Effect of catecholamines and inhaled drugs on the growth and virulence of bacterial respiratory pathogens

Dr Priti N. Kenia

(MBBS, MD (Paediatrics), DNB, MRCPCH)

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Abstract

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Background: *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Burkholderia cenocepacia (B. cenocepacia)*, a member of the *Burkholderia cepacia complex* (*Bcc*) are common biofilm forming pathogens in Cystic Fibrosis (CF) patients. *Bcc* contributes to mortality in CF lung transplantees. Drugs such as catecholamines and salbutamol are known to interact with bacteria in general. In CF and post transplanted patients, both these drugs are commonly used and can possibly interact with *P. aeruginosa* and *B. cenocepacia,* and enhance their virulence.

Aim: 1. To evaluate whether catecholamines affect the growth and virulence of *B, cenocepacia. 2.* To evaluate whether Salbutamol affects the growth and virulence of *B. cenocepacia* and *P. aeruginosa*. 3. To evaluate whether there was any interaction of Burkholderia with the ciliary epithelium and if the drugs catecholmaines and salbutamol affected this.

Methods: *In vitro* methods for growth, attachment and biofilm formation were carried out for *B. cenocepacia* with drugs, catecholamine and salbutamol, and for *P. aeruginosa* with salbutamol. To give a clinical context, biofilm formation on endotracheal tubes and *ex vivo* studies on healthy and CF airway epithelial cultures with and without supplemental drugs were carried out.

Results: *B. cenocepacia* were found to be catecholamine responsive organisms. Catecholamines increased the growth and biofilm formation of *B. cenocepacia*. Salbutamol did not influence the growth of either *B. cenocepacia* or *P. aeruginosa*, but increased the cell to cell aggregation. On endotracheal tubes both drugs enhance the formation of mature biofilms. *B. cenocepacia* infection on airway cultures did not affect ciliary beat frequency but attached to ciliary tips by five hours and was able to form mature biofilms and this was enhanced in presence of catecholamines and in CF epithelial cultures.

Summary: Catecholamines increase the growth, attachment and biofilm forming ability of *B. cenocepacia*. Salbutamol, a commonly used respiratory drug has an influence on the cell to cell aggregation for bacteria. *B. cenocepacia* produce biofilm within 5 hours after infection of the airway epithelium especially in CF epithelial cultures and more so with catecholamines. This observation is relevant to CF patients to guide clinical practice and detect mechanism of bacterial infection which may offer a therapeutic target for CF patients.

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I would like to dedicate this thesis to my beloved husband Anish, my lovely daughter, Prisha and my parents. May dad's soul rest in peace.

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Abbreviations

ASL	Airway surface liquid
AHL	acyl-homoserine lactone
AI	Auto Inducer
$\alpha_{1,}\alpha_{2}$	$Alpha_{1,2}$
ALI	Air liquid interface
ATP	Adenosine transphosphatase
Bcc	Burkholderia cepacia complex
$\beta_{1,}\beta_{2,}\beta_{3}$	$Beta_{1,2,3}$
CBF	Ciliary beat frequency
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CF	Cystic fibrosis
CFU	Colony Forming Unit
CO_2	Carbon dioxide
Cpm	Counts per minute
Dop	Dopamine
E.coli	Eschericia coli
Epi/Ad	Epinephrine/Adrenaline
EPS	Extracellular polymeric substance
ET	Endotracheal Tube
Fe	Ferric nitrate
Hz	Hertz
IL-13	Interleukin-13
IgG	Immunoglobulin G
LB	Luria Bertani broth
Lf	Lactoferrin
MCC	Mucociliary clearance
NK	Natural killer
NE/ NA	Norepinephrine /Noradrenaline
PBS	Phosphate Buffered Saline
QS/QSe	Quorum Sensing
Rpm	Revolutions per minute
SEM	Scanning Electron Microscopy
Tf	Transferrin

W/v	Weight/ volume
mM	Millimole
µM	Micromole
Mg	Milligram
µg	Microgram
µL	Microlitre
Ng	Nanogram
Ml	Millilitre
L	Litre
M	Mole
IU/ml	International units/ millilitre
Mm	Millimetre
µCi	Microcurie
^{o}Celcius	Degrees Celcius

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Chapter One

Introduction

1.1 Overview

The lung is constantly exposed to the environment. It is at risk of exposure to a large number of airborne pathogens and other toxic agents from the environment as a result of inhalation of several thousands of litres of air per day. The lungs have a large epithelial surface area and the respiratory epithelium lined with several types of cells and mucus that contains cytokines and protective macromolecules plays an important role in the host defence system. The respiratory epithelium is a highly effective barrier to microbes. Conducting airways are lined with pseudostratified columnar epithelial cells that become cuboidal as the branches extend to the alveoli. The pseudostratified epithelium primarily consists of ciliated, basal, goblet and other secretory cells, including Clara cells.

The ciliated respiratory epithelium forms a natural barrier to invasion and injury by inhaled pathogenic organisms and particulate material. The epithelium is lined by the periciliary fluid, sol, and the mucus gel layer, which together constitute the airway surface liquid (ASL). The ASL provides an ideal environment in which the cilia beat. The mucus gel layer is cleared from the airway by the highly coordinated ciliary beating. This process, known as mucociliary clearance (MCC), is an essential factor in pulmonary defence. Effective MCC depends on the structural and functional integrity of the cilia and quantitative and qualitative properties of the ASL. Alterations in any of these components may result in impaired MCC that leads to mucus retention and increased susceptibility to airway infection.

It is now widely accepted that the lungs are not sterile and house a microbial community consisting of a complex variety of microorganisms found in the lower respiratory tract

particularly on the mucous layer and the epithelial surfaces. These microorganisms include bacteria, fungi, viruses and bacteriophages and constitute the lung microbiota. The harmful or potentially harmful bacteria are also detected routinely in respiratory specimens. In disease states, the human innate system is impaired and changes in the microbial community composition allow pathogenic bacteria to become significant and cause disease progression. *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus*, and *Burkholderia cenocepacia* (*B. cenocepacia*) are found most often in CF patients and in patients who have had lung transplantation and form biofilm within their lungs.

Due to its direct access, the lungs make an attractive route for delivery for inhaled drugs such as salbutamol which is predominantly used in respiratory diseases such as asthma and CF. Many drugs also undergo metabolism in the lungs and their end products can be found dissolved in lung fluid. Medications such as salbutamol used in these diseases, are known to affect the ciliary function and also interact with bacterial pathogens, affecting their growth, virulence and biofilm formation. Catecholamines such as dopamine and norepinephrine are used as medications for low blood pressure in septic patients on intensive care units and in post transplantation patients are known to increase the growth of bacteria. They are naturally found in high concentrations in disease states such as CF and CF patients post lung transplantation and can potentiate the growth of bacteria present in these patients.

The lungs thus provide a stage to orchestrate the interplay between host mechanisms, pathogens and drugs. In patients with CF and post lung transplantation, there is plenty

of substrate and opportunities for complex interaction between host, pathogens and drugs and potentially affecting the outcome of disease.

This work focusses on investigating the growth, virulence and biofilm formation of *P. aeruginosa* and *B. cenocepacia*, involved in CF and lung transplantation patients, and whether drugs such as catecholamines and salbutamol have the potential to exacerbate the damage caused by these problematic opportunistic pathogens.

1.2 Structure of ciliated respiratory epithelium and function of cilia

1.2.1 Summary

This section focusses on the structure and function of human ciliated respiratory epithelium, the ASL layer, the structure of the cilia and the MCC in health. The human ciliary structure and function is described in some greater detail followed by an overview of factors that regulate ciliary function.

1.2.2 Structure of human respiratory epithelium

The respiratory epithelium consists of many distinct cell types that are characteristic of the proximal conducting or cartilaginous airways. The epithelial morphology changes to single columnar or cuboidal epithelium at the level of the terminal bronchioles and cuboidal or squamous at the alveolar level. The respiratory epithelium is lined by ciliated pseudo stratified columnar epithelium which has several type of cells- (Breeze, et al, 1977, Spina et al, 1998, Pucehelle et al, 1998)- ciliated columnar cells, secretory cells (including mucus producing goblet cells and Clara cells) and basal cells Submucosal glands are present along the tracheal-bronchial tree. The epithelium is lined by the airway surface liquid layer in which the cilia beat freely and in a highly coordinated fashion moving along pathogens or particulate materials for clearance.

The epithelial layer forms a tight barrier and the luminal junctional complex that is composed of the most apical tight junctions and adherens junctions that attach cell wall to adjacent basal membrane or to adjacent cell membranes. This complex is disturbed by inhaled micro-organisms and in a variety of airway diseases, including asthma and CF. This impaired epithelial barrier function also impacts susceptibility to respiratory infections.

Figure 1.1 gives a schematic diagram and a histology cross section view of the human respiratory pseudostratified epithelium.

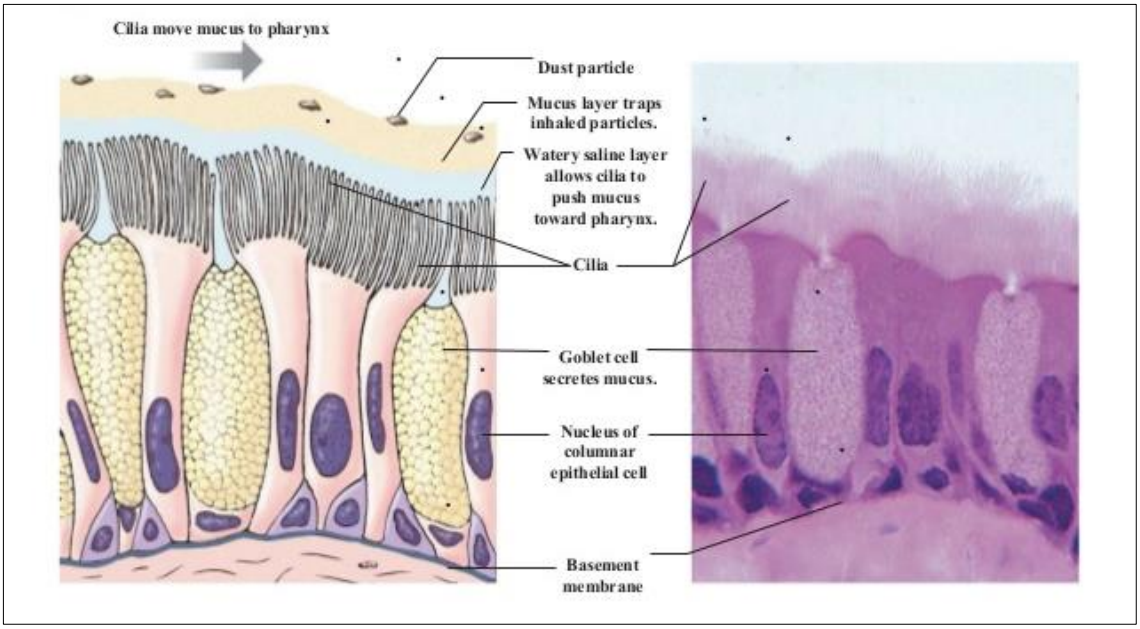


Figure 1.1: Schematic diagram and histology picture of a cross section of the human pseudostratified respiratory epithelium showing various types of cells, cilia and overlying airway surface liquid.

A brief description of the structural respiratory epithelial cells is given below.

1.2.2.1 Ciliated columnar respiratory epithelial cells

The well differentiated ciliated columnar epithelial cell accounts for >50% of all the cells (Spina et al, 1998) and extend up to the apical surface. (Antunes and Cohen 2007),

Each cell possesses approximately 200-300 cilia that project from the apical cell surface (Rhodin et al, 1966, Harkema et al, 1991) as shown in Figure 1.2. The large number of mitochondria present in the cells is involved in provision of energy for normal ciliary function.

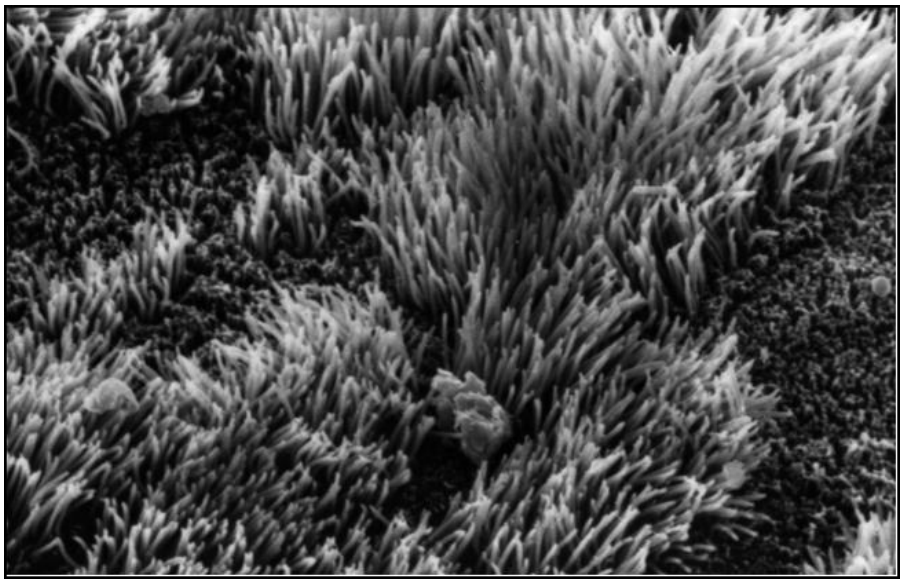


Figure 1.2: Scanning electron microscopy (SEM) image of ciliated respiratory epithelium of human nasal mucosa. Scale bar = 7µm. This image was taken by Mr. Andrew Rutman, University of Leicester and has been used with his permission.

1.2.2.2 Basal cells

Basal cells are pyramidal in shape and are abundant in the epithelium of the conducting airways, decreasing in numbers towards the distal airways. Basal cells play an important role in maintaining normal epithelial structural integrity. They are believed to be multipotent airway progenitor cells which help contribute to epithelial regeneration following airway injury, by developing into terminally differentiated epithelial cells.

1.2.2.3 Goblet cells (mucus cells)

Goblet cells are interspersed among the ciliated cells and contain mucin rich granules which release mucus into the luminal surface. Quantitative and qualitative alterations in

airway mucus may play a role in the pathophysiology of diseases such as asthma and CF.

1.2.2.4 Clara cells

Clara cells are present in both proximal and distal airways. They have a secretory role and they produce surfactant within the alveoli. Clara cell secretory protein expressing cells are also recognised to be multipotent airway progenitor cells in the bronchiolar epithelium.

1.2.3 Airway surface liquid (ASL)

ASL is made up of the periciliary fluid, sol and the mucus gel layers (Wanner et al, 1996, Girod et al, 1992, Boucher et al, 1994) and covers the underlying epithelium. ASL is rich in water, mucus, cytokines, and macromolecules such as lysozymes, lactoferrin, immunoglobulins, glycoproteins and lipids, many of which are important in the innate defence (Travis et al, 1999, Widdicombe et al, 1995). The periciliary fluid lubricates the epithelial surface and is an ideal environment for the cilia to function within. The depth of the periciliary fluid is important for effective ciliary function. Epithelial ion channels such as sodium channel (ENaC), calcium dependent chloride channel and the Cystic Fibrosis Transmembrane Regulator (CFTR) protein are involved in the production of the periciliary fluid. Cytokines and other stimuli in the microenvironment regulate these channels and affect the depth of this layer (Danahay et al, 2002, Galietta et al, 2000, Galietta et al, 2002). The overlying mucus layer (Boucher et al, 2003, Tarran et al, 2001) and the shear forces created by the beating cilia (Boucher et al, 1994 et al, Sleigh et al, 1998) both also contribute to maintaining the depth and the

spread of the periciliary fluid. Many of these factors are affected in presence of infection, by drugs and in diseases such as CF.

The mucus layer (gel layer) lies above the periciliary fluid and forms a protective barrier for the underlying epithelium (Girod et al, 1992, Houtmeyers et al, 1999). It is predominantly made up of water and other components include proteins, mucins such as MUC5AC and MUC5B, salts (Tarran et al, 2001) and enzymes and immunoglobulins (Houtmeyers et al, 1999). Factors such as acidity, osmolality and water content influence the mucin hydration and in turn the maintenance of the mucus layer (Houtmeyers et al, 1999).

1.2.4 Structure and function of cilia

Cilia are microscopic hair-like structures arising from basal bodies in the epithelial columnar cells and protruding in the luminal surface of the cells. There are two types of cilia in mammals: Primary cilia and motile cilia, and they differ in ultrastructure, location and function (Satir et al, 2007).

The primary cilia are usually non-motile and are located as sensory organelles in the pancreas, thyroid, renal tubule and bile duct (Veland et al, 2009). The only motile primary cilia are found in the embryonic node in early development and are thought to have an important role in determining left-right asymmetry of the internal organs (Nonaka et al, 1998, McGrath and Brueckner, 2003, Okada et al, 2005).

The motile cilia are found on the epithelial cells in the respiratory epithelium, ependymal cells lining the brain ventricles and the female oviduct (Schidlow et al,

1994). The motile cilia are present throughout the lung epithelial surface area with approximately 200 cilia per cell (Rhodin et al, 1966). The cilia in the proximal airways are approximately 6µm long and 0.3µm wide (Breeze et al, 1977, Lee 1977, Sleigh et al, 1988). The cilia are also surrounded by microvilli that measure approximately 1-3µm in length and 0.1-0.3µm in width (Busuttil et al, 1977, Satir, et al 1990). The cell apparatus contains mitochondria that provide energy for ciliary function (Breeze et al, 1977, Lee et al, 1997, Rhodin et al, 1966) (Figure 1.3).

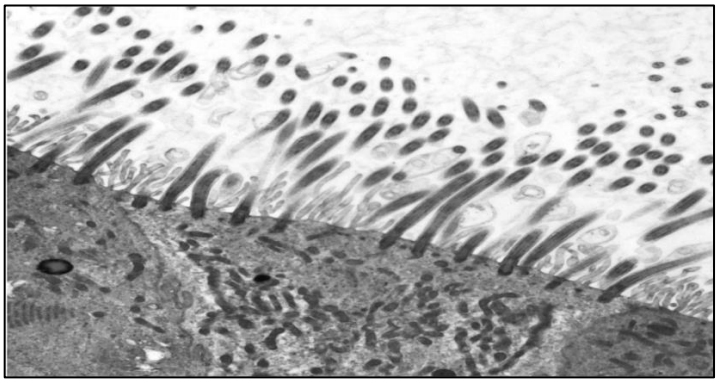


Figure 1.3: Transmission electron microscopy (TEM) image of human ciliated respiratory epithelium showing healthy ciliated epithelial cell with cilia and microvilli. This image was taken by Mr. Andrew Rutman, University of Leicester and has been used with his permission.

Cilia in the human respiratory epithelium beat in a highly coordinated fashion. The ciliary beat frequency (CBF) is 11-14 Hz at body temperature (Wanner et al, 1996, Sleigh et al, 1998, Chilvers et al, 2003). The beat pattern of cilia is asymmetric and consists of a fast ‘forward’ stroke followed by a slower ‘backward’ recovery stroke (Chilvers and O'Callaghan, 2000a) (Figure 1.4). This backward and forward coordinated motion moves the overlying mucus to enable clearance of particles, allergens, and organisms and maintains the health of the lungs, forming the mucociliary clearance mechanism.

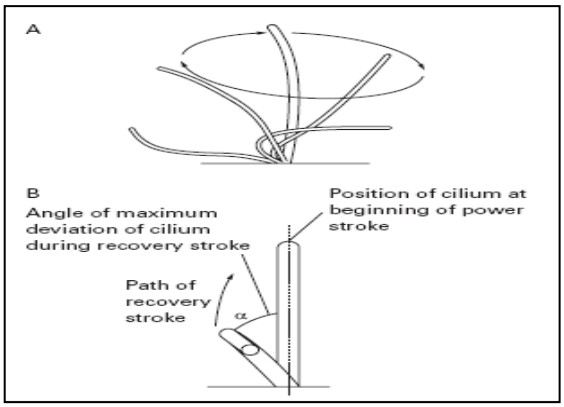


Figure 1.4: Planes of view used to observe and record the ciliary beat cycle and beat frequency. (A) Side profile of the ciliary beat pattern. (B) The cilium is viewed beating forward from the plane of the paper. Taken from (Chilvers and O'Callaghan 2000a).

The motile cilia has an axoneme which consists of nine doublet microtubules, dynein arms and radial spokes surrounding a central pair of singlet microtubules (Figure 1.5). This forms the 9+2 arrangement which extends from the basal body held in the cell cytoplasm. In contrast, the primary cilia have a 9+0 ciliary axoneme where the central microtubular pair is absent and they do not possess dynein arms with the exception of the primary nodal cilium. The cilia develop by a process called ciliogenesis which involves a series of events including the development of the basal bodies followed by generation of the ciliary axoneme with assembly of multi-protein complexes that are transported along the length of the axoneme by a process called intra-flagellar transport (Pan et al, 2007).

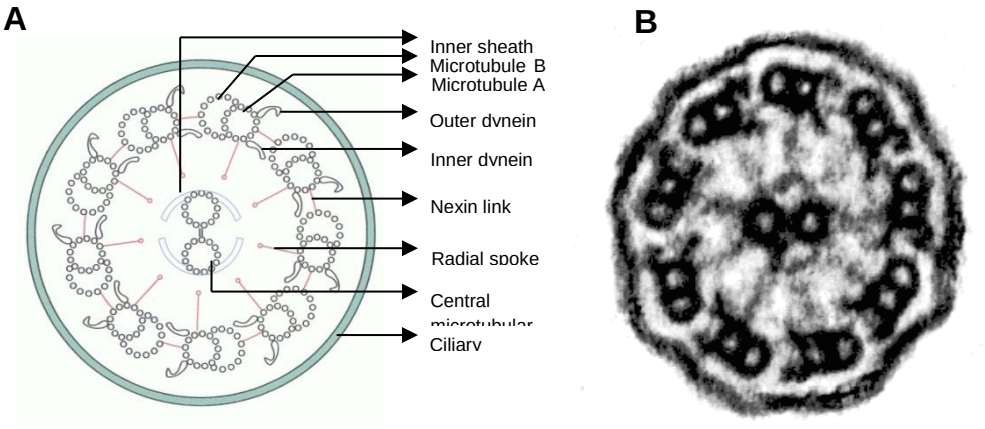


Figure 1.5: A – Schematic diagram of cross sectional image of human respiratory cilium, illustrating the classical '9+2' arrangement (taken from PhD thesis of Dr. Mina Fadaee-Shohada, University of Leicester, 2010, with permission)**.** B – Cross sectional transmission electron microscopy image of human respiratory cilium. This image was taken by Mr. Andrew Rutman, University of Leicester and used with his permission.

1.2.5 Factors regulating ciliary beat frequency

Ciliary beat frequency (CBF) is regulated by major intra-cellular messengers including calcium (Braiman et al, 1998, Zagoory et al, 2001), cyclic adenosine monophosphate (cAMP) (Lansley et al, 1992, Wyatt et al, 2005), cyclic guanosine monophosphate (cGMP) (Wyatt et al, 2005, Schmid, et al 2007) and also nitric oxide (Uzlaner et al, 1999, Salathe et al, 2007). Mechanical shear forces and mechanical stimulation by the overlying mucus layer mediating its effect via calcium pathways also affect the CBF (Felix et al, 1998, Sanderson et al, 1990). Both sympathomimetic and para-sympathomimetic agents enhance ciliary beat frequency (Verdugo et al, 1980, Wong et al, 1988) by activating the second messenger systems.

Ciliary activity is also affected by a variety of internal and external stimuli. The temperature (Green et al, 1995, O'Callaghan et al, 1995), pH (Clary-Meinesz, et al 1998), tonicity (Luk et al, 1983, van de Donk et al, 1980), viscosity (Luk et al, 1983), humidity (Horstmann et al, 1977, Mecke et al, 1976) and pressure of the overlying

periciliary fluid are all known to influence CBF (Calvet et al, 1999). Inflammatory mediators present in the airway epithelium in disease states can enhance (E.g.: Histamine, acetylcholine) (Wanner et al, 1975) or reduce (E.g.: Platelet-activating factor and eosinophil major basic protein) (Del Donno et al, 2000) the CBF.

A wide variety of pharmacological agents have also been shown to exert an effect on ciliary function (Abanses et al, 2009, Hofmann et al, 2010, Mallants et al, 2007, Mallants et al, 2008, O'Callaghan et al, 1994, Centanni et al, 1998). The diverse effect of medications on CBF has been extensively reviewed by Rusznak et al (Rusznak et al, 1994) and the majority of the medications are depressive. However, two of the commonest inhaled medications, short and long-acting Beta$_2$-agonists (β_2-agonists), salbutamol and salmeterol respectively, have been found to increase ciliary beat frequency (Hasani et al, 2003). β_2-agonists are known to improve pathologically reduced mucociliary function in patients with asthma (Pavia et al, 1987) or chronic bronchitis (Sackner et al, 1978). The long-acting β_2-agonist, formoterol is a powerful ciliary stimulant (Lindberg et al, 1995) that has been shown to increase mucociliary clearance by 46% after 6 days of treatment in patients with chronic bronchitis (Melloni et al, 1992). Inhaled corticosteroids do not appear to directly affect MCC (Sackner et al, 1977, Duchateau et al, 1986) and the improved MCC observed with inhaled corticosteroids in asthma patients is thus most likely a result of their anti-inflammatory properties (Agnew et al, 1984, Messina et al, 1993).

The CBF is tightly regulated by maintaining the microenvironment, and slight fluctuations in any of these factors do not have any adverse effects on the ciliary function or the mucociliary clearance. However, alterations in these factors are likely to

be exaggerated in disease states significantly affecting the ciliary function. CBF alteration in CF and lung transplantation, in presence of pathogens such as *P. aeruginosa* and *B. cenocepacia* and in presence of drugs such as catecholamines and salbutamol are discussed in detail under each relevant section.

1.2.6 Mucociliary clearance (MCC)

Approximately 20-30ml of ASL is produced every day. The cilia beat in this layer in a coordinated manner moving the overlying mucus and trapped particles (Boucher et al, 1994a, 199b, Wanner et al, 1996) (Figure 1.6). The ASL traps any particles or pathogens >5µm that reach the airways and move these upwards towards the pharynx where they are either swallowed or expelled via coughing (Antunes and Cohen, 2007, Stanke et al, 2015, Sleigh et al, 1988). This transport system to clear the mucus and foreign particles is called mucociliary clearance (MCC) (Figure 1.6). This is probably one of the most important innate defence mechanisms of the lungs. For this system to function effectively, it requires integrity of structure and function of ASL and cilia (Wanner et al, 1996).

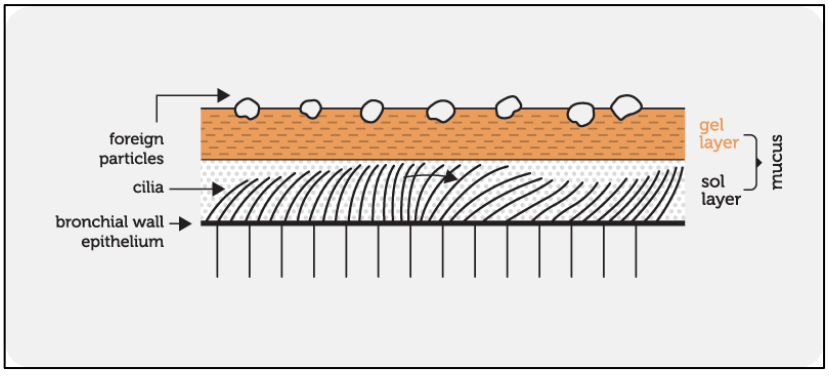


Figure 1.6: Diagrammatic representation of the mucociliary clearance showing the air surface liquid sol and gel layers, overlying the ciliated respiratory epithelium and coordinated sweeping movement of the cilia moving entrapped foreign particles

1.3 Lung microbiome

The microbiome is defined as the "ecological community of commensal, symbiotic and pathogenic organisms that share our body space" (Lederberg J et al, 2001). Medical texts refer to a sterile lung environment but it is clear that microbiota exists and interacts with the lung (Beck et al, 2012).

The lung is an organ constantly exposed to microbiota either through inhalation or subclinical micro aspiration from birth. The microbiome of the lung has relatively less bacterial biomass when compared to the lower gastrointestinal tract yet displays considerable diversity (Sassone-Corsi, 2015, Savage, 1977, O'Dwyer et al, 2016). There is growing appreciation for the fact that the gut commensal microbiota is an important regulator of the innate immune system forming the gut-lung axis (Abrahamsson et al, 2012, Bruzzese et al, 2014, Clarke, 2014) (Figure 1.7). There is evidence to support a crucial early period during life where intestinal microbiome development is important for the regulation of an appropriate immune response in the lung. CF and asthma are examples of chronic lung disease where disease course and susceptibility are influenced by shifts in the composition of the gut microbiota and could be the result of dysbiosis leading to altered airway microbiota and disproportionate inflammation.

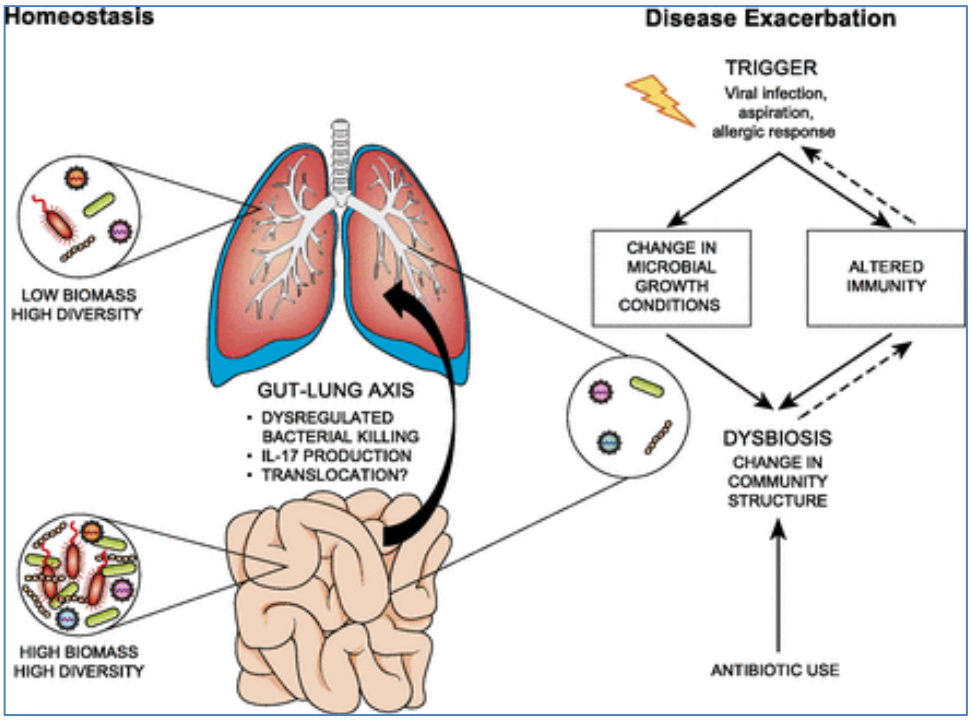


Figure 1.7: The gut-lung axis and dysbiosis causing inflammation and contributing to chronic lung disease.

The bacterial part of the microbiota has been more closely studied and consists of a core of nine genera consisting of aerobic, anaerobic and aerotolerant bacteria (Morris et al, 2013).. The bacterial microbiota is highly variable in particular individuals (Beck et al, 2012). There are a mean of 2000 bacterial genomes per square centimetres surface.

The composition of the lung microbiome is determined by elimination, immigration and relative growth within its communities and this changes dramatically during acute and chronic lung disease [O'Dwyer et al, 2016). Consequently, the community membership of the lung microbiome is altered in disease states with a shift in community composition away from the *Bacteroidetes* phylum, which dominates the healthy lung microbiome, towards *Proteobacteria*, the phylum that contains many familiar lung-associated gram-negative bacilli (Cox et al, 2012, Schenck et al, 2016).

The harmful or potentially harmful bacteria are detected routinely in respiratory diseases. *P. aeruginosa*, *Staphylococcus aureus,* and *Burkholderia cepacia* found most often in CF patients (Surette et al, 2012). The chronic colonization of the lower airways by bacterial pathogens is the leading cause of morbidity and mortality in patients with CF.

In CF, the altered airway milieu results in a thick mucus secretion that impairs normal innate immune defence, including impaired mucociliary clearance (O'Sullivan et al, 2009, Boucher, 2007). Consequently, the lower airways become colonized by bacteria. Traditionally, only a few organisms have been associated with chronic airway infections in CF and there is a progression of these from early childhood to adulthood. *Haemophilus influenzae* is an early colonizer of the CF airways, followed by *Staphylococcus aureus* (Surette et al, 2012). *P. aeruginosa* is the most common CF airway pathogen, affecting up to three-fourths of adults, and is the primary CF pathogen (Lyczak et al, 2002, Lipuma et al, 2010). *Bcc* bacteria are emerging CF pathogens and are a significant cause of morbidity and mortality especially in CF patients post lung transplantation (Surette et al, 2012). It is now widely recognized that the lower airways of patients with CF are colonized by a more complex polymicrobial community (LiPuma, 2012, Lynch and Bruce, 2013, Sibley et al 2006, Rogers et al, 2010). Mounting evidence supports ways in which microbiota dysbiosis can influence host defense and immunity, and in turn may contribute to disease exacerbations. Thus, the key to understanding the pathogenesis of chronic lung disease may reside in deciphering the complex interactions between the host, pathogen and resident microbiota during stable disease and exacerbations.

1.4 Lung infections in childhood

Acute lower respiratory tract infection or pneumonia is the leading single cause of mortality in children aged less than 5 years (GBD collaborators, 2017). Determining the etiology of these clinical manifestations is a challenge, and so empirical therapy is therefore adopted in most cases (Ball et al, 2002). The causative organisms are believed to be mainly respiratory viruses but bacteria, *Streptococcus pneumoniae* and *Haemophilus influenzae* in young children and *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* in older children are also commonly causative (Woolf and Daley, 2007).

Most CF patients experience acute symptoms during episodes of pulmonary exacerbations, and develop progressive lung disease caused by both chronic airway infections and host inflammation leading to development of chronic suppurative lung disease (Boucher et al, 2012). It is increasingly apparent that the microbial constituents of the lower airways in CF exist in a dynamic state (Surette et al, 2012). Indeed, while changes in prevalence of various pathogens occur through ageing, differences exist in different cohorts of patients from different regions and in different institutes. Classical pathogens are *P.* aeruginosa, *Bcc* and *Staphylococcus aureus* (Surette et al, 2012).

P. aeruginosa, the dominant airway pathogen, chronically infects up to 60–75 % of adult CF patients (Kerem et al, 1990), and is strongly associated with inflammation, decline in lung function and increased mortality (Nixon et al, 2001, Li, 2005).

Staphylococcus aureus, on the other hand, is the most prevalent organism during childhood, and often the first one isolated in CF children (Razvi et al, 2009). The

overall prevalence of *Staphylococcus aureus* infections has increased over time, both with methicillin sensitive (*MSSA*) and methicillin resistant (*MRSA*) (Razvi et al, 2009). Several studies have examined the clinical impact of *Staphylococcus aureus* infections, on infants and children [Wong et al, 2013, Glikman et al, 2008, Sly et al, 2009). Infection in young children is associated with a decline in lung function but as patients transition from adolescence into adulthood, it's prevalence decreases gradually and are not consistently associated with poor prognosis, but perhaps even better survival especially post-transplantation (Liou et al, 2001, 2007). The clinical significance of *Staphylococcus aureus* in adolescent and adult CF patients remains unknown.

Haemophilus influenzae is frequently present in respiratory tract cultures of young patients with CF with prevalence decreasing in adulthood. *Haemophilus influenzae* can be part of normal respiratory flora in healthy children, but is a significant pathogen in other disorders. *Haemophilus influenzae* is not consistently associated with rapid decline in lung function in CF. A study by Vandenbranden suggested that *Haemophilus influenzae* was one of the factors associated with decline in lung function in CF (Vandenbranden et al, 2011) while another showed preserved lung function in *Haemophilus influenzae* colonised individuals (Hecter et al, 2016).

As the clinical relevance of *Staphylococcus aureus* and *Haemophilus influenza* is as yet fully unclear in CF and these bacteria mainly affect the younger CF population without clear evidence of lung disease progression, I chose not to study these organisms within my study at this stage. *P. aeruginosa* and *Bcc* are more pathogenic and have a significantly associated with lung disease progression, morbidity and even mortality in

CF as well as post transplantation, were the two bacteria that I decided to work with due to their greater clinical relevance.

1.5 Bacteria studied in this work

1.5.1 Summary

Lung diseases such as CF are of interest to health care professionals as they are associated with a high morbidity and mortality related to chronic infection and eventual respiratory failure. Lung transplant is a treatment option for end stage respiratory failure in CF. Despite advances in management, infection remains the major cause of death in patients post lung transplantation. The two major pathogens strongly associated with CF and in those with lung transplantation are *P. aeruginosa* and *B. cenocepacia*. Both these are biofilm forming organisms which freely interact with the diseased lungs and with environmental stimulants. A brief review of the organisms, *P. aeruginosa* and *B. cenocepacia* is included in this section.

1.5.2 *Pseudomonas aeruginosa (P. aeruginosa)*

P. aeruginosa is a motile, Gram-negative rod that is ubiquitously present in the environment and has ability to adapt to diverse growth conditions leading to infections in many species, including humans (Silby et al, 2011, Rahme et al, 1995). It does not require growth factors and uses a wide variety of compounds as carbon sources. *P.aeruginosa* produces a variety of pigments including a fluorescent yellow-green pyoverdin as well as a blue-green pigment pyocyanin which have a role in iron acquisition (Todar et al, 2009).

It is an opportunistic pathogen and healthy humans do not typically suffer from *P. aeruginosa* infection. Immunocompromised individuals with burns, HIV-infection, cancer patients on chemotherapy are commonly infected, often with severe life threatening infections (Davis et al, 2003, Lederberg et al, 2000). It is also a prevalent organism in many community acquired infections but it may also simply inhabit an individual without causing infection in this set up. *P. aeruginosa* is one of the main leading causes (18-20%) of nosocomial lung infections (Spencer et al, 1996, Koulenti, et al 2009, Walker et al, 2015, Fujitan et al, 2011). Much of the transmission in hospital settings is from biofilms that have colonized on medical devices such as central venous catheters, endotracheal tubes, urinary catheters, prosthetic heart valves, and orthopaedic implants (Costerton et al, 1999; Stewart et al, 2001, Trautmann, et al 2005, Bee et al, 2006, Adair et al, 1999, Richards et al, 2000). Another group of patients commonly infected by *P. aeruginosa* are those with lung disease such as CF and *P. aeruginosa* causes chronic infection with biofilm formation in such patients.

1.5.2.1 Virulence mechanisms for *P. aeruginosa:*

P. aeruginosa adherence to the respiratory tract is a complex process. Generally, the pathogenesis of *Pseudomonas* is complex and multifactorial, since the bacterium is both invasive and toxigenic. The effective factors involved in the development of infection by this bacterium can be divided into two categories: Extracellular virulence factors and structural virulence factors (Figure 1.8) (Veesenmeyer et al, 2009). The bacteria reduce the tracheal mucus velocity and this in turn prolongs the persistence of viable bacteria in airways and thus it counteracts its own clearance (Wanner et al, 1996). *P. aeruginosa* possess pili, flagella and lipopolysaccharides that help motility and allow it to directly adhere to the cilia and the mucoid strain is 10-100 times more

efficient at adhering to cilia than the non-mucoid strains (Marcus and Baker, 1985). In addition to direct ciliary adhesion, secretory products of *P. aeruginosa* induce dyskinesis and ciliostasis (Wilson et al, 1985) and *P. aeruginosa* elastase causes axonemal protein degradation (Hingley et al, 1986). Extracellular factors include pigments that are active in iron absorption, prevent the growth of other bacterial species and degrade factors including protease, hemolysins and toxins. Thus, *P. aeruginosa* produces a variety of cellular structures and products involved in the ability to cause disease through enforcing the adhesion, protecting the phagocytosis, modifying the immune response or destroying the host tissue (Kipnis et al, 2006).

