucoid isolates.

agreement should be >90% for the accuracy to be acceptable³⁰¹⁻³⁰³. Overall there was 83% correlation between the Vitek 2 system and the reference methods (agar dilution). Very major errors indicating false susceptibility were at 6.6%, major errors indicating false resistance were at 1.8% and minor errors (susceptible/intermediate and resistant/intermediate discrepancies) were at 8.9%.

Two disc diffusion methods were used as reference methods, piperacillin/tazobactam and cefepime, they correlated 94% and 87% respectively. The remaining five antimicrobials were examined using agar dilution as the reference method, correlations ranged from 65-92%. Very major errors ranged from 13.9% for ciprofloxacin to 1.3% for amikacin. Major errors ranged from 0.6% for amikacin and ceftazidime to 3.8% for cefepime, Table 3.7. Minor errors ranged from 0% for piperacillin/tazobactam to 23% for gentamicin.

The overall best correlation was 94% for piperacillin/tazobactam. The worst correlation was for gentamicin, this is mainly due to the high number of minor errors (23%).

The antimicrobial susceptibilities for MSU isolates are routinely determined in the Clinical Laboratory in SJH using disc diffusion methods. Of the eleven MSU isolates included in this study, disc diffusion results from the Clinical Laboratory in SJH were only available for ten of the isolates. These results were compared with agar dilution (reference method) for three antimicrobials, ciprofloxacin, meropenem and gentamicin. There was a 10% correlation for ciprofloxacin between disc diffusion methods and the reference method. Very major errors, false susceptibility, for ciprofloxacin were at 20%. Major errors, false resistance were at 70%. There was a no correlation between disc testing methods and agar dilution. Major errors, false resistance, were at 90%. Correlation for gentamicin was at 20%, with 80% major errors.

Table 3.7 Comparison of susceptibility results determined by agar dilution and disc diffusion compared to Vitek 2 for NCF (n=158) *P. aeruginosa* isolates

Antimicrobial	Method ^a	Interpretation ^b			No. (%) errors ^c			Correlation ^d %	<i>P</i> value ^e
		R	I	S	VM	M	m		
Piperacillin-Tazobactam	Vitek 2	4	0	154	6	4	0	94	-
	Reference	8	0	150	(3.8)	(2.5)			
Ceftazidime	Vitek 2	16	9	133	12	1	13	84	-
	Reference	28	11	119	(7.6)	(0.6)	(8.2)		
Cefepime	Vitek 2	9	15	134	0	6	15	87	-
	Reference	6	1	151	0	(3.8)	(9.5)		
Meropenem	Vitek 2	17	4	137	16	2	8	84	0.0068
	Reference	31	10	117	(10)	(1.3)	(5)		
Gentamicin	Vitek 2	13	18	127	16	3	37	65	0.0005
	Reference	31	29	98	(10)	(1.9)	(23)		
Amikacin	Vitek 2	4	5	149	2	1	10	92	-
	Reference	4	5	149	(1.3)	(0.6)	(6.3)		
Ciprofloxacin	Vitek 2	23	13	122	21	4	17	73	-
	Reference	44	6	108	(13.2)	(2.5)	(10.8)		

^aReference: agar dilution or disc diffusion method, ^bDefined by CLSI, R: resistant, I: intermediate, S: susceptible, ^cVM: very major errors (false susceptibility), M: major error (false resistance), m: minor error (susceptible/intermediate and resistant/intermediate discrepancies), ^dCalculated by dividing the number of correct correlations by the total number of test, ^e*P* values (by Fisher's exact test) are a comparison between the two antimicrobial susceptibility testing methods with regards to resistant/intermediate results and susceptible results, and are shown only where *P* ≤ 0.05.

3.2.4.2 Comparison of susceptibility results determined by agar dilution compared to disc diffusion results for CF isolates

Disc diffusion testing methods are routinely used to determine antimicrobial susceptibility for CF *P. aeruginosa* isolates in SVH. This technique is preferred due to the slow growth of CF *P. aeruginosa* isolates. The disc diffusion results from SVH

microbiology laboratory were compared with agar dilution methods. Overall there was a 55.5% correlation between disc diffusion and the reference method (agar dilution). There was an extremely significant difference ranging from ($P = 0.0001-0.0005$) between disc testing and agar dilution. Very major errors (false susceptibility) were at 16.5%, major errors (false resistance) were at 4.2% and minor errors were at 24%, Table 3.8.

The best correlation was seen in colistin (74%). The worst correlation was seen in ciprofloxacin (36%), with very major errors of 14.1%, major errors of 8% and minor errors of 41.4%. There was an extremely significant difference, ranging from ($P=0.0001-0.0068$) between the disc diffusion method and the agar dilution method for all of the antimicrobials included.

Table 3.8 Comparison of susceptibility results determined by agar dilution compared to disc diffusion in CF (n=99) *P. aeruginosa* isolates

Antimicrobial	Method ^a	Interpretation ^b			No. (%) errors ^c			Correlation ^d	<i>P</i> value ^e
		R	I	S	VM	M	m	%	
Ceftazidime	Disc	31	0	68	23	5	19	53	0.0001
	Reference	43	20	36	(23.2)	(5)	(19.1)		
Meropenem	Disc	28	0	71	20	2	11	67	0.0001
	Reference	44	15	40	(20.2)	(2)	(11.1)		
Gentamicin	Disc	33	7	59	23	6	32	38	0.0001
	Reference	52	30	17	(23.2)	(6)	(32.3)		
Amikacin	Disc	28	1	70	9	3	23	65	0.0005
	Reference	32	22	45	(9)	(3)	(23.2)		
Ciprofloxacin	Disc	19	0	80	14	8	41	36	0.0001
	Reference	20	42	37	(14.1)	(8)	(41.4)		
Colistin	Disc	5	50	94	9	1	16	74	0.0001
	Reference	13	16	70	(9)	(1)	(16.1)		

^aReference: agar dilution or disc diffusion method. ^bDefined by CLSI, R: resistant, I: intermediate, S: susceptible. ^cVM: very major errors (false susceptibility), M: major error (false resistance), m: minor error (susceptible/intermediate and resistant/intermediate discrepancies). ^dCalculated by dividing the number of correct correlations by the total number of test. ^e*P* values (by Fisher's exact test) are a comparison between the two antimicrobial susceptibility testing methods with regards to resistant/intermediate results and susceptible results, and are shown only where $P \leq 0.05$.

3.2.5 Antimicrobial susceptibilities in relation to antimicrobial consumption rates at St. James's Hospital

The rates of antimicrobial resistance were examined in relation to the rates of antimicrobial consumption in SJH for hospital acquired (HA) isolates. The rates of antimicrobial consumption are expressed as defined daily doses per 100 occupied bed days; these data were provided by Colm Mc Donald, Antimicrobial Stewardship Pharmacist, SJH. The results are presented in Table 3.9. Unfortunately the dispensary computer system does not record antimicrobials under individual patients, only by ward. So it is not possible to retrospectively examine the amount of certain antimicrobial dispensed for *P. aeruginosa*, in this regard the data is limited. Furthermore, these data only includes hospital prescribing antimicrobial rates and therefore only relates to hospital acquired *P. aeruginosa* isolates. With this in mind resistant hospital acquired isolates are only included in Table 3.9.

The Table 3.9 illustrates the increased rate of consumption of piperacillin-tazobactam, imipenem, meropenem and gentamicin from 2006 to 2007 to 2008. Table 3.10 illustrates the levels of MDR resistance among the HA isolates, there is an increase in MDR from 2006-2008.

Imipenem in SJH is prescribed as imipenem/cilastatin (Primaxin), which contains equal quantities of imipenem and cilastatin. Imipenem is the active antimicrobial agent, if administered alone it is rapidly degraded and can cause kidney damage, cilastatin which has no antimicrobial activity acts to block the renal enzymes. The rate of resistance amongst the HA isolates against imipenem decreased from 2006-2007, at the same time there was an increase rate of consumption of imipenem from 0 in 2006 to 0.01564 DDD/100 OBD it would appear that imipenem was initially effective. From 2007-2008 there was an increase in consumption of imipenem which coincided with an increase rate of resistance among the HA isolates, there was also an increase rate of MDR HA isolates from 2006-2007-2008. For imipenem it would appear that the initial increase in consumption had the negative effect on the bacteria, increase consumption may have contributed to the emergence of increased levels of resistance.

From 2006-2008 there was a steady increase in the rate of consumption of meropenem, this coincided with an overall increase rate of resistance. Piperacillin-tazobactam consumption increased from 4.6170-6.1643 DDD/100 OBD from 2006-2008, the rates of resistance increased from 2006-2007 but returned to 2006 levels in

2008. It is a similar case for gentamicin, where levels of consumption increased from 0.80-1.642 DDD/100 OBD from 2006-2008. Levels of resistance initially increased but then decreased. It appears that lower levels of consumption stimulated resistance among the HA isolates but increased levels of consumption appeared to be effective, with levels of resistance decreasing. The amount of ticarcillin-clavulanic acid, ceftazidime, ciprofloxacin and levofloxacin dispensed fell from 2006-2008. Decreasing levels of consumption of levofloxacin coincided with decreasing levels of resistance among the HA isolates. For ticarcillin-clavulanic acid, ciprofloxacin and ceftazidime decreasing levels on consumption actually resulted in increased levels of resistance from 2006-2007, in 2008 the levels of resistance decreased. It appears that the initial reduction of consumption of these antimicrobials did not have an immediate effect on the levels of resistance.

Cefepime and piperacillin were not dispensed in 2006, 2007 or 2008. Yet there was an increase in the rates of resistance to piperacillin, this could be due to the increase levels of MDR isolates from 2006-2008.

From 2006-2007 the rate of consumption of amikacin increased from 0.0325-0.1654 DDD/100 OBD, the rate of resistance also increased during this time period. When the rate of consumption decreased from 2007-2008 the levels of resistance also decreased.

The reduction in the amount of colistin dispensed from 2006-2007 correlated with the reduction of resistance in the HA isolates. In 2008 there was an increase in the amount of colistin dispensed with correlates with an increase in resistance.

Table 3.9 Rates of antimicrobial consumption at St. James’s Hospital in comparison to rates of resistance from HA NCF *P. aeruginosa* isolates

Antimicrobial	2006	2007	2008	Resistance (%) ^b		
	DDD/100 OBD ^a	DDD/100 OBD	DDD/100 OBD	2006		
				n=32	n=22	n=16
Piperacillin	0	0	0	4 (13)	7 (32)	7 (44)
Piperacillin/ tazobactam	4.6170	5.4776	6.1646	2 (6)	2 (9)	1(6)
Ticarcillin/ clavulanic acid	0.0085	0.0024	0.0005	6 (19)	8 (36)	5 (31)
Ceftazidime	0.0664	0.0609	0.0583	8 (25)	6 (27)	4 (25)
Cefepime	0	0	0	0	1 (5)	0
Imipenem	0	0.0156	0.0422	20 (63)	12 (55)	11 (69)
Meropenem	1.4939	2.0381	2.5049	9 (28)	10 (45)	7 (44)
Gentamicin	0.8077	1.2873	1.6428	9 (28)	11 (50)	6 (38)
Amikacin	0.0325	0.1654	0.1353	1 (3)	3 (14)	1 (6)
Ciprofloxacin	10.4554	10.0266	5.5117	15 (47)	12 (55)	8 (50)
Levofloxacin	0.6760	0.3774	0.1372	16 (50)	10 (46)	3 (19)
Colistin	0.0648	0.0474	0.0801	5 (16)	3 (14)	5 (31)

^aDDD/100 OBD: Defined daily dose/100 occupied bed days. Red indicates an increase, yellow a decrease. ^bResistant, includes isolates which are fully resistant and intermediately resistant according to CLSL guidelines. Isolates included HA (n=80).

Table 3.10 Rates of MDR among the HA NCF *P. aeruginosa* isolates

HA isolates n= 70	2006 n=32 (%)	2007 n=22 (%)	2008 n=16 (%)
MDR	41	50	56

3.3 Discussion

The emergence of *P. aeruginosa* as a major opportunistic pathogen is a consequence of its ability to survive on minimal nutritional requirements and to tolerate a variety of physical and chemical conditions ^{14,304-305}.

Pseudomonas aeruginosa is a prevalent cause of nosocomial infection and a predominant cause of chronic infection, ultimately leading to increased morbidity and mortality in cystic fibrosis patients worldwide ^{298,306-308}. With this in mind, the monitoring and reporting of antimicrobial resistance is essential in order to provide an informed guide for antimicrobial therapy. Early diagnosis and appropriate antimicrobial therapy are essential factors in reducing the acquisition and spread of resistance genes in bacterial pathogens, thereby improving the prognosis of the patient and the overall cost of treatment ³⁰⁹⁻³¹¹. The life expectancy of patients with CF has steadily improved over the last number of decades, so much so that it is now estimated that a child born today with CF will survive into their late forties ²¹. The improved survival rate has been attributed to a number of factors, one of which is the effective use of antipseudomonal antimicrobial therapies ^{41,312-314}. However, due to prolonged and frequent antibiotic treatments antimicrobial resistant *P. aeruginosa* isolates have emerged. The prevalence of these resistant strains in the clinical setting have so far only been looked at in a relatively small number of European studies in recent years ^{256-258,315}.

Overall the levels of resistance were higher for all antimicrobials tested in the CF population as compared to the NCF population. High prescribing rates to piperacillin-tazobactam, meropenem, ciprofloxacin and levofloxacin (which were routinely prescribed in SJH) corresponded to higher levels of resistance in the HA group compared to the HCA and CA group. Interestingly piperacillin resistance was highest in the HA group even though there was no reported dispensing of piperacillin from SJH pharmacy, one explanation for this is the higher rate of MDR isolates among the HA group followed by the CA and HCA group. Resistance to the cephalosporins (ceftazidime, cefepime), aminoglycosides (gentamicin, amikacin) and imipenem was highest in the CA group reflecting possible higher levels of consumption of these antimicrobials in the community.

The rate of MDR was highest in the CF population at 88%. The next highest level was seen in the HA isolates at 41% followed by the CA and HCA. The majority of CF isolates were MDR, whereas the majority of HA, HCA and CA isolates were classed

as resistant, at a rate of 46 %, 60% and 56% respectively. The development of MDR phenotype increases mortality and monetary costs associated with treating bacterial infection ^{198,316-317}. Based on these data MDR appears at a higher (but not statistically significant) rate in the HA group compared to CA and HCA group. There is an extremely significant level of MDR in the CF population compared to the NCF isolates (HA, HCA, CA). In the NCF population 40% of MDR isolates are resistant to four different drug classes, 31% are resistant to three drug classes, 14% to five drug classes, 12% to six drug classes and 3% to seven drug classes. The majority of NCF isolates which were MDR to four drug classes were resistant to a combination of fluoroquinolones, aminoglycosides, carbapenems and penicillins. Seven out of the 20 isolates which were MDR for three drug classes were resistant to fluoroquinolones, aminoglycosides and carbapenems. Six out of the eight isolates which were MDR to six drug classes were resistant for fluoroquinolones, aminoglycosides, carbapenems, cephalosporins, penicillin's and β -lactam/ β -lactmase inhibitor combinations. No distinct pattern was seen amongst the isolates MDR to five drug classes. In the CF group, 25% of MDR isolates were resistant to four drug classes followed by 24% to six drug classes, 22% to five drug classes, 21% to three drug classes and 8% to seven drug classes. A total of 18/21 isolates which were MDR for six drug classes were resistant to fluoroquinolones, aminoglycosides, carbapenems, cephalosporins, penicillin's and β -lactam/ β -lactmase combinations, similar to NCF population. The majority of CF isolates which were MDR to three drug classes were resistant to fluoroquinolones, aminoglycosides and carbapenems this is also similar to NCF population.

Collectively resistant levels in the NCF population to the twelve antimicrobials tested ranged from 3-59%. Comparison of the results from the NCF collection to other similar published studies, TEST (The Tigecycline Evaluation and Surveillance Trial) which looks at antimicrobial resistance among bacterial isolates with hospital infections from 266 centres in Asia/ Pacific rim, North America, Latin America and Europe (including Ireland) ²⁵². MYSTIC (Meropenem Yearly Susceptibility Test Information Collection) which looks at antimicrobial susceptibility in 40 European centres (not including Ireland) from patients hospitalized with serious infection ²⁴⁹, a Japanese study which looked at hospital 371 isolates²⁵⁰ and a collection of USA surveillance studies ^{251,255,259-262,318}, Table 3.11. EARSS was not included as only blood culture isolates are included in the data. It is also important to note that some studies did not use the same breakpoints as were used in this study.

Piperacillin resistance was higher in the NCF collection than the other studies including a UK study²⁵³. Piperacillin-tazobactam and cefepime resistance was lowest in the NCF collection compared to TEST, MYSTIC, Japanese, United States and significantly lower than the UK study where cefepime resistance was at 38.9 %. The rates of resistance to imipenem, meropenem and gentamicin were generally higher when compared to the MYSTIC, Japanese and USA surveillance studies. The levels of resistance to imipenem and meropenem were similar to the UK study, with gentamicin resistance lower in the NCF group. Amikacin resistance levels were similar to the levels found in the TEST European study and Japanese study. Levofloxacin resistance was also similar to the levels found in the TEST European study. Overall in the NCF group resistance was highest for imipenem (59%), this is a similar result to the Japanese study where resistance was highest for the antimicrobials tested.

Table 3.11 Rates of resistance among NCF *P. aeruginosa* isolates from hospitals and ICU's

Antimicrobials ^a	NCF	TEST ^c				MYSTIC ^d	Japan ^e	USA ^f	
	n=194	n=5128				n=1449	n=371	n=589	n=606
	2006-2008 % R ^b	2004-2006 % R				2002-2006 % R	2002- 2003 % R	2005 % R	2006 % R
		Asia/Pacific Rim	North America	Latin America	Europe				
Pipercillin	19						9.4		
Pip-Tazobactam	5	11.2	9.6	13.8	10.6	15.1		9	11
Ceftazidime	26	26.9	17.2	40.6	21.8	19.1	7.3	10	13
Cefepime	3	29.6	21.8	42.1	24.2			5	6
Imipenem	59	18.6	15.4	34	18	24.6	37.5	7	11
Meropenem	25					18.1	18.3	7	6
Gentamicin	35					29.3	21.8	12	12
Amikacin	6	14.3	2.8	28	6.5	24.2	6.5		
Ciprofloxacin	33					27.3	12.1	22	21
Levofloxacin	31	36.4	36.7	34.7	33		13.5	22	22

^a Colistin, Ticarcillin-clavulanic acid excluded from comparison as they weren't included in TEST, MYSTIC, Japan or USA study.

CLSI in used to interpret % resistant.

^b Based upon CLSI interpretive breakpoints.

^c TEST: The Tigecycline Evaluation and Surveillance Trial, nosocomial isolates from blood, respiratory tract, urine, skin, wound and body fluids²⁵².

^d MYSTIC: Meropenem Yearly Susceptibility Test Information Collection, nosocomial isolates from blood, sputum, urine, cerebrospinal fluid, swabs²⁴⁹.

^e Japan: Japanese study, clinical isolates, n = 199 from respiratory system, n = 59 from urine, n = 98 from pus or secretion, n =12 from blood and n = 3 from other²⁵⁰.

^f USA: ^{251, 255, 259-262, 318}.

There is evidence that *P. aeruginosa* can exist as a biofilm in the lungs of CF patients^{63,319-325}. A number of groups have hypothesised that biofilm susceptibility testing would be a more accurate measure of *in vivo* resistance for chronic *P. aeruginosa* infections³²⁶⁻³³⁰. With this in mind various groups have attempted to develop biofilm assays³²⁶⁻³³⁰. As yet there is a lack in clinical evidence to support the belief that treatment regimes based on biofilm susceptibility testing results have an effective clinical response.

During acute exacerbation of CF lung infection it has been suggested that two bacterial states exist in the CF lung. The first is a biofilm plaque; bacteria growing in biofilm are known to be more resistant to antimicrobials^{63,325}. This biofilm plaque acts as a reservoir for the second state, planktonic bacteria which 'bloom' from the biofilm⁸³. These planktonic bacteria can be treated effectively with antimicrobials, but the existence of a biofilm ensures the persistence of an infection even when a clinical response to antimicrobials is observed. Over time and exposure to antimicrobials these planktonic bacteria may also develop resistance which would result in exacerbation of CF lung infection. Based on the currently available data, agar dilution was chosen as the reference method for examining susceptibility patterns in the CF *P. aeruginosa* isolates.

The susceptibility profiles of the 99 CF isolates were compared with similar studies from Europe²⁵⁶⁻²⁵⁸, Table 3.12. Among the CF isolates in this study the highest rate of resistance was seen against imipenem and meropenem (83% resistant). The highest levels of resistance in the UK study were similarly seen to gentamicin and meropenem. Cephalosporin resistance was the most prevalent amongst the isolates from Turkey. Amikacin and ciprofloxacin were the least effective antimicrobials amongst the Italian patients. Lower levels of resistance were seen in the CF collection compared to the UK study with the exception of piperacillin and colistin. Overall the levels of resistance were higher for the antimicrobials tested compared to the Turkey and Italian study. Disc diffusion susceptibility testing is a method employed by SVH to determine the pattern of resistance. Based on these data, disc diffusion method showed unacceptable correlation agreement ranging from 67-65% for all of the antimicrobials examined. Very major errors ranged from 9-23%, which were well over the accepted $\leq 3\%$ limit²⁹⁴. A European consensus statement for antibiotic therapy against *P. aeruginosa* in CF was published in the year 2000³³¹. It recommended that disc diffusion method should be used as the standard for antimicrobial susceptibility. The data presented here suggest that the disc diffusion method is unreliable.

Table 3.12 Rates of resistance among CF *P. aeruginosa*

Antimicrobials ^a	CF	UK ^c	Turkey ^e	Italy ^g				
	n=99	n=315 isolates, 76 patients	n=49, 22 patients	n=1315,224 patients				
	2006-2007	2001-2008 % R ^d	2003	1993-1997				
	% R ^b		% R ^f	% R ^h				
				1993	1994	1995	1996	1997
Pipercillin	47	31		21	18	31.5	27.5	23
Pip-Tazobactam	22	73	14					
Tic-Clavulanic acid	58	77						
Ceftazidime	64	77	39	23.2	18.4	21.7	24.8	15.5
Cefepime	37		39					
Imipenem	83		18	13.4	11.8	23.2	23.3	26.8
Meropenem	60	72	16					
Gentamicin	83	79						
Amikacin	55	69	20	29.1	34	60.1	70.3	37.8
Ciprofloxacin	63	70	39	29.1	24.9	45.4	47.6	35.9
Colistin	30	16						

^a Levofloxacin not excluded for comparison. CLSI in used to interpret % resistant.

^b resistance based on CLSI guidelines for agar dilution, 99 CF isolates from 99 patients.

^c UK study, 315 multidrug isolates from 76 Scottish CF patients²⁵⁶.

^d % resistant based on disk diffusion methods.

^e 49 *P. aeruginosa* isolates from 22 patients at Istanbul Medical Faculty, Turkey²⁵⁷.

^f % resistant based on disk diffusion methods.

^g 1315 *P. aeruginosa* isolates from 224 patients from CF centre of Gaslini Children's Hospital, Italy²⁵⁸.

^h % resistant based on disk diffusion methods.

The overall correlation agreement was 83% (due to high level of minor errors) for the Vitek 2 system with the antibiotics tested. The correlation agreement should be >90% for the accuracy to be acceptable. Results from the Vitek 2 automated system show that the very major error (VMA) rates for ceftazidime, meropenem, gentamicin and ciprofloxacin were higher than has been previously published^{294,302,332-333} these data suggest that the Vitek system was unable to detect high level resistance against these antimicrobials. Previously reported minor error rates (m) for meropenem, cefepime and ceftazidime were 21.7-23.5%, 14.4-18.3% and 16.6-21.7% respectively these data which were higher than those reported in this study^{294,302,332-333}. Unacceptable correlation with the reference method was due to high rate of VM errors in ceftazidime, meropenem and ciprofloxacin. A high rate of minor errors was responsible for the poor correlation agreement for cefepime and gentamicin, suggesting that the Vitek 2 system was unreliable at detecting intermediate level resistance. In previous evaluations of Vitek 2³³²⁻³³⁴ the VM errors for piperacillin-tazobactam ranged from (10.2-27%) which higher than what was found in this study but similar to²⁹⁴. The VM errors for amikacin were below <3% and the M and m errors were <10%. The overall correlation agreement was 92% for amikacin, similar to²⁹⁴. Based on these data, although the number of isolates tested was not large the Vitek 2 system was accurate at detecting piperacillin-tazobactam and amikacin resistance, but was unable to detect high level resistance in ciprofloxacin, meropenem and ceftazidime. A high rate of minor errors mainly contributed to the correlation agreement for cefepime, suggesting that the Vitek 2 system was capable at detecting high level resistance rather than intermediate level resistance. The combination of VM and minor errors for gentamicin meant that the Vitek 2 system was unreliable at detecting low and high level resistance.

The average age of CF patients in the collection was 28 years and 62 years in the NCF patient collection.

The highest levels of resistance were seen to aminoglycosides, carbapenems and penicillins in 38-48 year age group. The most effective antimicrobials in this age group were the fluoroquinolones and colistin. In the 27-37 year age group the most effective antimicrobials were the aminoglycosides and the β -lactam/ β -lactamase inhibitor combinations. Carbapenems, cephalosporins and penicillins were most effective in the 16-26 year age group. The differences seen in the antimicrobial resistant profiles in the each age group presumably reflects the different antimicrobial regimes. Penicillins, cephalosporins and carbapenems are the first line antimicrobials used to treat CF

patients. Antimicrobials such as ciprofloxacin and colistin are often reserved for more serious *P. aeruginosa* infections. It is also important to point out that the severity of disease in each patient varies, often CF patients do not receive antimicrobial therapy and are instead treated with physiotherapy.

In the NCF collection, the highest levels of resistance were seen in the 49-59 year age group, where the highest levels of resistance to aminoglycosides, carbapenems and β -lactam/ β -lactamase inhibitor combinations were seen. It was presumed that overall antimicrobial resistance would increase with age, (assuming elderly patients would have poorer general health as compared to younger individuals and therefore have increased exposure to antimicrobials). However, resistance was spread across all age groups. As demonstrated in the 16-26 year age group which had the highest level of resistance to cephalosporins and the second highest level of resistance to aminoglycosides. These antimicrobials (cephalosporins and aminoglycosides) were most effective in the 71-81 year age group and the 60-70 age group. Polymyxin, specifically colistin is prescribed for bowel decontamination and not routinely prescribed in SJH as a nebulised form. It is interesting to see higher levels of resistance to this antimicrobial in the 27-37 and 49-59 age group.

Previous studies indicated that mucoid and non-mucoid phenotypes of *P. aeruginosa* are frequently isolated simultaneously from CF patients⁸⁰⁻⁸¹. Since the mucoid phenotype is not seen in the environment it is assumed that most patients are initially colonized by environmental strains displaying wild-type phenotypes^{77,335-337}. In the CF lung non-mucoid isolates can convert to the mucoid phenotype, this conversion is caused by mutations in regulatory genes mainly in the *mucA* gene⁷⁸. Mucoid isolates produce an excess of alginate, which has been associated with increased tolerance to the host immune defence^{63,338}, and described in connection with biofilm formation³³⁹. The antibiotic susceptibility patterns of the CF isolates in this study showed that the mucoid isolates were generally more susceptible than the non-mucoid isolates. These results are similar to previously published data⁸⁰⁻⁸². There was a significant difference ($P=0.0347$) in the susceptibility between mucoid and non-mucoid for cefepime and an extremely significant difference ($P=0.0001$) between susceptibility to gentamicin.

Non-mucoid *P. aeruginosa* isolates are thought to be involved in the initial and intermittent colonisation of the CF lungs⁷⁷ and appear to do little damage to the CF lung^{60,80-82,340}. Initially acquired non-mucoid *P. aeruginosa* strains can reside in the CF lung for many years³⁴¹. The conversion to the mucoid form is indicative of the

overproduction of a capsule- like polysaccharide called alginate which is believed to be involved in biofilm formation the development of anti-*P. aeruginosa* antibodies and poor prognosis for the patient ⁵⁹. Since mucoid and non-mucoid isolates are simultaneously isolated from CF lungs it would be reasonable to assume that non-mucoid and mucoid isolates may co-exist in a biofilm of chronically infected patients. One explanation for the difference in susceptibilities could be the greater selective pressure on the non-mucoid isolates outside the biofilm. While antimicrobial therapy may not eradicate biofilms, clinical evidence has shown that antimicrobials with little activity in anaerobic biofilms can provide a clinical benefit in the management of acute *P. aeruginosa* infection by acting on planktonic *P. aeruginosa*.

Continued antimicrobial surveillance is essential to improving antimicrobial prescribing and implementation of effective infection control. The single most important control measure in the containment of antimicrobial resistant bacteria in the hospital setting is infection control ⁸⁷, this is especially important in hospitals which treat CF patients.

Chapter 4 Genotypic Analysis of Resistance Mechanisms in *Pseudomonas aeruginosa*

4.1 Introduction

Bacterial resistance to antimicrobials is the result of a number of factors: antimicrobial inactivation/modification, target alteration, the acquisition of plasmid-encoded genes, and reduced accumulation due to decreased permeability and/or increased efflux^{96,144,162,342-344}. All of the mechanisms reduce or eliminate the efficiency of an antimicrobial.

β -lactams are amongst the most widely used antimicrobials in the clinical setting¹⁸⁵. Since their introduction in the 1940's a large number of divergent β -lactams have been developed, including penicillins, cepheems (cephalosporins and cephamycins) and carbapenems. They all have a common four atom structure known as the β -lactam ring and exert their antimicrobial activity by binding to penicillin binding proteins, thereby inhibiting the cross linking of peptidoglycan (an essential cell wall component). β -lactamases are enzymes which are produced by bacteria which hydrolyse the β -lactam ring. A huge variety of β -lactamases exist in bacteria, at least 120 have been identified in clinical isolates of *P. aeruginosa*¹⁵¹. β -lactamases are divided into four classes based on their amino acid sequence. Class A, C and D possess a serine group at their active site. Class B enzymes require divalent cations to function and are known as metallo- β -lactamases (M β L). Five groups of M β Ls have been found in *P. aeruginosa* to date, IMP, SPM, GIM, VIM, AIM-1¹⁵¹. Extended spectrum β -lactamases known as ESBLs, and are capable of hydrolyzing broad spectrum cephalosporins³⁴⁵. Of the 200 ESBLs discovered in Gram-negative bacteria, 32 have been detected in *P. aeruginosa* belonging to Class A and D, (TEM, SHV, CTX-M, PER, VEB, GES, BEL-, OXA-type)¹⁹⁰. Resistance to β -lactams, including advanced cephalosporins and carbapenems is a growing clinical problem and can be the result of intrinsic resistance, and/or the derepression of the chromosomally encoded AmpC β -lactamase^{152,346}, low permeability and/or efflux³⁴⁷⁻³⁴⁸. Acquired β -lactam resistance involves plasmid and integron mediated β -lactamases^{183,349-350}. Both intrinsic and acquired mechanisms of resistance are often present at the same time, thereby conferring multi-drug resistance³⁵¹.

Aminoglycosides are an important component of anti-pseudomonal chemotherapy, especially for the treatment of pulmonary infections in CF patients³⁵². They are often used in combination with β -lactams as it is thought they exhibit synergy with β -lactams²²². Amikacin is the most frequently used of the aminoglycosides for *P.*

aeruginosa infections. Isolates which are resistant to amikacin also exhibit a relatively high level of resistance to other aminoglycosides such as gentamicin, netilmicin, tobramycin and isepamicin ³⁵³. Resistance to aminoglycosides, including gentamicin and amikacin is observed in all parts of the world but especially in Europe and Latin America ³⁵⁴. Aminoglycoside resistance may be caused by one or more of the following:

- 1) Presence of aminoglycoside-modifying enzymes, ANTs (aminoglycoside nucleotidyltransferases), AACs (aminoglycoside acetyltransferase), APHs (aminoglycoside phosphotransferase) ²²².
- 2) Structural alterations in the ribosomal target of the antimicrobial ²²².
- 3) Decreased cell permeability ²²².
- 4) Export of aminoglycosides by efflux pumps such as Mex XY ^{146,148,274}, MexAB-OprM ^{140,144}.

