Impact of Pseudomonas aeruginosa Liverpool Epidemic Strain (LES) on Cystic Fibrosis patients

Thesis submitted in accordance with requirements of
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by Abdul Ashish

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Declaration

The design of the work presented in this thesis was that of the author and his supervisors, Dr Martin J Walshaw and Professor Craig Winstanley. The author performed all experimental work. Statistical support was provided by Matthew Shaw and James Mcshane

Dr Abdul Ashish  

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Abbreviations

ASL: Airway Surface Liquid
ATP: Adenosine Triphosphate
ABC: ATP binding cassette
BAL: Broncho-alveolar Lavage
BMI: Body mass index
CBAVD: Congenital bilateral absence of the vas deferens
CF: Cystic Fibrosis
CFTR: Cystic Fibrosis Transmembrane Regulator
CFQ: Cystic Fibrosis Questionnaire
CFRD: Cystic Fibrosis Related Diabetes Mellitus
ENaC: Extracellular sodium channel
FEV1: Forced Expiratory Volume in 1 s
PCL: Peri-ciliary Layer
HRQoL : Health Related Quality of Life
LES : Liverpool Epidemic Strain
HM: Hypermutable phenotype
Aux: Auxotrophy
TOB: Tobramycin;
COL: Colistin
CEF: Ceftazidime
CIP: Ciprofloxacin
MER: Meropenem;
TAZ: Tazobactam / pipericillin
GM: Green mucoid
GNMS: Green Non-mucoid smooth
Mtr: Mucoid transparent
MWO: Mucoid white opaque
PCR: Polymerase Chain Reaction
PFGE: Pulse field Gel Electrophoresis
Psa: Pseudomonas Aeruginosa
QS: Quorum Sensing
RM: Red mucoid;
SNMS: Straw coloured Non-mucoid smooth.
TIVAD: Totally Indwelling Venous Access Device
Abstract

Cystic Fibrosis (CF) is the commonest life limiting inherited disease illness in the western world. Over the last few decades there have been many advances in the diagnosis and management of this condition. Patients born with the disease now are living into their fourth decade, which is a statement to the progress made over time.

Along with the progress there have been new challenges in the world of CF. In the last two decades there have been several studies reporting the presence of transmissible Pseudomonas Aeruginosa (Psa) strains in CF clinics worldwide. The first one to be reported in UK Liverpool paediatric clinic was later identified as the Liverpool Epidemic Strain (LES). Previous studies have demonstrated chronic infection with LES can result in accelerated fall in lung function, increased hospitalisation and antibiotic requirements. This thesis looks at the effect of chronic infection of adults with CF and its implications on healthcare institutions caring for such patients. In particular on an individual level i investigated the health related quality of life associated with patients chronically infected with LES and compared it those with unique or no Psa strains. I have demonstrated that chronic infection with LES strain significantly worsens health related quality of life compared to those with unique or no Psa strains. Patients infected by transmissible Psa strains had worse physical functioning, respiratory symptoms, treatment burden, vitality, role, health perception and emotion than those with unique Psa strains (p<0.01), and significantly poorer physical functioning, respiratory symptoms, treatment burden, body image, weight, role, and emotion than those without any Psa infection (p<0.05).
To understand the susceptibility of LES strains to common anti-pseudomonal antibiotics I studied the antibiograms of patients infected with LES strain over a 5-year period and compared the change in susceptibility to those infected with unique Psa strain. LES exhibited significantly more resistant isolates in 2004 (p<0.0001). There was an increase in antibiotic resistance in both LES and other Psa strains over time (p<0.001). Cox proportional hazards analysis of both unmatched (n=125) and matched (n=56) patients in 2004 revealed that LES infected patients were more likely to develop antibiotic resistant isolates over time (hazard ratio 8.1, p<0.001). Fewer LES isolates were classed as fully sensitive in both matched and unmatched groups at the end of study period (p<0.001).

I then looked at the phenotypic characteristics exhibited by LES strain in comparison with unique Psa strain during an infective episode to elucidate whether it is a specific character of LES assisting to survive in harsh CF environment. We analyzed sets of 40 sputum samples isolates from five CF patients each chronically infected with a different non-LES strain of *P. aeruginosa*. For each sample (two per patient), diversity was assessed by characterising nine phenotypic traits. All *P. aeruginosa* populations were highly diverse. The majority of phenotypic variation found was due to within-sample variation. I demonstrated that maintenance of diverse populations in the CF lung is a general feature of *P. aeruginosa* infections rather than a unique characteristic of LES. To assess the healthcare resource implication on institutions caring for such patients we carried out an economic analyses of healthcare utilisation of patients chronically infected with LES to those with unique Psa strain over a four-year period. Ascertainable costs were correlated in these two groups of patients. The mean cost per patient per year was higher for LES patients for inpatient care (£4393.37 v £1817, p=0.0006), outpatient
attendance (£3764 v £2515.91, p=0.0035) and also hospital antibiotic therapy (£980 v £505, p=0.001). Regular prescription costs were similar in both groups. Overall, the healthcare cost of caring for an adult CF patient with LES chronic infection was significantly more (1.6 times) than that for a matched patient with unique Psa strain chronic infection.

Finally, I looked the effects of segregation policies instituted at Liverpool adult Cf centre in limiting cross infection. Regular genotypic surveillance of sputum samples of all patients was instituted to monitor cross infection rates. This study elucidated the cross infection policies over a 7-year period and looked at the yearly number of patients with LES and other strains. There was a decline in the proportion of patients with LES (71% to 53%) and an increase in those with unique strains (23% to 31%) and without Psa infection (6% to 17%) over the study period.

Areas of potential future research based on this thesis are also outlined.
1.1 Epidemiology:

Cystic fibrosis (CF) is the most common life shortening, autosomal recessive disorder in the Caucasian population. In the UK the incidence is about 1 in 2500 live births and about 1 in 25 are carriers. Following major advances in understanding of the disease, genetics, microbiology and multidisciplinary approach to management, the average lifespan of a patient with CF has increased. The median predicted survival now is between 37-47 years (UK CF registry 2013, Canadian CF registry 2009). This is indeed a dramatic leap from four decades ago, when only 10% of CF patients were over the age of 18 years (Yankaskas et al 2004) and the focus of the disease management was on managing end-stage disease. As the survival of patients born with CF has increased over the years, the number of adults with CF is expected to surpass the number of cases in paediatric settings, which indicates the changing landscape of the average age of a CF patient. In the UK there is an increasing proportion (>70%) of CF diagnosis made within the first three months of life, with 90% occurring within the first year (UK CF registry 2013). It is now not uncommon for CF patients to enjoy facets of life which were previously limited to “normal” individuals, such as completing education, holding jobs and having a family. The recent UK CF registry (2013) suggests that in the UK 71% of all adults with CF are in full time jobs or education, and that this is improving year on year. However there are still problems that need to be overcome to achieve a normal lifespan and quality of life for these individuals. Chronic infections with Pseudomonas aeruginosa (Psa), which adversely affects patients with CF, is still a problem experienced by all CF centres in the world. Despite all these advances the median age at death still remains at a sobering 29 years (UK CF registry 2013), which still suggests that there is still a
long way to go and efforts at improving outcomes for these patients need to be continued tirelessly.

1.2 CF genetics

CF is an autosomal recessive condition caused by mutations in a single gene, located on the long arm of chromosome 7, which encodes a 1480 residue transmembrane glycoprotein called the CF transmembrane conductance regulator (CFTR) (Davies et al 1996, Welsh MJ & Smith 1993). It is the most common life-limiting genetic disease seen in the western world. There are about 1500 reported mutations, many of which are rare and may not result in clinical signs or symptoms. The majority of mutations involve less than three nucleotides leading to predominantly amino acid substitutions, frame shift or nonsense mutations (Gibson et al 2003). Of these 1500 mutations, only 22 have been identified with frequency of at least 0.1% known alleles (CF Consortium 1990) and have been demonstrated by direct evidence to cause sufficient loss of CFTR function to cause CF clinically and would be accepted for a genetic basis of diagnosis. Most of the other mutations are rare, restricted to a limited population and don’t usually result in clinical disease. The first identified mutation and the most common has a three base pair deletion that codes for phenylalanine at position 508 of the CFTR protein. This p.Phe508del mutation is the most common in the Caucasian population, accounting for over 90% of CF-related alleles (UK CF Registry 2013). The various mutations affect the function of CFTR in different ways; some resulting in abnormal CFTR production and others affect the intracellular processing of CFTR channel function or a combination of these (Zielenski & Tsui 1995) (Table 1). These specific mutations can have characteristics of more than one class. These mechanisms of CFTR dysfunctions are a simplistic framework in understanding the molecular basis of
epithelial abnormalities in CF. Most of the diagnosis however requires a combination of clinical disease associated with CF sweat chloride testing or nasal potential difference in combination of gene testing (Farrell et al 2008). There are newborn screening tests, which are available in the western world to aid early diagnosis. Patients without clinical spectrum of the disease but positive diagnostic tests or vice versa are usually followed up as they are at a higher risk of complications associated with CF.

Table 1 Classes of CFTR mutations

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<td>Defective protein production with premature termination of CFTR Protein production. No functioning CFTR chloride channels seen</td>
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<tr>
<td>Class 2 mutation</td>
<td>Defective trafficking of CFTR so that it does not reach the apical surface membrane where it is intended to function</td>
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<tr>
<td>Class 3 mutation</td>
<td>Defective regulation (opening and closing) of the CFTR chloride channel</td>
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<tr>
<td>Class 4 mutation</td>
<td>Defective conduction (passage of chloride ions through the channels)</td>
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<td>Class 5 mutation</td>
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1.3 Function of CFTR

CFTR acts as a Cyclic adenosine monophosphate regulated chloride channel at the apical membrane of various epithelial cells at sites including the sweat glands, salivary glands, airways, nasal epithelium, vas deferens in males, bile ducts, pancreas, intestinal
epithelium as well as many other sites (Welsh, MJ 1995). The function of CFTR is important in many of these organs as its defectiveness, mislocalisation or absence causes disease (Davies et al 1996). CFTR mediates transepithelial salt transport, liquid movement and ion concentrations in these organs (Tummler et al 1997). The structure of CFTR places it in the family of transport proteins called ATP binding cassette (ABC) transporters. (Cheng et al 1990) Members of this family utilise the energy of ATP hydrolysis to transport substrates across cell membranes.

CF reflects the absence of a functional CFTR protein at a cellular level (Welsh et al 1993). The various CFTR mutations are outlined in Table 1). The most common CF mutation, ΔF508, has been demonstrated to exhibit a problem in polypeptide maturation and translocation to the appropriate cellular domain, e.g. the apical membrane (Cheng et al 1990).

CFTR also regulates the movement of other ions across these trans-membranous channels; i.e stimulation of other chloride channels or inhibiting the epithelial sodium channel resulting in salt and water transport across epithelial barriers. CF is characterised by a wide variety of clinical expression relating to CFTR defects: dehydration and hyperviscosity of mucous secretions result in plugging of the ducts of exocrine glands which then predispose to multi-organ clinical manifestations, particularly within gastrointestinal, hepato-biliary, reproductive and respiratory tracts (Boucher R et al 2004). The main clinical features may include chronic suppurative lung disease, exocrine pancreas insufficiency, intestinal obstruction, gallstones, biliary cirrhosis and male infertility. However the most important site of disease that accounts for much of the morbidity and mortality is the lung.

1.4 Abnormal innate lung defense in CF

Recent studies have demonstrated that bronchiectasis can be seen in 22% of newborn
with CF (Stick et al 2009). BAL studies in these infants, as early as 3 months, has shown infection with microorganisms (Mott et al 2011). Early infections in CF occur with more benign bacteria such as *Haemophilus influenza* and *Staphylococcus aureus*. By adulthood 80% are infected with *Pseudomonas aeruginosa* (Kerem et al 1990). At a very basic level, CF lung disease could be seen as a failure of the innate defense mechanisms of the lung against inhaled microorganisms (Knowles et al 2002). Absence of functional CFTR, which causes problems with ion transport, leads to further problems in the defense mechanisms of the lung, which may result in persistence of bacterial pathogens in the lung leading to a chronic cycle of inflammatory response and lung damage.

Two popular theories have been developed to link the ion transport defects to the innate defense of airways against inhaled bacterial pathogens: firstly the failure of mechanical clearance of the bacteria and secondly the ion transport abnormalities causing alteration of the depth and tonicity of the periciliary fluid. Normally, once a bacterial pathogen is inhaled, mechanical clearance has been viewed as the primary innate defense mechanism. Mucus clearance provides the mechanical clearance that removes bacteria from the airways within few hours under normal conditions (Wanner et al 1996). Airway surface liquid (ASL) is composed of mucus and an underlying watery periciliary liquid (PCL). The promotion of efficient mucus transport requires coordination of events such as the efficient ciliary activity and the regulation of adequate quantity of ions and water on airway surfaces via the trans-epithelial transport channels. The capacity of the epithelium to maintain the PCL layer at the appropriate height requires the adjustment of ASL volume, a process that is believed to be mediated by isotonic volume transport (Matsui et al 1998, Tarran et al 2001). The microorganisms are usually trapped in the mucus layer. The cilia move from the small
distal to the larger proximal airway carrying along with it the mucus, periciliary surface liquid and the microorganism eventually leading to its clearance from the lung. The second hypothesis of airway defense was formed in 1990s for airways defense that focused on the role of antimicrobial peptides in ASL to provide a chemical shield on the airway surface as the primary defense (Goldman et al 1997, Smith JJ et al 1996). For this shield to be effective, two processes must be normally regulated by the airway epithelium. First, there must be secretion of the appropriate quantities of antimicrobial, salt-sensitive peptides (defensins), and secondly, the ASL must be modified, i.e. made hypotonic (w50 mM NaCl), so that antimicrobials are active. It has also been demonstrated that in the airway antimicrobial substances such as lactoferrin and lysozyme are present, which suppress bacterial growth over time. (Cole et al 1999) The mucus layer serves to trap inhaled material during the clearance process from the airways. The mucus layer uses two mechanisms to remove virtually all inhaled particles that deposit on airway surfaces: 1) mucus flow is "turbulent", so materials are mixed into the mucus layer and enmeshed/ trapped during clearance (Worlitzsch et al 2002) mucin molecules exhibit a layer of carbohydrate epitopes to ensure low affinity binding to most particles (Lamblin et al 2001).

To maintain a steady state in PCL needs a balance of Na⁺ absorption and Cl⁻ secretion. The epithelial cells express the epithelial Na⁺ channels (ENaC) and pumps Na⁺/K⁻/ATPase to mediate intracellular Na⁺ absorption. When ENaC is inhibited there are electrical driving forces that initiate the Cl⁻ secretion. This chloride secretion is mediated by CFTR. Lately the importance of both the accelerated Na⁺ absorption and the failure to initiate Cl⁻ secretion to the abnormal ASL volume homeostasis in CF has become clear. These abnormalities in both processes ultimately lead to depletion of the PCL layer and formation of thickened mucus plugs adherent to CF airways. This
reduction in ASL has been demonstrated to cause the spontaneous airway inflammation. A reduction in ciliary-dependent clearance results from thickening of the mucus rendering it less favorable for transport. Regnis (1994) have demonstrated reduced mucociliary clearance in CF patients with normal lung function compared with age-matched normal subjects.

1.5 Mucus hypoxia and bacterial colonization

Mucin secretion from goblet cells and submucosal glands continues despite this highly dysregulated mucus transport in patients with CF. This continued secretion of mucins into an already thickened mucus layer eventually lead to formation of mucus plaques and plugs on airway surfaces. As these plaques can reach heights of 1 cm in adult patients, the areas of these plaques near the CF cell surface become oxygen depleted (Worlitzsch et al 2002). In normal circumstances the turbulence of a normally functioning mucus layer will trap the microorganism leading to its clearance. However, as the mucus transport is impaired, some bacteria such as *P. aeruginosa* can easily penetrate into this thickened mucus plaque and migrate into hypoxic zones. *P. aeruginosa* adapts to this hypoxic environment by slowing its growth and by production of alginate. This formation of alginate reflects the conversion from the planktonic growth seen in the environment to one that is suited more to the anaerobic CF environment (i.e biofilm mode).

With these successful adaptations to the hostile CF environment by *P. aeruginosa* the scene is set for persistent infection. This mode of anaerobic growth provides a lot of advantages to the microorganism. Primarily the neutrophil mediated damage is limited in such an environment as it may be difficult for migratory neutrophils to penetrate into the thickened mucus plaques. Also, the diffusion of antimicrobial activities into the thickened mucus plaques may be limited. This failure of the secondary defence
mechanisms to fight *P. aeruginosa*, and the persistence of bacteria in the “protective environment” of biofilm, leads to a persistent state of infection of the adherent mucus (Worlitzsch et al 2002). This proliferant bacterial growth over time renders the mucus layer totally oxygen deficient. This anaerobic state of mucus layer and persistent bacterial colonization has implications in CF treatment. The minimal inhibitory capacity of the antimicrobial agents in an anaerobic biofilm environment may not be well represented by the laboratory condition usually employed during routine testing (De Kievit et al 2001).

**1.6 Clinical Manifestations of Mutations in CFTR Defects**

Most of the clinical manifestations seen in CF are due to the pathophysiological effects of the thickened mucus produced by the epithelial layer of apical acinar cells (Sheppard et al 2002). The destruction of the exocrine pancreas is attributed to tissue damage due to lytic enzymes of the acinar tissue following plugging of the pancreatic ducts by the thickened secretions of the pancreatic acinar cells leading to fibrosis and fatty replacement. Older patients have an increasing incidence of diabetes mellitus, associated with destruction of the pancreas (Costellessa et al 2000). The gastrointestinal manifestation of malabsorption of essential fats is due to reduced intestinal fluid secretion combined with inadequate pancreatic secretion. This leads to failure to thrive in newborns and gastrointestinal blockage known as meconium ileus, seen in approximately 5 to 10% of patients (Murshed et al 1997). Rectal prolapse can also be seen in up to 20% of untreated patients with CF in the first 5 years of life (Kulczycki et al 1958). Older patients can present with a clinical picture very similar to bowel obstruction, wherein distal intestinal obstruction syndrome can develop if fluid intake is poor or there is a failure to take pancreatic enzymes. Similarly, obstruction of the hepatic ducts has been hypothesized to be the etiology of the liver disease in CF. Bile
stasis and periportal fibrosis can often result in cirrhosis of the liver and ensuing complications such as portal hypertension and varices can also be seen (Roy et al 1982). Patients can also present with pan-sinusitis due to the mucociliary apparatus failure and electrolyte abnormalities, especially in summer months due to loss of electrolytes in sweat. Females are sub-fertile due to altered cervical viscosity. In males however there is congenital bilateral absence of the vas deferens (CBAVD), which renders most males infertile (Anguiano et al 1992). A primary defect in calcium metabolism and increase in urinary oxalate excretion has been linked to patients developing kidney stones (Matthews et al 1996). Many patients with CF suffer with malnutrition because of defects in their pancreatic and gastro-intestinal function. They have poor weight gain, muscle mass and short stature. Osteoporosis, low bone mass and fractures are common in both children and adults with CF (Sheppard et al 2002). Most cardiovascular manifestations are due to right heart failure and pulmonary hypertension (Stern et al 1980). Myocardial necrosis and fibrosis can lead to sudden cardiac arrest in childhood (Żebrak et al 2000). Neuro-pathological changes in CF resemble those of vitamin E deficiency, including dystrophic axons and demyelination. In animal models replacement of vitamin E has been shown to halt these changes (Sung et al 1980).

1.7 Airways in Cystic Fibrosis

The main clinical feature of CF lung disease is chronic airway infection and repeated infective exacerbations leading to irreversible lung damage and death (Kerem et al 1990). Although the CF lung is normal in utero and during first few months of life (Sturgess et al 1982), many infants become infected with bacteria and develop airway inflammation. Mucosal plugging of small airway may be the earliest indication of lung disease. Infants with CF get infected by microorganisms within the first few months of life. There is evidence, as discussed below, to suggest that such chronic colonisation of
microorganisms is preceded by vigorous inflammatory response by the immune system. Early infections are deemed more due to pathogens such as *S. aureus* and *H. influenzae*, but by adulthood 80% of CF patients are chronically colonised with *P. aeruginosa*, which ultimately causes the morbidity associated with CF. The inflammatory responses within the lungs to these microorganisms, the chronology of chronic colonisation, and the adaptations by microorganisms to enable survival in CF lungs will now be discussed.