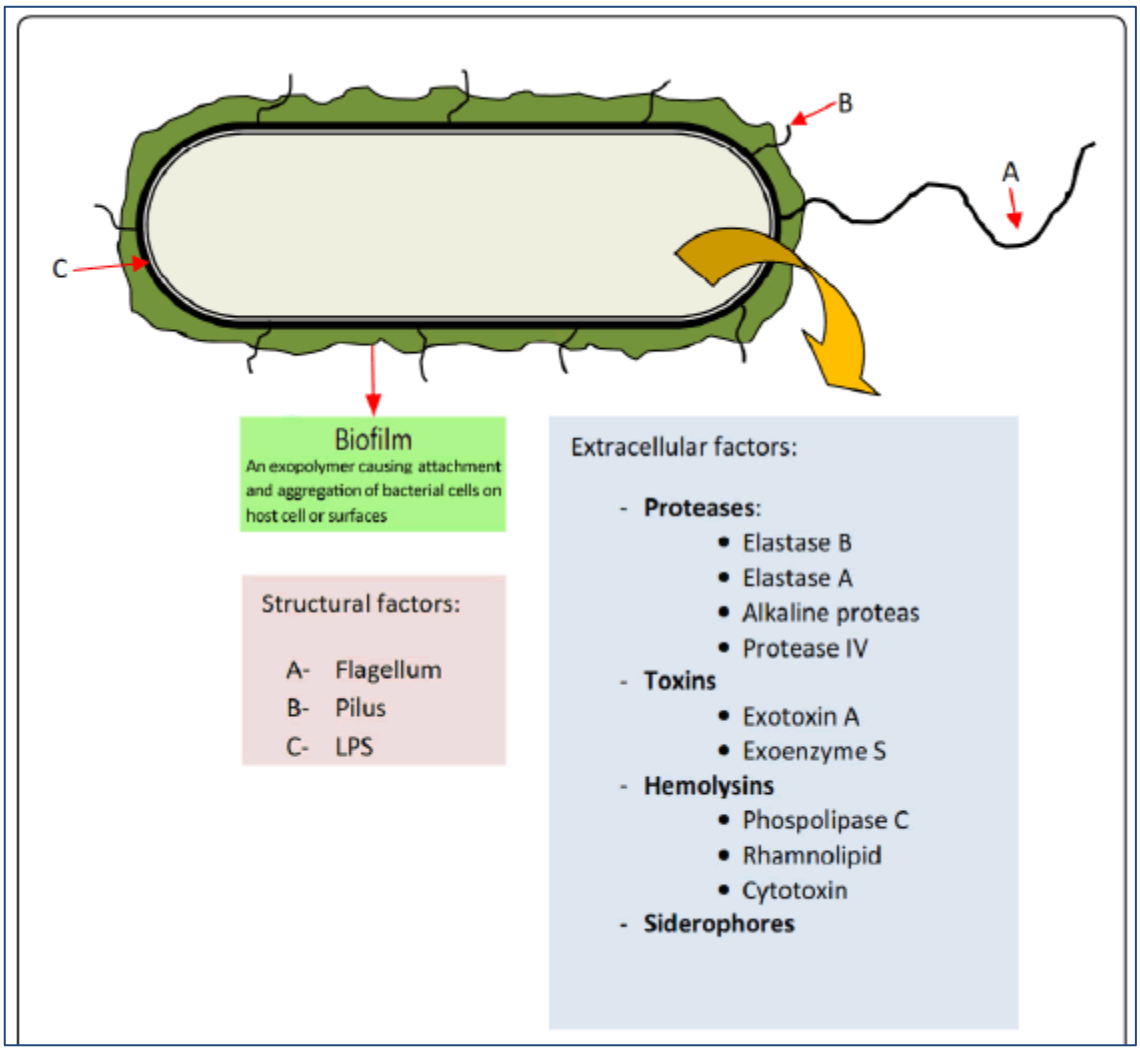


Figure 1.8: Schematic diagram showing various *P. aeruginosa* virulence mechanisms **(**Moghaddam et al, 2014)

Over and above this, *P. aeruginosa* tends to form biofilms whereby the bacteria become enmeshed in extracellular polysaccharide matrixes (Costerton et al, 1999, 2000, Hoiby et al, 2010) and this enables it to stay protected and persist in the CF lung. Early isolates from CF patients of *P. aeruginosa* are flagellated, planktonic and highly motile (Folkesson et al, 2012). Over the course of a CF infection, *P. aeruginosa* loses its flagellar motility (Burns et al, 2001, Folkesson et al, 2012) and also undergoes a mucoid conversion and biofilm formation phenotype (Folkesson et al, 2012). In biofilms, *P. aeruginosa* are embedded in a polymer matrix mainly consisting of the polysaccharide alginate (Hoiby et al, 2010). Biofilms are protective structures defending against both immune cell and antimicrobial attack. Mature biofilms release planktonic bacteria, which are dispersed to spread the infection further and contribute to persistence within the CF lung.

Many of the pathogenic mechanisms of *P. aeruginosa* are controlled by quorum sensing (QS) systems. Two main QS systems have been identified in *P. aeruginosa*: LasR- LasI and Rh1R – RhlI systems which regulate the virulence gene expression and the production of a number of secondary metabolites. This is explained in more detail under the section on QS. *P. aeruginosa* is resistant to many antibiotics such as beta-lactams, macrolides, tetracyclines, co-trimoxazole and most fluoroquinolones and sensitive to aminoglycosides (tobramycin, gentamicin), third fourth generation cephalosporins, and carbapenems. *P. aeruginosa* nosocomial infections have shown an increasing trend of antimicrobial resistance and multidrug resistance. General mechanisms attributing to the antibiotic resistance include blockade of entry, active efflux from the cell by multidrug efflux pumps, beta-lactamase activity, target structure alteration and down regulation of outer membrane porins (Schweizer et al, 2003, Driscoll et al, 2007).

1.5.3 *Burkholderia* species

Burkholderia species were originally classed as part of the *Pseudomonas* genus until they were given their own genus name – *Burkholderia* (Yabuuchi et al, 1992). *Burkholderia are* a group of gram-negative, aerobic, motile rods which were first identified as plant pathogens in 1950s (Mahenthiralingam et al, 2008). While *Burkholderia* bacteria have many beneficial roles in the environment, particularly in growth promotion of plants (Yabuuchi et al, 1992), their pathogenicity in susceptible human beings is a cause for concern.

The *Burkholderia* genome is similar to that of *Pseudomonas*, and has over 700 genes, allowing the genome to be easily altered (Mahenthiralingam et al, 2008). *Burkholderia* also contain three chromosomes and a genomic island (Tyler et al, 1996, Mahenthiralingam, 2008). Based on their genetic similarity, Burkholderia genus is classified into at least 43 species, which are extremely diverse and adaptable (Vandamme et al, 2000).

Most strains are not found to affect healthy humans, but are commonly infectious in patients with CF, chronic granulomatous disease (CGD), (Isles et al, 1984), in immunocompromised individuals correlated with contaminated surfaces and central venous access (Katsiari et al, 2012, Bressler et al, 2007) and in lung transplantation patients (Stephenson, et al 2012). There are historical reports of nosocomial outbreaks of *B.cepacia* in non-CF populations causing bacteraemia and increased mortality (Woods et al, 2004). One study reported the occurrence of bacteraemia following use of a contaminated salbutamol spray, and that concomitant use of the inhaled medication budesonide dramatically enhanced the risk of infection in non- CF patients (Ghazal et

al, 2006). Many *Burkholderia* infections could be avoided by using proper infection control and aseptic technique (Mann et al, 2010, Lucero et al, 2011).

Vandamme et al identified 9 distinct species within the *Burkholderia* genus which were termed genomovars I to IX (Vandamme et al, 1997) and share a high degree of 16S rDNA sequence similarity (98-99%). These are collectively named *Burkholderia cepacia* complex *(Bcc)* and consist *B. multivorans, B. cenocepacia, B. stabilis, and B. dolosa* among others. They are further subdivided into classes, for example, *B.cenocepacia* is subdivided into IIIA, IIIB, IIIC, IIID (Mahenthiralingam et al, 2008, Drevinek et al, 2008).

All *Bcc* have been isolated from human clinical and environmental sources. They emerged as significant CF pathogens in the 1980s (Isles et al, 1984). *Bcc* infects 2 to 8% of CF patients worldwide (Lynch et al, 2009) and cause significant health problems as they are difficult to eradicate from the CF lungs. There are at least 15 species within the *Bcc* which have been isolated from CF patients (Coenye et al 2001, 2003). The most commonly observed infectious agents are *B. cepacia* genomovars *B.cenocepacia, B. multivorans, B. gladioli* and *B. fungoru* (Coenye et al, 2003, 2004). *B. cenocepacia* is the most predominant; it accounts for between 50 and 80% of the cases of *Bcc* infection. The species *B. gladioli* is not classified under *Bcc* and can be isolated in lower frequency in patients with CF (Clode et al, 1999).

The outcome of lung affection by *Bcc* can vary from a stable respiratory function to rapid decline in lung function and mortality and this can be more severe than that due to *P. aeruginosa* (Courtney et al 2004). *Bcc* infections can manifest as a systemic disease

called “cepacia syndrome”, characterized by fever, pneumonia and bacteraemia (Isles et al, 1984).

Another high-risk group for development of serious *Bcc* infections are lung transplant patients. The *Burkholderia* species commonly associated with lung transplant patients include *B.cenocepacia* genomovars III and ET12 and *B.multivorans* (Lipuma et al, 2001). Mahenthiralingam et al found patients infected with *B.cenocepacia* genomovar III had a much more aggressive disease course compared to those with *B. multivorans* or *P. aeruginosa* (Mahenthiralingam et al, 2008). The risk of severe infection is increased if infected both preoperatively (30%) (Snell et al, 1993) or following transplantation (80%) (Lipuma et al, 2001). CF patients colonised with *B.cenocepacia* undergoing lung transplantation appear to be at greater risk of septicemia, pericarditis, pneumonia and empyema related to *B. cenocepacia* (Chaparro et al, 2001).

Bcc are particularly virulent strains due to their characteristic plasticity property (Drevinek et al, 2008, Tyler et al, 1996, Mahenthiralingam et al, 2008), high level of multi-resistant antimicrobial resistance, inherent virulence mechanisms and production of virulence factors and formation of biofilms which make them particularly difficult to treat and increase their ability to survive in the host. Baldwin et al, have shown that over 20% of isolates can be acquired from the environment (Baldwin et al, 2007). Patient to patient transmission of *Bcc* occurs and has led to outbreaks, and *B. multivorans* and *B. cenocepacia* are known to be highly transmissible (Lipuma et al 1998). The exact mode of spread is not fully known but direct contact is implicated. The UK CF Trust guidelines recommend that segregation measures should be put in place for all patients

with *Bcc* infection, regardless of species or strain and a recent systematic review supports this view (CF Trust, 2004).

1.5.3.1 Virulence mechanisms for *Bcc*

Bcc interacts with host cells by mechanisms that are common for many pathogens. These include host cell attachment, invasion and intracellular survival (McClean et al, 2009). Further pathogenesis is mediated by a range of virulence mechanisms that elicit their effects on the epithelial cells. Pathogenic mechanisms include acquisition of iron from host sources, production of lipopolysaccharide and enzymes, development of antimicrobial resistance and biofilm formation. *Bcc* has siderophores such as pyochelin, salicylic acid, ornibactins and cepabactin allowing effective iron uptake and this is utilised for many bacterial functions (Sousa et al, 2011, Thomas et al, 2007, Darling et al, 1998). It has been observed that lipopolysaccharide of *Bcc* is 4 to 5 times more endotoxic than that of *P. aeruginosa*, and induces neutrophil burst and release of Interleukin-8 from epithelial cells (Reddi et al, 2003, Hughes et al, 2008; Govan et al, 1995). They also cause neutrophil infiltration and pro-inflammatory cytokine production leading to tissue damage (Bjarnsholt et al, 2009, Pohl et al, 2014). I*n vitro* studies show that *Bcc* can replicate in CF and CGD macrophages which contributes to its pathogenicity and persistence (Valvano et al, 2015, Al-Khadar et al, 2014). A recent study by Mesureur et al showed for the first time *in vivo* evidence that macrophages are critical for multiplication of *B. cenocepacia* in the host, and subsequent induction of pro-inflammatory fatal infection (Mesureur et al, 2017). The *B. cepacia* genomovar IIIA and ET12, both implicated in lung transplant patients, encode for the cable pilus. Bacteria with the cable pili more readily adhere to the mucosal surface of lower respiratory tract causing colonisation (Saijan et al, 2000, Schwab et al, 2002). *Bcc*

bacteria also possess flagella for motility and adhesion, (Drevinek et al., 2008) extracellular lipases, metalloproteases and serine proteases all of which allow interaction with the epithelial cells of the respiratory tract (Leitao et al, 2010). *Bcc* makes siderophores (Thomas et al, 2007) which it utilises in acquiring iron for its growth. Interestingly, although a potential source of iron for CF pathogens, the mucosal iron sequestering protein lactoferrin also has the ability to reduce biofilm formation in *Bcc* bacteria (Caraher et al, 2007). *Bcc* has mechanisms to utilise iron from ferritin in iron-depleted media *in vitro* and also when in the CF lungs which are rich in ferritin (Whitby et al, 2006).

Iron uptake helps with the cell-cell aggregation, biofilm formation and host cell invasion of *Bcc* isolates (Berlutti et al, 2005). Approximately 80% of *Bcc* species isolated from CF patients form biofilms, which are essential for patient colonisation and pathogenesis (Conway et al, 2004). *Bcc* strains form thick biofilm in-vitro with a production of acyl-homoserine lactone (Conway et al, 2004) and exopolysaccharide. The biofilm formation causes destruction of glycocalyx layer produced by lung epithelial cells (Schwab et al, 2002; Mario and Dianella, 2007). The thick mucus in CF lungs provides a surface for bacteria to adhere to and is poorly cleared by the impaired mucociliary mechanism in CF (Drevinek et al, 2008, Hart and Winstanley, 2002).

QS plays a role in bacterial biofilm formation (Leitao et al, 2010) and ensures that there are appropriate bacterial numbers to avoid eradication by host defences. *Bcc* bacteria have a QS system known as the CepIR system (McKeon et al, 2011), which is similar to the LasIR/RhIR QS systems of *P. aeruginosa* (Tomlin et al, 2005). The role of *Bcc* QS is described in further detail in the next section.

1.6 Quorum sensing (QS) - Intra-kingdom signalling:

QS is a key behaviour-coordination mechanism by which many bacteria regulate gene expression in accordance with cell population density through the use of signalling molecules, known as autoinducers. QS is used by bacteria to communicate and coordinate their group interactions in infection processes.

QS was first described by Nealson *et al* in 1970 in the symbiotic relationship of *Vibrio fischeri*, and bobtail squid (*Euprymna scolopes*). Since this initial description, QS systems have been identified involved in gene regulation in both Gram-negative and Gram-positive bacteria (Asad and Opal, 2008).

QS pathways include bacteria populations, signal molecules, protein activators and target genes. In this system, bacteria secrete the signal molecules, autoinducers into the environment which accumulate as cell population increases. Once a certain threshold concentration of autoinducers is reached, it sets of a cascade that activates target gene expression which ultimately regulates various behaviours, such as virulence factors of the organism (Miller & Bassler, 2001). QS can occur within a single bacterial species as well as between diverse species, serving as intra- and inter- species signalling and communication network (Diggle *et al*., 2007).

The acyl homoserine lactones (AHLs) and autoinducing peptides (AIPs) are the main classes of known bacterial signalling QS molecules in Gram- negative and Gram positive bacteria respectively (Miller and Bassler, 2001). Gram-negative QS bacteria such as *Vibrio fischeri* and *P. aeruginosa* communicate through AHL mediated systems, which are the products of LuxI-type autoinducer synthases. LuxI protein is an

autoinducer enzyme and LuxR protein is a promoter binding protein. On secretion, the autoinducer protein binds the partner promoter protein, binds DNA and activates transcription of target QS genes (Bassler, 2002, Lade et al, 2014). Figure 1.9 is a schematic diagram to show the QS cascade in Gram negative bacteria.

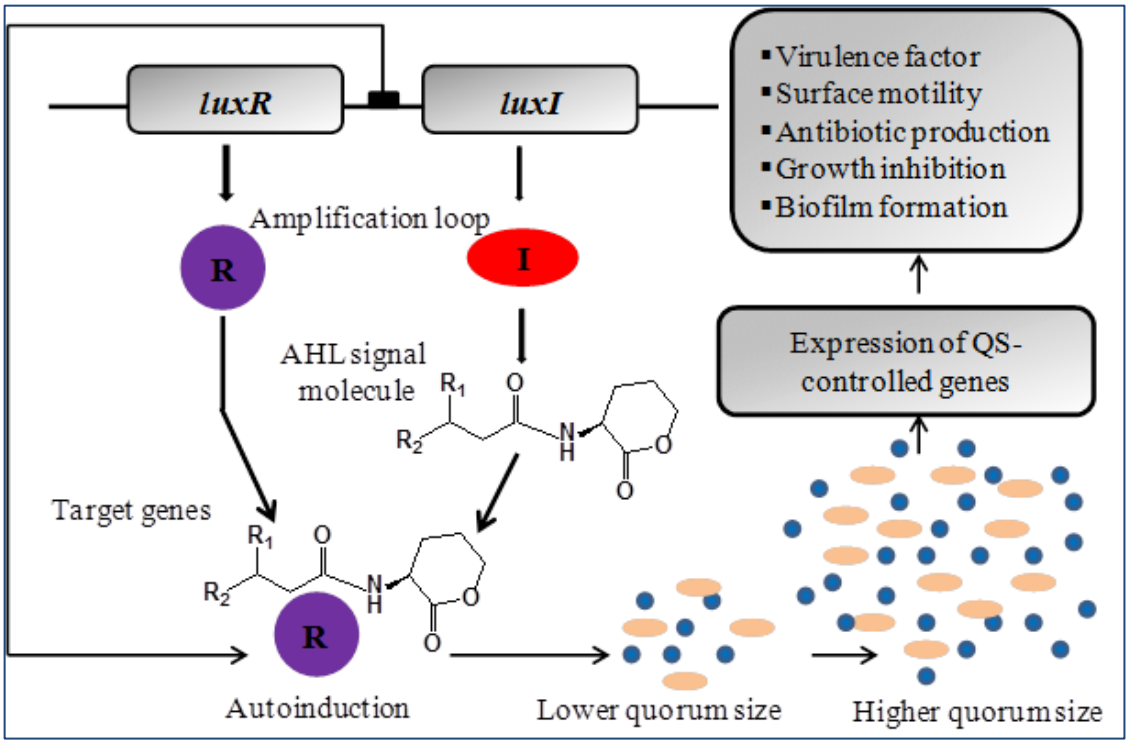


Figure 1.9: This schematic diagram shows the LuxR/AHL-mediated QS regulation of multiple gene expressions in Gram-negative bacteria. The 'R' protein is the AHL receptor and signal transducer while 'I' protein is AHL signal synthase responsible for production of AHLs. After synthesis, AHLs get diffused or pumped out of the bacterial cell into the surrounding medium and are taken up by nearby bacterial cells. At a certain level of bacterial cells, the QS system becomes fully activated leading to expression of QS target genes (Lade et al, 2014).

Gram-positive QS bacteria, such as *Streptococci*, *Staphylococci* and *Bacilli* mainly communicate with chemically modified short AIPs (Bassler and Lossick, 2006, Asad and Opal, 2008). Signalling proteins bind to membrane-bound sensor histidine kinases and signal transduction occurs by phosphorylation which ultimately affects DNA binding transcription factors responsible for regulation of target genes (Bassler and Lossick, 2006).

Two main AHL QS systems have been identified in *P. aeruginosa*, lasRI and rhlRI systems, which regulate the virulence gene expression and the production of a number of secondary metabolites (Pearson et al, 1997). Las gene controls the production of virulence factors such as lasB elastase, lasA elastase, alkaline protease and toxin A. The rhl gene controls the production of factors such as rhamnolipids, elastase, pyocyanin, cyanide (Lade et al, 2014, Davies1998, Pesci et al, 1997, Seed et al, 1995).

Multiple distinct QS systems have been identified in the *Bcc*. The first identified pathway, cepIR, *is a homologue of the* lasIR/rhlIR systems of *P. aeruginosa* and controls the expression of virulence factors as proteases and iron acquisition machinery (Tomlin et al, 2003, Lewenza et al, 1999, Mallot et al, 2003). The *cepI* gene encodes an autoinducer synthase, which is responsible for the production of *N*-hexanoyl-acylhomoserine lactone and *N*-octanoyl-acylhomoserine lactone signaling molecules. *B.cenocepacia* also has two other QS systems: an AHL-independent QS system known as CepR2 system (Malott et al, 2009) and CciIR system which also has many virulence regulatory systems (McKeon et al, 2011, Tomlin et al, 2005, Coenye et al, 2010). *B. cepacia* produces several extracellular virulence factors, including protease (McKevitt et al, 1989), lipase and four types of siderophores: salicylic acid, ornibactin, pyochelin, and cepabactin (Meyer et al, 1989, Sokol et al, 1992, Baldwin et al, 2004).

QS signalling systems have been considered to play an important role in biofilm formation by controlling EPS production in a number of bacteria such as *Vibrio cholera* and *P. aeruginosa* (Leitao et al, 2010, Davies *et al*., 1998, Hammer and Bassler, 2003, Sakuragi and Kolter, 2007). QS systems have been shown to be involved in all phases of biofilm formation and population density and the metabolic activity is controlled

through QS sensing within the mature biofilm according to the nutrient and resources availability (O'Toole et al, 2000, Asad and Opal, 2008). When cell densities in biofilms become high, QS signals are used to reduce the production of EPS to permit bacteria to escape the biofilm (Davies et al, 1998). QS also plays an important role in the pathogenesis of bacteria such as *P. aeruginosa* (Whitley et al, 1999, Davies et al, 1998), *Staphylococcus aureus* (Tenover and Gaynes, 2000) and *Bcc* (Conway et al, 2003, Cunha et al, 2004). Figure 1.10 shows the schematic representation of the association between QS and biofilm formation.

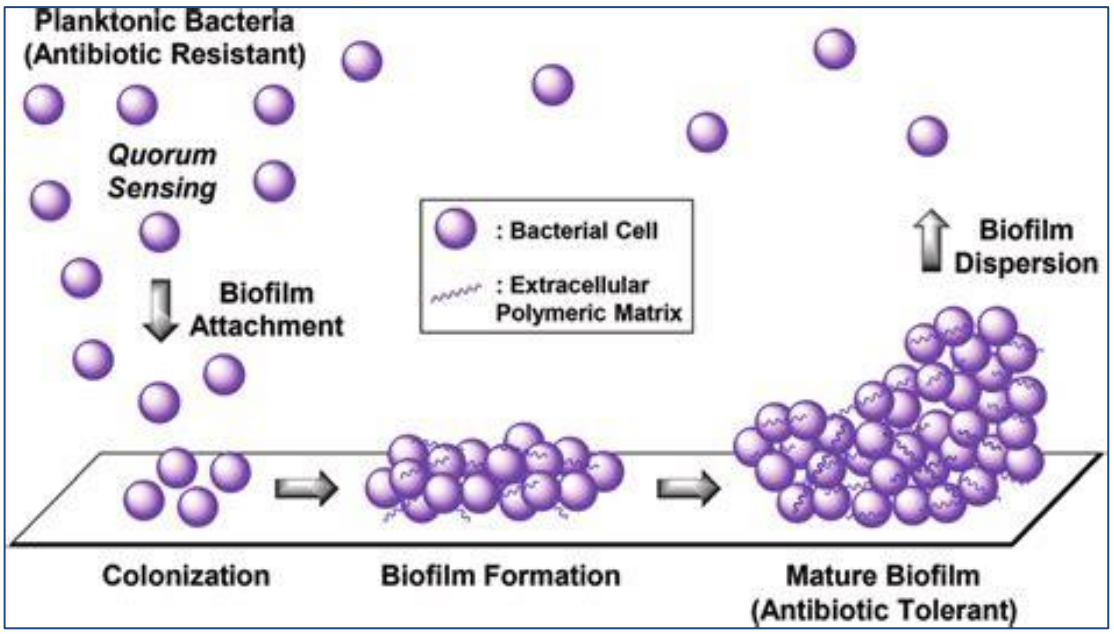


Figure 1.10: Free-floating planktonic bacteria use QS to attach to a surface and form a bacterial biofilm. A mature biofilm can disperse planktonic cells back into its surrounding environment and propogate itself using QS signalling pathways (Basak et al, 2017)

Bcc also have the ability to associate with *P. aeruginosa* in CF patients, as they share the same environmental niches, possibly use the same QS systems, frequently exchange genetic material with each other and suppress the growth and virulence of other co habiting CF pathogens (Costell et al, 2014, Eberl and Tummler, 2004, Braganzi et al, 2012). Schwab et al suggested that *Bcc* biofilm can inhibit *P. aeruginosa* biofilm development (Schwab et al, 2014).

Overall, QS regulates many phenotypes in Gram- positive and Gram- negative bacteria such as biofilm formation, sporulation, biosurfactant synthesis, antibiotic production, conjugation, competence, bioluminescence, motility, clumping, DNA transfer, secretion of nutrient-sequestering compounds and virulence determinants (Bassler and Miller, 2001, Bassler, 2002, Greenberg, 2003, Diggle et al, 2007). Understanding the process of QS will enable development of targeted treatment options.

1.7 Respiratory conditions studies

1.7.1 Cystic fibrosis (CF)

CF is an autosomal recessive genetic disorder caused by mutations in the CFTR (Hart and Winstanley, 2002).The absence of chloride transporters on epithelial membrane surfaces effects the mucosal secretions resulting in the production of viscous, and sulphated mucus in many organs, such as the lungs (Hart and Winstanley, 2002). This mucus is unable to remove bacteria, which become encapsulated in the excess mucus enabling bacteria to cause infection, in lower sterile airways (Hart and Winstanley, 2002).

The microbial community of CF patients is constantly evolving with one or more organisms becoming more prominent and suppressing growth of other organisms (Lipuma et al, 2010). The most common pathogens affecting CF patients include *Staphylococcus aureus*, *Haemophilus influenzae* and *P. aeruginosa but* opportunistic pathogens such as *B. cenocepacia* and *B. gladioli* (Lipuma et al, 2010) also cause chronic infection in these patients. These infections are commonly treated with antibiotics. However, ultimately due to limited treatment options patients require lung transplants or die as a result of infection (Murray et al, 2008).

P. aeruginosa is a Gram-negative motile bacterium and is one of the prominent pathogens for patients with CF and causes biofilms in these patients (Hart and Winstanley, 2002). Early *P. aeruginosa* infection, particularly before 5 years of age, is strongly associated with severe CF lung disease later in life (Pittman et al, 2011, Kosorok et al, 2001). *Bcc* bacteria are troublesome to the CF community as these bacteria have to ability to spread by person to person spread via droplets and contact (Mahenthiralingam et al, 2008, Chaparro et al, 2001). Of the *Bcc* strains the most common species that effect CF patients are *B. multivorans* (37%), *B. cenocepacia* (31%) and 15% of *Bcc* infected patients are infected with *B. gladioli* (Mahenthiralingam et al, 2008). *Bcc* cause chronic biofilms and lung disease in CF patients varying from mere colonization to severe decline in lung function (Courtney et al, 2004) or acute 'cepacia syndrome' (Isles et al, 1984).

1.7.2 Lung transplantation

Lung transplantation is an accepted treatment option for patients with end stage lung disease. The overall survival following lung transplantation remains poor particularly for CF patients (Burch et al, 2004) and majority of the deaths within the first year post lung transplantation are attributable to infection. Emerging evidence points towards a potential role of infections (bacterial, viral or fungal) in the pathogenesis of bronchiolitis obliterans, which is the major cause of death by five years post lung transplant (Scott et al, 1991, Sharples et al, 1996, Benden et al, 2007). The lung transplant recipients' increased susceptibility to respiratory infections may be multifactorial in causation. There is a suggestion that MCC is impaired following lung transplantation. Studies show that MCC particularly in the early post transplantation period may be a contributory factor in transplant failure (Edmunds et al, 1969, Brody et

al, 1972, Dolovich et al, 1987). Studies have not shown any difference in the CBF from the transplanted lungs compared to healthy individuals (Read et al, 1991, Norgaard et al, 1999, Dolovich et al, 1987) but Veale et al (Veale et al, 1993) showed impaired MCC in lung transplant recipients accompanied by a reduction in ciliary beat frequency. Ultrastructural abnormalities in the airway epithelium occur beyond the anastomosis site in lung transplanted patients several months after the surgery (Thomas et al, 2012).

Medications used at time of transplant can affect the MCC. MCC was significantly reduced in transplant patients, compared with healthy subjects, but acute inhalation of salbutamol significantly improved MCC in transplant patients (Laube et al, 2002). Studies in rats showed that dopamine is highly effective in limiting tissue damage and reducing complications after transplantation (Hanusch et al, 2008). Respiratory infection with *P. aeruginosa* and *B. cenocepacia* is known to be associated with increased morbidity and mortality particularly in post lung transplant patients (Snell et al, 1993, Chaparro et al, 2001). Bronchoalveolar lavage samples taken from lung transplant patients showed the presence of N-acylhomoserine lactones (AHLs) QS signaling molecules in both active infection and stable health states. AHLs not only control the expression of bacterial virulence genes but are also involved in stimulating the maturation of antibiotic resistant biofilms and host chemokine release (Tomlin et al, 2005, Coenye et al, 2010). It has been shown that there could be potential links between infection, rejection, and allograft deterioration (Ward et al, 2003). Within a few days, *P. aeruginosa* invades the host microbiota in cases of lung transplant in previously chronically *Pseudomonas* colonised CF patients. *P. aeruginosa* also undergoes phenotypic adaptation resulting in biofilm formation and swimming motility (Beaume et al, 2012).

Stephenson et al, 2012, studied 580 patients who had received a lung transplant. It was described that patients with *Bcc* infection had a median survival of 3.3 years compared with 12.36 years in those without infection. Likewise the risk of death after transplant in cases infected with *B. cenocepacia* was highest within the first year but also after that period compared with patients without *B. cenocepacia* infection (Stephenson et al, 2012, Gilljam et al, 2017).

1.8 Biofilms

1.8.1 Summary

Biofilms are colonies of organisms enmeshed in a matrix attached to a surface and are ubiquitously found in nature in various settings. Medical biofilms are relevant to my study and the discussion in this section pertains to medical bacterial biofilms. Organisms such as *P. aeruginosa* and *B. cenocepacia* are biofilm forming pathogens and cause chronic infection and colonisation on medical devices and in the lungs in diseases such as CF. A generalised definition of a biofilm is 'a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances (EPS), previously commonly known as exopolysaccharides, that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription' (Costerton et al, 1978, Donlan et al, 2002).

Biofilms were first discovered in the 17th century on dental surfaces and further studies explained the mechanisms and the relevance of these multicellular communities (Costerton et al, 1987, 1995, Donlan et al, 2002). Biofilms are characterised by the production of EPS, antimicrobial resistance, structural heterogeneity, community

interactions and genetic diversity (Donlan et al, 2002, Stoodley et al, 2002). Biofilms are ubiquitously present and are relevant in a number of fields like medicine, dentistry, bioremediation, water technology, engineering and food science (De Beer and Stoodley, 2006).

1.8.2 Medical biofilms

Biofilms can be beneficial as seen in the commensal organisms lining a healthy intestine and the female genitourinary tract but are more widely studied in the context of infections in the body, as over 80% of all human infections are biofilm-related (NIH, 2002, Davies et al, 2003). Biofilms form easily on damaged tissue or medical devices and cause chronic, persistent and often serious infections such as native valve endocarditis, lung infections in CF, periodontitis and colonisation of a wide variety of devices and implants (Donlan and Costerton, 2002, Hall-Stoodley et al, 2004).

Biofilms are of particular clinical interest as they are usually difficult to eradicate and contribute to development of chronic inflammation, antibiotic resistance, and spread of infectious emboli (Bryers et al, 2008, Hall-Stoodley et al, 2004). The bacteria within biofilms have been shown to be up to 1000 times more resistant to antibiotics (Parsek and Singh, 2003). They are also much less obvious to the immune system, because the extracellular matrix hides antigens more effectively than free planktonic bacteria (Parsek and Singh, 2003).

Medical devices are responsible for about 60–70% of hospital-acquired infections, particularly in critically ill patients and the three main sites are urinary tract, respiratory tract and blood stream (Darouiche et al, 2001, Bryers et al, 2008). Biofilm formation on

the medical devices or surgical implants starts by direct migration of the organisms from the skin along the catheter surface or by tissue damage and clot formation at site of surgical implantation (Donlan et al, 2001, 2002, Rodrigues et al, 2011). The initial adhesion begins as soon as a biomaterial is implanted into the body and a thin film of organisms and plasma-derived proteins forms altering the chemistry of the biomaterial. Further, fibronectin, collagen, albumin, immunoglobulins, mucins, haemoglobins, platelets and red blood cell fragments (Bayston et al, 2000) get lodged and the organisms which are not cleared by phagocytosis multiply and eventually form plaques; which further develop into microcolonies, produce EPS and form mature biofilms (Bayston et al, 2000). Some of these microcolonies can get dislodged and cause septic thromboemboli causing life threatening infections (Bryers et al, 2008). Commonly associated organisms include *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, P. aeruginosa* and yeast (Donlan et al, 2001).

1.8.3 Formation of biofilms

Biofilm development occurs in three main steps: attachment, growth and detachment. A five stage universal biofilm development cycle has been proposed largely on the basis of proteomic studies in *P. aeruginosa* (Sauer et al, 2002) (Figure 1.11). Motile planktonic biofilm bacteria reach a surface and localise to it by flagellar motility or by expression of adhesins (Lemon et al, 2008). This initial attachment is also called adsorption and is the first step in biofilm formation (Costerton et al, 1995, Stoodley et al, 2002). The extent of attachment depends on environmental factors, like nutrient levels, temperature, and pH, and genetic factors, including presence of genes encoding motility functions, presence of fimbriae and flagellae (Donlan et al, 2002). This initial process may be

reversible or irreversible. After attachment, the bacteria grow in number and spread as a monolayer on the surface to form microcolonies and start producing EPS matrix and developing the complex architecture of a mature biofilm (Finkel et al, 2003, Lemon et al, 2008) and become largely irreversible. Single cells detach from the mature biofilm due to shear stress, enzymatic degradation of surface binding proteins or EPS release (Hall-Stoodley et al, 2004) and the dispersed bacteria re-enter the planktonic mode and repeat the biofilm cycle when conditions are favourable (Costerton et al, 1995, Danese et al, 2000).

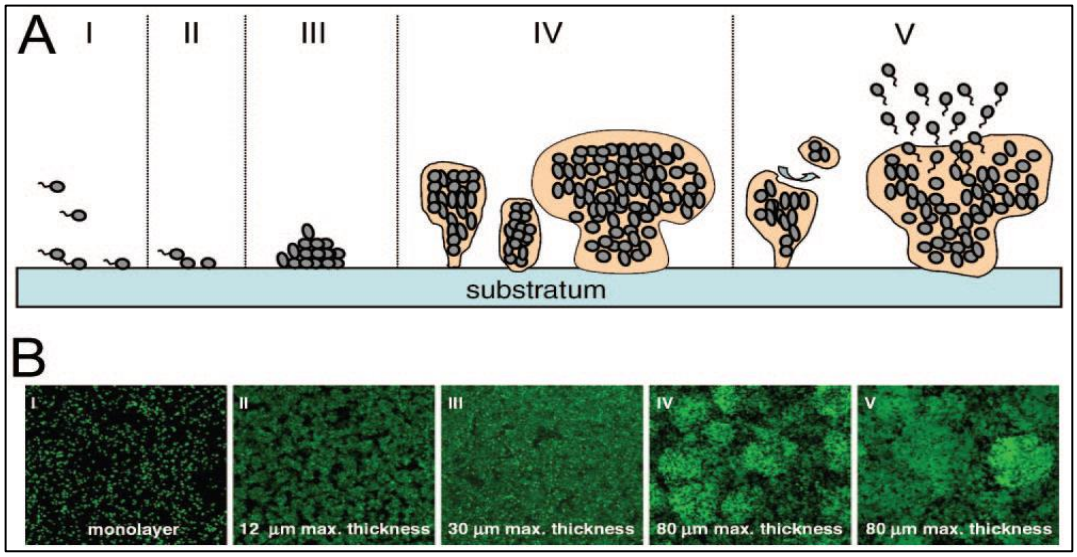


Figure 1.11: The biofilm development stages 1: Individual cells colonise the surface. 2: EPS is produced and attachment becomes irreversible. 3 & 4: biofilm architecture develops and matures. 5: single cells are released from the biofilm in the detachment stage. (Adapted from Wagner et al, 2008)

1.8.4 Biofilm structure

Although each bacterial biofilm is unique there are some general common architectural attributes (Tolker-Nielsen and Molin, 2000). The basic structural unit of the biofilm is the microcolony and microcolonies are separated from each other through 'interstitial voids' or water channels, resting on a base film all enmeshed in the matrix. Biofilm is the spatial arrangement of bacterial cell colonies, pores, water channels, EPS and

particulates. The structure of biofilms varies and ranges from simple planar as seen on dental plaques (Wimpenny et al, 2000), to columnar stacks seen mainly in water distribution systems (Keevil and Walker, 1992), to three dimensional complex mushroom shaped structures revealed by confocal scanning laser microscopy and fluorescent particles (Stoodley et al, 1994, De Beer et al, 1994) as seen in medical biofilms.

Medically-relevant biofilms are usually made up of microcolonies of single organisms as against those occurring in nature which are composed of various species of micro-organisms (Palmer and White 1997, Stoodley et al, 2002). The matrix forms the immediate environment of cells and is the supporting skeletal structure accounting for nearly 90% of the dry biomass of the biofilm (Allison et al, 2003, Flemming and Wingender, 2010). The matrix EPS is produced by the bacteria and is composed of water and other molecules such as polysaccharides, variety of proteins, glycoproteins, glycolipids, extracellular DNA many of which have a role in cell aggregation, surface adhesion, and biofilm formation (Sutherland et al, 2001, Flemming et al, 2007, Pamp et al, 2007).

1.8.5 Regulation of biofilm development

External factors affecting biofilm growth include the nature of the surface, duration of use of device, flow conditions of the surrounding medium, number and type of organisms, nutrient availability and presence of genes coding for surface adhesion proteins (Donlan et al, 2001, Conway et al, 2004, Stoodley et al, 2002, Hall-Stoodley et al, 2004). In suitable environmental conditions, the structure and composition of the biofilm itself lends the means to stabilise, protect, persist and propagate it. Biofilm-

dwelling organisms are protected against internal and external eradication processes such as desiccation, effect of biocides, antibiotics, ultraviolet radiation, and host immune defences by the physical and chemical nature of the biofilms. EPS plays an integral role in the stability and protection of biofilms and is responsible for attachment, detachment, mechanical strength, antibiotic resistance, biofilm architecture and enzymatic degradation activity (Flemming and Wingender, 2010). EPS produces EPS-degrading enzymes regulated by QS, acts as a nutrient source and an external digestive system (Flemming and Wingender, 2010). EPS also pushes the younger cells up and out for obtaining better nutrients (Xavier and Foster, 2007), suffocating neighbouring non-EPS producers, thereby benefitting the principal biofilm organisms.

The variation in gene expression in biofilm bacteria can range from 1% to 38% of the total genome and is increased when compared with planktonic bacteria (Sauer et al, 2003). In *P. aeruginosa* biofilms, genes up-regulated were involved in motility, attachment, translation, metabolism, transport and regulatory functions (Whiteley et al, 2000). Proteomics studies have revealed significant differences between planktonic and biofilm mode of growth in bacteria (Sauer et al, 2002, Sauer and Camper, 2001, Oosthuizen et al, 2002). Differentially expressed proteins are involved in oxidative damage, production of exopolymeric substances, aerobic and anaerobic metabolism, and membrane transport (Sauer et al, 2002).

Biofilm formation and propagation is also governed by the environmental signals between same species (intra-kingdom signalling), different bacterial species (inter-species communication) and those produced by eukaryotic cells (inter-kingdom communication) (Davies et al, 1998, Hall-Stoodley et al, 2004, Jayaraman et al, 2008).

QS enables bacteria to regulate gene expression according to the density of their local population by producing and releasing signalling molecules. *P. aeruginosa* and *B. cenocepacia* both release QS signalling molecules (called autoinducers) that contribute to biofilm development (Miller and Bassler, 2001). Chemical factors which are host-derived can also contribute to bacterial biofilm development. Inter-kingdom signalling mediates symbiotic and pathogenic relationships between bacteria and their hosts (Freestone et al, 2008, Pacheco et al, 2009). The hormonal communication between microorganisms and their hosts has been named as 'microbial endocrinology' and falls under the umbrella of inter-kingdom signalling (Freestone et al, 2008). Many studies have shown that stress hormones, such as the catecholamines can stimulate bacterial growth and virulence and directly affect biofilm formation.