Fluoroquinolones are one of the most effective antibiotics available for both parenteral and oral treatment of *P. aeruginosa* infections ²⁰⁸. However, the extensive use of fluoroquinolones both clinically and agriculturally has lead to high rates of resistance amongst bacteria ³⁵⁵. Resistance to fluoroquinolones involves two main mechanisms; alterations to the molecular targets of the quinolones and decreased accumulation due to impermeability of the outer membrane and/or overexpression of drug efflux systems ^{107,208}. Both of these mechanisms are chromosomally mediated. The first involves alterations in the quinolone resistance determining regions (QRDR) within topoisomerase II (GyrA and GyrB subunits) and topoisomerase IV (ParC and ParE subunits) ^{107,356-357}. The second type of mechanism involved in fluoroquinolone resistance is the expression of drug efflux pumps and loss of permeability. To date 12 potential efflux systems belonging to the resistance nodulation-division (RND) family have been identified in *P. aeruginosa* genome ¹⁴. Of these, four have been characterised as antimicrobial transporters ^{96,110}. MexAB-OprM (which is constitutively expressed) is

known to efflux fluoroquinolones, β -lactams, β -lactam/ β -lactamase inhibitor combinations, and imipenem.

The MexCD-OprJ and MexXY-OprM efflux systems are known to efflux fluoroquinolones, piperacillin, cefepime and meropenem but not ceftazidime or imipenem. MexXY-OprM is responsible for adaptive resistance to aminoglycosides^{143,358}. Fluoroquinolones are known substrates of MexEF-OprN and more recently MexPQ-OprM and MexVW-OprM³⁵⁸.

Recently three plasmid-mediated quinolone resistance determinants have been described^{275,359}. The first is *qnr*, initially described in *Klebsiella pneumoniae*²¹³. The plasmids carrying the *qnr* genes are capable of being transferred horizontally. Five *qnr* genes have been described so far; *qnrA*, *qnrB*, *qnrS*, *qnrC* and *qnrD*²¹⁸. These genes code for pentapeptide repeat protein that protects type II topoisomerases from quinolones²¹⁸. The second plasmid-mediated quinolone resistance determinant is *acc* (6')-Ib-cr gene, which is a variant of aminoglycoside acetyltransferase, it is capable of modifying ciprofloxacin and reducing its activity and has been shown to be associated and often co-located with *qnr* genes²¹⁸. The third type of plasmid-mediated quinolone resistance determinant is the quinolone efflux pump gene *qepA*³⁶⁰. To date none of these plasmid-mediated quinolone resistance determinants have been found in *P. aeruginosa*.

The aim of the study presented in this chapter was to investigate the molecular mechanisms involved in non-cystic fibrosis (NCF) and cystic fibrosis (CF) *P. aeruginosa* antimicrobial resistance. It is important to understand at a molecular level the basis of antimicrobial resistance in order to maximise the efficacy of antimicrobials which are used to combat infection caused by *P. aeruginosa*. Based on the phenotypic resistance patterns presented in Chapter 3, NCF and CF *P. aeruginosa* isolates were examined by:

- 1) Amplification and sequencing of genes associated with β -lactam, fluoroquinolone and aminoglycoside resistance
- 2) Quantitative real-time PCR (qRT-PCR) was used to establish the relative expression of efflux genes, the depression of the chromosomal AmpC and the loss of the outer membrane protein OprD, all of which contribute to β -lactam, aminoglycoside and fluoroquinolone resistance

- 3) The degree of genetic relatedness among the multidrug resistance isolates was examined using the DNA typing method; pulsed field gel electrophoresis (PFGE).
- 4) And finally, the presence of three epidemic clones, the Liverpool, Manchester and Midlands 1 epidemic strains, which have been found in many CF centre across Europe, was investigated by PCR amplification, sequencing and PFGE.

4.2 Results

4.2.1 Molecular characterisation of resistance mechanisms to the β -lactam antimicrobials

All isolates resistant to ≥ 1 β -lactam were screened for the presence of various acquired resistance genes in order to establish the genetic basis of resistance. A schematic representation of all resistance mechanisms to the β -lactams that were investigated in this study are summarised in Fig 4.1.

4.2.1.1 Resistance mechanisms to the β -lactams

Isolates which were resistant to imipenem and or meropenem were phenotypically screened for the presence of M β LS, as outlined in Chapter 3. A total of 23 NCF and 30 CF isolates demonstrated the presence of Class B β -lactamases (M β L). These isolates were screened for the *bla*_{VIM}, *bla*_{IMP}, *bla*_{SPM}, *bla*_{SPM} β -lactamase genes (the most common transferable M β LS)^{181,361} by PCR amplification reactions and sequencing, as outlined in Section 2.3.4.2. The *bla*_{VIM} gene was detected in 6 of these isolates. A representative gel is shown in Fig 4.2. Sequencing confirmed the presence of *bla*_{VIM-2}. Two isolates, NCF.2006.26 and NCF.2006.46 which according to the phenotypic test possessed a M β L, did not possess the *bla*_{VIM}, *bla*_{IMP}, *bla*_{SPM}, *bla*_{SPM} genes. These isolates did have Class 1 integron fragments according to PCR amplification, a representative gel can be seen in Fig 4.2.

Based of phenotypic results from Chapter 3, three isolates were screened for the presence of ESBL genes, Fig 4.3. None of the genes selected for analysis were detected.

4.2.2 Molecular characterisation of resistance mechanisms to fluoroquinolones

All NCF and CF isolates which were resistant to ≥ 1 fluoroquinolone were screened for the presence of chromosomal mutations in the DNA gyrase and topoisomerase IV genes, along with various acquired resistance genes, in order to investigate their genetic mechanism (s) of resistance. A schematic representation of all resistant mechanisms to fluoroquinolones that were investigated in this study are summarised in Fig 4.1.

4.2.2.1 Mutations in the QRDR are associated with fluoroquinolone resistance

A total of 78 (40%) NCF and 67 (68%) CF isolates were resistant to one or both of the fluoroquinolones tested (ciprofloxacin, levofloxacin). All of the isolates were screened for the presence of substitutions in the QRDR of GyrA, GyrB (DNA gyrase) and ParC, ParE (topoisomerase IV) subunits by PCR amplification reactions and sequencing, as outlined in Section 2.3.4.3. Representative agarose gels of *gyrA*, *gyrB*, *parC*, *parE* genes fragments amplified by PCR are shown in Fig 4.4, 4.5. The GyrA, GyrB, ParC and ParE subunits are present in all *P. aeruginosa*. The prevalence of the substitutions which were detected are summarised in Table 4.1, 4.2, 4.3, 4.4 and Fig 4.6, 4.7 for NCF isolates and CF isolates.

Substitutions in the QRDR of GyrA and ParC were detected in 71 and 16 NCF isolates respectively. No substitutions were detected in the GyrB or ParE subunit. The most common substitution (69 isolates) detected in the GyrA subunit was T83I substitution. Other substitutions were seen in GyrA at positions P79, G81, D87, A93, Q94, D104 and Q106. The most common substitution seen in the ParC subunit was the S87L substitution. Other substitutions were seen in the ParC subunit at positions G44, K46, Q49, R50, A53, A55, S57, K66, H67, G75, H84 and S87. In the CF population the majority of QRDR substitutions were seen in the GyrA subunit. A total of 29 isolates had the GyrA T83I substitution; other substitutions in GyrA were D87N and Q106K. Ten isolates had the S87L substitution in ParC. Seven isolates had a substitution at position R377 substitution in ParE.

***P. aeruginosa* isolates**

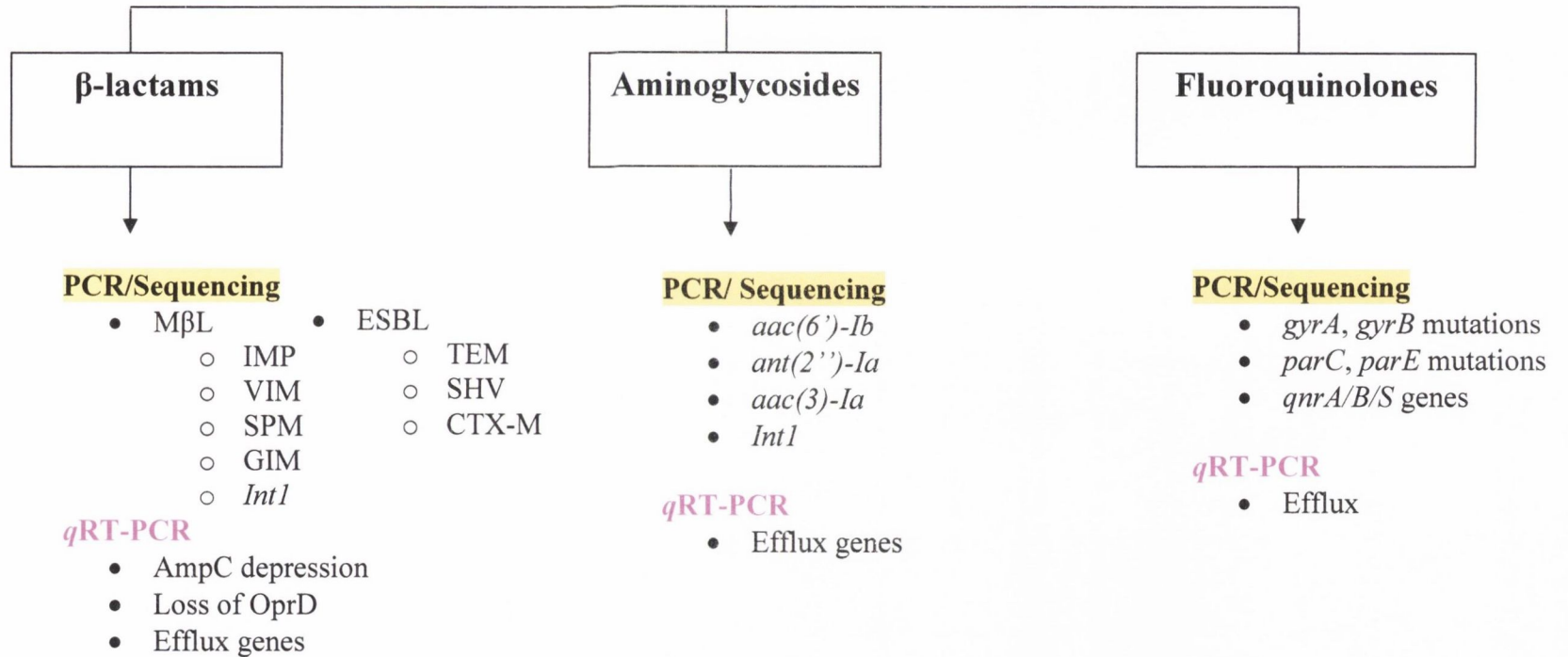


Fig 4.1 Resistance mechanisms in *P. aeruginosa* to β-lactams, aminoglycosides and fluoroquinolones as determined by PCR, sequencing and qRT-PCR.

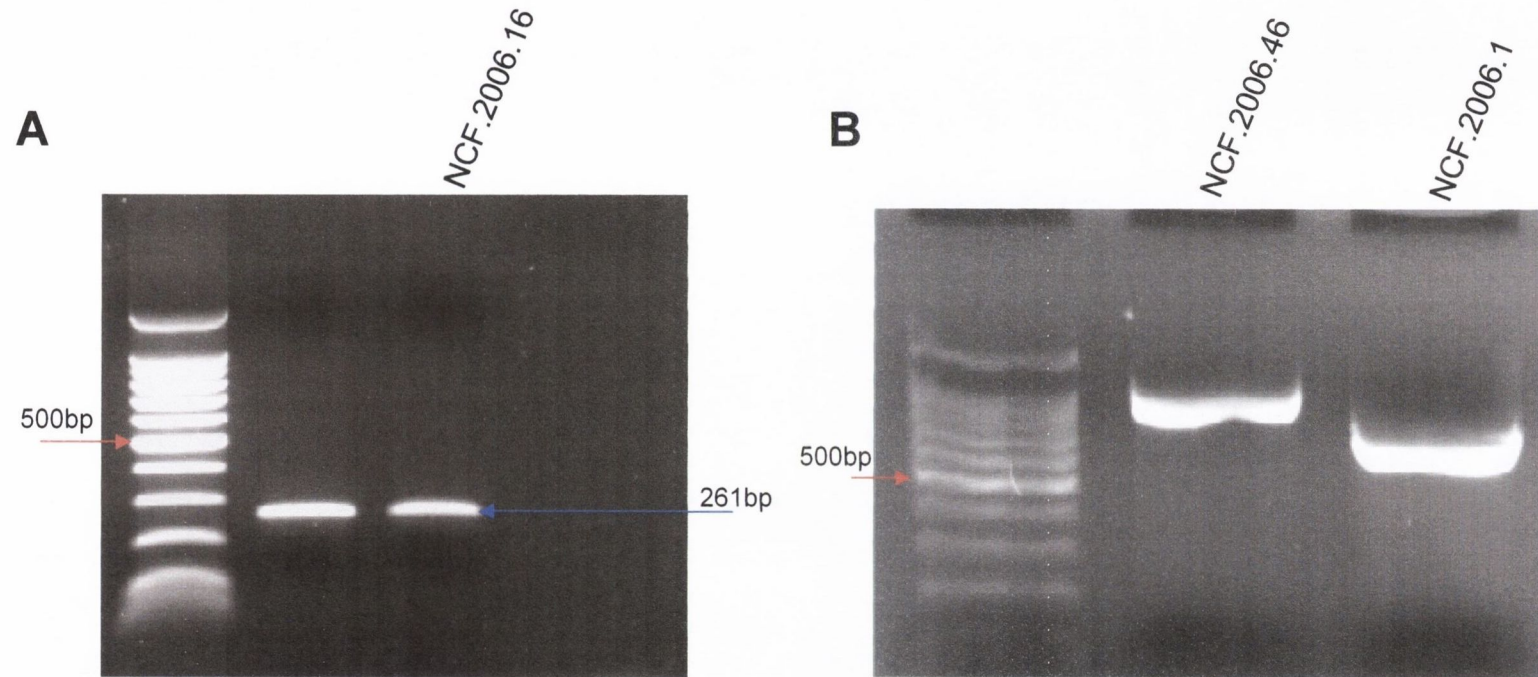


Fig 4.2 Detection of *bla*_{VIM} gene and class 1 integrons. **(A)** . Detection of *bla*_{VIM} gene (261bp). Lane 1: 100bp ladder, control, lane 2: *bla*_{VIM} *P. aeruginosa* positive control. **(B)** Detection of class 1 integrons (variable size) PCR products from two isolates NCF.2006.46 and NCF.2006.1 after amplification with integron primers (5'-CS and 3'CS), the primers are specific to the 5'- and 3'- conserved segments of class 1 integrons.

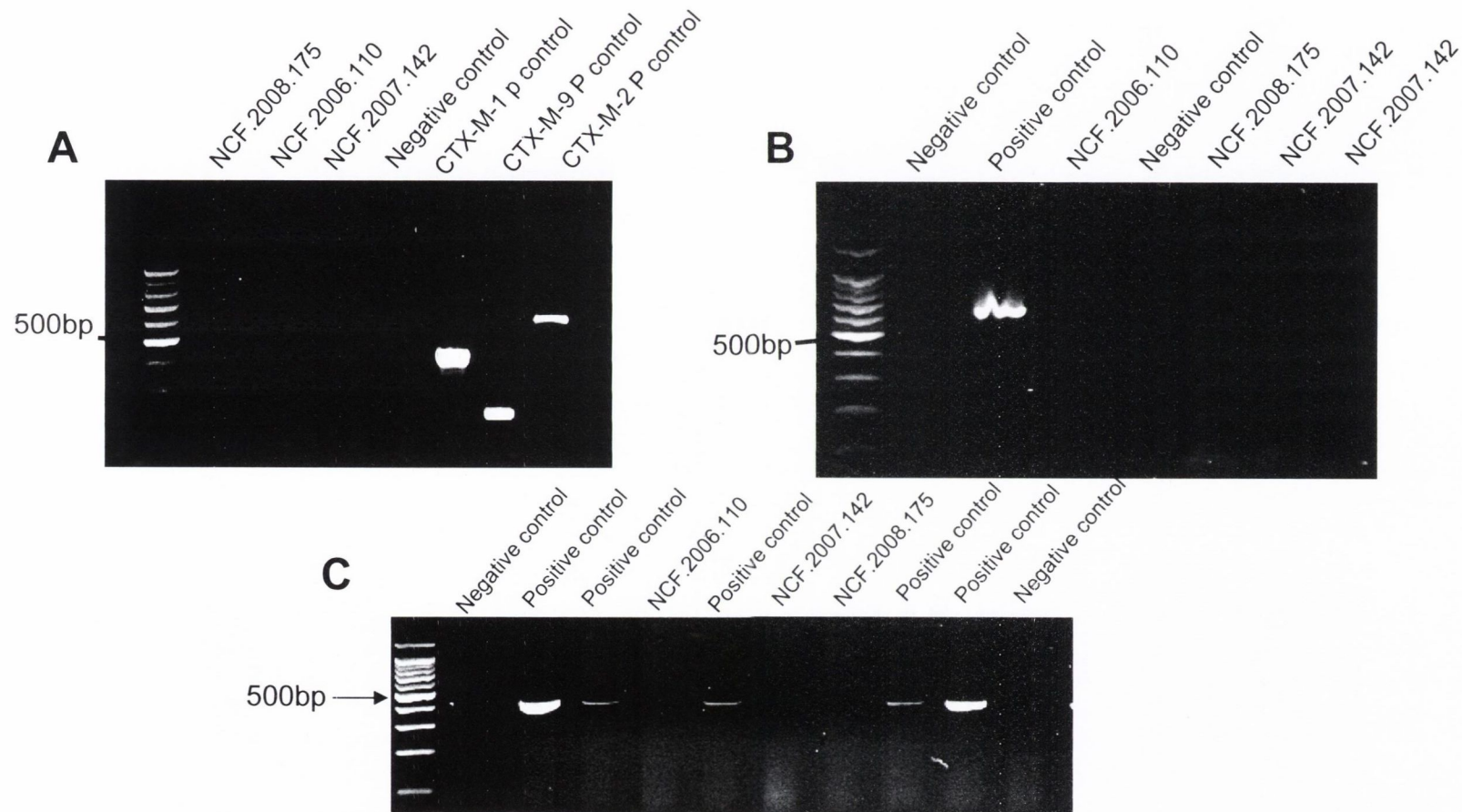


Fig 4.3 Detection of ESBL genes. **(A)** Detection of $bla_{CTX-M-1, 2, 9}$ genes by multiplex PCR. Lane 1: 100bp ladder, lane 6: CTX-M-1 *E. coli* positive control (415bp), lane 7: CTX-M-9 *Enterobacter cloacae* positive control (205bp), lane 8: CTX-M-2 *E. coli* positive control (552bp), **(B)** Detection of TEM β -lactamase. Lane 1: 100bp ladder, lane 3: *E. coli* ATCC 35218 positive control (516bp). **(C)** Detection of SHV β -lactamase. Lane 1: 100bp ladder, lane 3: *K. pneumonia* ATCC 700603 positive control (392bp), lane 4: *E. coli* clinical isolate, positive control, lane 6: *E. coli* clinical isolate, positive control, lane 10: *K. pneumonia* ATCC 700603 positive control (392bp).

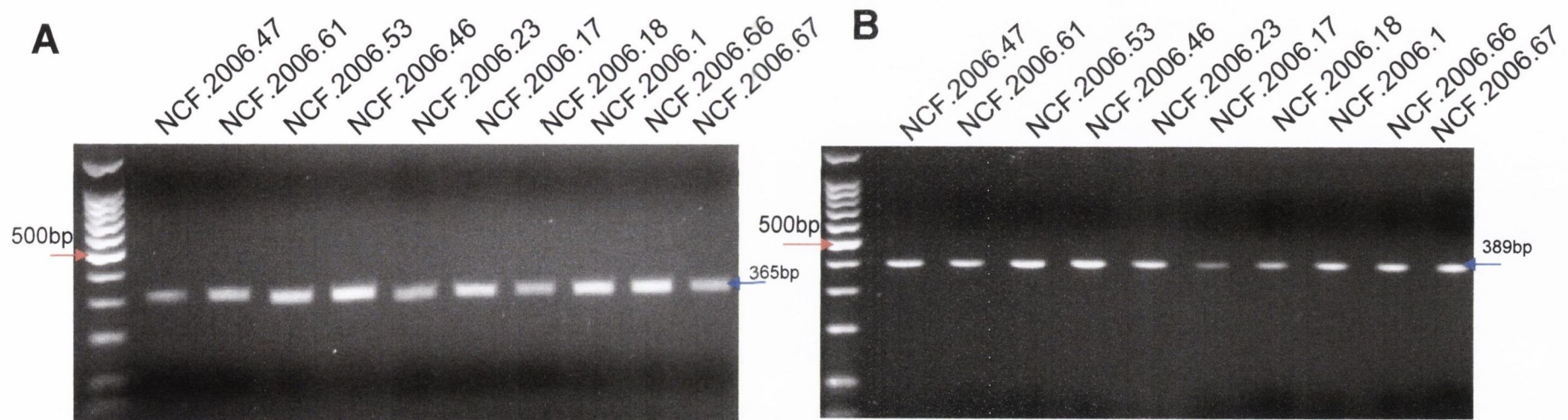


Fig 4.4 Amplification of *gyrA* and *gyrB*. **(A)** Agarose gel of *gyrA* gene fragments at 365bp, amplified by PCR. Lane 1: 100bp ladder. **(B)** Agarose gel of *gyrB* gene fragments at 389bp, amplified by PCR. Lane 1: 100bp ladder. No amplification was seen in the negative control, not shown.

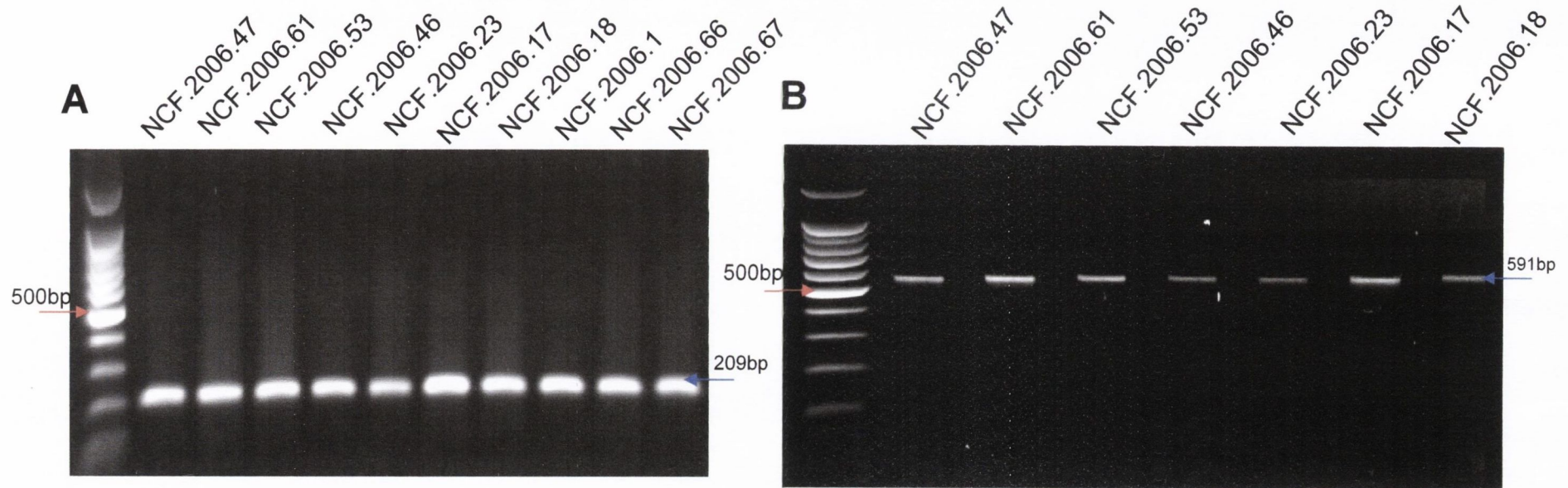


Fig 4.5 Amplification of *parC* and *parE*. **(A)** Agarose gel of *parC* gene fragments at 209bp, amplified by PCR. Lane 1: 100bp ladder. **(B)** Agarose gel of *parE* gene fragments at 591bp, amplified by PCR. Lane 1: 100bp ladder. No amplification was seen in the negative control, not shown.

	+79	+81	+83	+87	+93	+94	+104	+106																															
PAO1 <i>gyrA</i>	I	G	K	Y	H	P	H	G	D	T	A	V	D	T	I	V	R	M	A	Q	P	E	S	L	R	Y	M	L	V	D	G	Q	G	N	F	G	S	V	D
NCF.2006.106		R																																					
NCF.2006.79		R																																					
NCF.2006.47			I																																				
NCF.2006.53											N																												
NCF.2008.175						T																																	
NCF.2007.115										G																													
NCF.2006.61										G																													
NCF.2006.106																										A													
NCF.2006.113																																			K				

Fig 4.6 Sequence alignment of substitutions in the QRDR of *gyrA* in *P. aeruginosa*. The amino acid sequence of *P. aeruginosa* PAO1 (GenBank ID:NP_251858.1) is aligned with *P. aeruginosa* isolates which were resistant to fluoroquinolones. Amino acid residues in PAO1 GyrA subunit where substitutions occur are highlighted in red and bold, residues in the *P. aeruginosa* isolates that differ from those in PAO1 GyrA subunits are shown in bold. Dotted line indicates identical sequences.

Table 4.1 NCF *P. aeruginosa* substitutions in the QRDR of GyrA and ParC subunits

GyrA n=71	Substitution	ParC n=16	Substitution
1	P79R	1	G44S
1	G81R	1	K46I
69	T83I	1	Q49H
11	D87N	1	Q49I
1	D87T	1	R50L
1	A93G	1	A53L
8	Q94G	2	A55P
2	D104A	1	S57N
1	Q106K	2	K66L
7	No substitutions	2	K66H
		1	H67S
		2	G75R
		1	H84Q
		13	S87L
		1	S87W
		62	No substitutions

Table 4.2 CF *P. aeruginosa* substitutions in the QRDR of GyrA, ParC and ParE subunits

GyrA n=32	Substitution	ParC n=9	Substitution	ParE n=7	Substitution
29	T83I	5	S87L	1	R377P
				2	R377A
9	D87N	2	S87L	1	R377A
				1	R377P
2	Q106L	1	S87L	1	R377P
23	T83I	23	No	23	No
	D87N		substitutions		substitutions
	Q106K				
1	No	1	S87L	1	No
	substitutions				substitutions
1	No	1	S87L	1	R377A
	substitutions				
35	No	58	No	60	No
	substitutions		substitutions		substitutions

Table 4.3 Prevalence of substitutions in the QRDR of GyrA and ParC subunits and the associated ciprofloxacin and levofloxacin MICs from NCF *P. aeruginosa*

No. of isolates	GyrA Substitutions	ParC Substitutions	Ciprofloxacin MIC range mg/L	Levofloxacin MIC range mg/L
7	WT		0.25-64	
62		WT		1-64
33	T83I	WT	0.5-64	0.5-64
1	Q94G	WT	2	8
3	T83I,D87T	WT	2-16	4-32
6	T83I,D87N	WT	4-32	0.5-64
2	T83I, A93G	WT	4-8	8-16
5	T83I,Q94R	WT	4-8	0.25-16
1	T83I,D104A	WT	32	8
1	T83I,Q106K	WT	2	1
1	D87N,Q94R	WT	32	32
1	P79R,T83I,D104A	WT	4	8
10	T83I	S87L	1-16	1-32
1	T83I	S57N,K66L,H84Q,S87W	8	8
1	T83I	Q49H,S87L,T57N,K66H	32	64
1	T83I	G44S,K46L,Q49I,R50L,A53L,A55P,K66H,H67S,G75R	16	32
1	T83I,Q94G	A55P,K66L,G75R	8	16
1	G81R,T83I	S87L	32	64

Table 4.4 Prevalence of substitutions in the QRDR of GyrA, ParC and ParE subunits and the associated ciprofloxacin and levofloxacin MICs from CF *P. aeruginosa*

No. of isolates	GyrA Substitutions	ParC Substitutions	ParE Substitutions	Ciprofloxacin MIC range mg/L	Levofloxacin MIC range mg/L
32	WT	WT	WT	1-8	2-16
19	T83I	WT	WT	1-16	2-16
1	WT	S87L	WT	4	2
3	T83I	S87L	WT	2-16	4-32
1	D87N	WT	R377P	2	16
1	WT	S87L	R377A	2	32
1	T83I	S87L	R377P	2	32
3	T83I,D87N	WT	WT	2	4
1	T83I,D87N	S87L	R377P	4	8
1	D87N,Q106L	S87L	R377P	2	8
1	T83I,D87N	WT	R377P	4	16
1	T83I,D87N,Q106L		R377P	4	4

A

PAOI <i>parC</i>	+44+46 +49+50 +55 +57 +66 +67 +75 +84 +87	DGL K PV Q R R I V Y A MSELGLDAD S K H K SARTV G DVLGKF H P H G D SACYEAM
NCF.2006.102		.S
NCF.2006.102		... L
NCF.2006.72		 H
NCF.2006.102		 I
NCF.2006.102		 L
NCF.2006.80		 P
NCF.2006.102		 L
NCF.2006.1		 N
NCF.2006.61		 L
NCF.2006.72		 H
NCF.2006.102		 S
NCF.2006.80		 R
NCF.2006.1		 Q
NCF.2006.1		 W
CF.2006.198		 L

B

PAOI <i>parE</i>	+377	VSGVVKDAFSLWLNEHAEIGLQLAELAISNAG R RLKAGKKVERKKITQGPA
CF.2008.274		 P

Fig 4.7 Sequence alignment of substitutions in the QRDR of *parC* and *parE*. **(A)** Sequence alignment of ParC subunits of *P. aeruginosa* isolates. The amino acid sequence of PAO1 ParC subunit in *P. aeruginosa* (GenBank ID: NP_253651.1) is aligned with *P. aeruginosa* NCF and CF isolates which are resistant to fluoroquinolones. Amino acid residues in PAO1 where substitutions occur are shown in red and bold, residues in *P. aeruginosa* NCF and CF isolates which differ from PAO1 are shown in bold. **(B)** Sequence alignment of ParE subunit of *P. aeruginosa* CF isolate. The amino acid sequence of PAO1 ParE in *P. aeruginosa* (GenBank ID: YP_709477.1) is aligned with a CF *P. aeruginosa* isolate which is resistant to fluoroquinolones. Amino acid residues in PAO1 where substitutions occur are shown in red and bold, residues in *P. aeruginosa* NCF and CF isolates which differ from PAO1 are shown in bold. Dotted line indicates identical sequences.

