THE ROLE OF IMPAIRED SERUM BACTERICIDAL ACTIVITY IN CHRONIC *PSEUDOMONAS AERUGINOSA* INFECTION IN NON CYSTIC FIBROSIS BRONCHIECTASIS.

by
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A thesis submitted to the University of Birmingham for the degree of
DOCTOR OF MEDICINE

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July 2017

ABSTRACT

Non Cystic Fibrosis bronchiectasis is characterised by perpetual neutrophilic inflammation in the lungs. The ongoing vicious cycle of bronchiectasis leads to further damage to already damaged airways and is a culmination of repeated infection, inflammation and failure of the host response to maintain sterility of the airway, despite a sophisticated innate and adaptive immune system.

Pseudomonas aeruginosa commonly colonises the lungs of patients with bronchiectasis. I hypothesised that the concept of inhibitory antibodies in the serum may be a feature of *Pseudomonas* colonisation through a specific interaction between the host adaptive immune system and strain specific features.

Here I have identified a mechanism where some patients colonised with *Pseudomonas aeruginosa* produce IgG2 antibodies specifically against the O antigen of bacterial LPS, which rather than promote complement-mediated killing actually inhibits it.

To my family.

ACKNOWLEDGEMENT AND STATEMENT OF CONTRIBUTORSHIP

I would first like to sincerely thank Professor Stockley for giving me the opportunity to carry out this research and thank everyone at ADAPT for their support while this work was carried out especially Patti for all of her help processing lab samples.

I would also like to thank my supervisors Professor Stockley and Professor Henderson for their help and guidance. I would especially like to thank Dr Timothy Wells for teaching me how to work in a laboratory and his time and patience.

I would like to thank the patients for providing the samples to be analysed here but also for their willingness to participate in this research.

This work was carried out as part of a collaboration with Professor Ian Hendersons lab. I have performed all of the experiments presented in this thesis alongside and with the supervision of Dr Wells. Assistance was provided by Margaret Goodall for the IgG2 extraction. I consented all of the patients and collected samples from them.

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LIST OF ABBREVIATIONS

CF	**Cystic Fibrosis**
COPD	**Chronic Obstructive Pulmonary Disease**
ELF	**Epithelial Lining Fluid**
EPS	**Extracellular Polymeric Substance**
IL1β	**Interleukin 1 Beta**
IL-8	**Interleukin 8**
LTB4	**Leukotriene B4**
MASPs	**MBL Associated Proteins**
MBL	**Mannose Binding Lectin**
MPO	**Myeloperoxidase**
MRC	**Medical Research Council**
NADPH	**Nicotinamide Adenine Dinucleotide Phosphate**
ROS	**Reactive Oxygen Species**
SLPI	**Secretory Leukoprotease Inhibitor**
SGRQ	**St Georges Respiratory Questionnaire**
TNFα	**Tumour Necrosis Factor Alpha**

CHAPTER 1 INTRODUCTION

1.1 Bronchiectasis

Non Cystic Fibrosis Bronchiectasis is defined as an abnormal and irreversible dilation with damage of bronchi and bronchioles due to recurrent episodes of airway infection and repeated cycles of inflammation (Reid 1950; O'Donnell 2000; Barker 2002). This condition manifests in both adults and children with variable severity of symptoms. Classically, however, patients suffer from chronic production of sputum even in the stable clinical state. This is frequently purulent or mucopurulent in appearance, with frequent exacerbations of the condition with superimposed lower respiratory tract infections, and associated dyspnoea also typical of this condition (Whitters and Stockley, 2012). As a consequence there is significant morbidity and poorer quality of life in patients with bronchiectasis, (Martínez-García *et al*., 2007), and in developing countries it continues to be a significant cause of childhood morbidity (Karadag *et al*., 2005). Allied to the daily symptoms experienced by patients, chronic bacterial colonisation with pathogenic organisms exacerbates these symptoms and provides clinicians with a major challenge in management of bronchiectasis. The failure to maintain sterility of the airways implies a failure of host defences and there are a number of host and bacterial factors that contribute to bacterial colonisation and perpetuate the resulting on going inflammation characteristic of the lung in bronchiectasis.

1.1.1 Incidence and Prevalence of Bronchiectasis

Bronchiectasis increases in prevalence with age. Data from the United States of America estimates that it affects 4.2 per 100,000 people aged 18 to 34 and increases to 271.8 per

100,000 in those over the age of 75 (Weycker *et al*., 2005). British data has similarly shown an increased prevalence with age and greater in females. Somewhat surprisingly there has been an increased prevalence recorded year on year (Quint *et al*., 2015). This is contrary to the previously held belief that bronchiectasis was predominantly caused by childhood illness, and would become less common as a result of vaccination, antibiotic therapies and improved living standards. It may be that the increasing use of high resolution computed tomography (HRCT) scanning is identifying more patients, and that in an aging population there are causes of adult onset bronchiectasis which were previously unidentified or lead to increased burden of symptoms as patients age, resulting in reporting/presentation to health care systems. Moreover, bronchiectasis is increasingly recognized as with a feature of Chronic Obstructive Pulmonary Disease (O'Brien *et al*., 2000; Martínez-García *et al*., 2007), which is diagnosed in one million people in the UK alone although thought to be an underestimation (National Clinical Guideline Centre 2010).

1.1.2 Causes of Bronchiectasis

Bronchiectasis is often classified as idiopathic as, despite detailed medical history and investigation, an underlying cause is not identified in up to 50% of patients (Pasteur *et al*., 2000; Guan *et al*., 2015; Lonni *et al*., 2015; Dimakou *et al*., 2016; Gao *et al*., 2016).

Identifying an underlying cause (if possible) is considered important in the management of bronchiectasis as it can, in some cases, alter treatment. For example screening suitable patients for Cystic Fibrosis (CF) variants as recommended by British Thoracic Society guidelines 2010 (Pasteur, Bilton and Hill, 2010), and identifying these patients affected can significantly alter their treatment, with opportunities for more specialist care and therapies. Similarly identifying an underlying immunoglobulin deficiency may lead to effective

treatment with immunoglobulin replacement. The causes and associations of bronchiectasis are shown in table 1.1 (King *et al.,* 2006; Godoy *et al*., 2008; Rosenzweig 2008; Kilpatrick *et al*., 2009; MacFarlane *et al*., 2012; Whitters and Stockley, 2012; Chalmers *et al*., 2013; Araujo and Sucena, 2015; Parikh *et al*., 2015). Many of these conditions are rare and the broad category of post infective causes are the most commonly identified and hence assumed to be a causative factor, and this seems to be irrespective of geography and the patient population studied (Pasteur *et al*., 2000; Guan *et al*., 2015; Lonni *et al*., 2015; Gao *et al*., 2016).