1.9 Microbial Endocrinology

Microbial endocrinology is a research discipline that interrogates the connection between microbiology, endocrinology and neurophysiology in order to examine and understand the interaction of microorganisms with their host in health and disease states. A fundamental tenet is that "microorganisms carry the molecular machinery for sensing host hormones, which provide environmental cues that trigger growth and enhancement of pathogenic processes" (Lyte, 1993a, Lyte, 2004, Freestone et al, 2008). Lyte and Ernst, 1992 were the first to observe the effect on bacterial growth in presence of stress related catecholamine hormones (Lyte and Ernst, 1992). Catecholamine stress hormones interact with pathogens and this relationship is studied under the purview of microbial endocrinology. The interaction of catecholamines with bacteria and with the ciliated respiratory epithelium is also discussed in this section.

1.9.1 Implications of stress

Stress can be physical or emotional (Reiche et al, 2004) and stimulates the sympathetic nervous system and the hypopituary-adrenocortical axis to release several adaptive hormones including catecholamines, corticosteroids and adrenocorticotropin.

The sympathomimetic catecholamine hormones adrenaline (epinephrine) and noradrenaline (norepinephrine) are an integral part of the stress response in higher animals and their interplay with other hormones determines the physiologic "fight and flight" stress responses (Freestone et al, 2008a). The correlation between stress and susceptibility to infection is well recognised (Reiche et al, 2004) and stress hormones can also have a direct effect on infectious agents (Lyte et al, 2004, Freestone et al, 2008).

1.9.2 Use of catecholamine and related compounds in medical practice

Catecholamines are adrenergic amines derived from tyrosine characterized by presence of a benzene ring and two adjacent hydroxyl groups with an opposing amine side chain (Figure 1.12). Epinephrine (Epi), norepinephrine (NE), and dopamine (Dop) are the endogenously produced sympathetic-agonists which function both as neurotransmitters and hormones. Other exogenous sympathomimetic medicational compounds are salbutamol, phenylephrine, isoprenaline and dobutamine among many others. Dobutamine and isoprenaline are used in intensive care settings to regulate heart function and blood pressure (Smythe et al, 1993, Goldstein et al, 2003, www. bnf.org.uk). Salbutamol is widely used as a bronchodilator in acute and chronic respiratory diseases.

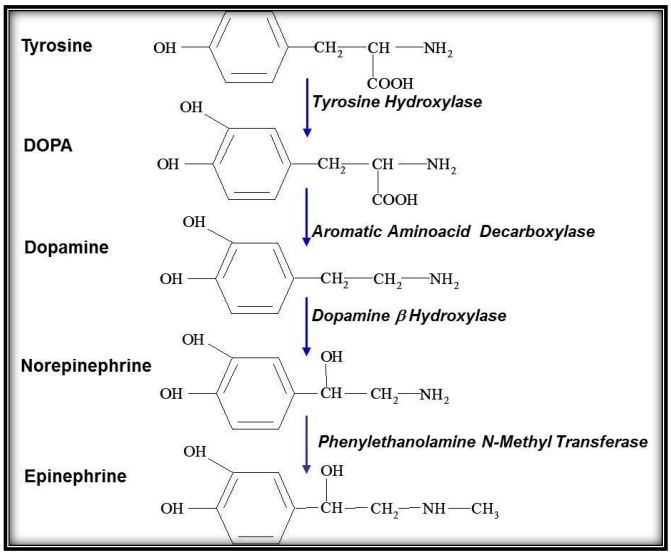


Figure 1.12: Catecholamine structure and biosynthetic pathway: Catecholamines are effector compounds derived from tyrosine and characterized by a benzene ring with two adjacent hydroxyl groups and an opposing amine side chain (Adapted from Sharaff & Freestone, 2011). Catecholamine synthesis is regulated by enzyme tyrosine hydroxylase and involves cAMP regulated protein kinase activity (Goldstein et al, 2003).

Dopamine is the first catecholamine to be synthesised, acting as the precursor molecule for synthesis of noradrenaline and adrenaline (Sharaff and Freestone, 2011, Freestone et al, 2008). Catecholamines regulate behaviour, cardiovascular function, metabolism and maintain homeostasis during exposure to stressors. Epinephrine and norepinephrine are rapidly secreted after stimulation of the sympathetic system and manifest their effects via specific protein-coupled adrenergic alpha (α) and beta (β) receptors in mammalian cells. The receptors are further divided into subclasses, α_1, α_2 and β_1, β_2 and β_3 which are variably distributed in various sites in the body. Catecholamines trigger a specific response in the effector organs by interacting with the adrenoreceptors that they express. Their main effects are on the cardiovascular system mediating a variety of physiological responses such as vasodilation and vasoconstriction, heart rate modulation, regulation of lipolysis, and blood clotting (Freddolino et al, 2004). In stress-related health problems, catecholamines are linked to disorders such as hypertension, myocardial infarction and stroke (Lundberg et al, 2005).

Receptors for catecholamine are also present in most immune function cells. Catecholamine and glucocorticoid hormones released during stress impair immune cell function, T-cell proliferation, NK cell activity, neutrophil phagocytosis, release of lysosomal enzymes from neutrophils and stimulate macrophage activity, B-cell antibody production and cytotoxic T-cell function (Reiche et al 2004). Although structurally similar to the catecholamines, dopamine acts via different receptors – D_1 and D_2 receptors (Missale et al, 1998) with different effects depending on the effector organ.

1.9.3 Effects of catecholamines on growth and virulence of bacteria

Catecholamine stress hormones are known to interact with pathogenic organisms. Studies show increase in growth of a number of gut inhabiting microbes, those causing respiratory infection, periodontal disease and skin associated bacteria (Freestone et al, 1999, 2008, Neal et al, 2001, Lyte and Ernst, 1992). *In vitro* studies closely mimicking the *in vivo* conditions show several log fold increase in the growth induction of bacteria such as *P. aeruginosa* (Lyte and Ernst, 1993, Freestone et al, 1999, 2008, 2009, 2012).

Many infectious bacteria utilise siderophores that possess high affinity for iron and scavenge iron to promote growth. However, products such as transferrin (Tf) in blood and Lactoferrin (Lf) in mucosal secretions have a high affinity for iron and reduce the total available iron for bacteria to use (Freestone et al, 2008, Sharaff and Freestone, 2011). Catecholamines form complexes with Tf and Lf weakening their affinity for iron, thus making more iron available for Gram-positive and Gram-negative bacteria that lack the system to obtain this iron otherwise (Freestone et al, 2000, 2002, 2003, 2007a, b, 2008, Lyte et al, 2003, Neal et al, 2001). The study by Sandrini et al showed

that the catecholamine-Tf complex causes loss of a valency of ferric to ferrous, and this in turn is not as easily taken up by Tf or Lf making it more available to the bacteria (Sandrini et al, 2010). Thus, catecholamine-facilitated iron provision from host iron binding proteins to many bacteria is one of the main mechanisms of inducing their growth. In the growth context, catecholamines also induce the production of Norepinephrine-induced autoinducer (NE-AI) which induces bacterial growth to a similar magnitude as the catecholamines (Lyte et al, 1996, Freestone et al, 1999, Lyte and Freestone, 2008).

Besides stimulating bacterial growth, NE also enhances the production of virulence factors. Lyte et al showed that NE increases the production of Shiga toxin by Enterohaemorrhagic *E. coli* O157:H7 (Lyte et al, 1996). Recent studies have shown increased bacterial attachment to host tissues in presence of catecholamines (Freestone et al, 2008, Hegde et al, 2009, Vlisidou et al, 2004, Bansal et al, 2007).

Patients on intensive care often receive inotropic support with catecholamines during their hospital stay (Smythe et al, 1993). Catecholamines accelerate planktonic growth and accelerate biofilm formation by *Staphylococcus epidermidis* on catheter-grade plastics (Lyte et al, 2003).

Studies with *Streptococcus pneumoniae* have shown increased growth but reduced adhesion to host cell surface via iron dependent mechanism (Gonzales et al, 2014). Sandrini et al showed enhanced and increased *Streptococcal* biofilm formation *in vitro* in presence of NE (Sandrini et al, 2014).

Many studies show that in presence of catecholamines, *P. aeruginosa* showed increased growth, virulence and increased biofilm formation on endotracheal tubing (Freestone et al, 2008, 2012). NE increases PA14 growth, virulence factor production, invasion of HCT-8 epithelial cells, and swimming motility in a concentration-dependent manner (Hegde et al, 2009). Li et al made an interesting observation and showed that *P. aeruginosa* growth is increased but the virulence factor production is reduced by supplying iron from transferrin and suggests the presence of a trade-off between the growth induction and virulence reduction (Li et al, 2011).

There is very limited information of the effect of catecholamines on *Bcc* growth or virulence. A study reported interaction between epinephrine and *Burkholderia pseudomallei*, the causative agent of melioidosis. This was the first study to report *in vitro* enhanced bacterial motility, transcription of flagellar genes and flagellin synthesis and also affected genes coding for superoxide dismutase (*sodB*) and the malleobactin receptor (*fmtA*) causing resistance to superoxide (Intarak et al, 2014).

1.9.4 Catecholamines in CF and lung transplantation

CF patients seem to have an alteration in catecholamine metabolism which is reflected in higher plasma dopamine levels (Schöni et al, 1985, 1986). This may be due to variable activity of enzymatic activity to plasma catecholamines. An interesting study in the context of lung transplantation studied alveolar fluid samples collected at bronchoscopy in post lung transplant patients (Dickinson et al, 2015). The procedure was carried our routinely when well or for an acute illness. The analysis showed that catecholamines (norepinephrine and epinephrine) were present in the alveolar fluid. There was a strong correlation between high catecholamine concentrations with

decreased community diversity in the bacterial lung microbiome, a feature that could predispose to pneumonia (Dickinson et al, 2015). The community membership showed that high-catecholamine alveolar fluids were enriched with *P. aeruginosa*. They proposed that a positive feedback loop of inflammation, selective bacterial growth, alveolar inflammation, increased catecholamines and further growth among catecholamine responsive species exists and that relationship is proportional to the catecholamine levels (Dickinson et al, 2015).

1.10 Salbutamol, an adrenergic agonist

Salbutamol is widely used in respiratory diseases as inhalational therapy. Patients with CF use salbutamol for its bronchodilatory effect and to aid airway clearance. The inhaled salbutamol drug easily disperses in the airway fluid (Mehta et al, 2010). Salbutamol is structurally similar to catecholamines (Lemke et al, 2013) and is an adrenergic receptor agonist. It is known to affect the host chemokine responses (Capelli et al, 1993, Otenello et al 1996, Gross et al, 2010), alter ciliary function (Devalia et al, 1992, Izeboud et al, 1999) and interact with bacteria directly and indirectly (Maris et al, 2006, Vandevelde et al, 2015, Zhang et al, 2011).

1.10.1 Structure and pharmacological preparations of salbutamol

Despite similarity to the classical catecholamine agonists, salbutamol has a hydroxymethyl group in the meta-position of the aromatic ring, instead of a hydroxyl group (Lemke et al, 2013) (Figure 1.13). This means that the aromatic ring of salbutamol occupies a different space in the β_2- adrenoreceptor and thus induces more selective β_2-receptor stimulation. Its chemical formulation is $C_{13}H_{21}NO_3$ and it has a molecular weight of 239.31.

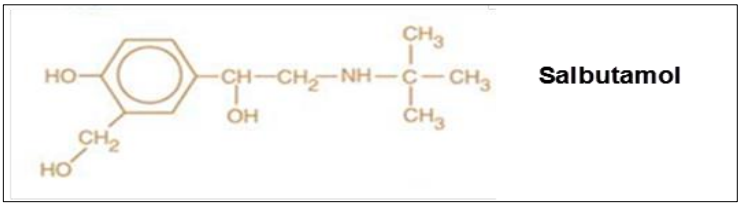


Figure 1.13: Salbutamol structure (Adapted from Xiaojie et al, 2006). Salbutamol has a hydroxymethyl group in the meta-position of the aromatic ring, instead of a hydroxyl group (Lemke et al, 2013).

Salbutamol is available as oral, intravenous and inhaled (metered dose inhaler, MDI or nebulized) preparation used for medication purposes (https://www.bnf.org/). The inhaled preparation is widely used for a variety of respiratory diseases such as asthma, chronic obstructive pulmonary disease and CF. Inhaled preparations are available as salbutamol sulfate delivered as a metered dose aerosolized preparation with HFA (hydrofluro alkane) or as a ready to use nebulized solution with water, sodium chloride and sulfuric acid (to adjust pH) (https://www.bnf.org/).

The nebulized drug is a racemic mixture of R-isomer and S-isomer (Mehta et al, 2010). The R-isomer is responsible for the pharmacological activity while the S-isomer has been associated with potentially causing airway hyper-reactivity and inflammation (Mehta et al, 2010). Salbutamol is commercially available as Ventolin, as 1mg/ml and 2 mg/ml differing only in concentration of salbutamol. I used 1 mg/ml solution for my studies.

The lung is a very attractive target for drug delivery for inhaled medications used in treatment of respiratory conditions such as asthma, chronic obstructive pulmonary disease and CF. The lung drug dosimetry is affected by deposition kinetics and clearance mechanisms in the respiratory tract (Mehta et al, 2010).

Aerosolised drugs contain heterogeneity of aerosol sizes. Majority of particles are larger than 10 μm and get deposited in the oropharyngeal region and do not reach the lung. Only 10-20% of the drug reaches the lower airways and gets distributed peripherally (Newhouse et al, 1978, Newman et al, 1982). Once the drug aerosol has been deposited onto the lung surface, a freely solubilized drug, such as salbutamol, will rapidly diffuse into the epithelial lining fluid and become available for action within 5-10 minutes. Eventually it will get degraded by enzymatic action and finally excreted from the body, but very little drug gets metabolised in the lungs (Ball et al, 1991, Anderson et al, 1994). The action lasts for 4-6 hours before it is finally excreted. Due to its quick onset of action and short duration of action, it is classed as a short acting β_2-agonist.

The total liquid volume available for dissolution in the human lung is approximately 10–30 mL. Lung clearance can be altered in disease (Olsson et al, 2011), but salbutamol is a highly soluble drug and no or only small differences in pharmacokinetics are expected unless the regional deposition is substantially different such as in CF or asthma. A study done by Atabai et al measured the alveolar fluid concentration in patients after therapeutic nebulisation of salbutamol and this study provides reliable data on alveolar fluid concentrations of the drug (Atabai et al, 2002). When given in therapeutic doses of 2.5-5mg nebulised salbutamol in the 6 hours prior to the measurement, median alveolar fluid concentrations of 1,250ng/ml (around 10^{-6}M) in patients with hydrostatic pulmonary oedema and slightly lower in those with pulmonary oedema from acute lung injury was noted. Plasma salbutamol levels were much lower, with a median of 5.2ng/ml (0.01 x 10^{-6}M) in patients with hydrostatic pulmonary oedema and 3.1ng/ml (0.01 x 10^{-6}M) in patients with pulmonary oedema from acute lung injury. Median level after dose adjustment per milligram of salbutamol was

500ng/ml/mg (Range 156 – 2,000ng/ml/mg) in the hydrostatic pulmonary oedema group and no significant difference in the acute lung injury group (Atabai et al, 2002). For my study, I used a range of concentrations to reflect the therapeutic levels that were likely to be reached in lower airways and in larger airways where more direct deposition after nebulisation was possible.

1.10.2 Pharmacological effects of salbutamol

Salbutamol acts by stimulating the β_2-receptors and the main action is bronchodilatation. β_2-receptors are mainly present on the bronchial smooth muscles and salbutamol stimulation causes bronchial smooth muscle relaxation and bronchodilatation (Goldstein et al, 2003). Once stimulated, β_2-receptors cause formation of ATP to cAMP (Lou et al, 2001) and this begins a cascade of actions ending with inhibition of myelin phosphorylation and lowering the intracellular concentration of calcium ions leading to muscle relaxation and bronchodilatation. cAMP and protein kinase production also affect ciliary function. cAMP also influences inflammatory cells and cytokine release in the airways and also interacts with pathogens.

1.10.3 Effects of salbutamol on bacteria

Salbutamol has been found to reduce inflammatory cytokine production on allergen stimulation (Romberger et al, 2016). Ozugul et al showed that salbutamol exerted protective effects on sepsis and lung injury in a sepsis model of rat (Ozugul et al, 2015). Salbutamol modulates cytokine production and affects the Th-cell priming ability and could be causative for autoimmune disease pathogenesis (Manni et al, 2011). Neutrophil respiratory burst activity (Otenello et al, 1996) and exocytosis (der Poll et al, 1996) were shown to be attenuated by β_2-agonist treatment. Bacterial killing and superoxide

anion release by alveolar macrophages was strongly suppressed by both salbutamol and formoterol (Capelli et al, 1993). These mechanisms will indirectly affect the clearance of organisms.

The racemic mixture of salbutamol attenuates cytokines by attenuating IL-13 thereby reducing the effect of *Mycoplasma pneumoniae* (Gross et al, 2010). Maris et al showed that both salmeterol and salbutamol inhibited the clearance of nontypeable *Haemophilus influenzae*, and the effect is mediated by stimulation of β_2-receptors (Maris et al, 2006). Ipratropium and, to a lesser extent, salbutamol were found to cooperate with antibiotics for bacterial clearance and disassembly of *Pneumococcal* biofilms (Vandevelde et al, 2015). Some studies show no difference to bacterial growth with salbutamol (Neal et al, 2011).

P. aeruginosa interaction with salbutamol is not well documented although there are several studies reviewing the interaction with the longer acting β_2-receptor, salmeterol, which has an overall anti-inflammatory and protective effect in presence of bacteria (Dowling et al, 1997, Kanthakumar et al, 1994, Coraux et al, 2004). Zhang et al showed a dose responsive increase in bacterial growth of *E.coli* and *P. aeruginosa* in air surface fluid on human airway carcinogenic cell line air liquid cultures with addition of salbutamol to culture medium. This growth was related to reduced anti-bacterial products (Zhang et al, 2011). Nothing is known about the interaction of *Bcc* with salbutamol.

1.10.4 Effect of salbutamol on ciliary function

Salbutamol increases the MCC, which is the first line defence mechanism in healthy individuals (Devalia et al, 1992, Izeboud et al, 1999). However, this effect may vary in diseases such as asthma and CF as tested with other short acting β_2-agonists (Mortensen et al, 1993, 1994). Also larger doses than those used therapeutically may be required to achieve MCC and this effect is immediate and is not seen at 24 hours after inhalation (Bennett et al, 1993). Cleary et al studied the effect of chronic inhalations of nebulized levalbuterol, the R-isomer of salbutamol on MCC. Levalbuterol did not improve MCC in healthy subjects, compared to albuterol or placebo (Cleary et al, 2006). Salbutamol and other β_2-agonists affect alveolar fluid clearance (Berthiaume et al, 1987, Cott et al, 1986, Sakuma, et al 1997, Foster et al, 1976, Bennett et al, 1993).

Several studies in animal and human epithelial cells have conclusively shown salbutamol to increase CBF (Devalia et al, 1992, Fazio et al, 1981, Yanuara et al, 1981, Iboue et al, 2013, Boek et al, 2002, Boon et al, 2016). Likewise, salbutamol has been shown to increase CBF in 20 subjects with chronic bronchitis using a radionuclide scan (Fazio et al, 1981) and in mice model in chronic allergic pulmonary inflammation (Toledo et al, 2011). The (R)-salbutamol enantiomer increased CBF significantly compared to the racemic mixture which only just increased the CBF. The effect on CBF may be mediated by β_2-receptor stimulation and protein kinase pathway (Frohock et al, 2002). Another mechanism of action of salbutamol associated increased CBF was felt to be via the cAMP pathway (Yanuara et al, 1981).

Thus, studies suggest that β_2-receptor agonists such as salbutamol have complex anti-inflammatory actions within the lungs and interact with bacteria.

1.11 Rationale for studying interaction of catecholamine, salbutamol and bacteria- *P. aeruginosa* and *B. cenocepacia* in CF

At a personal level, as a respiratory paediatrician by background, the rationale for studying infections in CF was pertinent to my field of interest. Chronic colonisation with *P. aeruginosa* and *B. cenocepacia*, which are the most pathogenic organisms in CF, causes significant morbidity and mortality in CF. Several factors have been known to influence the virulence of these biofilm forming bacteria but the influence of routinely used drugs such as salbutamol is not known and this was one of the questions of my study.

P. aeruginosa interaction with salbutamol is not well documented. Zhang et al showed a dose responsive increase in bacterial growth of *E.coli* and *P. aeruginosa* in air surface fluid on human airway carcinogenic cell line air liquid cultures with addition of salbutamol to culture medium. This growth was related to reduced anti-bacterial products (Zhang et al, 2011). Nothing is known about the interaction of *Bcc* with salbutamol.

During my early years of training, I was also introduced to the field of medical endocrinology through previous research involvement. The role of stress in infection is being elucidated and bacteria are catecholamine responsive. In patinets with CF there are high levels of catecholamines that are naturally produced in the body and those used as medications for low blood pressure on intensive care units for post-transplnatation patients. It is possible that when CF patients are stressed as happens with infective exacerbations, the usually quiescent colonies of *Pseudomonas* and *Burkholderia* flare up causing more inflammation and further decline in lung function. I decided to explore

the question of whether there was any effect of catecholamines on these bacteria in CF as another main aspect of my study.

Patients with CF have an alteration in catecholamine metabolism which is reflected in higher plasma dopamine and slightly elevated NE levels (Schöni et al, 1985, 1986). There may also be a suggestion of correlation of CF with the severity of the disease and the extent of lung involvement, as assessed by chest radiographic scores (Schöni et al, 1986). The adrenal glands of patients with CF show hyperplasia and Bongiovanni et al observed increased catecholamine levels particularly epinephrine in urine of patients with CF at autopsy (Bongiovanni et al, 1961) as did Barbero and Braddock (1967). The quantities of cortisol and 11-deoxycortisol in adrenal glands of patients with CF appear to be higher than found at post-mortem when other diseases were present (Bongiovanni et al, 1961). The exact implication of the higher catecholamine levels in CF is not known and it may be that they are responsible for an autonomic dysfunction in CF or reduced β_2-adrenergic responses (Gallant et al, 1981). *P. aeruginosa* and *B. cenocepacia* are particularly associated with CF patients causing chronic infection and biofilm formation and affecting the outcome of these patients, and *P. aeruginosa* has been shown to be highly catecholamine responsive (Freestone et al, 2012).

Lung transplantation is an accepted treatment option for children with end stage CF lung disease but post-transplant survival is not good and although this is multifactorial, infection remains a major factor for this (Burch and Aurora, 2004). Pulmonary infection with *B. cenocepacia* is associated with poor clinical outcome after lung transplantation in CF (LiPuma et al, 2001) and high mortality is reported after infection with *B. cenocepacia* (DeSoyza et al, 2010). However, these patients also spend time in intensive

care (Fuehner et al, 2012) where they are exposed to high levels of endogenous stress hormones and additionally to exogenously administered catecholamines that are used as inotropes.

Bacteria are responsive to catecholamines as shown in several studies from the field of microbial endocrinology (Freestone et al, 2008, Sandrini et al, 2015) and exposure to these drugs can increase both growth and biofilm formation of pathogenic microorganisms. Clinically, it is observed that during times of stress when blood catecholamine levels are elevated, human beings are more susceptible to infections (Reiche et al, 2004). Salbutamol is a structural analogue of catecholamine and is known to interact with bacteria. It is widely used in the setting of CF and post lung transplant to aid airway clearance. It is likely that in CF transplanted, interactions can occur between the freely available catecholamines and drugs such as salbutamol and the common CF pathogens, *P. aeruginosa* and *B. cenocepacia*, increasing their growth, virulence and biofilm formation and affecting the outcomes of these patients.

1.12 Aims and Objectives

The overall hypothesis was to determine if commonly used drugs such as catecholamines and salbutamol affect the growth and virulence of airway pathogens such as *B. cenocepacia* and *P. aeruginosa* in patients with CF.

The specific aims of the study were to:

1. To evaluate whether stress hormones affect the growth and biofilm formation of *B. cenocepacia* species.
2. To explore the role of iron in *B. cenocepacia* growth.
3. To evaluate whether salbutamol affects the growth and biofilm formation of *B. cenocepacia*.
4. To evaluate whether salbutamol affects the growth and biofilm formation of *P. aeruginosa*.
5. To assess the interaction of *B. cenocepacia* with the human airway epithelium in air liquid interface cultures form healthy individuals and CF patients.
6. To assess if catecholamines affected the interaction of *B. cenocepacia* with the human airway epithelium in air liquid interface cultures form healthy individuals and CF patients.
7. To assess if salbutamol affected the interaction of *B. cenocepacia* and *P. aeruginosa* with the human airway epithelium in air liquid interface cultures form healthy individuals and CF patients.

A variety of in vitro and ex vivo experiments were carried out to study the specific aims of the study and are detailed in various chapters.

Chapter Two

Materials and Methods

2.1Summary

Experiments were conducted *in vitro* and *ex vivo*, using air liquid interface cultures of human ciliated airway epithelial cells, to study the effects of various catecholamines such as dopamine, epinephrine and norepinephrine and a respiratory sympathomimetic medication, salbutamol, on the growth, attachment and biofilm formation of *P. aeruginosa* and *B. cenocepacia* commonly seen in post transplantation CF patients.

The *in vitro* experiments were conducted using clinical *P. aeruginosa* and clinical and reference *B. cenocepacia* with and without the presence of supplemental catecholamines, salbutamol and iron (Fe) used as positive control. The growth and attachment assays were carried out followed by assessment of biofilm analysis. To give a clinical context, medical grade plastic from endotracheal tubes was used for the biofilm assessment.

Alongside the *in vitro* analyses, to enhance the clinical relevance, I performed *ex vivo* studies using air liquid interface (ALI) cultures of human nasal airway epithelial cells from healthy individuals and bronchial epithelial cells from paediatric CF patients following lung transplantation at time of surveillance bronchoscopy done at Great Ormond Street Hospital for Children, London. The experiments performed were aimed at obtaining information about the early effect of infection and effect of drugs on bacteria on normal and CF host cell epithelial cultures over short time courses (five hours) and various measurements were made. Further advanced microscopy was used to obtain structural information about the early biofilm formation, if any, on the human epithelium.

2.2 Materials

2.2.1 Bacterial Strains (Table 2.1)

Bacterial Strain	Type	Reference	Obtained from
Pseudomonas aeruginosa	Clinical isolate	Clinical isolate	Patient with pneumonia
Burkholderia cenocepacia genomovar IIIA cbIA positive	Clinical strain	H60931240	Health Protection Agency, UK
Burkholderia cenocepacia reference strain	Reference strain	LMG 18863	Health Protection Agency, UK

2.2.2 Culture preservation

Frozen stock cultures were made from subculture plates and preserved at -80°C in cryoprotectant medium [25 % (w/v) sterile glycerol, 75 % (w/v) Luria broth (LB)] until required. Aliquots were thawed and cultured on plates, once every 24 hours for three days before use in the experiments to check the validity of the bacteria. Bacterial strains required for routine work were maintained through weekly re-plating the stock cultures on to Tryptone soya agar (TSA) plates.

For all growth related experiments, bacteria were incubated overnight at 37°C with shaking at 180 rpm by inoculating 5ml of Tryptone soya broth (TSB) or LB with 50µL of liquid inoculums or few colonies from agar plates. This culture was used and number of viable bacteria was determined by colony counting on TSA or LB plates for each experiment.

2.2.3 Neurochemical preparation

Catecholamines were prepared shortly before use as a 5mM stock solution in deionised

nano pure water, and then filtered through a 0.2µm pore size syringe unit filter and stored in the dark until used at -80^{o} Celsius.

- L(-)-Norepinephrine-(+)-bitartrate salt A9512 (NE), Sigma-Aldrich, UK
- Dopamine hydrochloride H8502 (Dop), Sigma-Aldrich, UK
- Epinephrine hydrochloride E4642 (Epi), Sigma-Aldrich, UK

2.2.4 Salbutamol

Salbutamol was used in the original chemical form to establish the methods of the *in vitro* experiments, but to make the results of my study more clinically relevant, the commercially available form, Ventolin nebules; GlaxoSmithKline UK, bought from local hospital pharmacy was used.

- Salbutamol, S8260, 25 mg, Sigma-Aldrich, UK
- Salbutamol sulphate, Ventolin nebules, 1mg/ml, GlaxoSmithKline, UK

They were available as nebules containing 2.5ml of a sterile 0.1% or 0.2% w/v solution of salbutamol (as salbutamol sulphate BP) in normal saline. All salbutamol-related experiments were performed using concentrations ranging from 0.01µg/ml to 100µg/ml. The salbutamol concentrations used were derived from data studying lung alveolar fluid after inhalation of therapeutic concentrations of this drug (Atabai et al, 2002) which reported median level as 1,250 ng/ml (1.25µg/ml of salbutamol). I used a range of concentrations above and below these levels as it is likely that concentration in the airways, particularly the major airways may be higher and those in diseased areas not adequately ventilated, may be much lower.

2.2.5 Culture Media

Most chemicals unless specified further in the report were bought from Sigma-Aldrich Ltd , UK, tissue culture media and stains from Invitrogen, UK and Sigma-Aldrich Ltd, UK and bacterial growth media from Oxoid Ltd, UK. All routine laboratory reagents and chemicals were purchased from Sigma-Aldrich, Poole, UK unless specified.

Media used in the study:

- **Luria Broth (LB) -** 1% (w/v) Tryptone, 0.5% (w/v) yeast extract, & 0.5% (w/v) NaCl, adjusted to pH 7.0 with 1M NaOH
- **Luria Agar (LA) -** LB solidified with 1.5% (w/v) agar
- **Tryptone Soya Broth (TSB) -** 30g/L
- **Tryptone Soya Agar (TSA) -** TSB solidified with 1.5% (w/v) agar
- **Standard American Petroleum Institute (SAPI) medium -** 2.7mM glucose, 6.25mM ammonium nitrate, 1.84mM KH_2PO_4, 3.35mM KCl, 1.01mM $MgSO_4$, adjusted to pH 7.5 with NaOH or KOH
- **Serum-SAPI -** 30% (v/v) Bovine serum in 70% (v/v) SAPI medium
- **Dulbecco Modified Eagles Medium (DMEM)** - DMEM (D596, Sigma, Poole, UK)
- **M9 Media**- 5x M9 salts ($Na_2HPO_4,7H_2O$, KH_2PO4, NaCl, NH_4Cl), 1M $MgSO_4$, 1M $CaCl_2$, 0.4% glucose
- **SAPI-Tris medium**- SAPI supplemented with 100mM Tris-HCl pH 7.5 (Freestone et al, 2001)

Culture media were prepared in deionised nano pure water, autoclaved at 121°C for 15 minutes and stored in sterile conditions at room temperature until used. Except serum,

all the other solutions were made up to 1 litre using distilled sterile water. Agar plates for bacteria culture were prepared by melting the solid media followed by cooling to 50°C and poured into sterile 90mm plastic Petri dishes and surface dried before use.

2.2.6 Stains and Dyes

- Crystal Violet biofilm stain, BDH Chemicals, UK
- FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Catalog no. L10316), Invitrogen™ Molecular Probes, Inc, Fisher Scientific, Loughborough, UK.
- SYPRO Ruby Biofilm Matrix stain (Catalog no. F10318), Invitrogen™ Molecular Probes, Inc, Fisher Scientific, Loughborough, UK.

2.2.7 Buffers and Solutions

- 1.0% Crystal violet
- 95% ,100% Ethanol
- **Phosphate buffered solution** (PBS) - 0.14M NaCl, 27mM KCl, 8.5mM Na_2HPO_4, 18mM KH_2PO_4, pH 7.4
- **Ferric nitrate (Fe),** 10 μM preparation

2.2.8 Proteins

- **Human lactoferrin** (partially saturated) (L-3309), Sigma-Aldrich
- **Peroxidase conjugated ChromPure human lactoferrin** (Cat.no 009-030-050), Jackson ImmunoResearch laboratories, Suffolk, UK
- **Anti-human lactoferrin made in rabbit** (primary antibody) (L-3262), Sigma, UK

- **Anti-rabbit IgG peroxidise conjugate made in goat** (secondary antibody) (A0545), Sigma, UK

2.2.9 Radiolabelled Iron [^{55}Fe] Lactoferrin preparation

- **Citrate:** 0.8M Sodium citrate titrated to pH7.5 with 1M Citric acid
- **Iron–citrate mix (Fe-c):** 0.5ml of $^{55}FeCl_3$ (500µCi), 3µl of 10mg/ml $FeCl_3$ (filter sterilised), 50µL 0.8M citrate,pH7.5
- **Lactoferrin labelling mixture:** 30µl 1M Tris-HCl pH 8.0, 55µl Iron-citrate mix made up to 210µL with dH_2O, 40µL Apo-LF (sigma)

2.2.10 Western blocking solutions

- Tris- Buffered Saline **(TBS):** 150mM NaCl, 10mM Tris-HCl at pH 7.5
- **TBS-T: Mixture of TBS and Polysorbate 20 (also known as Tween 20),** 5ml of Tween-20 brought up to 1 litre with TBS
- **Western blot blocking buffer:** 5% (w/v) Bovine Serum Albumin protein (BSA) and TBS-T

2.2.11 Culture Plates

12-well, 24-well and 96-micro-titre plates with and without lids (Nunc, Thermo Fisher, UK) were used for the in vitro experiments unless specified otherwise.

2.3 Methods for *in vitro* experiments

2.3.1 Bacterial overnight cultures

Bacterial cultures for experiments, unless specified otherwise, were grown in TSB or LB overnight at 37°C with shaking at 180 rpm in an incubator by inoculating 5ml of TSB or LB with 50µL of liquid inoculums or few colonies from agar plates.

2.3.2 Plating

LA plates were used to culture *P. aeruginosa*, while TSA plates were used to plate out *B. cenocepacia* strains.

2.3.3 Bacterial growth assays

Bacterial overnight cultures grown in LB were serially diluted in PBS to around 10^{-5} to 10^{-8}-fold into Serum-SAPI; the initial inoculum size was around 50-100 colony forming units per ml (CFU/ml)/ml. These low initial numbers of bacteria are intended to mimic the likely infectious doses occurring *in vivo* (Freestone et al, 1999, 2008). Aliquots of 1ml of inoculate was added to 24 well plates. Bacteria in each well were supplemented with Dop, Epi and NE at 5, 10 50µM final concentrations. Serum-SAPI without catecholamine supplementation acted as a negative control and 10µM Fe was added as positive control. Cultures were incubated statically at 37°C with 5% CO_2 in a humidified incubator for up to 24 hours. Cultures were mixed well after incubation and aliquots were removed and serially diluted using PBS from 10^{-7} to 10^{-6} in 96-well microtitre plates. Three 10µl aliquots were plated on TSA plates and incubated at 37°C. Each assay was carried out in at least duplicate and all sets of experiments were repeated on at least four different occasions. The results presented are of one representative experiment. The bacterial cells were counted and viable bacterial count

was expressed as CFU/ml. The CFU/ml was determined by the equation y x 10^d x 10, where y is the average colony count in 10µl and d is the dilution factor. The precise values of initial inoculum were determined by a similar method. The experiments were repeated using salbutamol in concentrations of 0.01, 0.1, 0.375, 1.25, 5 and 10µg/ml.

2.3.4 Bacterial growth kinetics time course analysis

Overnight cultures grown in TSB were diluted 1:100 with Serum-SAPI medium. The catecholamines, 100µM Dop, Epi and NE were added along with positive and negative controls as described above. 200µL of culture media was added in triplicate to a flat-bottom 96 well plate, incubated statically at 37°C. These were placed in a Varioskan multimode microplate spectrophotometer reader (Thermo Scientific, UK) which was set to shaking mode (background, on for 14.57 seconds, off 3 seconds, speed 420, diameter 1mm). Growth was measured over 24 hours at an absorbance or optical density of 595nm (denoted as OD_{595}). This time course of growth was done in triplicate on at least three different occasions. The experiments were repeated using salbutamol in concentrations of 0.01, 0.1, 0.375, 1.25, 5 and 10µg/ml.

2.3.5 Bacterial microtitre attachment assay

Bacterial attachment was investigated by measuring attachment to polystyrene plastic 96 well microtitre plates according to the method of O'Toole et al, 1999 and Stepanovic et al, 2007. This method has also been used in *Burkholderia* experiments by Conway et al, 2002. The test bacteria were grown overnight in LB or TSB and diluted using Serum-SAPI to an optical density of 0.1 at 595 nm (OD_{595}~0.1) and supplemented with different concentrations of catecholamine inotropes (Dop, Epi, NE), Fe as positive control and maintaining negative control using Serum-SAPI alone. Aliquots of 200µL

of each culture were added to a 96 well flat bottomed plate and incubated statically at 37°C, 5% CO_2 for 24 and 48 hours. After incubation, non-adherent bacteria were removed and planktonic cell levels measured by recording the optical density at 595 nm before the removal of the medium. The adhered bacteria were gently washed with 200µl PBS and air dried for 30 minutes. 125µL of 0.1% crystal violet stain was added and the plates were incubated at room temperature for 15 minutes. The stain was then removed by washing the wells thrice with water or PBS and left facedown to dry. The adherent crystal violet stain eluted with 200µl of 95% ethanol and optical density measured at 595 nm on a microplate reader (Bio-Rad, UK). The correction for background staining was made by subtracting the value for crystal violet bound to un-inoculated media controls (O'Toole et al, 1999, Stepanovic et al, 2007). The experiments were repeated using salbutamol at concentrations of 0.01, 0.1, 0.375, 1.25, 5, 10 µg/ml.

2.3.6 Fluorescence microscopy analysis

Bacteria were grown overnight in LB, and where appropriate diluted into 1:100 in LB or serum-SAPI and catecholamines were added. Positive and negative controls were maintained as before. 150 µl volumes of the cultures were added to a 96 well polystyrene microtitre flat bottom plate (Corning Costar, Cat. No. 450653), and incubated statically at 37°C for up to 48 hours. After incubation the planktonic bacteria were removed and the adherent cultures were stained with FilmTracer™ LIVE/DEAD Biofilm Viability kit or with SYPRO ruby biofilm matrix stain according to the manufacturer's protocol. The stain was washed off with 4%w/v Para formaldehyde. Stained wells were observed under NIKON Ti inverted fluorescence microscope at 40x objective at 480 excitation and 500 nm emission spectra (LIVE/DEAD stain) and 450 excitation and 610 emission spectra (matrix stain) using fluorescein isothiocyanate

(FITC) and tetramethylrhodamine isothiocyanate (TRITC) filters. The resulting TIFF image files were analysed using Image-J software.

A Nikon Eclipse Ti 2000 inverted microscope (Figure 2.1) connected to Nikon's Original imaging software, NIS-Elements captured the images of the 96 well plates under fluorescence and phase contrast modes. NIS-Elements provides an integrated control of the microscope, cameras, components and peripherals of the microscope and allows the programming of automated imaging sequences. Scanned images of the microscopic fields of the wells were merged using the Nikon NIS-Elements software.

The image analysis programme, Image-J analysed the merged images to quantify the area coverage of fluorescent emission of the attached bacteria. The integrated density or mean grey value of biofilm coverage of each well was measured using Image J functions. "Integrated Density" is defined as the sum of grey value pixel within an image. Total image fluorescent intensities per bottom surface of the wells was measured as the sum of the integrated densities of a selection, minus the background (Ma and Bryers et al, 2010, Burgess et al, 2010).

Integrated density readings (quantification of light emitted) of the green fluorescent (biofilm stain) or red fluorescent (SYPRO Ruby) images was measured and plotted to quantify biofilm formation to study the differences in biofilm formation with and without the catecholamines.