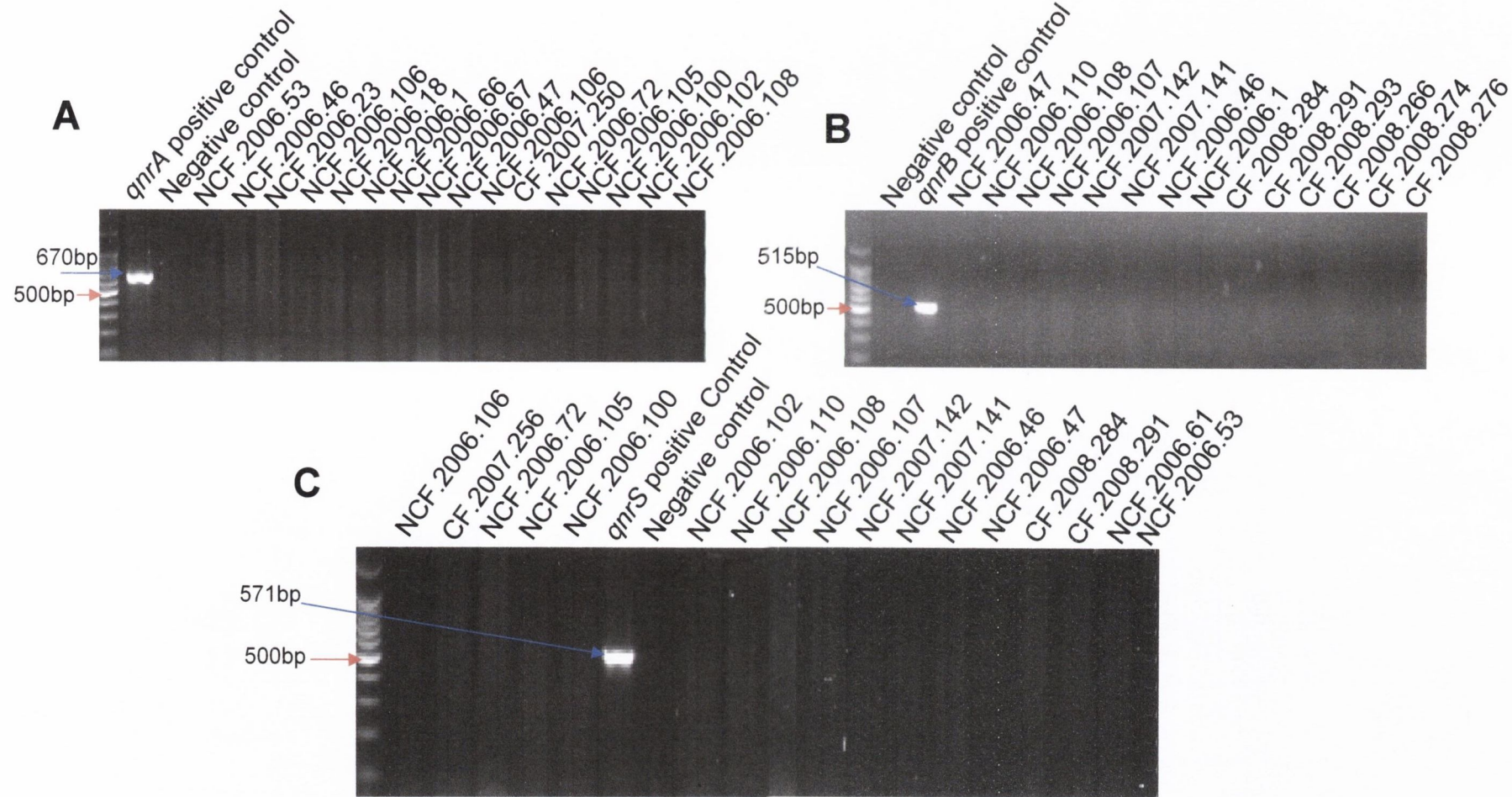


Fig 4.8 Detection of *qnrA*, *qnrB*, *qnrS* gene fragment amplified by PCR. **(A)** Detection of *qnrA* (670 bp), Lane 1: 100bp ladder, *qnrA* positive control (*K. pneumonia*). **(B)** Detection of *qnrB* (515 bp) gene fragment amplified by PCR. Lane 1: 100bp ladder, lane 2: negative control, lane 3: *qnrB* positive control (*K. pneumonia*). **(C)** Detection of *qnrS* gene fragment (571 bp) amplified by PCR. Lane 1: 100bp ladder, lane 7: *qnrS* positive control (*K. pneumonia*).

Thirty four NCF isolates encoded a single substitution in QRDR of GyrA subunit only. These isolates had an MIC from low to high (0.05-64mg/L) for ciprofloxacin and levofloxacin. Nineteen NCF isolates had just two substitutions in GyrA subunit, the MICs for these isolates ranged from 2-32mg/L for ciprofloxacin and 0.5-64mg/L for levofloxacin. A total of 10 NCF isolates had one substitution in GyrA and ParC, the MICs for ciprofloxacin ranged from 1-16mg/L and 1-32mg/L levofloxacin. Three NCF isolates had one substitution in GyrA and $\geq$ four substitutions in ParC, the MICs for ciprofloxacin ranged from 8-32mg/L and 8-64mg/L for levofloxacin. Two NCF isolates had ≥ 2 substitutions in GyrA and ≥ 1 substitution in ParC, the MICs for ciprofloxacin ranged from 8-32mg/L and 16-64mg/L for levofloxacin. Only seven isolates out of 71 tested had no substitutions in GyrA, GyrB, ParC or ParE.

In the CF population nineteen CF isolates had a single substitution in GyrA, MICs ranged from 1-16mg/L for ciprofloxacin and levofloxacin. A total of three isolates had just two substitutions in GyrA, the MICs for these isolates were 2mg/L for ciprofloxacin and 4mg/L for levofloxacin. In the CF collection three isolates had a single substitution in GyrA and ParC. The MICs for these isolates ranged from 2-16mg/L for ciprofloxacin and 4-32 for levofloxacin. One isolate had just a single substitution in GyrA and ParE, the MIC for this isolate were 2mg/L for ciprofloxacin and 16mg/L for levofloxacin. A single isolate had substitutions in ParC and ParE, (2mg/L-ciprofloxacin, 32mg/L-levofloxacin). One isolate had substitutions in three QRDR subunits (GyrA, ParC and ParE), the MIC for this isolate was 2mg/L for ciprofloxacin and 32mg/L for levofloxacin. A total of two isolates had ≥ 1 substitution in all three QRDR subunits (GyrA, ParC and ParE), the MICs for these isolates ranged from 2-4mg/L for ciprofloxacin and 8mg/L for levofloxacin. Just two isolates had ≥ 1 substitutions in GyrA and ParC, the MICs for these isolates were 4mg/L for ciprofloxacin and 4-16 for levofloxacin. A total of thirty five resistant fluoroquinolone isolates tested had no substitutions in QRDR.

NCF isolates had a statistically greater number of substitutions in *gyrA* compared to CF isolates ($P = 0.001$), T83I was the most prevalent substitution in both NCF and CF isolates but there was no statistically significant difference between the two populations. There was no statistically significant difference between the numbers of NCF fluoroquinolone resistant isolates which had substitutions in *parC* compared to CF fluoroquinolone resistant isolates which also had substitutions in *parC*. The most

prevalent substitution in *parC* for both populations was S87L. A statistically significant number of CF isolates had substitutions in *parE* ($P = 0.0038$) compared to NCF isolates which had no substitutions in *parE*.

4.2.2.2 Analysis of acquired fluoroquinolone resistance genes

The *qnr* genes are believed to be responsible for low level resistance but their presence favours the selection of other resistance mechanisms²¹⁸. All of the NCF and CF isolates had a MIC of ≥ 0.25 mg/L to both ciprofloxacin and levofloxacin and were therefore screened for the presence of *qnrA*, *qnrB* and *qnrS* acquired resistance genes, by PCR amplification as outlined in Section 2.3.4.4. A representative agarose gel of *qnr* gene fragments amplified by individual PCR is shown in Fig 4.8. All 194 NCF and 99 CF isolates tested did not have any of the three *qnr* genes. All of the NCF and CF isolates had previously undergone successful PCR amplified for *gyrA*, *gyrB*, *parC* and *parE* genes, therefore it can be said that the negative result is not due to template damage.

4.2.2.3 Resistance mechanisms to the aminoglycosides

All NCF and CF isolates which were resistant to ≥ 1 aminoglycoside were screened for the presence of aminoglycoside modifying enzymes. A systematic representation of the resistance mechanisms to aminoglycosides that were investigated in this study are summarised in Fig 4.1.

In the NCF collection 68/194 (35%) of the isolates tested were resistant to ≥ 1 of the aminoglycosides tested. In the CF collection 83/99 (84%) of the isolates tested were resistant to ≥ 1 of the aminoglycosides tested. Isolates which were resistant to either amikacin and/or gentamicin were screened for aminoglycoside modifying enzymes, *aac(6')-Ib*, *ant(2'')-Ia* and *aac(3)-Ia*, (Fig 4.9). Of the 68 isolates tested in the NCF population the *aac(6')-Ib* and *ant(2'')-Ia* genes were detected in two isolates, (Fig 4.9). In the CF population the *aac(6')-Ib* gene was detected in only one isolate. The two isolates which had the *aac(6')-Ib* gene had MICs ranging from 4-32mg/L for amikacin and 32-64mg/l for gentamicin. The isolate which had the *ant(2'')-Ia* gene had an MIC of 4mg/L for amikacin and 32mg/L for gentamicin.

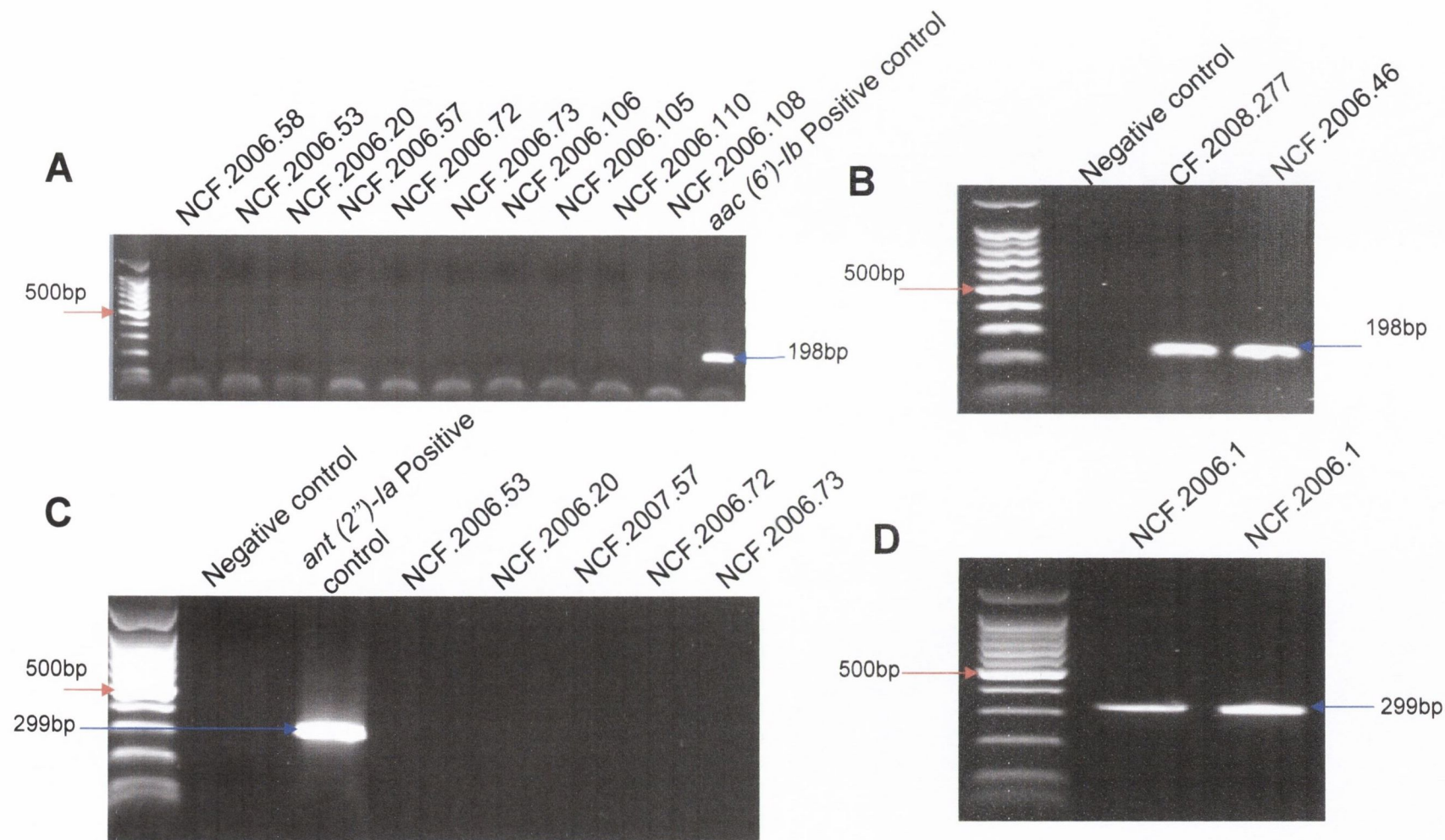


Fig 4.9 Detection of aminoglycoside modifying enzyme gene *aac(6')-Ib*, *ant(2'')-Ia*. **(A)** Lane 1: 100bp ladder, lane 12: *aac(6')-Ib* positive control (Clinical *P. aeruginosa* isolate). **(B)** Lane 1: 100bp ladder. **(C)** Lane 1: 100bp ladder, lane 3: *ant(2'')-Ia* positive control (clinical *P. aeruginosa*). **(D)** Lane 1: 100bp ladder.

4.2.3 Relative expression levels of efflux pump genes, the outer membrane protein OprD and AmpC β -lactamase by qRT-PCR

A group of 33 NCF (15) and CF (18) isolates were selected for further analysis using qRT-PCR. These isolates had a high level of resistance to at least one antimicrobial in each of the following drug classes; fluoroquinolones, aminoglycosides and carbapenems, (Table 4.5, 4.6). This group of isolates is referred to as the very multi-drug resistant (vMDR) group²⁵⁵.

4.2.3.1 Selection of genes of interest

In order to determine the expression of the MexAB-OprM, MexCD-OprJ and MexEF-OprN efflux systems, the first gene in each efflux operon was chosen; *mexA*, *mexC* and *mexE*. OprM forms the outer membrane component of the MexAB-OprM efflux system, the MexXY system is known to recruit OprM, for this reason the *oprM* gene was also included in the efflux study¹⁴⁴. The loss of the outer membrane protein OprD and the derepression of the chromosomal AmpC β -lactamase are associated with carbapenem and β -lactam resistance, for expression levels of the *oprD* and *ampC* genes were also determined by qRT-PCR. It has previously been shown that the expression of *ampC* and *oprD* genes correlate with β -lactamase activity and OprD protein levels¹⁰².

4.2.3.2 qRT-PCR analysis

The primers used for the PCR amplification of cDNA are listed in Table 2.2, Section 2.1.3. Representative agarose gel of the genes of interest are shown in Fig 4.10. The optimal primer concentration for each gene, along with their corresponding amplification efficiency and slope are documented in Table 4.7. Standard curves were prepared for each gene for verification of the efficiencies of the reactions for comparative quantification, Fig 4.11. Quantitative RT-PCR was performed on cDNA from NCF and CF *P. aeruginosa* isolates from the vMDR group for each gene of interest, relative to endogenous control, *rpsL*, as outlined in Section 2.4.1 and Section 2.4.2. The results are presented as ratios of gene expression between the target gene (target) and the reference gene (*rpsL*), using the following equation: $\text{Ratio} = (E_{\text{target gene}})^{\Delta C_t}_{\text{target(PAO1-test strain)}} / (E_{\text{rpsL}})^{\Delta C_t}_{\text{rpsL (PAO1-test strain)}}$,³⁶². E is the real-time PCR efficiency for the gene and C^t is the PCR cycle number at which the fluorescence meets the threshold on the amplification plot. An effect on gene expression was considered significant when the corresponding ratios were >2.5 or <0.4 ¹⁰².

Table 4.5 Genotypic profile of NCF isolates selected for efflux analysis by qRT-PCR.

Isolates	MIC(mg/L) ^a										
	PEN	β -lactam/ β -lactamase inhibitor		Cepaholsporins		Carbapenems		Aminoglycosides		Fluoroquinolones	
	Pipercillin	Pipercillin-tazobactam ^b	Ticarcillin-clavulanic acid ^b	Ceftazidime	Cefepime ^b	Imipenem	Meropenem	Gentamicin	Amikacin	Ciprofloxacin	Levofloxacin
NCF.2007.142	>256	S	R	64	S	128	128	32	32	16	32
NCF.2006.107	>256	S	S	8	S	64	16	64	8	64	64
NCF.2006.47	256	R	R	128	R	32	32	32	4	8	32
NCF.2006.1	256	R	R	128	S	32	16	32	4	16	16
NCF.2006.105	256	S	R	128	S	64	32	128	8	64	64
NCF.2007.141	256	S	R	4	S	32	64	64	16	4	2
NCF.2006.102	128	R	R	128	S	128	128	64	8	16	32
NCF.2008.193	64	S	R	>128	R	64	64	16	32	4	8
NCF.2006.72	32	S	R	64	S	16	8	128	128	32	32
NCF.2006.53	64	S	S	128	S	16	2	64	64	16	32
NCF.2006.110	8	S	S	32	S	64	8	64	8	32	64
NCF.2006.46	32	S	S	128	S	64	64	32	4	16	32
NCF.2006.106	32	S	S	4	S	64	16	16	8	4	8
NCF.2006.108	32	S	S	4	S	64	16	128	8	32	64
NCF.2006.100	16	S	S	8	S	16	4	32	8	32	8

^a MICs in the range of resistant (according to CLSI) are highlighted in red, isolates in the range of intermediate (according to CLSI) are highlighted in yellow, MICs in the fully susceptible range are not highlighted. Isolates are considered resistant if they fall in the intermediate or resistant categories. ^b Susceptibilities determined by disc diffusion.

Table 4.6 Genotypic profile of CF isolates selected for efflux analysis by qRT-PCR.

Isolates	MIC(mg/L) ^a										
	PEN	β -lactam/ β -lactamase inhibitor		Cepaholsporins		Carbapenems		Aminoglycosides		Fluoroquinolones	
	Pipercillin	Pipercillin-tazobactam ^b	Ticarcillin-clavulanic acid ^b	Ceftazidime	Cefepime ^b	Imipenem	Meropenem	Gentamicin	Amikacin	Ciprofloxacin	Levofloxacin
CF.2006.215	>256	R	R	>128	R	128	32	64	64	4	2
CF.2006.216	>256	R	R	>128	R	128	32	64	64	4	8
CF.2006.222	256	R	R	>128	R	128	32	128	>128	2	8
CF.2008.274	>256	R	R	128	R	4	16	64	128	4	8
CF.2007.255	>256	R	R	16	R	32	16	16	64	2	32
CF.2008.284	>256	R	R	32	S	32	64	16	32	4	8
CF.2007.256	>256	R	R	16	R	16	16	16	64	2	32
CF.2008.293	256	S	R	>128	R	128	128	64	>128	4	4
CF.2006.204	>256	S	R	64	R	128	32	32	32	2	8
CF.2006.212	>256	S	R	128	S	128	128	32	16	16	8
CF.2006.198	>256	S	R	64	S	128	128	32	32	16	32
CF.2007.251	256	S	S	>128	R	32	>128	>128	64	4	32
CF.2008.266	256	S	S	128	R	128	128	16	16	4	2
CF.2008.288	256	S	S	128	S	32	8	128	32	8	16
CF.2008.292	128	S	S	16	S	32	4	64	128	4	16
CF.2008.291	32	S	S	4	S	32	2	32	128	4	4
CF.2006.234	8	S	S	1	S	32	1	32	64	2	16
CF.2006.214	8	S	S	2	S	32	2	16	32	2	8

^a MICs in the range of resistant(according to CLSI) are highlighted in red, isolates in the range of intermediate (according to CLSI) are highlighted in yellow, MICs in the fully susceptible range are not highlighted. Isolates are considered resistant if they fall in the intermediate or resistant categories. ^b Susceptibilities determined by disc diffusion.

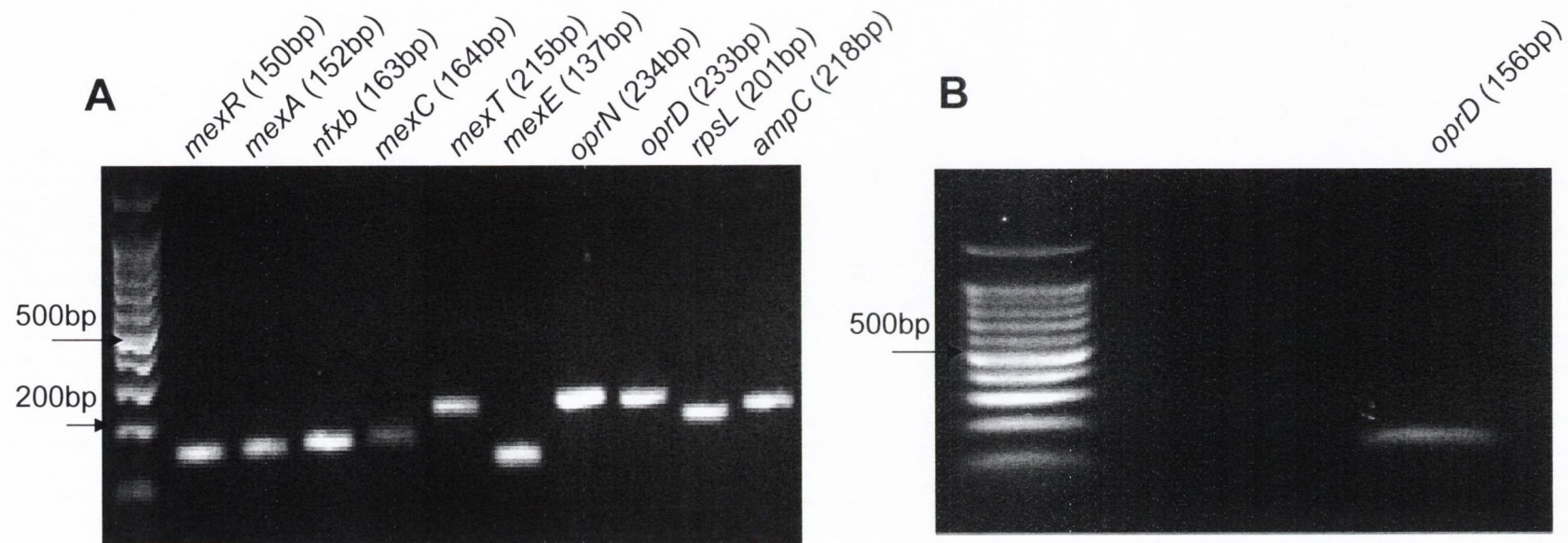


Fig 4.10 Agarose gel of gene fragments of the genes of interest. **(A)** PCR fragments of *mexA*, *mexC*, *mexE*, *ampC*, *rpsL* amplified in *P. aeruginosa* PAO1. **(B)** PCR fragment of *oprD* amplified in *P. aeruginosa* PAO1

Table 4.7 Optimal qRT-PCR conditions for the genes of interest

Gene name	Optimal primer (μ M)	Efficiency (%)	Slope
<i>ampC</i>	0.3	85	-3.36
<i>rpsl</i>	0.1	94.4	-3.4
<i>mexA</i>	0.1	96.9	-3.9
<i>mexE</i>	0.1	104	-3.3
<i>mexC</i>	0.1	90	-3.45
<i>oprD</i>	0.1	103	-3.3
<i>oprM</i>	0.1	108	-3.3

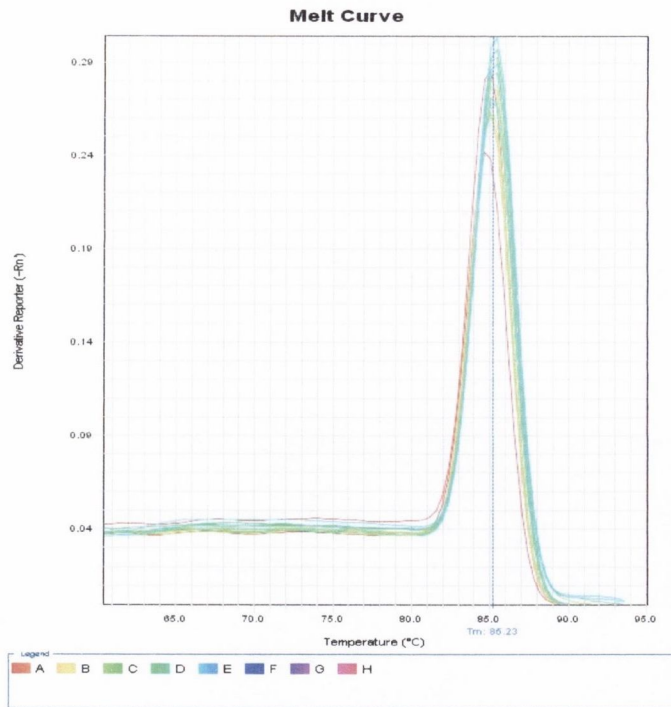
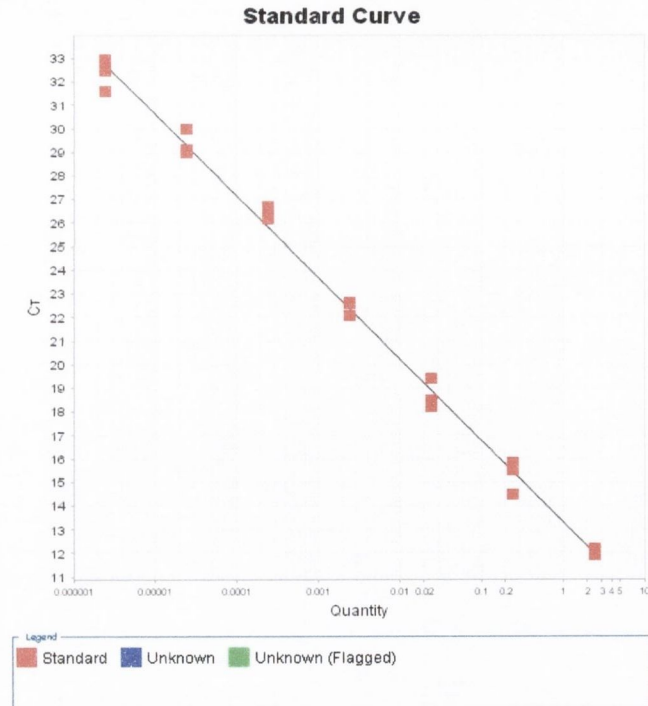
A**B**

Fig 4.11 Melt curve, standard curve for *rpsL*. **(A)** The melt curve is a plot of data collected during the melt curve stage. Peaks in the melt curve indicate the melting temperature of the target, multiple peaks indicate non-specific PCR amplification. **(B)** The standard used for the standard dilution series is a cDNA sample of known quantity. The standard curve dilution series extends above and below the expected abundance of the target (pg-fg range).

4.2.3.3 Relative expression levels of efflux genes

The relative expression levels of *mexA*, *mexC*, *mexE* and *oprM* were determined in the vMDR group, (Table 4.8). The expression levels of these genes to four previously characterised *P. aeruginosa* isolates, JFL30, JFL28, JFL10 and JFL05, which are known to hyperexpress MexAB-OprM, MexCD-OprJ, MexXY and MexEF-OprN respectively were also determined, (Table 4.8, 4.9), ³⁶³. JFL05, which is known to hyperexpress the efflux pump MexEF-OprN, expression levels in *mexE* were 131 times greater than in PAO1, *mexC* and *oprM* expression levels were 12 and 7 times higher than in PAO1. In JFL10, which is known to hyperexpress the efflux pump MexXY (MexXY does not contain a gene coding for an outer membrane protein, so it recruits OprM of the MexAB-OprM efflux pump to form a functional tripartite efflux system, ¹⁴⁴, expression levels of *oprM* were 161 times higher than in PAO1. Expression levels to *mexA* were 4.6 times higher than PAO1 and expression levels to *mexE* were 0.05 times lower than PAO1. JFL28 is an isolate which is known to hyperexpress MexCD-OprJ, expression levels to *mexC* were 56.7 times higher than PAO1, expression levels to *mexA* and *oprM* were 3.8 and 20.6 times higher than PAO1 respectively, expression to *mexE* was 0.2 times lower than PAO1. The fourth *P. aeruginosa* isolate, JFL30 is known to hyperexpress MexAB-OprM. Expression levels to *mexA* and *oprM* were 68.6 and 159 times higher than PAO1. Among the MDR group, expression levels for *mexA* ranged from 2.52-19.34 times higher than PAO1 for 11 of the MDR isolates. Of these 11 isolates, seven also had an increased expression level of *oprM* ranging from 3.03-10.52 times higher than PAO1. In one isolate, CF.2006.214, the expression of *oprM* was 0.27 times lower than PAO1. In the other three isolates, CF.2006.222, CF.2007.251 and CF.2008.292 expression levels of *oprM* were similar to *P. aeruginosa* PAO1. In three isolates, NCF.2006.108, NCF.2006.110 and CF.2008.284 expression of *oprM* ranged from 3.28-4.45 times greater than in *P. aeruginosa* PAO1, expression of *mexA* was not significant in isolates NCF.2006.108 and NCF.2006.110, expression of *mexA* was 0.03 times lower in isolate CF.2008.284. Five isolates had expression levels ranging from 0.27-0.41 times lower than *P. aeruginosa* PAO1 for *oprM*. Expression of *mexC* ranged from 2.29-111.17 times greater than *P. aeruginosa* PAO1 in 13 of the MDR isolates. Four isolates, NCF.2006.106, CF.2006.215, CF.2006.216 and CF.2008.284 had expression levels ranging from 0.05-0.37 lower for *mexC*, the remaining isolates had expression levels which were not considered significantly different from the WT PAO1. A total of 8 isolates had expression levels for *mexE* ranging from 2.55-174.70 times

greater than PAO1. A group of six isolates had expression levels which were lower than PAO1 and ranged from 0.01-0.30 times lower than PAO1. The remaining isolates had expression levels which were not considered significantly different from the wild type *P. aeruginosa* PAO1, (Table 4.8, 4.9).

Table 4.8 Relative expression levels of efflux genes associated with antimicrobial resistance as determined by qRT-PCR from NCF isolates

Isolates ^a	Gene ^b			
	<i>mexA</i>	<i>mexC</i>	<i>mexE</i>	<i>oprM</i>
PAO1	1.0	1.0	1.0	1.0
JFL05	0.81	12.09	131.3	7.16
JFL10	4.57	0.53	<u>0.05</u>	161.46
JFL28	3.84	56.76	<u>0.2</u>	20.63
JFL30	68.66	0.97	<u>0.06</u>	159.04
NCF.2006.1	<u>0.37</u>	1.19	0.96	0.41
NCF.2006.46	2.18	0.55	1.30	1.34
NCF.2006.47	0.64	4.93	6.72	2.39
NCF.2006.53	2.62	0.95	4.24	3.58
NCF.2006.72	1.71	0.90	0.45	<u>0.31</u>
NCF.2006.100	0.65	24.07	1.25	<u>0.33</u>
NCF.2006.102	2.52	111.17	2.75	9.88
NCF.2006.105	5.91	0.62	0.93	10.52
NCF.2006.106	0.48	<u>0.05</u>	0.45	0.84
NCF.2006.107	0.91	0.81	1.14	1.55
NCF.2006.108	0.77	1.12	1.74	3.30
NCF.2006.110	0.15	1.07	1.24	4.45
NCF.2007.141	0.80	3.32	0.50	0.45
NCF.2007.142	2.77	0.88	1.16	3.03
NCF.2008.193	1.98	1.68	0.88	0.43

^aJFL05: MexEF-OprN-hyperexpressing isolate, JFL10: MexXY-hyperexpressing isolate, JFL28: MexCD-OprJ- hyperexpressing isolate, JFL30: MexAB-OprM- hyperexpressing isolate

³⁶³ ^bValues represent fold change (mean of triplicate) in comparison with the transcription level in wild type PAO1. Values <0.4 are shown underlined, values >2.5 are shown in bold. qRT-PCR, quantitative real-time-PCR.