1.1.3 Clinical Features

The characteristic feature of bronchiectasis is a chronic cough productive of sputum ranging from a mucoid appearance to mucopurulent/ purulent (Stockley *et al*., 1984). Many patients seen in secondary or tertiary care will describe daily symptoms whilst those with milder disease may only experience these symptoms during exacerbations. The increased purulence of sputum is due to release of myeloperoxidase (MPO) contained within the azurophilic granules of airway neutrophils. The green pigmentation of this protein infers that increasing sputum purulence relates to increased numbers of inflammatory cells in the sputum as a result of increased neutrophil recruitment reflecting host response (Stockley *et al*., 2001).

Patients frequently recognise that exacerbations of their chronic disease is associated with increasing sputum purulence or volume - essentially with a worsening of their symptoms from their perceived "normal" baseline state. The isolation of a pathogenic organism is not necessarily diagnostic of an exacerbation as patients will often be chronically colonised with organisms and thus these will often be isolated on standard bacterial culture when the patient

Post infective causes	Host immune defects	Impaired mucociliary clearance	Inflammatory disease associations	Others
Post viral (measles) **Pertussis ('whooping cough')** **Post tuberculosis** **Bacterial pneumonia**	**Primary Antibody Deficiency** - **Common variable immune deficiency (CVID)** - **X linked agammaglobulinaemia** - **IgG subclass deficiency** - **Selective IgA deficiency** - **X linked hyper IgM syndrome.** **Complement deficiency** - **Mannose Binding Lectin deficiency.** - **L- Ficolin deficiency** **Inate immune defects** - **Chronic granulomatous disease** - **Defective oxidative burst** **Acquired immune deficiency** - **Chronic lymphocytic leukaemia.** - **Multiple myeloma** - **Protein losing states.**	**Primary ciliary dyskinesia** **Kartageners syndrome** **Youngs syndrome**	**Chronic obstructive pulmonary disease (COPD)** **Rheumatoid arthritis** **Inflammatory bowel disease.** **- Ulcerative colitis** **- Crohn's disease.**	**Alpha-1-antitrypsin deficiency** - **ZZ phenotype associated with increased severity of bronchiectasis.** **Middle lobe syndrome** **Foreign body inhalation** **Gastro oesophageal reflux disease (GORD)** **Yellow nail syndrome**

Table 1.1. Causes and Association of Non Cystic Fibrosis Bronchiectasis

is in, what they believe to be, their stable state. An increase in sputum purulence, indicative of increased neutrophil recruitment in response to worsening infection is therefore a useful clinical tool in identifying a clinical exacerbation. For the purposes of this thesis, and recruitment of patients to this study, I have considered an exacerbation to be defined by an increase in purulence or volume of sputum in comparison to the patients' normal baseline level.

The average frequency of exacerbations per year is reported to be between 2.1 and 6 (Smith *et al*.,1996; Wilson *et al*., 1997a; Wilson *et al*., 1998; Tsang *et al*., 1999). It should be noted that a consensus is required in the definition of an exacerbation as this can vary. Recognition and appropriate management of exacerbations are imperative in the treatment of bronchiectasis as more frequent and severe infections are associated with an accelerated rate of decline in lung function (Martínez-García *et al*., 2007). Repeated infections are thought to contribute to an ongoing inflammatory process in bronchiectasis and intuitively increased frequency of exacerbations would seem likely to be associated with poorer quality of life. The Bronchiectasis Severity Index (BSI) is a clinical predictive tool that identifies patients at risk of future mortality, hospital admissions and exacerbations. It identifies three or more exacerbations as an independent risk factor for increased mortality in bronchiectasis. Furthermore increased severity of disease as defined by BSI and an increased number of exacerbations were associated with worsening quality of life as assessed by the St Georges Respiratory Questionnaire. (Chalmers *et al.,* 2014)

Other symptoms associated with bronchiectasis include haemoptysis that can be life threatening (though rarely) and most often occurs during an exacerbation. Increased dyspnoea is often reported (Nicotra *et al*., 1995; Habesoglu, Ugurlu and Eyuboglu, 2011)

with impaired pulmonary function usually demonstrating mild to moderate airflow obstruction (King, 2011). There is some correlation between severity of dyspnoea and the extent of bronchiectasis measured by HRCT scanning and the degree of lung function impairment (Smith *et al*., 1996; Guan *et al*., 2014). Decline in lung function as measured by FEV_1 has been demonstrated in patients with idiopathic bronchiectasis over time (King *et al*., 2005). There are often additional other non specific symptoms such as chest pain and fatigue.

1.1.4 Pathological Findings

The basis for much of the knowledge regarding the macroscopic changes seen in the bronchiectatic bronchi is based on post mortem findings from the 1950s (Whitwell, 1952). These showed inflammatory changes of the thickened bronchial wall and dilated bronchi often containing pus. Evidence of the destructive nature of bronchiectasis was demonstrated by the finding of loss of muscle and cartilage in cases of advanced disease with granulation tissue noted in areas of ulcerated epithelium indicative of healing tissue. These findings identified bronchiectasis with three distinct pathological types, (cylindrical, cystic and varicose). These descriptions continue to provide the basis for radiological descriptions of bronchiectasis with features of thickened irregular bronchial walls, areas of bronchial dilatation, and mucus plugging often identifiable on HRCT. Cylindrical bronchiectasis is characterised radiologically by bronchi that fail to taper normally with the ‘ signet ring’ sign characteristic of this. This is most commonly seen and is often clinically associated with the least severe symptoms. Varicose bronchiectasis is where the dilated bronchi can be seen to have areas of relative narrowing, or a beaded appearance on CT scan. Cystic bronchiectasis is the most severe form with cyst like appearances of the bronchi, often extending to the pleural surface, relative destruction of the normal bronchial architecture, with visible air-fluid

levels also seen. This can vary in severity with localisation of disease or can be diffuse throughout both lungs and is partially dependent on the underlying aetiology. For example post tuberculous infection bronchiectasis may be confined to the previously affected area while bronchiectasis due to severe immunodeficiency may be more diffuse and that due to CF or ABPA may be more proximal.