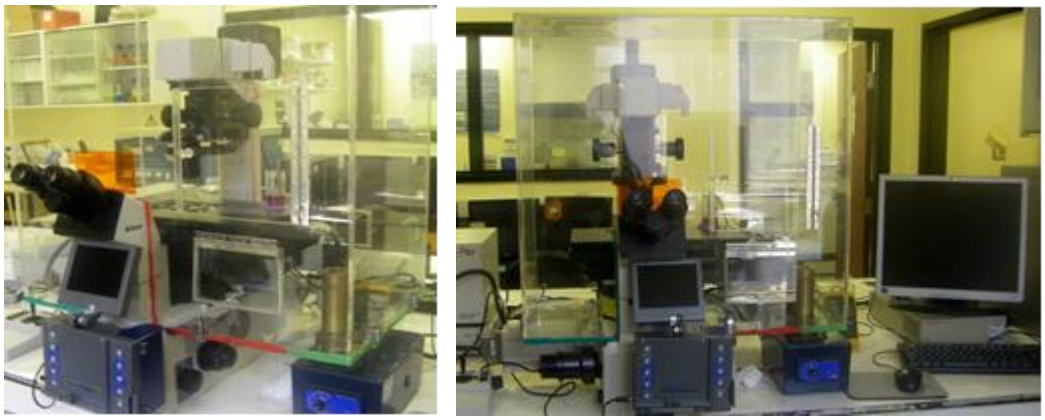


Figure 2.1: Nikon Eclipse TE 2000 inverted fluorescent microscope setup. This microscope was used for microtitre plate biofilm formation capture and analysis.

2.3.7 Bacterial cell-cell association (aggregation)

B. cenocepacia or *P. aeruginosa* overnight cultures grown in LB were serially diluted to around 10^{-5} to 10^{-8}-fold in Serum-SAPI to given an initial inoculum size of around 50-100 CFU/ml. Aliquots of 1ml of inoculate was added to 24 well plates. Bacteria in each well were supplemented with Dop, Epi and NE at 5, 10 and 50 µM final concentrations. Serum-SAPI without catecholamine supplementation acted as a negative control and 100µM Fe was added as positive control. Cultures were incubated statically at 37°C with 5% CO_2 in a humidified incubator for 24 hours. Clumping of cells, which represents bacterial cell aggregation was identified on the bottom of the wells and was digitally photographed at x100. The experiments were repeated using salbutamol in various concentrations – 0.01, 0.1, 0.375, 1.25, 5, 10 µg/ml.

2.3.8 Scanning electron microscopy of bacterial attachment to clinical plastics

The Portex endotracheal tubes (Smith's Medical, USA) made of medical grade plastic are routinely used in most intensive care units for intubation to provide ventilatory support. A 5mm diameter Portex endotracheal tube (Ref: PO100/166/050) was used in this experiment to assess if *P. aeruginosa* or *B. cenocepacia* strains attached to this

medical grade material, and if this attachment was enhanced in the presence of catecholamines or salbutamol.

To ensure similarity in growth rates between control and catecholamine or salbutamol-treated cultures, a higher bacterial inoculum of 10^5 CFU/ml was added to serum-SAPI medium with the Dop, Epi and NE additions as for growth assays (5-50μM) and for salbutamol, the concentrations 0.01, 0.1, 0.375, 1.25, 5, 10μg/ml. The bacteria were then incubated at 37°C in a 5% CO_2 humidified incubator for up to 48 hours in a 24 well plate along with 5x5 mm sections were cut out from the endotracheal tube (ET). After incubation, ET tube sections were transferred using sterile forceps into another 24 well plate and then gently washed three times in 2 ml PBS to remove any non-attached bacteria and then once with 2 ml of 0.1M sodium phosphate buffer (pH 7·2), to remove residual serum protein which would give artefacts on scanning electron microscopy (SEM) microgaps. Biofilms on the ET sections were fixed with cold 2.5% glutaraldehyde followed by dehydration in a graded series of ethanol to 100% and finally infiltration with hexamethyldisilizane and air drying. The dry endotracheal tube sections were each mounted onto aluminium stubs, cool sputter coated with gold and visualised by scanning electron microscopy using a Hitachi S3000H scanning electron microscope (performed by Dr. Stefan Hyman, School of Biological Sciences, University of Leicester).

2.3.9 Lactoferrin analyses

Bacterial overnight culture cell densities were measured with a spectrophotometer at OD_{595} nm and normalised to an OD_{595} of 1.0. Cultures were centrifuged at 10,000 revolutions per minute (rpm) for 10 minutes and the supernatant was discarded. The

pellet was washed twice in 50mM Tris-SAPI (pH 7.5) and resuspended in 1ml of 50mM Tris-SAPI pH 7.5 and incubated statically for 30 minutes at 37°C 5% CO_2 allowing the cells to adjust to the new medium. Aliquots of 10µg/µl of lactoferrin (Lf) were then added to the cultures which were incubated statically at 37°C 5% CO_2 for 1 hour. The control and Lf-treated cultures then underwent 2 fold dilutions 'dotted' in 10µl aliquots onto nitrocellulose membrane. Once dry the membrane was blocked overnight in blocking buffer at 4°C on a rocking platform set at 20 rpm. The blotted membranes were washed thrice in TBS-T for 10 minutes each on a rocker at 70 rpm. The nitrocellulose was exposed to 1:10000 dilution of anti-human lactoferrin (primary antibody) for 2 hours on a rocker set at 25 rpm, ensuring that the membrane was fully covered by the antibody solution. After the incubation, the nitrocellulose was washed thrice in TBS-T on a rocker at 70 rpm. The membrane was exposed to the secondary antibody at 1:30,000 for 1 hour at 25 rpm, ensuring that the membrane was fully covered by the antibody. The membrane was washed 3 times with TBS-T for 10 minutes each followed by another wash for 10 minutes with TBS on a rocker at 70 rpm until developing. Detection of antibody cross-reactivity using enhanced chemiluminsence and autoradiography on X-ray film was done (Freestone et al, 2000, 2003).

2.3.10 *B. cenocepacia* Lactoferrin iron uptake analysis

2.3.10.1 ^{55}Fe labelling of lactoferrin

^{55}Fe Lactoferrin (^{55}Fe-Lf) was prepared as described by (Freestone et al, 2000). Here 2mg of Apo-lactoferrin (Apo-Lf) was incubated at 37°C for 15 hours with labelling mixture containing 25 µCi of $^{55}FeCl_3$ in a reaction mixture containing a total of 1.5µg of Fe per mg of Lf protein, using 2mM sodium citrate acting as the iron donor. Any ^{55}Fe

that did not incorporate with Lf was separated using spin column chromatography (Micro Bio-spin 6 columns, BIO-RAD, UK, 732-6221).

2.3.10.2 Bacterial ^{55}Fe Lactoferrin uptake analysis

B. cenocepacia reference and clinical overnight cultures were diluted to around 50-100 CFU/ml into Serum-SAPI and supplemented with 50μM NE and 5μl/ml of ^{55}Fe-Lf (200,000 cpm radioactivity) and statically incubated at 37°C, in a humidified 5% CO_2 incubator for 24 hours. Bacterial cells were then centrifuged at 10,000 rpm for 10 minutes and the pellet was washed once in PBS followed by re-centrifuging at 10,000rpm for a further 10 minutes. *B. cenocepacia* bacterial samples where resuspended with 100μl PBS, and transferred into 4 ml scintillation tubes to which 2ml of Optiphase Safe scintillation fluid (Canberra-Packard, UK) was then added. The tubes were left overnight, enabling cell lysis, and ^{55}Fe incorporation was measured on a LKB WAKKAC 1219 Rackbeta liquid scintillation counter set on a tritium counting channel.

2.3.11 *B. cenocepacia* ^{3}H-NE uptake analysis

Overnight *B. cenocepacia* clinical and reference cultures were diluted to around 50-100 CFU/ml in Serum-SAPI and supplemented with 50μM NE or 100μM Fe and 1μCi of ^{3}H-NE; cultures were statically incubated at 37°C with 5% CO_2 for 24 hours. Bacterial cells then further processed as described for the ^{55}Fe uptake assays in section 2.3.10.

2.4 Human airway epithelial cell-bacterial interactions

Air liquid interface (ALI) cultures were prepared from respiratory epithelia taken healthy donors and CF patients to assess the effects of exposure of bacteria to the respiratory epithelium, basal cells and ciliated epithelium, in the presence of

catecholamines and salbutamol. Nasal healthy and nasal and bronchial epithelial cells from CF transplanted individuals were obtained by ciliary brushings. The epithelial cells were cultured to develop into ciliated ALI cultures.

2.4.1 Nasal and Bronchial ciliary brushing

Nasal ciliary brushings were collected from healthy controls (n=10) who were recruited from staff and students affiliated to the University of Leicester. Normal subjects were non-smokers who had no history of respiratory disease or respiratory infection for at least 6 weeks prior to the time of the brushing. The study protocol was approved by the Leicestershire and Rutland Regional Ethics Committee (Appendix 3) and written informed consent was obtained from all subjects using the consent form included in Appendix 1.

The CF epithelium was collected from the nasal and bronchoscopy samples of lung transplantation patients who attended Great Ormond Street Hospital for Children (GOSH), (London, United Kingdom) for their clinical care. Surveillance bronchoscopy is performed at 1 week and 1, 3, 6 and 12 months post-lung transplantation, as part of the standard treatment protocol. Bronchoscopy may be performed on clinical grounds in between times and after the first year after transplantation.

Over a two year period (2009-2011), samples from 10 CF individuals (<18 years age) with lung-transplantation were obtained at a time when they were free from respiratory infections requiring rescue use of antimicrobials for at least 6 weeks prior to the sample

being taken. Considerable time was spent in travelling to London and I was involved in consenting, collecting and processing these samples. Epithelial nasal brushings were also taken by me using the method described below. The bronchial brushings were collected by the lung transplant consultant from the native bronchus, 2-3 cms proximal to the transplant airway anastomosis line. The study collection protocol was approved by the Institute of Child Health and Great Ormond Street Hospital Research Ethics Committee (Appendix 3). Participating children and young persons provided assent and written informed consent was also obtained from parents (Appendix 2).

All individuals, healthy and post transplanted CF patients, had been free from respiratory infections requiring rescue use of antimicrobials for at least 6 weeks prior to the sample being taken.

The bronchial epithelial samples were from post lung transplant CF patients (n=10) and done at the time of routine surveillance flexible bronchoscopy at Great Ormond Street Hospital. Local ethics was sought and approved. Informed consent was obtained from all subjects (Appendix 2). The study protocol was approved by the Leicestershire and Rutland Regional Ethics Committee (Appendix 3).

Brushings were done using a 2mm nylon cytology brush rubbed against the epithelium (nasal or bronchial) in a back and forth direction. Epithelial cell strips obtained were then dislodged by agitating in 2ml of 20mM Hepes buffered medium 199 (pH 7.4, containing penicillin (100 IU/ml), streptomycin (100 µg/ml) and fungizone (2.5 µg/ml)]. The sample was kept in the fridge overnight allowing time for the antibiotics and

fungizone to work and clear any potential deep cellular infections and used for preparation of ALI cultures.

2.4.2 Culture of human epithelial airway cells

Various reagents and collagen coated plates were required as detailed below.

2.4.2.1 Collagen coated plates

For cell culture work, PureCol solution of collagen was prepared as a 1% w/v solution in PBS to cover the surface of plates, flasks, glass-slides and wells used in airway epithelial cell cultures and experiments. After incubating for 5 hours at room temperature, they were washed with nano pure water, air dried and stored at room temperature for later use.

2.4.2.2 Reagents and media used

The details of the reagents used for the respiratory cell cultures are given in Table 2.2

Table 2.2: List of reagents and media used for the epithelial cell cultures

Reagents	**Source/Catalogue number/info.**
Hepes buffered medium 199	Invitrogen, UK, 21180-021
PureCol solution (collagen)	Nunclon, Holland, 5409
Bronchial epithelial cell base medium (BEBM)	Lonza, Switzerland, CC-3171
Trypsin/EDTA	Sigma, UK, T3924

Basal epithelial growth medium (BEGM) Single Quots contains: Bovine pituitary extract (BPE) Insulin, bovine Hydrocortisone (HC); Gentamicin Sulfate and Amphotericin-B (GA-1000) Retinoic Acid Transferrin Tri Iodothyronine (T3) Epinephrine Epidermal growth factor, human recombinant (hEGF)	Lonza, Switzerland,T3924 CC-4009 CC-4021 CC-4031 CC-4081 CC-4085 CC-4205 CC-4211 CC-4221 CC-4230
Nunc 8-well tissue culture chambers	Thermo Fisher, UK, 177402
T80 Flasks	Sigma, UK, 156499
Nunc 12-well plates	Thermo Fisher, UK, 150628

2.4.2.3 Preparation of air liquid interface (ALI) culture

An adaptation of a previously described method was used to grow respiratory basal cells (Gray et al, 1996). The contents of the nasal brush biopsy were grown to 90-100% confluence in a well of a collagen coated 12 well plate with 1ml of basal epithelial growth medium (BEGM) containing antimicrobials at 37ºC. The cells were then washed with Trypsin/EDTA solution, recovered, centrifuged, and pellet re-suspended in BEGM. The cell suspension was then added to a T80 collagen-coated flask containing 14ml BEGM till they were 90-100% confluent. Cells were recovered with the same process and the pellet re-suspended in 1.8ml BEGM. The basal cell suspension was used for air liquid interface cultures. The suspension was divided into four to six collagen-coated transwell inserts (0.4 µm pore size, 1.2 cm^2 diameter) of a 12-well plate with 1ml BEGM on the basolateral side (Figure 2.2).

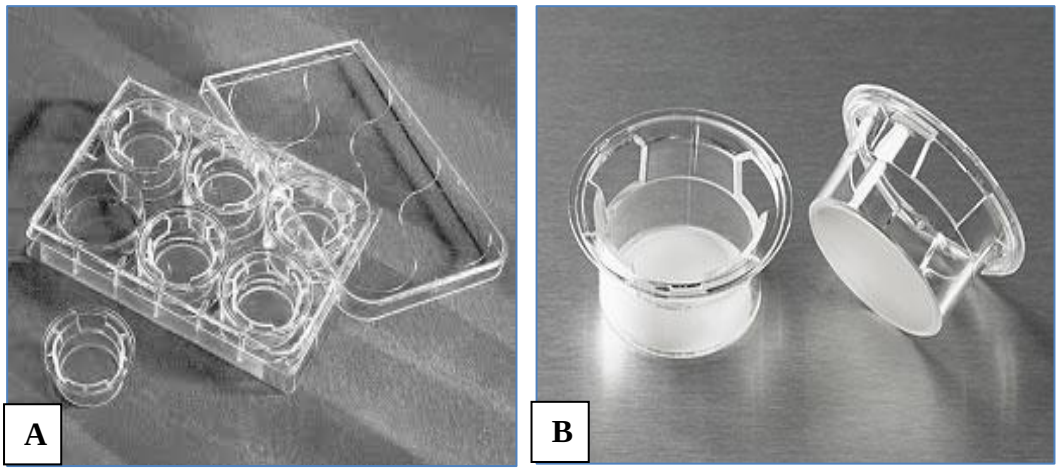


Figure 2.2: Picture of a well of a (A) 12 well plate with transwells and of (B) The transwell, used for ALI culture. The transwell sits in the well and has a basal permeable polyester transmembrane. The transwell inserts are used by first adding medium to the multiwell plate, followed by adding the transwell inserts, and lastly adding the medium and cells to the inside compartment. The transmembrane allows cell adhesion and the pores allow the nutrients to percolate to the cells. The medium level should be checked periodically and fresh medium added as required. Transwell inserts have three openings for standard pipet tips to allow samples to be added or removed from the lower compartment.

Until they reached confluence, the epithelial cells were fed with BEGM every other day by replacing medium on the apical and basal surfaces (300µl and 850µl respectively). When the basal cells became confluent the medium was removed from the apical surface, exposing the cells to air and the medium was also removed from the basolateral well and replaced with 700µl of air liquid interface (ALI) medium. Mucus was produced from the apical surface usually within the first two weeks and the first cilia emerged from cultures from ~day 16 onwards. Fully mature cilia were present after ~3 weeks in culture and the transwell contained ~ 2 x 10^5 cells with 30-50% of the surface ciliated. Figure 2.3 shows a schematic diagram of a transwell with ciliated epithelium.

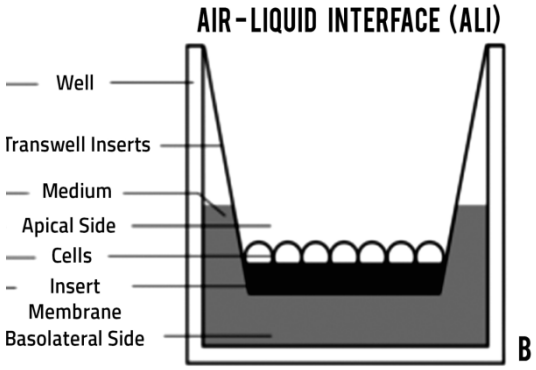


Figure 2.3: A schematic diagram of an ALI culture well with the transwell insert. Usually by day 28, ciliated pseudo stratified airway epithelial cells line the transmembrane and the cell cultures are maintained in this stage for the experiment. (Diagram adapted from Lee et al, 2016)

2.4.2.4 Bacterial infection of ALI cultures

Before infecting the basal and respiratory cells, ALI medium containing antibiotics was replaced with ALI medium free of antibiotics, retinoic acid, hydrocortisone and adrenaline for at least 12 hours prior to the infection. In addition, the cells were washed with BEBM and then infected with *P. aeruginosa* or *B. cenocepacia* (10^6 CFU/ml). Catecholamines were added at 10µM concentration, which is the level reached within the circulation of inotrope medicated patients (Freestone et al, 2012) and readings were carried out at 0, 3 and 5 hours for cilia beat frequency and cell integrity. Qualitative information was obtained to assess bacterial adhesion to cilia, bacterial aggregate formation and presence of debris including dead cells and mucus and comparison made between control (no bacteria added) and infected wells. At the end of the 5 hours, the experiment was completed. The supernatant was removed and 10µL plated for bacterial count. The transwell was washed to remove any adherent bacteria and fixed with 4%w/v Para formaldehyde for immunohistochemical staining and confocal microscopy.

2.4.3 Measurement of ciliary beat frequency (CBF)

CBF was measured as previously described (Mohammed et al, 1999, Hirst et al, 2000a). All CBF measurements were made at temperature between 36.5 and 37.5°C and the pH between 7.35 and 7.45. Ciliary beating was recorded using a Troubleshooter 1000 high speed video camera (Lake Image Systems Ltd, UK) at 250 frames per second (Figure 2.4). CBF was calculated by playing back the video and timing a pre-selected number of individual ciliary beat cycles using at least 50% cells present in that particular video. Ciliary beat was directly measured from movement of the ciliary tips. Groups of beating cilia were identified and the number of frames required to complete 5 beats was recorded. This was converted to CBF by a simple calculation (CBF = [(250/number of frames for 5 beats) x5].

CBFs were recorded from 5 random ciliated areas per well and these areas were marked by XY coordinates so that the same areas could be recorded for each time point. Five CBF readings were taken per well per time point at various XY coordinates - baseline (24 hours before experiment started) and at T0, T3 and T5. The 15 readings per time point per well were then averaged to provide a mean CBF per time point per well.

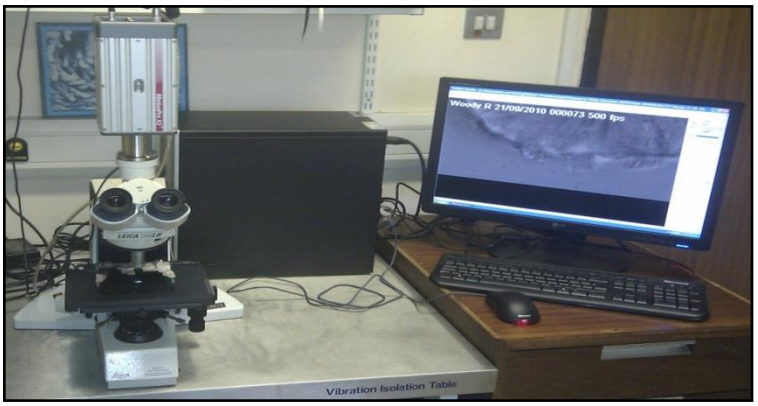


Figure 2.4: Digital high speed video microscopy imaging system used to analyse ciliary beat frequency.

2.4.4 Measurement of bacterial aggregation (cell-cell association) in ALI cultures

Note was made of the presence of any bacterial attachment to cilia and presence of bacterial aggregates or biofilm during each time point. Still pictures were obtained from the video files of each of the 5 ciliated areas studies at time point T5. It was difficult to separate the cell debris, bacterial aggregates or biofilms from each other and thus the area covered by all these elements was measured together. A transparent acetate sheet with a 10cm x 10cm printed grid containing 0.5 cm^2 squares was used for this purpose. The grid contains 100 squares and each square represents 0.2% of the total area measured.

This grid was placed on each still image and total area covered by cell debris, bacterial aggregates and biofilms was noted for each of the five areas. The total such area covered in each well was determined and expressed as a percentage of the total area measured.

2.4.5 Fluorescent labeling of ciliated cells infected with *B. cenocepacia*

At the end of the experiment, some wells were fixed with 4% w/v paraformaldehyde overnight at 4°C to fix the cells for immunofluorescence experiments. The next day, cells were washed with 200µl PBS for 20 minutes with three buffer changes. After the last wash 1ml 3% w/v BSA in PBS was added and left for 10 minutes at room temperature and then washed three times with PBS. Cilia were labelled with mouse anti-acetylated tubulin antibody (Sigma, UK, T6793) diluted 1:1000 and *B. cenocepacia* labelled with rabbit anti-*Pseudomonas* antibody (Abcam, ab68538) diluted 1:40 in 1% w/v BSA in PBS. A 200µl solution of these primary antibodies diluted in 3% w/v BSA in PBS was added to the cells for 2 hours at 37°C. After three washes in 200µl PBS,

antibodies bound to the tubulin protein of cilia were detected using goat anti-mouse alexafluor 594 (Invitrogen, UK, A-11020) diluted 1:250 in 1% w/v BSA in PBS. Antibodies bound to *Pseudomonas* were detected using FITC goat anti-rabbit IgG (Abcam, ab6717) diluted 1:250 in 1% w/v BSA in PBS. A 200µl solution of these secondary antibodies diluted in 1% w/v BSA in PBS was added to the cells for 2 hours at 37°C. During the final 10 minutes, a 1:1000 dilution of Hoechst stain (Sigma Aldrich, UK, H6024) was added to stain the cell nuclei. After 3 washes with PBS, the membranes were excised from inserts using a scalpel and mounted in an inverted orientation onto slides. After adding a few drops of mountant (80% v/v glycerol, 3% w/v n-propyl gallate in PBS) onto the membrane the cells were covered with a size 1.5 coverslip and sealed with nail varnish.

The respiratory ciliated cells were viewed with a Leica TCS SP5 laser scanning confocal microscope (X 63 Plan-APOCHROMAT 1.4 numerical aperture oil DIC lens or X 40 Plan- NEOFLUAR 1.3 numerical aperture oil DIC lens). The lasers for scanning were an Argon laser (488 nm), DPSS (561 nm) and blue diode laser (405 nm).

2.4.5.1 Image analysis software

At five hours, some of the ALI culture wells were fixed in 4% paraldehyde for confocal microscopy. After 48 hours, the supernatant was replaced with PBS and further stained for confocal microscopy as above. To create a 3D surface representation of confocal z stack series from ciliated cells a ‘3D volume render image’ using the blend mode of Imaris software was generated (Bitplane AG, Switzerland, http://www.bitplane.com).

Using the Surpass feature of Imaris it was possible to create 3D surfaces for each fluorescence channel. In this study 3D surfaces were only created for the cytoplasm (red-DiI) and nuclei (blue, Hoechst). Also, using the 'spot object' feature of Surpass, 1µm fluorescent objects (bacteria) were automatically recreated as spots, regardless of the overall intensity. Thus, it was possible to define each bacterium adhered to the cells as a 1µm spot. Imaris detects an automatic threshold at which to insert the spots. The number of spots in each image is calculated automatically. Using a Leica laser scanning confocal microscope (x63 objective) five random areas were imaged by obtaining z sections. Each z section was approximately 0.4µm. Only bacteria in close proximately to the red channel were included in the calculation. It was possible to locate any adhered bacteria and biofilm if any. This was visually quantified. Web training for each feature used is available at http://www.bitplane.com/go/web-training.

2.5 Data Analysis

Experiments were performed in duplicate on at least 3 separate occasions. Graphs were plotted using Graph Pad Prism Program (GraphPad, San Diego, CA, USA). Where appropriate, statistical analysis was carried out using one-way ANOVA (Analysis of Variance) or two-way ANOVA using GraphPad Prism Program. Statistical significance was indicated by a P value of less than 0·05.

All the microscopic were analysed using "Image J" version 1.46m software (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland; http://rsb.info.nih.gov/ij/) image processing program.

Chapter 3

Effect of catecholamines on growth, virulence and biofilm formation of *B. cenocepacia* species

3.1 Overview

Catecholamines are used to maintain heart and kidney function in critically ill patients, including those undergoing major surgery such as lung transplants. Tissue trauma also results in NE release, and it has been shown that the respiratory environment is naturally richly innervated and catecholamines are present within respiratory secretions (Freestone et al, 2012). Catecholamines such as epinephrine are also administered directly at μM levels into intubated patients to reduce airway inflammation (Stannard and O'Callaghan, 2002). Studies have shown altered metabolism of the catecholamines in CF patients and higher serum levels in these patients. Bacteria inhabiting the upper airways will therefore encounter both endogenous and exogenous epinephrine and NE. This section investigates if there are effects of catecholamines on *B. cenocepacia* species, as these pathogens are often implicated in chronic lung disease in CF patients and failure of transplanted lungs in CF patients

Catecholamines are known to interact with bacteria and increase their growth and biofilm forming ability (Neal et al, 2001, Freestone et al, 2002). They have the ability to improve nutrient availability to the bacteria and also affect the cell to cell inter-kingdom signalling ability (Kendall et al, 2014, Sperandio et al, 1999). Previous work done by Freestone et al (1999, 2012) and others (Lyte and Ernst, 1992, Belay and Sonnenfeld, 2002, O'Donnell et al, 2006) shows that *Pseudomonas* has the ability to increase growth and biofilm formation in the presence of 5-100 μM catecholamine concentration. These concentrations are achievable after therapeutic use of these drugs. A study done with *Burkholderia pseudomallei* showed that it responded to catecholamines (Intarak et al, 2014) and this was the first study to report this interaction with any *Burkholderia* species.

To study the effect of catecholamines on *B. cenocepacia* species associated with CF lung disease, I carried out work exploring effect of catecholamines on the growth and biofilm formation on *B. cenocepacia* strains based on methods described in Chapter 2, Materials and Methods.

3.2 Results

3.2.1 Effect of catecholamines on *B. cenocepacia* growth

The effects of clinically relevant concentrations of catecholamines Epi and NE on growth of clinical and reference *B. cenocepacia* strains was investigated in this section. Serum-SAPI medium was used since it provides a host like environment with limited availability of nutrients, iron and presence of stressful antibodies and complement. It is a preferred medium for testing catecholamine responses (Freestone et al, 1999).

The clinical and reference *B. cenocepacia* were serially diluted to 10^{-7} dilutions (around 10 CFU/ml) in Serum-SAPI medium enriched with 5μM, 10μM and 50μM concentrations of catecholamines plus 10μM Fe (as a positive growth control for tolerance to serum) and no additions (negative control). Viable bacterial count (expressed as CFU/ml) of cultures were carried out after overnight incubation (18 hours static growth in a humidified CO_2 incubator set at 37^oC).

Figures 3.1A and B show a representative graph of the catecholamine NE with clinical and reference strains of *B. cenocepacia* at -6 log dilution. The catecholamine growth effect was bacterial cell density dependent. There was a significant increase in bacterial numbers in the catecholamine enriched inocula compared to control in the -6 and -7 log dilutions ($p<0.05$). It could also be seen that iron treated cultures showed a significantly higher growth compared to control but not significantly different to catecholamines. This was probably due to the iron-restricted nature of serum caused by the presence of the ferric iron sequestering protein transferrin (Freestone et al, 1999). The catecholamine increases in growth were generally similar in magnitude with induction

of growth occurring at levels as low as 5µM, which is interesting as this level is reached clinically in surgical patients (Thompson et al, 1999). These results clearly show that *B. cenocepacia* strains are catecholamine responsive microbes.

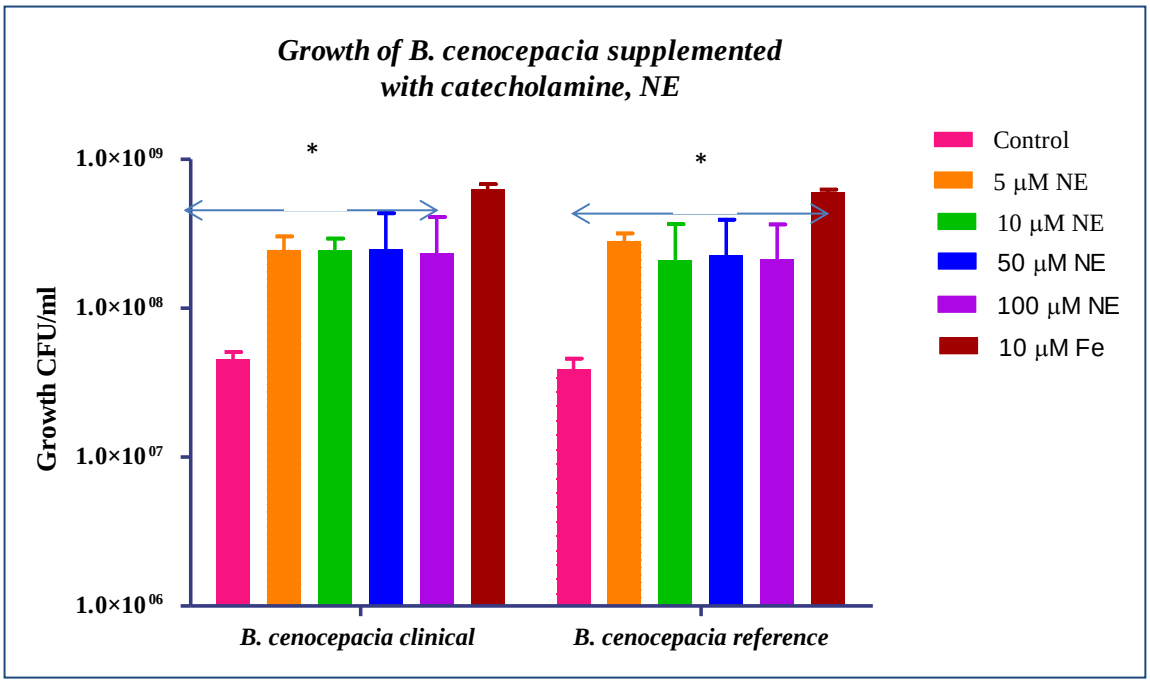


Fig 3.1 A and B: Growth of *B. cenocepacia* in presence of catecholamine, NE: Overnight cultures of the strains were diluted with serum-SAPI with or without the catecholamines at various concentrations and grown for 18 hours statically at 37^{o}C. Bacteria were then enumerated by serial dilutions and plating to obtain the growth measured as CFU/ml. Values represent the means and standard deviations of triplicate platings from duplicate cultures. Key: Control, bacteria no drug added; NE, noradrenaline; Fe, ferric nitrate. The symbol * denotes significance, $p<0.05$ and the bars denote SD around mean.

3.2.2 Effect of catecholamines on growth kinetics of *B. cenocepacia*

The reference and clinical strains of *B. cenocepacia* were cultured in serum-SAPI medium along with different concentrations of catecholamines and iron and continuous readings of cell density were taken using a Varioskan spectrophotometer (Thermo Scientific, UK), over a 24 hour period. The resultant time course showed a catecholamine dose dependant increase in the growth of the two bacteria, more evident with the reference strain and maximal with 50µM NE (OD_{595} of 0.40) after 12 hours. This was comparable to the growth achieved with Fe and significantly higher than the

un-supplemented control (OD_{595} of 0.30). Figure 3.2 shows the representative data for the clinical *B. cenocepacia* strain with NE at various concentrations.

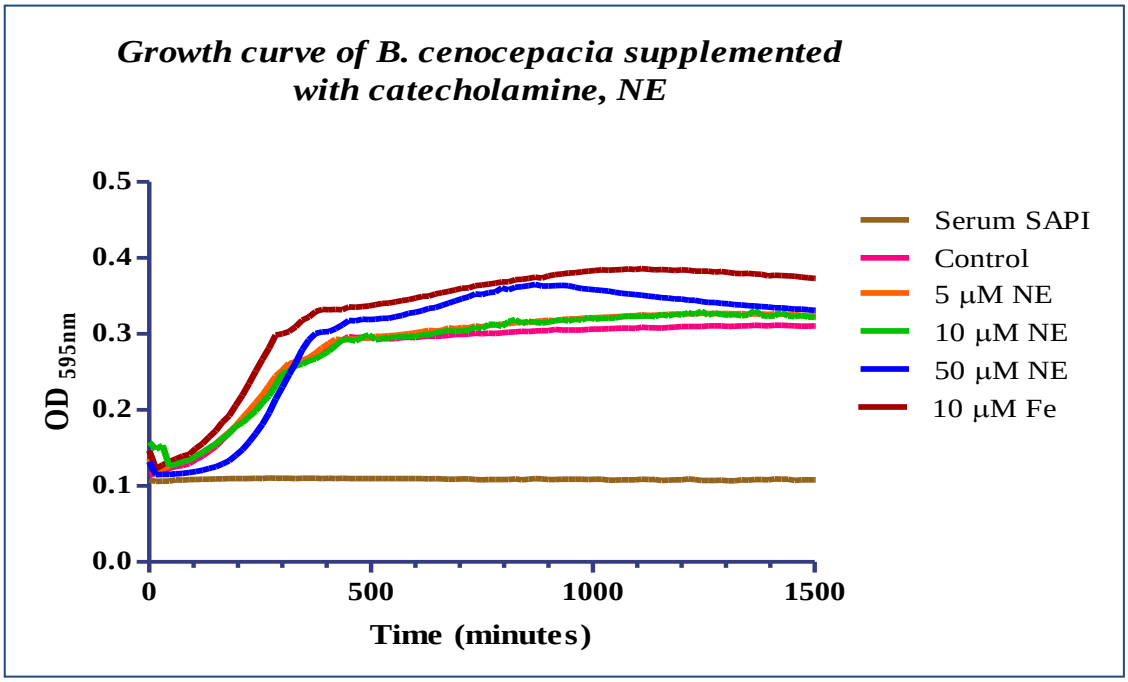


Figure 3.2: Time course of growth of *clinical B. cenocepacia* strain in the presence of catecholamine, NE. Overnight cultures were diluted 1:1000 in Serum-SAPI medium and growth kinetics measured over 24 hours. Key: Control, bacteria no supplemented drug; NE, norepinephrine; Fe, ferric nitrate.

3.2.3 Effect of catecholamines on *B. cenocepacia* on initial attachment

The initial stages of biofilm formation of the *B. cenocepacia* strains were studied by crystal violet microtitre plate attachment assay wherein samples were incubated in Serum-SAPI for 24 and 48 hours in a 96 well microtitre plate and attachment of bacteria measured with crystal violet staining.

The presence of catecholamines showed significantly increased attachment of *B. cenocepacia* strains at the various catecholamine concentrations. Figure 3.3 shows the representative data for the attachment with clinical *B. cenocepacia* with catecholamine,

NE. There was a dose significant related increase in attachment between 5 and 50µM NE and concentrations at 48 hours.

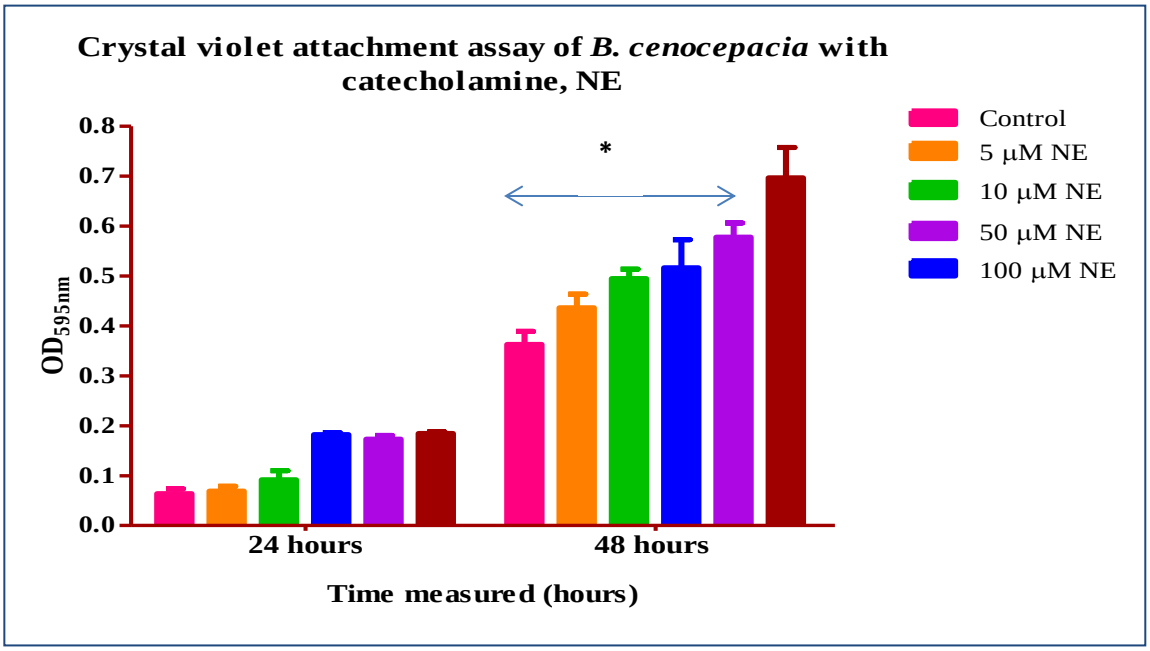


Figure 3.3 Catecholamine, NE increases *B. cenocepacia* attachment on crystal violet assay. The graph show significantly increased attachment of *B. cenocepacia* to polystyrene following NE exposure. Overnight cultures were diluted 1:100 into the indicated media and bacteria cultured and attachment measured at 48 hours with crystal violet. Data represent means and SD of 4 biological replicates. Key: Control, bacteria no supplemented drug; NE, norepinephrine; Fe, ferric nitrate. The symbol * denotes significance, $p<0.05$ and the bars denote SD around mean.

3.2.4 Effect of catecholamines on *B. cenocepacia* attachment

Further biofilm aspects of the *B. cenocepacia* strains were studied by using a fluorescence microtitre plate attachment assay. Bacteria were incubated in Serum-SAPI for 24 and 48 hours in a 96 well microtitre plate in the presence and absence of catecholamines and attachment of bacteria stained with LIVE-DEAD fluorescent stain.

It was seen that at both 5 and 50 µM concentrations of the inotropes, there was increased attachment of both the *B. cenocepacia* strains. Representative data for the clinical and reference strain with 50µM concentrations of catecholamines, Dop, Epi and NE is shown in Figure 3.4. Visually under the microscope, there appeared to be more

clearly formed bigger microcolonies of *B. cenocepacia* particularly at the higher concentrations of catecholamines.

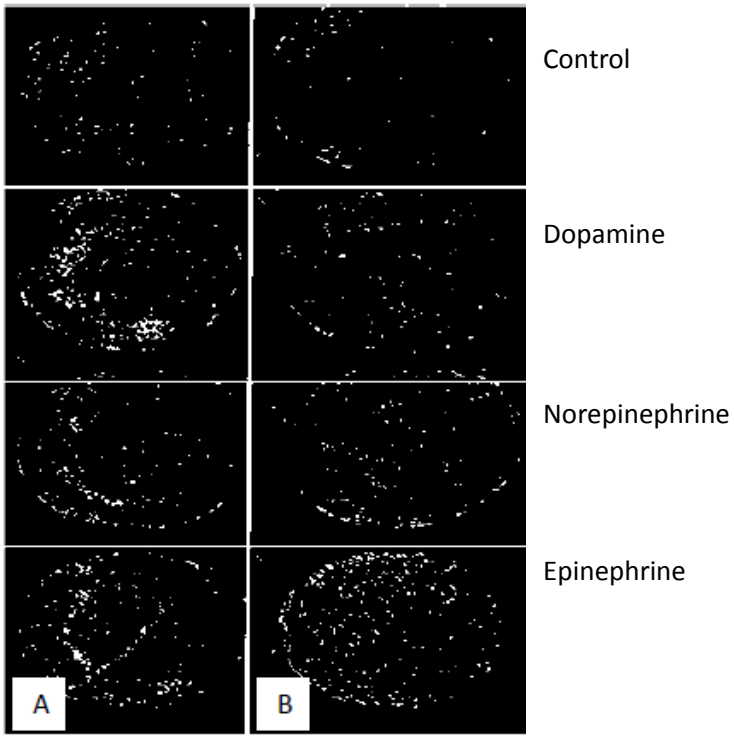


Figure 3.4: Bacteria were incubated for 48 hours without drug (Control) and with addition of the catecholamines- 50µM Dop, NE and Epi. The 96 well microtitre plate bottom was stained with a LIVE-DEAD stain and observed under NIKON Eclipse T-inverted fluorescence microscope (10x). The initial biofilm attachment is seen as small aggregates (green in original picture, changed to white for easy visibility in this picture). **A**. Experiment with clinical *B. cenocepacia* strain and **B.** Experiment with reference *B. cenocepacia* strain.