Table 4.9 Relative expression levels of efflux genes associated with antimicrobial resistance as determined by qRT-PCR from CF isolates

Isolates ^a	Gene ^b			
	<i>mexA</i>	<i>mexC</i>	<i>mexE</i>	<i>oprM</i>
PAO1	1.0	1.0	1.0	1.0
JFL05	0.81	12.09	131.3	7.16
JFL10	4.57	0.53	<u>0.05</u>	161.46
JFL28	3.84	56.76	<u>0.2</u>	20.63
JFL30	68.66	0.97	<u>0.06</u>	159.04
CF.2006.198	1.27	2.68	1.19	0.60
CF.2006.204	1.52	1.36	2.18	1.88
CF.2006.212	1.01	0.55	0.73	0.62
CF.2006.214	6.00	46.35	<u>0.25</u>	<u>0.27</u>
CF.2006.215	6.08	<u>0.37</u>	19.04	3.89
CF.2006.216	1.39	<u>0.11</u>	<u>0.06</u>	0.80
CF.2006.222	2.89	1.70	0.63	1.41
CF.2006.234	19.34	15.79	1.19	2.99
CF.2007.251	5.40	59.66	2.55	1.07
CF.2007.255	0.91	3.74	<u>0.29</u>	1.33
CF.2007.256	0.66	0.53	<u>0.30</u>	0.67
CF.2008.266	1.52	2.29	8.44	1.60
CF.2008.274	1.79	2.91	15.41	0.79
CF.2008.284	<u>0.03</u>	<u>0.34</u>	1.34	3.28
CF.2008.288	2.15	0.76	<u>0.01</u>	8.03
CF.2008.291	3.18	2.29	<u>0.20</u>	7.00
CF.2008.292	3.35	3.91	174.70	1.22
CF.2008.293	0.62	5.04	0.55	0.66

^aJFL05: MexEF-OprN-hyperexpressing isolate, JFL10: MexXY-hyperexpressing isolate, JFL28: MexCD-OprJ- hyperexpressing mutant, JFL30: MexAB-OprM- hyperexpressing mutant ³⁶³.

^bValues represent fold change (mean of triplicate) in comparison with the transcription level in wild type PAO1. Values <0.4 are shown underlined, values >2.5 are shown in bold. qRT-PCR, quantitative real-time-PCR.

4.2.3.4 Relative expression levels of *ampC*

In Chapter 3 it was reported that 30 NCF and 46 CF isolates showed rapid hydrolysis of chromogenic cephalosporin (1-2s) in combination with resistance to piperacillin and ceftazidime, indicating hyperproduction of AmpC or other cephalosporinases. Of the 30 NCF and 46 CF isolates, ten from the NCF and ten from the CF collection were included in the vMDR group. The phenotypic profiles of the twenty isolates and the expression levels of *ampC* can be seen in Table 4.10. A total of 9 isolates (5 NCF, 4 CF) had expression levels for *ampC* ranging from 2.53-5.72 times higher than PAO1. Two isolates, NCF.2007.142 and CF.2008.266 had expression levels ranging from 0.26-0.40 times lower than PAO1. The remaining 10 isolates had expression levels which were not significantly different from the wild type PAO1.

Table 4.10 Relative expression levels of *ampC* associated with β -lactam resistance as determined by qRT-PCR

Isolates	Penicillin	Cephalosporins		Gene expression
	Piperacillin	Ceftazidime	Cefepime	<i>ampC</i>
NCF.2006.1	256	128	S	5.72
CF.2008.293	256	>128	R	5.71
CF.2006.216	>256	>128	R	3.38
CF.2006.204	>256	64	R	4.99
NCF.2006.47	256	128	R	3.03
CF.2008.266	256	128	R	<u>0.26</u>
CF.2008.274	>256	128	R	2.53
CF.2008.288	256	128	S	0.99
NCF.2007.142	>256	64	S	<u>0.40</u>
CF.2008.284	>256	32	S	0.64
NCF.2008.193	64	>128	R	2.00
CF.2007.255	>256	16	R	1.63
NCF.2007.141	256	4	S	1.69
NCF.2006.53	64	128	S	3.84
NCF.2006.46	32	128	S	1.27
NCF.2006.72	32	64	S	0.87
NCF.2006.110	8	32	S	2.86
NCF.2006.100	16	8	S	3.79
CF.2008.291	32	4	S	1.47
CF.2006.234	8	1	S	1.40

^aMIC values highlighted in red indicate resistant phenotype, according to CLSI, values highlighted in yellow indicate intermediate resistant phenotype according to CLSI. ^bValues represent fold change (mean of triplicate) in comparison with the transcription level in wild type PAO1.Values <0.4 are shown underlined, values >2.5 are shown in bold. qRT-PCR, quantitative real-time-PCR.

4.2.3.5 Relative expression levels of *oprD*

The loss of the outer membrane protein OprD is associated with imipenem and meropenem resistance. The 33 vMDR isolates examined were resistant to at least one of the carbapenems examined. MICs to imipenem among the 33 vMDR isolates ranged from 4-128 mg/L, MICs to meropenem ranged from 2->128, (Table 4.11).

Expression levels of *oprD* ranged from 0.01-0.33 lower than the wild type *P. aeruginosa* PAO1 in 20 of the isolates. There was an increase in the expression of *oprD* (5.13 times higher than PAO1) in one isolate NCF.2006.106. In the remaining 12 isolates there was no significant difference in the expression level of *oprD* compared to the wild type *P. aeruginosa* PAO1, (Table 4.11).

Table 4.11 Relative expression levels of *oprD* as determined by qRT-PCR in the vMDR NCF and CF isolates

Isolates	Carbapenems ^a		Gene expression ^b
	Imipenem	Meropenem	<i>oprD</i>
	MIC (mg/L)	MIC (mg/L)	
NCF.2007.142	128	64	<u>0.35</u>
CF.2006.198	128	128	<u>0.23</u>
CF.2006.215	128	32	<u>0.33</u>
CF.2006.216	128	32	<u>0.10</u>
CF.2006.222	128	32	<u>0.17</u>
CF.2008.293	128	128	<u>0.29</u>
NCF.2008.193	64	64	<u>0.27</u>
CF.2008.266	128	128	<u>0.16</u>
NCF.2006.105	64	32	<u>0.17</u>
NCF.2006.46	64	64	<u>0.19</u>
NCF.2006.47	32	32	<u>0.07</u>
NCF.2006.1	32	16	<u>0.24</u>
CF.2007.256	16	16	<u>0.16</u>
NCF.2006.110	64	8	<u>0.01</u>
CF.2008.288	32	8	<u>0.25</u>
NCF.2006.72	16	8	<u>0.33</u>
CF.2006.214	32	2	<u>0.03</u>
NCF.2006.53	16	2	<u>0.27</u>
NCF.2006.100	16	4	<u>0.02</u>
CF.2008.274	4	16	<u>0.03</u>
CF.2006.204	128	32	0.82
CF.2006.212	128	128	0.41
CF.2007.251	32	>128	0.35
NCF.2006.102	128	128	0.96
NCF.2006.106	64	16	5.13
NCF.2006.107	64	16	1.04
NCF.2006.108	64	16	1.79
NCF.2007.141	32	64	0.63
CF.2008.284	32	64	0.92
CF.2007.255	32	16	1.56
CF.2006.234	32	1	0.59
CF.2008.291	32	2	0.46
CF.2008.292	32	4	0.42

^aMIC values highlighted in red indicate resistant phenotype, according to CLSI, values highlighted in yellow indicate intermediate resistant phenotype according to CLSI. ^bValues represent fold change (mean of triplicate) in comparison with the transcription level in wild type *P. aeruginosa* PAO1. Values <0.4 are shown underlined, values >2.5 are shown in bold. qRT-PCR, quantitative real-time-PCR.

4.2.4 Are the vMDR isolates clonally related?

In order to determine if the vMDR isolates were clonally related, pulsed field gel electrophoresis (PFGE) was carried out. A total of thirty three MDR isolates were selected for PFGE analysis based on their genotypic profiles, (Fig 4.5, 4.6). PFGE is an established DNA typing technique considered to be the 'gold standard' because it is highly discriminatory and reproducible ^{291,364-366}. *Xba*I digestion was performed successfully on all but two of the selected 33 isolates despite the use of addition proteinase K and 50 μ M thiourea (prevents the premature degradation of DNA by reactive Tris radicals), ³⁶⁷ and increased *Xba*I incubation from 18-24 hours. In total six different clusters (>80% similarity) were detected, (Fig 4.12, Table 4.12, 4.13). Band patterns that were >80% identical were considered related conform the Tenover criteria, ^{291,368}, which states that a 2-3 band difference indicates very closely related strains. The phenotypic and genotypic profiles of these 8 clusters are shown in Table 4.12, 4.13, 4.14, 4.15, 4.16, 4.17.

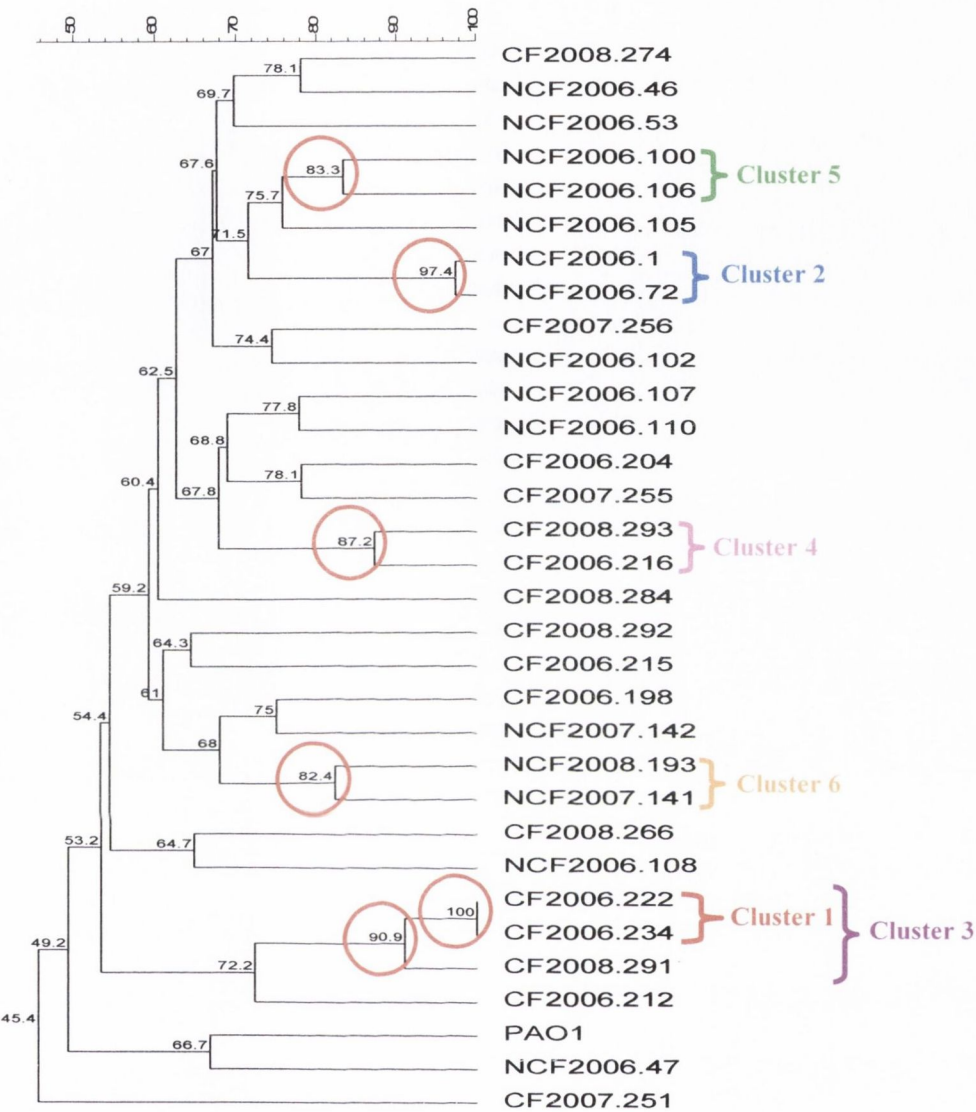


Fig 4.12 Dendrogram of pulsed-field electrophoresis (PFGE) profiles from 31 of the 33 MDR isolates. Two isolates CF.2006.214 and CF.2008.288 were untypable. A percentage similarity is shown above the dendrogram. A total of 6 clusters (>80% genetic similarity) were found, circled in red and gathered into 6 cluster groups. Band patterns that were more than >80% identical were considered related according to Tenover criteria (2-3 band difference indicates related strains).

Table 4.12 PFGE % similarity profiles of clusters 1-3 of vMDR isolates compared to genotypic and phenotypic results.

Isolate	PFGE % Similarity	Date of specimen collection	Phenotypic results ^a						Genotypic results ^b								
			Carbapenems		Aminoglycosides		Fluoroquinolones		MβL		Aminoglycoside Modifying enzymes				QRDR		
			Imipenem	Meropenem	Gentamicin	Amikacin	Ciprofloxacin	Levofloxacin	Imp	Vim	<i>aac(6')-Ib</i>	<i>ant(2'')</i>	<i>aac(3)-Ia</i>	<i>gyrA</i>	<i>gyrB</i>	<i>parC</i>	<i>parE</i>
CF.2006.222	Cluster 1	02/08/2006	128	32	128	>128	2	8						D87N Q106L	S87L	R377P	
CF.2006.234		23/08/2006	32	1	32	64	2	16					D87N		R377P		
NCF.2006.1	Cluster 2	04/04/2006	32	16	32	4	16	16		√		√		T83I	S87N K66L H84Q S87W Q49H		
NCF.2006.72		10/10/2006	16	8	128	128	32	32						T83I	S87L T57N K66H		
CF.2006.222	Cluster 3	02/08/2006	128	32	128	>128	2	8						D87N Q106L	S87L	R377P	
CF.2006.234		23/08/2006	32	1	32	64	2	16						D87N		R377P	
CF.2008.291		15/08/2008	32	2	32	128	4	4						T83I Q106L D87N		R377P	

Table 4.13 PFGE % similarity profiles of clusters 4-6 of vMDR isolates compared to genotypic and phenotypic results

Isolate	PFGE cluster	Date of specimen collection	Phenotypic results ^a						Genotypic results ^b									
			Carbapenems		Aminoglycosides		Fluoroquinolones		MβL		Aminoglycoside Modifying enzymes				QRDR			
			Imipenem	Meropenem	Gentamicin	Amikacin	Ciprofloxacin	Levofloxacin	Imp	Vim	<i>aac(6')-Ib</i>	<i>ant(2'')</i>	<i>aac(3)-Ia</i>	<i>gyrA</i>	<i>gyrB</i>	<i>parC</i>	<i>parE</i>	
CF.2006.216	Cluster 4	28/07/2006	128	32	64	64	4	8										
CF.2008.293		18/08/2008	128	128	64	>128	4	4										
NCF.2006.100	Cluster 5	24/10/2006	16	4	32	8	32	8							T83I D104A			
NCF.2006.106		16/11/2006	64	16	16	8	4	8							P79R T83I D104A			
NCF.2007.141	Cluster 6	01/06/2007	32	64	64	16	4	2							T83I			
NCF.2008.193		14/08/2008	64	64	16	32	4	8							T83I			

^a MICs highlighted in red (resistant according to CLSI) and yellow (intermediately resistant according to CLSI) are categorized as resistant, as seen in Chapter 3, unhighlighted MICs are categorized as susceptible according to CLSI guidelines. ^b √: The presence of a gene or mutation within a gene

Table 4.14 PFGE % similarity profiles of clusters 1-3 of vMDR isolates compared to phenotypic and qRT results for OprD and AmpC

Isolate	PFGE cluster	Date of specimen collection	Phenotypic results ^a					Genotypic results ^b	
			Carbapenems		Penicillin	Cephalosporins		qRT	
			Imipenem	Meropenem	Pipercillin	Ceftazidime	Cefepime	<i>oprD</i>	<i>ampC</i>
CF.2006.222	Cluster 1	02/08/2006	128	32				0.17	
CF.2006.234		23/08/2006	32	1	8	1	S	0.59	1.40
NCF.2006.1	Cluster 2	04/04/2006	32	16	256	128	S	0.24	5.72
NCF.2006.72		10/10/2006	16	8	32	64	S	0.33	0.87
CF.2006.222	Cluster 3	02/08/2006	128	32				0.17	
CF.2006.234		23/08/2006	32	1	8	1	S	0.59	1.40
CF.2008.291		15/08/2008	32	2	32	4	S	0.46	1.47

Table 4.15 PFGE % similarity profiles of clusters 4-6 of vMDR isolates compared to phenotypic and qRT results for OprD and AmpC

Isolate	PFGE cluster	Date of specimen collection	Phenotypic results ^a					Genotypic results ^b	
			Carbapenems		Penicillin	Cephalosporins		qRT	
			Imipenem	Meropenem	Pipercillin	Ceftazidime	Cefepime	<i>oprD</i>	<i>ampC</i>
CF.2006.216	Cluster 4	28/07/2006	128	32	>256	>128	R	0.10	3.38
CF.2008.293		18/08/2008	128	128	256	>128	R	0.29	5.71
NCF.2006.100	Cluster 5	24/10/2006	16	4	16	8	S	0.02	
NCF.2006.106		16/11/2006	64	16	32	4	S	5.13	
NCF.2007.141	Cluster 6	14/08/2008	32	64	256	4	S	0.63	1.69
NCF.2008.193		01/06/2007	64	64	64	>128	R	0.27	2.00

^a MICs highlighted in red (resistant according to CLSI) and yellow (intermediately resistant according to CLSI) are categorized as resistant, as seen in Chapter 3, unhighlighted MICs are categorized as susceptible according to CLSI guidelines. ^b √: The presence of a gene or mutation within a gene.

Table 4.16 PFGE % similarity profiles of clusters 1-3 of vMDR isolates compared to phenotypic and qRT results for efflux genes

Isolate	PFGE cluster	Date of specimen collection	Phenotypic results ^a						Genotypic results ^b			
			Carbapenems		Aminoglycosides		Fluoroquinolones		qRT			
			Imipenem	Meropenem	Gentamicin	Amikacin	Ciprofloxacin	Levofloxacin	<i>MexA</i>	<i>MexC</i>	<i>MexE</i>	<i>OprM</i>
CF.2006.222	Cluster 1	02/08/2006	128	32	128	>128	2	8	2.89	1.70	0.63	1.41
CF.2006.234		23/08/2006	32	1	32	64	2	16	19.34	15.79	1.19	2.99
NCF.2006.1	Cluster 2	04/04/2006	32	16	32	4	8	8	0.37	1.19	0.96	0.41
NCF.2006.72		10/10/2006	16	8	64	128	32	64	1.71	0.90	0.45	0.31
CF.2006.222	Cluster 3	02/08/2006	128	32	128	>128	2	8	2.89	1.70	0.63	1.41
CF.2006.234		23/08/2006	32	1	32	64	2	16	19.34	15.79	1.19	2.99
CF.2008.291		15/08/2008	32	2	32	128	4	4	3.18	2.29	0.20	7.00

Table 4.17 PFGE % similarity profiles of clusters 4-6 of vMDR isolates compared to phenotypic and qRT results for efflux genes

Isolate	PFGE cluster	Date of specimen collection	Phenotypic results ^a mg/L						Genotypic results ^b			
			Carbapenems		Aminoglycosides		Fluoroquinolones		qRT			
			Imipenem	Meropenem	Gentamicin	Amikacin	Ciprofloxacin	Levofloxacin	<i>MexA</i>	<i>MexC</i>	<i>MexE</i>	<i>OprM</i>
CF.2006.216	Cluster 4	28/07/2006	128	32	64	64	4	4	1.39	<u>0.11</u>	<u>0.06</u>	0.80
CF.2008.293		18/08/2008	128	128	64	>128	4	4	0.62	5.04	0.55	0.66
NCF.2006.100	Cluster 5	24/10/2006	16	4	32	8	32	8	0.65	24.07	1.25	0.33
NCF.2006.106		16/11/2006	64	16	16	8	4	8	0.48	<u>0.05</u>	0.45	0.84
NCF.2007.141	Cluster 6	01/06/2007	32	64	64	16	4	2	0.80	3.32	0.50	0.45
NCF.2008.193		14/08/2008	64	64	16	32	4	8	1.98	1.68	0.88	<u>0.43</u>

^a MICs highlighted in red (resistant according to CLSI) and yellow (intermediately resistant according to CLSI) are categorized as resistant, as seen in Chapter 3, unhighlighted MICs are categorized as susceptible according to CLSI guidelines. ^b Values represent fold change in comparison with the transcription level in wild type *P. aeruginosa* PAO1. Values ≤ 0.4 are shown underlined, values ≥ 2.5 are shown in bold.

PFGE cluster 1 consists of two CF isolates, CF.2006.222 and CF.2006.234 which were 100% identical. Both isolates had a non-mucoid morphology. The first isolate, CF.2006.222 was collected on the 02/08/2006 the second, CF.2006.234 was collected on the 23/08/2006. Isolate CF.2006.22 has higher resistant MIC for both carbapenems and aminoglycosides compared to isolate CF.2006.234. They both have similar resistant MICs for ciprofloxacin, isolate CF.2006.234 has a higher resistant MIC for levofloxacin. Isolate CF.2006.222 (which was collected first) has substitutions in three of the QRDR subunits (*GyrA*, *ParC* and *ParE*), in *gyrA* there were two substitutions, D87N and Q106L, in *parC*, S87L and *parE* R377P. Isolate CF.2006.234 had just one substitution in *gyrA*, D87N and one in *parE*, R377P. Isolate CF.2006.222 had a higher resistant MIC for imipenem and meropenem compared to CF.2006.234 which had a susceptible MIC for meropenem, the expression level of *oprD* was 0.17 for isolate CF.2006.222 compared to 0.59 for isolate CF.2006.234, indicating that *oprD* expression was reduced in isolate CF.2006.222 which had a higher resistance to the carbapenems.

In PFGE cluster 2, isolates NCF.2006.1 and NCF.2006.72 were 97.4% similar. NCF.2006.1 was collected on the 04/04/2006 from a sputa sample. Isolate NCF.2006.72 was collected on the 10/10/2006 from a swab sample. Isolate NCF.2006.1 (first collected) had higher resistant MIC compared to isolate NCF.2006.72 for the carbapenems; it also had the *bla*_{VIM-2} and *ant* (2'')-Ia genes. Both isolates have the same substitution in *gyrA* gene, T83I. Isolate NCF.2006.1 had four substitutions in *parC*, S57N, K66L, H84Q and S87W. Isolate NCF.2006.72 had four different substitutions in *parC*, Q49H, T57N, K66H and S87L. Isolate NCF.2006.1 had higher MICs for the carbapenems and piperacillin compared to NCF.2006.72. The expression level of *orpD* was 0.24 for NCF.2006.1 compared to 0.33 for NCF.2006.72. The expression level of *ampC* was 5.72 for NCF.2006.1 compared to 0.87 for 0.87 for NCF.2006.72; *ampC* derepression confers resistance to penicillins and most cephalosporins in *P. aeruginosa*.

PFGE cluster 3, which had 90.9 % similarity, consisted of three isolates, CF.2006.222 which was collected on the 02/08/2006 and had a non-mucoid phenotype, CF.2006.234 which was collected on the 23/08/2006 and had a non-mucoid phenotype and CF.2008.291 which was collected on the 15/08/2008 and had a mucoid phenotype. Isolate CF.2006.222 had a higher resistant MIC for imipenem, meropenem and gentamicin. CF.2006.234 has a higher MIC for levofloxacin compared to CF.2006.222 and CF.2006.234. Isolate CF.2006.222 has two substitutions in *gyrA*, D87N and Q106L,

one substitution in *parC*, S87L and one substitution in *parE*, R377P. CF.2006.234 has one substitution in *gyrA* D87N and one substitution in *parE*, R377P. Isolate CF.2008.291 has three substitutions in *gyrA*, T83I, D87N and Q106L and one substitution in *parE*, R377P. Isolate CF.2006.222 has a higher MIC for imipenem and meropenem compared to the other two isolates. The expression level of *oprD* is 0.17 for CF.2006.222, the expression level of *oprD* was not significantly different from WT PAO1, the expression level of *oprD* was 0.46 for CF.2008.291. Both CF.2006.234 and CF.2008.291 had susceptible phenotypes to meropenem, piperacillin, ceftazidime and cefepime and the expression level of *ampC* was not significantly different to wild type *P. aeruginosa* PAO1.

PFGE cluster 4 consists of two isolates CF.2006.216 and CF.2008.293 which were 87.2% similarity. Isolate CF.2006.216 was the first collected on the 28/07/2006, it had a mucoid phenotype. Isolate CF.2008.293 was collected on the 18/08/2008, it had a non-mucoid phenotype. Isolate CF.2008.293 had a higher resistant MIC for imipenem, meropenem and amikacin. The isolates had identical MICs for gentamicin, ciprofloxacin and levofloxacin. Neither isolate had substitutions in QRDR. Isolate CF.2006.216 had a higher MIC for imipenem. Both isolates had similar MICs for piperacillin, ceftazidime and cefepime. The expression of *oprD* was 0.10 for isolate CF.2006.216 and 0.29 for CF.2008.293. The expression level of *ampC* for CF.2006.216 was 3.38 and 5.71 for CF.2008.293, indicating a derepression of *ampC*.

PFGE cluster 5 consisted of two isolates, NCF.2006.100 which was isolated from a swab sample collected on the 24/10/2006 and NCF.2006.106 isolated from a sputa sample collected on the 16/11/2006. Both isolates were resistant to imipenem, gentamicin, ciprofloxacin and levofloxacin. Isolate NCF.2006.100 had a higher MIC to gentamicin and ciprofloxacin. Both isolates were susceptible to amikacin. Isolate NCF.2006.100 had two substitutions in *gyrA*, T83I and D104A. Isolate NCF.2006.106 had three substitutions in *gyrA*, P79R, T83I and D104A. Both isolates were resistant to imipenem and were susceptible to piperacillin, ceftazidime and cefepime. The expression level of *oprD* was 0.02, the expression level of *orpD* was 5.13 for isolate NCF.2006.106, suggesting *oprD* was upregulated.

PFGE cluster 6 consisted of two isolates which were 82.4 % similar, NCF.2007.141 which was isolated from a sputa sample collected on the 01/06/2007, NCF.2008.193 isolated from a sputa sample collected on the 14/08/2008. Both isolates were resistant to imipenem, meropenem, gentamicin and ciprofloxacin. Isolate

NCF.2008.193 had a higher MIC to imipenem and meropenem. Isolate NCF.2008.193 was resistant to amikacin and levofloxacin, isolate NCF.2007.141 was susceptible to amikacin and levofloxacin. Both isolates had the same single substitution in *gyrA*, T83I. Both isolates were resistant to imipenem, meropenem and piperacillin. Isolate NCF.2007.141 was resistant to ceftazidime and cefepime, isolate NCF.2008.193 was susceptible to ceftazidime and cefepime. Isolate NCF.2008.193 had an expression level to *oprD* of 0.27, in isolate NCF.2007.141 there was no significant difference in the expression of *oprD* compared to WT PAO1. There was no significant difference in the expression levels of *ampC* in both isolates compared to PAO1.

4.2.5 Detection of CF epidemic clones, Liverpool epidemic strain (LES), Manchester 1 epidemic strain (MES) and the Midlands 1 epidemic strain, frequently found in CF centres

All CF isolates were screened for the presence of the three most widely studied cystic fibrosis epidemic *P. aeruginosa* strains; the Liverpool epidemic strain (LES), the Manchester epidemic strain (MES) and the Midlands 1 epidemic strain^{282-285,289,369-371}. All 99 CF isolates were screened by multiplex PCR for the presence of two LES markers, PS21 (364bp)²⁸² and LESF9 (431bp)²⁸³, a Midlands 1 marker, MIDI (649bp)²⁸⁴ and a marker for MES, MAI5 (308bp)^{285,289}, positive controls were provided by Professor Winstanley (Fig 4.13).

Of the 99 tested, two isolates had PCR gene fragments which corresponded to one or other of the LES markers, PS21 and LESF9, (Fig 4.13). The phenotypic profiles of the isolates are shown in Table 4.17. After PCR amplification, isolate CF.2006.197 had a band corresponding to one of the LES markers, LESF9 (431bp), isolate CF.2008.288 had a band corresponding to LES-PS21 (364bp), sequencing confirmed this. False positives have been noted previously for the PS21 LES marker^{282-285,289,369-371}, as a result a second marker was identified, LESF9²⁸³. Neither PS21 nor LESF9, which map two separate genomic islands (LESGI-3 and LESGI-1, respectively) are not thought to be 100% specific but the combination of the two has not been reported previously in any non-LES strains. Because CF.2006.197 and CF.2008.288 did not possess both markers it was assumed they were not LES. Pulsed field gel electrophoresis was carried out on CF.2006.197 for further confirmation, (Fig 4.14).

4.3 Discussion

P. aeruginosa is naturally resistant to an array of structurally unrelated antimicrobial agents³⁶³, this is due in part to the low permeability of its outer membrane which is about 1/100 of the permeability of *E. coli* outer membrane³⁷², resistance is also due to the expression of efflux pumps which have a wide substrate specificity^{96,110,358,373-374} and the naturally occurring chromosomal AmpC β -lactamase. β -Lactams, fluoroquinolones and aminoglycosides are among the most widely used antimicrobials in the clinical setting, but resistance to these classes of antimicrobials is now common in many parts of the world^{87,229}. *P. aeruginosa* is responsible for between 10-15% of the nosocomial infections worldwide²⁹⁹ and is a major pathogen in CF patients³⁷⁵. Although there are many papers describing the different types of antimicrobial mechanisms present in *P. aeruginosa*, there are few studies detailing the prevalence of these mechanisms in clinical isolates of *P. aeruginosa*.

VIM-2 has been found widely across Europe and the rest of the world^{342,361,376-384,385,386-390}. The *bla*_{VIM-2} gene was first reported in Ireland in 2008³⁹¹. After initial phenotypic screening for M β Ls by Etest, six NCF isolates were confirmed by PCR and sequencing as carrying the *bla*_{VIM-2} gene, all six isolates also had an associated Class 1 integron. Although these isolates were not believed to be associated with an outbreak, the presence of mobile genetic elements carrying a resistance gene could pose a threat to infection control. A large number of the NCF and CF isolates which phenotypically demonstrated the presence of M β L, according to the M β L Etest, did not possess any of the M β L genes investigated in this study, a recently published paper³⁶¹ also found a relatively high proportion of false positives when using the M β L Etest.