1.1.5 Pathogenesis of bronchiectasis.

As described it is often difficult to attribute the development of bronchiectasis to a particular underlying disease or immune state. Post infective related are the most common subgroup and it is believed that infective insults to the lung not only initiate the development of the features of bronchiectasis but that repeated infection maintains and increases it. The vicious cycle hypothesis of bronchiectasis describes an initial breach in the lung host defence, usually due to infection, with subsequent neutrophilic inflammation, impaired mucociliary clearance facilitating bacterial survival and an ongoing inflammatory response with airway destruction, resulting chronic bacterial colonisation perpetuating the cycle (Cole and Wilson, 1989).

There are other factors that may also impact on this cycle. For example a genetic predisposition such as cystic fibrosis, or impaired innate or adaptive immune response will also facilitate bacterial survival and perpetuate the ongoing inflammatory response (Whitters and Stockley, 2012) as outlined in Fig 1.1.

1.1.6 Bacterial Colonisation

Bacterial Colonisation in lung disease is generally defined as at least two positive sputum cultures separated by at least 3 months in a year (Pasteur *et al*., 2000). The prevalence of

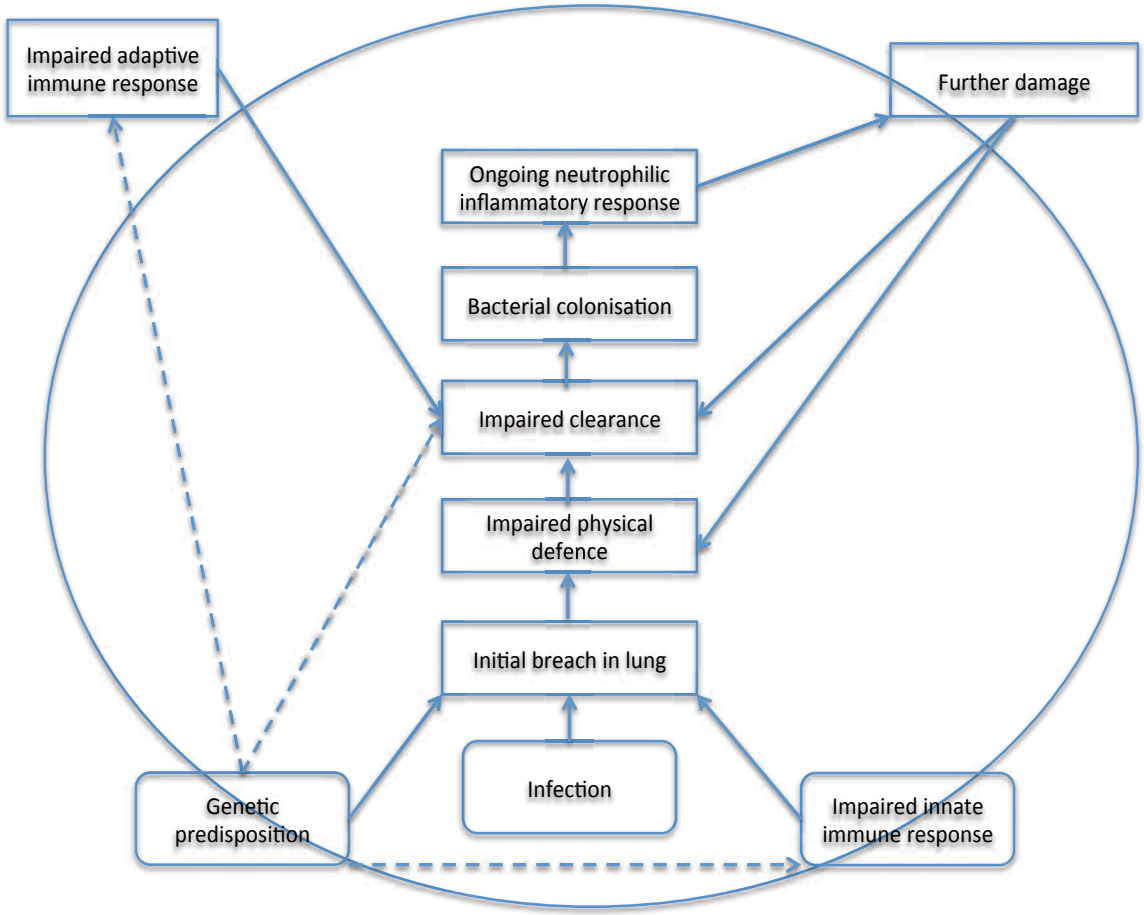


Figure 1.1 The initial breach in the lung in bronchiectasis is usually thought to be caused by infection. Predisposing defects in innate immunity or primary lung defence mechanisms may predispose to this initiation including genetic predisposition as in cystic fibrosis. This establishes a chain of events culminating in bacterial colonisation and perpetuated by tissue damage as a consequence of the subsequent inflammatory response. In addition other inherited conditions such as primary ciliary dyskinesia will impair clearance of secretions, and immune deficiencies impair innate and adaptive immune responses (Cole and Wilson,1989; Whitters and Stockley,2012).

bacterial colonisation varies in the literature and many published studies are based on pathogen isolation on only one occasion rather than repeated growth over time. Colonisation is reported to be high amongst patients with bronchiectasis. Studies defining colonisation as positive sputum at different time points report rates of colonisation amongst patients of between 35.5% up to 66% (Angrill *et al*., 2002; Pasteur *et al*., 2000; King *et al*., 2007; Borekci *et al*., 2016). *Haemophilus influenzae (H. influenzae)* and *Pseudomonas aeruginosa (P. aeruginosa)* are reported to be the most common colonising organisms isolated. *P. aeruginosa* colonisation is reported in 9-24 % of patients with bronchiectasis with *H.influenzae* reported in 11 -35% in these same studies. Other potentially pathogenic colonising organisms include *Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Klebsiella pneumonia, acinetobacter sp* and *Moraxella cattarhalis* (Pasteur *et al,*. 2000; King *et al*., 2007). Non-tuberculous mycobacteria (NTM) are also significant pathogens found in patients with bronchiectasis with meta-analysis showing a prevalence of NTM of just over 9% in patients with bronchiectasis (Chu *et al*, 2014).