### 1.8 Exaggerated inflammatory response in airways

CF infants develop bacterial infections early and the airway epithelial cells respond by mounting a vigorous inflammatory response (Stick et al 2009). CF disease involves a whole host of inflammatory mediators including cytokines such as IL-6, IL-8, granulocyte macrophage stimulating factor (GM-CSF) and Tumour necrosis factor (TNF)-α. A large influx of neutrophils occurs into the lungs. Some of these mediators, such as TNF-α and interleukins (IL)-1β, stimulate the epithelial cells to produce further pro-inflammatory cytokines using different signalling pathway than accessed by bacterial products, which leads to a overwhelming influx of these mediators in the lungs. In a study of children under the age of 12 with CF, all the children had evidence of pathogens in the lungs (Konstan et al 1994). The BAL of CF lungs predominantly were found to be infiltrated with neutrophils (57%, compared to 3% of controls). The mean concentration of neutrophils was found to be 380 times of that of controls, which is indicative of the exuberant inflammatory response seen in CF (Muhlebach et al 1999). The neutrophils in the lungs may also survive for longer in the CF lungs due to the presence of excess GM-CSF and lack of IL-10 (Bonfield et al 1995, Bonfield et al 1999). The neutrophils and their products such as elastase when present in excess further diminish the host’s ability to fight infections, causing active lung damage. This
also impairs the function of neutrophil mediated, complement directed, phagocytosis of 
*P. aeruginosa*, hence impairing the two most important mechanisms of clearing the 
bacteria. Studies in children have also suggested that they have a positive antibody 
response 12 months prior to first cultures being positive for *P. aeruginosa* (Heijerman et 
al 2005). A study by Khan et al where BAL samples of 16 infants with CF were 
examined, showed evidence of high levels of IL-8 even when cultures were negative for 
microorganisms, suggestive of exaggerated immune response even in infants with 
relatively normal lungs (Khan et al 2011). Airway macrophages were suggested as the 
source of IL-8, which further leads to neutrophil influx and long term lung damage. 
Bonfield et al. demonstrated excess of macrophages in CF lungs, which causes an 
increase in proinflammatory cytokines such as IL-8, IL-1, tumour necrosis factor (TNF) 
–alpha, IL-6 (Bonfield et al. 1999). There was also a reduction of IL-10, which is the 
cytokine synthesis factor inhibitor leading to an unregulated concentration of 
inflammatory cytokines and to enhanced lung damage. Eventually because of CFTR 
defects the bacteria are retained within the CF lung, causing these inflammatory 
responses to be florid and resulting in long term damage of the lungs.

**1.9 Adaptation by bacteria to survive in CF environment**

In CF, chronic bacterial infections display a limited spectrum. During early years, 
organisms such as *H. influenzae* and *S. aureus* dominate, but by adulthood 80 % of the 
CF patients are colonised by *P. aeruginosa*. (Gilligan et al 1991, Bauernfeind et al 
1987, Gilligan et al 1991, Rosenfield et al 1999). *P. aeruginosa* is not generally able to 
cause infections in normal hosts, but becomes pathogenic in patients with weakened 
defences. The pathogen can cause a wide range of infections in patients whose natural 
defences are in some way compromised, including burns infections, wound infections, 
eye infections, urinary tract infections, infections in Intensive Care Units or Neonatal
Units, as well as infection associated with lung disease, most notably CF (Pitt, T.L 1986, Lyczak et al 2002). The CF lung is a very hostile and heterogeneous environment in which the colonising microorganisms have to adapt to survive. Over time *P. aeruginosa* undergoes many genotypic and phenotypic changes to adapt to the CF environment. It has been shown that some strains of *P. aeruginosa* have the ability to mutate rapidly in the lungs of patients with CF (Oliver et al 2000). These “hypermutable strains” have been demonstrated to show resistance to multiple classes of antibiotics. Yet another important mechanism of survival of *P. aeruginosa* in CF lungs is the production of the exopolysaccharide alginate, which leads to a mucoid colony phenotype and is a marker for the establishment of chronic infection (Govan & Deretic 1996). In CF lungs a steep concentration gradient exists between airway lumen and interior of mucus (Worlitzsch et al 2002), which further aids the survival of this organism (Govan et al 1993). This adaptation also protects the bacteria from antibiotics and renders the organism multiresistant and aids its survival. There are some other well-studied phenotypic changes seen in CF isolates of *P. aeruginosa* to aid survival. These include the loss of O-side chains on lipopolysaccharide (LPS) (Hancock et al 1983), flagella-dependent motility (Luzar et al 1985), distinctive acylation of LPS (Ernst et al 1999), and increased auxotrophy (Thomas et al 2000). The ability of *P. aeruginosa* to form biofilms is also important for their success in the CF lungs (Silby et al 2011, Høiby et al 2010). *P. aeruginosa* also undergoes genotypic changes for its survival. It has one of the largest bacterial genomes and this offers it the potential to adapt to varied environments.

1.10 Microbiological aspects of cystic fibrosis lung infection

In the healthy respiratory system, the upper respiratory tract is usually colonized by a host of microorganisms that make up the normal commensal flora while the lower respiratory tract is kept in a sterile state by the various defense mechanisms of the host.
There are various innate defense mechanisms, which an organism has to overcome prior to mounting an infection/inflammatory response. A robust immune system is active at all times to keep us protected against infection. These barriers can be classed as physical barriers such as skin, epithelium or phagocytic barriers such as the macrophages or neutrophils at a more cellular level. Failure of these innate defense systems results in susceptibility to infection of our respiratory tract. Patients with CF are prone to such infections due to a combination of many factors such as CFTR defect, exaggerated immune response, and predilection to and persistence of certain microorganisms in the respiratory tract, eventually leading to recurrent infections. Infants with CF are often unable to expectorate sputum, which is usually derived from secretions in their lower respiratory tract, and therefore oropharyngeal or throat swab cultures, which target the upper respiratory tract secretions, are generally performed to detect pathogens. In reality, these cultures detect organisms, including potentially pathogenic ones, present in the throat and not necessarily in the lungs. There has been some correlation between the microbial content of upper and lower respiratory tract secretions, as studied by Ramsey et al. In a study of 250 non-expectorating CF infants under the age of 18 months, the positive predictive value of a positive oropharyngeal culture was approximately 95% and negative predictive value was 44%, which is suggestive that negative cultures in this cohort were unreliable. *S. aureus* is commonly the first pathogen to infect the lungs of CF patients (Ramsey et al 1991). *H. influenzae* is also a common respiratory coloniser seen in the early years of a CF patient. Since the advent of potent antibiotics and the emergence of more pathogenic organisms these organisms are now not considered to be so important in the microbiology of CF. *S. aureus* is often found as a colonizer in the anterior nares of patients, which is a subsequent risk factor for future infections (Perl et al 2002, Wenzel et al 1995). Following treatment with
intravenous antibiotic the prevalence of *S. aureus* colonization is reduced (Goerke et al 2000). It often remains unclear whether isolation of such organism reflects benign colonization or pathogenic disease state. CF patients may be infected with same strain of *S. aureus* for up to 2 years (Branger et al 1996). Lately an increase in prevalence of MRSA has been noted in CF clinics, especially in USA (CF registry 2002). It is reported that upto 23% of patients in CF clinics in the USA have had MRSA isolated from respiratory specimens. MRSA acquisitions have mostly been reported in hospitalized patients, suggestive of a potential reservoir of this bacterium. (Bell et al 2000). Although the implications of MRSA colonization remain unclear, it has been reported that children with chronic colonization required increased intravenous antibiotics and had a poorer chest radiograph score with no effect on lung function or growth (Miall et al 2001). Therefore some CF clinics advocate using prophylactic antibiotic on isolation of *S. aureus*. However, whilst there is a consensus among clinicians about a beneficial effect from treatment of staphylococci associated with clearance of the organism from the sputum, there are no data indicating that this treatment leads to improved lung function or other clinical benefit (Smyth & Walters, Cochrane database review, 2012).

A 3-year study of over 3000 CF patients in the European Registry of Cystic Fibrosis demonstrated that continuous anti-staphylococcal prophylaxis increases the rate of patients converting from a non-*P. aeruginosa* to *P. aeruginosa*-positive status (Ratjen et al 2001). The patients in this study were categorized both according to their age and whether they received continuous (200 or more days per year), intermittent (only during acute exacerbations), or no anti- staphylococcal therapy. This study demonstrated that children under the age of 6 receiving continuous anti-staphylococcal therapy had a higher prevalence of *P. aeruginosa* in their sputum sample than in those receiving no or
only intermittent therapy. This difference was not significant over the age of 12. There was no significant difference in other prognostic markers of disease severity such as BMI & FEV\textsubscript{1} between the three antibiotic treatment groups. This observation linked the possibility of long term anti-staphylococcal antibiotic prophylaxis resulting in \textit{P. aeruginosa} acquisition (Govan et al 1992, Emminger et al 1987, Geddes et al 1988). This has important implication as long-term outcomes for CF patients are much better in patients without \textit{P. aeruginosa} infection (Huang et al 1987, Hudson et al 1993). However, further studies are needed to better understand the clinical importance of clearing \textit{S. aureus} and the potential consequences for \textit{P. aeruginosa} infection (Smythe & Walters, Cochrane database review, 2012).

Burns et al demonstrated that there was evidence of \textit{P. aeruginosa} in BAL (Broncho-alveolar lavage) samples in 72% of children under the age of 3 years. When combined with serological testing they found evidence of \textit{P. aeruginosa} in 97.5% of patients with \textit{P. aeruginosa}, which contradicts the hypothesis that staphylococcal infection is a precursor for future \textit{P. aeruginosa} colonization (Burns et al 2001). It is notable that 80% of the \textit{P. aeruginosa} isolates in this study were non-mucoid variant which were susceptible to antibiotics rather than the mucoid variant which is the classic variant associated with morbidity in CF chronic infections. The \textit{P. aeruginosa} undergo the phenotypic changes to mucoid variant over time as a survival response to adapt to hostile CF environment.

Recent studies have shown that the microbiology of the CF lung is far more complicated than was originally thought, with many bacterial species, including anaerobes, co-existing as part of a complex CF “microbiome” (Blainey et al 2012). The clinical relevance of this is not yet understood.

\textbf{1.11 \textit{P. aeruginosa} in CF}
a) Initiation and establishment of *P. aeruginosa* infection:

*P. aeruginosa* is a ubiquitous organism found in the environment. It is a commensal of the upper respiratory tract in 5-10% of the normal population and asymptotically exists in the GI tract of CF patients in up to 20% of cases (Speert et al 1993). This presence of *P. aeruginosa* in the environment means that CF patients are constantly exposed to this opportunistic pathogen. Although CF patients can acquire *P. aeruginosa* in their respiratory tracts at any time, studies have shown that 70 to 80% of CF patients are chronically infected with *P. aeruginosa* by their teen years. Once *P. aeruginosa* chronically colonizes the lung, it results in long-term lung infections generally requiring intravenous antibiotics. The diversity of *P. aeruginosa* strains isolated from CF patients suggests that most clinical isolates are acquired from the surrounding environment (Burns et al 2001, Sogbanmu et al 1980).

b) Progression of chronic infection

Although there is a highly varied clinical course for CF patients based on acquisition of various well-known prognostic factors, the establishment of chronic infection with *P. aeruginosa* is perhaps the most significant. Long-term lung function is best preserved in individuals who remain uninfected with *P. aeruginosa* for as long as possible. Prior to the conversion to chronic infection, there is an opportunity to eradicate the *P. aeruginosa*. Indeed there has been considerable progress in this respect, with the wide scale use of Early Eradication Therapy (Smyth &Walters, Cochrane database review, 2012). Once the mucoid phenotype of *P. aeruginosa* emerges, this correlates with the onset of significant deterioration in lung function (Pedersen et al 1992, Demko et al 1995). The mucoid variant is known to be due to the overproduction of an exopolysaccaride, alginate, which is a negatively charged, linear copolymer of partially O-acetylated β-1,4-linked d-mannuronic acid and its C5 epimer, α-l-guluronic acid (Govan
This alginate production is a key factor in the pathogenic picture, along with several other molecular changes to *P. aeruginosa* isolates from CF patients. Curiously, mucoid *P. aeruginosa* strains almost all produce a LPS, rendering the organisms susceptible to the bactericidal effects of complement (Schiller et al 1984, Pier et al 1984). However, effective complement levels in the lungs are below those capable of killing mucoid *P. aeruginosa* strains. The conversion of *P. aeruginosa* microcolonies from a non-mucoid to a mucoid phenotype marks the transition to a more persistent state, characterized by antibiotic resistance and accelerated pulmonary decline (Pedersen et al 1992, Govan and Deretic, 1996, Lyczak et al 2002). Ineffective clearance by both innate and acquired immune responses leads to chronic infection, establishing mucoid *P. aeruginosa* as the major pathogen of CF airway disease (Lyczak et al, 2002).

One other key aspect of the pathogenesis of chronic lung infection in CF is the ability of *P. aeruginosa* to grow as a biofilm, which increases bacterial resistance to phagocytic killing and antibiotics (Meluleni et al 1995). Biofilms, which are microcolonies of bacteria normally attached to a surface and encased in a biopolymeric matrix, form within the airway and protect the bacteria from host immune factors and antimicrobial agents (Mah and O’Toole, 2001; Dunne et al 2002, Parsek and Singh, 2003). Mucoid-microcolony formation may have profound effects on the ability of the host to cope with the pathogen. Impaired phagocytosis, not capable of clearing the organism has been suggested (Krieg ey al., 1988, Meshulam et al 1982, Cabral et al 1987). As a result, the large numbers of neutrophils are attracted by inflammation mediated chemotaxis, with the ultimate consequence of lung damage directly from excessive neutrophil elastase activity and indirectly from immune system-mediated tissue damage resulting indirectly from immune complexes (Lucey et al 1985, Bruce et al 1985). Once this stage is
reached it is impossible to clear *P. aeruginosa* from the lungs. However, evidence from the analysis of explanted lungs from infected CF patients suggests that *P. aeruginosa* form mucoid aggregate biofilms, rather than surface-attached biofilm (Bjarnsholt et al 2009). In addition, mucoid and non-mucoid variants of *P. aeruginosa* often co-exist in chronically infected patients, suggesting that the adaptive behavior is not as simple as a switch of the whole population from non-mucoid to mucoid (Mowat et al 2011).

Quorum sensing (QS) is a cell density monitoring mechanism, and is a generic phenomenon described in many bacteria. Bacteria are able to sense their own immediate environment and react appropriately; however QS gives them the ability to sense their own cell density and to communicate with each other, and behave as a population instead of individual cells (Fothergill & Winstanley 2009). In *P. aeruginosa* QS has been widely studied because of its importance in the control of many virulence factors. *P. aeruginosa* uses two signaling system known as a las and rhl systems for QS. It has been suggested that QS plays a pivotal role in the initial stages of infection, but that its role in pathogenicity diminishes over time. Some studies have highlighted the accumulation of mutations within *P. aeruginosa* populations over time, potentially leading to loss of virulence (Schaber et al 2004; Smith et al, 2006). In particular, it has been proposed that lasR mutants, defective in QS, occur as an adaptation in chronic infections (D'Argenio et al, 2007), and that the QS system may have a negative impact on the organism's long-term fitness (Heurlier et al 2006). Bollinger et al. also showed that QS regulation of gene expression in *P. aeruginosa* was complex, dynamic and dependent on the growth state of the cells and nutritional factors (Bollinger et al 2001). They further suggested a role for QS products in negative regulation of gene expression in biofilm formation. There is some evidence that QS plays an important role in viability of *P. aeruginosa* in anaerobic biofilms (Winstanley et al 2009), though it
seems likely that there are two probably independent, pathways to biofilm formation in *P. aeruginosa*: QS-dependent or alginate dependent forms independent of each others existence.

Although many of the virulence factors controlled by QS (such as secreted elastase, protease, pyocyanin, cyanide, rhamnolipid) are considered to be more relevant to acute infections, and *lasR* mutants are common in CF populations of *P. aeruginosa*, both secreted virulence factors (such as pyocyanin) and QS signaling molecules can still be detected in the sputa of chronically infected CF patients, suggesting that QS may play an important role beyond the early stages of infection (Fothergill & Winstanley, 2009).

It is apparent that the acquired host immune response to mucoid *P. aeruginosa* is ineffective at eliminating this pathogen. This ineffective response most likely contributes significantly to the inflammatory process that damages the lungs of CF patients. It is hypothesized that immune responses to the alginate antigen of mucoid *P. aeruginosa* consist mostly of antibodies that fail to mediate opsonification mediated phagocytic killing of the organism: so-called nonopsonic antibodies. Although these nonopsonic antibodies are found in all humans and are not unique to CF patients, the general titres are higher in CF patients (Pier et al 1987, Ames et al 1985). CF patients clearly do produce antibodies that can mediate the opsonification mediated killing of mucoid *P. aeruginosa*, but specificity to the alginate antigen is lacking (Parad et al 1999). In an in vitro biofilm model these non-alginate antibodies have failed to demonstrate the killing of mucoid *P. aeruginosa* (Meluleni et al 1995), potentially explaining their lack of effectiveness at protecting CF patients from chronic *P. aeruginosa* infection. However in some patients there are naturally occurring opsonic antibodies to alginate generated by an unknown mechanism that seem to have a better response in fighting these alginate antigens. However vaccinations of humans with such
antigens generated from mice/ rabbit models have not consistently demonstrated protective antibodies (Pier et al 1994) demonstrating this defect in our innate immune mechanisms in fighting alginate antigens.

1.12 Epidemic strains in CF

a) Europe and worldwide

In the last two decades several CF clinics worldwide have reported the presence of transmissible *P. aeruginosa* strains (Fothergill et al 2012). *P. aeruginosa* is an organism commonly found in the environment and capable of surviving in various niches. In general, each CF patient within a cohort will acquire a different strain and the acquisition of *P. aeruginosa* in CF patients has generally been attributed to the environment, although there is little direct evidence to confirm or dispute this. Detailed analyses of several large collections of isolates from both environmental and human samples have suggested that the wider population of *P. aeruginosa* has a largely non-clonal but epidemic structure, with some dominant clones present (Wiehlmann et al 2007, Pirnay et al 2005). For example, the “clone C” strain has a worldwide distribution and has been found in CF patients and the CF environment, as well as in geographically distinct areas and associated with a wide variety of clinical outcomes (Romling et al 2005). Although CF patients normally have unrelated strains of which the original source remains unknown, initial indications of cross infection were reported by a Danish CF centre, where a *P. aeruginosa* strain identified in 1973 (low proportion) by phage-typing was isolated in 1986 in 42% of the patient population; by 1986 this particular strain was noted to be multiresistant (Pedersen et al 1986). Selective use of antibiotics and patient isolation was suggested based on this centre’s experience. The argument for isolation was strengthened by yet another Danish study whereby a winter camp of children with CF resulted in cross infection of all the attendees with a clonal
strain of *P. aeruginosa*, confirmed by pulsed field gel electrophoresis (PFGE) (Ojeniyi et al 2000). Various other epidemic strains have now been identified in CF clinics. For example, epidemic strains have been reported in several CF clinics in Australia (Armstrong et al 2003, Bradbury et al 2008). There have been at least five different epidemic strains reported in Australia, known as Australian epidemic strains (AES) 1-3 and Sydney strains S1 and S2. AES-1 and AES-2 were described in Melbourne and Brisbane respectively (Armstrong et al 2003, Bradbury et al 2008, O'Carroll et al 2004, Tingpej et al 2007). AES-1 was found in both adult and paediatric CF populations in both Melbourne and Brisbane, and resulted in higher hospitalizations and mortality among those with chronic infection (Tingpej et al 2010). AES-2 is much less common and has been associated with increased antibiotic resistance and a fall in lung function. AES-3 is a strain found in Tasmania. Patients harbouring AES-3 suffered significantly more exacerbations requiring hospitalization and increased multi-antibiotic resistance compared with other strains (Bradbury et al 2008). These strains have also been isolated in CF centres in the Czech Republic and New Zealand (Nemec et al 2010, Schmid et al 2008). Gene expression characteristics during biofilm and planktonic growth have been analysed for strains AES-1 and AES-2 showing enhanced biofilm formation and upregulated type III secretion system genes respectively (Manos et al 2009, Manos et al 2008). These virulence factors have been cited as plausible explanations for success of these strains.

There have been other transmissible strains described in Norwegian and Dutch CF centres. In 1995, in a Dutch study cross-infection after a summer camp was found to be negligible (<2%), and authors advocated free intermixing of children with and without *P. aeruginosa*. Indeed, the authors concluded that the risk of cross infection was trivial compared to the joys of a summer camp (Hoogkamp et al 1995). However another
Dutch group described the risk of transmission among CF children attending summer camps as considerable following the finding of two (type 18 and 23) subtypes of *P. aeruginosa* in previously uninfected children, following which segregation measures were instituted (Brimicombe et al 2008). Details regarding the morbidity caused by these strains or plausible reasons for transmissibility have not been reported.

A similar sequence of events has been reported from Canada where Speert et al (2002) reported an “extremely low risk” of transmission of *P. aeruginosa* between patients. Their conclusions were based on evaluation of sputum isolates from a CF clinic in Canada. Of the 157 genotypes isolated from 174 patients evaluated by PGFE, 123 were found to be unique to individual patients. A total of 34 types were shared by more than one patient; epidemiological evidence linked these individuals only in the cases of 10 siblings and 2 unrelated patients. They concluded that there was no significant evidence of cross infection or transmissibility in these patients. However two other studies have demonstrated evidence of epidemic strains in Canada. In the first study (Beaudoin et al 2010), two clonal strains of *P. aeruginosa*, type A and type B, were found to infect more than 20% of CF patients in Ontario, Canada. Four isolates of each type were subjected to further analysis. All 8 isolates expressed high levels of antibiotic resistance. Subsequently, in 2010 Aaron et al reported 25% infection with clonal strains A and B (Aaron et al 2010). Interestingly, strain A was found to be genetically similar to the Liverpool Epidemic Strain (LES) strain (see next section). Compared with patients infected with unique strains of *P. aeruginosa*, patients infected with the LES (strain A) or strain B had similar declines in lung function, but the 3-year rate of death or lung transplantation was greater in those infected with the LES. This was unequivocal evidence of presence of a well-studied epidemic strain, which demonstrated adverse
outcomes in infected patients. More recently, another transmissible strain (the Prairie Epidemic Strain, PES) has also been described in Canada (Workentine et al 2013).
b) The LES in the UK:

Evidence of an epidemic strain in UK CF clinics was first brought to light by Cheng et al. (Cheng et al 1996) at a Liverpool paediatric CF clinic. 77% of children in this clinic showed presence of isolates resistant to ceftazidime, when only a handful of these children had ever had been given ceftazidime. Using genomic fingerprinting techniques it was demonstrated that 90% of these isolates were the same strain of *P. aeruginosa*, now known as the Liverpool Epidemic Strain (LES), indicating the presence of an epidemic strain in this CF population that exhibited some antibiotic resistance.