Compared with the CF group, there were many more substitutions in the GyrA and ParC subunits in the NCF group. Although the most common substitutions in the Gyr A (T83I, D87N) and ParC (S87L) subunits were found in both NCF and CF isolates. The *gyrA* T83I, D87N and *parC* are among the most common substitutions found, and are known to contribute to fluoroquinolone resistance^{206-211,356,392-397}. The *gyrA* Q106K substitution was found in two NCF and 2 CF isolates, alterations at position 106 has been observed in another study by Lee *et al*²⁰⁸ and has been shown to be responsible for quinolone resistance in *E. coli*^{356,394}. Less common substitutions found in the GyrA subunit were always accompanied with either T83I or D87N substitution, further confirming the importance of this mutation in fluoroquinolone

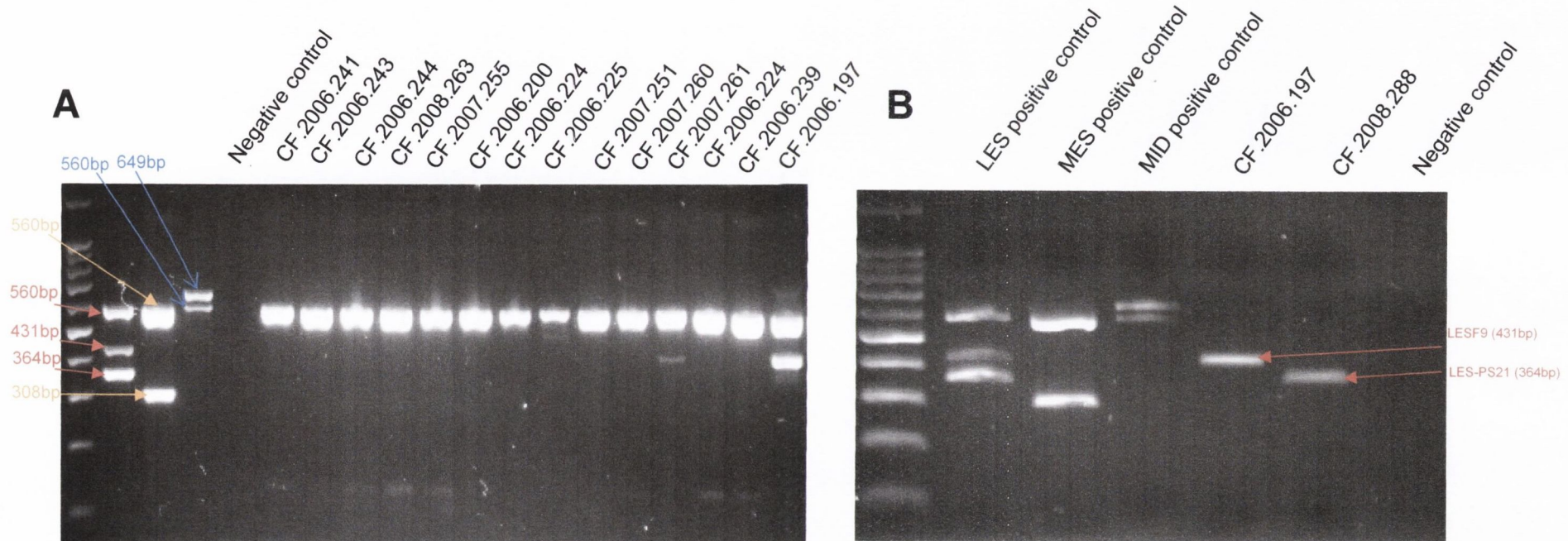


Fig 4.13 Detection of three UK CF epidemic strains, the Liverpool epidemic strain (LES), Midlands 1 and the Manchester epidemic strain (MES) by multiplex PCR. **(A)** Lane 1: 100bp ladder, lane 2:LES positive control (560bp product=16s RNA target, 431bp product=LESF9 target, 364bp= LES-PS 21 product), lane 3: MES positive control (560bp product=16s RNA target, 308bp = MAI5 target) lane 4: Midlands 1 positive control (560bp product=16s RNA target, 649bp=MIDI). **(B)** lane 1: 100bp ladder.

Dice (Opt:0.50%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
Xba1

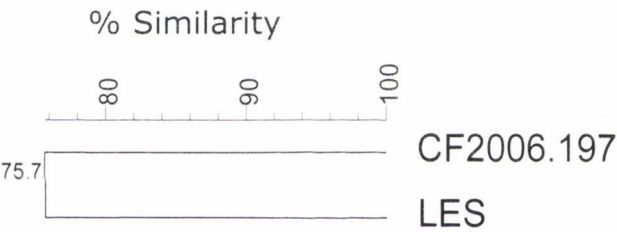


Fig 4.14 Dendrogram of pulsed-field gel electrophoresis (PFGE) profiles of CF.2006.197 and *P. aeruginosa* LES positive control, isolate CF.2008.288 was untypable by PFGE. There is a 75.7% similarity between CF.2006.197 and the LES isolate, they are not closely related.

resistance. Overall fluoroquinolone resistance in the NCF group was due to mutations found in the QRDR. Seven fluoroquinolone resistant isolates did not possess substitutions in the QRDR, it is possible that another mechanism may have contributed to their resistance. In the CF group, although the percentage of resistance to fluoroquinolones is higher than in the NCF group, a total of 32 resistant isolates did not have any substitutions in the QRDR, the cause of their resistance is undetermined. Four of these isolates are included in the MDR group which were examined for relative expression of efflux genes as determined by qRT and will be discussed later. The presence of plasmid-mediated *qnr* genes which have been shown to contribute to quinolone resistance in *Enterobacteriaceae*^{275,359,398} were not detected, although *qnr* genes have yet to be found in *P. aeruginosa*.

The presence of the most common aminoglycoside modifying enzymes²²² were investigated as part of this study. Only two genes were found, *aac(6')-Ib* and *ant (2'')-Ia* in three isolates, these isolates also possessed a Class 1 integron and it is likely that the aminoglycoside modifying enzymes were carried on these integrons, as has previously been shown³⁷⁹. That leaves 66 NCF and 83 CF isolates which do not possess the common aminoglycoside modifying enzymes, what could have caused aminoglycoside resistance in these isolates? It has been reported previously^{232,399-405} the significance impermeability plays in aminoglycoside resistance, especially in isolates from CF patients^{233,399,406-407}, where it is often the most common resistant mechanism. Impermeability could be due to changes in the structure of the OM of *P. aeruginosa*, the expression or repression of various outer membrane proteins or changes in LPS structure. Efflux could also play a part in the resistance of the isolates and will be discussed later. It could also be possible that another, less common aminoglycoside modifying enzyme is present, there are three known types of aminoglycoside-modifying enzymes; aminoglycoside nucleotidyltransferases (ANTs), aminoglycoside acetyltransferase (AACs) and aminoglycoside phosphotransferase (APHs). At least 50 different genes for aminoglycoside-modifying enzymes are known in bacteria⁴⁰⁸. The majority of the NCF and CF isolates which were resistant to the aminoglycosides were also resistant to at least one or more of the other antimicrobial classes, so there may be multiple factors present which resulted in cross resistance.

Quantitative real-time PCR (qRT-PCR) was employed to investigate the expression of the efflux genes along with outer membrane protein OprD and the

chromosomal β -lactamase AmpC because of its excellent sensitivity and specificity^{102,409}. The important role that efflux systems play in the resistant profile of *P. aeruginosa* has been known for a number of years^{96-97,100-104,358,410-411}. The Mex efflux systems are considered to be the most clinically relevant because of their exceptional broad substrate specificity³⁵⁸. Four of the Mex efflux systems (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) have been best characterised as antimicrobial transporters in *P. aeruginosa*³⁵⁸. The MexAB-OprM and MexXY-OprM efflux pump are expressed at basal level in WT strains, but are overexpressed in resistant isolates^{97,144,274,412}. MexCD-OprJ and MexEF-OprN are not normally expressed in WT strains but their expression is up-regulated in resistant isolates^{131,149,413}. The upregulation of at least one efflux related gene was seen in 23 out of the 33 MDR isolates examined. Since these isolates were resistant to a number of antimicrobials, it is possible that these isolates are expressing multiple efflux pumps, it has been shown previously that isolates can simultaneously overproduce multiple efflux pumps which broaden their resistance profile²⁷³. Generally among the isolates examined, when there was an increase in expression of *mexA* there was also an increase in expression of *oprM*, with a number of exceptions where there was an increase in expression of *mexA* but not *oprM*. In these isolates the expression of *mexC* and *mexE* was increased, it is likely that the MexAB-OprM efflux system was not the main efflux pump involved in resistance. There were also a number of isolates where expression of *oprM* was greater than *mexA*, in these isolates no other efflux gene was overexpressed. This is unusual as *oprM* is located on the same operon as *mexA*, the reason for this is unclear there was possibly a mutation in *mexA* which did not allow it to be transcribed in full. The expression of *mexC* and *mexE* has been shown to be down-regulated in wild type strains,^{131,412}. The *mexC* gene was upregulated in 14 isolates; it was the only gene upregulated in five isolates suggesting that the MexCD-OprJ was the most prominent of the three efflux pumps examined. *mexE* was upregulated in 8 isolates but it was always upregulated in combination with a least one other efflux pump gene. When comparing the efflux gene expression results with PFGE results it is observed that the expression of the efflux genes is different in each isolate, so it appears that efflux plays a greater or lesser part in each isolate.

Carbapenems, especially imipenem and meropenem are known to diffuse through the OprD porin⁴¹⁴. OprD has been shown to be regulated at a transcriptional¹³²

and posttranscriptional level¹³³ studies have found a good correlation between gene expression and protein expression^{102,415}. All isolates examined were resistant to either one or other or both of the carbapenems (imipenem, meropenem). Expression of *oprD* was down-regulated in 29 isolates which was expected. *oprD* expression levels were similar to PAO1 in three isolates, of these three two isolates had at least one efflux gene up-regulated, the other isolate did not. Interestingly one isolate, NCF.2006.106 over-expressed *oprD*, yet this isolate was resistant to imipenem and meropenem.

Chromosomal AmpC β -lactamase confers resistance to penicillins and most cephalosporins. Its expression can be partially or completely derepressed because of changes in the regulatory loci⁴¹⁶, overproduction of AmpC enzyme can also occur through induction of *ampC* gene during exposure to specific β -lactams (e.g. cefoxitin, imipenem) but is reversible after the agent is removed². AmpC expression was upregulated to some degree in most isolates with the exception of a few isolates where AmpC was downregulated, it is possible that AmpC derepression is not a resistance mechanism in these isolates.

The use of qRT-PCR in the hospital laboratory setting as a rapid and sensitive method for the determination of expression profiles of resistant isolates has been suggested by Dumas *et al*¹⁰². This study found that, although qRT-PCR is a very useful research tool, the technique itself requires extensive optimization and lengthy preparation of samples, furthermore reagents can be very expensive. Its use in the clinical laboratory setting as a rapid tool for the identification for the overproduction of efflux systems therefore may not be appropriate.

Variation in DNA fingerprinting profiles derived from PFGE analysis is often the result of insertions and or deletions rather than nucleotide polymorphisms⁴¹⁷⁻⁴¹⁸ but as it has been shown strains are able to acquire genomic islands and plasmids without the disruption of its overall genomic structure⁴¹⁹. PFGE results revealed the presence of 6 clonal groups among the MDR group. Cluster 1 and 3 consisted of CF isolates and clusters 2, 5 and 6 consisted of NCF isolates ranging in clonality between 87-100% for the CF isolates and between 83-97% for the NCF isolates. A cut off point of >80% similarity was chosen, as isolates above this threshold were considered to be closely related according to previous reports,^{291,368}. All of the isolates examined were vMDR, although the isolates within each cluster were closely related, the degree of resistance to each antimicrobial varied. A number of substitutions in the QRDR were maintained within the isolates in each cluster, in particular the T83I and D87N substitution in *gyrA*,

indicating that this is a stable substitution which contributes to fluoroquinolone resistance. Efflux, *oprD* and *ampC* gene expression also varied within each isolates. What can be deduced from this is that although the isolates are closely clonally related, they have been isolated from separate individuals and presumably are responding to the specific host environment that they are in. CF isolates from clusters 1, 3 and 4 were collected anywhere from 15 days to 2 years apart. All of the CF isolates have come from CF patients treated in the same centre so it is not surprising that certain *P. aeruginosa* isolates are shared and persist in the small CF population.

NCF isolates belonging to clusters 2, 5 and 6 were collected between 14 days to 14 months apart. The isolates in these clusters had either come from sputa or swab samples and with the exception of cluster 6; all of the isolates were hospital acquired. Cluster 6 isolates, NCF.2007.141 and NCF.2008.193 had both come from sputa samples. NCF.2007.141 was hospital acquired whereas NCF.2008.193, which was collected 14 months later was community acquired. It could be argued that the hospital environment is acting as a selective environment for resistant bacteria which can then be potentially disseminated in the community. In order to prevent the spread of MDR *P. aeruginosa* within the hospital environment, strict infection control procedures must be adhered to.

The CF isolates were screened for the presence of three widely studied cystic fibrosis epidemic strains^{282-285,289,369-371}, the Liverpool, Midland 1 and Manchester epidemic strains were not found amongst the CF isolates in this study. The Liverpool epidemic strain (LES) has been found to be highly transmissible and virulent⁴²⁰⁻⁴²³, establishing infection not only in CF patients but also non-CF individuals⁴²¹ and even cats⁴²⁰. It is associated with greater morbidity⁴²³ and appears to have enhanced survival in the air droplets⁴²⁴. It is the most common clone found amongst CF patients in England and Wales⁴¹⁷, its also been identified in Scotland⁴²⁵ and more recently in Canada⁴²⁶. It is believed that an individual travelling to a CF camp in Canada either brought the LES with them or picked it up in Canada and brought it back to the UK, this assumption is based on earlier observations of *Burkholderia cenocepacia* which was found in CF patients from Scotland, Manchester and Canada⁴²⁶. It is unknown how many Irish CF patients travel to the UK for treatment or to attend meetings, but as yet the LES, Midland 1 and Manchester epidemic strains have not been reported in the Republic of Ireland.

From this chapter it can be surmised that the *P. aeruginosa* isolates from CF and NCF patients are phenotypically and genotypically diverse, with the exception of the six PFGE clusters. Continued antimicrobial surveillance along with infection control is essential in preventing clonal outbreaks.

Chapter 5 Hypermutation in *Pseudomonas aeruginosa*

5.1 Introduction

Mutators or hypermutators are microorganisms which have a mutation rate which can be up to 1000-fold higher than wild-type strains ^{402,427}. The mutator phenotype results from a defect in the DNA repair or error avoidance systems. The mismatch match repair (MMR) is the most important of these systems, alterations due to the failure of the MMR system are the most common cause of hypermutation in bacterial populations ⁴²⁸⁻⁴²⁹. The MMR system detects and repairs errors which occur during replication, including short insertions, mismatches and deletions, thereby ensuring genetic fidelity in each generation. The genes involved in the MMR system are *mutS*, *mutL*, *mutH*, *uvrD* or *mutU*, inactivation of any of these genes has been known to increase the rate of mutation from 100-1000 fold in *E. coli* ⁴³⁰.

Generally speaking in most natural bacterial populations, the majority of isolates have a low mutation frequency (10^{-1}); this presumably is because long term genetic stability is favoured in wild type populations over hypermutable isolates which may accumulate deleterious mutations ⁴³¹. However, hypermutation may be advantageous in certain environments. Over the last number of years experiments have shown that the mutator phenotype confers an evolutionary advantage during bacterial adaption to new environments ⁴³²⁻⁴³³ such as, the host immune system ⁴³⁴ and to antimicrobial treatment ⁴³⁵.

The CF lung is a challenging environment for bacteria to colonise and survive in; it has a fluctuating inflammatory responses and may have varied exposure to different antimicrobials often for prolonged periods of time. These factors make the CF lung an ideal environment for the establishment of a stable hypermutator phenotype ^{82,436-437}. The association between antimicrobial resistance and hypermutability in chronic CF infections has been reported previously. However, only a few of these studies have examined the mechanisms of hypermutation ^{82,436-443} and only one study has examined hypermutation in non-CF MDR isolates ⁴⁴⁴. In that study, the mechanisms of hypermutation were not investigated.

Since few resistance genes were detected in the MDR group it is postulated that hypermutation could be the cause of the MDR phenotype.

The aim of this study presented in this chapter was;

- 1) To establish the prevalence of the hypermutator phenotype in the multidrug resistant (MDR) group which includes both NCF and CF clinical isolates
- 2) To determine the presence of mutations in the *mutS*, *mutL* and *uvrD* genes which may be responsible for the hypermutator phenotype
- 3) To relate the hypermutator phenotype to mutations in QRDR and relative expression of efflux genes

5.2 Results

5.2.1 Are mutators present in the MDR group?

In Chapter 4 thirty three isolates were categorised as very multi-drug resistance (vMDR). The NCF and CF isolates which made up the vMDR group were investigated to determine if they were hypermutators.

5.2.1.1 Determining mutation frequencies of MDR *P. aeruginosa*

Mutation frequencies were determined by plating on rifampicin media, based on the method described by Oliver *et al*⁴³⁶ as outlined in Section 2.1.2.4. Briefly, three independent overnight cultures from each isolate were serially diluted and inoculated onto MH plates with and without 300mg/L rifampicin. The plates were examined after 36h, mutant isolates were re-streaked on to rifampicin plates. Isolates were considered to be hypermutators if they had a mutation frequency 20-times greater than that of *P. aeruginosa* PAO1⁴³⁶ ($\geq 4.8 \times 10^{-8}$). In Fig 5.1 and Table 5.1, 5.2 the mutation frequencies for the MDR group are shown along with colony morphology and PFGE grouping. Fifteen NCF isolates were included in the MDR group and all had a non-mucoid morphology. As outlined in Chapter 3 the isolates have come from a variety of sources. In total, two isolates had come from MSU, four from wound swabs and nine from sputa samples. One isolate, NCF.2006.47 had a mutation frequency of 12.7×10^{-1} , which was the highest mutation frequency seen amongst the MDR group.

Seen CF isolates were included in the MDR group, nine isolates were non-mucoid and nine isolates had a mucoid morphology. A total of twelve CF isolates were categorised as hypermutators. Of these 12, six were non-mucoid and six were mucoid. According to PFGE results five of these hypermutator isolates belonged to PFGE groups, as defined in Chapter 4. Isolates CF.2006.216 (which was mucoid) and CF.2008.293 (non-mucoid) belonged to Group 3, isolates CF.2006.222 (non-mucoid), CF.2006.234 (non-mucoid) and CF.2008.291 (mucoid) belonged to Group 4. Isolate CF.2008.274 (mucoid) belonged to Group 8 which included the NF isolate NCF.2006.102 which was not a hypermutator. All the CF isolates had come from sputa samples.

Table 5.1 Mutation frequencies for NCF *P. aeruginosa* isolates

Isolate	PFGE Cluster ^a	Mutation Frequency ^b
<i>P.aeruginosa</i> PAO1		4.8 x 10 ⁻⁸
NCF.2006.47		12.7 x 10 ⁻¹
NCF.2006.1	2	1.4 x 10 ⁻⁸
NCF.2006.53		1.87 x 10 ⁻⁸
NCF.2006.102		2.1 x 10 ⁻⁸
NCF.2006.105		2.5 x 10 ⁻⁸
NCF.2006.107		2.9 x 10 ⁻⁸
NCF.2006.46		3.6 x 10 ⁻⁸
NCF.2006.106	5	4.0 x 10 ⁻⁸
NCF.2007.142		4.0 x 10 ⁻⁸
NCF.2006.110		5.4 x 10 ⁻⁸
NCF.2007.141	6	6.1 x 10 ⁻⁸
NCF.2006.72	2	6.3 x 10 ⁻⁸
NCF.2008.193	6	6.8 x 10 ⁻⁸
NCF.2006.108		7.3 x 10 ⁻⁸
NCF.2006.100	5	8.6 x 10 ⁻⁹

^aPFGE groups as in Chapter 4. ^bMutation frequencies, each isolate was tested in triplicate (mean value shown).

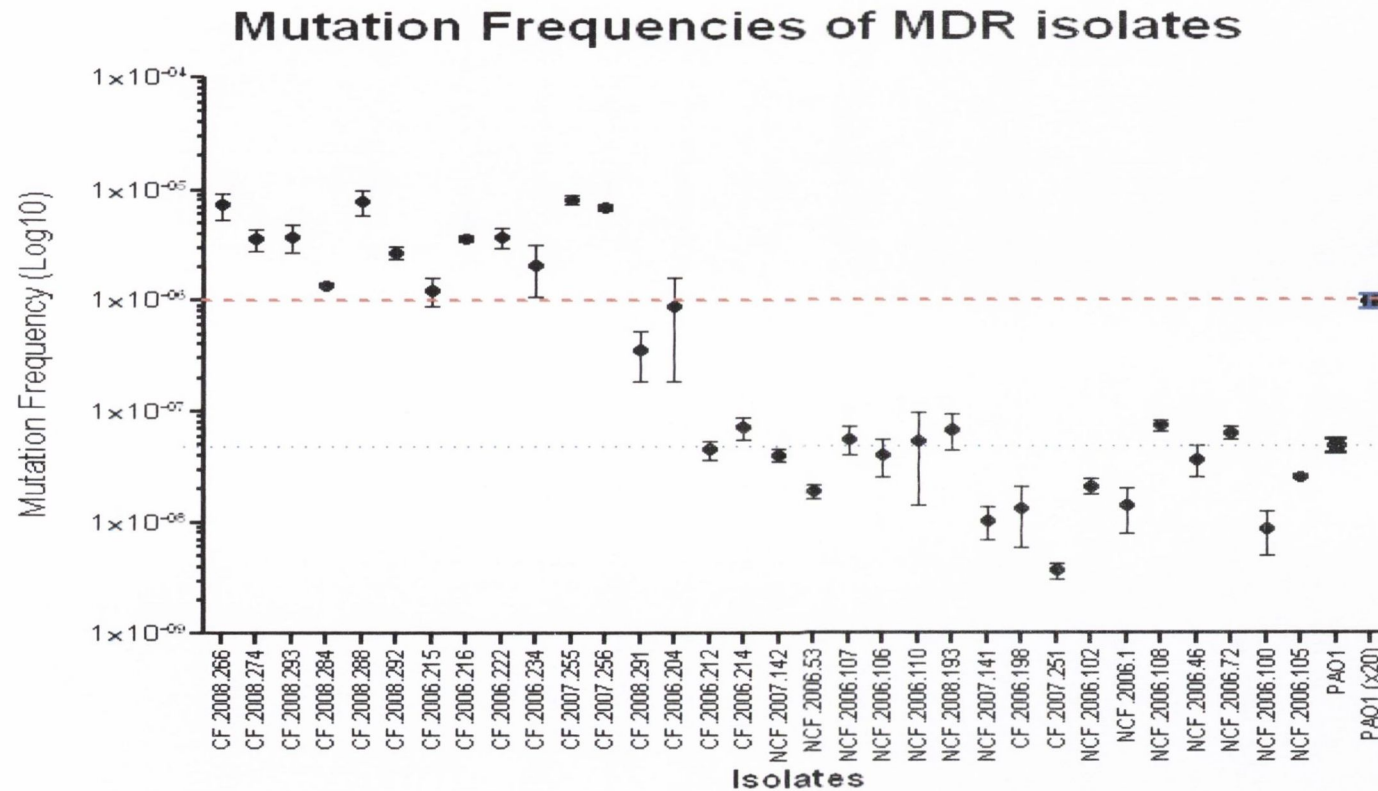


Fig 5.1 Mutation frequencies for 14 NCF and 18 CF MDR *P. aeruginosa* isolates. All strains were tested in three independent cultures. The blue dashed line represents the mutation frequency of the laboratory reference strain *P. aeruginosa* PAO1. The dotted red line represents the breakpoint for mutators, isolates were considered to be strong mutators if they had a mutation frequency of $\geq 1 \times 10^{-6}$ (approximately 20 times greater than PAO1). Isolate NCF.2006.47 was not included as it was not believed to be a hypermutator.

Table 5.2 Mutation frequencies for CF *P. aeruginosa* isolates

Isolate	Morphology	PFGE Cluster ^a	Mutation Frequency ^b
<i>P.aeruginosa</i> PAO1			4.8 x 10 ⁻⁸
CF.2006.215	Mucoid		1.2 x 10 ⁻⁶
CF.2008.284	Non-Mucoid		1.3 x 10 ⁻⁶
CF.2006.234	Non-Mucoid	1, 3	2.0 x 10 ⁻⁶
CF.2008.292	Mucoid		2.6 x 10 ⁻⁶
CF.2006.216	Mucoid	4	3.5 x 10 ⁻⁶
CF.2008.274	Mucoid		3.5 x 10 ⁻⁶
CF.2006.222	Non-Mucoid	1, 3	3.7 x 10 ⁻⁶
CF.2008.293	Non-Mucoid	4	3.7 x 10 ⁻⁶
CF.2007.256	Mucoid		6.8 x 10 ⁻⁶
CF.2008.266	Non-mucoid		7.2 x 10 ⁻⁶
CF.2008.288	Mucoid		7.8 x 10 ⁻⁶
CF.2007.255	Non-Mucoid		8.0 x 10 ⁻⁶
CF.2008.291	Mucoid	3	3.5 x 10 ⁻⁷
CF.2006.204	Non-Mucoid		8.1 x 10 ⁻⁷
CF.2006.198	Non-Mucoid		1.3 x 10 ⁻⁸
CF.2006.212	Mucoid		4.5 x 10 ⁻⁸
CF.2006.214	Mucoid		7.1 x 10 ⁻⁸
CF.2007.251	Non-Mucoid		9.5 x 10 ⁻⁸

^aPFGE groups as in Chapter 4. ^bMutation frequencies, each isolate was tested in triplicate (mean value shown) highlighted in red indicate mutators.

5.2.2 Investigation of rifampicin resistance in mutator isolates

Isolate NCF.2006.47 had an extremely high mutation frequency. It was speculated that this isolate may be highly resistant to rifampicin *per se*, which contributed to the high mutation frequency. Isolate NCF.2006.47 was inoculated on to triplicate plates containing 300µg/ml rifampicin and grown at 37°C for 24h, after which time the plates were examined. Large colonies were seen on both the MH and the rifampicin containing plates. All mutator strains were included in the following study prior to exposure to rifampicin, to ensure that the hypermutator phenotype was not caused by mutations in the *rpoB* gene.

5.2.2.1 Sequence analysis of *rpoB* gene in mutator isolates

Rifampicin resistance is associated with mutations in the *rpoB* gene, in order to investigate this, the DNA region corresponding to the rifampicin cluster I, II and II of *E. coli rpoB* gene was amplified in isolate NCF.2006.47 and the other mutator isolates, (Fig 5.2), isolates were taken from the MH plate. Mutations associated with rifampicin resistance are almost exclusively located in the *rpoB* gene and have been linked to amino acid alternations found in three regions of the β -subunit of RNA polymerase (known as cluster I-III), mutations in the N-terminal region of the gene are rare^{287,445-447}. Clusters I, II and III are highly conserved in prokaryotes⁴⁴⁷⁻⁴⁴⁸. A paper published recently by Jatsenko *et al*²⁸⁷ revealed a high conservation of these regions in *P. aeruginosa* PAO1. No mutations or deletions were detected in the *rpoB* gene in the other 12 mutator isolates. Isolates NCF.2006.47 had a mutation in cluster I (D521G) which is known as a mutational hotspot and has been associated with high level rifampicin resistance in *P. aeruginosa*²⁸⁷.

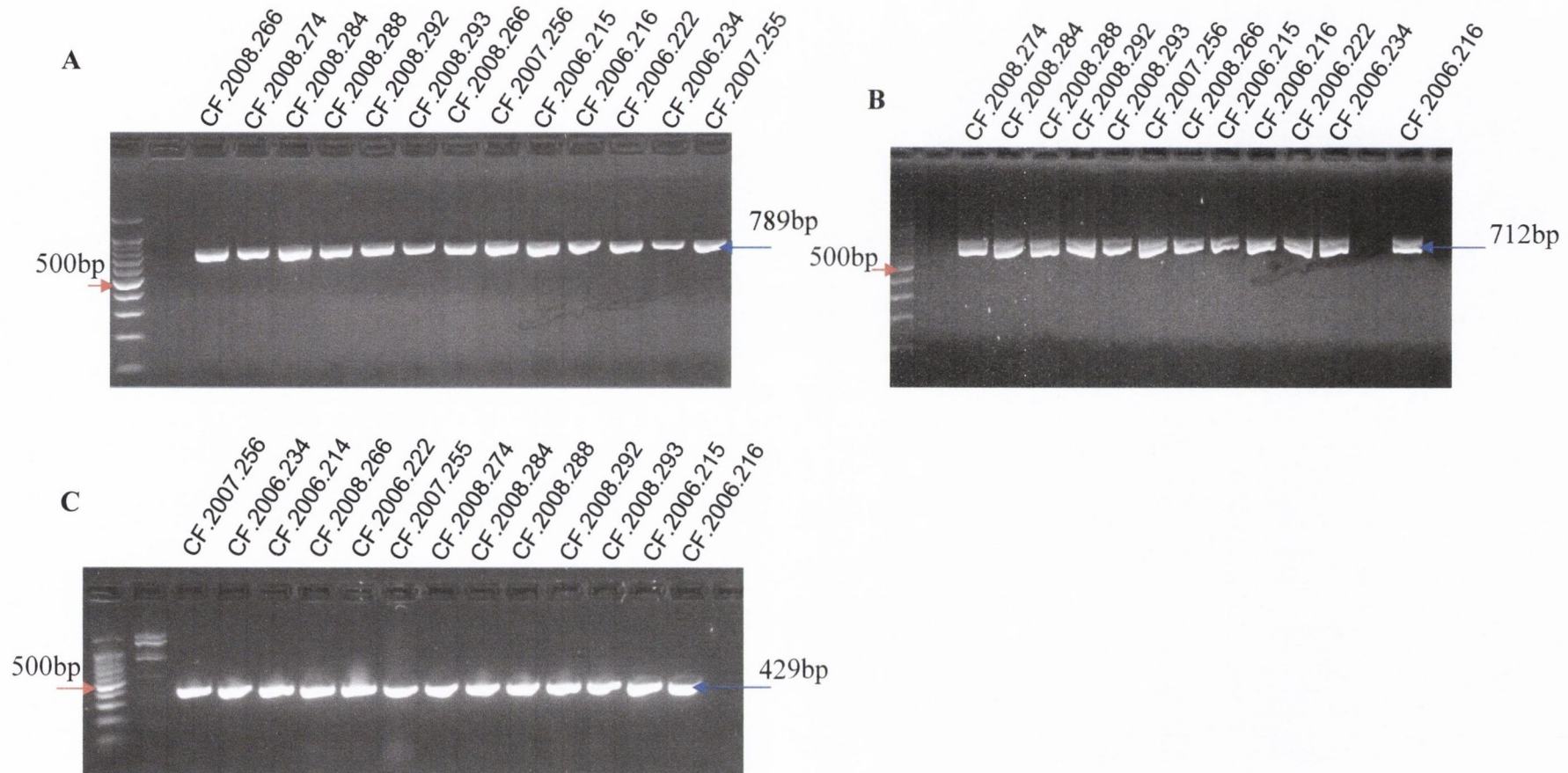


Fig 5.2 PCR amplifications of the *rpoB* gene. **(A)** PCR amplification of DNA corresponding to covering cluster I, II, region in-between II and III, III of *rpoB* gene (789bp), lane 1:100bp ladder, lane 2: negative control. **(B)** PCR amplification of DNA to nucleotide sequence at position 2-26, 694-713 relative to ATG initiation codon of *rpoB*. lane 1: 100bp ladder, lane 2: negative control. **(C)** PCR amplification covering between 1649-2078bp (429bp), lane 1: 100bp ladder, lane 2: unrelated isolate.

5.2.2.2 How does a high mutation frequency affect antimicrobial resistance?

Out of the 12 hypermutators found in the MDR group, a statistically significant number ($P=0.0001$, by Fischer's exact Test) originated from CF *P. aeruginosa* isolates, none had come from the NCF *P. aeruginosa* isolates. The high proportion of hypermutators present in CF population in comparison to NCF is similar to previously published results^{438,442,444}. Mutations in the quinolone resistant determining region (QRDR) of the fluoroquinolone resistant hypermutator isolates are presented in Table 5.3. A total of 5/12 of the hypermutator isolates has mutations in the GyrA subunit, the most common mutations were T83I (3/5 isolates) and D87N (3/5 isolates). These mutations have been shown previously to contribute to fluoroquinolone resistance^{206-211,356,392-397}. Mutations were not found in the GyrB subunit. One common mutation was seen in the ParC subunit, in 5/12 isolates (S87L). A total of 5/12 isolates had a mutation in the R377 amino acid in ParE. Overall the level of resistance to the fluoroquinolones was relatively low, (ranging from 2-32mg/L). Mutations in the QRDR contribute to resistance in some of the isolates but there are a number of hypermutator isolates which do not have mutations in QRDR.

The relative expression levels of the efflux genes, *mexA*, *mexC*, *mexE*, *oprM*, the outer membrane protein OprD and AmpC β lactamase in the hypermutator isolates are presented in Table 5.4. As discussed in chapter 4, the MexAB-OprM, MexCD-OprJ, MexEF-OprN efflux systems are capable of effluxing a variety of structurally unrelated antimicrobials. A total of 10/12 hypermutators displayed an increased expression of at least one of the efflux genes examined. A decrease in expression of the *oprD* gene was observed in 8/12 hypermutator isolates, the loss of the outer membrane protein is associated to carbapenem resistance¹⁰².

The relative expression levels of *ampC* were examined in twenty isolates in Chapter 3, of those isolates, 8 were hypermutators, *ampC* expression was shown to be increased in 3/8 of the hypermutators.