1.1.7 Pseudomonas aeruginosa Colonisation

The mechanisms influencing colonisation with *P. aeruginosa* will be discussed in detail as this forms the basis of the studies reported in this thesis. Not only is this one of the two most common pathogenic organisms that colonise patients with bronchiectasis but it is resistant to many of the commonly used antibiotics. Resistance patterns of *P. aeruginosa* in cystic fibrosis have been studied in detail (Doring *et al*., 2000; Oliver *et al*., 2000; Pitt *et al*., 2003;) but less is known about resistance of strains isolated in non-CF bronchiectasis. It is perceived to be less common, perhaps because in comparison to CF patients, those with bronchiectasis are less likely to be on long term antibiotics.

Chronic colonisation with *P. aeruginosa* has been identified as an independent risk factor for an accelerated rate of decline in lung function in bronchiectasis (Martínez-García *et al*., 2007). Other studies have identified it in association with more severe impairment of lung function though whether this is cause or effect is unclear (Davies *et al*., 2006; McDonnell *et al*., 2015).

Hospital admissions have been shown to be higher than with other colonising organisms (McDonnell *et al*., 2015) and it is associated with increased numbers of exacerbations and poorer quality of life (Wilson *et al* 1997; Martínez-García *et al*., 2007; Goeminne *et al*., 2012; Rogers *et al*., 2013; Guan *et al*., 2014; Rogers *et al*., 2014; McDonnell *et al*., 2015). BSI has also shown that *P. aeruginosa* colonisation is an independent predictor of hospital admission (Chalmers *et al*, 2014. Further meta analysis has also shown *P. aeruginosa* to be associated with a higher risk of death, hospital admission and exacerbations (Finch et al, 2015).

1.1.8 Diagnosis and Management

In clinical practice the diagnosis of bronchiectasis is usually made by identification of classical symptoms confirmed by radiological features of bronchiectasis on HRCT. Investigations are then aimed to try and establish an underlying cause and identify any pathogenic organisms in the sputum. Measurements of serum immunoglobulin are taken (IgA, IgM and IgG) to establish any adaptive immune deficiency. The usefulness of checking IgG subgroup levels are uncertain and not routinely recommended. No significant association with IgG subclass deficiency and bronchiectasis has been identified (Hill *et al*.,

1998; Stead *et al*., 2002; King *et al*., 2006).

Baseline specific antibody levels against tetanus toxoid, and the capsular polysaccharides of *H. influenzae* and *S. pneumoniae* are measured although the colonising species of *H. influenzae* is not capsulated. If these antibody levels are low, vaccination is undertaken with the relevant antigen followed by repeat measurement at least three weeks later. If levels remain low it may indicate a failure of the adaptive immune response. Both tests of the immune system are recommended (Vendrell *et al*., 2005) and indeed are considered routine clinical practice in a specialist bronchiectasis clinic. Pulmonary function testing is usually undertaken particularly in cases where a concurrent diagnosis of COPD or asthma is suspected. In addition to these standard tests, specialist investigation such as nasal nitric oxide (nNO) can be undertaken where primary ciliary dyskinesia is suspected. Measurements of nNO are lower in patients with PCD in comparison to healthy patient and those with idiopathic bronchiectasis (Wodehouse *et al*, 2003). Ciliary biopsy would be undertaken where primary ciliary dyskinesia is suspected and younger patients should be screened for cystic fibrosis variants as recommended by the British Thoracic Society Bronchiectasis Guideline (2010) (Pasteur, Bilton and Hill, 2010), particularly when other features suggestive of CF are present.

The management of bronchiectasis includes treatment of the underlying cause where identified and where a suitable treatment exists. Irrespective of the underlying cause patients are taught airway clearance techniques and encouraged to perform these exercises daily as sputum clearance thought imperative to the management of this condition. A Cochrane systematic review deemed airway clearance techniques safe although requiring further study and evaluation of short and long term benefits (Lee *et al*., 2015). Evidence to support the use

of inhaled bronchodilators, inhaled steroids and mucolytics in bronchiectasis is lacking. Inhaled therapies only have a role where there is coexistant airway obstruction in line with their utilisation in COPD (Whitters and Stockley, 2013). Antibiotics form the mainstay of current management of bronchiectasis and courses of between 10-14 days of a suitable antibiotic targeted to the organism found in the patients sputum is recommended during exacerbations (Pasteur, Bilton and Hill, 2010). Where patients exacerbate frequently, are colonised with a pathogenic organism or chronically produce purulent sputum to suggest a high bacterial load, long term antibiotic therapy, either nebulised or oral may be initiated in an attempt to lower the bacterial load. This has largely not been subjected to placebo-controlled trials although sequential studies indicate clear benefit to sputum purulence and patient well being (Stockley, Hill and Burnett, 1985). More recently confirmation of benefit has been shown with inhaled gentamicin in patients colonised with *Pseudomonas aeruginosa* (Murray *et al*., 2011).

1.2 The Host Immune System

The vicious cycle of bronchiectasis implies failure of host defences leading to ongoing inflammation and damage. The innate immune system responds immediately to inhaled pathogens and is the first barrier to infection. It comprises the physical epithelial barriers and mucociliary clearance in addition to secreted anti inflammatory proteins and polypeptides, with subsequent recruitment of phagocytes to the site of inflammation and specific antibody production. Locally produced IgA and IgG in addition to diffusion of IgA and IgG from plasma form the basis of this immediate response.

The adaptive immune response has two main pathways. The humoral response mediated by B-lymphocytes and their production of antibody. Cell mediated immunity is mediated by T lymphocytes. The role of cell mediated immunity in bronchiectasis is not fully understood although increased CD4 lymphocytes have been identified in the airways of bronchiectasis patients (Gaga *et al*., 1998), whilst bronchial biopsy specimens have shown a marked inflammatory infiltrate with a CD8+ and CD4+ T cells indicating that cell mediated inflammation is activated and may play a role in bronchiectasis pathogenesis (Silva *et al* 1989). The humoral response and its relevance to the development and perpetuation of bronchiectasis is better established and relevant to the central hypothesis of this thesis.