Suppression subtractive hybridization was used to identify sequences present in the LES but absent from other strains of *P. aeruginosa* (Parsons et al 2002). This technique was then used to develop a diagnostic PCR test for identification of the strain from colonies or directly from sputum samples (Parsons et al 2002, Panagea et al 2003). Evidence for the adverse effects of chronic infection with this strain were first demonstrated by Al–Aloul (2004), who prospectively followed a cohort of 12 patients chronically infected with LES for 5 years and demonstrated that chronic infection with the LES causes increased annual loss of lung function (mean FEV1 difference between groups −4.4% (95% CI −8.1 to −0.9; p<0.02)) and poor nutrition (mean BMI 19.4 v 22.7, p<0.02).

FEV1 and BMI have been proven as important indicators of the health status in patients with CF (Al-Aloul et al 2004). Deterioration in these important predictors was the first evidence that LES adversely affects the health of CF patients. However this study only included a small number of patients at a single centre in UK. The LES had also been reported to show some unusual features not previously reported in *P. aeruginosa* strains in CF. In a study by McCallum et al, genotypic identification uncovered evidence of superinfection by the LES strain whereby the LES was able to replace previously established unique strains of *P. aeruginosa* (McCallum et al 2001). No evidence of
environmental contamination was found, but all patients became super-infected after contact with colonized individuals during inpatient stays. This phenomenon was noted to affect healthy individuals as well.

McCallum et al. (2002) reported that the LES was able to cause infection in the previously healthy parents of a patient with CF (McCallum et al. 2002). Once infected with the LES, these previously healthy individuals had hospitalization and required treatment with intravenous antibiotics repeatedly. LES has also been shown to spread from an infected individual to a pet cat (Mohan et al. 2008).

In a nationwide survey to establish the distribution of *P. aeruginosa* strain genotypes among CF patients in the UK, the LES was found to be the most prevalent strain, accounting for over 11% of isolates from over 120 hospitals (Scott & Pitt, 2004). Fluorescent amplified fragment length polymorphism (FAFLP) analysis revealed some microheterogeneity among strains of the LES genotype. This microheterogeneity may indicate the adaptations this strain has been undergoing over time to survive in the hostile environment of CF lungs. This report was the first indication to suggest that the LES was widely transmissible and that cross-infection with *P. aeruginosa* has occurred both within and widely between CF centres in England and Wales. A more recent study confirmed that the LES remains widespread amongst the UK CF community (Martin et al. 2013). Studies up to now had only demonstrated that the LES is transmissible and that chronic infection with LES could worsen the general health of the individuals. These previous studies were largely limited to the UK, and mostly from a single centre with a small number of patients. There had been no evidence from elsewhere or indicators to suggest that infection with LES leads to increased mortality. However, the study by Aaron et al. (2010), focusing on the LES-like strain in Canada, demonstrated just this. A 3-year prospective observational cohort study of adult patients cared for at
CF clinics in Ontario was carried out with regular sputum genotyping. 15% patients in this centre were found to be infected with a LES-like strain. In patients infected with the LES, the 3-year rate of death or lung transplantation was significantly greater than those infected with unique strains.

c) Other epidemic strains in the UK

Given the emerging evidence from CF centres in the UK for the presence of transmissible epidemic strain, which had adverse effects on the health of the CF population, detailed multi-centre surveys were carried out. To assess the prevalence of such strains in UK CF centres. Scott & Pitt (2004) undertook genetic analysis of sputum samples from over 120 hospitals. Although the majority of patients harbored unique strains, there was evidence of several epidemic strains. 11% of all isolates from over 15 centres in England were identified as the LES. However, other abundant strains, such as Midlands1, Manchester Epidemic Strain and clone C were identified.

**Midlands1 strain:** this was the second most prevalent strain after LES in the survey conducted by Scott & Pitt (2004). In a further study by Chambers et al. (2005) this strain was found in 30% of individuals at one CF centre in UK. However this strain was not found to have adverse effect on patient’s health compared to non-epidemic *P. aeruginosa* or to have enhanced antibiotic resistance. No further studies regarding this strain have been reported.

**Manchester strain:** Jones et al (2004) found 14% of patients with chronic *P. aeruginosa* had isolates with similar genotypes at their centre in Manchester. The shared isolates showed unusual phenotypic characteristics and resistance to a number of antipseudomonal antibiotics. This was the first report suggestive of cross-infection by a multiresistant *P. aeruginosa* strain at this centre. An 8 year prospective observational study by Jones et al (2010) demonstrated that patients infected with epidemic strains
required more intravenous antibiotic treatment requirement and hospitalization without any fall in lung function, BMI or increase in mortality.

**Other Epidemic strains:**

There have been isolated reports of epidemic strains in various geographically diverse regions of UK. For example, in Sheffield the MR strain was identified (Edenborough et al 2004), and in Leeds a colistin resistant transmissible strain was reported (Denton et al 2002). In both of these cases there was a need for increased antibiotic requirement but without any other adverse effects reported for patients.

**1.13 Spread of epidemic strains:**

Numerous studies have attempted to identify the initial source of *P. aeruginosa* in CF patients, but it still remains unknown for most patients. *P. aeruginosa* can survive for prolonged periods. Different phenotypic variants of *P. aeruginosa* exhibit different periods of survival on inanimate objects. In one study it was reported that non-mucoid strains suspended in saline could survive on dry surfaces for 24 h, whereas mucoid strains could survive 48 h or more (Doring et al 1996, Zimakoff et al 1983), and some strains such as the LES, suspended in the sputum of CF patients, can survive on dry surfaces for up to 8 days (Doring et al 1996).

*P. aeruginosa* has been recovered from both inpatient and outpatient environmental sources. Various strains exhibiting genetic variability have been isolated from immediate environment of CF patients (Doring et al 1996, Romling et al 1994), toys, baths, and hand soaps (Zimakoff et al 1983), and lung function equipments and hospital drains (Speert et al 1987). Isolation of such strains from the environment however doesn’t help to identify the source despite the matching characteristics of the strains seen in the environment and healthcare setting (Wolz et al 1989, de Soyza et al 2001, Doring et al 1996). However several other studies have not been able to detect any
environmental contamination in a centre where there was evidence of epidemic strains despite multiple cultures (McCallum et al 2001). There has been direct evidence of *P. aeruginosa* strains in cultures taken from hands of healthcare professionals and nebulizers used by patients suggestive of indirect sources of spread of such strains (Speert et al 2002, Doring et al 1991). Other potential sources of *P. aeruginosa* include whirlpools, hot tubs (Berrouane et al 2000), swimming pools, or dental equipment (Jensen et al 1997). Droplet transmission has been demonstrated by isolating *P. aeruginosa* from agar plates placed 1.25 to 3 ft from a coughing CF patient (Doring et al 1996, Zimakoff et al 1983). Air samples from CF clinics and hospital rooms have yielded contradictory results (Speert et al 1987, Wolz et al 1989) and therefore no clear conclusions can be drawn regarding environmental spread.

Evidence of patient-to-patient transmission of *P. aeruginosa* has been studied extensively. The best documentation of shared strains of *P. aeruginosa* among CF patients has been noted between siblings with CF (Grotheus et al 1988, Wolz et al 1989). There have been several reports of shared strains of *P. aeruginosa* among CF patients linked to mingling of patients in recreational camps (Fluge et al 2001, Ojeniyi et al 2000).

Panagea et al. (2005) conducted an environmental survey at the Liverpool adult CF clinic to determine the extent of environmental contamination with the LES, and to identify possible reservoirs and routes of cross-infection. Samples were collected from staff, patients and the environment commonly used by the patient (drains, bath tubs, showers, dry surfaces, respiratory equipment and air in both inpatient ward and outpatient clinic). *P. aeruginosa* strains were tested by using PCR assays specific for the LES. The LES was isolated from patients' hands, clothes and bed linen.

Environmental contamination with LES was only detected in close proximity to
colonized patients (external surfaces of their respiratory equipment, and spirometry machine tubing and chair) and was short-lived. No environmental reservoirs were found. LES was detected in the majority of air samples from inside patients' rooms, the ward corridor and the outpatient clinic. Survival of LES on dry surfaces was significantly longer than that for some other strains tested. A similar survey of the CF environment was carried out in the Manchester CF clinic, including room air sampling, and identification of individual *P. aeruginosa* by genotyping (Jones et al 2005). The typing patterns were compared with those of epidemic strains responsible for cross infection among the patients. Epidemic *P. aeruginosa* strains were isolated from room air when patients performed spirometric tests, nebulisation, and airway clearance, but were not present in other areas of the inanimate environment of the CF centre. These studies do suggest that aerosol dissemination may be the most important factor in patient-to-patient spread of epidemic strains of *P. aeruginosa* in CF centres with cross infection.

### 1.14 Controversies in morbidity due to epidemic strains

Various studies have been carried out to assess the impact of infection with transmissible strains with respect to patient morbidity. In one study, Al-Aloul *et al.* (2004) looked at two cohorts of CF patients attending the Liverpool CF service whose LES status was identified by genomic fingerprinting technique. 12 adult CF patients infected with LES were matched in 1998 for age, spirometric parameters, and nutritional state to those without and their clinical course was followed for 5 years. Patients with LES had a greater annual loss of lung function than those without (mean difference between groups 24.4% (95% CI –8.1 to 20.9; \(p=0.02\)), and by 2002 their percentage predicted forced expiratory volume in 1 second (FEV\(_1\)) was worse (mean 65.0% v 82.6%, \(p=0.03\)). Their nutritional state also deteriorated over the study period
(mean difference between groups in body mass index 20.7 (95% CI −1.2 to 20.2; 
p=0.01)), such that by 2002 they were malnourished compared with LES negative 
patients (mean BMI 19.4 v 22.7, p=0.02) (Al-Aloul et al 2004). This illustrates that 
chronic infection with the LES strain in CF patients confers a worse prognosis than 
infection with other non-LES strains. However this was a study, which was conducted 
with a small study population. The study by Chambers et al. (2005) done at a different 
CF centre in the UK demonstrated similar findings in terms of the morbidity caused by 
the LES. Patients infected with the LES exhibited enhanced antibiotic resistance, 
increased need for intravenous antibiotics, renal failure (Al-Aloul et al 2005) and 
hospitalization. These studies demonstrate that the LES is a problem in more than one 
centre in the UK. More recently there has been evidence to suggest that LES is a 
problem not just limited to the UK. There has been a Canadian study reported by Aaron 
et al. (2010) who studied 446 patients with CF. 102 were discovered to be infected with 
one of two common transmissible strains of P. aeruginosa. 15% patients in this centre 
were found to be infected with a strain genetically similar to the LES. Compared with 
patients infected with unique strains of P. aeruginosa, patients infected with the LES 
(strain A) had a rapid decline in lung function (difference in decline in percent predicted 
FEV1 of 0.64% per year [95% CI, −1.52% to 2.80% per year] and 1.66% per year [95% 
CI, −1.00% to 4.30%], respectively). The 3-year rate of death or lung transplantation 
was also greater in those infected with the LES (18.6%) compared with those infected 
with unique strains (8.7%) (adjusted hazard ratio, 3.26 [95% CI, 1.41 to 7.54]; P=.01) 
(Aaron et al 2010). This study is further evidence of the fact that the LES causes 
increased morbidity and mortality in adult CF patients. This study was conducted with a 
larger number of patients compared to the previous study, with a long period of follow 
up demonstrating the hazards of chronic infection with LES. These studies have
conclusively proved that chronic infection with LES causes a rapid decline in health
status in these individuals leading to higher healthcare needs and mortality. However,
this problem is not universal with other known epidemic strains. It has been suggested
that changes to infection control programmes in relation to frequent epidemic clones
should be based on their frequency, virulence across all age groups and mode of
acquisition (Fothergil et al 2012). The Australian epidemic strain-1 (AES-1) and AES-2
are common in CF clinics in mainland eastern Australia, but not in the environment.
A large CF clinic in Australia examined 258 P. aeruginosa isolates from 112
participants using genotyping by PFGE. Ninety-eight patients were followed up for 1
year and associations sought between infection with a frequent epidemic clone, clinical
outcome and antibiotic resistance. Four frequent P. aeruginosa epidemic clones affected
almost 50% of participants. Three of these four were associated with increased
exacerbations and hospital-admission days, but only one strain showed increased
resistance to antibiotics (Armstrong et al 2003). Patients infected with another epidemic
strain from Canada ST-439 not previously identified in patients with CF, were followed
up for a period of 3 years and there were no greater risk of hospitalization or lung
transplant. (Aaron et al 2010). Other Epidemic strains isolated in UK (Tubbs et al 2001,
2002) haven’t shown evidence of morbidity or increased mortality, which is further
evidence to support the assumption that not all epidemic strains are harmful. Hence
assertions that epidemic strains are always harmful and that measures should be
implemented to protect patients with such chronic infections do not hold true
universally.
1.15 Previous studies describing phenotypic and genotypic characteristics of the LES

It has been demonstrated in infection models that there is a big variation in the virulence characteristics of various sub-types of the LES. Four different LES subtypes displayed varied virulence in a mouse respiratory infection model (LES431, LESB65, LES400, LESB58), with LES431, which is associated with the spread of infection to the non-CF parent of a CF patient, being by far the most virulent (Carter et al 2010). It has been shown that some LES isolates, including LES431, have enhanced expression of virulence factors controlled by QS (Salunkhe et al 2005). In particular, such isolates produce high levels of pyocyanin (Fothergill et al 2007). Pyocyanin and its precursor phenazine 1-carboxylic acid (PCA) have a number of toxic effects on patients with CF. (Fothergill and Winstanley, 2009) For example, pyocyanin inhibits the beating of human respiratory cilia in vitro and induces neutrophil apoptosis and impairs neutrophil induced host defenses. Increased concentration of pyocyanin results in further reduced expression of CFTR in lung and nasal epithelium, further potentiating the genetic effects of CFTR. Certain genetic mutations within LES variants such as the LES 431 lead to an up-regulated QS regulon as well as other transcriptional changes (including transcription of AmpC beta-lactamase), characteristics that could contribute to the success of the LES. (Salunkhe et al 2005). Some phenotypic features such as ability to survive on dry surfaces for prolonged period may also explain its success.

The earliest archived isolate of the LES (named LESB58, from 1988) was genome sequenced in 2009 (Winstanley et al 2009). The main genomic differences between the LES and other strains of P. aeruginosa are due to the presence in the genome of a number of genomic islands and prophages, and it was shown that some genes within
these prophage regions play an important role in the competitiveness of the strain in a rat model of chronic lung infection. Hence prophage regions are also implicated in the success of this strain.

Adaptations typical of \textit{P. aeruginosa} during CF infections have been mentioned earlier. However, recently it has become apparent that populations of the LES harbor extensive phenotypic diversity during chronic infections in the CF lung. Phenotypic and genotypic diversity in LES populations has been studied by analysis of multiple isolates per sputum sample (Fothergill et al 2010, Mowat et al 2011). These studies have revealed extensive within-population variations in the phenotypes of the LES, including auxotrophy, hypermutability, production of pyocyanin and susceptibilities to a range of antibiotics. Each patient population is diverse but also dynamic (Mowat et al 2011).

This has important consequences with respect to the potential for adaptation (because \textit{P. aeruginosa} populations can harbor extensive diversity, enabling them to adapt quickly), and the relevance of antimicrobial susceptibility tests based on single isolates. The considerable diversity in antibiograms obtained when analyzing multiple isolates from the same CF sputum sample suggests that results based on single isolates may be flawed (Mowat et al 2011). This diversification could be important in the emergence of subtypes better able to exhibit transmissibility, which may be due to improved ability to colonise, and replace established \textit{P. aeruginosa} (superinfection), enhanced survival/persistence in the environment, or a combination of these. At the start of the project described in this thesis, it was not known whether this diversification was widespread or a particular feature of the LES. Subsequent publications suggest the former (Workentine et al 2013).

As a part of the work undertaken here, I aim to address the issue of whether diversification is a common feature of \textit{P. aeruginosa} populations in CF. In addition, I
investigate other reasons that may be contributing to the persistence of LES. Although
there have been studies proving an association between the LES and increased patient
morbidity, there has been no study proving that the LES exhibits enhanced antibiotic
resistance. In addition, there are unanswered questions regarding how chronic infection
with the LES affects an individual’s Health Related Quality of Life (HRQL). Intuitively
it may appear that chronic infection with LES should adversely affect both of these
factors, but so far it has not been studied or reported. It may also be the case that
institutions with patients chronically infected with the LES have higher economic and
healthcare resource implications; however, this has also not been reported in the past.
Since adverse effects of the LES are now known, our CF centre has instituted
segregation measures to limit its numbers and to protect patients with no previous P.
aeruginosa growth from the harmful health hazards resulting from acquisition of the
LES. It is however unknown if such segregation measures are useful in limiting the
numbers of LES infection and prevention of superinfection.

1.15 **Aims of this study.**

1. To compare the health related quality of life of LES-infected patients with those CF
patients infected with non LES *P. aeruginosa*.

3. To compare antibiotic resistance exhibited by the LES with the resistance of unique
(non LES) *P. aeruginosa* strains.

3. To determine whether extensive diversification is a common feature of *P.
aeruginosa* populations during respiratory infections in CF.

4. To analyze the healthcare costs associated with chronic infection with the LES.

5. To review the results of cohort segregation policies at the Liverpool adult CF unit in
limiting the spread of the LES strain.

1.16 Format of the thesis

The thesis contains a general introduction chapter (Chapter 1) at the start and then a
general discussion chapter (Chapter 7) at the end. The other chapters (Chapters 2 – 6)
are in the form of five studies written for publication:

- The work presented in chapter 2 has been published JR Soc Sh Rep 2012; 3:12.
  DOI 10.1258/shorts.2011.011119
- The work presented in chapter 3 has been published in Journal of Cystic
  Fibrosis, volume 11, issue 3, May 2012 173-179
- The work presented in chapter 4 has been published in Journal of Cystic
  Fibrosis, volume 12, issue 6, December 2013, pages 790-793
- The work presented in chapter 6 has been published in J R Soc Sh Rep 2013; 4:1
  DOI 10.1258/shorts.2012.012018
Chapter 2 : A Cohort Study of Health Related Quality Of Life Outcomes in Adult CF patients chronically infected With Transmissible Pseudomonas aeruginosa Strains.

Authors: Abdul Ashish, Matthew Shaw, James McShane, Martin J. Ledson, Martin J. Walshaw

2.1 Abstract:

Background: Although adult CF patients harbouring epidemic Pseudomonas aeruginosa (Psa) strains (particularly the Liverpool epidemic strain, LES) have a rapid decline in physical markers of disease severity and increased mortality compared to other CF patients, it is not known if they also have a poorer health-related quality of life (HRQoL).

Methods: 157 clinically stable adult CF patients (93 with transmissible Psa [median age 26 years (IQ range 26-31), 38 females]; 44 with unique Psa [22 (20-28), 16 females], and 20 without Psa infection [21(19-29), 9 females]) completed a validated CF HRQoL questionnaire (CFQ-UK, Teen/Adult revised, version 1) prior to being seen by a health professional at a routine CF clinic. Those with Burkholderia cepacia and atypical CF were excluded. Kruskal–Wallis and Mann Whitney’s U tests were used to analyse the data.

Results: Patients infected by transmissible Psa strains had worse physical functioning, respiratory symptoms, treatment burden, vitality, role, health perception and emotion than those with unique Psa strains(p<0.01), and significantly poorer physical functioning, respiratory symptoms, treatment burden, body-image, weight, role, and emotion than those without any Psa infection (p<0.05). Furthermore, in a matched cohort of 39 patients, those with LES infection reported significantly worse physical
functioning, treatment burden, respiratory symptoms and health perception than those with unique Psa infection (p<0.02).

Conclusion: Chronic infection with transmissible Psa strains, particularly LES, confers a worse quality of life in adult CF patients. Coupled with the established poorer clinical outcome, this reinforces the need to prevent the spread of such strains in CF community.

2.2 Introduction:
Cystic fibrosis (CF) is the commonest potentially lethal inherited disease in the Western World. Although it is incurable, survival in CF has improved over the years and current epidemiological data suggest that the average life expectancy of an individual born with CF in the UK is now 38 years. (1833 UK 2008) However, this is at the expense of a high treatment burden, particularly in those chronically infected with Pseudomonas aeruginosa (Psa), which is known to confer, increased morbidity and mortality (Henry et al 1992, Kerem et al 1990) and may therefore diminish their quality of life (QoL).