The fluorescent intensity of the entire well was quantified to give the 'integrated density' for each well. The histogram shows the integrated densitometries for each of the above wells (Figure 3.5). The biofilm formation was enhanced with the addition of catecholamines compared with the control at ~48 hours ($p<0.05$).

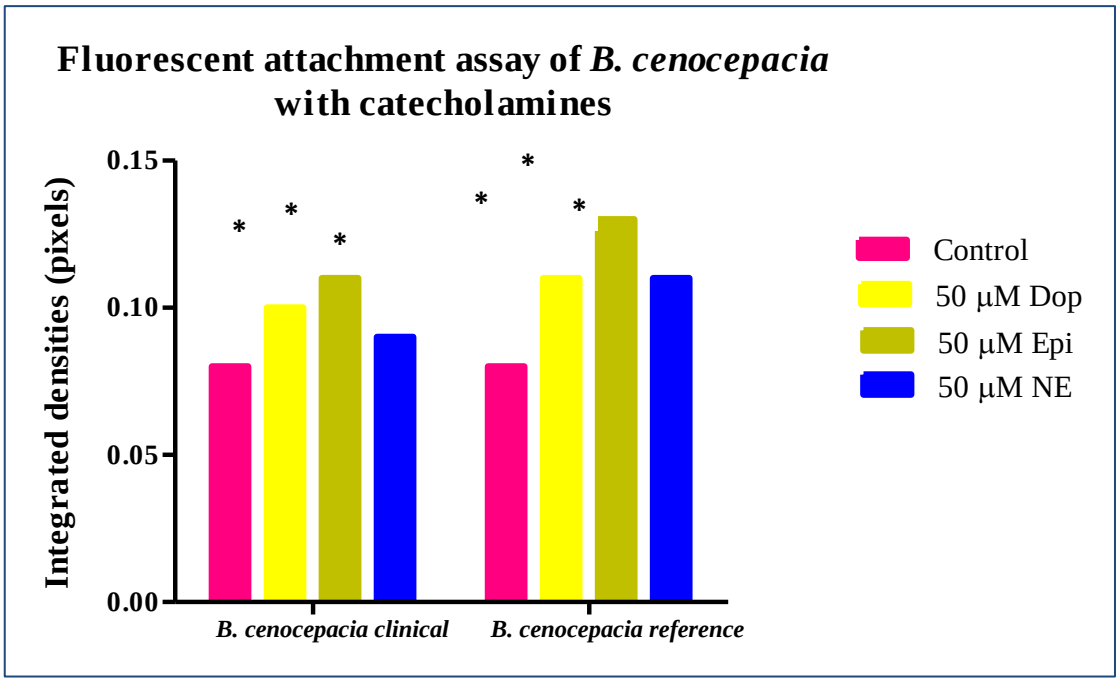


Figure 3.5: Histogram showing fluorescence intensities measured as 'integrated densities' of attached bacteria for each well. The symbol * indicates statistical significance of $p<0.05$.

This suggests that in addition to enhancing growth and attachment, the inotropes were also increasing *B. cenocepacia* biofilm development.

3.2.5 Effect of catecholamines on *B. cenocepacia* cell-cell association

When conducting the growth assays in section 3.2.1, it was noticed that the bacterial cells formed clumps in the wells of the plastic ware when in serum-SAPI medium. It is known that bacteria aggregation (cell to cell association) is one of the intermediate steps of biofilm formation (Sauer et al, 2002). A visual assessment of the plates under a 20x objective by light microscope was undertaken and representative pictures are shown in Figures 3.6.

It can be seen that there was 'clumping' noted in the control well without addition of any drugs, but the amount and size of bacterial clumps appeared much greater in the

catecholamine enriched culture. The clumps were made of aggregated bacteria within a mesh with some loose fibrous connections to the walls of the well and the clumps were loosely floating on the surface of the medium. It was possible to easily tease the mass apart at this stage. Examples of the images of control clinical *B. cenocepacia* cultures (without supplemented drug) and those with addition of 10µM Epi and NE and Control are shown in Figures 3.6 A, B and C respectively.

The clumping denotes the first stage of biofilm formation. The catecholamines had clearly increased the bacterial aggregation and matrix production, one of the key developmental steps in biofilm formation.

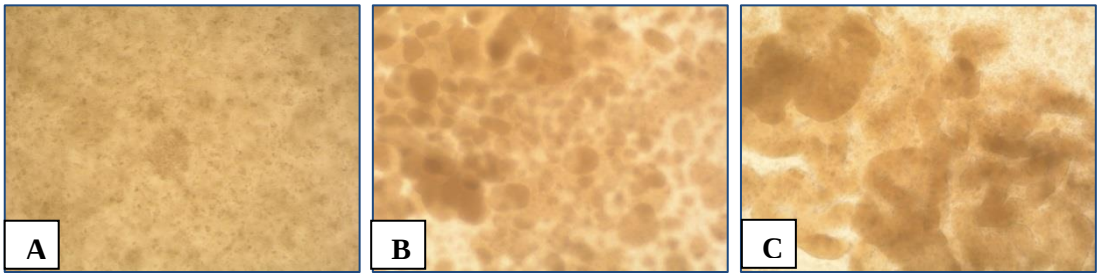


Figure 3.6A, B, C: Representative images of light microscopy of a -6 dilution well of a 24 hour *B. cenocepacia* culture in Serum-SAPI medium with **A.** Control, bacteria only- no added drugs **B.** 10µM Epi and C**.** 10µM NE.. The drug supplemented wells showed significantly increased aggregation of bacteria and the aggregates were much larger in size.

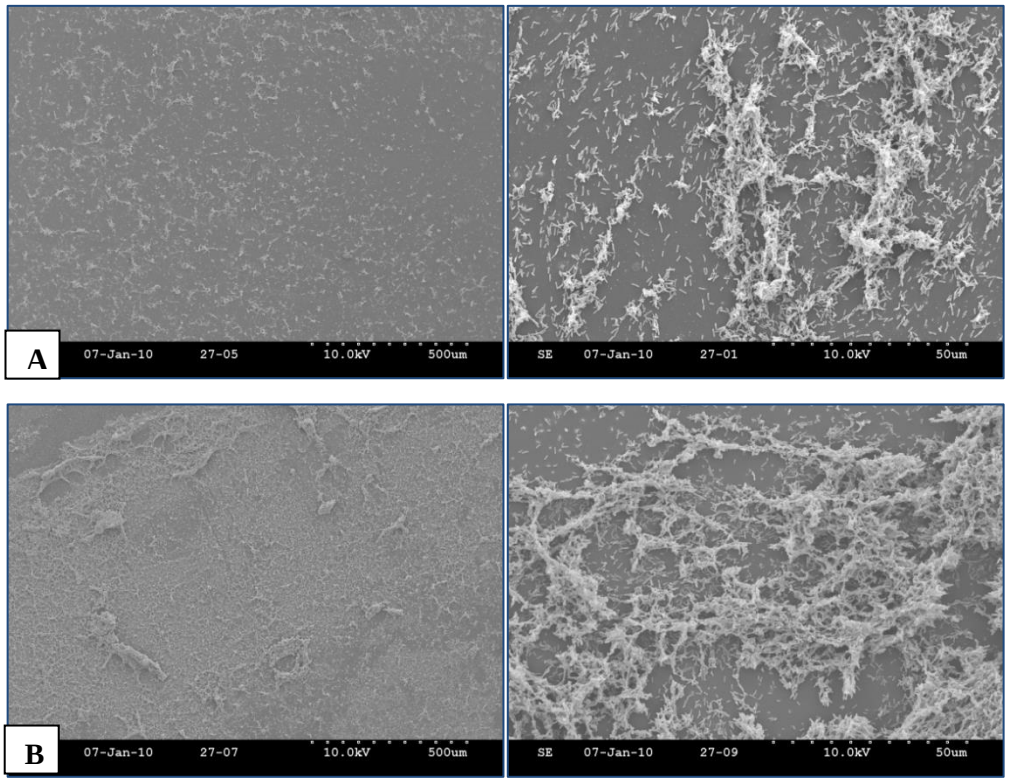
3.2.6 Catecholamines effect on *B. cenocepacia* biofilm on endotracheal tubes

The endotracheal (ET) tube is an important nidus for bacterial seeding and biofilm formation and thus can be a focal source of respiratory infection in ventilated patients (Adair et al, 1993, 1999, Freestone et al, 2012). Patients undergoing lung transplantation may be intubated for a considerable length of time in their post-operative recovery period. This investigation was thus done in a more clinically relevant context to study the possibility of inotrope effects on endotracheal tube biofilm formation. The ability of the *B. cenocepacia* strains to produce biofilms and the effect of catecholamines on ET

tube colonisation by *B. cenocepacia* was assessed using scanning electron microscopy (SEM) as per the methods described in Chapter 2.

Figures 3.7 show the SEM images of the surface of the ET sections after 48 hours of incubation with *B. cenocepacia* with and without supplementation with NE at lower magnification (scale 500μm) and higher magnification (scale 50 μm) .

There is relatively little biofilm formation seen on the ET surface in the un-supplemented serum-SAPI *B. cenocepacia* cultures compared to that seen with 5μM NE and higher concentration 50μM NE supplemented cultures. Both these concentrations are therapeutically achievable in bodily fluids in health and disease states in individuals with CF. There is a dose dependent increase in the density and structure of the biofilms. Similar findings were observed with other catecholamines and at different drug concentrations (images not shown).



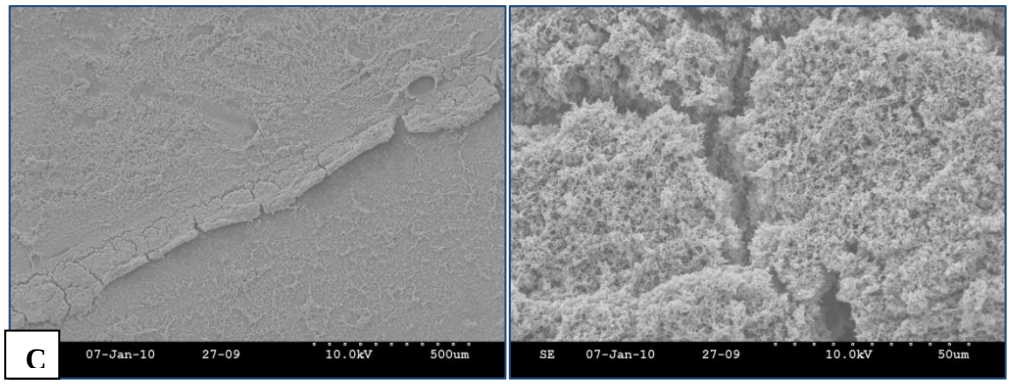
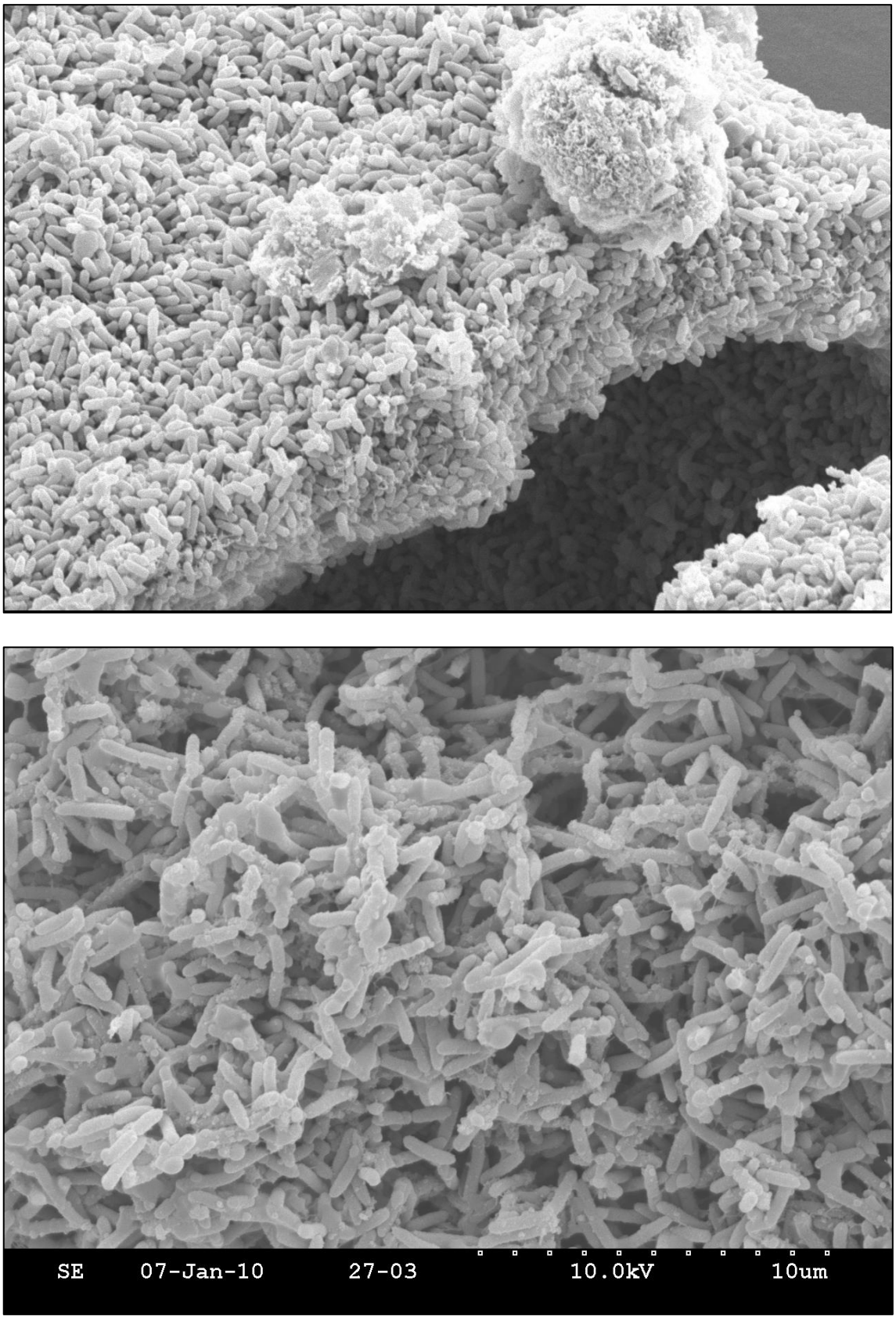


Figure 3.7A, B and C: Representative SEM images of catecholamine effects on the morphology of *B. cenocepacia* biofilm noted on ET tube segments after 24 hours in serum SAPI compared to control without drug. Data is representative of 3 biological replicates of control and norepinephrine supplemented bacterial cultures at 500µm and 50µm magnification **Row A**. Control, bacteria only no drug added, **Row B.** 5µM norepinephrine, and **Row C.** 50µM norepinephrine.

To obtain a clearer view of the 3-dimensional structure and EPS matrix with enmeshed bacteria, the 50µM norepinephrine supplemented bacterial culture image is shown in Figure 3.8 and 3.9.

The biofilm appears variable in thickness in these scanning electron microscopy images and it is likely that due to the multiple dehydration steps involved in the preparation of the ET sample that larger heavier chunks of biofilm could have become dislodged (a problem reported by Freestone et al, 2012). Collectively, the experiments detailed in this chapter shows that catecholamines induce copious mature *B. cenocepacia* biofilm on medical grade plastics.



Figures 3.8 and 3.9: Magnified images (at 10µm scale) of the bacterial culture supplemented with 5µM norepinephrine. There is clear visibility of a dense 3-dimensional biofilm structure with EPS matrix formation with enmeshed bacteria.

3.3 Mechanisms of catecholamine growth induction

3.3.1 *B. cenocepacia* can bind lactoferrin

Lactoferrin (Lf) is a glycoprotein present in various secretory fluids of the body including the airway epithelial fluid and has anti-microbial properties forming one of the innate mechanisms of the lung. Its main role is to sequester free iron and in doing so it removes essential substrate required for bacterial growth (Farnaud et al, 2003). Lf binds to bacterial walls, and forms peroxides affecting the stability and permeability of the cell wall causing cell breakdown (Andrés et al, 2005). This lethal action is unrelated to the iron steal action. Investigations were therefore carried out to check if *B. cenocepacia* cells were able to bind to lactoferrrin. This was done for the clinical and reference *B. cenocepacia* strains and analysed using the whole cell dot blotting method described in Chapter 2.

Lf binding (Figure 3.10) was observed in *B. cenocepacia* strains analysed using anti-Lf antibodies coupled to horse radish peroxidase followed by enhanced chemiluminsence and image capture on x-ray film. The image shows that Lf is bound by the bacteria, which is the first time this has been demonstrated. The fainter circles in the bacteria only controls in Figure 3.10 are due to the endogenous peroxidase(s) present in the bacteria activating the enhanced chemiluminsence reagents.

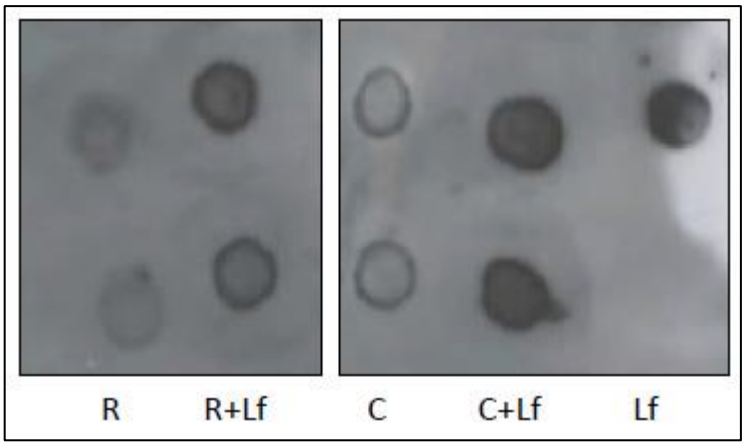


Figure 3.10: *B. cenocepacia* clinical and reference strains can bind lactoferrin. Lactoferrin binding assays was carried out as described in Chapter 2, Materials and Methods. Key: **C**, Clinical strain, no lactoferrin, **R**, Reference strain, no lactoferrin, **C** and **R +Lf** contain lactoferrin. **Lf** is the lactoferrin only positive control.

3.3.2 Radioactive iron labelled lactoferrin (^{55}Fe-Lf) uptake is increased by *B. cenocepacia* in presence of catecholamines

To determine if Lf iron was being used by the *B. cenocepacia*, uptake of ^{55}Fe from radiolabelled ^{55}Fe-Lf (Lf labelled with radioactive ^{55}Fe as described in Chapter 2) was investigated. *B. cenocepacia* cultures were grown in serum-SAPI without and with 50μM NE, to allow the elucidation of the impact of catecholamines on iron uptake from Lf, and grown at 37^{o}C for 24 hours. Bacteria were counted for cell numbers, harvested, washed and measured for ^{55}Fe-incorporation as described in Chapter 2.

Figure 3.11 shows the ^{55}Fe levels of the control and NE-supplemented cultures. It was found that there was an increase in iron acquisition as a result of NE administration compared to controls; increases in iron uptake were significant $p<0.05$ with the students T-test. This shows that *B. cenocepacia* can utilise iron from Lf and iron uptake from the iron-sequestering protein is increased in the presence of a catecholamine.

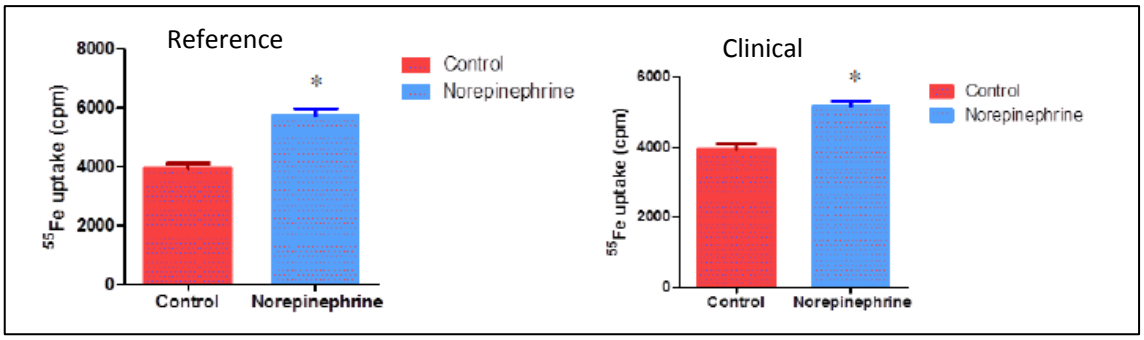


Figure 3.11 Catecholamines increase *B. cenocepacia* uptake of ^{55}Fe from ^{55}Fe-lactoferrin. Histograms show bacterial ^{55}Fe uptake from ^{55}Fe-lactoferrin is enhanced when a catecholamine is present for reference and clinical *B. cenocepacia* isolates, respectively ($p>0.05$). * denotes significance value of $P<0.05$ and error bars denote mean +/- SD; n=3.

3.3.3 Radioactive norepinephrine (^{3}H-NE) uptake analysis shows that catecholamines are internalised by *B. cenocepacia*

^{3}H-NE uptake was analysed with *B. cenocepacia*. The uptake of ^{3}H-NE helps determine whether the catecholamine is actually taken into the bacteria or is merely adsorbed to the outside of the bacteria. Bacteria were cultured in Serum-SAPI medium and various dilutions of the culture were processed to check whether *B. cenocepacia* took up NE. As shown in Figure 3.12, a significant increase in ^{3}H-NE uptake occurred in both clinical and reference strains at similar levels. Interestingly, for the clinical strain ^{3}H-NE uptake was enhanced when non-labelled NE was present ($p<0.01$).

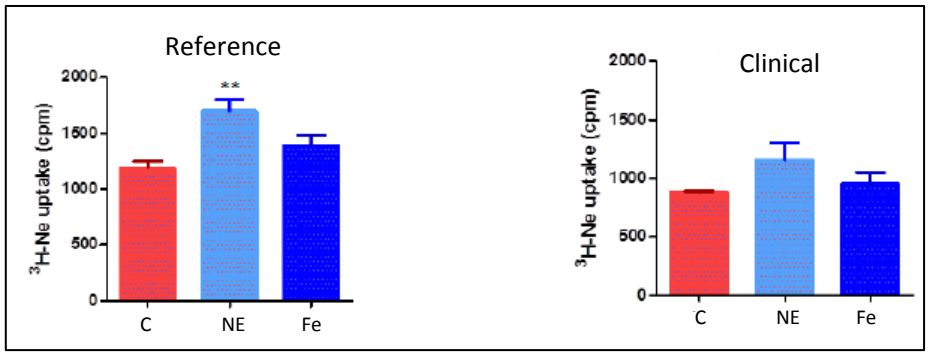


Figure 3.12 *B. cenocepacia* uptake of ^{3}H-NE. Uptake of ^{3}H-NE by reference and clinical *B. cenocepacia* incubated with Control, NE or Fe supplementation. Values shown are means of triplicate assays. The symbol ** indicates statistical significance of $p<0.01$ and error bars show SD of the mean; n=3.

3.4 Discussion

Patients with CF have a predisposition to infection with bacteria such as *Burkholderia* which form chronic infections aided by biofilms which can lead to significant morbidity and mortality. In end stage CF, patients who undergo lung transplantation are at higher risk of death due to *Burkholderia* infection (Schöni et al, 1985, 1986) and post lung transplanted CF (Dickinson et al, 2015) patients have higher endogenous catecholamine production. Also, whilst in the post-operative period, CF lung transplantees are exposed to exogenous inotropic catecholamines. Patients with CF and those post lung transplantation CF patients have indwelling plastic central lines for long term intravenous antibiotics and those who are ventilated have endotracheal tubes. The respiratory tissues, via the endotracheal tube, may also be used as a direct site for systemic administration of inotropes (Raymondos et al, 2000) and procedures such as ET tube suctioning of patients have shown to contribute to significant systemic increase in NE and epinephrine (Schmidt and Kraft, 1995). Catecholamines such as epinephrine may also be directly administered via the ET (Stannard and O'Callaghan, 2002). Dopamine and NE are also naturally present within respiratory mucus in mammalian animals (Lucero et al, 1998). This suggests that the bacteria inhabiting the ET and upper airways are likely to come into contact with both endogenous and exogenous catecholamines and to a greater extent in CF and post-lung transplantation patients. Patients exposed to catecholamine-based inotropic drugs have been shown to induce growth of both pathogenic and commensal bacteria (Neal et al, 2001, Freestone et al, 2002) and stimulate biofilm formation in *Staphylococcus epidermidis* (Lyte et al, 2003). Studies have shown that NE and other catecholamines stimulate *P. aeruginosa* growth at 5-100μM concentration, which are easily attainable in presence of endogenous and

exogenous catecholamines (Lyte and Ernst, 1992, Freestone et al, 1999, Belay and Sonnenfeld, 2002, O'Donnell at al 2006).

Burkholderia are biofilm forming organisms and in CF lungs this lends to their pathogenesis of lung damage and relatively high resistance to antibiotics (McClean et al, 2009, Conway et al, 2002). Previous studies have mainly focused on culturing, identification and antibiotic resistance of *Burkholderia* biofilms (Sousa et al, 2011, Thomas et al, 2007, Darling et al, 1998). There is very limited information of the effect of catecholamines on *Burkholderia* growth or virulence. A study reported interaction between epinephrine and *Burkholderia pseudomallei*, the causative agent of melioidosis. This was the first study to report *in vitro* effect of catecholamines on *Burkholderia* species (*B. cenocepacia)* in this case. There was enhanced bacterial motility, transcription of flagellar genes and flagellin synthesis, increased genes coding for superoxide dismutase (*sodB*) and the malleobactin receptor (*fmtA*) causing resistance to superoxide (Intarak et al, 2014).

In this chapter an investigation on the effects of catecholamines, freely present in the lungs of CF patients, on *B. cenocepacia* growth and biofilm formation was carried out. The *B. cenocepacia* strain was clinically relevant for this group of patients. The clinical context was also brought in by way of endotracheal tube studies. In this study, it has been shown that catecholamine inotropes could stimulate *B. cenocepacia* growth and biofilm formation even at lower concentrations (5µM) and this is clinically relevant. *B. cenocepacia* strains form thick biofilm *in vitro* and biofilm formation causes destruction of glycocalyx layer produced by lung epithelial cells (Schwab et al, 2002; Mario and Dianella, 2007). All bacteria require iron for growth *in vivo*, and for this reason iron

restriction in the host body fluids by iron binding proteins transferrin and lactoferrin is a primary host defence (Freestone et al, 2008). Catecholamine inotropes have shown to stimulate growth of a number of bacteria by scavenging iron from serum transferrin (Freestone et al, 2000, 2002, 2003, and 2007). In this study, I observed thick mature biofilm forming on clinical plastics such as endotracheal tube. In this study it also became clear that the mechanism of growth stimulation by the catecholamines in host like media involved the catecholamine enabling *B. cenocepacia* to acquire and internalise the iron bound within iron sequestering proteins such as lactoferrin and resulted in internalisation of both ^{55}Fe-Lf and the inotrope (NE in the case of this study). That *B. cenocepacia* can use a catecholamine to increase its uptake of ^{55}Fe-Lf is important, as lactoferrin is a major protective protein in mucosal secretions.

Collectively, all the tests done in this chapter have showed that *B. cenocepacia* growth and biofilm formation was increased by catecholamines and catecholamines enable the bacteria to access host iron to facilitate this. Exposure to *B. cenocepacia* aided by catecholamines allows them to overcome the iron restriction in the host and causes severe or chronic infection in vulnerable patient groups such as CF and post lung transplantation patients.

Chapter 4

Effect of salbutamol on the growth, virulence and biofilm formation of *P. aeruginosa* and *B. cenocepacia*

4.1 Overview

Pseudomonas and *Burkholderia* are respiratory pathogens strongly associated with lung disease such as CF and cause chronic infection and biofilm formation in these patients; *Burkholderia* is particularly associated with mortality in CF patients who have undergone lung transplantation. The work done by Freestone et al (2012) and Sharaff (PhD University of Leicester, 2012), suggests that *Pseudomonas* responds to catecholamines and has increased growth, virulence and biofilm formation. Salbutamol, an adrenergic drug similar in structure to catecholamines and widely used in lung disease, is also known to interact with bacteria (Gross et al, 2010, Maris et al, 2006, Vandevelde et al, 2015). A recent study by Zhang et al showed a dose responsive decreased in air surface fluid antibacterial activity by 5 to 8-fold with salbutamol thereby resulting in increased bacterial growth of *E. coli* and *P. aeruginosa* in airway surface fluid on human airway carcinogenic cell line air liquid cultures (Zhang et al, 2011).

Salbutamol is routinely used in CF for bronchodilatation and airway clearance. The doses used clinically are 2.5 to 5 mg nebulised or 1000 µg inhaled delivered by a metered dose inhaler. The nebulised drug is readily dissolvable in airway fluid and easily dispersible into the circulation. It is deposited in the larger airways and also reaches the alveolar spaces and achieves a concentration of 1.25µg/ml in alveolar fluid after inhalation of therapeutic doses (Atabai et al, 2002). In my work, I have used a range of concentrations between 0.1µg/ml to 50µg/ml. The higher concentrations were used to try and represent the greater deposition in the larger airways and on endotracheal tube surfaces after therapeutic inhalation. Salbutamol was used in the original chemical form to establish the methods and determine concentrations of the in

vitro experiments, but to make the results of my study more clinically relevant, the commercially available form, Ventolin nebules; GlaxoSmithKline UK, bought from local hospital pharmacy was used.

It is possible that salbutamol can interact with *P. aeruginosa* or *B. cenocepacia* in the CF patients and affect their growth and virulence. This question has not been explored previously and the aim of this project is to study if there is an effect of clinically relevant salbutamol concentrations on growth and biofilm formation of *P. aeruginosa* and *B. cenocepacia in vitro.*

4.2 Results

4.2.1 Effect of salbutamol on *P. aeruginosa* growth

The effects of various concentrations of salbutamol on growth of clinical *P. aeruginosa* strain was investigated. The bacteria were serially diluted to 10^{-8} dilutions (to around 10 CFU/ml) in Serum-SAPI medium enriched with various concentrations from 0.1 to 50µg/ml of a clinical preparation of nebulised salbutamol (Ventolin, 2.5mg/ml) and unsupplemented bacterial culture was used as control . The viable bacterial count (expressed as CFU/ml) of the overnight cultures were carried out for the different initial inoculum dilutions. Figure 4.1 shows the growth (expressed as CFU/ml) of *P. aeruginosa* in control (unsupplemented) cultures and those with additional salbutamol at various concentrations. The results show no significant increase in viable bacterial numbers in the salbutamol enriched inocula compared to the control after incubation.

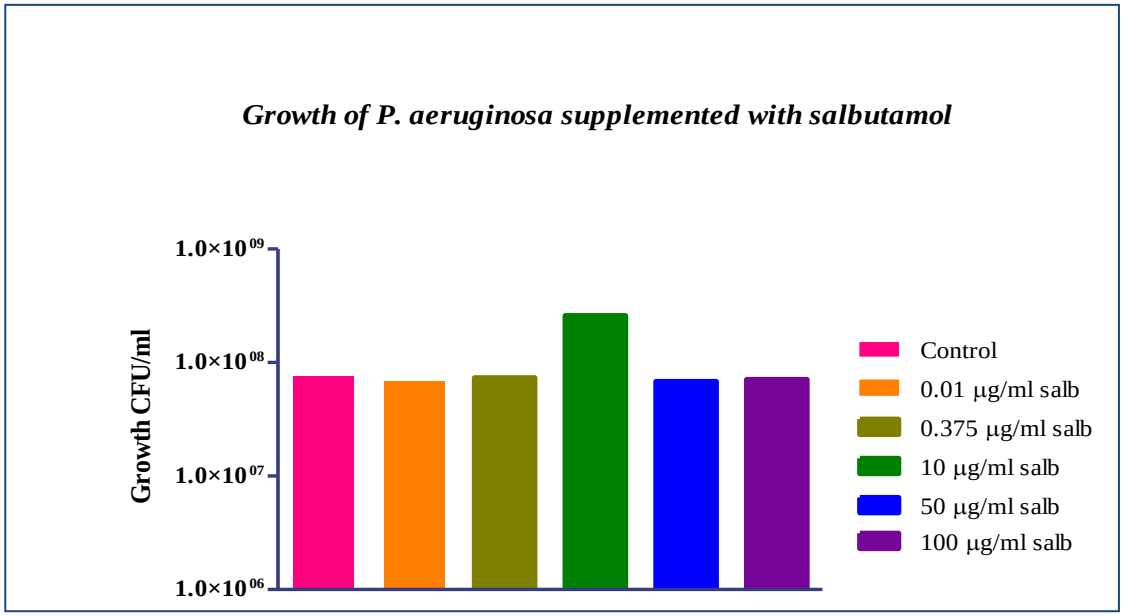


Fig 4.1: Growth of *P.aeruginosa* in presence of salbutamol: Overnight cultures of the strains were diluted in 10-fold steps with serum-SAPI with and without supplemented salbutamol at various concentrations and then incubated statically at 37°C for 24 hours. Bacteria were then enumerated by serial dilution and plating to obtain the final CFU/ml. Values represent the means and standard deviations of triplicate platings from duplicate cultures. Key: Control, bacteria no drug added.

4.2.2 Growth kinetics for *P. aeruginosa* in the presence of salbutamol

P. aeruginosa was cultured in serum-SAPI medium along with different concentrations of salbutamol and continuous readings were taken using the Varioskan spectrophotometer at OD_{595} over a 24 hour period. The resultant time curve showed no significant difference in the growth profiles of the two bacteria. The representative data for the clinical isolate with higher concentrations of additional salbutamol is shown (Figure 4.2) and similar observations were made with the lower salbutamol concentrations.

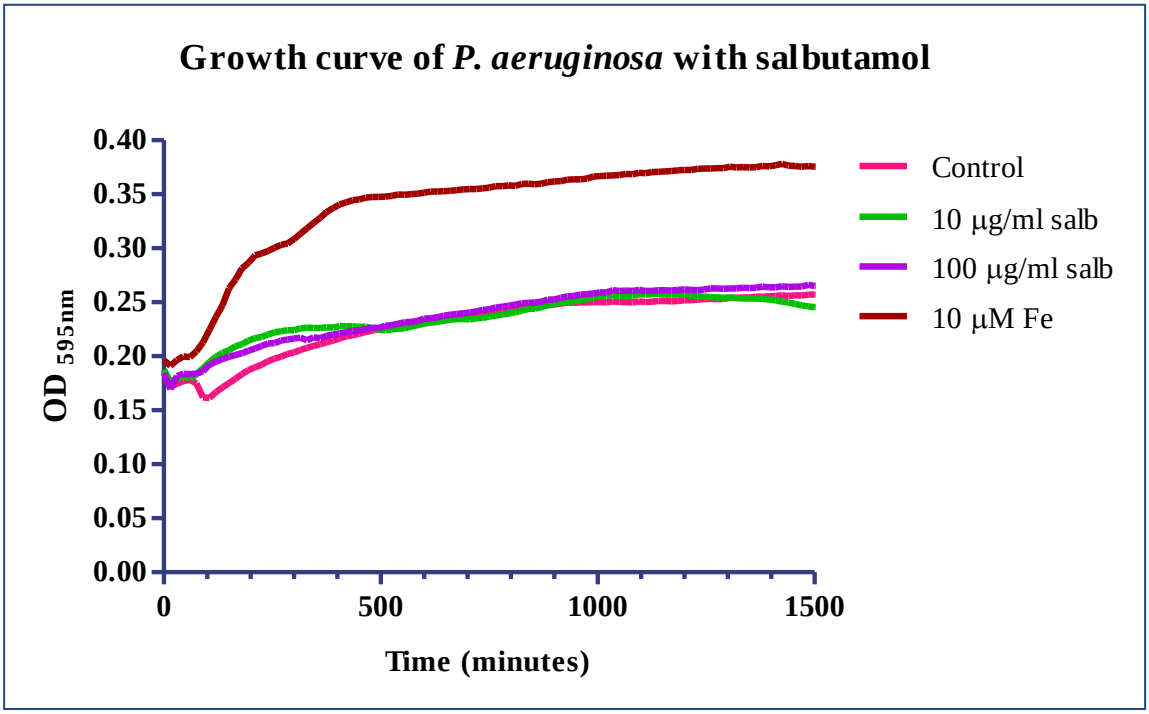


Figure 4.2: Time course of growth of *P.aeruginosa* strains in the presence of salbutamol. Overnight *P. aeruginosa* cultures were diluted 1:1000 in Serum-SAPI medium and growth kinetics measured over 24hours. Key: Values show the means of three separate experiments; control, bacteria no drug added; n=3.

4.2.3 Effect of salbutamol on *P. aeruginosa* initial attachment

The initial stages of biofilm formation of the *P. aeruginosa* was studied by crystal violet microtitre plate attachment assay wherein samples were incubated in Serum-SAPI for 24 and 48 hours and attachment of bacteria measured using crystal violet staining.

Biofilm formation on polystyrene with the clinical strain and in response to salbutamol in serum-SAPI medium at 24 and 48 hours is shown in Figure 4.3. The data shows that presence of salbutamol did not increase the initial attachment of *P. aeruginosa* at any salbutamol concentration either at 24 or 48 hours.

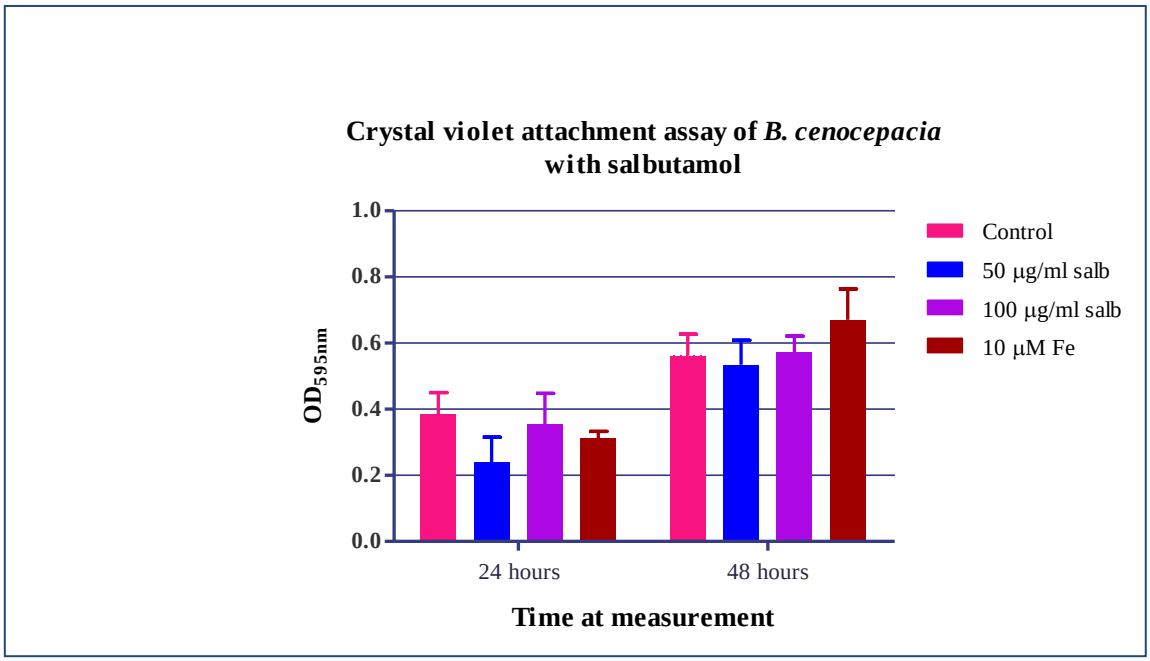


Figure 4.3 *P. aeruginosa* attachments on crystal violet assay with and without supplementation with salbutamol. The histogram shows no difference in attachment of *P. aeruginosa* to polystyrene stained with crystal violet following higher concentrations of salbutamol exposure compared to the un-supplemented control at 24 and 48 hours. Data represent means and SD of 4 biological replicates. Key: Control, bacteria no drug added.