1.2.1 The Innate Immune System of the Lung

The innate immune defence of the lung is the immediate defence against inspired pathogens. This comprises physical barriers with the mucociliary escalator the initial barrier to infection, and airway secretions containing anti infective proteins and polypeptides forming a local response to invading pathogens. Immunoglobulins form part of this initial response with secretary IgA the main local antibody found in the respiratory tract contributing to the initial immune response.

1.2.2 Mucociliary Escalator

This physical barrier consists of ciliated epithelium that lines the airway from larynx to the terminal bronchioles. It consists of goblet cells that secrete mucus and ciliated columnar cells (Clarke and Pavia, 1980). Particles greater than 2-3μm are trapped on the mucus layer that covers these cells and are then removed by mucociliary clearance and cough (Nicod 1999;

Loebinger, Bilton and Wilson, 2009). It is failure of mucociliary clearance that affects patients with conditions such as primary ciliary dyskinesia predisposing to bronchiectasis. For pathogens that evade this initial barrier in the lung there are further local immune responses to aid clearance and modulate the local inflammatory response.

1.2.3 Immune Proteins

Epithelial lining fluid (ELF) forms a thin fluid level lining the airways (Rogan *et al*., 2006; Rodvold, George and Yoo, 2011). This consists of airway secretions containing anti infective proteins and polypeptides including lysozyme, lactoferrin and Secretory Leucoprotease Inhibitor (SLPI) amongst others (Travis, Singh and Welsh, 2001; Rogan *et al*., 2006). When pathogens invade the lung sub mucosal gland epithelial cells and resident macrophages respond by making and secreting these antimicrobial proteins. The most abundant are lysozyme, lactoferrin and SLPI.

Lysozyme is stored in both primary and secondary neutrophil granules. It has bactericidal and bacteriostatic effects and acts by lysis of the bacterial cell wall (Rogan *et al*., 2006).

Lactoferrin is released from neutrophil secondary granules and has both antimicrobial and anti-inflammatory effects. It also has bactericidal and bacteriostatic effects with additional antifungal and antiviral properties (Rogan *et al*., 2006). Lactoferrin effects antibacterial activity in several ways. Firstly, it limits the amount of free iron available (which is essential for growth of microorganisms) by binding to iron and has been shown to enhance the antibacterial effects of some antibiotics (Leitch and Willcox, 1999). Furthermore, it may inhibit biofilm formation by bacteria – a defence mechanism employed by bacterial

populations, including *P. aeruginosa* whereby bacteria are embedded within an extracellular polymeric substance (EPS), increasing resistance to antibiotics and host killing (Singh *et al*., 2002). It has also been shown to have key anti-inflammatory actions by inhibition of production of interleukin-8 (IL-8), in human endothelial cells (Elass *et al*., 2002). Lactoferrin acts in synergy with lysozyme and SLPI in bacterial killing – each acting to enhance the effect of the others (Singh *et al*., 2000). Increased levels of lactoferrin and lysozyme in bronchoalveolar lavage of patients with chronic bronchitis have been reported, in comparison to that seen in healthy smokers and healthy controls suggesting that both contribute to the inflammatory response. However they did not correlate with neutrophil levels suggesting that recruited neutrophils were not the main source (Thompson *et al*., 1990). This instead suggests that the majority of production of these proteins are from other airway cells including sub epithelial glands. Whether a similar process plays a role in bronchiectasis is unclear though it may be overwhelmed by the exaggerated neutrophilic response associated with bronchiectasis.

SLPI is produced by the mucous glands, macrophages. neutrophils and airway epithelial cells and has antifungal, antiviral and antibacterial properties (Rogan *et al* 2006). It has anti bacterial effects against both gram negative and gram-positive organisms (Heimstra *et al*., 1996). The Anti protease activity of SLPI acts to inhibit neutrophil elastase activity thereby opposing its damaging elastolytic activity as it is released from neutrophils in areas of inflammation and infection. It is reduced by the presence of elastase activity (Sullivan *et al*., 2008) and is subject to cleavage and inactivation in areas of lung affected by pneumonia, leaving neutrophil elastase less likely to be inactivated and hence exacerbating further inflammation and damage (Greene *et al*., 2003). It has also been shown to be reduced in the lungs of patients with chronic bronchitis during the time of an exacerbation, improving to

baseline level, following treatment of the exacerbation. (Hill *et al*., 1999; White *et al*., 2003). Whether similar mechanisms exist in bronchiectasis with chronic inflammatory changes is unknown although it may be relevant that it has been shown to be lowest in patients with the greatest degree of neutrophilic inflammation in the stable state. (Hill, Bayley and Stockley, 1999)

The secretion of lysozyme, lactoferrin and SLPI by neutrophils is modulated by the inflammatory response in the lungs. In response to chemoattractants, neutrophils are recruited to areas of inflammation with the release of their immune proteins from preformed granules, during phagocytosis and production of reactive oxygen species (ROS) at the site of inflammation. (Stockley, 2002; Cowburn *et al*., 2008; Whitters and Stockley, 2012).

Bronchiectasis is characterised by neutrophilic inflammation irrespective of the underlying causes of the disease. Neutrophil counts measured in bronchoalveolar lavage or sputum have repeatedly been demonstrated to be elevated (Angrill, Agusti and Torres, 2001; Watt *et al*., 2004; Guran *et al*., 2007), with a further increase noted in association with isolation of potentially pathogenic organisms (Angrill, Agusti and Torres, 2001). Increased neutrophils have also been noted in the airway tissue of bronchiectasis patients to further support the role of a predominantly neutrophilic inflammatory response (Zheng *et al*., 2001). It is this continual inflammatory response by neutrophils which perpetuates the vicious cycle as previously described. Although other inflammatory cells including B and T cells, natural killer cells and mast cells are also implicated in the inflammatory response, (Eller *et al*.,1994; O'Shaughnessy *et al*., 1997; Gaga *et al*.,1998; Sepper *et al*.,1998 Zheng *et al*.,2001) in bronchiectasis neutrophils are the most abundant cells and mediator of damage and hence are

discussed in more detail below.