The WHO has defined health as “a state of complete physical, mental and social well being and not merely the absence of disease or infirmity” (WHOQOL 1995), and therefore, in CF managing the patient’s psychological and psycho-social wellbeing, which influence their QoL, is as important as managing the physical aspects of their disease.

Health-related quality of life (HRQoL) questionnaires can describe health outcomes in ways that are meaningful to patients and families as well as to healthcare professionals, and recently a disease-specific HRQoL questionnaire (the CFQ-US) has been developed for patients with CF, and a UK version of this (the CFQ-UK) has been validated for use without the loss of its psychometric properties. (Quittner et al 2005)
Although several studies have looked at QoL in CF, few have investigated how an individual patient’s disease state, particularly infection with Psa, may impact on this. Recently, transmissible Psa strains have been identified, the most important of which is the Liverpool Epidemic Strain (LES) (Cheng et al 1996) now widespread throughout UK CF centres (Scott & Pitt, 2004), which has also been reported in Canada (Aaron et al 2008). LES is associated with a worse clinical outcome (Al-Aloul et al 2004). We have a high incidence of LES Psa infection in CF patients attending our adult clinic: we were therefore interested to look at the effect of Psa infection, and in particular that due to LES, on the quality of their lives and used the CFQ-UK for this purpose.

2.3 Methods:

All 204 patients in a clinically stable state (i.e. no exacerbation within the previous 4 weeks) attending a routine outpatient visit between August 2009 and March 2010 formed the potential study population. Those with atypical CF or chronically infected with Burkholderia cepacia complex were excluded. Of the remaining 168 patients, 11 (5%) refused to take part. Following informed consent, 157 patients completed the CFQ-UK (Teen/Adult revised, version 1) (Quittner et al 2005) prior to being seen by a healthcare professional. Clinical details (sputum microbiology, lung function, nutritional state, and diabetes mellitus) were retrieved from the case records to aid subsequent data analysis. The regional ethics committee approved this study.

(Study Ref no -09/H1013/52: Oldham, Tameside & Glossop & Salford & Trafford Research Ethics Committees)
2.3.1 Psa Infection state

All patients submit microbiological samples at every clinic visit/inpatient stay: chronic infection with Psa is defined by at least 3 positive sputum samples within a 6-month period. (Hoiby et al 2000) Our unit has pioneered Psa genotyping methodology in CF, and we regularly genotype the Psa isolates from our patients to aid cross infection control measures within the clinic: those without known LES infection undergo genotyping using PCR every 3 months to identify unique and other transmissible Psa strains (Fothergill et al 2008), whilst those infected with LES undergo a genotypic check (using PCR and primers PS21 & F9) (Smart et al 2006) on a yearly basis. Using this system, we are aware of the Psa genotypes infecting our CF patients at all times.

Based on this, we divided the surveyed patients into 3 groups: 93 with LES (all infected prior to transfer from the paediatric sector), 44 with other Psa strains (43 infected prior to transfer from the paediatric sector), and the remaining 20 without Psa infection or with persistently negative cultures (6 cases had undergone successful Psa eradication since transfer from the paediatric sector) (Table 2.1).
Table 2.1: Total study population demographics (n=157)

<table>
<thead>
<tr>
<th></th>
<th>Psa Strain</th>
<th>No Psa Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LES</td>
<td>Other</td>
</tr>
<tr>
<td>Number of patients</td>
<td>93</td>
<td>44</td>
</tr>
<tr>
<td>Females (%)</td>
<td>38 (51)</td>
<td>16 (38)</td>
</tr>
<tr>
<td>Median age, years (IQR)</td>
<td>26 (26-31)</td>
<td>22 (20-28)</td>
</tr>
<tr>
<td>Mean FEV1% (SD)</td>
<td>65 (23)</td>
<td>69 (23)</td>
</tr>
<tr>
<td>Mean BMI (SD)</td>
<td>21.7 (3.5)</td>
<td>22.6 (3.6)</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>46 (49)</td>
<td>13 (29)</td>
</tr>
<tr>
<td>Enteral feed</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>TIVAD (%)</td>
<td>45 (48)</td>
<td>8 (18)</td>
</tr>
</tbody>
</table>
2.3.2 Statistical analysis and patient matching

The Kruskal-Wallis test was used to determine differences between the 3 groups on all 12 CFQ-UK domains, and where significant differences were noted individual comparisons between groups were made using the Mann-Whitney U test.

In our initial unmatched cohort although there were no significant differences in age or FEV1 between groups, patients chronically infected with LES had a higher disease burden as evidenced by more with CFRD (Cystic Fibrosis Related Diabetes Mellitus), TIVAD (Totally Indwelling Venous Access Device) implantation and the use of enteral feeding (all p<0.005 compared to other groups). To overcome this, using all available patient data we developed a multivariate logistic regression model and generated a propensity score for LES group membership (Blackstone et al 2002, Kuss, O 2002) in order to allow matching with patients infected with other Psa strains. A full nonparsimonious model was developed that included FEV1, BMI, age and gender as independent variables and treatment category as the dependent variable. The goal is to balance recorded patient characteristics between treatment groups by incorporating everything recorded that may relate to either systematic bias or simply bad luck.

The regression model had an appropriate fit (Hosmer–Lemeshow goodness-of-fit $\chi^2$ 12.4 and p=0.13) and acceptable discrimination (c statistic, 0.78) indicating an acceptable ability to differentiate between patients with or without LES. We then used a macro (available at: http://www2.sas.com/proceedings/sugi29/165-29.pdf) to perform propensity-matching. Before matching, the median propensity scores for patients with and without LES were 0.70 and 0.66, respectively (Wilcoxon signed-rank test p=0.036). After matching, the the median propensity scores for patients with and without LES were 0.67 and 0.66, respectively (Wilcoxon signed-rank test p=0.81)
Using this technique we were able to match 39 patients chronically infected with LES with 39 patients infected with other Psa strains. We did not attempt to match LES patients with those without Psa since Psa infection per se is known to confer a worse morbidity and mortality.

A p<0.05 was considered to be significant. All statistical analysis was performed using SAS for Windows Version 8.2.

2.4 Results:

The demographic details and clinical characteristics of the 157 patients who completed the CFQ–UK questionnaire are given in Table 2.1. Patients chronically infected with LES had a higher disease burden compared to those with other Psa strains or no Psa infection.

Using the Kruskall–Wallis test, comparison across all 3 groups of patients produced significant differences in 9 of the 12 HRQoL domains. Individual comparisons between groups using the Mann-Whitney U test across these 9 domains revealed that patients harbouring LES infection had significantly worse physical functioning, respiratory symptoms, treatment burden, vitality, role, health perception and emotion than those with unique Psa strains, and significantly poorer physical functioning, respiratory symptoms, treatment burden, body-image, weight, role, and emotion than those without any Psa infection. However patients infected with unique Psa strains only reported a worse perception of body image than those with no Psa infection (see Table 2.2).

Table 2: Difference in HRQoL* domains between LES infection, other infection and no Psa infection groups: Mann-Whitney U test [median score (IQR)] (*higher scores indicate better outcomes) – Unmatched groups Psa
<table>
<thead>
<tr>
<th>Microbiology</th>
<th>Other Psa</th>
<th>No Psa</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical function</td>
<td>79 (58-96)</td>
<td>75 (56-94)</td>
<td>NS</td>
</tr>
<tr>
<td>Role</td>
<td>83 (58-100)</td>
<td>83 (62-100)</td>
<td>NS</td>
</tr>
<tr>
<td>Vitality</td>
<td>58 (50-75)</td>
<td>58 (33-91)</td>
<td>NS</td>
</tr>
<tr>
<td>Emotion</td>
<td>80 (66-93)</td>
<td>83 (63-93)</td>
<td>NS</td>
</tr>
<tr>
<td>Body image</td>
<td>78 (55-100)</td>
<td>100 (100-100)</td>
<td>0.008</td>
</tr>
<tr>
<td>Respiratory symptoms</td>
<td>67 (50-83)</td>
<td>77 (67-86)</td>
<td>NS</td>
</tr>
<tr>
<td>Treatment burden</td>
<td>67 (55-76)</td>
<td>72 (33-83)</td>
<td>NS</td>
</tr>
<tr>
<td>Health perception</td>
<td>67 (55-78)</td>
<td>67 (0-89)</td>
<td>NS</td>
</tr>
<tr>
<td>Weight</td>
<td>100 (67-100)</td>
<td>100 (100-100)</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LES</th>
<th>Other Psa</th>
<th>No Psa</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>75</td>
<td>75</td>
<td>0.03</td>
</tr>
<tr>
<td>75</td>
<td>83</td>
<td>83</td>
<td>0.04</td>
</tr>
<tr>
<td>50</td>
<td>58</td>
<td>58</td>
<td>NS</td>
</tr>
<tr>
<td>73</td>
<td>83</td>
<td>83</td>
<td>0.05</td>
</tr>
<tr>
<td>67</td>
<td>100</td>
<td>100</td>
<td>0.0009</td>
</tr>
<tr>
<td>55</td>
<td>77</td>
<td>77</td>
<td>0.0002</td>
</tr>
<tr>
<td>55</td>
<td>72</td>
<td>72</td>
<td>0.01</td>
</tr>
<tr>
<td>55</td>
<td>67</td>
<td>67</td>
<td>NS</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LES</th>
<th>Other Psa</th>
<th>No Psa</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>75</td>
<td>75</td>
<td>0.0003</td>
</tr>
<tr>
<td>75</td>
<td>83</td>
<td>83</td>
<td>0.01</td>
</tr>
<tr>
<td>50</td>
<td>58</td>
<td>58</td>
<td>0.008</td>
</tr>
<tr>
<td>73</td>
<td>80</td>
<td>80</td>
<td>0.02</td>
</tr>
<tr>
<td>67</td>
<td>77</td>
<td>77</td>
<td>NS</td>
</tr>
<tr>
<td>55</td>
<td>67</td>
<td>67</td>
<td>0.002</td>
</tr>
<tr>
<td>55</td>
<td>67</td>
<td>67</td>
<td>0.01</td>
</tr>
<tr>
<td>55</td>
<td>66</td>
<td>66</td>
<td>0.01</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>100</td>
<td>NS</td>
</tr>
</tbody>
</table>
Furthermore, in the matched cohort of 39 patients (see Table 2.3), those with LES infection reported significantly worse physical functioning, treatment burden, respiratory symptoms and health perception than those with unique Psa infection (see table 2.4), and 5 further domains (role, vitality, body image, and emotional and social functioning) approached statistical significance.

Table 2.3: Univariate Demographics in LES infected and other Psa infected - Matched Group

<table>
<thead>
<tr>
<th></th>
<th>Other Psa infection (n=39)</th>
<th>LES infection (n=39)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female (%)</td>
<td>14 (37)</td>
<td>11 (28)</td>
<td>0.36</td>
</tr>
<tr>
<td>Age (years)</td>
<td>28 (8)</td>
<td>28 (9)</td>
<td>0.66</td>
</tr>
<tr>
<td>BMI (SD)</td>
<td>22.6 (3.6)</td>
<td>22.3 (3.1)</td>
<td>0.80</td>
</tr>
<tr>
<td>FEV1 (SD)</td>
<td>71 (22)</td>
<td>69 (21)</td>
<td>0.77</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>11 (28)</td>
<td>18 (46)</td>
<td>0.15</td>
</tr>
<tr>
<td>Enteral feeding</td>
<td>1</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>TIVAD (%)</td>
<td>12 (31)</td>
<td>21 (53)</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Continuous variables are shown as mean (standard deviation)
Categorical variables are shown as a number (percentage)
Table 4: HRQoL outcomes* in LES infected and Other Psa infected Matched Groups: Mann-Whitney U test

<table>
<thead>
<tr>
<th>QoL Dimension</th>
<th>Other Psa infection (n=39)</th>
<th>LES infection (n=39)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical Function</td>
<td>84 (63 – 100)</td>
<td>72 (33 – 88)</td>
<td>0.02</td>
</tr>
<tr>
<td>Role</td>
<td>84 (67 – 100)</td>
<td>76 (50 – 92)</td>
<td>0.06</td>
</tr>
<tr>
<td>Vitality</td>
<td>59 (50 – 75)</td>
<td>51 (33 – 67)</td>
<td>0.06</td>
</tr>
<tr>
<td>Emotional Function</td>
<td>81 (67 – 93)</td>
<td>74 (53 – 93)</td>
<td>0.07</td>
</tr>
<tr>
<td>Social Function</td>
<td>79 (61 – 83)</td>
<td>68 (50 – 83)</td>
<td>0.07</td>
</tr>
<tr>
<td>Body Image</td>
<td>89 (56 – 100)</td>
<td>68 (44 – 100)</td>
<td>0.09</td>
</tr>
<tr>
<td>Eating</td>
<td>100 (89 – 100)</td>
<td>100 (78 – 100)</td>
<td>0.16</td>
</tr>
<tr>
<td>Treatment Burden</td>
<td>68 (56 – 78)</td>
<td>57 (44 – 67)</td>
<td>0.02</td>
</tr>
<tr>
<td>Health Perception</td>
<td>68 (56 – 89)</td>
<td>57 (33 – 78)</td>
<td>0.01</td>
</tr>
<tr>
<td>Weight</td>
<td>100 (67 – 100)</td>
<td>100 (33 – 100)</td>
<td>0.36</td>
</tr>
<tr>
<td>Respiratory Symptoms</td>
<td>73 (50 – 83)</td>
<td>62 (44 – 72)</td>
<td>0.006</td>
</tr>
<tr>
<td>Digestion</td>
<td>89 (67 – 100)</td>
<td>89 (67 – 100)</td>
<td>0.70</td>
</tr>
</tbody>
</table>

* Higher scores indicate better outcomes
Variables quoted as median (inter-quartile range)
2.5 Discussion:

Although it is known that age, sex, FEV1, nutritional state, the use of indwelling venous devices, pulmonary exacerbations and the presence of diabetes can adversely affect the QoL in CF patients (Gee et al 2003, Gee et al 2005, Abbott et al 2009), little work has been done on the effect of chronic infection in this group. Studies looking at B cepacia infection have given variable results. Duff (Duff et al 2002) showed that segregation from other patients caused feelings of isolation, anger and of being a microbiological “leper” but did not use objective QoL assessment tools, and although Gee (Gee et al 2005) did not demonstrate any excess effect of B cepacia infection compared to other organisms, no details of disease severity were recorded and the patient numbers were small.

As regards Psa infection, whilst Goldbeck (2007) suggested that new infection had an independent negative effect on QoL, the study was small and did not achieve statistical significance. Although previous studies addressing the effect of chronic Psa colonisation have failed to show any adverse effects, the study by Britto et al (2004) had poorly matched groups, used a generic QoL questionnaire (sf-36) and only a third of the participants were adults, and the study by Havermans et al (2009) in 57 adult CF patients did not use patient matching and no information regarding Psa strain types was provided.

To our knowledge this is the first study evaluating the health-related quality of life in a substantial number of Psa-infected adult CF patients including those chronically infected with the most important such transmissible strain, LES. For the first time, we have shown that infection with LES confers a worse quality of life in CF than infection with other Psa strains and those without any Psa, and this was confirmed in matched groups of patients. This is an important finding that needs to be heeded by the CF healthcare community.
LES first came to light in 1996 at our local paediatric CF centre (Cheng et al 1996), and is now widespread throughout UK CF clinics (Scott & Pitt, 2004). It has also been reported at a CF centre in Canada. (Aaron et al 2008) It is highly transmissible – it can super-infect patients already possessing other Psa strains, (McCallum et al 2001) can spread to non-CF relatives (McCallum et al 2002), and also cross-infect other species (Mohan et al 2008). We have a large cohort of such patients, mainly inherited from the paediatric sector and have already shown that these patients have a poorer prognosis (Al-aloul et al 2004) and increased treatment burden (Ashish et al 2010) than other (matched) patients. It is therefore not surprising that these individuals also have a poorer quality of life.

Furthermore, LES-infected patients not only scored more poorly in those domains assessing physical well being (physical function, treatment burden and respiratory symptoms) but also in those assessing psychological functioning (vitality, emotion, health perception and body image). The impairment of physical domain QOL indicators are expected as LES is known to cause a more rapid decline in lung function, weight, and increase the need for IV antibiotics, triggering the need for extra therapy which in turn results in a higher treatment burden. However, the poorer scores in the psychological domain indicators suggest that LES has a more profound effect on these individuals than that expressed by physical deterioration alone. This combination of poor perceived physical parameters (poor physical function, treatment burden, weight, and respiratory symptoms scores) and higher psychological burden (poor vitality, emotion, health perception and body image scores) in patients with LES may result in an overall worse HRQoL compared to those infected with other Psa strains or without Psa infection.

However, the cohort of LES-infected patients in our study were older and had poorer pulmonary function and had a higher disease burden as evidenced by higher incidence of CFRD, those with
TIVAD’s and needing enteral feeds than the remainder, suggesting that at least some of these differences in quality of life could be due to this. It was for this reason that we compared matched groups, obtained using a validated statistical propensity scoring system. In this matched group of patients infected with Psa strains, those with LES still demonstrated a significantly worse perception of physical (physical function, treatment burden, respiratory symptoms) and psychological (health perception) well being compared to patients with other Psa strains. Furthermore, although other aspects of psychosocial well being (role, vitality, emotional function, social function & body image) failed to achieve statistical significance, there were strong trends, indicating that these changes are a true reflection of the patients’ LES infection status. It was for this reason that we opted not to use alpha correction (e.g. Bonferroni) for group matching since the differences between the matched groups (without correction) were close to the level of significance: this is highly unlikely to be a chance occurrence.

In contrast, patients infected with other Psa strains only had an altered body image compared to patients without Psa infection, suggesting that acquisition of sporadic Psa may not significantly impact on the psychological well being of the individual.

It has already been shown that depression and anxiety are more common in CF (Riekert et al 2007) and can adversely affect their QoL: anxious patients report more respiratory symptoms, have a poorer perception of their health and treatment burden, and poorer social and emotional functioning, whilst those with depression show a poorer body image, eating disturbances and emotional functioning. (Haverman et al 2008) Although our study was not designed to address the prevalence of anxiety and depression among our patients, it may be that these factors also contribute to the poorer QoL outcomes in patients infected with epidemic strains and this merits further study.
This large cross-sectional QoL study adds further evidence to the poor outcomes associated with chronic infection with transmissible Psa strains in CF: those harbouring LES reported worse scores across most HRQoL domains compared to patients without such infection.

There are limitations to our study: we only looked at one centre with a high prevalence of one transmissible Psa strain (LES) and used a cross-sectional design, and it may be that the results cannot be generalised to other transmissible Psa strains or the CF population at large. Nevertheless, LES is the most prevalent transmissible strain in the UK and has already spread to units elsewhere, so our findings will be of relevance to many CF clinics. Also, this is the largest study of the measurement of QoL in adult CF patients, using a new validated tool, which is increasingly being employed in the holistic assessment of this chronic disease group. Although longitudinal studies with repeated QoL measurements before and after chronic infection with such transmissible epidemic strains are needed to address their true effect on QoL in CF patients, our study does highlight the physical as well as psychosocial limitations faced by patients infected with such epidemic strains.

Prevention of cross infection with transmissible strains is essential to ensure better physical and psychological outcomes for these patients, and such strategies should be adopted by all CF centres.

Contributorship statement

MJW designed the study and edited the manuscript. AA conducted the study and wrote the draft of the manuscript. MS, JM carried out the statistical analysis and edited the manuscript. MJL edited the manuscript.
Acknowledgements

We thank Dr Alexandra Quittner for allowing us to use CFQ-UK and providing us with all the necessary software required to use the questionnaire.
Chapter 3 Extensive diversification is a common feature of *Pseudomonas aeruginosa* populations during respiratory infections in cystic fibrosis

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3.1 INTRODUCTION

Chronic lung infections with *Pseudomonas aeruginosa* are the most common cause of the morbidity and mortality associated with cystic fibrosis (CF). Such infections can persist for long periods, during which *P. aeruginosa* adapts by accumulating mutations that lead to phenotypic adaptations, such as the switch to a mucoid phenotype. It has been clear that mutations can lead to populations of *P. aeruginosa* composed of multiple clones with differing antimicrobial susceptibility profiles. (Foweraker et al 2009, Fothergill et al 2010) In a previous study, (Mowat et al 2011) we analyzed *P. aeruginosa* sputum populations from ten CF patients each infected with an important transmissible strain, the Liverpool Epidemic Strain (LES) (Winstanley et al 2009), which is widespread in the UK and has also been reported in North America (Fothergill et al 2012). We showed, based on 15 different phenotypic and genotypic characteristics, that LES populations were highly diverse and dynamic during CF infections (Mowat et al 2011).

Other groups have used genome sequencing or transcriptomics approaches to study sequential isolates from infections of CF patients, and demonstrated the accumulation of particular
mutations that indicate adaptation to the CF lung in terms of altered or reduced virulence, as well as antimicrobial susceptibility (Smith et al 2006, Brago et al 2009, Huse et al 2010). A recent study of strain DK2, infecting multiple patients in Denmark, presented evidence suggesting that after the accumulation of mutations during the early stages of infection, a homogeneous population of DK2 emerged (Cramer et al 2011). This appears to contradict our study with the LES, suggesting that the maintenance of extensive diversity may be a strain-specific feature of infections with the LES. However, similar analyses using the widely distributed Clone C and PA14 strains indicated that diversity may be dependent upon the emergence of hypermutators amongst the P. aeruginosa population (Yang et al 2011).