Table 5.3 Fluoroquinolone resistance in hypermutator vMDR isolates

Isolate	PFGE Cluster	Phenotypic results ^a		Genotypic results ^b			
		Fluoroquinolones		QRDR			
		Ciprofloxacin	Levofloxacin	<i>gyrA</i>	<i>gyrB</i>	<i>parC</i>	<i>parE</i>
CF.2008.288		8	16	X	X	X	X
CF.2008.292		4	16	T83I	X	X	X
CF.2008.284		4	8	X	X	X	X
CF.2008.274		4	8	T83I	X	S87L	R377A
				D87N			
CF.2007.255		2	32	T83I	X	S87L	R377A
CF.2007.256		2	32	X	X	S87L	R377A
CF.2006.234	1,3	2	16	D87N	X	X	R377P
CF.2006.222	1,3	2	8	D87N	X	S87L	R377P
				Q106L			
CF.2008.293	4	4	4	T83I	X	X	X
CF.2006.216	4	4	8	X	X	X	X
CF.2006.215		4	2	X	X	X	X
CF.2008.266		4	2	X	X	S87L	X

^aMICs highlighted in red (resistant according to CLSI) and yellow (intermediately resistant according to CLSI), as seen in Chapter 3, unhighlighted MICs are categorized as susceptible according to CLSI guidelines. ^bX: The absence of a mutation within a gene.

Table 5.4 Phenotypic profile of vMDR hypermutators compared to qRT results for *mexA*, *mexE*, *oprM*, *oprD* and *ampC*

Isolate	PFGE Cluster	Phenotypic results ^a						Genotypic results ^b					
		Carbapenems		Aminoglycosides		Fluoroquinolones		qRT					
		Imipenem	Mero	Genta	Amikacin	Cipro	Levo	<i>mexA</i>	<i>mexC</i>	<i>mexE</i>	<i>oprM</i>	<i>oprD</i>	<i>ampC</i>
CF.2008.293	4	128	128	128	>128	4	4	0.62	5.04	0.55	0.66	0.29	5.71
CF.2006.222	1, 3	128	32	128	>128	2	8	2.89	1.70	0.63	1.41	0.17	
CF.2008.216	4	128	32	64	64	4	8	1.39	0.11	0.06	0.80	0.10	3.18
CF.2006.215		128	32	64	64	4	2	6.08	0.37	19.04	3.89	0.33	
CF.2007.255		32	16	16	64	2	32	0.91	3.74	0.29	1.33	1.56	1.63
CF.2007.256		16	16	16	64	2	32	0.66	0.53	0.30	0.67	0.16	
CF.2008.284		32	64	16	32	4	8	0.03	0.34	1.34	3.28	0.92	0.64
CF.2008.288		32	8	128	32	8	16	2.15	0.76	0.01	8.03	0.25	0.99
CF.2008.274		4	16	64	128	4	8	1.79	2.91	15.41	0.79	0.03	2.53
CF.2008.266		128	128	16	16	4	2	1.52	2.29	8.44	1.60	0.16	0.26
CF.2008.292		32	4	64	128	4	16	3.35	3.91	174.70	1.22	0.42	
CF.2006.234	1, 3	32	1	32	64	2	16	19.34	15.79	1.19	2.99	0.59	1.40

^aMICs highlighted in red (resistant according to CLSI) and yellow (intermediately resistant according to CLSI), as seen in Chapter 3, unhighlighted MICs are categorized as susceptible according to CLSI guidelines. Mero: meropenem, Gent: gentamicin, Cipro: ciprofloxacin, Levo: levofloxacin. ^bValues represent fold change (mean of duplicate samples) in comparison with the transcription level in wild-type *P. aeruginosa* PAO1. Values ≤ 0.4 are shown in italics, values ≥ 2.5 are shown in bold. qRT-PCR, quantitative real time PCR chain reaction.

5.2.3 Involvement of *mutS*, *mutL* and *uvrD* genes in hypermutation

5.2.3.1 Determining common mutations in the *mutS* gene

Sequence homology to the *E. coli* MMR genes, *mutS*, *mutL* and the DNA helicase II (UvrD) have been found in *P. aeruginosa*, in contrast no homologues have been found to the *E. coli* endonuclease MutH^{427,436,449-450}. After repeated attempts, *mutS* amplification was only successful for 5/13 hypermutators, (Fig 5.3 and Table 5.5). Amino acid substitutions were found in *mutS* for 3/5 isolates. Isolate CF.2008.293 and CF.2006.216 both had the same non-synonymous mutation (A545T), this mutation has been reported previously⁴³⁷ in a clinical epidemic isolate which had come from Dublin, both isolates are clonally related, (Table 5.5). CF.2008.293 has two additional non-synonymous mutations, E693G which was also reported previously by Kenna *et al*⁴³⁷, A545 and E693 are part of the V domain of MutS which is involved in ATPase activity^{427,451-452}. These mutations could explain the elevated mutation frequencies observed. The third mutation in isolate CF.2008.293 was located in the region beyond helix-turn-helix domain (K810E), as far as I am aware this mutation has not been reported previously, it is located in the C-terminal domain region of *P. aeruginosa* which is thought to be involved in MutS oligomerization⁴⁵³, this mutation could contribute to elevated mutation frequency seen in this isolate. CF.2006.234 was the third isolate with a non-synonymous mutation (E672K), also located in ATPase domain^{427,451-452}, as far as I am aware this mutation has not been reported previously but could contribute to elevated mutation frequency seen in this isolate.

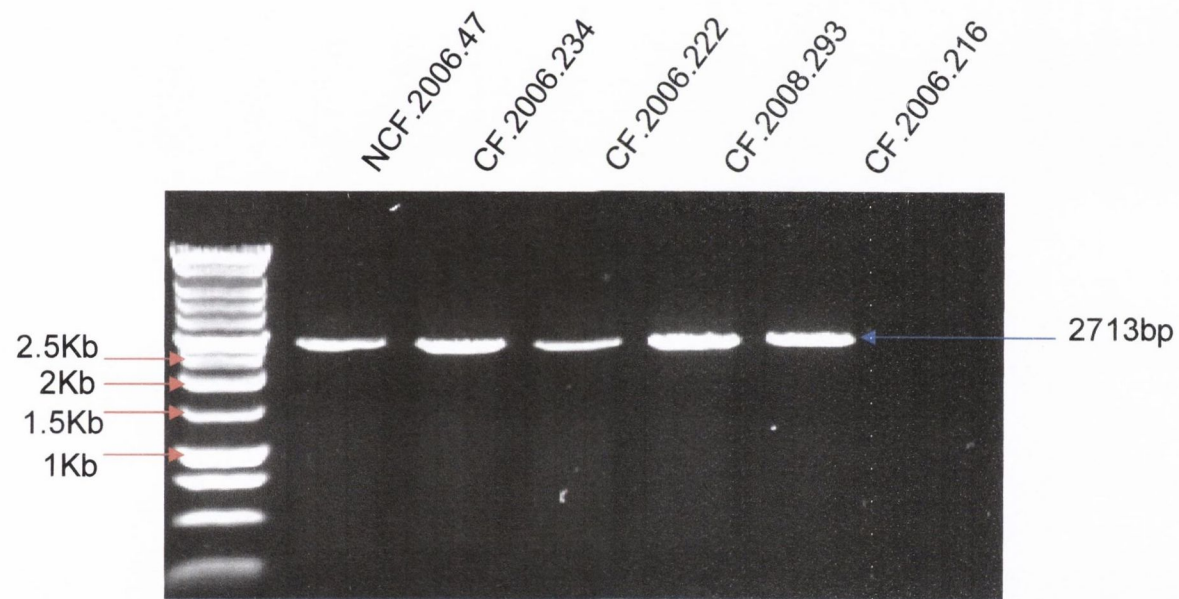


Fig 5.3 Amplification of *mutS* gene (2713bp). Lane 1: 1Kb DNA ladder, lane 6: negative control (dH₂O).

Table 5.5 Characterization of mutator *P. aeruginosa* isolates

Isolate	Morphology	PFGE Cluster ^a	Mutation Frequency ^b	Gene ^c		
				<i>mutS</i>	<i>mutL</i>	<i>uvrD</i>
NCF.2006.47	Mucoid		12.7 x 10 ⁻¹	/	/	K344R
CF.2006.215			1.2 x 10 ⁻⁶	NA	1bp insertion (→T)	1bp insertion (→T)
CF.2006.216			3.5 x 10 ⁻⁶	A545T	/	/
CF.2006.222	Non-Mucoid	1, 3	3.7 x 10 ⁻⁶	/	/	/
CF.2006.234	Non-Mucoid	1, 3	2.0 x 10 ⁻⁶	E672K	/	/
CF.2007.255	Non-Mucoid		8.0 x 10 ⁻⁶	NA	1bp insertion (→T)	/
CF.2007.256	Mucoid		6.8 x 10 ⁻⁶	NA	/	/
CF.2008.266	Non-mucoid		7.2 x 10 ⁻⁶	NA	NA	NA
CF.2008.274	Mucoid		3.5 x 10 ⁻⁶	NA	/	/
CF.2008.284	Non-Mucoid		1.3 x 10 ⁻⁶	NA	/	10bp deletion
CF.2008.288	Mucoid		7.8 x 10 ⁻⁶	NA	/	1bp insertion (→T) (→C)
CF.2008.292	Mucoid	4	2.6 x 10 ⁻⁶	NA	/	I279V
CF.2008.293	Non-Mucoid		3.7 x 10 ⁻⁶	A545T E693G K810E	/	/

^aPFGE groups as in Chapter 4. ^bMutation frequencies, each isolate was tested in triplicate (mean value shown) highlighted in red indicate mutators. ^c /: No mutations detected, NA: not amplified. A 1bp insertion (→T) in isolate CF.2006.215 after nucleotide 345. A 10bp deletion in isolate CF.2008.284 in the upstream region of *uvrD* 200bp region. Isolate CF.2008.288 has 1bp (→T) and (→C) after nucleotides 572 and 598.

5.2.3.2 Detection of less common mutations in *mutL* gene

In *E. coli*, the recognition of a mismatch by the MMR system relies on Dam-methylation. Briefly, MutS recognises and binds to the mismatch, MutL interacts with MutS and together they activate the endonuclease MutH which cleaves the non-methylated strand⁴³⁹. UvrD (helicase II) unwinds the DNA strands and excision and repair can then proceed in either the 5' or 3' direction. *P. aeruginosa* does not have a Dam or MutH homologue, and therefore recognition of the daughter strand is not based on DNA methylation but alternative recognition pathways⁴³⁹. It has been shown that *P. aeruginosa* MutL has conserved 21 out of the 22 amino acids known to affect the functioning of *E. coli* MutL⁴⁵⁰ and the *P. aeruginosa* MutL has been shown to function in *E. coli*⁴⁵⁰. Amino acid sequences were analysed against *P. aeruginosa* PAO1 for 12/13 hypermutator strains, the *mutL* gene could not be amplified for isolate CF.2008.266. A 1bp insertion (→T) after nucleotide 560 was found in two isolates, CF.2006.255 and CF.2006.215, this 1bp insertion caused a premature stop codon at position 317 which resulted in a truncated *mutS* gene (34 amino acid long instead of 634 amino acid). These isolates were not clonally related. No other non-synonymous mutations were detected in the other ten MDR isolates, (Fig 5.4 and Table 5.5).

5.2.3.3 Are there mutations in the *uvrD* gene?

The MDR isolates were also screened for mutations in *uvrD* gene. A comparison between *uvrD* gene of *E. coli* and the *uvrD* gene in *Salmonella*, *P. aeruginosa* PAO1 and *P. aeruginosa* Liverpool epidemic strain are shown in Fig 5.5. PCR amplification was not possible for isolate CF.2008.266. A non-synonymous mutation was seen in isolate CF.2008.292 (I279V) The mutation was within motif IV, this conserved motif, VIRLEQNYRSTASILK, contains invariant residues (shown in bold) of the nucleotide binding site⁴⁵⁴. Isolate NCF.2006.47 had a substitution (K344R) located between motif IV and motif IVa, its role in the hypermutator phenotype is undetermined. A 10bp deletion in the 200bp upstream region of the *uvrD* gene was seen in isolates CF.2008.284. Furthermore, 1bp insertions were seen in isolate CF.2008.288 (→T after nucleotide 572, →C after nucleotide 598), located in motif VI. Isolate CF.2006.215 had a 1bp insertion after nucleotide 345 (→T), (Table 5.5). To the best of my knowledge these mutations have not been reported previously.

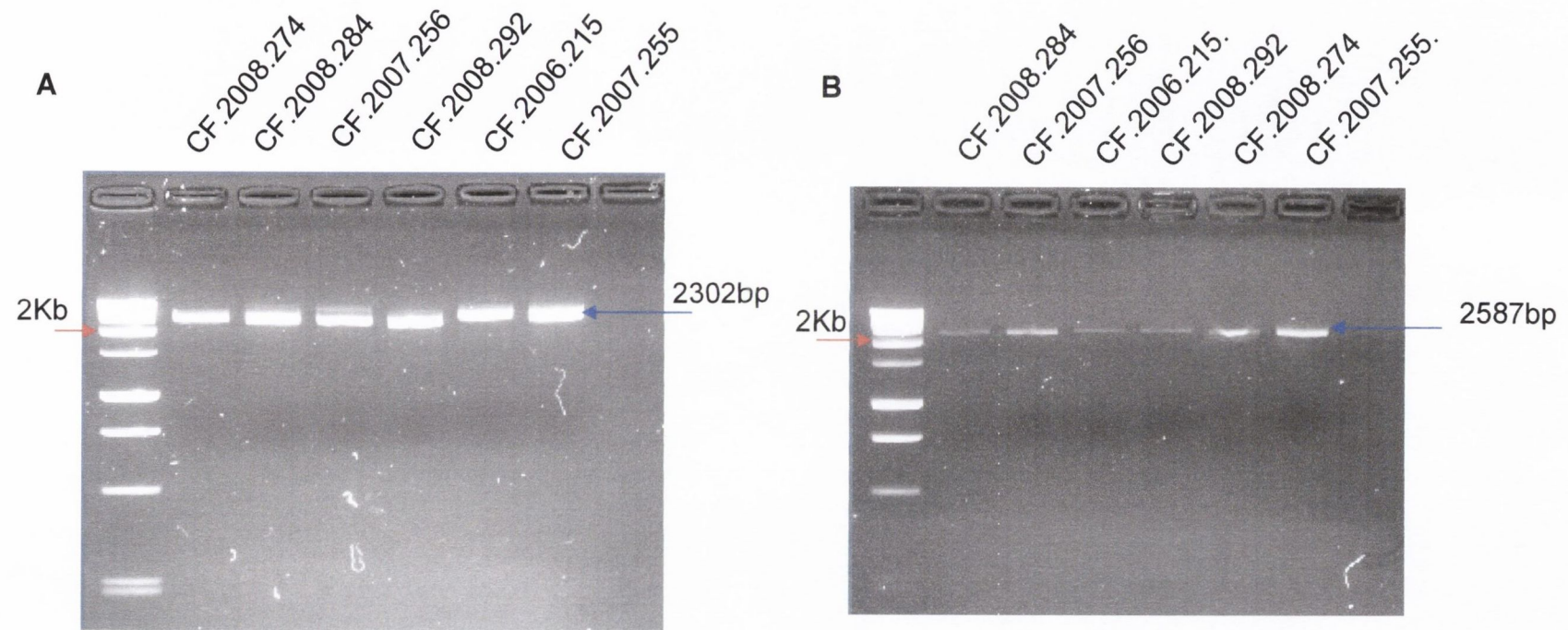


Fig 5.4 PCR amplification of *mutL* (2302bp) and *uvrD* (2587bp). **(A)** *mutL*, lane 1: 1Kb DNA ladder, lane 8: negative control. **(B)** *uvrD*, lane 1: 1Kb DNA ladder, lane 8: negative control.

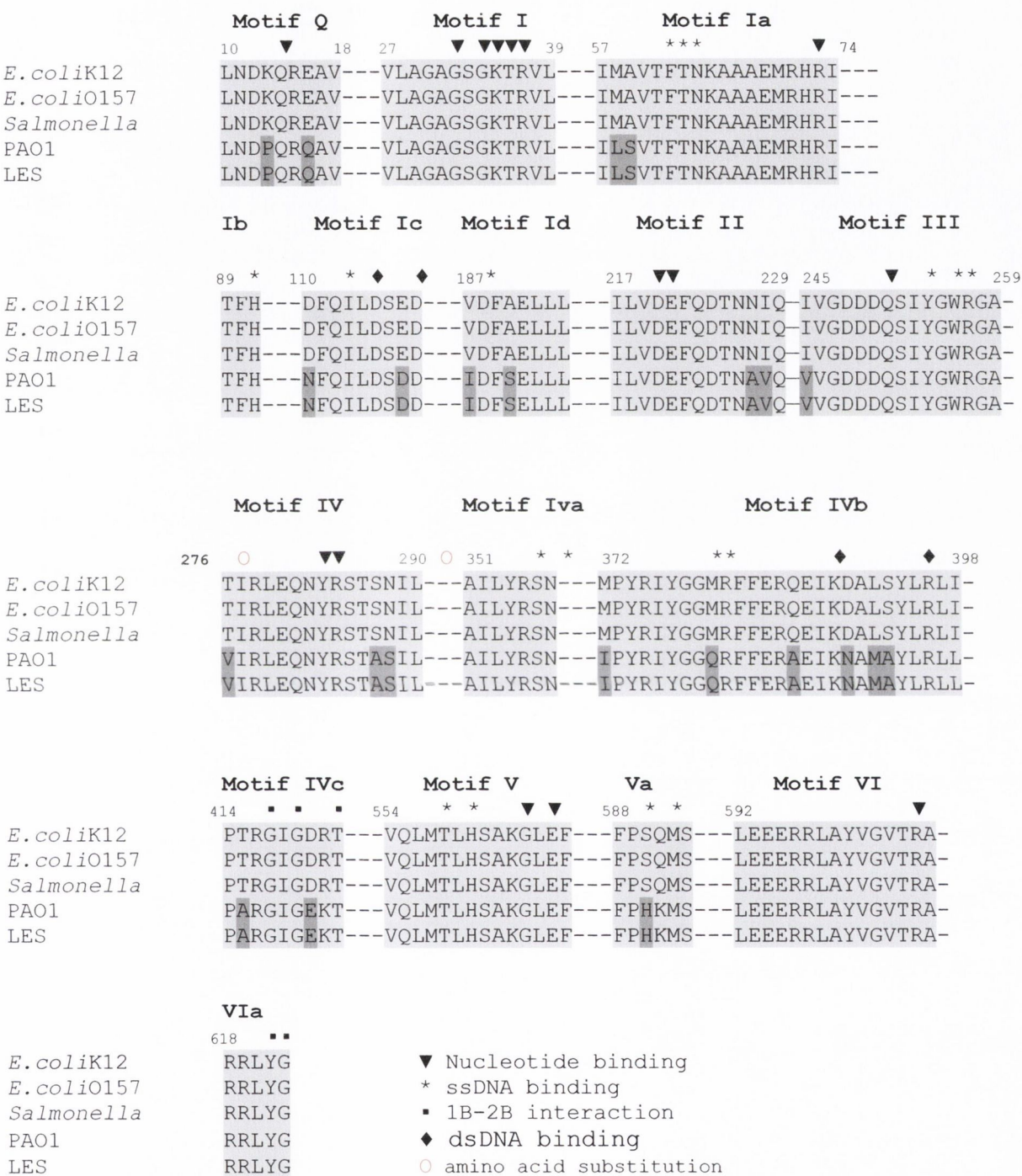


Fig 5.5 Sequence alignment of the eight ATPase motifs of *uvrD* gene in *E. coli* (0157 and BL21) compared to *P. aeruginosa* PAO1, *Salmonella enterica subsp. enterica serovar Typhimurium str. 14028S* and *P. aeruginosa* Liverpool epidemic strain (LES). The amino acid substitution (I279V) is seen in isolate CF.2008.292, a K344R substitution is seen in isolate NCF.2006.47.

5.2.4 Restoration of the function of *mutS* in hypermutator isolates by complementation

To explore the genetic basis for the mutator phenotype, complementation studies were performed. The plasmid pANK-M1, contained wild type (PAO1) *mutS* gene ²⁶⁹. The MICs of carbenicillin (a resistance marker for these plasmids) were determined by agar dilution method for the hypermutable isolates before electroporation. Depending on the level of resistance, transformants were selected on MH agar plates containing 200-250 mg/L carbenicillin. The vector pUCP20 ²⁶⁸ served as a negative control for all mutator complementation assays. The control vector and pANK-M1 were electroporated into separate independent hypermutable isolates as described in Smith *et al* ²⁹² and in Section 2.6. Initially, isolates for which no amplification of *mutS* could be achieved were examined. The MICs of the 8 isolates to carbenicillin were determined. In the case of four isolates, CF.2007.255, CF.2007.256, CF.2008.266 and CF.2008.284 complementation studies were not possible as they had an MIC of ≥ 500 mg/L to carbenicillin.

For the remaining four isolates, the pANK-M1 plasmid containing *mutS* gene was transformed into these isolates and the mutation frequencies were determined in three independent colonies from each series. The remaining hypermutator isolates were examined, isolate NCF.2006.47 was highly resistant to rifampicin and did not have any mutations in *mutS* so complementation was not carried out on this isolate. The complementation results for isolates CF.2006.216, CF.2006.222, CF.2006.234 and CF.2008.293 are shown in Table 5.6. After complementation with the *mutS* gene, all of the isolates examined had a reduced mutation frequency suggesting that the mutations present in these isolates contributed to their hypermutability.

Table 5.6 Complementation of *mutS* gene

Isolate	Mutation Frequency	Complementation ^a
CF.2006.215	1.2×10^{-6}	2.1×10^{-8}
CF.2008.274	3.5×10^{-6}	1.8×10^{-8}
CF.2008.288	7.8×10^{-6}	8.4×10^{-8}
CF.2008.292	2.6×10^{-6}	9.9×10^{-8}
CF.2006.216	3.5×10^{-6}	2.0×10^{-8}
CF.2006.222	3.7×10^{-6}	7.1×10^{-7}
CF.2006.234	2.0×10^{-6}	7.4×10^{-9}
CF.2008.293	3.7×10^{-6}	8.6×10^{-8}

^aComplementation was considered positive when the mutation frequency after transformation of pANK-M1 was decreased ≥ 2 logs, negative when the mutation frequency was decreased < 1 log, and partial if the reduction was 1 log ⁴⁴⁰.

5.3 Discussion

The presence of hypermutators in the MDR group, which included NCF and CF isolates, was investigated in this chapter. Out of the thirty three isolates examined, twelve were found to have an elevated mutation frequency. Not surprisingly, all 12 isolates had come from CF patients. The association of the hypermutator phenotype and antimicrobial resistance in CF patients has been examined previously^{82,427,436,440-441,443,455}. The presence of mutations in the MMR genes from *P. aeruginosa* has been examined in a number of studies^{427,436,441,456-458}, on the whole these studies have mainly only concentrated on *P. aeruginosa* isolates from CF patients.

In order to determine the cause of hypermutator phenotype, the *mutS* gene was examined in all hypermutator isolates, including the NCF isolate which was resistant to rifampicin. Amplification of the *mutS* gene was only successful in 5/12 isolates, after repeated attempts and use of alternative primers. It was speculated that these isolates, which had mutation frequencies ranging from 1.2×10^{-6} - 8.0×10^{-6} , may possibly have a disruption in the *mutS* gene. To investigate this, complementation with *mutS* from PAO1 was preformed successfully in 4/8 isolates, complementation could not be performed on the other four isolates because they were highly resistant to carbenicillin (antibiotic marker). A non-mutator phenotype was restored in the four isolates, suggesting that the speculated disruption in the *mutS* gene contributed to the hypermutator frequencies among these isolates. The loss of the *mutS* gene in these isolates may be the result of large chromosomal inversions. During the course of an outbreak, large chromosomal inversions have been detected in *Bordetella pertussis*, *Salmonella typhi* and *Neisseria meningitidis*, which suggests that genome rearrangements may create bacteria which are sufficiently different and therefore able to escape the hosts immune systems and cause infection^{269,459}. A high frequency (50%) of large chromosomal inversions have been found in *P. aeruginosa* isolates from CF patients⁴⁶⁰. It is possible that a large chromosomal inversion may have caused the loss of *mutS* in the isolate collection.

For the remaining hypermutator isolates, mutations in the *mutS* gene were found in three isolates. The A545T mutation, which had been reported previously in a clinical epidemic isolate from Dublin⁴³⁷ was detected in two of the three isolates. These isolates were clonally related according to PFGE patterns. Isolate CF.2006.216 was the first to be collected, had only one mutation in *mutS*, isolate CF.2008.293 was collected nearly

two years later and had two additional mutations in the *mutS* gene, E693G which was also reported previously⁴³⁷ and K810E which as far as I am aware has not been reported previously. These clonally related isolates have come from separate patients collected nearly two years apart, complementation with PAO1 *mutS* restored the non-mutator phenotype indicating that these mutation were important in establishing a hypermutator phenotype. Another non-synonymous mutation was detected in the third isolate; E672K which was located in an ATPase domain, complementation with *mutS* from PAO1 restored the non-mutator phenotype, also indicating that this mutation was important. The three isolates which had mutations in the *mutS* gene did not possess any mutations in the *mutL* or *uvrD* genes. No mutations were detected in the *mutS*, *mutL* or *uvrD* genes in isolates CF.2006.216, the isolate was included in the *mutS* complementation study to ensure the *mutS* gene in the isolate was functioning. The mutation frequency was partially reduced with the addition of *mutS* from *P. aeruginosa* PAO1 suggesting that other factors might be involved in the hypermutator phenotype in this isolate. Isolate CF.2008.266 which did not produce a PCR product from *mutS* or did not possess mutations in *mutS* were investigated for the presence of mutation in the *mutL* and *uvrD* genes. Isolate CF.2008.266, which had a mutation frequency of 7.2×10^{-6} did not held any PCR products for the genes. Complementation was not possible for this isolate due to high carbenicillin resistance, so it can only be speculated that large deletions in the *mutS*, *mutL* and *uvrD* genes are responsible for the high mutation frequency. A 1bp insertion in the *mutL* gene after nucleotide 560 was detected in two isolates, CF.2006.215 and CF.2007.255. Isolate CF.2006.215 also had a 1bp insertion in the *uvrD* gene. The 1bp insertions could have contributed to the mutation frequencies in these isolates, although complementation would need to be done to confirm this. Mutations in the *uvrD* gene are rarely seen in *P. aeruginosa*^{427,436-437,440-442,456,458}. A number of mutations and deletions were seen in the *uvrD* gene amongst the isolates examined. Isolate NCF.2006.47, which had a mutation in the *rpoB* gene also had a mutation in *uvrD* (K344R), which was located between motif IV and IVa, this mutation could have contributed towards the high mutation phenotype. A non-synonymous mutation was seen in isolate CF.2008.292 (I279V), this mutation was within motif IV of the *uvrD*, again this mutation to the best of my knowledge has not been reported previously and could have contributed to the hypermutator phenotype but would need to be confirmed with complementation studies. Isolates CF.2008.284 and CF.2008.288

both had deletions in the *uvrD* gene which could also have contributed to the hypermutator phenotype.

Non-mucoid CF *P. aeruginosa* isolates have long been associated with early colonisation, the appearance of the mucoid phenotype is indicative of a chronic infection. Yet mucoid and non-mucoid phenotypes have been shown to be isolated from the CF lung simultaneously, one group Kenna *et al*⁴³⁷ excluded mucoid isolates from its study of early colonising *P. aeruginosa* CF hypermutators, yet they found quite a high proportion of these 'early' non-mucoid isolates to be hypermutators. Data presented here found an equal proportion of hypermutators were mucoid or non-mucoid which suggests that both mucoid and non-mucoid are capable of developing a hypermutator phenotype in the CF lung. The results presented here found that all of the hypermutators had come from CF patients, which is similar to previous studies^{436,444}. Interestingly, the majority of the mutations and deletions were found in *uvrD* followed by *mutS*. The hypermutator phenotype in these isolates may have contributed to the MDR resistance also seen in these isolates. There are a number of isolates which do not have any mutations in the genes examined in this study, their mutator phenotype could be linked to defects in other DNA repair proteins. The role of another system involved in error repair, the DNA oxidative repair (GO) system, has been investigated and well characterised in *E. coli*⁴⁶¹ and more recently it has been examined in *P. aeruginosa*⁴⁶². Homologs to a number of the components in the GO system described in *E. coli* have been found in *P. aeruginosa*⁴⁶³. In a recent paper by Mandsberg *et al*⁴⁶², the development of antimicrobial resistance was investigated in *P. aeruginosa* GO deficient mutants, it was found that disruption or mutation in the genes involved in the GO system lead to increased hypermutation⁴⁶².

The link between the hypermutator phenotype and antimicrobial resistance should be considered a major negative clinical effect, as it limits the use of many commonly used antimicrobials. Interestingly it has been reported that colistin might be an exception to the strong linkage of hypermutators with antimicrobial resistance, colistin resistance among hypermutators has not been noted so far^{82,428,464}. However, two of the isolates in this study, CF.2006.216 and CF.2006.234, were resistance to colistin (MIC 4mg/L) the remaining isolates in this chapter which were classified as hypermutators were all colistin susceptible.

The best strategy in dealing with hypermutators and avoiding their appearance in the first place has yet to be established. As yet, it is unclear whether aggressive

antimicrobial therapy, which is often suggested in CF *P. aeruginosa* lung infection, would be adequate for this purpose.

**Chapter 6 Colistin resistance in *Pseudomonas*
*aeruginosa***

6.1 Introduction

The emergence of multidrug resistant bacteria and the decline in the discovery and development of novel antimicrobials has curtailed the treatment options available for many patients; as a result there has been renewed interest in older antimicrobials, such as polymyxins⁴⁶⁵.

Colistin (polymyxin E) is a member of a group of antimicrobials known as polymyxins. Colistin has been available for clinical use since the early 1960s, but its use was curtailed because of fears of nephrotoxicity and the introduction of newer broad spectrum antimicrobials. Polymyxins interact electrostatically with the outer membrane of Gram-negative bacteria, competitively displacing the divalent cation (Mg^{2+}) from the negatively charged phosphate groups of membrane lipids²⁴³. The result is a destabilising effect on the outer membrane, leading to cell leakage and eventual cell death²⁴⁴.

Data regarding the pharmacokinetic and pharmacodynamics of the drug were and still are limited. The lack of information about the pharmacokinetics and pharmacodynamics of colistin in humans means that appropriate colistin dosing strategies for the different routes of administration have yet to be established. A suboptimal dose of colistin creates the risk of the development of resistance.

Colistin is available for clinical use in two forms; colistin sulphate (for oral use; bowl decontamination or topically as a cream) and colistimethate sodium (sodium colistin methanesulphonate, colistin sulfomethate sodium) for parenteral use²³⁹. Colistimethate sodium is less toxic than colistin sulphate but it is also less effective²³⁹, colistimethate sodium is an inactive prodrug of colistin which is unstable *in vitro* and *in vivo* and is hydrolysed in aqueous media which creates a complex mixture of partially sulphomethylated derivatives with the potential to produce up to 32 different products, including colistin²³⁸⁻²³⁹. As recommended by CLSI and BSAC²⁷⁰⁻²⁷¹, colistin sulphate was used for susceptibility testing.