1.2.4 The Role of Neutrophils and Neutrophilic Inflammation in Bronchiectasis

As previously noted, neutrophils have three main roles –phagocytosis, the production of reactive oxygen species (ROS) and the release of immune proteins from preformed granules. The chemoattractants IL-8, leukotriene B4 (LTB4), tumour necrosis factor α (TNFα), and interleukin 1beta (IL-1β) are all drivers of neutrophil recruitment. IL8 in particular has been shown to have a crucial role during exacerbations (Gaga *et al*., 1998; Mikami *et al*., 1998; Watt *et al*., 2004). An increase in IL-8 is seen in increasingly purulent sputum with a higher chemotactic activity which may perpetuate the cycle of damage with continued neutrophil recruitment. (Mikami *et al*., 1998). Targeting therapies to this exaggerated response may be detrimental as at the time of an acute infection neutrophil chemotaxis is required. The immune proteins are released on activation of the neutrophil, through a hierarchical process of degranulation (Borregaard *et al*., 2001; Xu and Hakansson, 2002).

Azurophilic granules (primary granules) release (amongst others) myeloperoxidase, bactericidal/permeability increasing protein (BPI), defensins, neutrophil elastase, proteinase 3 and cathepsin G. Specific granules (secondary granules) release other key antibacterial proteins such as lactoferrin and cathelicidin and tertiary (gelatinase) granules contain, and release, gelatinase acetyltransferase and lysozyme (Rogan *et al*., 2006). It may be that the release of all these pro inflammatory mediators has a role to play in the development of bronchiectasis. However, in particular the activity of proteases released by neutrophil activation are thought to have a major role in the pathogenesis of bronchiectasis. The protease/antiprotease imbalance has been crucial to the understanding of the destructive

processes in other inflammatory lung conditions such as COPD (Stockley 1999), and it may be that unregulated activation of proteases has a similar role in bronchiectasis. Proteases have been said to cause bronchial dilatation – a diagnostic finding of bronchiectasis and thus unopposed protease activity may be relevant in the development of bronchiectasis (Khair *et al*., 1996; Zheng *et al*., 2000). Neutrophil elastase perpetuates the neutrophilic inflammation seen in the airways by upregulating the production of Interleukin 8 (IL-8) from epithelial cells (Devaney *et al*., 2003). It has also been shown to reduce ciliary beat frequency, and therefore inhibit mucous clearance, at high concentrations with epithelial damage evident at lower concentrations without slowing ciliary beat frequency (Amitani *et al*., 1991).

The respiratory burst, or oxidative burst, results in the release of ROS to facilitate bacterial killing. During phagocytosis, phagocytes including neutrophils increase oxygen consumption through the activity of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase that generates oxygen metabolites and formation of antimicrobial ROS (Dahlgren and Karlsson, 1999; Tung *et al*., 2009).

The release of uncontrolled ROS by neutrophils, and oxidative stress, may however be deleterious with a damaging effect on tissues (Tung *et al*., 2009). In the case of bronchiectasis this would exacerbate the existing process. It was previously believed that ROS exert a direct toxic effect on ingested pathogens but has been suggested by Reeves *et al* that respiratory burst sets in motion events which enhance the activity of proteases to digest the contents within the phagocytic vacuole (Reeves *et al*., 2002).

Chronic granoulomatous disease is a disease associated with bronchiectasis as shown in table 1.1. Patients have a defective gene for one of the subunits of NADPH oxidase and therefore are not able to produce ROS (Rosenzweig 2008). Patients with this condition frequently

develop infections and granuloma formation and inflammatory complications (Godoy *et al*., 2008).

It has been shown by King *et al* 2006, that phagocytosis was normal in bronchiectasis compared to control subjects, but that oxidative burst was lower (King *et al*., 2006), however, there was failure to identify any significant difference in respiratory burst in a detailed analysis of causative factors in bronchiectasis (Pasteur *et al*., 2000). Therefore, the role of ROS in neutrophilic inflammation and its significance or otherwise in the pathogenesis of bronchiectasis remains uncertain.

Finally neutrophils act by phagocytosis whereby they engulf and internalise the bacterial pathogen forming a phagosome. King *et al* have previously shown phagocytosis to be normal in patients with bronchiectasis (King *et al*., 2006). The pathogen is subject to opsonisation by binding of IgG via its Fab portion. The Fc portion binds to Fc receptors on the cell surface of the neutrophil facilitating the formation of a phagosome and destruction of the pathogen. The composition of immunoglobulins in the airways is discussed below.

1.2.5 Secreted Immunoglobulin

Secretary Immunoglobulin A (IgA) is the major antibody isotype found in mucosal secretions in the respiratory tract contributing to initial defence mechanisms whilst Immunoglobulin G (IgG) predominates in plasma (Pilette *et al*., 2006). Most lung IgA is produced locally within the pulmonary tissues by sub epithelial plasma cells. IgA in the airway is thought to inhibit adherence of antigens to the lung epithelium and behave as an immune barrier (Pilette *et al*., 2001). It is reported to bind to lectin-like bacterial adhesions, blocking epithelial colonisation

and operating as an immune barrier. Moreover, similar to IgG it is able to promote phagocytosis at least by airway macrophages. IgA enables delivery of antigens across the epithelium into luminal secretions, thereby removing locally formed immune complexes and limiting systemic access (Burnett *et al*., 1987). The structure and composition of IgA found in airway secretions is different to that in serum. In humans there are two subtypes of IgA (IgA1 and IgA2). Lung secretions have around 30% IgA2 compared to only 10-20% in blood. The majority of IgA in the airways is produced locally in epithelial cells although a proportion is still derived from blood. (Burnett 1986; Burnett, Crocker and Stockley, 1987; Pilette *et al*., 2001) In the presence of infection stability of IgA2 is maintained better than IgA1 as it is less susceptible to destruction by bacterial IgA proteases (Burnett, Crocker and Stockley, 1987; Pilette *et al*., 2001). In the presence of infection, an increase in the proportion of IgA2 has been demonstrated (Burnett *et al*., 1990). There does appear to be a local IgA response to pathogens with post mortem examination of the lungs of patients with bronchitis or bronchiectasis demonstrating and increased number of IgA positive cells compared with controls (Burnett, Crocker and Stockley, 1987).