In this study, we examined the diversity of P. aeruginosa populations in a set of five adult CF patients each chronically infected with a different non-LES strain of P. aeruginosa. We analysed sets of 40 isolates of P. aeruginosa from each of two sputum samples per patient for nine characteristics, including hypermutability, and susceptibility to commonly used antimicrobials. In order to test whether extensive diversification is a feature unique to the LES, or common to P. aeruginosa infections of CF patients in general, we compared LES and non-LES populations from matched chronically infected adult CF patients using the same set of characteristics.

3.2 MATERIALS AND METHODS

3.2.1 Patients and samples

Sputum samples were collected for routine diagnostic purposes from adult CF patients (CF20-CF24; Table 3.1) known to be chronically infected (> 5 years) with different non-LES strains of P. aeruginosa in 2009-2010. Strains were genotyped using an ArrayTube system (Wiehlmann et al 2007) and identified according to the hexadecimal code generated by this method as genotypes 2F82 (CF21), 2C1A (CF22; Midlands 1 strain (Fothergill et al 2008), 0F1A (CF23),
C80A (CF24) and AF9A (CF25). Samples taken during periods of exacerbation were subdivided into two categories: beginning of an exacerbation (acute 1), before intravenous antibiotic treatment had commenced; typically 3-7 days after admission (acute 2). Exacerbations were defined by a clear set of criteria as described previously (Goss et al 2007). For each patient included in this study, one acute 1 and one acute 2 sample was used. The five patients chronically infected with non-LES strains were matched with five LES-infected patients. All patients had been infected with *P. aeruginosa* for > 5 years (Table 1). Comparison data for LES-infected patients was taken from a previous study (Mowat et al 2011). Patients with similar age, lung function (FEV$_1$) and BMI were selected. We had difficulty in exactly matching non-LES infected patients to LES-infected patients in all clinical characteristics. In our experience patients infected with non-LES strains of *Psa* have less aggressive disease, preserved clinical characteristics and better overall outcome. Hence, patients infected with non-LES strains for > 5 years in general had better lung function when compared to patients infected with the LES. Even in an institution with caters for approximately 250 adult CF patients we could not recruit non-LES infected patients with exactly matching clinical characteristics to LES-infected counterparts. This study was approved by the Local Research Ethics Committee (REC reference 08/H1006/47).
Table 3.1. Patient details and phenotypic characteristic exhibited by sputum isolates from patients chronically infected with *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Patient characteristics&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total</th>
<th>Mutations (%)</th>
<th>Antibiotic resistance (%)</th>
<th>Colony morphology type (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, Age (y), FEV&lt;sub&gt;1&lt;/sub&gt; (%), BMI, n&lt;sup&gt;a&lt;/sup&gt; Sub</td>
<td>HM, Aux</td>
<td>TOB, COL, CEF, CIP, MER, TAZ, GM, GNMS, Mtr, MW, StN, MS, RM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-LES:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF20 M</td>
<td>26</td>
<td>41</td>
<td>23</td>
<td>80</td>
</tr>
<tr>
<td>CF21 M</td>
<td>30</td>
<td>43</td>
<td>15</td>
<td>80</td>
</tr>
<tr>
<td>CF22 M</td>
<td>21</td>
<td>38</td>
<td>16</td>
<td>80</td>
</tr>
<tr>
<td>CF23 M</td>
<td>21</td>
<td>38</td>
<td>21</td>
<td>80</td>
</tr>
<tr>
<td>CF24 M</td>
<td>22</td>
<td>54</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>All (Mean)</td>
<td>24</td>
<td>43</td>
<td>19</td>
<td>400</td>
</tr>
<tr>
<td>LES:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF3 F</td>
<td>22</td>
<td>66</td>
<td>28</td>
<td>80</td>
</tr>
<tr>
<td>CF4 M</td>
<td>33</td>
<td>45</td>
<td>19</td>
<td>80</td>
</tr>
<tr>
<td>CF7 F</td>
<td>24</td>
<td>40</td>
<td>17</td>
<td>80</td>
</tr>
<tr>
<td>CF8 F</td>
<td>27</td>
<td>39</td>
<td>18</td>
<td>80</td>
</tr>
<tr>
<td>CF10 F</td>
<td>24</td>
<td>40</td>
<td>17</td>
<td>80</td>
</tr>
<tr>
<td>ALL (Mean)</td>
<td>26</td>
<td>46</td>
<td>20</td>
<td>400</td>
</tr>
</tbody>
</table>
Abbreviations: FEV₁, Forced Expiratory Volume in 1 s; BMI, body mass index; HM, hypermutable phenotype; Aux, auxotrophy; TOB, tobramycin; COL, colistin; CEF, ceftazidime; CIP, ciprofloxacin; MER, meropenem; TAZ, tazobactam / pipericillin; GM, Green mucoid; GNMS, Green Non-mucoid smooth; Mtr, Mucoid transparent; MWO, Mucoid white opaque; RM, Red mucoid; SNMS, Straw coloured Non-mucoid smooth.

* nSub indicates the total number of different sub-types for each set of 80 isolates.
3.2.2 Microbiology and phenotypic/genotypic tests

Sputum samples were collected from adult CF patients at the Regional Adult Cystic Fibrosis Unit in Liverpool. These represented routine samples taken for diagnostic purposes, and clinicians determined the antimicrobial therapy. For each patient, two samples were taken: the first at the beginning of the exacerbation, before intravenous antibiotic treatment had commenced and the second at the end of the exacerbation when symptoms had resolved.

Exacerbation was defined by a set criteria, including drop in FEV1 (forced expiratory volume in 1 second), increased sputum or discoloration, temperature and increased cough (Goss & Burns, 2007). All of the patients studied were chronically infected with the LES. Sputum was treated with an equal volume of Sputasol (Oxoid), incubated at room temperature with shaking at 200 r.p.m. for 15 min, and then cultured on Pseudomonas selective agar with CN supplement (Oxoid). Forty single colonies were isolated per sputum sample, ensuring that each different colony morphology type was proportionately represented. Each of these 40 isolates was confirmed as *P. aeruginosa* LES using PCR tests for the amplification of the *P. aeruginosa* oprL gene (De Vos et al., 1997) and the two LES markers, PS21 (Parsons et al., 2002) and LESF9 (Smart et al., 2006), before being subjected to a range of phenotypic tests.

**PCR amplification assays:** PCR amplifications were carried out in a total volume of 25 ml. For each reaction, 1.25 U GoTaq polymerase (Promega), 16TaqMaster (Helena Biosciences), 300 nM each oligonucleotide primer (Sigma-Genosys; Table 16 Taq buffer, 2.5mM MgCl and 100mM nucleotides (dATP, dCTP, dGTP, dTTP) was used along with 1 ml DNA from boiled suspensions of colonies. PCR amplification was carried out for 30 cycles of 95 uC (1 min), the chosen annealing temperature (2 min) and 72 uC (2 min). Following this, a final extension step
of 72 uC for 10 min was carried out. In the case of LES prophages 2, 3, 4 and LESGI-5, additional PCR assays were performed using primers designed for the flanking regions of the insertions, and therefore only if the insertion was not present would the expected band be observed. For PCR amplification to detect free phages directly from sputum samples, the sputum was first treated with Sputasol, diluted (1 : 10) with sterile distilled water, filter-sterilized (0.2 mm) and DNase I treated as follows: addition of 3.5 U DNase I (Roche), 3.5 ml 106DNase I buffer and 3 ml sterile distilled water, followed by incubation for 15 min at room temperature. To stop the reaction 3.5 ml 25 mM EDTA (pH 8.0) was added and the solution was heated to 65 uC for 10 min. Following this, PCR amplification was carried out using 5 ml of the phage preparation. Each PCR amplification reaction was replicated twice.

**Colony morphology:** Colony morphology was assessed on Columbia agar plates (Oxoid), with each isolate assigned to one of six morphotypes: (1) green, non-mucoid and smooth (GNMS); (2) mucoid and transluscent (MTr); (3) mucoid and green (MG); (4) mucoid, white and opaque (MWO); (5) straw coloured, non-mucoid and smooth (StNMSm); (6) red/brown pigmentation and mucoid (RM).

**Antibiotic susceptibility:** Using current BSAC guidelines (Andrews et al 2009), antibiotic susceptibilities were determined by disk diffusion tests for six antibiotics that are commonly used to treat CF patients, namely: ceftazidime, colistin, ciprofloxacin, meropenem, tazobactam/piperacillin and tobramycin. The following amounts were used: 85 mg tazobactam/piperacillin, 10 mg meropenem, 10 mg tobramycin, 5 mg ciprofloxacin, 30 mg ceftazidime and 25 mg colistin sulphate (all from Oxoid). The sizes of the zones of inhibition (mm) were also recorded. Isolates were assigned as phenotype resistant or sensitive for all
antibiotics. For ciprofloxacin and meropenem an extra phenotype of intermediate resistance was included.

**Other phenotypic traits:** Auxotrophy was determined by testing the ability of isolates to grow on glucose M9 media and hypermutability was assessed by determining spontaneous mutation rates on Luria agar containing rifampicin (Sigma) (300 μg / ml) following overnight growth in Luria broth (Oliver et al 2000).

Each trait was assigned a number code (1-6 for morphology; 1 or 2 for all other tests with the exception of susceptibility to ciprofloxacin or meropenem [1-3]). A haplotype was defined as a specific combination of these trait values. For example, LES haplotype 1 has the code 1 for colony morphology, 2 for hypermutability, 2 for auxotrophy, 1 for susceptibility to tobramycin, 1 for susceptibility to colistin, 3 for susceptibility to ceftazidime, 2 for susceptibility to ciprofloxacin, 3 for susceptibility to meropenem, 3 for susceptibility to tazobactam/piperacillin. From the numerical profiles generated, the relatedness of each isolate was calculated using the eBurst algorithm available at the site http://eburst.mlst.net.

### 3.2.3 Statistical analyses

In order to estimate the population differentiation between patients, between samples within patients and between isolates within samples, we performed hierarchical analysis of variance using the ade4 package in R (r-project.org). We define a sub-type as a specific combination of trait values, and the phenotypic distance between a pair of sub-types as the number of traits that differed between them. Sub-type diversity was calculated as the probability of two randomly
picked isolates being the same sub-type based on the sub-type frequencies within a sample. Sub-type sharing between a pair of samples was calculated as the probability of a randomly picked isolate from each sample sharing the same sub-type. This sub-type sharing probability was normalised using a logit transform (log[p/(1-p)]).

3.3 RESULTS

3.3.1 Overall *P. aeruginosa* phenotypic diversity

High phenotypic diversity was apparent in the *P. aeruginosa* populations from each of the chronically infected CF patients. Based on the nine phenotypic traits analyzed, 400 isolates taken from 10 sputum samples from non-LES-infected patients comprised a total of 75 distinct phenotypic sub-types. The number of sub-types present within each patient is shown in Table 3.1, along with the frequency of each phenotypic characteristics measured. When the data from LES-infected and non-LES-infected patients were analyzed together, there were 152 sub-types of *P. aeruginosa* present in total, of which 97 were found only in LES-infected patients, 76 were found only in non-LES-infected patients, and 21 were shared between the two groups. There was a mean of 10 sub-types per set of 40 isolates for samples from non-LES-infected patients, compared to 10 for samples from LES-infected patients.

3.3.2 Sub-type variation in individual patients

Hierarchical analysis of variance was performed on LES and non-LES groups separately to estimate the proportion of phenotypic variation attributable to (i) variation among patients, (ii) variation among samples within patients, and (iii) variation among isolates within samples. In both groups, the greatest contribution to overall diversity was due to phenotypic diversity between isolates within samples (LES-infected patients, 83%; non-LES infected patients, 81%).
Overall, the LES and non-LES infections exhibited equivalent levels of diversity within a single sputum sample (Fig 3.1a) and an equivalent degree of correlation between different sputum samples taken from the same patient (Fig 3.1b) & (Fig 3S).
Figure 3.1. Comparisons between non-LES and LES samples.

(a) Diversity within LES and non-LES samples is calculated from the probability of two isolates within a sample being of the same sub-type ($p_{\text{share}}$). Circles and triangles represent samples from the beginning and end of an exacerbation, respectively. No statistically differences in diversity were observed, either between non-LES and LES samples or between samples from the beginning versus end of an exacerbation.

(b) Similarity between pairs of samples taken from the same patient. Similarity is calculated from the probability of two isolates from different samples being of the same haplotype ($p_{\text{share}}$). Circles represent pairs of samples taken at the beginning and end of an exacerbation and, hence, less than one month apart. Triangles represent pairs of samples taken at the beginning of separate exacerbations more than two months apart. Dotted lines indicate the mean level of similarity between samples from different patients within either the non-LES or the LES groups.

### 3.3.3 Frequency of phenotypic traits
There were differences between the groups (samples from non-LES-infected patients and from LES-infected patients) with respect to some of the phenotypes tested. There was a far greater range in prevalence of isolates exhibiting the hypermutability phenotype amongst the non-LES samples [Non-LES (0-61%, LES (2-12%)]. This was also observed for auxotrophy [Non –LES (0-100%), LES (4-52%)]. Susceptibilities to commonly used antibiotics varied considerably within patient samples, with the exception of colistin (no resistant isolates) (Table 3.1).

3.4 DISCUSSION

Within-host adaptation of P. aeruginosa during chronic CF infection results in the evolution of diversity within patients. Previous studies, which have tended to focus on small numbers of sequential isolates (Smith et al 2006, Bragonzi et al 2009, Huse et al 2010), have concluded that P. aeruginosa adapts in specific ways, including loss of virulence. Yet, it has been demonstrated that concurrent pairs of isolates (taken from the same sputum sample) can also vary considerably from each other (Chung et al 2012), and we have shown previously that some strains of P. aeruginosa, such as the LES, exhibit dynamic turnover of sub-types exhibiting different phenotypic and genotypic characteristics (Mowat et al 2011). It has been suggested that some strains reach an evolutionary plateau, characterized by low phenotypic variations (Cramer et al 2011), leading us to wonder whether the extensive diversification seen with chronic LES infections may be a particular feature of this epidemic strain. In this study we analyzed samples from multiple adult CF patients infected with non-LES P. aeruginosa strains and compared them with equivalent samples from LES-infected patients. Our observations of diversity in sub-type composition within single sputum samples from both the LES and the non-LES-infected patients suggest that the extensive diversity reported previously is a widespread feature of P. aeruginosa populations in the lungs of CF patients who have been chronically infected for long periods. For both groups (LES and non-LES), the greatest contribution to the diversity in sub-types observed was within individual sputum samples, rather than because of variation between patients, even though the non-LES group patients were each infected with a different strain.
The wider range of frequencies of some of the phenotypic traits measured between the LES-infected and the non-LES-infected groups is likely to be due to strain-specific variations in the non-LES group. Hence, although the extent of diversity was not different between the two groups, the actual phenotypes contributing to the variations did differ. For example, it was notable that isolates with the HM phenotype were present amongst the sets of 40 isolates analyzed in all of the samples from patients infected with the LES, but this was not the case for samples containing non-LES strains. However, there was no clear link between detectable HM phenotype and the number of subtypes identified in a patient. For example, although no HM isolates were identified amongst isolates tested from patient CF23, the *P. aeruginosa* population exhibited the highest number of different sub-types amongst the 80 isolates tested for each of the non-LES group.

Hence, this study provides further evidence that the CF lung, which constitutes a spatially heterogeneous environment with multiple discrete ecological niches, is able to sustain multiple divergent sub-types of *P. aeruginosa* simultaneously, and that this is a common feature of these kinds of infections.

**Acknowledgements**

We thank Paul Roberts for assistance with collection and archiving of isolates.
Fig 3.S1: Haplotype variation seen in LES and Non–LES patients. (As demonstrated by e-burst)

Pink – both LES and non-LES
Green – LES only
Black – non-LES only
Chapter 4: Increasing Resistance of the Liverpool Epidemic Strain (LES) of Pseudomonas aeruginosa (Psa) to Antibiotics in Cystic Fibrosis (CF) – a Cause for Concern?

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4.1 Abstract:

Background: Transmissible Pseudomonas aeruginosa (Psa) strains such as Liverpool Epidemic Strain (LES) are now widespread throughout UK CF clinics: its susceptibility to antibiotics is therefore important. To study this, we compared antibiogram patterns of Psa strains in our CF clinic over 5 years, looking at differences in resistance patterns between strains and changes to these over time.

Methods: The antibiograms of sputum samples collected at annual review between 2004 and 2008 from patients attending our centre were included. We compared Psa isolate antibiotic resistance (to six anti-pseudomonal antibiotics) patterns for patients infected with LES with those infected with other Psa strains, both in the total population in 2004 (125 patients) and 2008 (202 patients) and also longitudinally from 2004 to 2008 in matched and unmatched patients.

Results: LES exhibited significantly more resistant isolates in 2004 (p<0.0001). There was an increase in antibiotic resistance in both LES and other Psa strains over time (p<0.001). Cox proportional hazards analysis of both unmatched (n=125) and matched (n=56) patients in 2004 revealed that LES infected patients were more likely
to develop antibiotic resistant isolates over time (hazard ratio 8.1, p<0.001). Fewer LES isolates were classed as fully sensitive in both matched and unmatched groups at the end of study period (p<0.001).

Conclusion: This study shows a worrying trend in antibiotic resistance in the Psa isolates amongst those chronically infected with LES. This highlights the need to prevent cross infection through segregation and also the need to develop new strategies to treat these organisms.

4.2 Background:
Chronic respiratory infection with Pseudomonas aeruginosa (Psa) is the major cause of morbidity and mortality in CF (1797 Kerem 1990; 1831 Henry 1992), where most deaths are due to end stage lung disease (Zuckerman et al 1998). Treatment of these infections involves combinations of intravenous anti-Psa antibiotics, often given over prolonged periods, which may encourage antibiotic resistance (Cheng et al 1996, Pitt et al 2003) and also enhance toxicity (Katbamna et al 1998, Green et al 1985). Furthermore, the recent emergence of Psa clones that are highly transmissible between CF individuals and have been shown to confer a worse prognosis (Al-aloul et al 2004, Griffiths et al 2005, Bradbury et al 2008) has complicated antimicrobial management.

Despite this, there have been no systematic studies looking at antibiotic resistance over time in Psa in the CF community. We therefore undertook a 5 year prospective study of the antibiotic resistance of Psa in patients attending a large adult CF unit in the north-west of England, where there is a cohort of individuals chronically infected
with the Liverpool Epidemic Strain (LES) of Psa, the most prevalent and important such transmissible strain in the UK (Scott & Pitt 2004). We looked for any differences in resistance patterns between strains and whether these patterns have changed over time.

4.3 Patients and Methods:

4.3.1 Patient cohorts
The Liverpool Adult CF Unit provides specialist services for North Wales, Cheshire, Merseyside, and the Isle of Man: a catchment of approximately 3 million people. All adult CF patients have regular (at least bimonthly) sputum analysis for Psa and to prevent cross-infection we have regularly genotyped their strains for many years. Patients who were chronically infected with Psa (defined by at least 3 positive sputum samples within a 6 month period) (Fredericksen et al) formed the study population. For the purposes of this study, patients additionally chronically infected with Burkholderia cepacia complex and the only 2 with another known transmissible Psa strain (Midlands1) were excluded.

We looked at Psa isolates cultured from samples between 2004 and 2008: to standardise antibiogram selection, the results from the sputum sample closest to the anniversary of the patient’s birthday were used for each year for all patients.