4.2.4 Effect of salbutamol on *P. aeruginosa* cell-cell association (aggregation)

Clumping or aggregation of *P. aeruginosa* cells in the plastic culture wells in the presence of salbutamol was evident during growth response assays done in serum-SAPI medium. To review the nature of the aggregated clumps, a visual assessment of the plates under a 100x objective by light microscope was made and pictures were taken. Representative pictures are shown in Figure 4.4A demonstrating the aggregation of bacteria at -6 dilution cultures with various concentrations of salbutamol. The clumping of bacteria in the presence of salbutamol was greater than that without any drug

supplementation and that the clumps appeared denser at the higher drug concentrations. This observation was consistently observed in three separate experiments. The clumps or aggregates of bacterial are the intermediate steps of biofilm formation and it can be concluded that although salbutamol does not affect the growth of the *P. aeruginosa*, there is likely to be an influence on the biofilm formation by way of increased cell to cell binding and increase in the ability to form EPS.

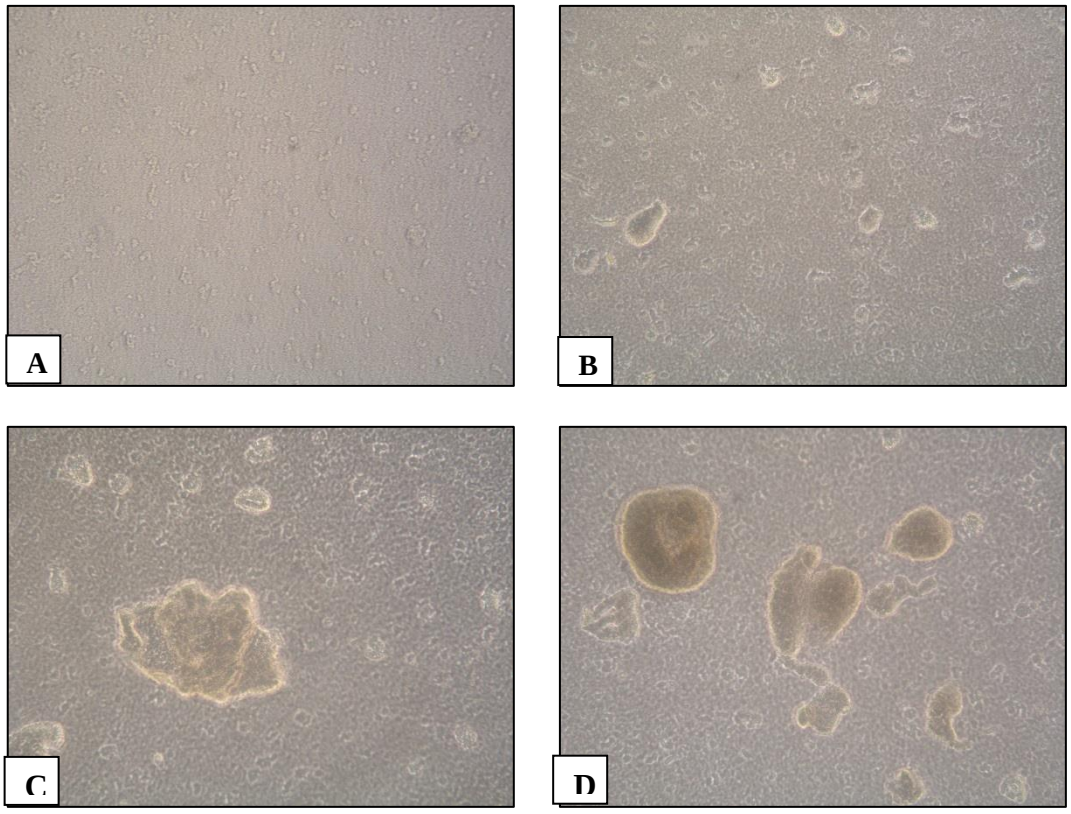


Figure 4.4 A and B: Light microscopy (x20) images of -8 (A) and -5 (B) dilution wells of 24 hour *P. aeruginosa* culture in Serum-SAPI. The images show **A.** Control-bacteria, no drug added; **B.** 0.10µg/ml salbutamol; **C.** 0.375µg/ml salbutamol and **D.** 1.0µg/ml salbutamol supplementation. Images are representative of three separate experiments and similar findings were seen at other concentrations.

4.2.5 Salbutamol effect on *P. aeruginosa* biofilm formation on endotracheal tubes

To translate the above findings to the clinical context, I then evaluated the effects of salbutamol exposure on biofilm formation on endotracheal (ET) tubes using scanning electron microscopy (SEM) as described in Chapter 2. Figures 4.5A and B show the

SEM images of the biofilm on the surface of the ET tube sections at 1mm and 10µm scale. There is relatively little biofilm formation in the un-supplemented control cultures (Figure 4.5A) compared to that seen with 50µg/ml salbutamol treated culture (Figure 4.5B). The higher magnification images give an indication of the thickness and degree of maturity of the biofilm as shown in Figure 4.6 A and B. Data is representative of three biological replicates.

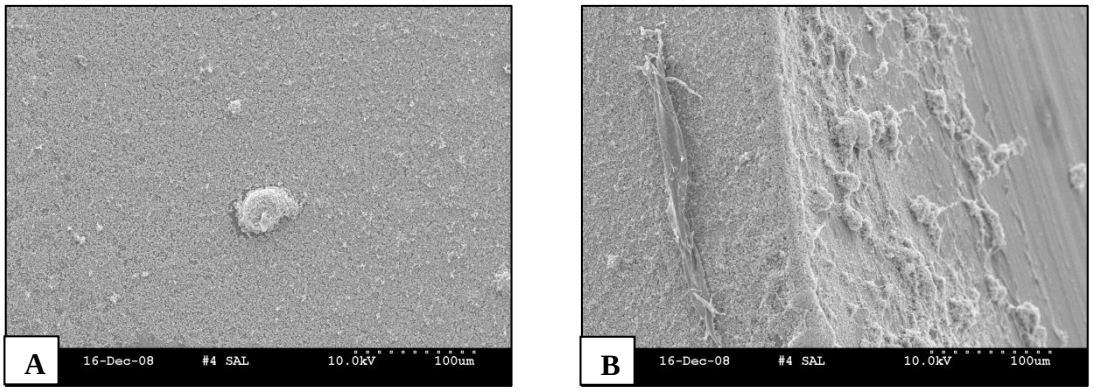


Figure 4.5 A, B: Representative SEM images of salbutamol effects on the extent and morphology of *P. aeruginosa* biofilm noted on ET tube segments after 24 hours incubation in serum SAPI compared to control without drug. **A**. Control, no drug added, **B**. 10µg/ml salbutamol supplementation.

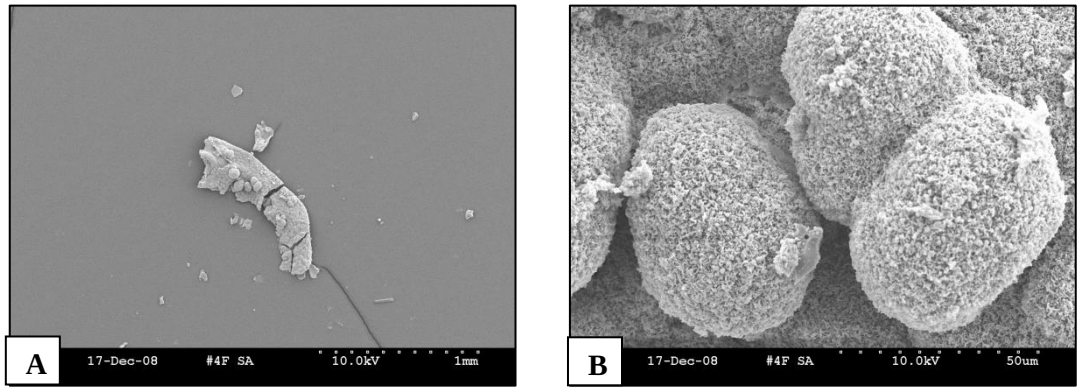


Figure 4.6 A and B: **A**. Fragment of salbutamol supplemented *P. aeruginosa* biofilm that was detached from the ET tube surface during preparation. **B.** The head on view shows the mushroom like structures indicating maturation of the biofilm.

This study showed that salbutamol exposure enhances *P. aeruginosa* biofilm formation with development of EPS matrix and enmeshed bacterial colonies and a mature 3-dimensional ‘mushroom like’ structure is already formed by 24 hours.

In conclusion, it was noted that salbutamol at varying fluid concentrations that are achieved after therapeutic inhalational doses, did not affect the growth of *P. aeruginosa* or its capacity to cause initial attachment to surfaces. Interestingly, however, salbutamol even at low concentrations had a tendency to cause increased *P. aeruginosa* aggregation after 24 hours incubation. Significant to the clinical setting, salbutamol enhanced the *P. aeruginosa* biofilm formation on medical grade plastic (endotracheal tubes) and the biofilm formed was mature and thick in only 24 hours.

4.2.6 Effect of salbutamol on *B. cenocepacia* growth

The effects of salbutamol at various concentrations on growth of clinical and reference *B. cenocepacia* strains was investigated in this section. The clinical and reference strains were serially diluted to -8 dilutions (around 10 CFU/ml) in Serum-SAPI medium enriched with various salbutamol concentrations from 0.1 to 50µg/ml of a clinical preparation of nebulised salbutamol (Ventolin, 2.5mg/ml) and compared against control bacteria (un-supplemented inoculum). The viable bacterial count (expressed as CFU/ml) of the overnight cultures were measured by serial dilution and plating onto LA. The data in Figure 4.7 shows the growth (CFU/ml) for clinical and reference *B. cenocepacia* strains in the presence and absence of salbutamol and reveal there was no significant increase in viable bacterial numbers in the salbutamol enriched inocula compared to the control.

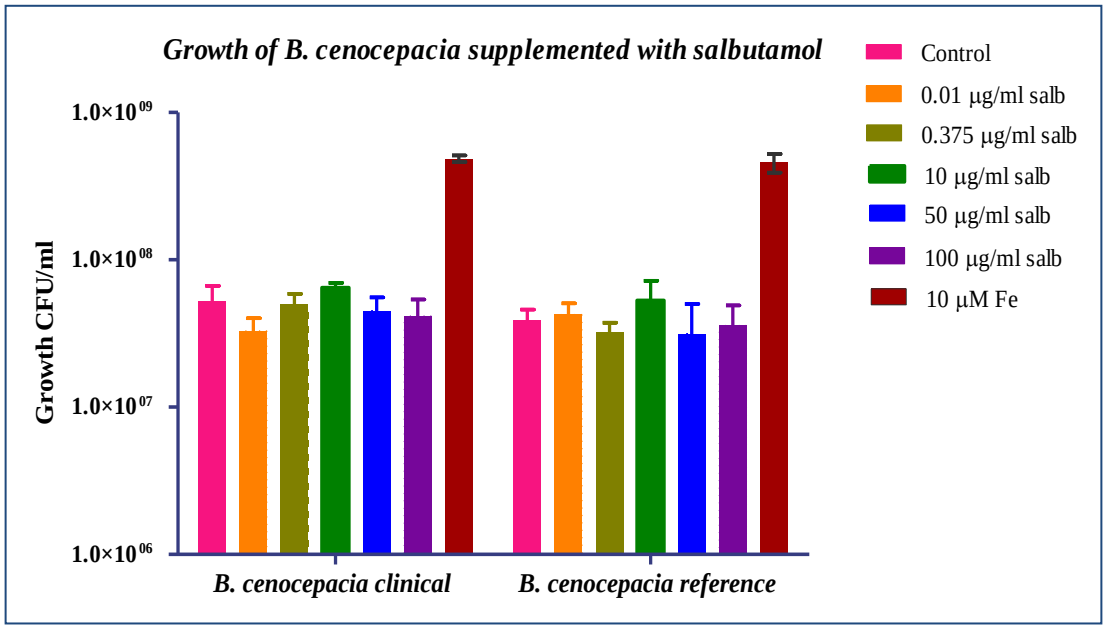


Fig 4.7: Growth of *B. cenocepacia* in presence of salbutamol: Overnight cultures of the clinical and reference strains were diluted in 10-fold steps with serum-SAPI with supplemented salbutamol at various concentrations and grown for 24h statically at 37^{o}C. Bacteria were then enumerated by serial dilutions of the gown cultures and plating to obtain the final CFU/ml. Values represent the means and standard deviations of triplicate platings from duplicate cultures. Key: Control, bacteria no drug added; Fe, iron supplemented.

4.2.7 Growth kinetics for *B.cenocepacia* in the presence of salbutamol

The clinical and reference strains of *B. cenocepacia* were cultured in serum-SAPI medium along with different concentrations of salbutamol and continuous readings were taken using the Varioskan spectrophotometer at OD_{595} over a 24 hour period.

The resultant time curve showed no significant difference in the growth profiles of the two bacteria. The representative data for the clinical isolate is shown at the higher concentrations of salbutamol (Figure 4.8) and similar results were seen with other concentrations of salbutamol.

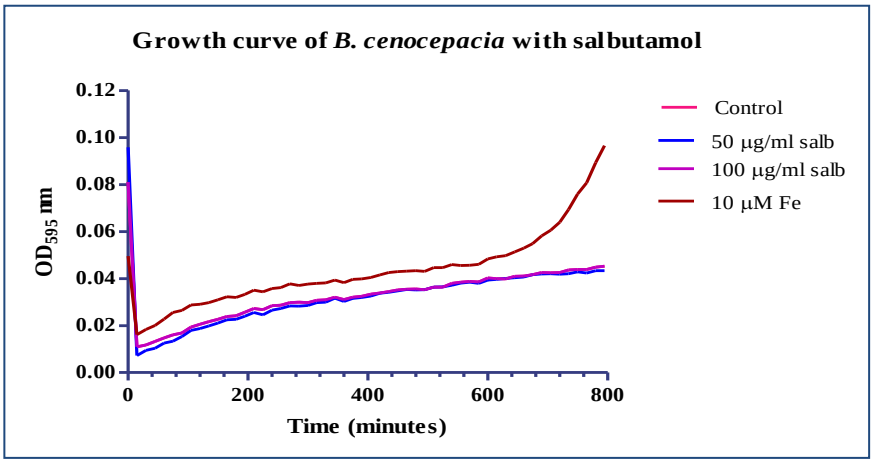


Figure 4.8: Time course of growth of *B. cenocepacia* clinical and reference strains in the presence of salbutamol. Overnight *B. cenocepacia* cultures were diluted 1:1000 in Serum-SAPI medium and growth kinetics measured over 24hours at optical density of 595nm (OD595nm). Key: Values show the means of three separate experiments. Control, bacteria no drug added; Fe, iron.

4.2.8 Effect of salbutamol on *B. cenocepacia* initial attachment

The effect of salbutamol on initial stages of biofilm formation of the clinical and reference *B. cenocepacia* strains were studied by crystal violet microtitre plate attachment assay wherein bacteria were incubated in Serum-SAPI for 24 and 48 hours in a 96 well microtitre plate and attachment of bacteria measured with crystal violet staining as described in Chapter 2. Biofilm formation on polystyrene with the clinical strain and in response to salbutamol in serum-SAPI medium at 24 and 48 hours is shown in Figure 4.9. The data shows that addition of salbutamol did not increase the initial attachment of *B. cenocepacia* strains at various concentrations either at 24 or 48 hours when compared to unsupplemented cultures; similar results were also observed with the reference *B. cenocepacia* strain (data not shown).

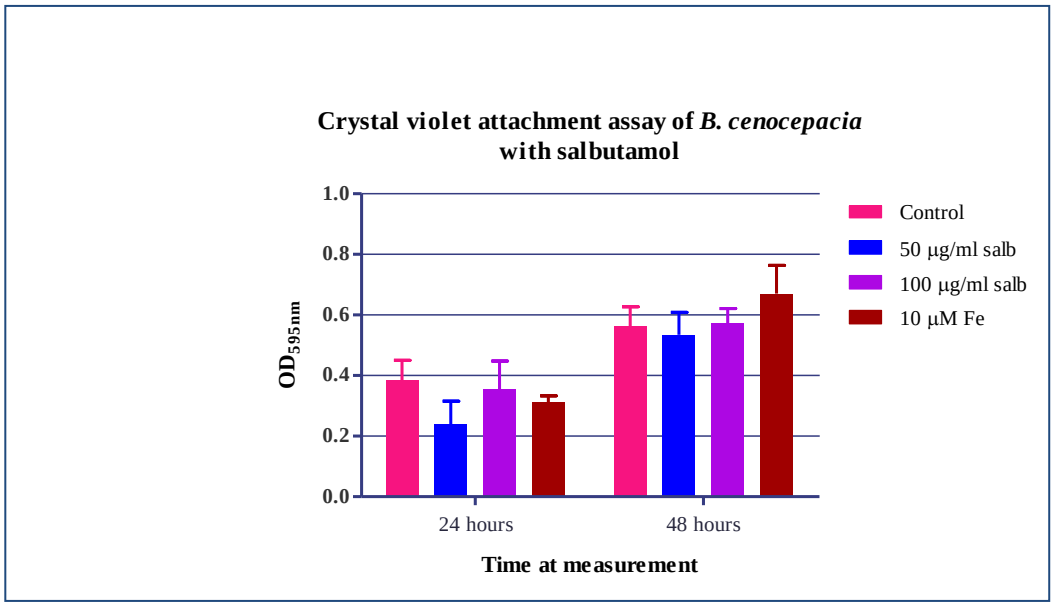


Figure 4.9: *B. cenocepacia* attachment on crystal violet assay with and without supplementation with salbutamol. Overnight cultures of the clinical strain were diluted 1:100 into the indicated media and bacteria cultured and attachment measured at 24 and 48 hours with crystal violet. The histogram shows no difference in attachment of *B. cenocepacia* to polystyrene following salbutamol exposure at greater concentrations at 24 and 48 hours. Data represent means and SD of 4 biological replicates. Key: Control, bacteria no drug added.

4.2.9 Effect of salbutamol on *B. cenocepacia cell-cell* aggregation

B. cenocepacia had a tendency to aggregate at 24 hours in Serum-SAPI medium plus salbutamol as evident under light microscopy. Figures 4.10 A and B show the representative images for unsupplemented control compared with 1.0µg/ml salbutamol supplementation respectively.

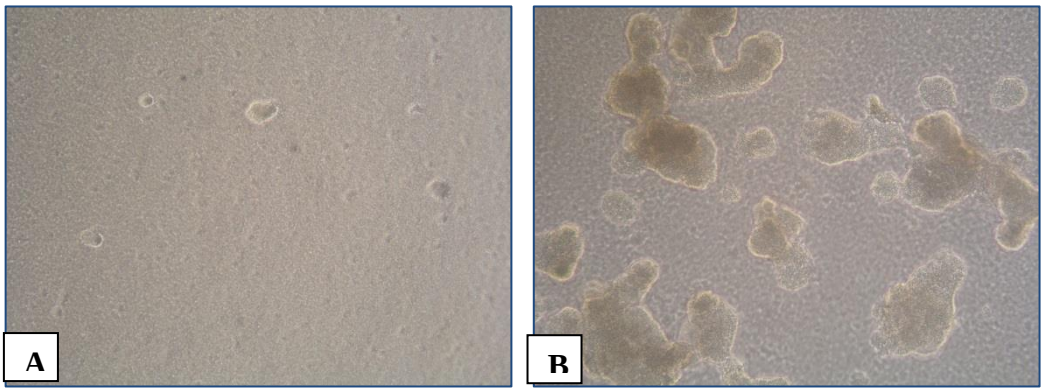


Figure 4.10: A and B: Light microscopy (x2) images of *B. cenocepacia* in Serum-SAPI after 24 hour cultures. The images show **A.** Control- bacteria only, no drug added; **B.** 1.0µg/ml salbutamol supplementation. Images are representative of three separate experiments and typical of various other salbutamol concentrations.

The density and the size of the aggregates were both increased with salbutamol supplementation even at 1µg/ml concentrations (Figure 4.11). Testing for live and dead staining was considered but not possible due to the fragile nature of the aggregates which broke down easily into the Serum-SAPI medium.

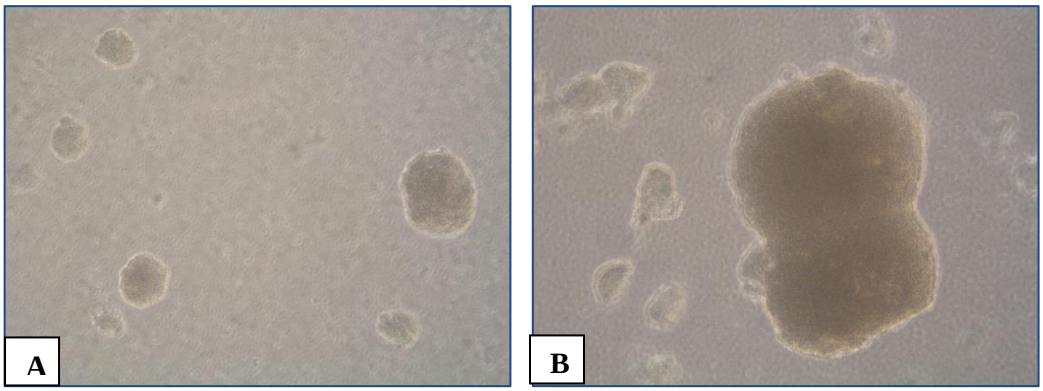


Figure 4.11: Images showing comparison of bacterial aggregate size. A. Control *B. cenocepacia* well, no drug and **B**. 1.0µg/ml salbutamol supplemented well.

4.2.10 Salbutamol enhances *B. cenocepacia* biofilm formation on endotracheal tubes

As there was evidence of increased aggregation, I then proceeded to check if there was enhanced biofilm formation and used ET tube segments to assess this. This would be relevant in those CF patients who have been transplanted and intubated post-operatively on intensive care. Biofilm formation was assessed on ET tube using scanning electron microscopy for *B. cenocepacia* with salbutamol enrichment.

Figure 4.12 A and B shows the SEM images of the surface of the ET tube sections without drug supplementation and with addition of 1.0µg/ml salbutamol. There is a distinct increase in the extent and nature of biofilm formation when salbutamol is added to the culture. The 10µm magnified image with salbutamol supplementation (Row B,

third image), shows that there is matrix formation with enmeshing of the bacteria. This pattern was visible at all concentrations of salbutamol in three separate experiments.

The spread and the extent of the biofilm formation were not as heightened as that seen with catecholamines but certainly were suggestive of very mature looking biofilms. This study showed that salbutamol had a tendency to enhance the *B. cenocepacia* biofilm after 24 hours of incubation.

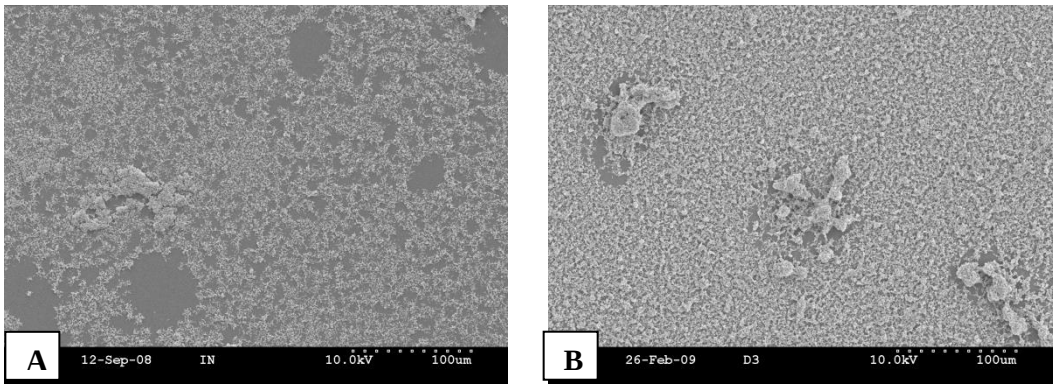


Figure 4.12 A and B: Representative SEM images of salbutamol effects on the extent and morphology of clinical strain *B. cenocepacia* biofilm ET tube segments after 24 hours incubation in serum-SAPI medium compared to control without drug. **A.** Control, no drug added, **B.** 1.0µg/ml salbutamol supplementation.

4.3 Conclusion

The study in this chapter suggests that salbutamol does not affect the growth or initial attachment of either *P. aeruginosa* or *B. cenocepacia.* Salbutamol has an effect on the biofilm forming ability of both bacteria and this effect is not dose dependent but is evident even at lower concentrations that are possibly achieved in the lungs alveolar spaces after therapeutic inhalation of the medication. The higher concentrations may represent that seen in the larger airways and again we note that biofilm formation increases at these concentrations and on ET tube surfaces which have clinical implications.

4.4. Discussion

Salbutamol is widely used as inhaled therapy in respiratory diseases such as asthma and in CF. Salbutamol is a highly soluble drug and is freely available for immediate action in the lungs and usual nebulised dosages of 2.5-5mg get deposited in the larger airways and in the peripheral alveolar spaces readily. Salbutamol has been found to affect the inflammatory cytokine and chemokine production on allergen stimulation (Romberger et al, 2016) and in autoimmune disease (Manni et al, 2011). Neutrophil respiratory burst activity (Otenello et al, 1996) and exocytosis (der Poll et al, 1996) were shown to be attenuated by β_2-agonist treatment. Bacterial killing and superoxide anion release by alveolar macrophages was strongly suppressed by both salbutamol and formoterol (Capelli et al, 1993). These mechanisms all indirectly affect the clearance of organisms.

Some studies show increased bacterial clearance (Vandevelde et al, 2015) and reduced virulence (Gross et al, 2010) with salbutamol whereas another study shows no difference in the bacterial growth (Neal et al, 2011). Contrarily, Maris et al showed that both salbutamol and long acting β_2- agonist salmeterol reduced the clearance of non-typeable *Haemophilus influenza* (Maris et al, 2006). Studies have found salmeterol to improve mucociliary clearance in patients with *Pseudomonas* colonisation (Dowling et al, 1997, Kanthakumar et al, 1994) and exert a protective effect on airway epithelial integrity that was otherwise impaired by *P. aeruginosa* exoproducts (Coraux et al, 2004). Zhang et al showed a dose responsive increase in bacterial growth of *E.coli* and *P. aeruginosa* in air surface fluid on human airway carcinogenic cell line air liquid cultures with addition of salbutamol to culture medium. This growth was related to reduced anti-bacterial products (Zhang et al, 2011). The effect of salbutamol on

Pseudomonas and *Burkholderia* growth and virulence has not been directly explored previously and for the first time is addressed in this study.

This study demonstrates that although salbutamol does not affect the bacterial growth, exposing the bacteria to varying concentrations of salbutamol resulted in increased biofilm forming ability of these organisms even at lower concentrations. The biofilms were mature and thick after only 24 hour after exposure and showed matrix formation. The *in vitro* conditions and exposure to salbutamol increase the ability of the bacteria to form biofilms and it is likely that salbutamol affects the cell to cell signalling pathways independent of growth.

In clinical settings, salbutamol usage is high in many respiratory diseases including CF where *Pseudomonas* and *Burkholderia* are common lung pathogens. Salbutamol is routinely used for bronchodilatation and to facilitate airway clearance in this group of patients. Those undergoing lung transplantation are intubated post-operatively and *Burkholderia* is associated with morbidity and mortality in this group. The respiratory tissues, via the endotracheal tube, may also be used as a direct site for administration of salbutamol. It is likely that salbutamol may have an effect on the bacterial cell to cell signalling and influence the bacterial aggregation and further biofilm formation.

The interaction between the bacteria, *Pseudomonas* and *Burkholderia,* and salbutamol can happen in the clinical setting and the findings of this study has potential implications to routine practice.

CHAPTER 5

Effect of catecholamines and salbutamol on interaction of *B. cenocepacia* and *P. aeruginosa* with human airway ciliated epithelium

5.1 Overview

B. cenocepacia and *P. aeruginosa* are commonly found in nature and do not usually affect the healthy lung but are commonly found to be pathogenic in CF patients (Marcus and Baker, 1985). Both these bacterial species cause chronic lung infection by forming biofilms. Lung transplant is a treatment offered for end stage CF disease and again *Burkholderia* are implicated in increasing morbidity and mortality in this group of patients (Stephenson et al, 2012).

Catecholamines are present within the lungs, and in vitro studies have shown they can also mediate access of transferrin and Lf iron to the bacteria and this iron is utilised by bacteria to increase their growth and biofilm formation (Freestone et al, 2003, 2008). In this study, I found *B. cenocepacia* to be responsive to catecholamines and this effect was mediated by accessing iron (Chapter 3, section 3.4). Studies show that the catecholamine levels are increased in CF and in lung transplantation patients due to altered metabolism (Schöni et al, 1985, 1986) and post lung transplanted CF (Dickinson, 2015), stress related to infection (Freestone et al, 2002) or procedures such as ET tube suctioning (Schmidt and Kraft, 1995) and use of exogenous inotropic agents post transplantation (Raymondos et al, 2000).

Salbutamol is widely used for bronchodilatation and airway clearance in CF and intubated patients on intensive care such as the post transplantation group. Salbutamol has been found to interact with bacteria but its overall effect on bacterial virulence and clearance is not conclusive (Maris et al, 2006, Vandevelde et al, 2015, Zhang et al, 2011). Investigations done as documented in Chapter 4 showed *B. cenocepacia* and *P. aeruginosa* formed increased aggregates and mature biofilm in presence of salbutamol.

Medications used at time of transplant can affect the MCC. MCC was significantly reduced in transplant patients (Edmunds et al, 1969, Brody et al, 1972, Dolovich et al, 1987), compared with healthy subjects. Acute inhalation of salbutamol significantly improved MCC in transplant patients (Laube et al, 2002). Likewise, in healthy individuals salbutamol can increase the ciliary function (Devalia et al, 1992, Izeboud et al, 1999), but this effect may vary in diseases such as CF (Mortensen et al, 1993, 1994).

As the lungs provide an environment for close interaction of bacteria such as *B. cenocepacia* and *P. aeruginosa* with drugs such as salbutamol and catecholamines especially in disease states, I chose to study this interaction using the air liquid interface (ALI) culture model with cells obtained from healthy and post-transplanted CF individuals was used in this part of the study. The Ali culture model provides an environment that is as similar to the human lungs as possible.

5.2 Methods for ALI culture experiments

5.2.1 Subjects

The epithelium samples were collected from post transplantation CF patients and healthy volunteers and processed for culture as described in Chapter 2.

5.2.2 Air liquid interface (ALI) culture preparation

ALI cultures were prepared as outlined in Chapter 2 for both nasal and bronchial epithelial samples. Unfortunately, over the period of the study, a large proportion of the samples succumbed to three massive bouts of infections and thus only a small proportion of the samples were finally suitable for any kind of testing. This was not expected events and unfortunately due to lack of time, no further samples could be

obtained. In all, only four CF post transplantation bronchial (CF-B) and 8 healthy nasal (HN) ALI cultures were available for this study.

5.2.3 Preparation of *B. cenocepacia* and *P.aeruginosa* for ALI culture interactions

Clinical and reference strains of *B. cenocepacia* were used and a clinical strain of *P. aeruginosa* was used. The methodology used for preparation of inoculum for ALI culture infection was as described in Chapter 2 (Section 2.3.1).

5.2.4 Layout of ALI cultures and exposure of epithelial cultures to bacteria and drugs

Preparation for the ALI culture infection experiments was started 24 hours prior to the experiment, as ALI cultures were fed with 700 µl of antibiotic free BEBM culture medium and the bacterial inoculum prepared. On the day of the experiment, baseline readings for CBF were taken from 5 areas per well prior to any intervention and the XY coordinates noted for each of these areas to ensure that the same cilia were observed throughout the study. Each ALI culture well was then rinsed with 200µl BEBM. Bacterial inoculum was diluted to obtain 10^6 CFU/ml and 500µl of each strain was added to the well. Drugs, either 10µM NE or 10µg/ml salbutamol were also added at this stage. For the control, primary epithelial cells were incubated with 500µl BEBM without any bacteria or additional drugs.

Each ALI culture 12-well plate had between 3 and 5 ciliated transwells. Allowing for a control well per culture, bacteria and drugs were added in the remaining wells as summarised in Table 5.1. Retrospectively, it would seem more useful to not use the *B. cenocepacia* reference strains due to the limited number of ciliated wells eventually

available for the experiments, this limitation was not anticipated and thus not planned for. It was felt necessary to use the reference strain in these experiments due to the highly novel nature of these experiments and to validate the clinical strain against it.

Table 5.1: Summary of the patient demographics and ALI experimental setup. Different combinations of bacteria and drugs were added to HN, healthy nasal ALI culture and CF-B, CF post-transplanted bronchial ALI culture.

Culture	Donor age/ sex	Medical details	Type of culture	Ciliated wells	Type of bacteria added	Drug added	Completed time course
HN1	34y/M	Nil of note	Healthy nasal	3	- *B. cenocepacia* clinical - *B. cenocepacia* reference	Nil	Yes
HN2	25y/F	Nil of note	Healthy nasal	4	- *B. cenocepacia* clinical	-NE 10 µM -Salbutamol 10µg/ml	Yes
HN3	40y/F	Nil of note	Healthy nasal	4	- *B. cenocepacia* clinical	-NE 10 µM -Salbutamol 10µg/ml	Yes
HN4	22y/F	Nil of note	Healthy nasal	4	- *B. cenocepacia* clinical	-NE 10 µM -Salbutamol 10µg/ml	Yes
HN5	30y/M	Nil of note	Healthy nasal	4	- *P.aeruginosa* clinical	-Salbutamol 10µg/ml	Yes
HN6	48y/F	Nil of note	Healthy nasal	5	- *B. cenocepacia* clinical - *B. cenocepacia* reference	-NE 10 µM -Salbutamol 10µg/ml	Yes
HN7	18y/M	Nil of note	Healthy nasal	5	- *B. cenocepacia* clinical -*P.aeruginosa* clinical	-NE 10 µM -Salbutamol 10µg/ml	Yes
HN8	23y/M	Nil of note	Healthy nasal	5	- *B. cenocepacia* clinical -*P.aeruginosa* clinical	-NE 10 µM -Salbutamol 10µg/ml	Yes
CF-B1	14y/F	CF	CF post-transplanted bronchial	4	- *B. cenocepacia* clinical - *B. cenocepacia* reference	-NE 10 µM	Yes
CF-B2	18y/F	CF	CF post-transplanted bronchial	4	- *B. cenocepacia* clinical	-NE 10 µM -Salbutamol 10µg/ml	No, only T0 done
CF-B3	10y/M	CF	CF post-transplanted bronchial	4	- *B. cenocepacia* clinical	-NE 10 µM -Salbutamol 10µg/ml	No, only T0 done
CF-B4	14y/M	CF	CF post-transplanted bronchial	5	- *B. cenocepacia* clinical -*P.aeruginosa* clinical	-NE 10 µM -Salbutamol 10µg/ml	Yes

5.2.5 ALI culture measurements

The measurements were taken 24 hours before any intervention was done to the ALI cultures to check. On the day of the experiment, three further readings were taken- immediately on addition of bacteria and drugs (T0), three hours after infection (T3) and then at five hours (T5). The experiment was stopped at this point. These time points were chosen based on previous (unpublished) laboratory observations to allow for growth and to ensure that it was possible to assess CBF without having the effect of destruction of the cells in the ALI culture due to infection. As per methods described in Chapter 2 , bacterial growth was determined by obtaining 10µl of supernatant at T5 and serially diluting these in PBS, followed by plating onto Luria agar; colony counts of viable bacteria were assessed after 24 hour incubation of the plates.

CBFs were recorded from 5 random ciliated areas per well and these areas were marked by XY coordinates so that the same areas could be recorded for each time point as described in Chapter 3. Five CBF readings were taken per well per time point at various XY coordinates - baseline (24 hours before experiment started) and at T0, T3 and T5. The 15 readings per time point per well were then averaged to provide a mean CBF per time point per well.

Additionally, notes were made of presence of ciliary dyskinesia, bacterial adhesion to cilia, biofilm formation and amount of debris. To quantify it, determination of the area covered by this debris and bacterial aggregates and biofilm was done by using a grid of 0.5 cm^2 squares placed on the picture of each of the five areas studied as described in Chapter 2. The final amount was represented as a percentage of the total area measured and averaged for the five wells.

An example of this technique is shown in Figure 5.1.

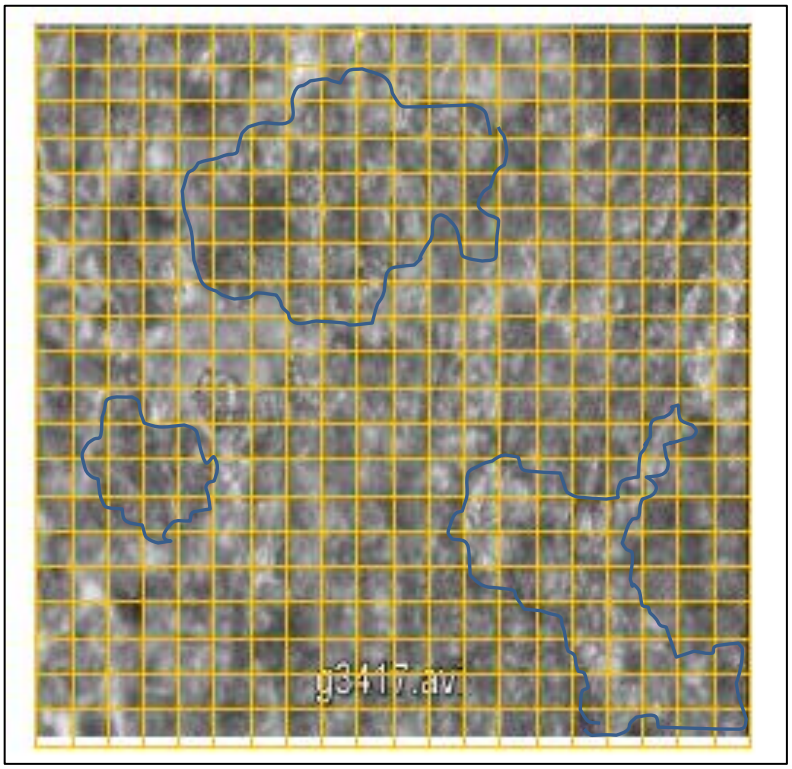


Figure 5.1: This is a still picture of one of the measured area with a grid placed on it. The area covered by cell debris, bacterial aggregates and biofilms was noted and represented as a percentage of the total area measured. In this example, some of the areas covered by bacterial aggregates are marked out in blue.

At five hours, the ALI infection experiment was stopped. The ALI culture was fixed in 4% paraformaldehyde and stained for fluorescent microscopy or confocal imaging as described in in Chapter 2. The confocal microscopy was a new process to me and took several hours per reading by the nature of the test. Due to this, two samples each from healthy nasal and CF bronchial epithelial cultures were processed for confocal and rest were used for fluorescent microscopy.

5.2.6 Statistical analysis

Sample size was calculated based on CBF as the main outcome measure. It was estimated that, to detect a mean difference in ciliary beat frequency of 2 Hz (with a

standard deviation of 1 Hz) between two groups, with a confidence interval of 95% and a power of 80%, a sample size of six (n=6) in each group would be required. As there were only four CF-B cultures, the study was underpowered for this group. Statistical analysis was performed using GraphPad Prism5. Non-parametric data were described as median (IQR). Groups were initially compared using the Friedman test and post-hoc analysis was performed using Dunn's method. A p value of <0.05 was considered statistically significant.

Comparison of the CBF and qualitative analysis was completed from the recorded videos by two observers independently (P Kenia and J Hayes), both of who were blinded to whether the recordings were from control or intervention wells and agreement between the two observers was excellent.

5.3 Results

5.3.1 CBF measurements

5.3.1.1 CBF readings over five hour time course for HN and CF-B ALI cultures

To determine if differences existed in CBF of respiratory epithelia between normal and CF patients, measurements of the beat frequency were taken at baseline, prior to all infection experiments. Baseline CBF readings were found to be within normal range (11-16Hz) for all ALI cultures except for 2, where one, HN5, was fast (17-22Hz) and one, CF-B2 was slow (6-7Hz).

Over the time course of five hours, there were further video recordings per ALI for each time point (T0, T3 and T5). CBF was calculated from five individual readings and a mean CBF reading was obtained per well per time point. The HN ALI cultures showed

that the mean CBF readings changed over the period of the experiment from Baseline to T5 and this change in CBF ranged from 5Hz lower than baseline to 6 Hz above baseline readings for the HN ALI culture experiments. The time course measurements for the 8 HN ALI cultures are shown in Figure 5.2.