IgG is the predominant antibody found in human serum. A more detailed discussion of its function is discussed later. Four subclasses of IgG exist in human sera and all have been detected in bronchoalveolar sampling from healthy subjects (Merrill *et al*., 1985). The majority of airway IgG is present due to diffusion from plasma (Reynolds 1988). Plasma cells producing IgG are however found in bronchial mucosa (Soutar 1977; Burnett 1986) and are the source of the locally produced IgG. The biological effect of IgG in the lung is poorly understood. In the knowledge that the majority of IgG in the airways is due to diffusion from plasma it seems intuitive that levels of IgG in the lung would be similarly affected by conditions such as hypogammaglobulinaemia with low serum immunoglobulin levels – perhaps contributing in part to the tendency to infection and bronchiectasis. Hill *et al* have

demonstrated increased levels of IgG in the sputum of patients with bronchiectasis. Local production was thought to account for some of this increased IgG. (Hill *et al*.,1998).

IgG antibody to *P. aeruginosa* and complement components are readily detectable in the sputum of patients with cystic fibrosis bronchiectasis where these antibodies are shown to opsonise colonising *P. aeruginosa* in the lung (Hann and Holsclaw 1976). Thus it seems likely that IgG found in the lung is involved in antibody mediated killing as a component of the local immune response. Similarly Hann *et al* demonstrated the presence of complement proteins in the sputum specimens recovered from the lung, suggesting local complement activation. This would imply that, it is highly likely that antibody-mediated killing is involved in the host defence against bacterial lung infection.

1.2.6 The Complement System

The complement system is a crucial component of host defence consisting of a highly regulated cascade of multiple plasma proteins which compliment the killing of bacteria by serum antibodies. Initially thought to have a primary role in innate immunity it has been shown to compliment bacterial killing by both innate and adaptive immune systems. It compliments killing by antibodies by opsonisation with activated complement proteins binding to pathogens and facilitating phagocytosis. C3b is the most potent of these proteins. Fragments of some complement proteins (C3a, C5a), are products of the sequential enzymatic cleavage and have a critical role. C3a and C5a are chemoattractants to phagocytes such as neutrophils attracting them to the site of inflammation or infection (Sarma and Ward, 2010). Finally there is the formation of the membrane attack complex C5b-9 (MAC), as the final product of the terminal components of the cascade. This can destroy certain pathogens by

disruption of the bacterial cell membrane. (Sahu and Lambris, 2001; Janeway *et al*., 2005; Sarma and Ward, 2010; Merle *et al*., 2015)

It has a role in both the innate and adaptive immune systems. There are three well defined pathways which constitute the complement system – the classical pathway, the mannose binding lectin (MBL) pathway and the alternative pathway. The stimulants to each of these three pathways is shown in figure 1.3. Briefly the classical pathway is activated by antibody and antigen binding by IgG or IgM whereas IgA is a poor initiator of complement response. The resultant IgG or IgM immune complex is then bound at the Fc portion by the C1 complex (consisting of C1q,C1r and C1s molecules) of the cascade, by C1q binding. This activates C1s and C1r to cleave C4 and C2 to form C3 convertase (C4bC2a) where the three pathways converge (Sahu and Lambris, 2001; Janeway *et al*., 2005; Sarma and Ward, 2011; Merle *et al*., 2015).

The mannose binding lectin (MBL) pathway is stimulated by carbohydrate on microbial surfaces binding to lectins (carbohydrate binding proteins) such as MBL or ficolin. Both lectins circulate normally in the serum as complexes with MBL – associated proteins. (MASPs) (Sørensen, Thiel and Jensenius, 2005; Wallis, 2007) Activation of this pathway again leads to formation of the C3 convertase C4bC2a via cleavage of first C4 to C4a and C4b and C2 to C2a and C2b (Sahu and Lambris, 2001; Janeway *et al*., 2005).

Finally, the alternative pathway is activated by lipids, carbohydrates and proteins found on cell surfaces. It also terminates in the formation of a C3 convertase (C3 (H_2O)Bb) (Sarma and Ward, 2011). During normal healthy condition it is the most relevant of the three pathways maintaining a low level of activity monitoring for pathogen invasion in a process referred to as "tick over" (Merle *et al*., 2015). This is essentially the constant hydrolysis of C3 found in plasma, to form C3 (H_2O). The change in configuration allows Factor B to then

bind to the reconfigured C3 where it is cleaved by factor D, then cleaved to Ba and Bb. Bb remains bound to C3 (H_2O) to form a C3 convertase (C3 (H_2O)Bb. (Sarma and Ward, 2011;Merle *et al*.,2015).

Following formation of C3 convertase the three pathways converge. This ultimately results in cleavage of C3 forming C3a and C3b. As stated previously C3b acts as an opsonin to facilitate phagocytosis and also binds C3 convertase to form C5 convertase which subsequently cleaves C5 to C5a and C5b. C5b is then able to bind to C6, C7, C8 and C9 resulting in the formation of activation products C3a, C3b, C5a and the membrane attack complex C5b-9 (MAC) and destroy pathogens by disrupting membrane integrity.

A summary of the major components of the three pathways is provided in figure 1.2. The broad functions of each complement protein is shown in table 1.2.*

1.2.7 Complement Deficiency and Bronchiectasis.

Inherited complement deficiencies are rare. They are reported to account for between 1-10% of primary immune deficiencies (Grumach and Kirschfink, 2014). As such their role in bronchiectasis is undetermined. Complement deficiency does however seem to increase the tendency to particular infections likely as a consequence of inadequate opsonisation and defects in cell lysis but the implications of deficiency of complement depend on which part of the pathway is affected. C3 is the most abundant complement protein and is cleaved by C3 convertase at the point of convergence of the three pathways. Case numbers are small but a review of reported cases by Skattum *et al* reports that deficiency of C3 seems to be associated with increased tendency to encapsulated bacteria, likely at least partly as a consequence of impaired opsonisation.

Binding to Immune complex and pathogen surface	Binding to mannose	Activating enzymes	Membrane binding protein and opsonins	Mediators of inflammation	Membrane attack proteins
C1q	MBL	C1r C1s C2b Bb Factor D MASP 1 MASP2	C4b C3b	C5a C3a C4a	C5b C6 C7 C8 C9

Table 1.2 shows functional protein classes in the complement system. *Regulatory proteins and complement receptors are not included (Janeway *et al*., 2005).