We compared Psa isolate antibiotic resistance patterns for patients infected with LES with those infected with sporadic Psa strains, both in the total population in 2004 (125 patients) and 2008 (202 patients) and also longitudinally from 2004 to 2008 in both matched and unmatched patient groups (see below).
4.3.2 Patient matching and statistical analysis

We used a propensity score (Black et al 2002) for LES group membership in order to allow matching with the other group. This was determined without regard to outcome, using multivariable logistic regression analysis with a full non-parsimonious model that included all available patient characteristics, balancing all items recorded that may relate to either systematic bias or simple chance. This model yielded a C statistic of 0.78, indicating an acceptable ability to differentiate between patients with or without LES. We then used a macro (www2.sas.com/proceedings/sugi29/165-29.pdf) to perform propensity matching. Using this model, in 2004 28 LES infected patients were matched with 28 infected with other Psa strains on clinical parameters (age, sex, FEV1, BMI, time since diagnosis of CF, hospital admissions, outpatient visits and baseline antibiotic resistance) (Table 4.1). 2004 patient demographics are shown in Table 4.1.
Table 4.1: Characteristics of 125 adult CF patients chronically infected with *P. aeruginosa* attending the Liverpool Adult CF Centre in 2004 (unmatched and matched groups)

<table>
<thead>
<tr>
<th></th>
<th>Unmatched</th>
<th></th>
<th></th>
<th>Matched (n=56)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Other Psa Infection (n=34)</td>
<td>LES Psa Infection (n=91)</td>
<td>p value</td>
<td>Other Psa Infection (n=28)</td>
<td>LES Psa Infection (n=28)</td>
<td>p value</td>
</tr>
<tr>
<td>Female (%)</td>
<td>43</td>
<td>53</td>
<td>0.44</td>
<td>46</td>
<td>43</td>
<td>0.79</td>
</tr>
<tr>
<td>Years since diagnosis</td>
<td>22 (16-27)</td>
<td>25 (23-29)</td>
<td>0.003</td>
<td>23 (18-29)</td>
<td>23 (21-26)</td>
<td>0.62</td>
</tr>
<tr>
<td>Age (years)</td>
<td>26 (21-34)</td>
<td>29 (24-36)</td>
<td>&lt;0.01</td>
<td>25 (23–34)</td>
<td>27 (25-32)</td>
<td>0.18</td>
</tr>
<tr>
<td>BMI</td>
<td>22 (20-24)</td>
<td>21 (19-23)</td>
<td>0.08</td>
<td>21 (20-24)</td>
<td>22 (19-25)</td>
<td>0.80</td>
</tr>
<tr>
<td>FEV1 (%)</td>
<td>64 (46-83)</td>
<td>64 (45-85)</td>
<td>0.88</td>
<td>63 (45~88)</td>
<td>77 (49-94)</td>
<td>0.45</td>
</tr>
<tr>
<td>Hospital admissions</td>
<td>4 (1-7)</td>
<td>8 (4-14)</td>
<td>&lt;0.001</td>
<td>4 (1-7)</td>
<td>6 (3-10)</td>
<td>0.45</td>
</tr>
<tr>
<td>Outpatients visits</td>
<td>33 (21-38)</td>
<td>36 (22-50)</td>
<td>0.29</td>
<td>33 (21-36)</td>
<td>31 (17-49)</td>
<td>0.89</td>
</tr>
<tr>
<td>Baseline antibiotic resistance (%)</td>
<td>11.4</td>
<td>46.2</td>
<td>&lt;0.001</td>
<td>14.3</td>
<td>10.7</td>
<td>&gt;0.99</td>
</tr>
</tbody>
</table>

Categorical variables shown as percentage, comparisons made with $\chi^2$ test;

Continuous variables shown as median (25th - 75th percentiles), comparisons made with Wilcoxon rank sum
Cox proportional hazards analysis was then used to determine changes in antibiotic resistance over time in both the matched and unmatched groups. Statistical significance was calculated using Fisher’s exact test and Chi squared tests with Yates correction: \( p < 0.05 \) was considered to be significant. Stata software version 2.0 was used to analyse the data.

### 4.3.3 Microbiological methods

Antibiotic susceptibility testing:

Sputum samples were treated with equal quantities of sputasol (oxoid), agitated at 200 rpm for 15 minutes at room temperature, plated onto blood & chocolate agar and MacConkey agar, and incubated at 37° C. The plates were examined at 48 hours for growth of Psa colonies, and those with distinct morphotypes were further cultured onto purity plates (chocolate agar). Each morphologically different subtype (up to 5 per sample) was tested for antibiotic susceptibility using BASC guidelines (Andrews et al 2009) by the disc diffusion method using the following: tazobactam/piperacillin 85 \( \mu \)g, meropenem 10 \( \mu \)g, tobramycin 10 \( \mu \)g, ciprofloxacin 5 \( \mu \)g, ceftazidime 30 \( \mu \)g and colistin sulphate 25 \( \mu \)g. The sizes of the zones of inhibition (mm) were recorded and susceptibilities were defined as per standard protocols. In total 9200 susceptibility patterns were analysed.

Sputum samples with multiple morphotypes were judged to be sensitive only if all morphotypes were sensitive to at least two of the three class of antibiotics tested (fluoroquinolones, aminoglycosides, and beta-lactams/carbapenems). Strains were defined as resistant if they were resistant to two of the three classes of antibiotics (CF Foundation 2004) and pan-resistant if resistant to all three classes of antibiotics.
4.3.4 Psa genotyping:
Oligonucleotide primers (Sigma-Genosys) used in PCR assays were (PASS, PS21R, PS21F, LESF9F, LESF9R). DNA for PCR amplification was prepared by making a suspension of a few colonies in 200 ml 5% Chelex-100 (Sigma) solution. After vigorous mixing, the suspension was boiled for 5–10 min. Following centrifugation, 150 ml of supernatant containing the DNA was removed and stored at -22°C. Typically, 1 ml of this DNA was used directly in 25 ml volumes containing 1.25 units Taq DNA polymerase (Promega), 16 TaqMaster (Helena Biosciences), 300 nM each primer, 16 Taq buffer, 2.5 mM MgCl2 and 100 mM nucleotides (dATP, dCTP, dGTP, dTTP). Amplifications were carried out in an Eppendorf MasterCycler thermal cycler for 30 cycles consisting of 95°C (1 min), annealing temperature (1 min) and 72°C (2 min), with an additional extension time at 72°C (10 min) following completion of the 30 cycles.

Psa and LES strains were identified using gel electrophoresis looking for bands representing specific amplicons (Panagea et al 2003, Panagea et al 2005). The PA-SS, primer pair, specific for all Psa, yields an amplicon of 956 bp; the LES-specific primer pairs LESF9 and PS21 yield amplicons of 461 bp and 364 bp respectively. PCR tests leading to the PA-SS amplicon only were deemed LES-negative. Tests leading to the presence of all three amplicons (PA-SS, LESF9 and PS21) were deemed LES-positive (Smart et al 2006, Fothergill et al 2008).

4.4 Results:

4.4.1 Cross-Sectional (Unmatched) Analysis of 2004 and 2008 cohorts
Analysis of the 2004 antibiograms showed that LES was more resistant to ceftazidime, meropenem, piperacillin/tazobactam (all p<0.001) and tobramycin (p=0.0001) than other Psa strains, which in turn were more resistant to ciprofloxacin
There was no resistance to colomycin in either group. Overall, pooled resistance was greater in the LES group (mean 18.1%) than the other Psa group (mean 7.2%), (p<0.0001).

Analysis of the 2008 antibiograms showed that, compared to 2004, the overall mean antibiotic resistance increased in both groups, but LES isolates became significantly more resistant (p<0.0001) to all antibiotics except colomycin when compared to other Psa isolates (Table 4.2). Although in 2008 the other Psa isolates showed increased mean resistance to ciprofloxacin (10.6% vs 20.6%, P=0.0002), tobramycin (3.5 vs 8.5, p=0.002) and colomycin (0 vs. 2.8, P=0.008), the LES isolates had increased mean resistance to all six antibiotics used (all P<0.0001 except piperacillin/tazobactam p=0.01). The proportions of resistant and pan-resistant strains were also significantly higher in the LES group (see fig 4.1).

Table 4.2 & fig 4.1
Table 4.2: Mean antibiotic resistance exhibited by LES Psa and Other Psa isolates to six commonly used antibiotics in 2004 & 2008

n = number of isolates

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>2004</th>
<th></th>
<th>2008</th>
<th></th>
<th>Change in antibiotic resistance within Psa type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Other Psa</td>
<td>LES Psa</td>
<td>p-value</td>
<td>Other Psa</td>
<td>LES Psa</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>10.6</td>
<td>5.1</td>
<td>0.002</td>
<td>20.7</td>
<td>34.4</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>9.3</td>
<td>39.3</td>
<td>&lt;0.0001</td>
<td>12.9</td>
<td>58.1</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>3.0</td>
<td>10.5</td>
<td>0.0001</td>
<td>8.5</td>
<td>35.1</td>
</tr>
<tr>
<td>Colomycin</td>
<td>0.0</td>
<td>0.0</td>
<td>NS</td>
<td>2.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Piperacillin/Tazobactam</td>
<td>10.9</td>
<td>26.3</td>
<td>&lt;0.0001</td>
<td>10.9</td>
<td>31.5</td>
</tr>
<tr>
<td>Meropenem</td>
<td>9.6</td>
<td>32.0</td>
<td>&lt;0.0001</td>
<td>11.7</td>
<td>43.1</td>
</tr>
</tbody>
</table>
Fig 4.1: Prevalence of strain resistance in patients infected with Psa followed from 2004 to 2008

(n = number of patients)

Number of patients infected with Psa followed from 2004 *

LES - 15 deaths and 3 transfers  Other Psa - 4 deaths and 1 transfer
4.4.2 Longitudinal follow up of 2004 cohort:

Unmatched cohort:

The 125 patients in the 2004 cohort were followed for up to 5 years. Compared to the other Psa infected group, in terms of baseline demographics the 91 patients chronically infected with LES had a significantly higher age, mean antibiotic resistance, time since diagnosis, and number of hospital admissions, but no difference in sex, FEV1, BMI or number of outpatient visits than those with unique strains (see Table 4.1). Although Cox proportional hazards analysis revealed that the LES infected patients were more likely to develop antibiotic resistant isolates over time (hazard ratio 8.1, p<0.001) (table 4.3), as were female CF patients (hazard ratio 1.56, p=0.045) and those with a lower baseline FEV1 (hazard ratio 0.99, p=0.03), BMI and time since the diagnosis of CF had no effect. At the end of the 5 year study period, there were 15 deaths and 3 transfers in patients with LES compared to 4 deaths and 1 transfer in those with other Psa, and fewer LES group patient isolates were classed as sensitive (9/73), compared to 25/31 in the other Psa group (p<0.001) (see fig 4.1).

Matched cohort:

Cox proportional hazards analysis of the 56 matched patients showed that LES infection (hazard ratio 6.1, p<0.001) and lower baseline FEV1 (hazard ratio 0.98, p=0.03) increased the likelihood of developing antibiotic resistant isolates over time, whilst female sex, BMI or time since diagnosis had no effect (table 3). Even when adjusted for those clinical variables predicting antibiotic resistance over time (age, sex, BMI, FEV1, number of hospital admissions), LES infected patients developed more antibiotic resistant isolates during the 5-year period (fig 4.2) (isolates classed as sensitive: LES infected group [21.7%] versus other Psa infected group 22 [88.5%], p<0.001).
Table 4.3: Cox proportional hazards for follow up antibiotic resistance in unmatched & matched cohorts

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% confidence interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unmatched population (n=125)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LES</td>
<td>8.1</td>
<td>3.79 to 17.39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female</td>
<td>1.56</td>
<td>1.01 to 2.40</td>
<td>0.046</td>
</tr>
<tr>
<td>2004 BMI</td>
<td>1.00</td>
<td>0.93 to 1.07</td>
<td>0.99</td>
</tr>
<tr>
<td>2004 FEV1 %</td>
<td>0.99</td>
<td>0.98 to 1.00</td>
<td>0.03</td>
</tr>
<tr>
<td>Time since diagnosis</td>
<td>1.00</td>
<td>0.97 to 1.00</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>Matched population (n=56)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LES</td>
<td>6.1</td>
<td>2.48 to 15.18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female</td>
<td>1.34</td>
<td>0.63 to 2.87</td>
<td>0.44</td>
</tr>
<tr>
<td>2004 BMI</td>
<td>1.12</td>
<td>0.98 to 1.27</td>
<td>0.09</td>
</tr>
<tr>
<td>2004 FEV1 %</td>
<td>0.98</td>
<td>0.96 to 1.00</td>
<td>0.03</td>
</tr>
<tr>
<td>Time since diagnosis</td>
<td>1.00</td>
<td>0.96 to 1.05</td>
<td>0.76</td>
</tr>
</tbody>
</table>
Fig 4.2: Antibiotic sensitivity profile of patients chronically infected with LES and other Psa strains attending Liverpool Adult CF Unit between 2004-2008.*

* Antibiotic sensitivity adjusted for number of patients in each group/year
4.5 Discussion:

For the first time to our knowledge we report a longitudinal analysis of the antibiotic sensitivity patterns of Psa strains in chronically infected adult CF patients, including those of the most common transmissible Psa strain in the UK, LES. Our results demonstrate a high prevalence of antibiotic resistance in Psa isolates in patients infected with LES and an increased rate of acquisition of antibiotic resistance over time, compared to unique Psa strains.

Morbidity in patients with CF results from chronic suppurative lung disease due to Psa, where repeated exacerbations cause progressive lung damage leading to respiratory failure and death in the majority of cases (Zuckerman et al 2007, Belkin et al 2006). Up to 70% of adult CF patients in the UK are chronically infected with Psa strains (UK CF registry 2008). Possibly due to selective antibiotic pressures, over time these organisms undergo genotypic changes leading to the emergence of treatment-resistant phenotypes: it was the appearance of such a ceftazidime-resistant phenotype at our local paediatric CF centre that led to the important discovery of the original transmissible clone of Psa, LES, in 1996 (Cheng et al 1996). LES is widespread throughout UK CF units (Scott & Pitt 2004) and has also been discovered elsewhere in the World (Aaron et al 2008): it causes increased morbidity, confers an increased healthcare burden, hastens clinical deterioration (Al-Aloul et al 2004, Ashish et al 2010) and can even spread to non-CF patients (McCallum et al 2002) and across species (Mohan et al 2008). Other transmissible strains have since been discovered (Griffiths et al 2005, Armstrong et al 2003, Brimicombe et al 2008, Fluge et al 2001, Dinesh et al 2003, Ojeniyi et al 2000, Jones et al 2005, Speert et al 2002, O'Carroll et al 2004, Tubbs et al 2001), and whilst some these have shown increased virulence and antibiotic resistance and can cause poorer clinical outcomes (Al-Aloul

Although a number of workers have shown the presence of resistant isolates of transmissible strains in their patients (Al-Aloul et al 2004, Griffiths et al 2005, Jones et al 2001, Denton et al 2002) none have demonstrated the presence of an excess of multiresistant strains, and there have been no previous studies looking at how the antibiotic sensitivity patterns of these epidemic strains may change over time. We have a large cohort of LES-infected CF patients, mainly inherited from paediatric practice, and this has given us the opportunity to study this strain over a prolonged period.

From our longitudinal study of up to 200 adult patients over 5 years, we have demonstrated that LES is not only more resistant to antibiotics, but also has an enhanced rate of acquisition of antibiotic resistance over time. We have previously shown that chronic infection with LES causes increased hospitalisation and IV antibiotic use (Al-Aloul et al 2004). However, this increased antibiotic resistance may not simply be a reflection of repeated antibiotic exposure leading to the emergence of resistance by natural selection, since the effect was still seen following adjustment for confounding factors, and antibiotic pressure only had a weak effect.

The ability of Psa to mutate rapidly (hypermutate) in the harsh environment of the CF lung that may give it a survival advantage (Mena et al 2008, Oliver et al 2000), has also been suggested as an explanation for the emergence of antibiotic resistance. However, although hypermutable Psa strains can occur in up to 36% of CF patients (Oliver et al 2000) we have found that hypermutability occurs less frequently amongst LES isolates (Fothergill et al 2007) suggesting an alternative explanation for its resistance to antibiotics is required.
Many isolates of LES, including those associated with transmission to non-CF patients, exhibit an unusual quorum sensing (QS) phenotype termed hypervirulence (Winstanley et al. 2009). This phenotype is characterized by a dysfunctional QS system, leading to overproduction of QS-regulated factors too early in the growth phase. These hypervirulent isolates produce high levels of pyocyanin and other QS-regulated exoproducts of relevance to CF infections (Fothergill et al. 2007), and also exhibit greater killing activity against *Drosophila* and *C. elegans* (Salunkhe et al. 2005). The phenotype has also been linked to increased resistance to some antibiotics (Fothergill et al. 2007) and has not been identified in other Psa isolates, whether in CF or other hosts. Hence, it seems feasible that it might play a role in the success of this epidemic clone, the greater morbidity associated with it, and the development of resistance.

LES has also been found to possess an unstable genotype: the subtypes within LES undergo deletions, insertions and rearrangement leading to significant phenotypic changes (Fowerekar et al. 2009, Fothergill et al. 2010). In a UK based survey looking at several transmissible strains, LES had greater microheterogeneity than other strains underlining its genetic variability.

There are limitations to our study. Firstly, we applied *in vitro* antimicrobial sensitivity testing to organisms existing in the special circumstances of the CF lung, where Psa lives in a complex anaerobic bio-film community (Hill et al. 2005), and exhibits extensive phenotypic divergence, including a mucoid form. Removing these organisms and growing them in planktonic media in aerobic conditions may not mirror sufficiently their natural habitat. Furthermore, many different subtypes may exist within the same sputum sample, each with a different antibiogram (Fowerekar et al. 2009; Fothergill et al. 2010). Nevertheless, we standardised our approach to the
assessment of antibiotic resistance by collecting samples at the same fixed time points for each patient, and utilising industry standard methodology (Andrews et al. 2009) towards antimicrobial testing. Furthermore, by using the same technique for all Psa isolates (both LES and other strains) over a prolonged period the results produced are comparable. Even though individual sputum sensitivities may not be representative of the true sensitivity in an infecting bacterial population, using longitudinal data over 5 years gives us a glimpse of the overall trends of the antibiotic resistance patterns in these patients, confirming the developing problem of antibiotic resistance in transmissible Psa in CF patients, particularly in those with LES infection. Secondly, we only studied LES and not other transmissible strains, so we cannot be sure that our conclusions are valid for all such strains. However, LES is the most prevalent transmissible strain in the UK and has already spread to units elsewhere, so our findings will be of relevance to all CF units.

In conclusion, this study shows a worrying trend in antibiotic resistance in Psa isolates in the CF population, particularly amongst those chronically infected with LES, which not only exhibits more antibiotic resistance but also develops resistance much more rapidly than other Psa strains. This highlights the requirement for new antimicrobial therapies and the paramount need to enforce stringent infection control and segregation policies in CF units to limit the spread of transmissible Psa strains to improve outcomes for CF patients.
Chapter 5: The increased healthcare economic burden associated with chronic infection with transmissible Pseudomonas aeruginosa strains in CF.

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5.1 Abstract:
Background: Although chronic infection with transmissible Pseudomonas aeruginosa (Psa) strains causes increased treatment requirements, morbidity, and ultimately mortality in CF patients, the burden this places on the healthcare economy, of increasing importance in these times of financial austerity, has not been studied.

Methods: To investigate this further, using logistic regression we matched (for age, sex, BMI, FEV1, and diabetes) 40 adult CF patients chronically infected with the commonest UK transmissible Psa strain (the Liverpool Epidemic Strain, LES) with 40 infected with unique Psa strains and compared their healthcare costs (inpatient and outpatient care, antibiotic therapy, and regular prescriptions) over a 4 year period. Statistical significance was calculated using the chi-squared test with Yates correction and Wilcoxon sign rank test for non-parametric data

Results: The mean cost per patient per year was higher for LES patients for inpatient care (£4393.37 v £1817, p=0.0006), outpatient attendance (£3764 v £2515.91,
p=0.0035) and also hospital antibiotic therapy (£980 v £ 505, p=0.001). Regular prescription costs were similar in both groups. Overall, the healthcare cost of caring for an adult CF patient with LES chronic infection was significantly more (1.6 times) than that for a matched patient with unique Psa strain chronic infection.

Conclusions: CF patients chronically infected with LES place an additional burden on the healthcare economy than that associated with chronic Psa infection. This has implications for the financial modelling currently underway in the UK to adequately resource CF units, and also underlines the need to prevent infection with transmissible Psa strains by effective cross infection control strategies.

5.2 Background:
Cystic fibrosis is the commonest potentially fatal inherited disease in the Western World, where a faulty gene interferes with salt and water transport in mucus producing cells, resulting in abnormally thick mucus, which causes organ damage and eventual failure. Although improvement in the diagnosis and treatment of patients with CF over the last two decades has resulted in better outcomes, the average life expectancy is still only 39 years (UK CF Registry 2008). This improvement in life expectancy is the direct result of various cost-intensive advances including the provision of expensive mucus thinning drugs and antibiotics, and the development of specialist centre care through dedicated multidisciplinary teams. However, chronic lung infection particularly due to organisms such as Pseudomonas aeruginosa (Psa) remains the major cause of morbidity and mortality in CF patients (Kerem et al 1990, Henry et al 1992) with the majority of deaths due to end stage lung disease. As patients with the disease live longer, new challenges to
both patients and clinicians have emerged, including cross infection with strains of Psa, which can spread between patients (Zuckerman et al 1998). The first reported such strain (the Liverpool Epidemic Strain, LES) can cause a rapid decline in clinical prognostic markers such as lung function and nutritional state, and increases the need for antibiotic therapy and hospital admission. (Cheng et al 1996, Al-Aloul et al 2004) Once acquired, it cannot be eradicated and can not only super-infect and replace existing Psa strains in CF patients but is also capable of infecting non-CF individuals (McCallum et al 2002, McCallum et al 2001) and crossing species (Mohan et al 2008). A UK based survey of CF centres demonstrated LES to be the most common strain, present in 11% of CF clinics and accounting for 48% of all epidemic strains (Scott & Pitt 2004): it has now also been reported in Canada (Aaron et al 2008) However, despite an increasing body of evidence establishing the ill effects of these epidemic strains in the UK and worldwide, there have been no systematic studies looking at the cost implications, healthcare resource utilisation or disease burden associated with their presence. Such an economic evaluation would require the analysis of both the costs and outcomes of two or more competing uses of healthcare resources in order to generate an overall comparative assessment.