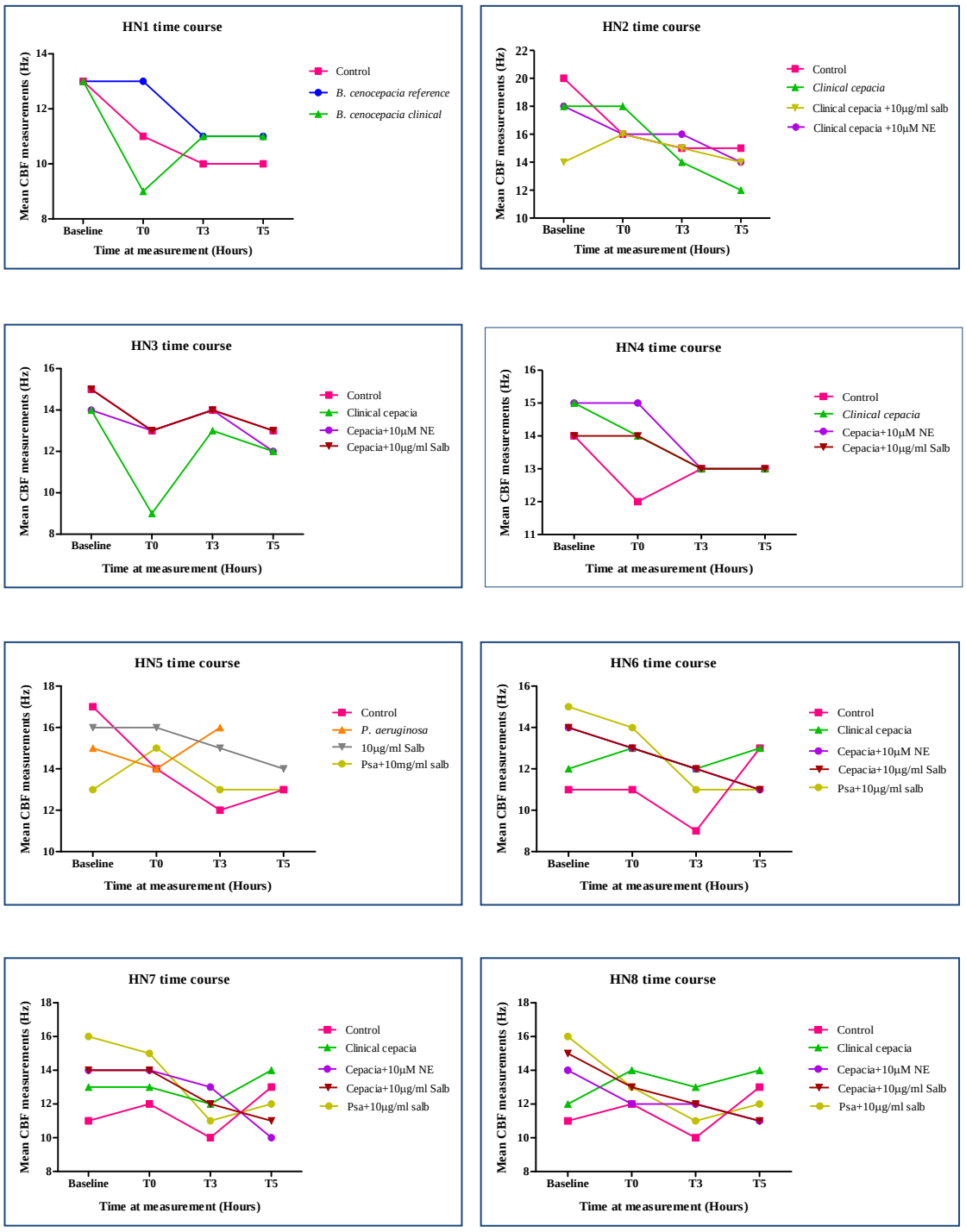


Figure 5.2: CBF readings over the 5 hour time course for the 8 healthy nasal (HN1-8) ALI cultures. Key: **Control** non- infected wells, no drugs added, ***Cepacia***-*Clinical B. cenocepacia*, **Psa**-*P. aeruginosa*, **NE**-norepinephrine, **Salb**-Salbutamol supplemented wells as summarised in Table 5.1. Mean CBF readings are plotted for **Baseline**, before infection, and at time of infection **(T0),** three hours after infection **(T3)** and five hours after infection **(T5).** There is no distinct pattern of change in the CBFs between infected wells with or without drugs added or when compared to control wells during the time course.

Two of the four CF post-transplant bronchial epithelial ALI cultures (CF-B) showed significant cell damage and debris formation, and no ciliated cells were available for CBF assessment at T3 and T5 readings for CF-B2 and CF-B3. Figure 5.3 A and B shows a representative area from a *B. cenocepacia* infected well of CF-B3 and a healthy area for comparison.

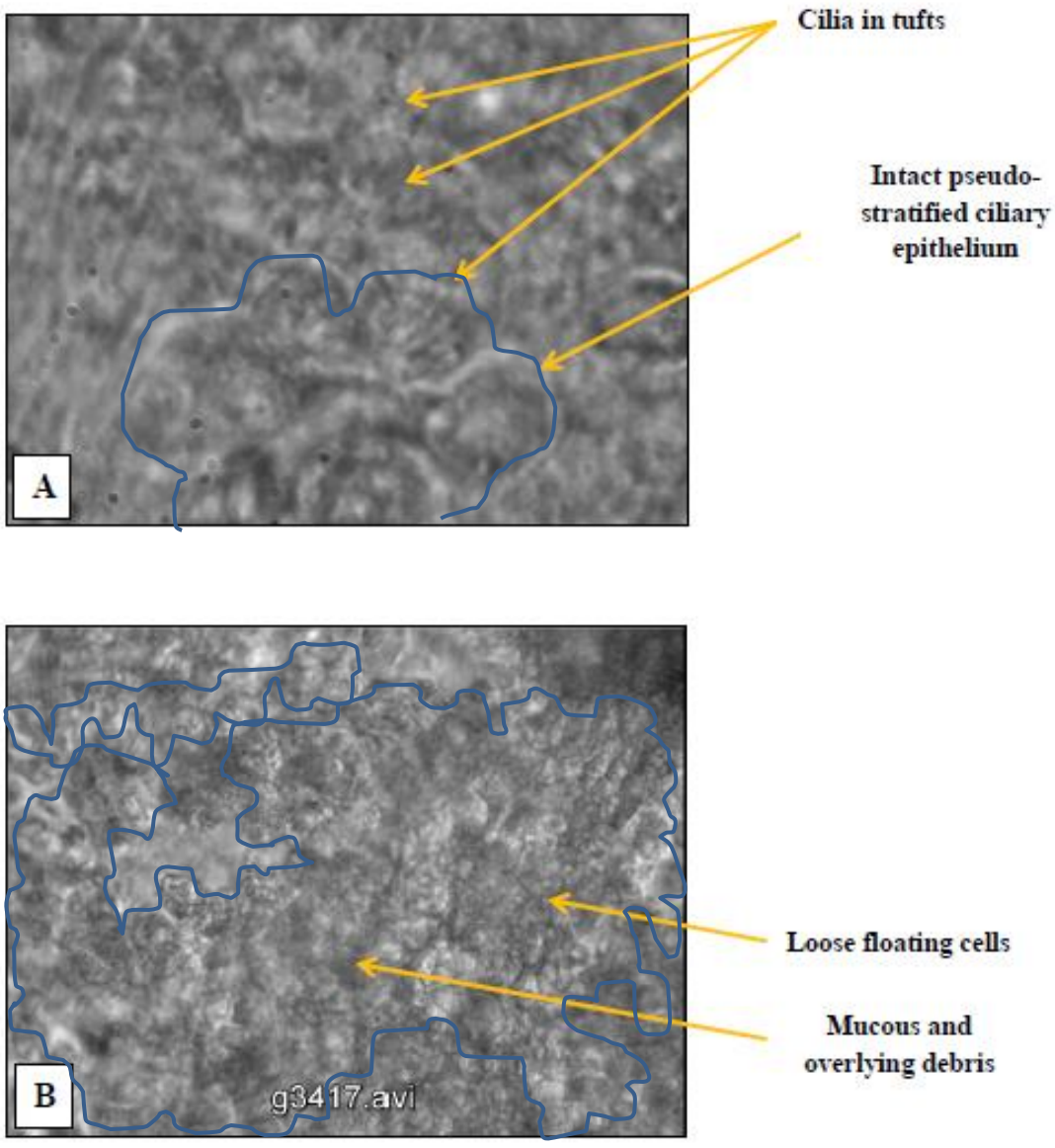


Figure 5.3 A and B: Figure A shows an area of a healthy well with normal stratified ciliary epithelium at T3 from CF-B1 and **Figure B** shows an area from a well of CF-B3 with loose cells and debris floating. Similar cell destruction was noted in all the wells of CF-B2 and CF-B3 at the time of the T3 reading.

In the remaining two CF-B ALI cultures, there were changes in the mean CBF readings ranging from 6Hz lower than baseline to 2Hz above baseline. The time course for the four infected CF post transplantation bronchial (CF-B) ALI cultures is shown in Figure 5.4. This study showed that there was no specific pattern of change in CBF after exposure to *B. cenocepacia.*

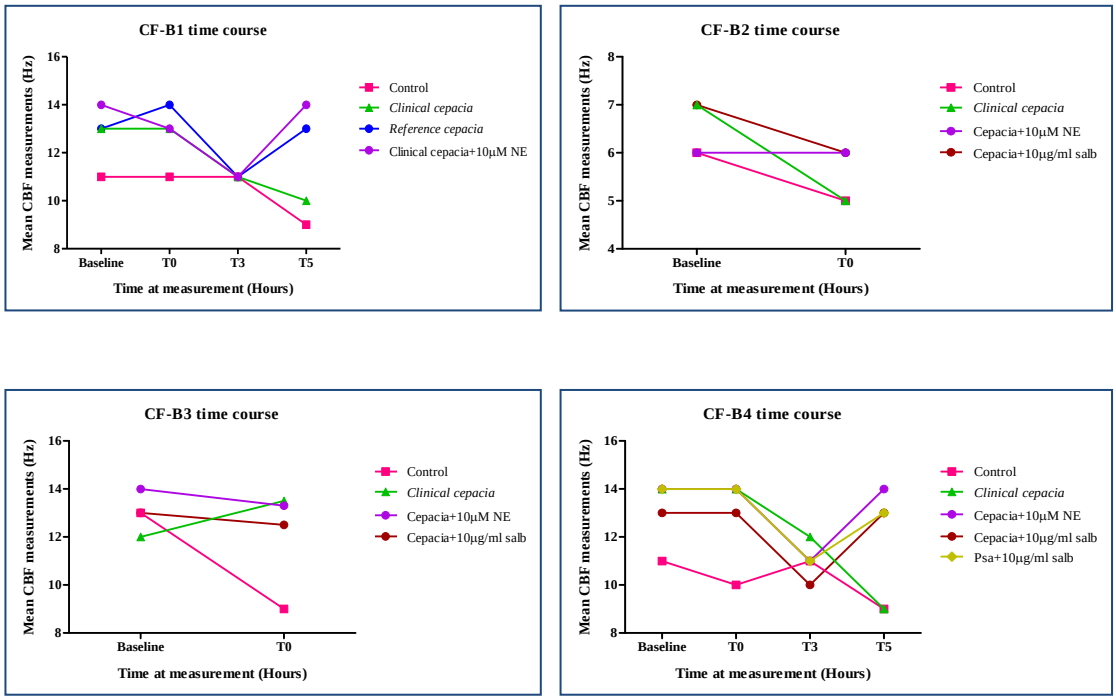


Figure 5.4: CBF measurements over the time course for the 4 CF-B ALI cultures. Key: **Control** non- infected wells, no drugs added, **Cepacia**-Clinical *B. cenocepacia*, **Psa**-*P. aeruginosa,* **NE**-Norepinephrine supplemented, **salb**-Salbutamol supplemented. Mean CBF readings are plotted for **Baseline**, before infection, and at time of infection **(T0),** three hours after infection **(T3)** and five hours after infection **(T5).** There is no distinct pattern of change in the CBFs between infected wells with or without drugs added or when compared to control wells during the time course.CF-B2 and B3 were damaged by the T3 and thus the set of CBF readings is not complete. There is no distinct pattern of change in the CBFs between infected wells bacteria with or without drugs added compared to control wells.

5.3.1.2 Comparison of the CBF measurements between healthy nasal and CF-post transplantation bronchial cultures after *B. cenocepacia* infection

This study included observations from two of the four CF-B ALI cultures and 7 HN

ALI cultures infected with the clinical *B. cenocepacia* strain. The difference in the mean CBF measurements (Δ Mean CBF) for each time point for all healthy and all CF ALI cultures were comparable for non-infected control wells, *B. cenocepacia* infected wells and those with additional salbutamol of NE supplementation. Figure 5.5 shows the data for the salbutamol enriched clinical *B. cenocepacia* wells at various time points and Figure 5.6 shows the data for the NE supplemented clinical *B. cenocepacia* wells at T0, T3 and T5. As there were only two full set of readings in the CF-B over the five hour time course, no significance could be deduced from this study. However, the observations suggested that there was no difference in the CBF measurements with *B. cenocepacia* infection in healthy compared to CF epithelium with and without salbutamol (Figure 5.5) or NE (Figure 5.6) supplementation.

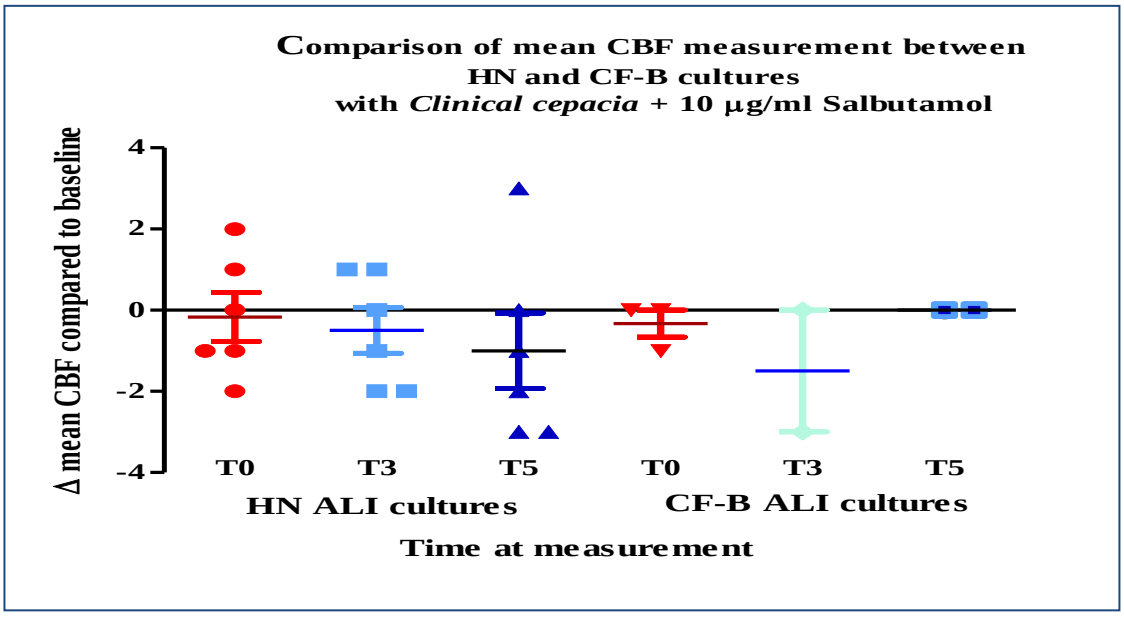


Figure 5.5: Comparison of Δ Mean CBF between clinical strains of *B. cenocepacia* and 10µg/ml salbutamol supplementation between healthy nasal cultures (HN) and CF post transplantation bronchial (CF-B) cultures over the time course. The mean CBFs between healthy and CF *B. cenocepacia* infected ALI cultures were comparable with salbutamol supplementation.

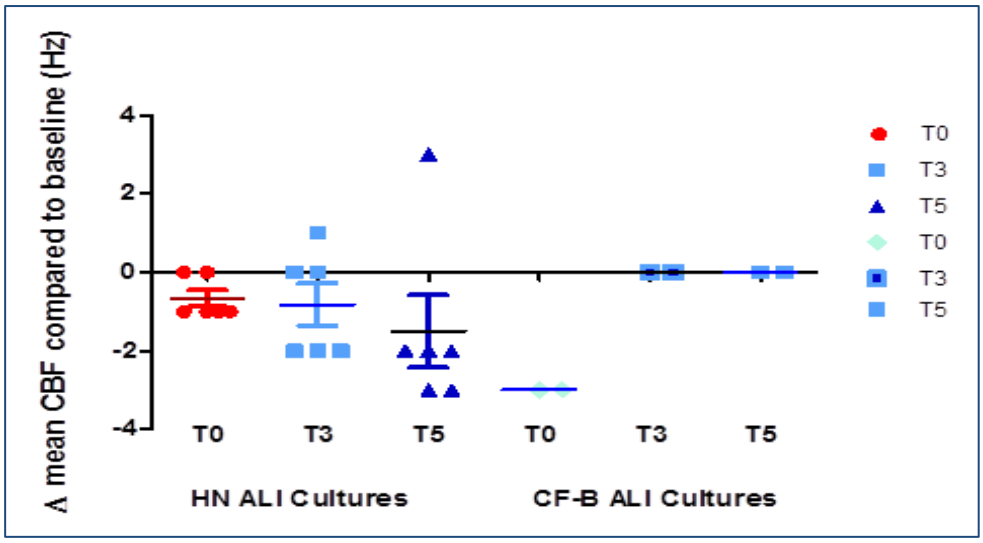


Figure 5.6: Comparison of Δ Mean CBF between clinical strains of *B. cenocepacia* and 10µM NE supplementation between healthy nasal cultures (HN) and CF post transplantation bronchial (CF-B) cultures over the time course. The mean CBFs between healthy and CF *B. cenocepacia* infected ALI cultures were comparable with NE supplementation.

5.3.1.3 CBF measurements after clinical and reference *B. cenocepacia* infection

The mean CBF differences (Δ Mean CBF) for each time point were compared to the baseline for clinical and reference *B. cenocepacia* strains. These measurements were plotted and compared to the control non-infected wells. The CBF measurements changed compared to mean baseline reading, ranging from 6Hz lower than baseline to 4 Hz above baseline (Figure 5.7A-C). However, the mean CBF changes were not significantly different (±2Hz) between the non-infected control wells and the *B. cenocepacia* infected wells at any of the time points (Figure 5.7A-C). This suggested that *B. cenocepacia* does not affect the CBF on introduction to human airway ciliated epithelium when studied up to 5 hours after infection.

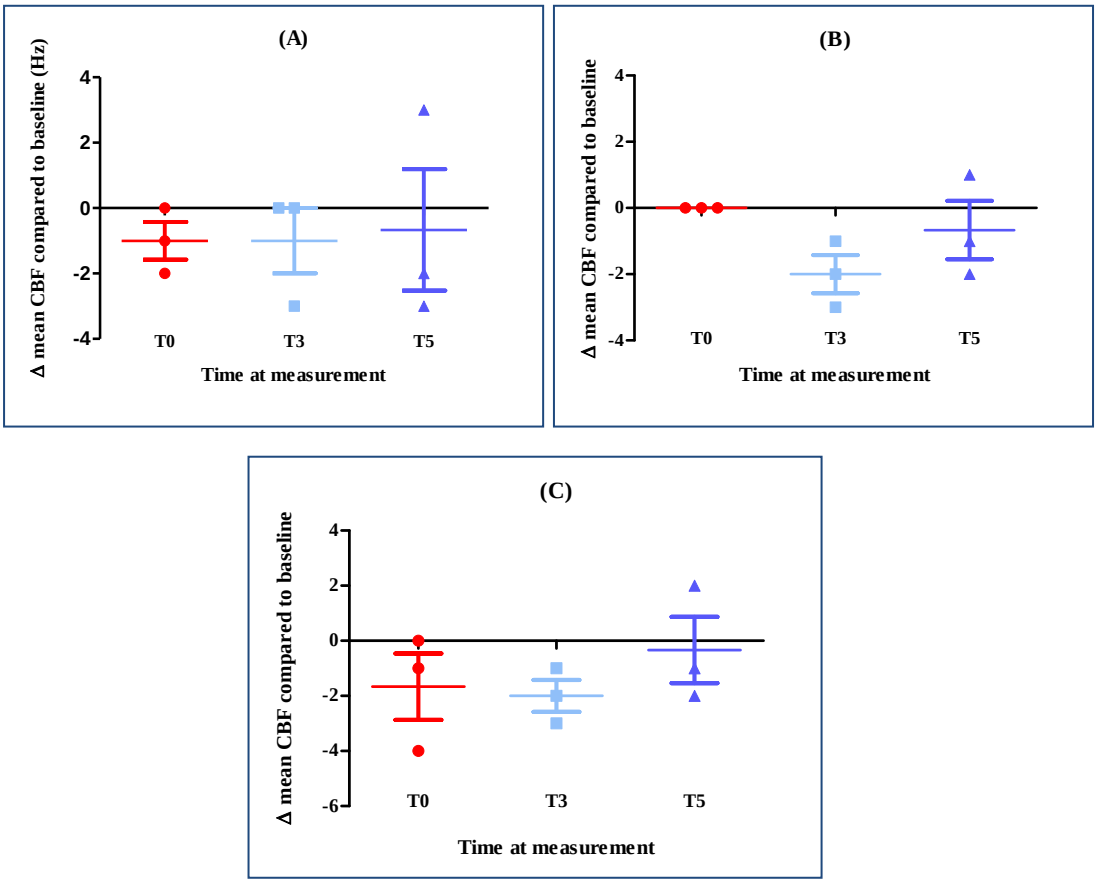


Figure 5.7A, B, C: Comparison of the Δ Mean CBF between **(A)** Control, non- infected wells, **(B)** Reference *B. cenocepacia* strain infected wells and **(C)** Clinical *B. cenocepacia* strain infected wells over the 5 hour time course. The mean CBF readings were comparable across all three groups with no significant difference.

5.3.1.4 Effect on CBF measurements after NE addition to *B. cenocepacia* infected ALI cultures

NE supplementation with clinical *B. cenocepacia* infection was possible in 9 of the 12 ALI cultures. The mean CBFs were measured at baseline, T0, T3 and T5 as before. With NE supplementation, there were significant differences in the CBFs over the time point measurements compared to mean baseline reading, ranging from 5 Hz lower than baseline to 2 Hz above baseline. There was no significant difference (±2Hz) when the mean CBFs were compared across the non-infected control wells, *B. cenocepacia* infected wells and with NE supplemented wells at any of the time points (Figure 5.8 A-C).

This indicated that *B. cenocepacia* did not significantly influence CBF even in presence of a catecholamine like NE, which was shown earlier to increase biofilm formation *in vitro* (Chapter 3, section 3.3.3-6).

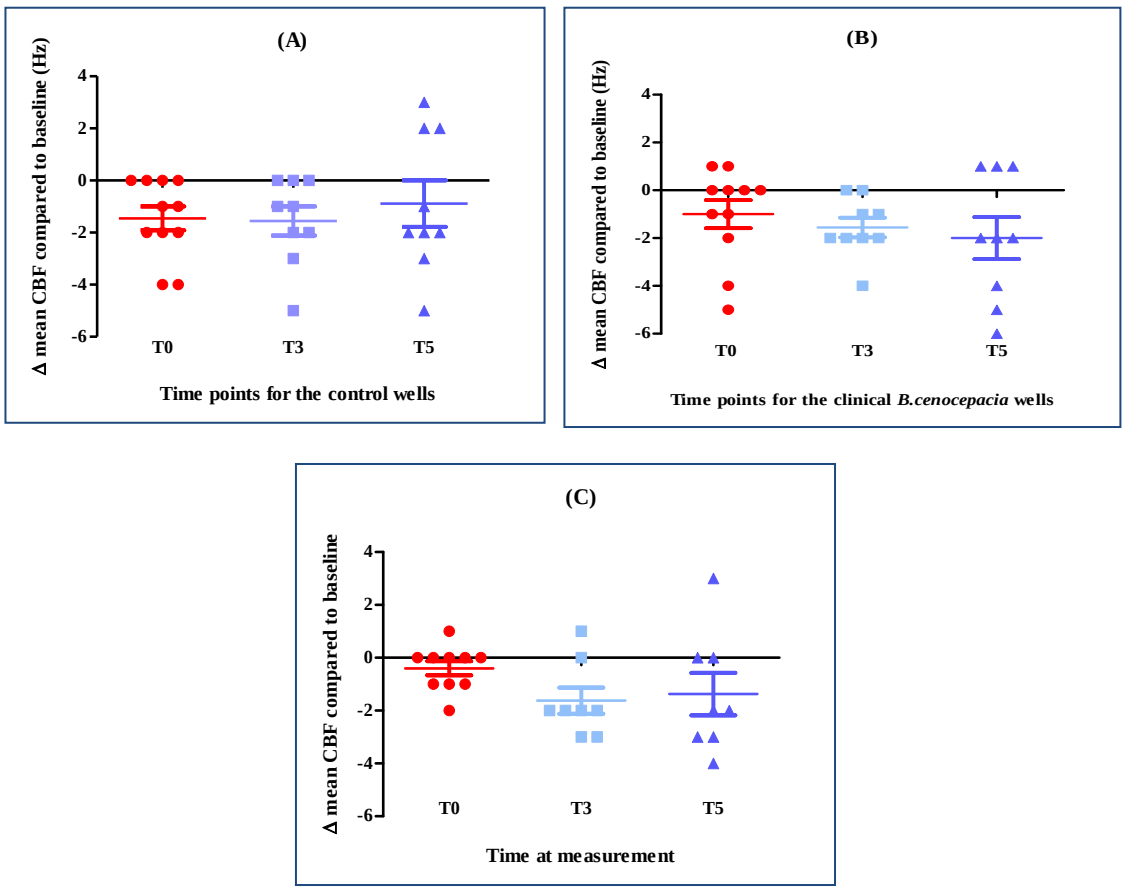


Figure 5.8 A, B, C: Comparison of the Δ Mean CBF between Control, non- infected wells, **(B)** *B. cenocepacia* strain infected wells and **(C)** *B. cenocepacia* with 10µM NE supplemented wells over the 5 hour time course. There was no significant difference in the Δ mean CBF between the non-infected and infected ALI culture wells or with NE supplementation.

5.3.1.5 Effect on CBF measurements after salbutamol addition to *B. cenocepacia* infected ALI cultures

Investigation of the effects of salbutamol supplementation with clinical *B. cenocepacia* infected ALI cultures was possible in 7 of the ALI cultures. The mean CBF readings measured with salbutamol supplemented cultures showed significant individual variation in CBF readings between time points. At five hours, the differences were 3 Hz below to 3 Hz above the mean baseline CBF readings for the *B. cenocepacia*

supplemented with salbutamol wells compared to 6 Hz below and 1 Hz above the mean baseline reading for the *B. cenocepacia* infected wells without salbutamol addition and 5 Hz below to 3 Hz above the mean baseline reading for the control non-infected wells (Figure 5.9 A-C). There was no significant difference (±2Hz) when the mean CBFs were compared across the control (non-infected), bacteria only and salbutamol supplemented wells at any of the time points (Figure 5.9 A-C).

I could conclude that the addition of salbutamol to the *B. cenocepacia* ALI cultures did not affect the ciliary beat frequency.

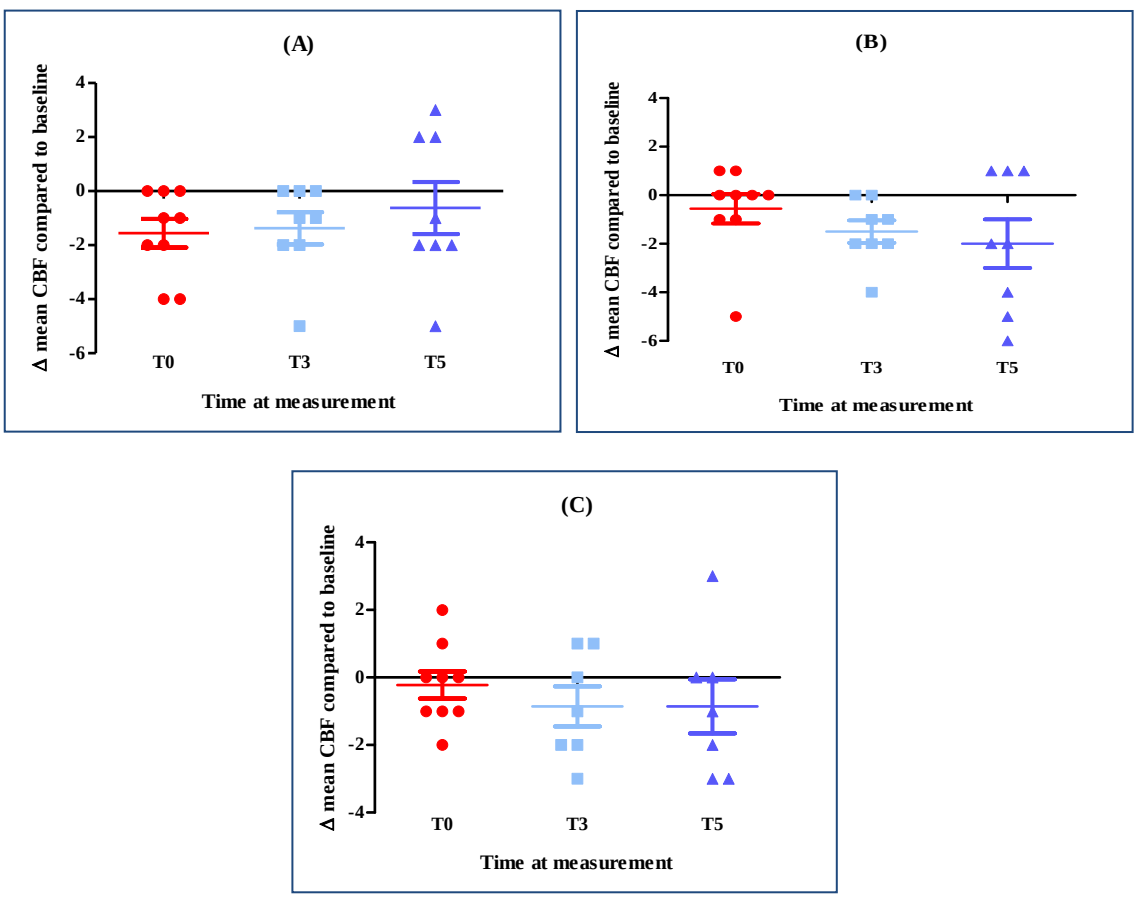


Figure 5.9A, B, C: Comparison of the Δ Mean CBF between Control, non- infected wells, **(B)** *B. cenocepacia* strain infected wells and **(C)** *B. cenocepacia* with 10µg/ml salbutamol supplemented wells over the 5 hour time course. There was no significant difference in the Δ mean CBF between the non-infected and infected ALI culture wells or with salbutamol supplementation.

5.3.1.6 Effect on CBF measurements after salbutamol addition to *P. aeruginosa* infected ALI cultures

In the three ALI cultures in which *P. aeruginosa* infected wells were supplemented with salbutamol, the Δ mean CBF with salbutamol supplementation was not significantly different from the control well (Figure 5.10A,B). This set of data suggests that there is no influence of salbutamol on the CBF readings following *P. aeruginosa* infection of the ALI cultures but the number of samples was not enough to infer the significance.

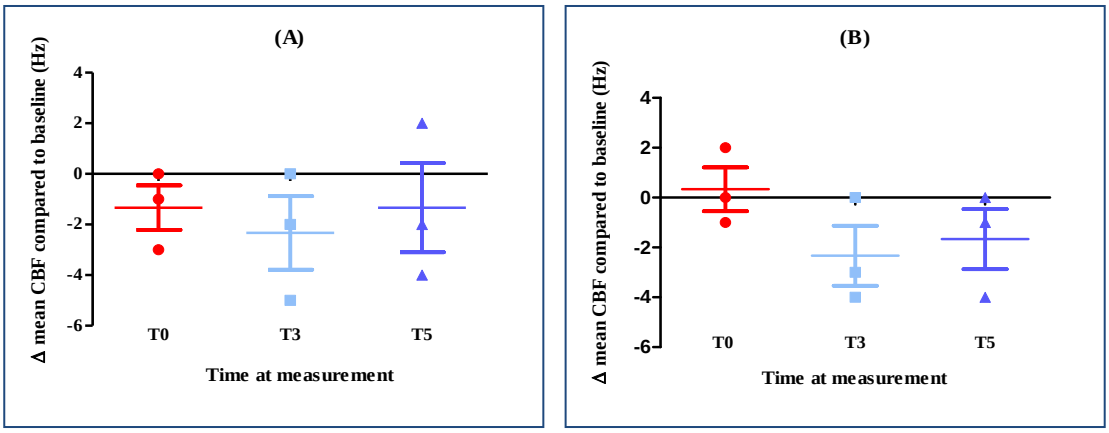
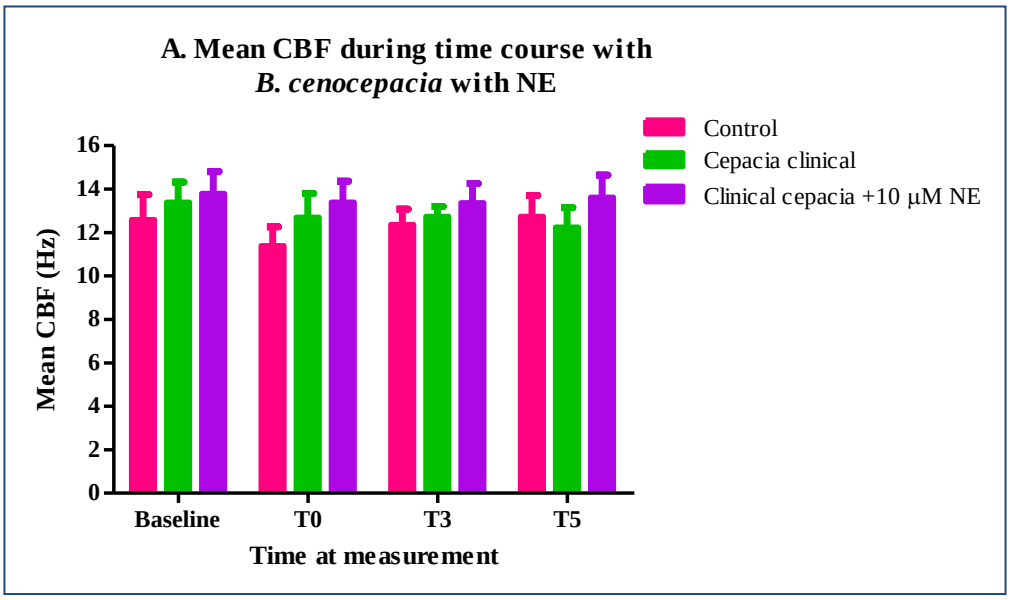


Figure 5.10A and B: Comparison of the Δ Mean CBF between **(A)** Control, non-infected wells and **(B)** Clinical *P. aeruginosa* supplemented with 10µg/ml salbutamol over the 5 hour time course. There was no significant difference in the mean CBF in either set of wells.

5.3.1.7 Comparison of the CBF measurements between salbutamol and NE supplementation of the *B. cenocepacia* infected ALI cultures

The 9 NE supplemented *B. cenocepacia* ALI cultures were compared with the 7 salbutamol supplemented *B. cenocepacia* ALI cultures. There was no difference noted in the mean CBF readings at Baseline, T0, T3 and T5 between the *B. cenocepacia* infected wells supplemented with 10µM NE (Figure 5.11A) compared with those enriched with 10µg/ml salbutamol (Figure 5.11B) when compared with bacteria alone or non-infected control wells.



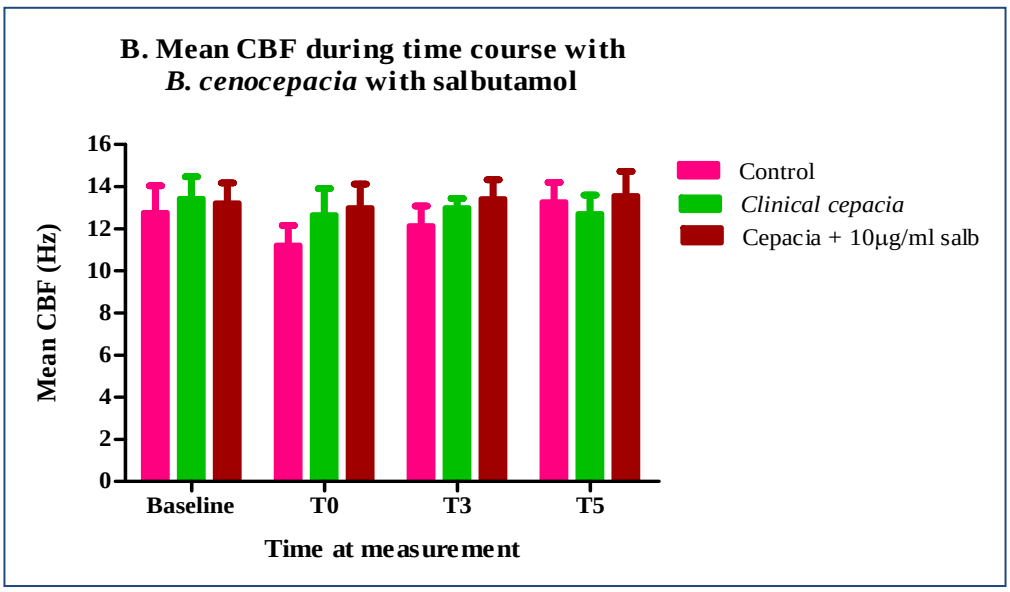


Figure 5.11 A and B: Comparison of the mean CBFs recorded at each time point, Baseline, T0, T3 and T5 with Control, non-infected wells, *B. cenocepacia* infected wells , *B. cenocepacia* with either **(A)** 10µM NE or **(B)** 10µ/ml salbutamol supplementation. There was no significant difference between the mean CBF measurements at any of the time point in either group.

With the studies done so far, it could be concluded that *B. cenocepacia* does not affect the CBF significantly (± 2Hz) in healthy nasal epithelial ALI cultures and the addition of NE or salbutamol does not influence this. Similar observations were made with the CF post transplanted bronchial epithelial ALI culture studies, but the number of samples was not enough to conclude this confidently. Overall, *B. cenocepacia* does not affect

the CBF up to 5 hours after initial infection and drugs, NE and salbutamol do not influence this.

5.3.2 Qualitative and quantitative analysis of the ALI cultures

The wells were assessed for presence of ciliary dyskinesia, bacterial adhesion, biofilm formation and amount of debris. At the end of the experiment, the area covered by bacterial aggregates or biofilms was noted and total percentage coverage was calculated and averaged for the five wells as described in Chapter 2.

5.3.2.1 Qualitative and quantitative analysis of the HN ALI cultures

The cilia in the control non-infected wells remained vigorous throughout the study period and there was good particulate clearance observed with insignificant occasional dyskinesia. There were few areas (<5% average) covered with floating loose cells or debris. For the *B. cenocepacia* and *P. aeruginosa* infected wells with and without drug addition, there was average 10% (range 0-12%) dyskinesia noted in all the ciliary measurements done. There were occasional bacteria freely floating in the fluid and bacteria attached to ciliary tips in 2 out of the 6 NE supplemented wells but no bacterial adhesion was observed in the other infected wells.

The aggregates ranged from small (covering <10% area) to medium- large (covering up to 40% of the area studied). Most large aggregates were in the NE supplemented wells and covered a mean of 36% of the total surface area measurements done compared to 16% with the unsupplemented bacterial wells. The salbutamol supplemented wells covered 20% of the total surface area but the aggregates were mainly small in size. Figures 5.12 A-D show representative areas of the wells with *B. cenocepacia* and with salbutamol and NE supplementation.