Resistance to polymyxin is mainly associated with decreased uptake into the bacterial cell which is the result of reduced capacity for initial binding²⁴⁵. Polymyxins interact electrostatically with the outer membrane of Gram-negative bacteria; they competitively displace the divalent cation (Mg^{2+}) from the negatively charged phosphate groups of membrane lipids²⁴³. The result is a destabilising effect on the outer membrane, leading to increased cell permeability and eventual cell death²⁴⁴. Adaptive

polymyxin resistance was first described in 1976⁴⁶⁶ when wild-type *P. aeruginosa* PAO1 were passaged through media containing limiting concentrations of Mg^{2+} and then exposed to increasing concentrations of polymyxin B. Since then it has been shown that under Mg^{2+} limiting conditions, lipid A component of LPS of *P. aeruginosa* undergoes modifications which have been associated with polymyxin resistance^{247-248,329}. The modification of lipid A, including the addition of 4-amino-4deoxy-L-arabinose, is known to be directed by the *arnBCADTEF-ugd* operon²⁴⁶. This operon in turn is known to be regulated by two distinct two-component systems, PhoP-PhoQ and Pmr-PmrB^{248,467-468}. Two-component systems function by responding to environmental changes and are involved in the expression of virulence factors, two-component systems consist of a sensor kinase and a response regulator that is phosphorylated by the sensor kinase via a transducing signal following an environmental stimulus⁴⁶⁹. Under Mg^{2+} limiting conditions PhoPQ and PmrAB autoregulate *oprH-phpPQ* and *pmrAB* operons respectively, while separately upregulating the expression of *arnBCADTEF* operon^{247-248,470-471}. Both the PmrAB and the *arnBCADTEF* operon have been shown to be independently upregulated in response to various cationic antimicrobial peptides in laboratory mutants²⁴⁶, however neither PhoPQ nor PmrAB seem to be essential for this upregulation²⁴⁷. More recently, a novel two component system, ParR-ParS was discovered⁴⁷², which has been shown to be involved in the activation of the *arnBCADTEF* and *PmrAB* operons⁴⁷⁰. Muller *et al*⁴⁷⁰ also found that the ParR-ParS system is involved in bacterial adaptation to four antimicrobial classes including polymyxins, aminoglycosides, fluoroquinolones and β -lactams through LPS modification, increased drug efflux and reduced porin pathway. A diagram explaining the role of two component systems in colistin resistance is shown in Fig 6.1.

Early studies of colistin resistance in *P. aeruginosa* were based on studies of *Salmonella enterica* serovar *Typhimurium*,^{247-248,468,473-474}. Extensive work has been done on polymyxin and antimicrobial peptide resistance on *Salmonella enterica* serovar *Typhimurium*⁴⁷⁵⁻⁴⁸¹. *Salmonella* is an intracellular pathogen that causes gastroenteritis in humans⁴⁸². After ingestion, *Salmonella* passes through the stomach and invades M cells in the Peyer's patches of the small intestinal epithelium⁴⁸³. After invasion, *Salmonella* is engulfed by macrophages from the hosts immune system⁴⁸⁴⁻⁴⁸⁵. Once inside the macrophage *Salmonella* is capable of replicating within a specialized compartment known as the *Salmonella*-containing vacuole⁴⁸⁶. Within the host cells, *Salmonella* is likely to encounter antimicrobial peptides from the hosts immune system

⁴⁸². Antimicrobial peptides are small (2-4 kDa) cationic molecules that have a similar mechanism of action to polymyxins ⁴⁸⁷⁻⁴⁸⁸, they form an important part of the host defence and are produced by macrophages, neutrophils and mucosal epithelial cells ⁴⁸⁹. Resistance to these antimicrobial peptides is in part mediated by modifications to lipid A (addition of 4-aminoarabinose), which in turn is controlled by the two-component systems, PhoPQ and PmrAB ^{477,479}. Papers by Brodsky *et al* ^{482,490} has found that the *mig-14* gene in *Salmonella*, which is dependant on PhoP for transcriptional activation within cultured macrophages ⁴⁹¹, is involved in polymyxin B resistance. The role of the Mig-14 homolog in *P. aeruginosa* (PA5003) has not yet been fully elucidated and has not been investigated for polymyxin resistance.

Recently Eswarappa *et al* ⁴⁹² have found that the *yejABEF* operon in *Salmonella* which encodes a putative ATP-binding cassette (ABC) transporter is involved in counteracting antimicrobial peptides. The homologues for this operon in *P. aeruginosa* are PA1807-PA1811, which also encodes a putative ABC transporter ^{14,493}, its involvement in polymyxin resistance has not been investigated.

The regulation and expression of complex bacterial genomes are tightly controlled by an array of two-component regulatory systems and transcriptional regulators, especially sigma factors ⁴⁶⁹. Basic initiation of transcription in bacteria is performed by RNA polymerase (multiunit, $\alpha_2\beta\beta'\omega$) which binds to sigma 70 (σ^{70}) ⁴⁹⁴. The sigma 70 proteins are responsible for promoter recognition and melting double-stranded DNA binding to core RNA polymerase ⁴⁹⁵. In *E. coli*, one housekeeping sigma factor is responsible for the majority of transcription during exponential growth and a pool of alternative sigma factors are responsible for transcription of specific regulons associated with environmental or physiological changes ^{469,496}. Recently Barchiesi *et al* ⁴⁹⁷ have found that the inactivation of the alternative sigma factor, RpoN, increases polymyxin resistance in *Salmonella* by a mechanism which is not linked to PmrAB-dependant pathway ⁴⁹⁷. In *P. aeruginosa* RpoN has been shown to play a role in the expression of virulence factors, the global regulation and negative control of quorum-sensing and antimicrobial tolerance ⁴⁹⁸⁻⁵⁰⁰. The role of *rpoN* in polymyxin resistance in *P. aeruginosa* has not yet been investigated.

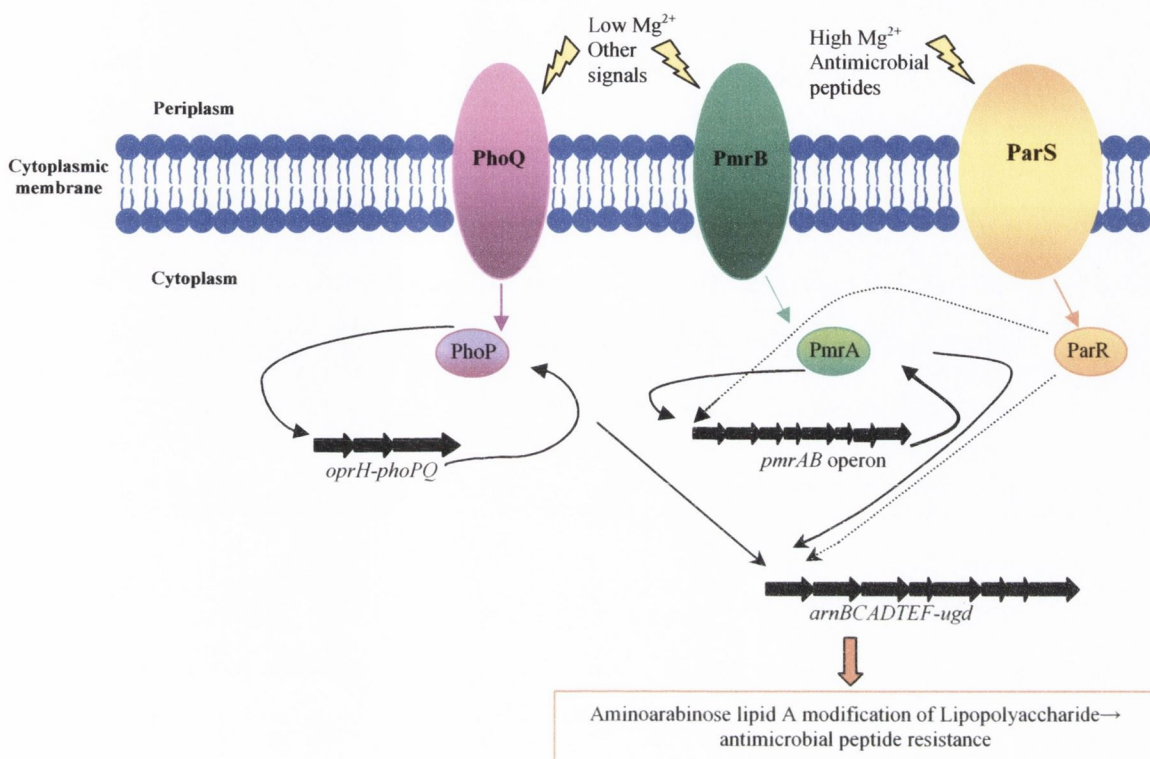


Fig 6.1 Schematic representation of *P. aeruginosa* PhoPQ, PmrAB and ParRS regulatory networks in resistance to cationic antimicrobial peptides. In Mg^{2+} limiting conditions the response regulators, PhoP and PmrA are activated (phosphorylated), they positively autoregulate the transcription of their respective operons as well as *arnBCADTEF-ugd* operon. The ArnBCADTEF system modifies lipid A, reducing the overall negative charge on lipopolysaccharide, consequently increasing resistance to polymyxin and cationic peptides. Conversely under high Mg^{2+} conditions, PhoP and PmrA are dephosphorylated and presumed inactive (not shown), however under high Mg^{2+} conditions and subinhibitory concentration of antimicrobial peptides, the ParRS regulatory system activates *arnBCADTEF-ugd* and *pmrAB* operons, thereby increasing resistance to antimicrobial peptides. Other genes regulated by PmrA and PhoP are not shown. Figure adapted from Gooderham *et al*²⁴⁸ and Muller *et al*²⁴⁷.

The aim of the study presented in this chapter was:

- 1) to investigate the prevalence of colistin resistance among NCF and CF *P. aeruginosa* isolates
- 2) to examine the mechanisms of colistin resistance by PCR and sequencing
- 3) To determine the expression of the *arnBCADTEF* operon by qRT-PCR in selected resistant/ sensitive isolates
- 4) to examine the level of clonality amongst the colistin resistant isolates
- 5) to investigate if other genes may be involved in colistin resistance

6.2 Results

6.2.1 Determination of colistin resistance

A phenotypic screen using three methods was used to determine the level of colistin resistance in the 194 NCF and 99 CF *P. aeruginosa* isolates.

6.2.1.1 Comparison of agar dilution, Etest and disc diffusion for testing resistance to colistin

Agar dilution, disc and Etest methods were used to determine the levels of resistance to colistin, according to CLSI and BSAC guidelines²⁷⁰⁻²⁷¹ and as outlined in Section 2.2. The negative controls used were *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922, they both fell within the susceptible range according to CLSI and BSAC guidelines for agar dilution and disc diffusion testing.

In relation to agar dilution, levels of resistance to colistin were higher in the CF collection than the NCF collection. Similarly the levels of intermediate resistance were also higher in CF collection, Table 6.1, 6.2. Using the Etest method, three NCF isolates were shown to be resistant to colistin, according to CLSI guidelines. No CF isolate was shown to be resistant to colistin according to Etest method. Disc diffusion tests were performed on the isolates according to BSAC guidelines; four isolates were resistant in NCF and CF collection.

The four isolates which were shown to be resistant using the disc diffusion method in the NCF collection had MIC of ≥ 64 mg/L according to agar dilution method, putting them in the higher end of the resistant category. Three of the four isolates had Etest MIC's of 64mg/L, 32mg/L and 16 mg/L. The fourth isolate had an MIC of 2mg/L, putting it in the susceptible range according to CLSI guidelines.

Table 6.1 Comparison of agar dilution, to Etest and disc diffusion for NCF (n=194) *P. aeruginosa* isolates

Reference	Interpretation			Comparison method	Error rates ^b			Correlation ^c %
	(%) ^a				(%)			
	R	I	S		VM	M	m	
Agar	14	9	171	Etest	11	0	9	89.6
	(7.2)	(4.6)	(88.2)					
	3		191					
	(1.5)	0	(98.5)					
	4		190	Disc	10	0	9	90.2
	(2)	0	(98)					

^aR: resistant, I: intermediate resistant, S: susceptible, according to CLSI guidelines. ^bVM: very major errors (false susceptibility), M: major error (false resistance), m: minor error (susceptible/intermediate and resistant/intermediate discrepancies) ^cCalculated by dividing the number of correct correlations by the total number of tests

Table 6.2 Comparison of agar dilution, to Etest and disc diffusion for CF (n=99) *P. aeruginosa* isolates

Reference	Interpretation			Comparison method	Error rates ^b			Correlation ^c %
	% ^a							
	R	I	S		VM	M	m	
Agar	13	15	71					
	(13.1)	(15.2)	(71.7)					
	0	0	99	Etest	28	0	0	71.7
			(100)					
	4	0	94	Disc	24	1	0	74.7
	(4.1)		(94.9)					

^aR: resistant, I: intermediate resistant, S: susceptible, according to CLSI guidelines. ^bVM: very major errors (false susceptibility), M: major error (false resistance), m: minor error (susceptible/intermediate and resistant/intermediate discrepancies) ^cCalculated by dividing the number of correct correlations by the total number of tests

Previous studies have shown that agar dilution can give higher colistin MIC's for *P. aeruginosa* isolates, providing greater sensitivity than other testing methods for the detection of polymyxin resistant isolates²⁴⁰. Therefore agar dilution was used as a reference method to determine the error rates⁵⁰¹⁻⁵⁰³.

The error rates in the NCF collection were similar for Etest and disc testing compared to agar dilution. Etest and disc diffusion correlated 89.6-90% with agar dilution method which is similar to what has been found previously⁵⁰¹⁻⁵⁰². In the CF collection there were more very major errors (false susceptibility) using the Etest method compared to disc diffusion method. Both Etest and disc had identical minor error rates. Over all the correlation between agar dilution and Etest, agar dilution and disc diffusion were similar in the CF collection. Etest and disc diffusion correlated better with the agar dilution method in the NCF isolates tested than the CF isolates tested.

6.2.1.2 Comparison of colistin susceptibilities in NCF, CF, vMDR and hypermutator isolates

There was a statistically significant (P value = 0.001, Fisher's exact Test) number of CF isolates which were resistant to colistin in comparison to NCF isolates, (Fig 6.2). Among the colistin resistant isolates in both groups, a smaller percentage ($n = 2$, 9%) of the isolates were vMDR in the NCF collection compared to the CF collection ($n = 6$, 21%), but this number was not statistically significant. As seen in the previous chapter, hypermutators were only detected in the CF group, and only two were colistin resistant. Overall in the CF colistin resistant isolates, nine were mucoid and 19 were non-mucoid.

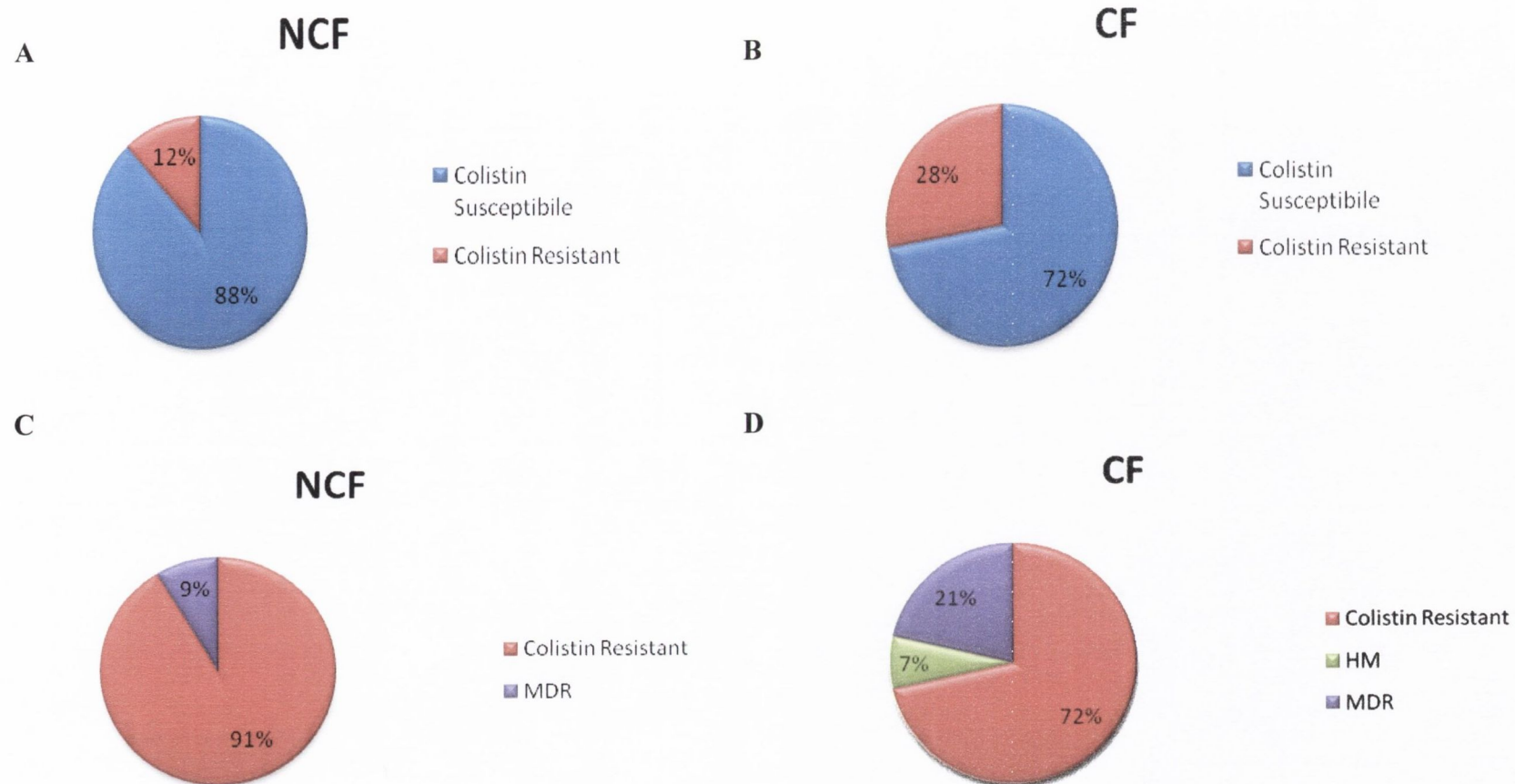


Fig 6.2 Percentage of colistin resistance in NCF, CF, vMDR and HM (hypermutator) groups. **(A)** Percentage of colistin resistance in the NCF group n=23. **(B)** Percentage of CF isolates which are colistin resistant, n=28 **(C)** Percentage of NCF colistin resistant which are MDR (vMDR) **(D)** Percentage of CF colistin resistant isolates which are MDR (vMDR) and hypermutators.

6.2.2 Detection of mutations in genes associated with colistin resistance

Isolates which were colistin resistant were screened for the presence of mutations and/ or deletions in *pmrB* and *rpoN* genes which may account for colistin resistance.

6.2.2.1 Identification of mutations in the *pmrB* gene from the PmrAB two component system

Previously it has been shown that a single amino acid substitution in *pmrB* has been associated with polymyxin resistance in *P. aeruginosa*⁵⁰⁴. The presence of this mutation and others recently found in *pmrB*⁵⁰⁵ were investigated among the colistin resistant isolates, (Fig 6.3). Mutations were not detected in the region examined.

6.2.2.2 Mutations in the alternative sigma factor gene, *rpoN*

The expression of genes in *P. aeruginosa* and most bacterial species is controlled by a multitude of transcriptional regulators, partially sigma factors⁴⁶⁹. RpoN is an alternative sigma factor in *P. aeruginosa*, it is not only a necessary part of the nitrogen metabolic pathway,⁵⁰⁶ but it has also been found to play important role in motility, the transport of nutrients, in the formation of pili, in mucoidy, cell-to-cell signalling and more recently, it has been associated with antimicrobial tolerance^{53,349,498-500,507-511}. A recent study by Barchiesi *et al*⁴⁹⁷ observed that the inactivation of *rpoN* markedly increased resistance to polymyxins in *Salmonella enterica* by a mechanism not linked to PmrAB pathway. *P. aeruginosa* shares 55% sequence homology with *E. coli* RpoN, (Fig 6.4). *E. coli* RpoN is divided into three functional regions⁵¹². Region 1 interacts with the activator protein to control promoter melting, Region 2 is involved in DNA binding and Region 3 recognises and binds the consensus promoter elements⁴⁶⁹. Amino acid substitutions were found at position S71T in isolates CF.2006.196, NCF.2006.149 and CF.2006.200 and N122S in isolate CF.2008.290. The amino acid substitutions are located within Region 1 of RpoN.

6.2.3 Relative expression levels of the first gene in the *arnBCADTEF-ugd* operon, *arnB*

Resistance to polymyxins is caused by the inhibition of the interactions between the antimicrobial and the lipid A on the LPS⁵⁰⁵. Due to modifications, directed by the *arnBCADTEF-ugd* operon, the lipid A moiety becomes more negatively charged⁵⁰⁵. It has been shown that under nutrient limiting conditions, 4-amino-4deoxy-L-arabinose is added to lipid A. This addition is directed by the *arnBCADTEF-ugd* operon which itself is modulated by two-component regulatory systems PhoPQ and PmrAB^{246,248,468,513}. To investigate if the *arnBCADTEF-ugd* operon is upregulated in the colistin resistant isolates, qRT-PCR was used to examine the relative expression of *arnB* in four resistant/susceptible isolates. Isolate NCF.2006.72 had a colistin MIC of >128mg/L (resistant), NCF.2006.78 >128mg/L (resistant), CF.2006.220 4mg/L (intermediate resistance), and NCF.2006.100 which had a colistin MIC of 2mg/L (susceptible). The *rpsL* gene was used as the housekeeping gene, the optimal primer concentrations and the percentage efficiency of the primers are shown in Fig 6.5. The standard curve and melt curve of *arnB* is shown in Fig 6.5.

The *arnB* gene was upregulated in isolates NCF.2006.72, CF.2006.220, NCF.2006.78 compared to PAO1, (Fig 6.6). To investigate if a non-lethal concentration (0.3µg/ml)⁵¹⁴ of colistin could induce the *arnBCADTEF-ugd* operon, the isolates were grown overnight (18-20h) in the presence of 0.3µg/ml in cation-adjusted Mueller-Hinton broth (CAMHB), the expression of *arnB* was then measured, (Fig 6.7). The *arnB* expression increased in isolate NCF.2006.78, which had a colistin MIC of >128mg/L. There was small increase in *arnB* expression in isolate NCF.2006.100, which had a colistin MIC of 2mg/L. Interestingly, *arnB* expression decreased in isolates NCF.2006.72 and CF.2006.220 which had a colistin MIC of >128mg/L and 4mg/L respectively.

These results would suggest that LPS modification may be responsible for moderate levels of resistance and that it is likely that other mechanisms are present in isolates NCF.2006.72 and CF.2006.220. Isolates NCF.2006.72 and CF.2006.220 both seem to express *arnB* at a higher level before incubation with colistin is interesting.

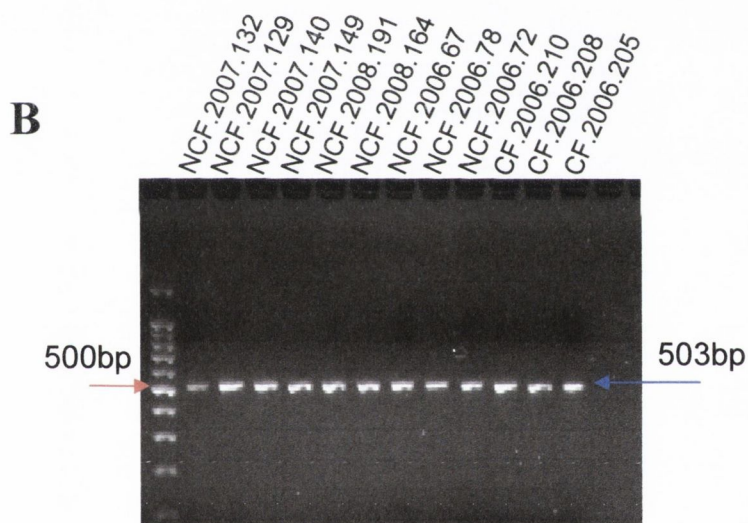
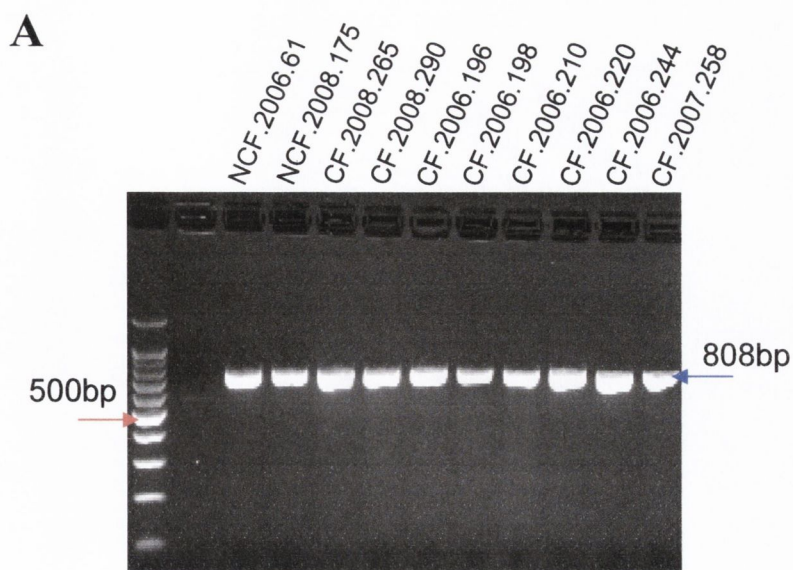
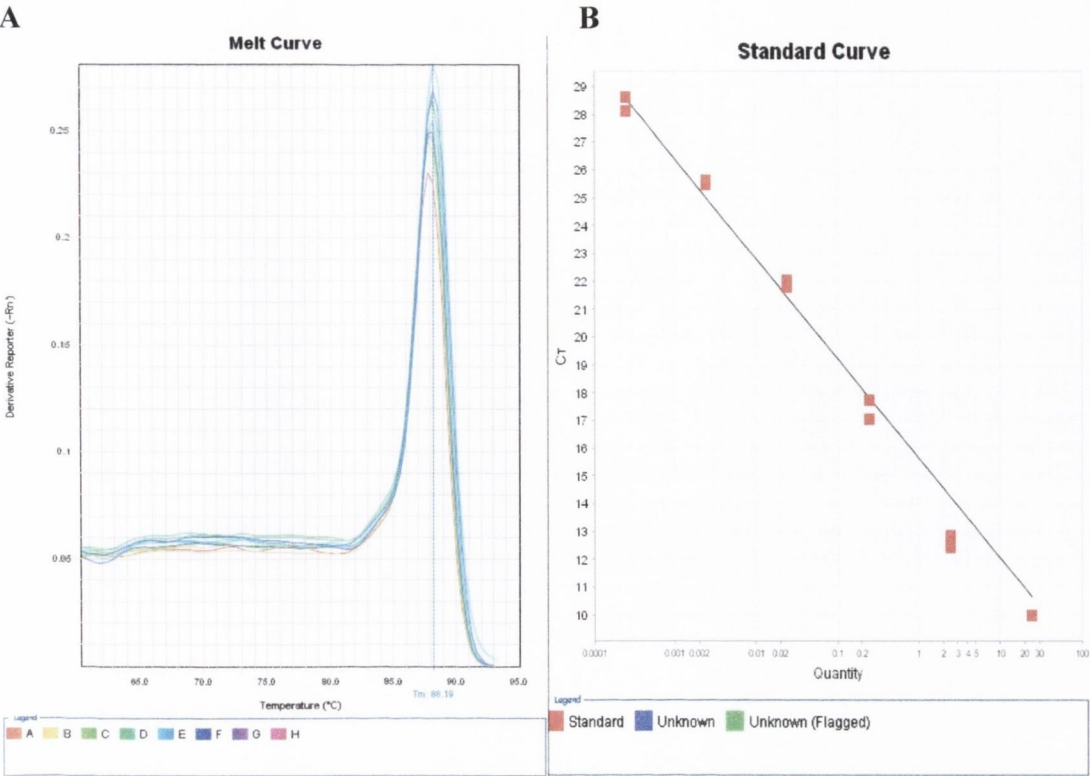


Fig 6.3 Amplification of *pmrB* and *rpoN*. **(A)** *pmrB* (808bp), Lane 1:100bp ladder, lane 2: negative control. **(B)** *rpoN* (503bp), Lane 1: 100bp ladder, lane 14: negative control.



C

Gene name	Optimal Primer (μ M)	Efficiency (%)	Slope
<i>rpsl</i>	0.1	94.4	-3.4
<i>arnB</i>	0.3	90	-3.5

Fig 6.5 Melt curve and standard curve for *arnB* primers. **(A)** Melt curve for *arnB* primers, a single peak indicates a single PCR product, which is essential in qRT experiments. **(B)** Standard curve of *arnB* primers, slope -3.5, % efficiency of the primers 90.5, standard dilution series (172pg-1.72fg). The relative standard curve is used to determine relative target quantity in samples. **(C)** Optimal qRT-PCR conditions for the genes of interest

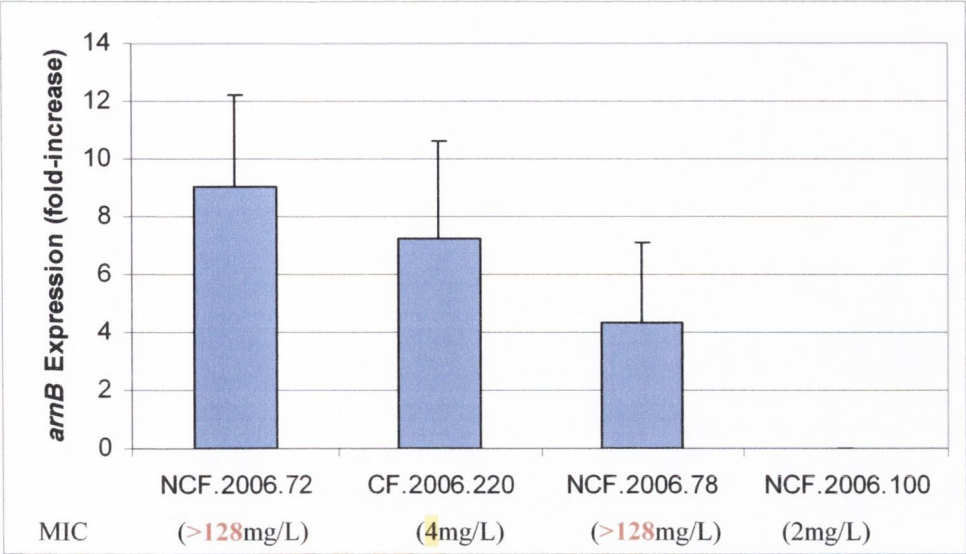


Fig 6.6 Expression of *arnB* in isolates NCF.2006.72 (>128mg/L), CF.2006.220 (4mg/L), NCF.2006.78 (>128mg/L), NCF.2006.100 (2mg/L) measured as fold change compared to expression of *arnB* in PAO1. Error bars represent the standard deviations of three biological repeats, performed in duplicate. Red indicates a resistant MIC, yellow an intermediate resistant phenotype and no colour indicates a susceptible phenotype.

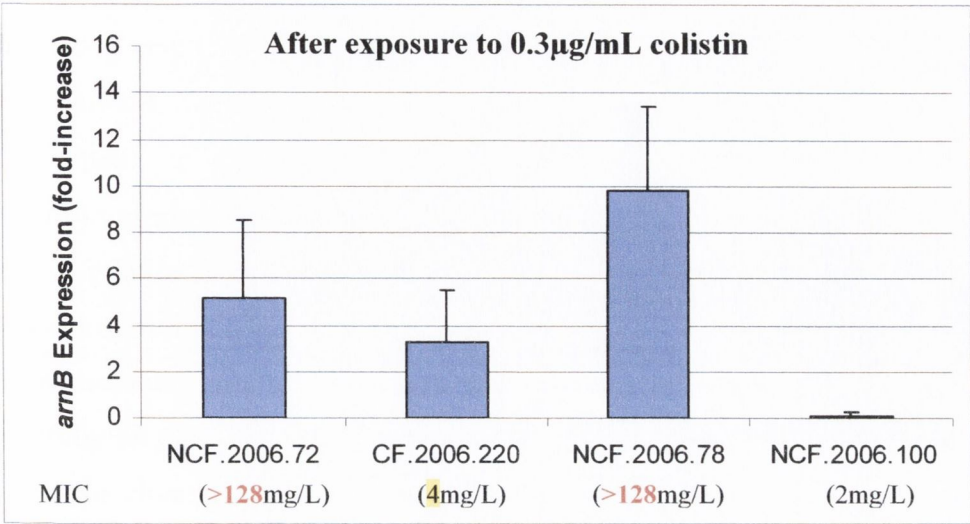


Fig 6.7 Effect of 0.3µg colistin mL⁻¹ on the expression of *arnB* in isolates NCF.2006.72 (>128mg/L), CF.2006.220 (4mg/L), NCF.2006.78 (>128mg/L), NCF.2006.100 (2mg/L) measured as a fold change compared to the expression of *arnB* in PAO1. Error bars represent the standard deviation of three biological repeats, each performed in duplicate. Red indicates a resistant MIC, yellow an intermediate resistant phenotype and no colour indicates a susceptible phenotype.