In our Specialist Adult CF Centre in the North-West of England, a post hoc analysis of our clinic population in 2000 revealed that 79% of those chronically infected with Psa harboured LES (Salunkhe et al 2005). We therefore undertook a 4-year study of the healthcare resource costs of our CF patients infected with LES, and compared them with to those infected with sporadic Psa strains.
5.3 Methods:

We prospectively collected data on treatment burden, complications and the total costs involved for patients infected with LES and those infected with sporadic Psa strains, longitudinally for the 4 year period 2005-2008 in matched patient groups (Table 5.1). Data regarding hospital spells, outpatient visits, radiology and blood tests were collected from the hospital database. Details of medication use were collected from medical and nursing notes, and the pharmacy database. Unit costs were determined from Department of Health national tariffs and drug costs from the pharmacy procurement files. Nursing and physiotherapy time required by patients each day were estimated from the experience of senior nursing and physiotherapy staff.

Patient demographics (matched and unmatched groups) are shown in Table 1

Table 5.1: Unmatched patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>LES Negative (n=42)</th>
<th>LES Positive (n=121)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>45.2</td>
<td>48.8</td>
<td>0.69</td>
</tr>
<tr>
<td>Diabetes</td>
<td>35.7</td>
<td>58.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Age (years)</td>
<td>28.3 (23.9 – 29.1)</td>
<td>26.9 (24.4 – 32.1)</td>
<td>0.48</td>
</tr>
<tr>
<td>FEV1</td>
<td>69 (47 – 84)</td>
<td>53 (37 – 77)</td>
<td>0.046</td>
</tr>
<tr>
<td>BMI</td>
<td>23 (20 – 26)</td>
<td>21 (18.9 – 23.3)</td>
<td>0.006</td>
</tr>
<tr>
<td>Mortality</td>
<td>7.1</td>
<td>9.9</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Categorical variables shown as percentage, comparisons made with $\chi^2$ test;

Continuous variables shown as median (25th–75th centiles), comparisons made with Wilcoxon rank sum test
Chronic infection with Psa was defined as its isolation in 3 or more consecutive sputum samples in a 12-month period. All patients with LES had acquired it prior to transfer from the paediatric sector, and a strict inpatient and outpatient segregation policy is in place to prevent cross infection. We have been carrying out genotypic identification of Psa strains in respiratory samples from all patients attending our service since December 2003, where LES and other epidemic Psa strains are identified using combined multiplex PCR (Smart et al 2006). Respiratory samples from patients known to be colonised with LES undergo an annual genotypic confirmation, whilst those from the remaining PSA infected patients undergo 3 monthly genotyping to look for the main UK epidemic strains (LES, Midland1 and Manchester strains). We therefore know the Psa genotypes of all our patients at all times.

The quality of life of patients with epidemic Psa strains in comparison to unique Psa strains has been addressed elsewhere. (Ashish et al 2010) We will however draw upon the overall conclusions in order to evaluate the relationships between QoL and costs.

5.3.1 Patient matching and statistical analysis:

To enable comparisons to be made between patients without the influence of underlying variations in characteristics and health state we developed a propensity score (Blackstone et al 2002) for LES group membership, which allowed matching with other groups. This was determined without regard to outcome, using a multivariable logistic regression analysis with a full non-parsimonious model that included all available patient characteristics, balancing all items recorded that may relate to either systematic bias or simple chance. This model yielded a C statistic of
0.78, indicating an acceptable ability to differentiate between patients with or without LES. We then used a macro (www2.sas.com/proceedings/sugi29/165-29.pdf) to perform propensity-matching. Using this model, in 2005, 40 LES infected patients were matched with 40 infected with other Psa strains on clinical parameters (age, sex, FEV1, BMI and diabetes (Table 5.2). We used mean values to compare costs between the different groups.

Table 5.2: Matched patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>LES Negative (n=40)</th>
<th>LES Positive (n=40)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>45.0</td>
<td>42.5</td>
<td>0.82</td>
</tr>
<tr>
<td>Diabetes</td>
<td>37.5</td>
<td>35.0</td>
<td>0.82</td>
</tr>
<tr>
<td>Age (years)</td>
<td>27.8 (23.9 – 38.8)</td>
<td>30.4 (25.0 – 34.7)</td>
<td>0.44</td>
</tr>
<tr>
<td>FEV1</td>
<td>69 (47 – 83.5)</td>
<td>68 (49.5 – 88)</td>
<td>0.83</td>
</tr>
<tr>
<td>BMI</td>
<td>22.5 (20 – 26)</td>
<td>22.5 (20.0 – 25.4)</td>
<td>0.81</td>
</tr>
<tr>
<td>Mortality</td>
<td>7.5</td>
<td>7.5</td>
<td>&gt;0.99</td>
</tr>
</tbody>
</table>

Categorical variables shown as percentage, comparisons made with \( \chi^2 \) test;

Continuous variables shown as median (25\(^{th}\) – 75\(^{th}\) centiles), comparisons made with Wilcoxon rank sum test
Statistical significance was calculated using the chi-squared test with Yates correction and Wilcoxon sign rank test for non-parametric data. p<0.01 was considered to be significant. Stata software version 2.0 was used to analyse the data.

5.4 Results:

Of the 163 patients in 2005, 121 were chronically infected with LES (74.2%).

Univariate analysis of the 2005 cohort revealed that patients with LES had a significantly lower FEV1, BMI and a higher prevalence of diabetes (table 5.1). The well-matched patient groups were followed up to analyse disease burden, overall costs and healthcare resource utilisation (table 5.3-5.4) continuous variables shown as median (25th–75th centiles), comparisons made with Wilcoxon rank sum test.
<table>
<thead>
<tr>
<th></th>
<th>LES Negative (n=40)</th>
<th>LES Positive (n=40)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospital care</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inpatient days</td>
<td>15.2 (5.2 – 32)</td>
<td>60.2 (31.7 – 99.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Inpatient admissions</td>
<td>2 (1 – 5)</td>
<td>6.8 (4 – 11.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nursing time</td>
<td>98.8 (33.8 – 208)</td>
<td>391.0 (205.9 – 644.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Physiotherapy time</td>
<td>5.7 (2 – 12)</td>
<td>22.6 (11.9 – 37.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Outpatient visits</td>
<td>4.7 (3.8 -7.0)</td>
<td>7 (4.6 – 8.2)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Home IV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean number/year</td>
<td>11.2 (0 – 44.8)</td>
<td>14 (0 – 56)</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
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<tr>
<td>Port</td>
<td>12.5</td>
<td>32.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Assisted feed PEG</td>
<td>0</td>
<td>5.0</td>
<td>0.49</td>
</tr>
<tr>
<td>Deranged liver enzymes</td>
<td>32.5</td>
<td>32.5</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Depression</td>
<td>2.5</td>
<td>12.5</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Repeat prescriptions</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Oral energy drinks</td>
<td>32.5</td>
<td>47.5</td>
<td>0.17</td>
</tr>
<tr>
<td>Macrolide</td>
<td>65</td>
<td>67.5</td>
<td>0.81</td>
</tr>
<tr>
<td>Median duration</td>
<td>15.5 (0 – 35)</td>
<td>22 (0 – 35.5)</td>
<td>0.91</td>
</tr>
<tr>
<td>Oral Steroids</td>
<td>12.5</td>
<td>30</td>
<td>0.056</td>
</tr>
<tr>
<td>Median oral antibiotics / year</td>
<td>1 (0.8 – 1.4)</td>
<td>1 (0.8 – 1.9)</td>
<td>0.36</td>
</tr>
<tr>
<td>Inhaled antibiotics</td>
<td>67.5</td>
<td>62.5</td>
<td>0.64</td>
</tr>
<tr>
<td>Median duration</td>
<td>16 (0 – 32.75)</td>
<td>21.5 (0 – 48)</td>
<td>0.60</td>
</tr>
<tr>
<td>DNAse</td>
<td>37.5</td>
<td>50</td>
<td>0.26</td>
</tr>
<tr>
<td>Median duration</td>
<td>12.2 (0 – 28.5)</td>
<td>14.6(0- 48)</td>
<td>0.49</td>
</tr>
<tr>
<td>Tests</td>
<td>1 (0.8 – 1.5)</td>
<td>1.5 (1 – 2.4)</td>
<td>0.05</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>---------------</td>
<td>---------------</td>
<td>------</td>
</tr>
<tr>
<td>Mean radiology tests / year</td>
<td>5 (3.6 – 6)</td>
<td>7.1 (5.9 – 10.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean microbiology tests / year</td>
<td>4 (2.5 – 6.1)</td>
<td>4.5 (3.6 – 5.6)</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Categorical variables shown as percentage, comparisons made with $\chi^2$ test
Table 5.4: Matched cohorts, patient costs

<table>
<thead>
<tr>
<th>Cost variable</th>
<th>Mean cost</th>
<th>95% confidence</th>
<th>Mean cost</th>
<th>95% confidence</th>
<th>p value</th>
</tr>
</thead>
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<td>Inpatient cost</td>
<td>1817</td>
<td>(807.2 - 2827).</td>
<td>4393.37</td>
<td>(3383- 5403)</td>
<td>p=0006</td>
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<td>Nursing cost</td>
<td>550</td>
<td>(244.6 - 856.5)</td>
<td>1331</td>
<td>(1025- 1637)</td>
<td>p=0006</td>
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<tr>
<td>Physiotherapy cost</td>
<td>116</td>
<td>(51.31 -179.7)</td>
<td>279</td>
<td>(215.0- 343.4)</td>
<td>p=0006</td>
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<tr>
<td>Outpatient cost</td>
<td>2515.91</td>
<td>(1933. - 3099)</td>
<td>3764</td>
<td>(3181 -4347)</td>
<td>p=0035</td>
</tr>
<tr>
<td>Cost of macrolide (azithromycin)</td>
<td>339</td>
<td>(235.8 - 441.9)</td>
<td>353</td>
<td>(249.8 - 455.8)</td>
<td>p=0.85</td>
</tr>
<tr>
<td>Cost of inhaled antibiotic</td>
<td>1042</td>
<td>(425.6 - 1658)</td>
<td>1119</td>
<td>(502.6 - 1735)</td>
<td>p=0.86</td>
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<tr>
<td>Cost of DNAse</td>
<td>1780</td>
<td>(950.5 -2609)</td>
<td>2121</td>
<td>(1302 - 2941)</td>
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<td>Cost of oral abx</td>
<td>80.5</td>
<td>(51.91 -109.0)</td>
<td>85.5</td>
<td>(56.94 -114.0)</td>
<td>p=0.81</td>
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<tr>
<td>Cost of home iv</td>
<td>225.5</td>
<td>(102.9 - 348.5)</td>
<td>295.24</td>
<td>(172.5 - 418.0)</td>
<td>p=0.43</td>
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<td>Cost of iv hospital abx</td>
<td>505</td>
<td>(363.8 - 647.1)</td>
<td>980</td>
<td>(842.2 - 1118)</td>
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<tr>
<td>Cost of blood tests</td>
<td>8.15</td>
<td>(6.8 -9.4)</td>
<td>10</td>
<td>(8.759 - 11.28)</td>
<td>p=0.03</td>
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<tr>
<td>Cost of microbiology</td>
<td>31.65</td>
<td>(25.30 - 37.97)</td>
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<td>(25.40 - 39.80)</td>
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<tr>
<td>Cost of TIVAD</td>
<td>51.31</td>
<td>(6.526 - 96.11)</td>
<td>105.62</td>
<td>(61.97 - 149.3)</td>
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<td>Cost of radiology</td>
<td>12</td>
<td>(3.069 - 21.02)</td>
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<tr>
<td>Mean cost /yr</td>
<td>9074.02</td>
<td></td>
<td>14893.83</td>
<td></td>
<td>p=0.001</td>
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</table>

Categorical variables shown as percentage, comparisons made with $\chi^2$ test;

Continuous variables shown as mean (25th–75th centiles), comparisons made with Wilcoxon rank sum test;
Compared to patients with other Psa strains, those with LES had a higher disease burden with higher hospital admission rates (median 6.8/pt/yr vs. 2.0, p<0.001), inpatient stays (median 60.2 days/pt/yr vs. 15.2, p<0.001), outpatient attendances (median 7.0/pt/yr vs. 4.7, p<0.01) and blood tests (median 7.1 vs. 5.0 p<0.001). There was a trend towards more TIVAD insertions, prevalence of depression and a need for radiological investigations in those with LES.

There was also a significantly greater healthcare resource burden as evidenced by the requirement for more inpatient nursing time (median 391.0 vs 98.8 hours/pt/yr) and physiotherapy time (median 22.6 vs. 5.7 hours/pt/yr), higher inpatient stay costs (median £3549.4 vs 896.8/pt/yr), inpatient IV cost (£7123.0 vs 3088.9) (all p<0.001), outpatient attendance costs (£3502.2 vs 1706.2, p=0.003), and requirement for blood tests (9.5 vs 6.7, p<0.001) and radiological examinations (2.0 vs 1.3. p=0.05)

Overall, the mean cost of caring for a CF patient with LES was more than that with other Psa strains (£14893.83 vs. £9074.02, p=0.001).

However, there was no significant difference in the mean cost or duration of chronic treatments including inhaled mucolytic and antibiotic therapy, and oral immunosuppressives including macrolides and steroids. There were also no significant differences in home IV therapy or procedural costs (Port or PEG insertion) in patients with LES compared to other Psa strains.

5.5 Discussion:
There have been many studies focusing on direct healthcare costs associated with CF, typically from the perspective of a hospital or third party payer, all retrospective in design. Robson et al estimated the annual cost incurred by a UK adult unit (Robson et al 1992) at £8241 per CF patient, with drugs (inpatient and outpatient) accounting for
57% of the total: costs for patients requiring regular intravenous antibiotics were 3 times that of the average patient, with up to 21 times in the final stages of the disease. A US paediatric CF centre conducting a similar analysis estimated the average annual patient cost at $13300, with older and more severely ill patients consuming more resource (Lieu et al 1999). However a German paediatric CF centre estimated the average yearly cost to be €23989 with outpatient drugs making up 47% of the total. (Baumann et al 2003) A regression-based analysis revealed that both FEV1 and Psa colonisation correlated with cost, with patients colonised with Psa costing three times more than the remainder (Johnson et al 1999). This important finding highlights the contribution of Psa infection and its treatment to the overall economic burden of cystic fibrosis, yet despite this there have been no systematic studies looking at the effect of colonisation with epidemic Psa strains per se. Other studies looking at the economic aspects of cystic fibrosis have focussed on the cost effectiveness of interventions.

Despite the advances in CF care over the last two decades, chronic respiratory infection with Psa still remains the main cause of morbidity and mortality, where 90% of the deaths are still due to end–stage respiratory failure. Over the last decade strains of Psa have emerged which are capable of cross-infecting other CF patients and other individuals. Some of these strains have been reported to possess characteristics, which make them more virulent, transmissible and adaptable to the CF lungs (Salunkhe et al 2005). The most common epidemic strain in UK CF clinics is the Liverpool Epidemic Strain (LES) which was first reported at the paediatric CF centre in Liverpool, when Cheng et al demonstrated the presence of a ceftazidime resistant Psa strain possessing a single genotype in 85% of the clinic population. (Cheng et al 1996) LES is the most
prevalent transmissible Psa strain; it is widespread throughout UK CF clinics and has now spread to Canada (Aaron et al 2010). We have already shown that LES can cause an accelerated fall in FEV1, BMI and results in increased hospital stays. A number of other clonal strains have now been reported in UK and worldwide (Bradbury et al 2008, Fluge et al 2001), many of which are associated with a poorer prognosis. However, chronic infection with such strains is not universally accepted as a poor prognostic factor (Tubbs et al 2001, Speert et al 2002, O’Carroll et al 2004). This may be because other factors (age, female sex, poor lung function and BMI, and CF related diabetes), which can also lead to higher morbidity and mortality in CF, have masked their effect.

Nevertheless, since Psa is a well established poor prognostic indicator in CF, and poorer outcomes have been demonstrated following chronic infection with transmissible strains (Armstrong et al 2003, Jones et al 2001, Al-Aloul et al 2004), a higher healthcare resource burden and cost for centres looking after such patients is expected. Thus, for the first time we have demonstrated the disease burden and costs associated with chronic infection with the most important and prevalent epidemic Psa strain (LES). This prospective study compares the costs of caring for 40 LES-infected CF patients over 4 years attending a large adult CF centre in the north-west of England, with 40 other Psa-infected CF patients well matched for the important prognostic indicators of age sex, lung function, and CF-related diabetes mellitus.

Using this substantial cohort, we have clearly demonstrated the increased healthcare disease burden and costs associated with LES infection: those with LES required 3 times as many hospital admissions, 4 times more inpatient days and twice as many outpatient visits compared to those with unique Psa strains. This increased hospital resource use by patients with LES is also reflected in significantly higher inpatient IV
antibiotic use, blood tests, radiology examinations and the cumulative time spent by allied health professionals to care for these patients which add to the healthcare resource burden on the provider. Based on our data, on average the cost of caring for a patient with LES is at least 60% more than for a patient with a unique Psa strain. Although there were no significant differences in the use of chronic treatments or their duration between the groups, there was a suggestion that those with LES are commenced on these treatments earlier, perhaps reflecting an increase in the severity of their illness following LES acquisition. Although an increase in treatment burden following infection with transmissible strains has previously been demonstrated (Jones et al 2001), our study is much more detailed and focussed on the most virulent epidemic Psa, LES. We believe these differences are more reflective of the true disease burden faced by patients with LES and indicative of healthcare resource burden placed on the healthcare provider. Furthermore, a longer follow-up period and greater sample size confirms the previous results from our centre (Al-aloul et al 2004).

The reasons for this apparent increased cost of LES infection is unclear: although we have already shown that LES exhibits increased virulence factors such as the overproduction of pyocyanin, a dysregulation of quorum sensing mechanisms and the presence of genomic islands which could contribute to this (Winstanley et al 2009, Fothergill et al 2007) further study is required. We have also demonstrated that LES is associated with a poorer quality of life in CF patients, and this may increase their interaction and need for more healthcare resources, over and above their physical requirements (Ashish et al 2012).
There are limitations to our cost assessment: not all medications (e.g. inhalers, pancreatic supplements, and insulin) could be completely documented, and many medications have formulations that vary in cost and in the amount taken by each patient. Since such prescriptions are individually tailored to the patient, it is difficult to calculate an average value for these items for the purpose of cost estimation. The capture of outpatient costs is also difficult, where the use of extra medications (such as oral antibiotics) relies upon adequate documentation by the prescribing clinician, and as a result some may been missed. Also, it is impossible to determine exactly how much nursing and physiotherapy time each patient has required over time: some patients carry out their own physiotherapy regimens or use expectoration devices and may not require as much time as others. Conversely, severely unwell patients may require much more attention than the stipulated time outlined in the study.

Furthermore, we have not incorporated the indirect costs associated with hospital admission or increased treatment (loss of income, travel costs, childcare costs etc), which may be attributable to poor health in an individual patient, estimated at up to 60% in CF (Pauly, MV 1983). Moreover the costs associated with running parallel streams of outpatient clinics, and inpatient facilities have not been included. These are cost-intensive strategies introduced to minimise the spread of transmissible strains and are an important aspect of care for these patients. However, such missed costs would almost certainly be greater in the LES-infected patients, and their inclusion would therefore strengthen the differences between the two groups. It may also have been interesting to compare the current financial allocation to CF trusts based on banding and the estimated costs as per our study but that was outside the scope of our study.

In conclusion, this study has shown that chronic infection with LES is an independent risk factor for a higher treatment burden in CF and therefore a greater cost to the
healthcare economy, than other strains of Psa. It also reinforces the need to prevent the spread of such transmissible Psa strains in CF clinics through stringent cross-infection control measures.

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Contributorship: AA collected all the data, collated the results drafted and edited the manuscript. MS collated and analysed the data and edited the manuscript. LH provided the data and edited the manuscript. CW analysed the results, microbiological methods and edited the manuscript. MW reviewed the results, drafted, edited and proof read the manuscript.

6.1 Abstract:

Objectives: To assess if cohort segregation policies are effective in preventing cross infection in CF (Cystic Fibrosis) clinics.

Design: A prospective cohort study.

Setting: A large adult Cystic Fibrosis Centre in Northwest England.

Participants: All CF patients cared for at the Liverpool Adult CF centre 2003-2009.

Methods: Regular sputum sampling with genotyping of Psa isolates led to a policy of inpatient and outpatient segregation by microbiological group.
Main outcome measures: Prevalence and cross-infection/super-infection rates of a transmissible Pseudomonas (Psa) strain i.e. the Liverpool Epidemic Strain (LES) in adult CF patients at the Liverpool Adult CF Centre from 2003-2009.

Results: There was a decline in the proportion of patients with LES (71% to 53%) and an increase in those with unique strains (23% to 31%) and without Psa infection (6% to 17%) from 2003 to 2009. There were two cases of LES super-infection and one case of new chronic Psa infection (with a unique strain). There were no cases of transmissible strain infection in patients previously un-infected by Psa.

Conclusions: Our segregation policy has halted the spread of the commonest highly transmissible strain in the UK (LES) in our clinic, without endangering patients who were not previously infected with Psa. It confirms that if genotypic surveillance is used, it is unnecessary to segregate patients infected with unique strains from those without Psa infection.