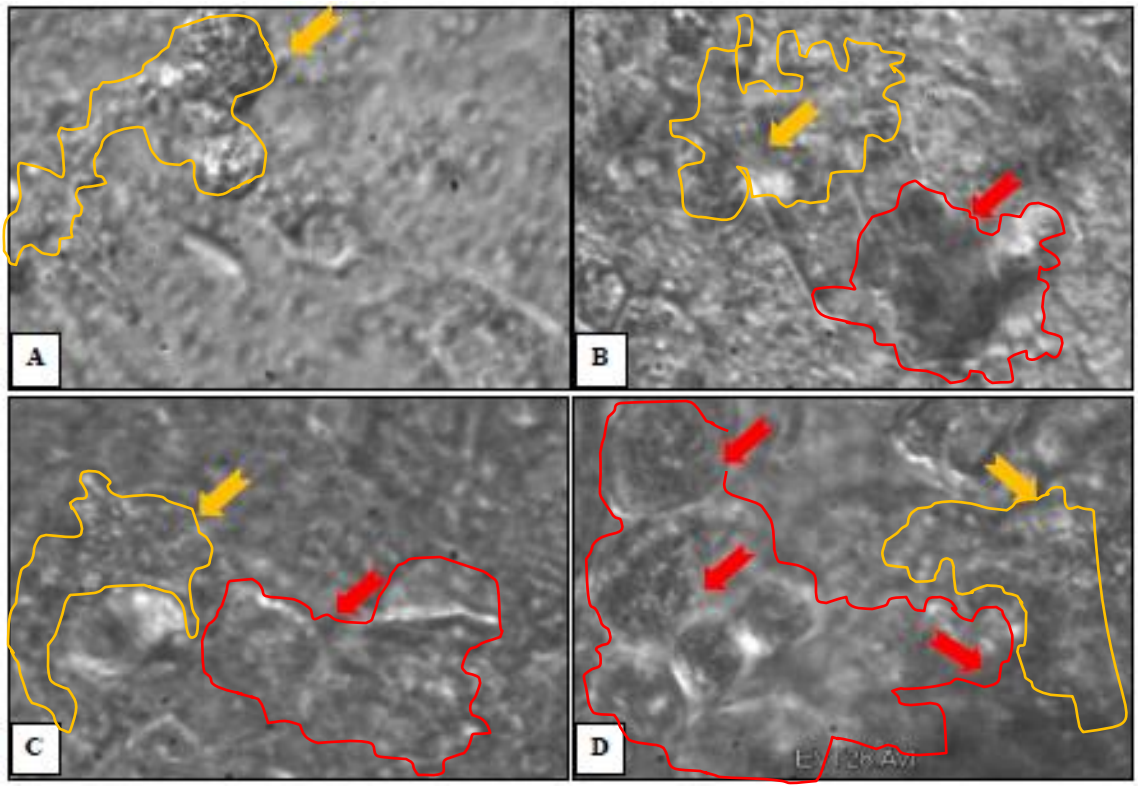


Figure 5.12 A-D: These are representative images of the aggregates from HN ALI cultures seen in **A.** *B. cenocepacia* infected well, **B.** *B. cenocepacia* with 10 µg/ml salbutamol and **C and D** *B. cenocepacia* with 10 µM NE supplemented wells. The yellow arrows and outlines show the biofilms and the red arrows and outlines show the debris and loose cells. The aggregates were larger and more surface area of the ALI culture was covered in the NE enriched wells.

There was presence of floating bacterial aggregates in clumps or with visible EPS formation in >80% of the infected areas reviewed (Figure 5.13 A, B).

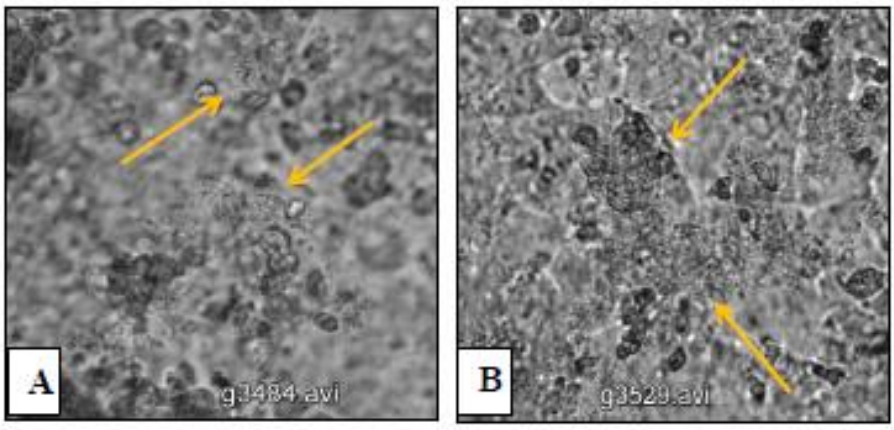


Figure 5.13 A and B: These figures show the early aggregates (yellow arrows) and the matrix floating in the supernatant fluid of the well along with cell debris.

Cell debris was visible in the bacterial infected wells after three hours. At five hours, the cell debris which would likely include loose cells, mucus and bacteria was present in most infected wells and more in the NE supplemented bacterial wells when compared to other wells.

5.3.2.2 Qualitative and quantitative analysis of the CF-B ALI cultures

Three of the four CF-B ALI cultures appeared healthy at the start whereas one had intact epithelium but slow beating cilia at baseline. Eventually, two of the ALI cultures deteriorated over the time course and the cells were lifted off completely by T3 reading. At three hours, there was significant cell debris, and the bacterial wells had large bacterial aggregates. It was not possible to quantify the aggregates due to the underlying debris as this would likely overestimate the quantity.

In the other two surviving cultures, the control wells appeared healthy with few loose floating cells and debris. In the bacterial wells, there was a high degree of cell destruction and biofilm and bacterial aggregate formation. This was much more evident in the NE wells compared to the non-supplemented wells and with the salbutamol supplemented wells (Figure 5.14 A-D). The cell destruction and bacterial aggregation was higher with the CF-B cultures compared to the HN cultures.

The aggregates covered 42% of the surface area of the ALI cultures in the NE supplemented wells (Figures 5.15 A-D).

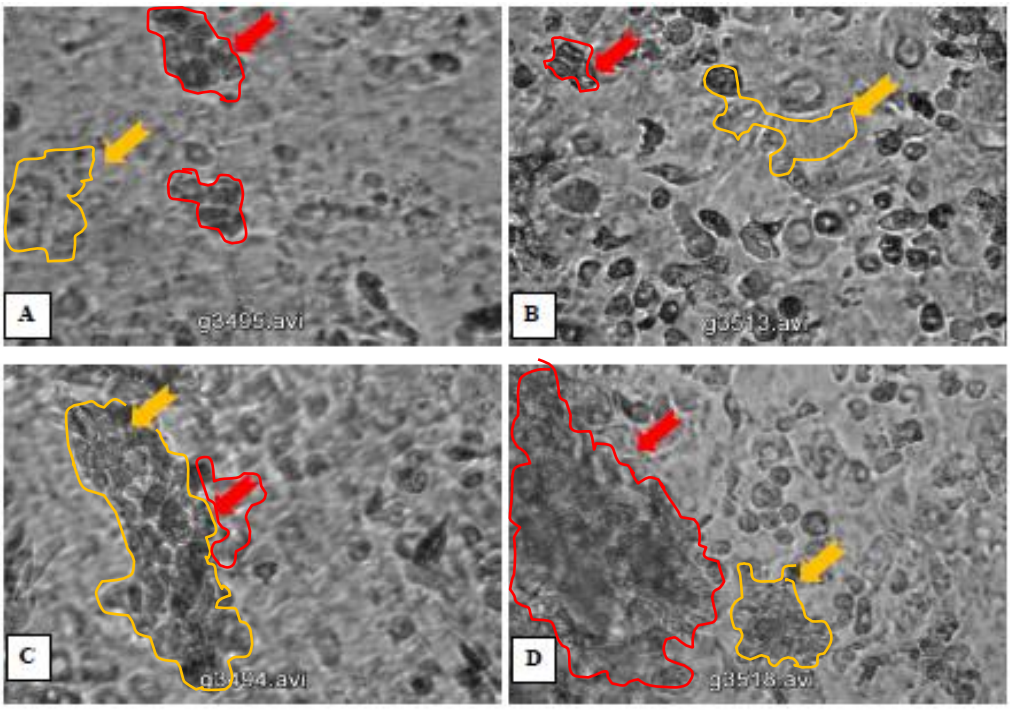


Figure 5.14 A-D: These are representative images of the aggregates from CF-B ALI cultures seen in **A.** *B. cenocepacia* infected well, **B.** *B. cenocepacia* with 10 µg/ml salbutamol and **C and D** *B. cenocepacia* with 10 µM NE supplemented wells for the healthy nasal (HN) cultures. The yellow arrows and outlines show the biofilms and the red arrows and outlines show the debris and loose cells. The aggregates were larger and more surface area of the ALI culture was covered in the NE enriched wells.

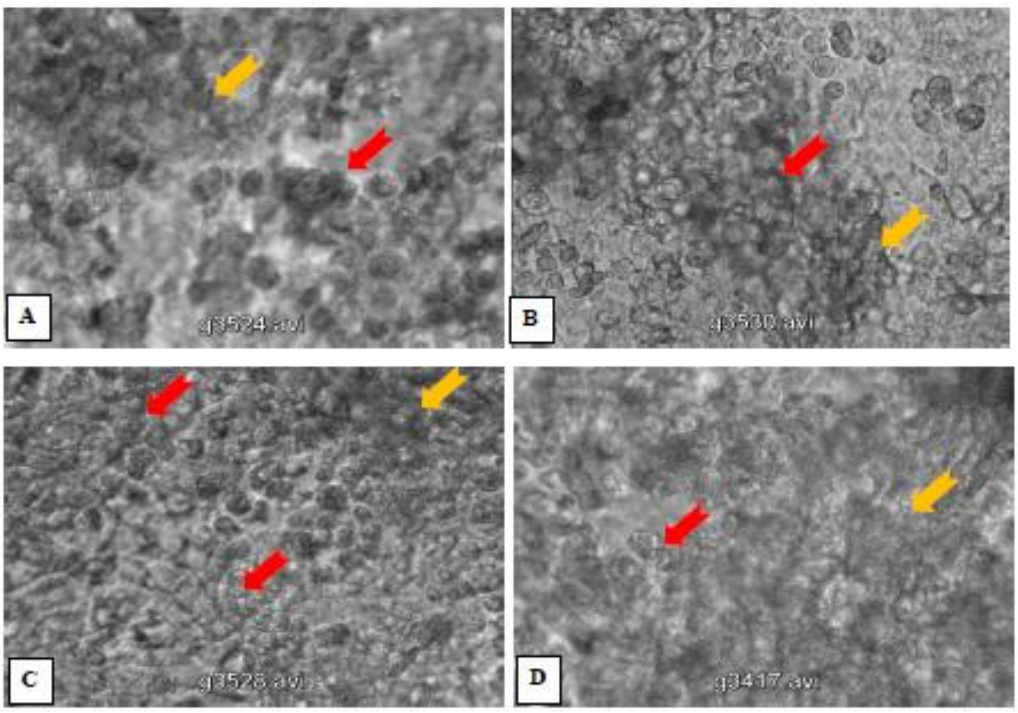


Figure 5.15 A-D shows that the bacterial aggregates and debris was more in the NE enriched *B. cenocepacia* wells from a CF-B ALI culture wells.

With these measurements, it could be concluded that there were clumps, aggregates and early biofilm formation at five hours after initial infection with *B. cenocepacia* and this is increased in the presence of NE and salbutamol supplementation. This occurrence was more pronounced in the CF-BALI cultures compared with the HN ALI cultures but the numbers were small to comment on the significance of this result. Bacterial adhesion occurs to ciliary tips and this was noted predominantly in the NE enriched cultures. Increased cell destruction and mucus formation occurs with *B. cenocepacia* infection and this is greater in the NE enriched wells and higher in the CF-B ALI cultures.

5.3.3 Confocal microscopy demonstrating bacterial adhesion and aggregate formation in infected ALI cultures

At the end of the experiment, cells were fixed and prepared for staining with fluorescent antibody stains for cilia and bacteria and the transmembrane mounted in an inverted orientation onto slides and imaged under a laser scanning confocal microscope as described in Chapter 2. Four samples, two each from HN and CF-B ALI cultures were analysed. Using Imaris software, the stacked images produce a 3D surface view demonstrating cilia (red), nucleus (blue) and adhered bacteria(green) was produced. Adherent bacteria and aggregate was only observed in one of the CF-B areas. The Figure 5.16 shows the 3D volume rendered image and staining of the various elements of the remnants of the ALI culture on the transmembrane of the NE supplemented clinical *B. cenocepacia* well of one of the CF-B ALI cultures.

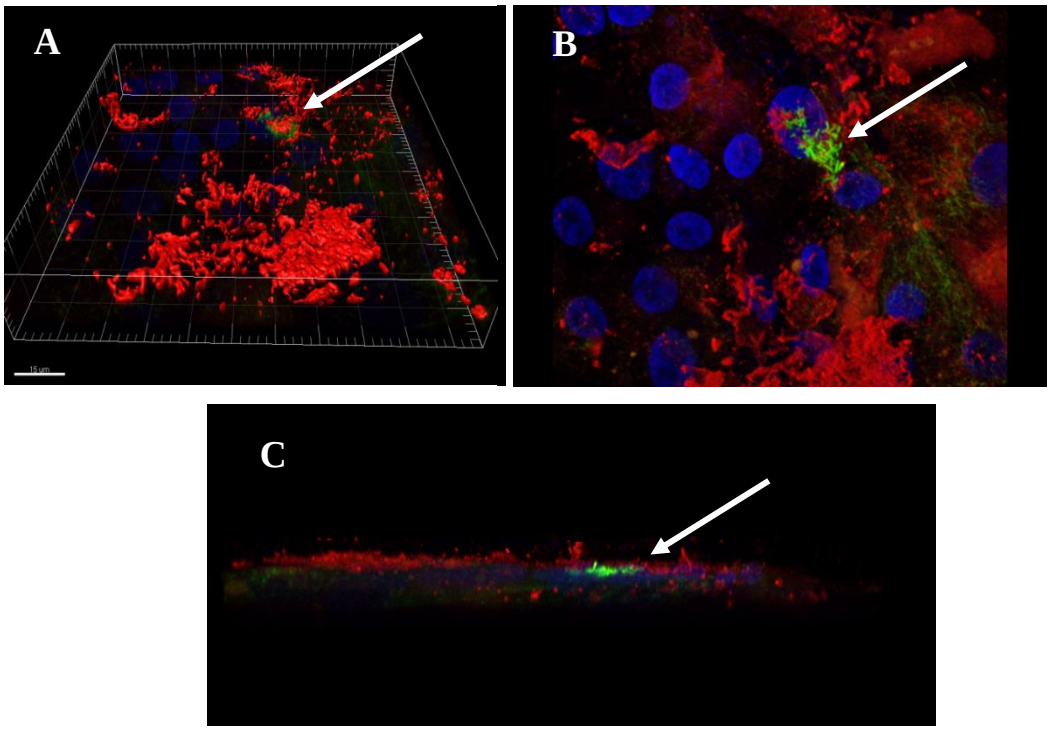


Figure 5.16: Confocal images of infected ciliated epithelium cells **A.** Imaris 3D rendering showing blue nucleus with red cilia and green spots which are the *B. cenocepacia* bacterial aggregates (the white arrows point to it). **B.** Image showing the bacterial aggregate in green attached to the ciliary tips with a head- on view and **C.** Side view of the stacked image which shows the height of the bacterial aggregate.

5.3.4 Bacterial growth after exposure to the airway epithelium

The bacteria from infected ALI cultures were cultured on agar plates as per the method mentioned in Chapter 2 (section 2.3.3) at T5 and bacterial growth expressed as CFU/ml. Figures 5.17 and 5.18 show the *B. cenocepacia* or *P. aeruginosa* growth for all the ALI culture experiments. There was no difference in the colony count of the bacteria between *in vitro* and ALI culture exposed bacterial cultures. There was no difference between the growth characteristics of the HN and CF-B ALI culture exposed *B. cenocepacia* (Figure 5.18) or *P. aeruginosa* (Figure 5.19) and these bacteria maintained their growth ability after exposure to the airway epithelium.

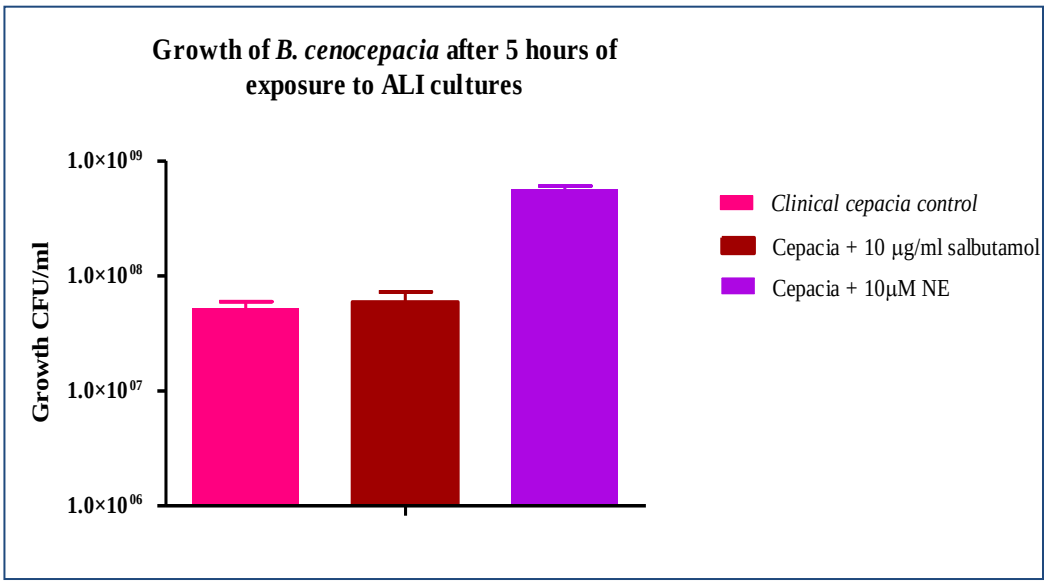


Figure 5.17: Growth of clinical *B. cenocepacia* exposed to the ALI cultures for 5 hours with and without supplemental drugs. Three 10µL amounts of the supernatant was plated on agar plates and growth of viable bacteria expressed as CFU/ml. Key: Control (non supplemented), NE- norepinephrine supplemented culture and salbutamol is the salbutamol supplemented culture.

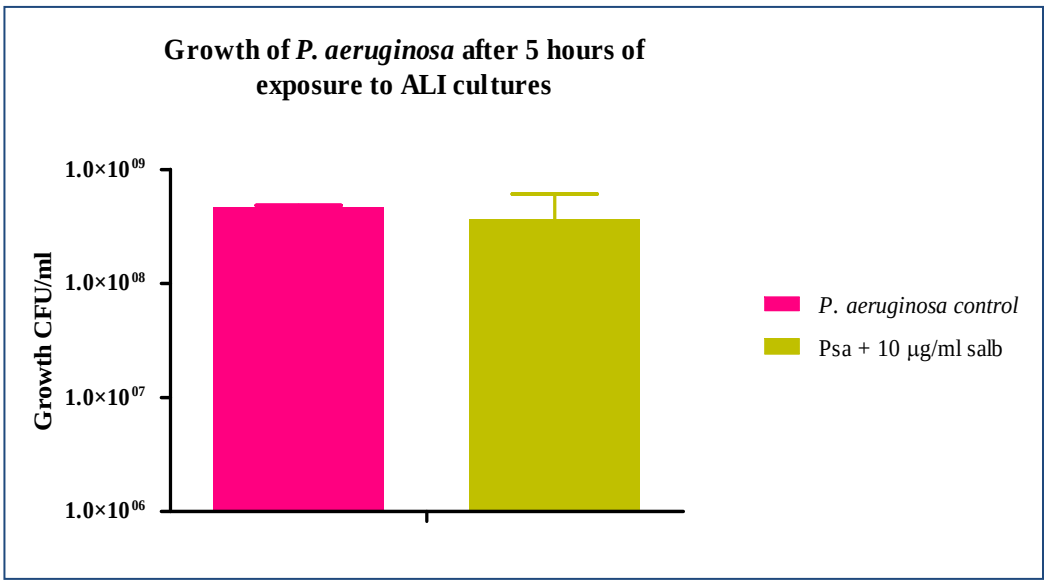


Figure 5.18: Growth of *P. aeruginosa* exposed to the ALI cultures for 5 hours with and without supplemental salbutamol. Three 10µL amounts of the supernatant were plated on agar plates and growth of viable bacteria expressed as CFU/ml. Key: Control (non-supplemented control) and salbutamol is salbutamol supplemented culture.

5.4 Conclusion

This study assessed the early interaction of *B. cenocepacia* with the human airway epithelial cells in health and CF and in presence of salbutamol, a commonly used respiratory drug and NE, an inotropic drug that is used in intensive care settings.

The *B. cenocepacia* did not affect the CBF in either healthy epithelial or in CF post transplantation cultures (Figure 5.7) and the changes in CBF after infection over a five hour period were not different from that seen in non-bacterial control ALI cultures (Figures 5.2 and 5.4). The addition of NE (Figures 5.8) and salbutamol (Figure 5.9) to the *B. cenocepacia* infected cultures did not influence the CBF and they were not different from each other either (Figure 5.11). Similarly, salbutamol supplemented *P. aeruginosa* infection did not affect the CBF (Figure 5.10).

There was evidence of *B. cenocepacia* attaching to the tips of cilia and forming bacterial aggregates and early biofilms within 5 hours after infection (Figure 5.14 and 5.17). The bacterial attachment was more observable in the CF-B epithelium (Figure 5.15). Although, clumps were present in all bacterial infected cultures and varied from small to large, the larger aggregates were present mainly in the catecholamine supplemented wells and covered more surface area compared to non-supplemented wells (Figure 5.15). Salbutamol also increased the aggregation but not to the same extent as NE (Figure 5.15, 5.16). The aggregate formation was more prominent in the CF-B ALI cultures than in the HN cultures (Figure 5.13 and 5.15).

Damage to the underlying epithelium was seen with the *B. cenocepacia and by* three hours and at five hours, there were significant number of damage noted in most infected areas of the ALI cultures (Figure 5.13 and 5.15). The damage was more prominent in

the NE supplemented wells ((Figure 5.15 C, D). The CF-B epithelium was more dramatically affected with two cultures very severely damaged after three hours of infection and the remaining two cultures showing more damage compared to the healthy epithelium (Figure 5.13 and 5.15). The biofilm was visibly attached to the ciliated cells after the five hours of infection as visualised on confocal microscopy in the CF-B NE supplemented ALI culture (Figure 5.17). This indicated that by five hours bacteria can attach to the airway epithelial surface and form strongly attached biofilm.

Bacteria continued to remain viable after the initial exposure to the airway epithelium and did not lose or modify their growth ability (Figure 5.18 and 5.19).

It can be concluded that there is very early virulent interaction of *B. cenocepacia* with the human ciliated airway epithelium and this seems to be more severe in the CF-B epithelium. Salbutamol at high concentrations, which are achieved only with supra-therapeutic usage or possibly achieved in the large airways after inhalation of a therapeutic dose and NE at concentrations achieved after therapeutic usage can increase the bacterial aggregation and associated cell damage on the human airway epithelial cultures. There was not enough number of experiments needed to demonstrate significance and further work will need to be carried out to do so.

5.5 Discussion

The ciliated epithelium along with the overlying surface liquid form the MCC escalator and is a key part of the lung's innate defence mechanism protecting it from the inhaled pathogens and particulate matter. The airway surface and fluid lining its surface is rich in chemokine and cytokines and various proteins which help kill pathogens, tackle allergens and protect the lung. The lung also forms an easy portal for drug delivery by

inhaled route and is an organ for first pass metabolism of many chemicals and drugs, and thus drugs can achieve steady concentrations within the lung fluid. The lungs thus allow an opportunity for pathogens to interact easily with human host cells and drugs can potentially influence this.

In disease such as CF, this interaction is likely to be more pronounced as the mucociliary clearance is reduced (Hart and Winstanley, 2002), there is presence of inflammation and greater endogenous catecholamine levels (Schöni et al, 1985, 1986, Dickinson 2015), more prominent single microbial communities of bacteria (LiPuma et al, 2010) such as *P. aeruginosa* and *B. cenocepacia* forming biofilms and therapeutic inhalation of drugs such as salbutamol. It is known that patients with CF infected with *P. aeruginosa* and *B. cenocepacia* are at risk of severe decline in lung function and increased morbidity and mortality especially in case of post-lung transplantation. The investigations using ALI human airway epithelial cultures provided an opportunity to study these interactions in an environment closely mimicking the human lungs. Cultures from CF post transplantation patients and healthy individuals helped understand the differences in responses in CF disease.

The effect of early infection with *B. cenocepacia* in airway epithelial cultures showed that these bacteria are capable of interacting with cilia and attach to their tips. *B. cenocepacia* have virulence factors including flagella, cable pili (Sousa, 2011) and adhesins, which allow it to freely float and adhere to surfaces within the host lung. Bacteria have the ability to adhere to the ciliary tips, mucus and overlying debris particularly in CF cultures and form aggregates. It is known that adherence occurs on epithelial cell layers and mucus (Tomlin et al, 2005) changing the behaviour of the bacteria from a planktonic to static form where clumping (bacteria-bacteria attachment

leading to microcolony formation) occurs. In healthy patients this is prevented by the defence mechanisms within the lung. The initial attachment of the *Burkholderia* is reversible occurring for approximately 8 hours and is favoured when there are flagella and cable pili present (Kobayashi et al, 2009, Coenye et al, 2010).

No significant difference was observed in the mean CBF readings between bacteria infected and non-infected ALI cultures and there was a large range of variation from baseline measurements with no consistent pattern of change. It could be concluded that *B. cenocepacia* do not affect CBF.

Floating or planktonic form bacteria were noted throughout the cultures and few were adherent to the ciliary tips. A large amount of floating and loosely adherent bacteria aggregates were noted especially in the CF cultures. The next stage of the biofilm formation is where the initial attachment is 'set', which prevents the removal of the biofilm. This kind of firm biofilm was noted on confocal scanning electron microscopy in case of the NE supplemented CF post-transplant bronchial ALI culture (Figure 5.16). *Burkholderia* produce EPS as building matrix for their biofilms and also use EPS products such as cepacian which enables thick biofilms production (Cunha et al, 2004). Biofilms are produced as they provide many advantages for bacteria including the ability to avoid immune system and antimicrobial attack (Savoia & Zucca, 2007) all of which promotes bacterial survival (Cunha et al, 2004).

Burkholderia also possess extracellular lipases, metalloproteases and serine proteases which allow interaction with the epithelial cells of the respiratory tract (Mahenthiralingam et al, 2008) and *Bcc* produce lipopolysaccharides resulting in a strong immune response (Leitao et al, 2010). These products may be the reason for the

significant and consistent amount of epithelial cell destruction, mucus formation and debris seen in the *B. cenocepacia* infected wells. The overwhelming damage destroys ciliated cells (Figure 5.15) and eventually cause reduced or absent mucociliary clearance and biofilm formation.

Burkholderia also has siderophores such as pyochelin, salicylic acid, ornibactins and cepabactin allowing effective iron uptake as iron is required for many bacterial functions (Sousa et al, 2011, Darling et al, 1998). Catecholamines mediate the access of iron to the bacteria and this iron is utilised by bacteria to increase their growth and biofilm formation. In my study, I found *B. cenocepacia* to be responsive to catecholamines and this effect was mediated by accessing iron (Chapter 3, section 3.4). Studies show that the catecholamine levels are increased in CF and in lung transplantation patients due to altered metabolism (Schöni et al, 1985, 1986) and post lung transplanted CF (Dickinson et al, 2015), stress related to infection (Freestone et al, 2002) or procedures such as ET tube suctioning (Schmidt and Kraft, 1995) and to use of exogenous inotropic agents post transplantation (Raymondos et al, 2000). Due to the heightened exposure of catecholamines in CF patients and also those post-transplant, there is a likelihood that the *ex vivo* phenomenon of enhanced bacterial cell aggregation could occur in the human lungs especially when unwell or following respiratory epithelial damage after viral infection. The increased bacterial aggregation and biofilm formation and cell destruction seen in the CF NE supplemented cultures shows that the interaction of the bacteria with this drug results in more bacterial virulence and damage to the host even in *ex vivo* environment.

Salbutamol, commonly nebulised or inhaled in CF patients has known interactions with bacteria (Maris et al, 2006, Vandevelde et al, 2015, Zhang et al, 2011). In Chapter 4,

the experiments done showed that salbutamol affected the *P. aeruginosa* and *B. cenocepacia* cell-cell attachment with increased aggregation and (Chapter 4, sections 4.3.3-5 and 4.3.8-10). Salbutamol inhalation significantly improved MCC in transplant patients and has anti-inflammatory properties (Laube et al, 2002); likewise in healthy individuals salbutamol can increase the ciliary function. Salbutamol did not influence the CBF but seemed to increase the biofilm aggregate formation on the human airway cultures. Higher salbutamol concentration was used mimicking those achieved in the larger airways or direct nebulisation in intubated patients to establish if there was any effect. These results cannot be directly applied to the infection in the lower airways but for intubated patients where larger salbutamol doses are nebulised directly into the large airways, more peripheral deposition can occur and this finding may be relevant and may also be relevant for biofilm formation on ET tubes.

With these experiments, I was able to demonstrate that there is early interaction of *B. cenocepacia* with the human healthy and CF epithelium and drugs, salbutamol and catecholamines influenced the biofilm formation and this is more pronounced in the CF epithelium and with catecholamines.

CHAPTER SIX

General Discussion

6.1 Discussion

The aims of this thesis were to: 1. To examine the effects of salbutamol on growth and virulence of *P. aeruginosa* and *B. cenocepacia* and 2. To study the effect of catecholamines on *B. cenocepacia* (a previous study led by Freestone et al (2012) had already investigated the clinical significance of *Pseudomonas*-catecholamine interactions) and 3. To study the effects of Burkholderia infection in the air liquid interface cultures of healthy and CF patients with and without drugs. The design of the *in vitro* and *ex vivo* experiments of the current study were framed to understand better bacteria-drug interactions in the clinical context of CF and lung transplantation by studying biofilm on medical grade plastic and using human airway epithelial cultures from CF and healthy individuals. The data generated from this study shows that salbutamol, a commonly used respiratory inhalational drug, does not affect bacterial growth or the initial bacterial attachment of biofilm formation, but does enhance the cell to cell aggregation for both *P. aeruginosa* and *B. cenocepacia*. Salbutamol at therapeutic levels also has the ability to rapidly influence formation of thick mature biofilm on medical grade plastic. The cell to cell association and early mature biofilm with EPS formation of *P. aeruginosa* and *B. cenocepacia* can be strikingly seen in the human epithelial cultures by 5 hours after infection, which explains why it may be so difficult to eradicate these bacteria from the CF patients' lungs if not identified early.

Salbutamol has been found to modulate inflammatory cytokine responses and affect allergen responses (Romberger et al, 2016). Salbutamol modulates cytokine production and affects the Th-cell priming ability and could be causative for autoimmune disease pathogenesis (Manni et al, 2011). Neutrophil respiratory burst activity (Otenello et al, 1996) and exocytosis (der Poll et al, 1996) were shown to be attenuated by β_2-agonist treatment. Bacterial killing and superoxide anion release by alveolar macrophages was

found to be strongly suppressed by both salbutamol and formoterol (Capelli et al, 1993). These mechanisms could therefore indirectly affect the clearance of microorganisms from the CF lungs. It was found that *P. aeruginosa* directly interacted with salmeterol, a longer acting beta-adrenergic agonist, to cause pyocyanin-induced slowing of ciliary beat frequency and mucociliary clearance in CF patients with *P. aeruginosa* colonisation (Dowling et al, 1997, Kanthakumar et al, 1994). On the other hand, in a more positive aspect, Salmeterol was shown to protect airway epithelial integrity that was otherwise impaired by *P. aeruginosa* exoproducts (Coraux et al, 2004). Zhang et al showed a dose responsive increase in bacterial growth of *E.coli* and *P. aeruginosa* in air surface fluid on human airway carcinogenic cell line air liquid cultures with addition of salbutamol to culture medium. This growth was related to reduced anti-bacterial products (Zhang et al, 2011). However, in my study this mechanism alone does not explain the *in vitro* findings which showed salbutamol in serum-based media (reflective of the lung environment) is not stimulatory to *P.aeruginosa* or *B. cenocepacia*.

The salbutamol effect seen in my studies was clinically important and there is a need to address the exact mechanism of action which was not studied here. It is possible that salbutamol influences the cell to cell signaling pathways leading to greater bacterial adhesion and EPS formation. This finding provokes several important questions that need to be addressed in future research that will help elucidate mechanisms of action and influence treatment choices for patients with CF who are particularly at risk of acquiring infection like those with severe lung disease or the post-lung transplanted CF patients. Microarray analysis, which is a snapshot of total gene expression in an organism, could be used to study which pathways of *Pseudomonas* and *Burkholderia* gene expression are being modulated by both the salbutamol and the catecholamine inotropes.

Catecholamines had a great influence on *B. cenocepacia* physiology causing tenfold more growth compared to controls without any drug. They also caused greater *B. cenocepacia* surface attachment, increased cell to cell association and induced more mature and thick biofilm on endotracheal tubes compared to unsupplemented bacteria. These effects were seen at low catecholamine concentrations that can be achieved after therapeutic usage. The ALI culture experiments showed that NE supplemented bacterial cultures had greater amount of biofilm with more cell damage and this effect was more pronounced in the CF cultures.

Mediating bacterial access to the host's iron stores was found to be a mechanism of action in terms of growth stimulation of *B. cenocepacia* in this study and for *Pseudomonas* in the Freestone et al study (Freestone et al, 2012). In the human body, the Tf and Lf pathways are highly important protective pathways against infection as they bind iron tightly providing very little or no iron to bacteria and inhibiting growth of most pathogenic bacteria in host tissue fluids (Baker & Baker, 2004). Lactoferrin also has the ability to reduce biofilm formation in *Burkholderia* bacteria (Caraher et al, 2007). As Lf is the main iron binding protein in mucosal secretions and Tf in the blood, this makes for a very effective system in preventing iron from being captured by bacteria (Baker and Baker et al, 2004, Freestone et al, 2008). Bacteria have therefore evolved a variety of mechanisms to acquire host iron, including specific Tf and Lf binding proteins, and the production of high affinity ferric iron binding molecules such as siderophores (Freestone et al, 2008). For CF patients, growth of bacteria within the lungs siderophore production is important, the main one being pyoverdin from *P. aeruginosa* has been shown to be able to remove Fe from transferrin (Lamont et al, 2009).

The relevance of the biofilm enhancing effect is in periods of ill health, physical stress and on intensive care unit (Freestone et al, 2008, Lyte et al, 2003). The findings from my study are of particular consequence in severely affected CF patients and post transplanted CF patients who are admitted to intensive care unit post operatively where the levels of endogenous catecholamines are high and those on intensive care unit may be given further exogenous catecholamines as treatment for low blood pressure and they are particularly vulnerable to infection with *Burkholderia* species. A study done with *Burkholderia pseudomallei* showed that it responded to catecholamines (Intarak et al, 2014) and this was the first study to report this interaction with any *Burkholderia* species.

The healthy lungs are rarely affected by long resident pathogenic bacteria and this may be due to the well balanced immune responses which help fight infectious bacteria without causing overwhelming cell damage. This was noticed in the healthy ALI epithelial cultures wherein the cell damage was minimal after infection and the *P. aeruginosa* and *B. cenocepacia* biofilms were smaller in bacterial clumps as noted by the surface area covered. In diseased state, particularly with CF, there is heightened inflammation and inflammatory responses and thus the effort to fight the infection could damage their own airway cells causing further inflammation and this was evident in the CF ALI cultures showing increased debris and larger aggregates. Hormones such as catecholamines released in the body during illness could aid the growth of bacteria and increase their virulence making the human more prone to acquiring infection and further forming biofilms.

6.2 Future Work

Technically, preparing ALI cultures from CF donors was very challenging, and any future studies which must include this patient group needs to factor in increased epithelial sample collection numbers to accommodate the apparently reduced robustness of the epithelial cell brushings. For time reasons, I also did not have the opportunity to fully examine the underlying mechanisms of bacterial interaction with drugs on the CF airway epithelium. Further detailed cytokine and chemokine analysis from airway epithelial cells in response to drugs would be needed to understand the mechanisms by which infection occurs and bacterial propagate and form biofilms and how drugs and microbial infection affect this. There is likely to be a complex interaction affecting the inter- and intra-kingdom signalling pathways of host and bacteria. Though there may not be a significant difference in the ciliary function between the healthy and CF airway, the potential effects of increased biofilm formation suggest altered host signalling responses between CF and healthy patients, and need further investigation.

The findings of this thesis of enhanced bacterial biofilm formation on airway epithelial cultures in the CF transplanted patients with salbutamol and stress hormones is of clinical relevance. The various experiments and use of airway epithelial model allow an opportunity to develop a detailed understanding of the mechanisms by which bacteria infect humans, the role of drugs and the magnitude and variety of host responses particularly in CF and post-lung transplantation patients who have increased susceptibility to microbial colonisation– a major cause of morbidity, lung function decline and eventual mortality in both non-transplanted severe and transplanted CF patients. Moreover, the usage of common place drugs used in patients would need a review as it is now clear from this and other studies (Freestone et al, 2012) that the clinical relevance of drug side effects are no longer solely confined to the patient.

The data in this thesis generates some important questions that need to be addressed in future research. Unraveling the cause(s) of the enhanced cell to cell association and biofilm formation with commonly used drugs in treatment of CF patients poses an interesting challenge for future researchers. Studies targeting the effect of medications commonly used post-lung transplantation may guide the optimal treatment regimen for post lung transplantation management and minimize any stimulatory effect on pathogens that might still be present which could compromise the success of the lung transplant.

Appendix 1- Consent form for the healthy volunteers

Consent Form for Healthy individuals Attending Leicester Royal Infirmary for a Ciliary Biopsy to determine if they have Primary Ciliary Dyskinesia

Title of Study: The Effect of Infection and Medications on the Ciliary Respiratory Epithelium for Patients with and without Primary Ciliary Dyskinesia

Chief Investigator:
Professor Chris O'Callaghan
Professor of Paediatrics
Department of Infection, Immunity & Inflammation
University of Leicester

You may contact the investigator by 0116 252 3269, or via e-mail on **ajb64@le.ac.uk**.

Please initial box

1. I confirm that I have read and understood Information Sheet Version 1 dated 07/03/06 for the above study, and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw without giving any reason, without any future medical care I may receive, or legal rights, being affected. ☐
3. I understand that I will not be identified in any documents relating to the trial. ☐
4. I agree to take part in the above study. ☐
5. I agree for my case notes to be reviewed at a later date if necessary. ☐

Name of Patient	Date:	Signature:
Name of Person Taking Consent:	Date:	Signature:
Researcher:	Date:	Signature:

ONE FOR PATIENT
ONE FOR RESEARCHER

Appendix 2- Consent form for the lung transplant patients

Consent Form for Patients with Lung transplant undergoing bronchoscopy

Title of Study: **Ciliated epithelium in paediatric lung transplant recipients**

Chief Investigator:
Professor Chris O'Callaghan
Professor of Paediatrics
Department of Infection, Immunity & Inflammation
University of Leicester

You may contact the investigator by 0116 252 3269, or via e-mail on ajb64@le.ac.uk

Please initial box

6. I confirm that I have read and understood the Information Sheet Version 1 dated 07/03/06 for the above study, and have had the opportunity to ask questions. ☐

7. I understand that my participation is voluntary and that I am free to withdraw without giving any reason, without any future medical care I may receive, or legal rights, being affected. ☐

8. I understand that I will not be identified in any documents relating to the trial. ☐

9. I agree to take part in the above study. ☐

10. I agree for my case notes to be reviewed at a later date if necessary. ☐

.....	.....	.....
Name of Patient	Date:	Signature:
.....	.....	.....
Name of Person Taking Consent:	Date:	Signature:
.....	.....	.....
Researcher:	Date:	Signature:

ONE FOR PATIENT
ONE FOR RESEARCHER

Appendix 3: Ethics application reference details

Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee

The Institute of Child Health
30 Guilford Street
London
WC1N 1EH

Telephone: 020 7905 2620
Facsimile: 020 7905 2201

01 June 2007

Dr Paul Aurora
Consultant and Honorary Senior Lecturer, Paediatric Respiratory Medicine and Lung Transplantation
Great Ormond Street Hospital for Children
Institute of Child Health
30 Guilford Street
London WC1N 1EH

Dear Dr Aurora

Full title of study: **Ciliated respiratory epithelium in paediatric lung transplant recipients**
REC reference number: **07/Q0508/38**

Thank you for your letter of 16 May 2007, sent on your behalf by Dr B Thomas, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

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