6.2.4 Are the colistin resistant isolates clonally related?

In recent years, there have been reports of clonal outbreaks of colistin resistant isolates in CF centres in Denmark and the UK ⁵¹⁵⁻⁵¹⁶. Since 28% of the CF isolates were colistin resistant, the clonal relationship of these isolates was examined.

Colistin is not an antimicrobial usually included in routine antimicrobial screening in clinical laboratories, yet 12% of the NCF isolates were colistin resistant, the clonal relationship of these isolates was also examined. Based on pulsed field gel procedures outlined in Section 2.5 (Materials and Methods), the 51 NCF and CF colistin resistant isolates were examined, (Fig 6.8). The *Xba*I digestion was performed successfully on all but two (NCF.2006.82 and CF.2006.210) of the 51 colistin resistant isolates. No clusters (>80% similarity) were detected. This result indicates that the isolates examined were not part of a clonal outbreak, and would suggest that colistin resistant isolates have not been transferred from patient-to-patient, but likely have arise independently in each patient.

6.2.5 Selection of mutants from Manoil *P. aeruginosa* mutant library which may be associated with colistin resistance

In the University of Washington, the Manoil laboratory has created a mutant library for *P. aeruginosa* PAO1. As discussed previously, resistance to antimicrobial peptides and polymyxins have been studied extensively in *Salmonella enterica* serovar *Typhimurium*. The possible involvement of other genes in *P. aeruginosa* colistin resistance was investigated. Using *Salmonella* as a model, genes known to have an association with antimicrobial peptides and / or polymyxin resistance in *Salmonella* were chosen from the Manoil *P. aeruginosa* mutant library, Table 6.3. The mutants were examined by agar dilution to determine their MIC to colistin. The host *P. aeruginosa* isolate used to create the mutant was chosen over the sequenced PAO1 ¹⁴ due to its wild-type twitching motility function.

The *mig-14* gene in *Salmonella* has been associated with polymyxin B resistance ⁴⁸², the Mig-14 homolog in *P. aeruginosa* (PA5003) had an intermediate MIC, according to agar dilution.

The PhoPQ and PmrAB two component systems have been shown to be associated with colistin resistance in *P. aeruginosa* ^{246,248,467-468,470-471}. At least one

mutant of *phoP*, *phoQ*, *pmrA* and *pmrB* was resistant to colistin. The *rpoN* gene in *P. aeruginosa* has been showed to be involved in the nitrogen metabolic pathway, motility, the transport of nutrients, in the formation of pili, in mucoidy, cell-to-cell signalling and more recently it has been associated with antimicrobial tolerance^{53,349,498-500,506-511}, the *rpoN* equivalent in *Salmonella* has been shown to be involved in polymyxin resistance⁴⁹⁷. At least one of the *rpoN* mutants had an elevated colistin MIC.

In *Salmonella*, the *yejABEF* operon is involved in antimicrobial peptide resistance, the homologs in *P. aeruginosa* PA1807-PA1811 all displayed a resistant colistin MIC. This is only a preliminary study of *P. aeruginosa* mutants, further work in this area is required and will be discussed later.

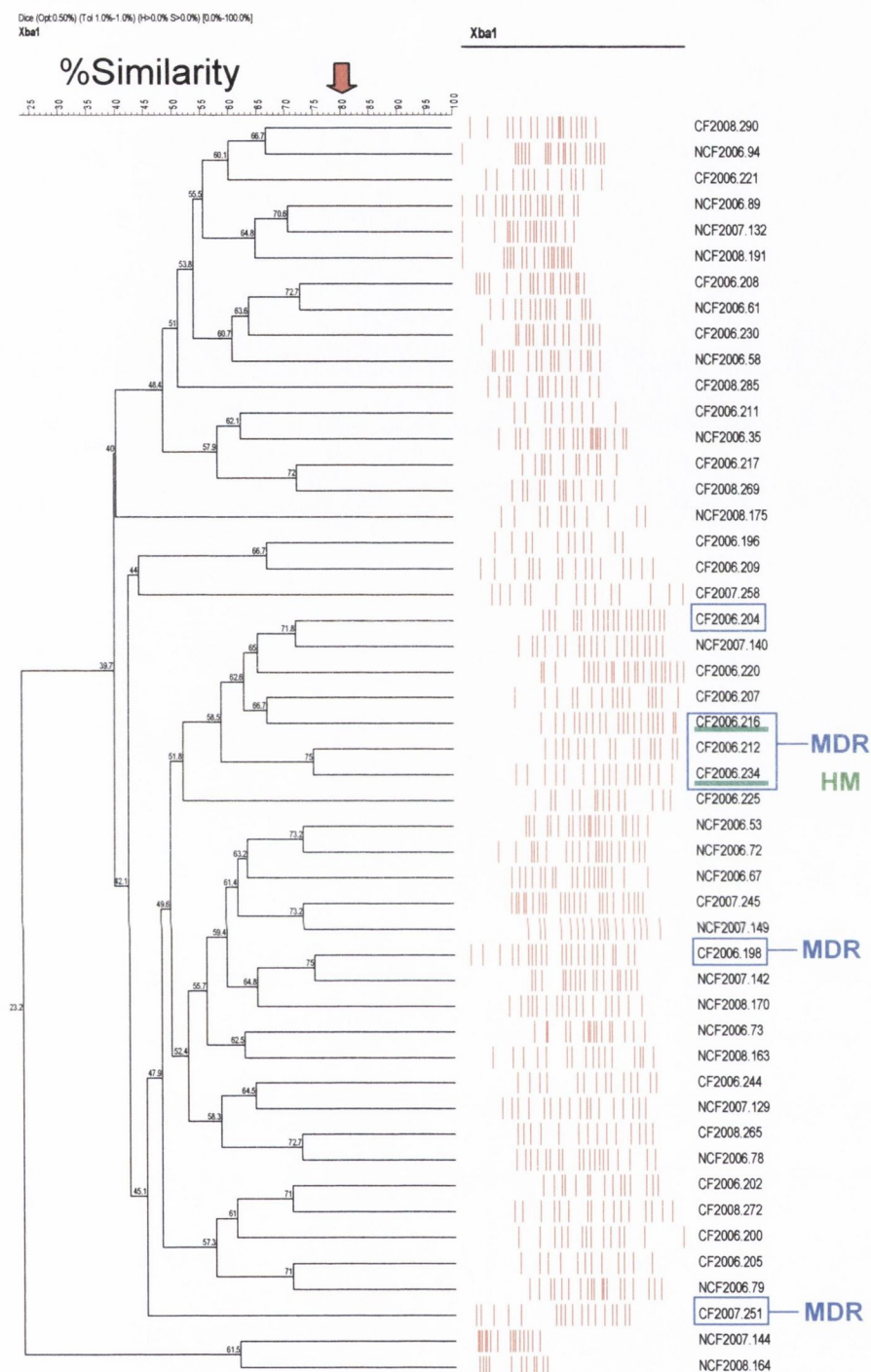


Fig 6.8 Dendrogram of pulsed-field electrophoresis (PFGE) profiles from 49 of the 51 colistin resistant isolates. Dendrogram of PFGE analysis. Two isolates NCF.2006.82 and CF.2006.210 were untypable. A percentage similarity is shown above the dendrogram. No clusters (>80% genetic similarity) were found. Band patterns that were more than >80% identical were considered related conform the Tenover criteria (2-3 band difference indicates related strains). Isolate boxed in blue were multi drug resistant (MDR), isolates underlined in green were hypermutators.

Table 6.3 Mutants screened for colistin resistance

Strain Name	Gene	Gene Product	Colistin Agar Dilution (mg/L) ^a
AO1			2
W9409	<i>mig-14</i> (PA5003)	Hypothetical protein	4
W3129	<i>phoP</i> (PA1179)	Two-component response regulator PhoP	16
W3128	<i>phoP</i> (PA1179)	Two-component response regulator PhoP	32
W3130	<i>phoP</i> (PA1179)	Two-component response regulator PhoP	16
W3131	<i>phoQ</i> (PA1180)	Two-component sensor PhoQ	16
W3132	<i>phoQ</i> (PA1180)	Two-component sensor PhoQ	4
W9677	<i>pmrA</i> (PA5159)	Multidrug resistance protein	4
W9676	<i>pmrA</i> (PA5159)	Multidrug resistance protein	2
W9679	<i>pmrB</i> (PA5160)	Drug efflux transporter	2
W9678	<i>pmrB</i> (PA5160)	Drug efflux transporter	4
W8507	<i>rpoN</i> (PA4462)	RNA polymerase sigma-54 factor	2
W8508	<i>rpoN</i> (PA4462)	RNA polymerase sigma-54 factor	8
W4183	<i>yef</i> homologues (PA1811)	Probable solute-binding protein	16
W4184	<i>yef</i> homologues (PA1811)	Probable solute-binding protein	4
W4181	<i>yef</i> homologues (PA1810)	Probable binding protein component of ABC transporter	4
W4182	<i>yef</i> homologues (PA1810)	Probable binding protein component of ABC transporter	8
W4180	<i>yef</i> homologues (PA1809)	Probable permease of ABC transporter	16
W4179	<i>yef</i> homologues (PA1809)	Probable permease of ABC transporter	4
W4178	<i>yef</i> homologues (PA1808)	Probable permease of ABC transporter	16
W4177	(PA1807)	Probable ATP-binding component of ABC transporter	16
W4176	(PA1807)	Probable ATP-binding component of ABC transporter	16

^aMIC values highlighted in red indicate resistant phenotype, according to CLSI, values highlighted in yellow indicate intermediate resistant phenotype according to CLSI.

6.3 Discussion

Colistin is the last line drug for many CF patients chronically infected with *P. aeruginosa*. Since its reintroduction into the clinical setting, although rare, resistance has been reported⁵¹⁵⁻⁵¹⁶. A small number of reports recently have described colistin resistant epidemic strains in CF centres in the UK and Denmark⁵¹⁵⁻⁵¹⁶. The aim of this chapter was to investigate the prevalence of resistance using agar dilution as the reference method, and comparing to the results from the disc diffusion and Etest method. Agar dilution has been found previously to be comparable to broth microdilution (for antibiotics other than colistin) and Etest has been shown to be comparable to agar dilution⁵¹⁷. However it has been reported that disc diffusion methods correlate poorly with agar dilution methods in non-CF *P. aeruginosa* isolates^{502,518-520}. More recently studies have shown that broth microdilution, Etest and disc diffusion methods may in fact underestimate the incidence of *P. aeruginosa* colistin resistance^{298,501,503,518-519,521}. In this study the overall correlation between disc and Etest in comparison to agar dilution, were similar, between 71-75% in the CF group and 89.6-90% for NCF group. The disc and Etest methods appear to correlate better in the NCF rather than CF group, although there was many NCF isolates which were defined as susceptible according to the disc diffusion methods which were resistant according to the agar dilution methods. Many clinical laboratories in Ireland rely on either disc or Etest methods to detect colistin resistance, because of the number of very major errors seen here and other studies^{298,501,503,518-519,521}, there is potential for colistin resistance to go undetected in Irish hospitals. In fact colistin is not usually included in routine susceptibility testing for non-CF patients, but this study found that 12% of NCF isolates were resistant to colistin, according to agar dilution methods. Underlying colistin resistance could pose a threat to CF patients. Although the CF and NCF colistin resistant isolates which were examined by PFGE were not found to be closely related in this study, if colistin resistant *P. aeruginosa* isolates are present in the environment then there is potential that these colistin resistant strains could spread to CF patients.

Sub-inhibitory concentrations of colistin have been shown to result in the upregulation of the *arnBCADTEF-ugd* operon in *P. aeruginosa* PAO1^{247,514}, depression of the *arnBCADTEF-ugd* operon is involved in colistin resistance^{247,467,471,516}. To examine if sub-inhibitory concentrations could result in a resistant phenotype (according to agar dilution), the level of *arnB* expression was examined in a number of

clinical strains. Isolate NCF.2006.72 and NCF.2006.78 were resistant to colistin and had an MIC of >128mg/L, isolate CF.2006.220 was intermediately resistant and had an MIC of 4mg/L, isolate NCF.2006.100 was susceptible to colistin and had an MIC of 2mg/L. Firstly the level of *arnB* expression was examined in all of the isolates and compared to *P. aeruginosa* PAO1, as expected the level of *arnB* expression was elevated in the colistin resistant isolates. When the isolates were exposed to a sub-inhibitory concentration of colistin, the level of *arnB* expression (compared to unexposed) increased in isolate NCF.2006.78 (>128mg/L) and the susceptible isolate NCF.2006.100, indicating that sub-inhibitory concentration could induce *arnB* expression and could have an effect on agar dilution result.

Suboptimal doses of antimicrobials have been shown to alter gene expression in bacteria^{514,522-524}. In fact suboptimal doses of antimicrobials have actually been shown to be advantageous to bacterial survival⁵²⁵. Since the pharmacokinetics and pharmacodynamics are not fully understood it is important to understand how suboptimal doses of colistin affect *P. aeruginosa*.

RpoN is an alternative sigma factor in *P. aeruginosa* and has been found to play an important role in the nitrogen metabolism pathway along with other roles in motility, transport of nutrients, the formation of pili, mucoidy, cell-to-cell signalling and more recently *rpoN* inactivation has been linked to antimicrobial tolerance^{53,469,498-500,507-511}. A deletion in the *rpoN* gene in *Salmonella enterica* has been shown to increase polymyxin resistance, independently of the PmrAB, PhoPQ pathway⁴⁹⁷. An *rpoN* mutant from the mutant library was resistant to colistin, the presence of mutations at position S71 and N122 were observed in a number of isolates, these mutations are located within Region 2 of the RpoN protein (based on *E. coli*) which is involved in DNA binding⁴⁶⁹, the role of these mutations in colistin resistance will need to be investigated further. Mutations in the *pmrB* gene which have been shown to contribute to colistin resistance were not detected in the isolates examined, but other mutations may be present.

Initial tests with the Manoil *P. aeruginosa* mutants revealed that disruption to PA5003 (*mig-14* homolog), PA1807-PA1811 (*yejABEF*) along with *rpoN*, *phoPQ* and *pmrAB* genes had an effect on colistin susceptibility in these isolates. The role of these genes, in particular *pa5003*, *pa1807-pa1811*, in colistin resistance in *P. aeruginosa* has not been examined previously; it would be interesting to see what effect they have on

colistin resistance. The role of PA5003 (*mig-14* homolog), PA1807-PA1811 has not yet been fully elucidated in *P. aeruginosa*.

Chapter 7 General Discussion

Antimicrobial resistance is a growing global problem. Infections caused by multidrug resistant bacteria present daily challenges to clinicians and their patients. Although multidrug resistant Gram-negative bacteria are an increasing problem, the development of novel antimicrobials has been slow. *P. aeruginosa* is recognised as a major and dreaded cause of nosocomial infection among patients with localised or systemic impairment of immune defences; it is a common cause of hospital-acquired pneumonia, bloodstream and urinary tract infections²⁶³. In CF patients, *P. aeruginosa* causes the most important bacterial complication leading to chronic colonisation²⁶³. Given this, monitoring the rates in antimicrobial resistance is an important factor for guiding empiric therapy.

P. aeruginosa isolates that are MDR render antimicrobial therapy less effective and costly and frequently result in increased mortality rates especially amongst the immuno-compromised³¹⁶. As shown in this thesis a greater proportion of CF isolates (88%) were resistant to more than one antimicrobial compared to NCF isolates (34%). A number of surveillance studies have been carried out which examine the level of *P. aeruginosa* resistance from CF²⁵⁶⁻²⁵⁸ and NCF *P. aeruginosa* isolates^{249-252,254-255,259-262,318}, but this is the first study to examine the resistance levels of *P. aeruginosa* isolates from two population groups in Ireland. Data shown here highlights the distinct differences in antimicrobial resistance between the two populations. As expected, greater antimicrobial resistance was seen in the CF population, although few resistance genes were detected. This cause of the high resistance without the presence of resistance genes is one avenue for future research.

As shown in this study, Vitek 2 had a poor overall correlation agreement when compared to agar dilution, which suggests that further optimisation of the Vitek 2 system is required. Although the Vitek 2 system is not considered appropriate for testing with CF *P. aeruginosa* isolates, as shown in this study, disc susceptibility testing is not accurate. A more accurate and possibly quicker method would be broth microdilution method, as outlined in the CLSI guidelines. This method has been shown to be comparable to agar dilution and would allow for many isolates to be examined simultaneously on 96 to 100 well plates.

Surveillance studies are important but there is a lack of standardisation and susceptibilities may vary depending on the breakpoints applied. This also makes the comparison of results from different studies difficult, currently EARSS uses the CLSI guidelines but there needs to be a European consensus on which guidelines to use.

The Cystic Fibrosis Association in Ireland commissioned an independent review of CF services in Ireland. A report titled 'The Treatment of Cystic Fibrosis in Ireland: Problems and Solutions' was published in 2005⁵²⁶. This review highlighted the lack of adequate CF services in Ireland and made two key recommendations; i) Urgent action to correct the dangerously inadequate staffing position and ii) Establishment of a small number of adequately staffed and fully supported CF centres. The report was particularly damning of the provision of CF services at St. Vincent's Hospital, which is the country's largest CF centre (60% of adult CF patients in Ireland are treated in St. Vincent's). Since the report was published a number of steps have been taken by St. Vincent's Hospital including the construction of a newly dedicated CF unit which is due to be completed in March 2012.

Following on from this, it is clear that there is a need for a *P. aeruginosa* Reference laboratory to be incorporated into a National CF Reference Laboratory. A National Reference Laboratory would provide essential surveillance on *P. aeruginosa* and other pathogens associated with CF. Considerable resources would be required to set up a National CF Reference Laboratory including; trained laboratory staff with expertise in antimicrobial susceptibility testing in addition to molecular detection and typing techniques, surveillance scientist, antimicrobial pharmacists as well as consultant microbiologists and infectious disease physicians. This reference laboratory would provide many benefits to the CF community and others suffering from chronic non-CF lung disease. The reference laboratory would provide essential information on the national *P. aeruginosa* antimicrobial resistance trends and would facilitate in the characterization of resistance determinants (e.g. β -lactamases and aminoglycoside modifying enzymes, typing of MDR isolates) from isolates received from regional centres. All of these data would aid our understanding of *P. aeruginosa* resistance and would guide clinicians on appropriate antimicrobial therapy thereby improving patient care and reducing overall healthcare costs.

Ideally, and without financial constraints a future avenue of research would be the use of high-through-put or next generation sequencing (NSG) to give insights into the mechanisms of *P. aeruginosa* infection and antimicrobial resistance. With the advent of NGS technologies, whole genome sequencing that previously took months or years can now be completed in days or weeks⁵²⁷⁻⁵²⁸. NGS is an umbrella term used to describe new sequencing technologies that deliver sequence data 100-1000 times more

cheaply than traditional approaches⁵²⁹⁻⁵³⁰. These new technologies have a number of applications which could be used in clinical setting. For instance, as demonstrated by a number of groups⁵³¹⁻⁵³⁴, NGS could be used to screen transposon libraries to identify genes and pathways that contribute to *P. aeruginosa* pathogenesis. NGS could also be used to examine the epidemiology of *P. aeruginosa* isolates. In a study by Lewis *et al*⁵²⁸ 454 whole genome sequencing was used to examine a number of multidrug resistant *A. baumannii* isolates involved in an outbreak. These isolates were indistinguishable using standard methods, but high-through-put sequencing identified three single-nucleotide polymorphisms (SNPs) in three loci, which allowed the investigators to discriminate between the isolates and provided evidence of transmission between patients. This type of analysis could also be used for community profiling of *P. aeruginosa* in samples of sputa. High-through-put sequencing has been used to detect the relative abundance of different bacterial species within surgical wounds, leg ulcers and chronic rhinosinustitis⁵³⁵⁻⁵³⁷. This data could be used to accurately report the prevalence of *P. aeruginosa* in various specimen types.

NGS is already changing the research landscape, it is only a matter of time before these technologies become cheap and fast enough to replace competing typing methods and diagnostic technologies.

Recently, a DNA expression microarray was developed by Walsh *et al*⁵³⁸ for *Enterobacteriaceae* and non-*Enterobacteriaceae*, which screens for the presence of antimicrobial resistance and virulence factor genes. Although only a few *P. aeruginosa* isolates were included in this study, the results are promising and there is further scope for development of this array to quickly and accurately screen for resistance and virulence determinants among clinical isolates.

Since the introduction of quinolones in 1962, only two novel classes of antimicrobials have been approved by the FDA: the synthetic oxazolidinones (linezolid) in 2000⁵³⁹ and the naturally occurring lipopeptide (daptomycin) in 2003. However, both of these drugs are only effective against gram-positive bacteria⁵⁴⁰. Two new anti-Gram-negative antimicrobials recently reached the market; the glycylcycline, tigecycline⁵⁴¹ and the carbapenem, doripenem⁵⁴². While tigecycline is active against *Acinetobacter* and many *Enterobacteriaceae*, it is not clinically effective against *Pseudomonas*³¹³. And although doripenem is active against *Pseudomonas*, there is significant cross-resistance with meropenem³¹³.

New anti-*Pseudomonas* drugs are scarce in the pharmaceutical pipelines and predominately only in the early stages of development ³¹³. As a result, a variety of approaches are being pursued, as summarised in Table 7.1.

Table 7.1 Current/emerging treatment regimes for *P. aeruginosa* infection

Current treatment	Emerging treatment options
Broad-spectrum antimicrobials: Fluoroquinolones, aminoglycosides, imipenem, meropenem, third generation cephalosporins	KB001, Humaneered TM high affinity antibody fragment ⁵⁴³ has demonstrated promising therapeutic activity in the prevention of serious lung infection caused by <i>P. aeruginosa</i> , KB001 binds to PcrV protein which is an essential protein involved in type-three secretion system. Phase II.
Gram-negative selective antimicrobials: Piperacillin, aztreonam, either alone or in combination with tazobactam ⁵⁴⁴⁻⁵⁴⁵	CXA-101, is a novel cephalosporin that has excellent potency against MDR <i>P. aeruginosa</i> ⁵⁴⁶ . Phase II under way. NXL104/ceftazidime is a cephalosporin-inhibitor combination being developed by Noxexel to treat Gram-negative hospital infections ⁵⁴⁷ . Phase II. BLI-489/Piperacillin is been evaluated by Wyeth as a successor to their very successful piperacillin/tazobactam combination, ³¹³ . It is active against class A and C and some class D β -lactamases ⁵⁴⁸⁻⁵⁴⁹ .Phase I Preclinical trials have shown the lipopeptide CB-182,804 has shown promise as a potent, bactericidal, parenteral antibiotic for Gram-negative infections.
Experimental treatment options	
Lactams and β-lactamase inhibitor combinations	
BAL30072 is a novel siderphore monobactams with activity against a broad range of Gram-negative bacteria ³¹³	
NXL 105, from the same chemical class as NXL-104, with additional substitutions confers additional activity as an inhibitor of essential penicillin-binding Gram-negative bacteria ³¹³	
BAL30376 is a novel triple combination comprising the siderphore monobactams BAL19764, BAL29880, a bridged monobactams specific of class C β -lactamases, a specific inhibitor of class A β -lactamases ³¹³ .	
Peptides and peptides mimetics	
Arenicin-3,is an antimicrobial peptide isolated from the marine lugworm <i>Arenicola marina</i> ³¹³	
POL7001 and POL7080 are synthetic protein epitope mimetics that mimic protegrin-I, a lytic antimicrobial peptide ³¹³	
RTA3 is a novel cysteine containing peptide derived from the commensal organism <i>Streptococcus mitis</i> , ³¹³	

Recent attempts to discover novel antimicrobial classes have made use of proteomics. By studying the expression levels of a multitude of proteins under a variety of different growth conditions, specific proteins become indicative of the particular physiological state of the cell ⁵⁵⁰. Therefore one application of proteomics in drug discovery is the identification of novel antimicrobial targets. Proteomic applications alone, or in combination with transcriptomics and other phenotypic methods could play an increasing role in target validation and mode of action determinants of novel compounds and variants of exciting compound classes ⁵⁵⁰. However, this technology is still in the early stages.

Therefore, the challenge facing us today is to curb the emergence of *P. aeruginosa* resistance either through the adaptation of existing antimicrobials and/or the use of combination therapies. The inclusion of further binding domains at the same target (e.g. telithromycin ⁵⁵¹⁻⁵⁵²) is an example of optimization of current drug classes. Another approach in dealing with *P. aeruginosa* infection involves treatment with a combination of antimicrobials. For example the combination of β -lactams with aminoglycosides is often used to successfully treat *P. aeruginosa* infection ². However, combination therapy does not always prevent the emergence of resistance and clinical failures are still a risk ^{178,553-555}.

The most effective strategy in dealing with *P. aeruginosa* infection is to limit or prevent infection. This is not always possible, Table 7.1 summarises the methods used to prevent and manage *P. aeruginosa* infection.

Table 7.2 Current strategies to prevent/manage *P. aeruginosa* infection in CF patients^a

Strategies	Aims/Outcome
Prevention/targeting early <i>P. aeruginosa</i> infection	
Including infection control and patient isolation	Prevents transmission of <i>P. aeruginosa</i> , moderately to highly effective ⁵⁵⁶
Early eradication via antimicrobial treatment	To eliminate <i>P. aeruginosa</i> infection as soon as it appears. Effective in uncontrolled clinical trials ⁵⁵⁷⁻⁵⁵⁹
Development of infection	
Immunotherapy	Innate immune response in CF patients is ineffective at clearing <i>P. aeruginosa</i> infection; adapt the acquired immune response through vaccination against known <i>P. aeruginosa</i> virulence factors. Moderately successful clinical trial of flagella vaccine ⁵⁶⁰ .

Targeting of established infection Antimicrobial therapy	Standard antimicrobial therapies reduce bacterial counts and inflammation ⁵⁶¹ . Inhaled tobramycin, TOBI®, received FDA approval and has been shown to reduce bacterial counts and inflammation ⁵⁶² Caystan®, aerosolized form of aztreonam received FDA approval in February 2010. BAYQ 3939, which is an inhaled form of ciprofloxacin) has entered Phase 2 in 2010. MP-376 (new aerosol formulation of levofloxacin) is under development, Phase 3 in 2010
Targeting of inflammatory consequences of infection Anti-inflammatory drugs	Ibuprofen, high doses demonstrated reduced lung function. Six other drugs in various stages of clinical trials ⁴¹
Targeting of defective CFTR gene Gene therapy CFTR modulation	Addition of normal copies of <i>cftr</i> gene to cells. Compacted DNA (PLASmin™): uses compacted DNA (non-viral) to introduce normal copies of the gene into CF airways. Phase 1 completed ⁵⁶³ These therapies aim to correct the function of defective CFTR protein. Clinical trials ongoing ^{556,564}

^a Adapted from ⁴¹ and <http://www.cff.org/research/DrugDevelopment>

It is for this reason that antimicrobial surveillance, infection control and antimicrobial stewardship are of the upmost importance in dealing with *P. aeruginosa* infection. Antimicrobial stewardship strategies are an essential part of improving patient care and therefore must be continuously monitored. Incorrect antimicrobial prescribing creates unnecessary pressure for the selection of resistant bacteria, thereby contributing to the accelerated emergence and spread of resistant bacteria ⁵⁶⁵. A number of strategies have been proposed to improve antimicrobial stewardship in Ireland ⁵⁶⁶. Which include ongoing education for prescribers on the appropriate use of antimicrobials, the creation of an antimicrobial formulary with restricted prescribing of targeted agents, (in response to the significant increase in fluroquinolone resistance in St. James’s Hospital, the prescribing of fluoroquinolones has been restricted since June 2008), and scheduled changes of antimicrobial classes used for empirical therapy. These strategies must be administered by multidisciplinary teams composed of consultant microbiologists,

infectious disease physicians, antimicrobial pharmacists, surveillance scientists and infection prevention and control nurses. It is also paramount that all members of staff are aware of the importance of hospital hygiene and infection prevention.

Limitations of this thesis

Future experiments to confirm the functional properties of the chromosomal mutations detected in this study would include cloning regions into a reporter plasmid vector, followed by transformation and expression in highly competent *P. aeruginosa* cells, and subsequently antimicrobial susceptibility testing^{292,567}. As entire genes were not fully sequenced in some cases, it is possible that other mutations that confer resistance may have been present in regions not amplified. The molecular techniques performed in this study do not discriminate between resistant determinants that are encoded on a plasmid, the chromosome or a transposable element. Future experiments to investigate if typical plasmid encoded elements were plasmid encoded or chromosomal encoded involve the Kado and Liu method⁵⁶⁸ to separate all plasmid DNA from chromosomal DNA followed by Southern blotting with a gene specific probe.

Due to lack of available patient information, it was not possible to prospectively gather information on the antimicrobial regimens of each patient received.

Table 7.3 Future Directions

Chapter 4	- Investigating the presence of other ESBLs and aminoglycoside modifying enzymes. - Determining if the overexpression of the efflux pumps was caused by a specific mutation in the efflux gene regulators.
Chapter 5	- Examine the role of the GO system in hypermutation amongst the isolates. Sequencing of the genes involved. - Investigate efflux gene regulators, to determine if mutations in these regulators have caused the overexpression of efflux pump genes - Determine if large chromosome inversions are the cause of the loss of <i>mutS</i> in a number of isolates
Chapter 6	- Sequencing the full length of <i>pmrA</i>, <i>pmrB</i>, <i>phoP</i>, <i>phoQ</i> of colistin resistant isolates. If mutations were found, carry out complementation experiments to determine if the mutation contributed to colistin resistance. If a common mutation was found, transfer the affected gene into a susceptible <i>P. aeruginosa</i> clinical isolate to determine the effect of the mutation on colistin resistance. - Elevated MICs were seen in mutants of <i>rpoN</i>, PA5003 (mig-14 homolog), PA1807-

PA1811 (*yefABEF* homolog), I would carry gene knockout experiments to determine what affect this would have on colistin resistance. I would then sequence the full length genes of *rpoN*, PA500., PA1807-PA1811 in colistin resistant isolates to determine what if any mutations are present.

- I would look at *arnB* expression in all colistin resistant isolates and I would also examine the LPS structure of the colistin resistant isolates by SDS-PAGE and silver staining.
-

Future projects

Many argue that conventional susceptibility testing is not appropriate for *P. aeruginosa* growing in biofilm^{326,328-330,569}. A number of biofilm assays have been developed by these groups^{326,328-330,569}, but one of the main problems with these studies is that no definitive conclusions of the actual clinician effectiveness of these assays can be drawn. Future research in the form of prospective trial comparing clinical outcomes is required.

P. aeruginosa growing in biofilm is not just a problem for CF patients. A study could be set up examining isolates from patients found to be colonized with *P. aeruginosa*, or have a serious *P. aeruginosa* infection. Detailed patient information would be gathered, including age, sex, primary source of infection, previous antimicrobial exposure and immune status. Four different susceptibility methods would be evaluated; agar, broth, disc diffusion and biofilm assay³²⁶. These data would provide vital clinical information on which method was most accurate at predicting clinical effectiveness of a given antimicrobial, thereby guiding clinicians on the appropriate antimicrobial to prescribe.

The research described in this thesis has added to the current understanding on the topic of *P. aeruginosa* in clinical isolates and serves as a platform for which further progress can be made.

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