6.2 INTRODUCTION

Although Cystic Fibrosis (CF) is the most common potentially lethal inherited disease in the Western World, due to improved management the life expectancy of CF patients has greatly improved in the last two decades and in the UK the median survival is 38 years (UK CF Registry 2008). However, the majority of patients still die from end stage lung disease, due to chronic bacterial infection with Pseudomonas aeruginosa (Psa) (Belkin et al 2006). Despite the success of aggressive therapy against acute infection/colonisation with this organism, most CF patients become chronically infected by early adulthood, and once established Psa is impossible to eradicate and can cause a rapid decline in clinical parameters, doubling their mortality. (Henry et al 1992, Fredereiksen et al 1999, Kerem et al 1990)
Initially it was believed that each individual harboured his or her own unique Psa strain which was incapable of cross infecting another individual (Speert et al 1987) with or without CF. However, in 1996 Cheng et al demonstrated the widespread presence of an antibiotic resistant Psa at the children’s CF centre in Liverpool, UK. Psa genotyping revealed the children to be infected with the same clone, and it was postulated that this particular variant may have the capability to spread to other individuals. Subsequently, we have shown that it can super-infect those with their own unique strains, (McCallum et al 2001) in many cases replacing them, and can also spread to non-CF patients (McCallum et al 2002) and across species (Mohan et al 2008). This variant, which has now been shown to be widespread throughout UK CF centres (Scott & Pitt 2004) and has also been reported in Canada, (Aaron et al 2008) is labelled the Liverpool Epidemic Strain [LES]. Reports of other transmissible strains have now been made, not only in the UK (Jones et al 2001, Al-Aloul et al 2004, Edenborough et al 2004, Tubbs et al 2001, Denton et al 2002) but also elsewhere. (Armstrong et al 2003, Griffiths et al 2005, O’Carroll et al 2004, Bradbury et al 2008, Speert et al 2002, Dinesh et al 2003)

The Liverpool Adult Centre receives patients from the paediatric centre, which reported the first outbreak of LES in 1996, and many of these children have grown up and transferred to the adult sector. A post-hoc analysis in 2000 (Panagea et al 2003) of sputum cultures from our CF patients identified LES in 79% of those chronically infected with Psa, demonstrating the widespread infection of patients inherited from the original reporting paediatric centre.

To control this epidemic, in 2003 we implemented a strict cohort-based segregation policy for all our CF patients alongside our paediatric centre, monitored by regular Psa genotyping which includes markers for the most common known UK
transmissible Psa strains. All patients submit microbiological samples at every clinic visit/inpatient stay, and Psa isolates from those without known LES infection undergo genotyping every 3 months. Psa isolates from patients infected with LES undergo a genotypic check on a yearly basis. In 2009, 1098 genotype tests were carried out on Psa-infected patients. Using this system, we are aware of the Psa genotypes infecting our CF patients at all times.

Based on this, those infected with LES are segregated together into a separate outpatient clinic and a 12-bedded purpose built inpatient facility, all with separate rooms. Patients infected with Burkholderia species or any known other transmissible Psa strains are isolated from all others, both as outpatients and inpatients. Other patients (including those infected without known transmissible strains of Psa and those without Psa infection) are not segregated from each other, since unique Psa strains by definition cannot spread between patients: this group are supervised in the same outpatient clinics and admitted to individual rooms on the same ward for inpatient treatment. Since patients known to be infected with transmissible strains attend outpatient clinics, radiology, lung function, and pathology and physiotherapy departments at different times and on different days to the remainder, we have ensured their complete segregation within the hospital environment.

Patients chronically infected with MRSA are kept within-group, and managed according to the standard cross infection prevention protocols for this organism active in the hospital at the time. All our patients with CF are given strict instructions counselling them against mixing with other CF individuals, and those admitted to hospital agree a contract, which includes this.
We have continued this prospective surveillance for Psa strains among our patients from 2003 to date, and we now report the results of our segregation policy at our centre for 7 years, up to 2009.

### 6.3 Microbiological Methods

Lower airway microbiological samples are obtained for culture at every clinic visit and frequently (at least weekly) during inpatient treatment stays by a qualified physiotherapist. Wherever possible, sputum samples are preferred to cough swabs. The samples are sent in sealed sterile containers to the routine microbiology laboratory where they are processed in the standard fashion, within 24 hours, including subculture for Psa. Representative Psa isolates from each sample are stored for future potential Psa genotyping at -80°C. Using our surveillance protocol (outlined above) isolates are batched for genotypic analysis and the clinical database updated accordingly.

Psa genotyping methods have evolved with time, and these have been incorporated into our laboratory protocol:

**2003-2005**

Psa genotyping was carried out using the RAPD (rapid amplification of polymorphic DNA) technique of sputum Psa isolates from all patients. (Mahentherelingam et al 1996) Ambiguous results were checked using PFGE (pulse field gel electrophoresis).

**2005-2008**
The method was modified during PCR by using the primers PA-SS (Pseudomonas specific bands at 956 bp) and PS21 (LES specific bands at 364 bp) to identify LES-positive isolates. (Panagea et al 2003) LES-negative isolates by PS21/PA-SS underwent further typing using RAPD for comparison with other common epidemic strains (Manchester and Midlands1).

In March 2008, by combining the specific primers and modifying the product mix we identified LES, Manchester and Midlands1 strains from one test (Combined Multiplex PCR). (Fothergill et al 2008) Primer F9 added to the PCR mix increased the specificity of the test to identify LES.

Chronic colonisation with Psa is defined as isolation of the organism in three or more successive samples taken at least 4 weeks apart over a 6 month period. Patients with Psa were classified as LES or other common known epidemic strain positive if any respiratory sample confirmed their presence on genotypic analysis, with the remainder defined as sporadic strains.

Longitudinal follow up of CF patients

Data on all existing patients and new patients joining the Liverpool adult CF centre from 2003 to 2009 were collected from hospital records and our departmental database.

6.4 Results

6.4.1 Clinic Demographics

Over time, the adult clinic population has grown from 148 in 2003 to 244 in 2009. The proportion of patients chronically infected with Psa has diminished year on year, from 84% in 2003 to 74% in 2009 (see Figure 6.1). Of these, 71% were chronically infected with LES in 2003 and this proportion fell steadily to 53% in 2009 (p<0.001).
The proportion of patients transferring into the clinic from the paediatric sector also altered, with progressively fewer patients chronically infected by Psa, and of these, progressively fewer chronically infected by LES (see Table 6.1). During the study period, 56 patients have left the clinic (40 died [30 LES], 12 transferred elsewhere [6 LES], and 4 lost to follow-up [all sporadic strains]. Patients who underwent lung transplantation remained with their original cohort since upper airway Psa infection with their original strain is presumed, whether or not the lower airway becomes re-infected with Psa.
Fig 6.1: Prevalence of chronic Psa infection at the Liverpool CF clinic 2003-2009
Table 6.1: Incidence and prevalence of LES, Other Psa Strains and No Psa Infection at Liverpool Adult Cystic Fibrosis Unit 2003-2009

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<td>LES</td>
<td>95 (71%)</td>
<td>105 (70%)</td>
<td>109 (68%)</td>
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<td>115 (58%)</td>
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<td>30 (23%)</td>
<td>31 (21%)</td>
<td>37 (23%)</td>
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<td>13 (9%)</td>
<td>15 (9%)</td>
<td>22 (12%)</td>
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<td></td>
</tr>
<tr>
<td>Non-Psa New</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>7</td>
<td>12</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Transfers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.4.2 Super-Infection

Over the 7-year period, 2 patients previously chronically infected with unique Psa strains have acquired LES (super-infection) (see Table 6.2). In both cases, these patients had undergone social contact with LES infected patients outside the hospital environment, despite appropriate counselling against this. There was no evidence of any nosocomial contact.
Table 6.2: Details of patients super-infected with LES attending the Liverpool Adult CF clinic 2003-2009

<table>
<thead>
<tr>
<th></th>
<th>Date of joining service</th>
<th>Sample type</th>
<th>Date of conversion to LES</th>
<th>Sample type</th>
<th>Known social contacts with other LES pts</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sept 1992</td>
<td>Sputum</td>
<td>Sept 2004</td>
<td>Sputum</td>
<td>Yes</td>
<td>Socialised with 2 LES patients</td>
</tr>
<tr>
<td>2</td>
<td>Aug 2005</td>
<td>Cough swab</td>
<td>July 2007</td>
<td>Sputum</td>
<td>Yes</td>
<td>Knew 2 LES patients from childhood, frequent socialisation</td>
</tr>
</tbody>
</table>
Pseudomonas Conversion

There has only been one case of new chronic Psa infection in the uninfected cohort, despite attempted eradication. This occurred in 2009 and genotyping revealed a unique Psa strain. A further 6 patients developed acute infections with Psa (unique strains) and these were successfully eradicated using recommended protocols. There were no cases of patients without Psa becoming infected with either a known transmissible or an existing unique strain over the time period.

6.4.3 Other Epidemic Strains

Two patients are infected with the Midlands1 transmissible strain, one since the start of the screening process and another on presentation to our unit in 2005. Both these patients are managed separately from the rest of the clinic population and remain uninfected with any other epidemic Psa strain.

6.5 Discussion:

Traditional thinking suggested that CF patients with chronic lung infections harboured their own unique organisms, and could not transmit these to similar individuals. (Speert et al 1987) However, this was shown to be incorrect with the outbreak of the Burkholderia cepacia complex (Bcc) epidemic in CF patients attending Summer Camps in the US in the late 1980s. (Pegues et al 1994) Not only could these organisms spread to Bcc naïve patients, (White et al 1995, Johansen et al 1998) but we also showed that different Bcc strains could spread to patients already infected to their detriment. (Ledson et al 1998) Subsequent stringent segregation of infected patients by the CF healthcare community halted this epidemic, (Muhdi et al 1996) and now few CF patients harbour these organisms.
However, at that time whether Pseudomonas aeruginosa might possess a similar ability was controversial since strains are indistinguishable by phenotypic methods alone. Although an increase in the incidence and prevalence of multi-resistant Psa was noted in a Danish CF centre in the 1980s, (Pedersen et al 1986) and following phenotypic cohort segregation there was a fall in the annual incidence of new infections, other control measures (early eradication therapy for Psa infection and elective intravenous treatment for those with chronic infection) were also employed. These, coupled with a lack of genotypic identification of the resistant Psa isolates, meant the link between cohort segregation and improving clinical outcomes could not be made.

Transmissible strains may be more antibiotic resistant and have been shown to confer a worse prognosis with increased treatment requirements, (Jones et al 2001, Bradbury et al 2008) inpatient hospital stays, worsening lung function and nutritional state, (Al-Aloul et al 2004) and will ultimately cause excess mortality (Armstrong et al 2002). Prevention of infection of CF patients with these strains is therefore paramount: since they do not survive long in the environment and nosocomial reservoirs have not been found, patient-to-patient contact is the likely source of their acquisition, such that patient segregation becomes the most important infection control measure.

Unfortunately, CF care is complex and requires the coordinated efforts of a multi-disciplinary team: whilst the grouping of patients together at dedicated centres is associated with improved outcomes, it also means they are potentially exposed to pathogens, in particular transmissible Psa strains (Mahadeva et al 1998).

However, the degree of contact necessary between CF individuals to allow transmission of organisms is unknown: although we have shown that LES-infected patients produce an aerosol of viable infected droplets that can be detected for several
hours in the environment, and this clone also has an enhanced ability to survive on hard surfaces compared to other strains, (Panagea et al 2005) the amount of exposure necessary for host acute infection/colonisation with Psa in general remains unclear. Contact density must be an important factor in predicting cross infection: in adult patients, the limited contact possible within the environment of an outpatient visit is unlikely to be sufficient, and where cross-infection has been documented, this has followed an inpatient stay (McCallum et al 2002, Jones et al 2001).

Nevertheless, complete avoidance of patient-to-patient contact is the gold standard to prevent the passage of organisms from one individual to another: under these circumstances, for inpatient care all CF patients would be accommodated in separate areas on different wards, and for outpatient care in separate clinics at separate times of the week.

As regards outpatient care, it is impossible to review all patients entirely separately and some units use the approach whereby each patient remains in a single clinic room and is visited in turn by members of the CF MDT (Saiman et al 2003), thereby ensuring that patient-to-patient contact within the clinic should not occur. However, such a strategy is time consuming, limits the number of patients that can be seen in any one session and even if patients adhere strictly to appointment times, since they are invited to attend the hospital at the same time some mixing cannot be prevented. Furthermore, it is irrational to stringently segregate outpatients but not inpatients, where the risk of cross infection is much greater.

Similarly as regards inpatient care, due to limited healthcare resources such segregation is difficult for most units, and many adopt the policy of admitting patients to the same facility, but in different rooms and with agreed rules of conduct whilst on the ward. However, children and young adults are gregarious and some mixing is
inevitable, especially at those social times in the evening and weekends which are
difficult to police, and it has been shown that this strategy ultimately results in cross-
infection (Jones et al 2010).

Many adult CF clinics therefore use cohort segregation as the most practicable way of
limiting cross-infection. However, Psa strains cannot be separated on phenotypic or
antibiogram patterns (Davies et al 2003) such that policies which rely on these criteria
will inevitably allow cross infection to occur. These include ones that segregate solely
on multi-resistance or the separation of Psa positive from Psa negative patient groups.
In the latter, those with sporadic strains cannot by definition cross-infect, and
therefore their separation from those who are Psa negative is illogical, but they can in
turn become super-infected by those in the Psa positive group with transmissible
strains with the potential for consequent clinical deterioration (McCallum et al 2001).

It therefore follows that, in order for any cohorting policy to be effective, the clinician
needs to be aware of the strains of Psa in their CF clinic population in real time. This
can only be achieved by Psa genotyping on a regular basis, allowing those with
transmissible strains to be segregated from all other individuals.

It is this policy we adopted in 2003 in our developing adult CF clinic, where
increasing numbers of patients already infected with transmissible Psa (LES) were
arriving from the local paediatric centre as they reached adulthood, and a cross-
sectional survey had shown a high prevalence of LES amongst our patients. Our
results show that by using regular genotypic surveillance of Psa strains and
segregating patient groups accordingly, we have prevented infection by nosocomial
contact. The very few patients who have developed super-infection all did so through
well documented social contact outside the hospital environment, despite advice to the
contrary. It is of note that other units undertaking Psa genotypic analysis but without
effective patient segregation measures in place have noted a high cross infection rate with LES during this period. (Jones et al 2010) Furthermore, although we have not separated those without Psa infection from those infected with sporadic Psa strains, there has been only one new case of chronic Psa infection in this group (with a unique strain), underlining that it is unnecessary to separate these patient cohorts. A further 6 cases of acute Psa infection with unique strains occurred, all of which were successfully eradicated – although previous studies (Frederickson et al 1999, Lee et al 2004, McKay et al 2009) reported a higher incidence of such acute infections, these were carried out in a largely paediatric population where the prevalence of chronic Psa infection is much lower.

There are financial consequences to adopting this genotypic surveillance protocol: each test costs approximately £20, and with the addition of technician time the yearly cost to our clinic is currently £22,000. This is likely to increase further, since although the relative numbers of patients infected with Psa strains is diminishing with time due to better cross-infection control and eradication therapy (particularly in paediatric practice), the absolute numbers continue to grow as more patients live longer.

Nevertheless, we believe that not only is the cost of this testing outweighed by the clinical benefit, but there are strong economic arguments for its use. Firstly, segregating patient by other methods would necessitate an alteration in clinic and ward infrastructure, which would be costly and for some units impossible, and secondly cross infection with transmissible strains has been shown to confer an increased healthcare cost burden (Ashish et al 2010). Finally, the emerging medico-legal consequences of allowing cross infection between CF patients within the hospital environment, which can be costly, are also avoided.
In conclusion, we recommend the use of genotypic surveillance of Psa strains, to allow rational segregation of CF patients. Using such a method, we have halted the epidemic in our clinic of LES, the most prevalent and important transmissible Psa strain within the CF community.
Chapter 7. General discussion:

7.1 Conclusions

In conclusion, this thesis has summarized some of the difficulties associated with chronic colonisation with the LES in the adult CF population. It has highlighted some of the problems faced by these patients, especially the quality of life and the psychosocial elements of chronic infection so often overlooked.

Some of the microbiological aspects previously suspected to be associated with LES, such as enhanced antibiotic resistance, have been proven beyond any doubt in this thesis. We have shown the enhanced antibiotic resistance expressed by LES but also demonstrated that LES acquires antibiotic resistance much faster than the non-epidemic strains. The reasons for such a characteristic of LES remain unexplained.

Other characteristics attributed to LES, such as phenotypic diversity, presumed to be a reason for its success, have proven to be general characteristics of *P. aeruginosa* during chronic lung infections in CF. Our study of patients chronically infected with Non-LES strains has revealed an extensive phenotypically diverse population. We believe it is a characteristic of *P. aeruginosa* strains in general rather than a specific trait of LES or particular Non-LES strain. This has been confirmed by others, who have also reported extensive within-population phenotypic diversity for *P. aeruginosa* in CF (Darch et al 2015, Workentine et al 2013). This raises the question as to why LES is so successful in colonising the CF lungs and causing morbidity and mortality. Research needs to address these questions at a molecular and basic microbiological level. A much more detailed understanding of LES is needed at a very basic level to further understand the reasons behind its dominance. There have been attempt to do this using genomics technologies. In addition to the genome of LESB58, genome sequence data is now available for a number of other LES isolates from the UK and
Canada. A recent study using genome sequencing suggests that chronically LES-infected patients in the Liverpool CF Unit often harbour co-existing but divergent sub-lineages of the LES (Williams et al 2015). The clinical significance of this is not yet understood, but it further emphasises the complexity of the infecting populations of bacteria in the CF lung.

Another feature of the LES that could help to explain its success in competition with other strains of *P. aeruginosa*, is its carriage of multiple prophages (Winstanley et al 2009). A recent study has demonstrated that these LES prophages are very active in the production of free phages in the sputum of CF patients. (James et al 2015) These represent considerable weapons against other competing strains and may contribute to the ability to cause superinfection.

The host-bacterium interactions also need to be studied at a more detailed level. Cytokine responses (possibly exaggerated), long-term host adaptation to recurrent infective episodes and the role of viral agents in initiating these exacerbations need to be addressed. We also need to elucidate as to why some patients have rather well preserved lung function, better general health and are less susceptible to infective exacerbations, despite having the same genetic mutation and colonising organisms. In order to understand these issues, we not only have to study LES in detail but have to examine different hosts to understand these variable response and health effects.

The thesis also sheds light on some of the larger issues faced by the institutions caring for CF patients, such as financial and infection control. It has been proven in the past that chronic infection with the LES in adult CF patients causes a rapid decline in lung function, BMI and overall health of an individual. This thesis also looks at the these effects further and investigates the burden LES places on the QoL of these individuals and wider resource implication at an institutional level caring for such patients. Our
institution looks after the largest number of patients chronically infected with this strain in the UK and possibly in the entire adult CF community. As a result of research within this thesis we have been able to show the health effects of chronic colonisation with this strain on the individual and at an institutional level. We have demonstrated that chronic colonisation with LES on an individual level causes not just physical decline in health but also results in a poor QoL on several measureable variables compared to individuals colonised with non-LES variant. This may further lead to poor compliance with medications, resulting in health decline that we witness with these individuals. Although now we know that the LES can affect HRQoL outcomes, we need to address this issue more proactively and perhaps encourage patients to do this questionnaire on a yearly basis and offer the psychological support in individuals where a need is identified. In this way perhaps we could address the psychological burden of this disease. At the same time it would be interesting to study whether such an intervention may have any positive benefit to the lung function and to the overall outcomes of physical health.

In previous studies we have identified that these strains are highly infectious and transmissible. This study looks at the preventative measures taken in our institution to control cross infection. We have demonstrated quite successfully that implementation of strict policies for elective and emergency hospital visits in a cohort of patients with various strains can be successfully implemented with minimal cross infection over a five-year period. The implementation of such policies requires a substantial monetary and personal investment. We need to be aware of the Psa genotype of all colonised individuals on a regular basis, especially those who harbour a non-LES strain. Cross infection issues such as those studied in CF, may also be relevant for other respiratory
infections, such as *P. aeruginosa* infections in non-CF bronchiectasis patients. Although one recent study, based on a single centre, suggested that cross infection might not be a common problem (De Soyza et al 2014), however further work is needed in this area.

It remains a challenge to ensure there is no cross-infection, especially since the advent of a variety of ways of virtual social interaction among CF patients. As young adults they may trivialize the education we provide in order to fulfil their social intuitiveness. However our patient cohort remains well informed of the physical decline in heath that is seen with LES infection. Most CF patients have a social life, which may lead to cross infection. At an institutional level this causes severe problems of cross infection and to be able to care for hundreds of adult CF patients with various strains in itself remains a challenge.
7.2 Areas for future work:

Despite an improved understanding of LES infections in patients with CF there are various aspects, which remain unclear. Some of the key questions that remain unanswered are:

1. Why do only some patients with LES infection have severe ill effects whereas some individuals despite chronic colonisation have minimal co-morbidities?

2. Why do female patients have a poorer outcome with LES infection?

3. Strains such as the LES have genotypic and phenotypic features specific to it, so could we develop targeted therapies to eradicate /treat LES infection?

4. What is the role of viral infections in acquisition of LES?

5. Cross infection issues in Non-CF bronchiectasis

More work needs to be carried out in this field, particularly in patients with chronic LES infection, to address these various questions which may help us look after these patients better, enabling them to lead a more fulfilling and normal life.
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