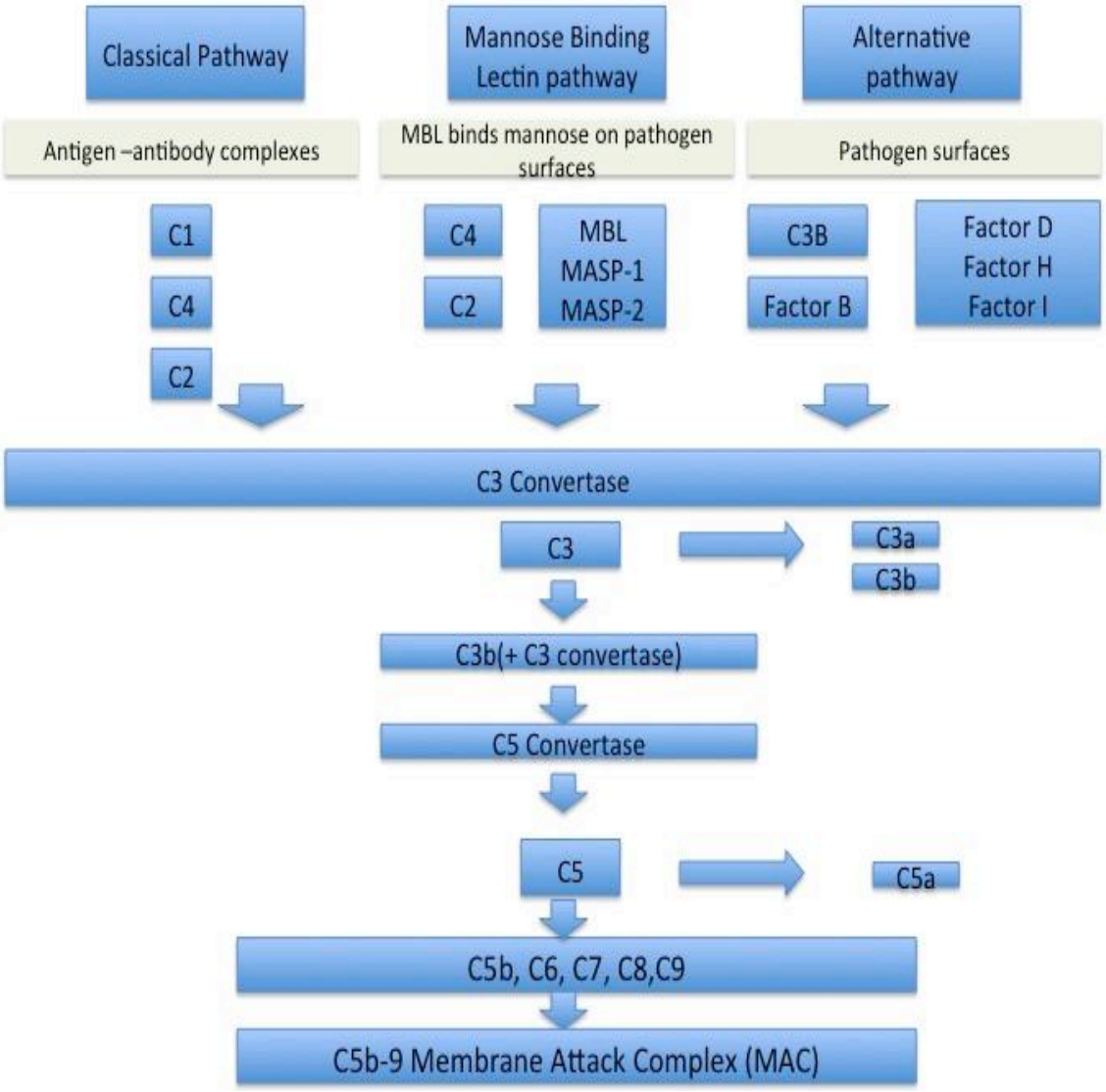


Figure 1.2 shows the major components and pathways of the three major complement cascade pathways – the classical, mannose binding lectin pathway and the alternative pathway. The stimulus to each pathway is depicted in the grey shaded boxes. The three pathways converge with the cleavage of C3 to C3a and C3b. The binding of C3b to C3 convertase forms C5 convertase whch subsequently cleaves C5 to C5a and C5b which is then able to bind to C6, C7, C8 and C9 resulting ultimately in the formation of the membrane attack complex C5b-9 (MAC).

Respiratory tract infections and meningeal infections are the most common seen in C3 deficiency. Respiratory tract infections are associated with defects of the classical pathway (C1q,C1r,C1s,C2,C3 and C4 deficiencies), due primarily to *Streptococcus Pneumoniae* while defects of the alternative pathway predispose to infection with *Neisseria.* (Skattum *et al*.,2011). Whether the prevalence of bronchiectasis is increased in these patients with a greater tendency to infection is not known.

Deficiencies of the mannose binding lectin pathway have been associated with an increased tendency to respiratory infection, particularly in childhood. (Koch *et al*., 2001;Cedzynski *et al*., 2004; Gomi *et al*., 2004).

Low or deficient serum Mannose-binding lectin (MBL) has also been associated with worsening severity of disease in cystic fibrosis with worse lung function, earlier acquisition of *P. aeruginosa* and increased rate of death (Chalmers *et al*., 2011). Results are conflicting in bronchiectasis with Chalmers *et al* reporting increased severity of bronchiectasis, including increased exacerbations, hospital stays and increased bacterial colonisation rates with a higher rate of *P. aeruginosa* colonisation compared to controls. The rate of MBL deficiency was not noted to be higher in patients with bronchiectasis compared with controls however. (Chalmers *et al*., 2013).

A similar study failed to demonstrate any worsening severity of disease symptoms in bronchiectasis patients. The incidence of MBL deficiency was again similar to the general population (MacFarlane *et al*., 2012). The conflicting results of these studies do seem to indicate similar rates of MBL deficiency in patients with bronchiectasis as in the general population which suggests it is not associated with a higher risk of developing bronchiectasis but whether it influences the severity and outcomes in the disease requires further study.

Perhaps of significance, measurement of serum L-ficolin and MBL in patients with stable bronchiectasis again found no difference in average MBL values between patients with stable bronchiectasis, healthy blood donors and patients admitted to hospital with pneumonia. L-ficolin deficiency however, was found more frequently in the bronchiectasis group suggesting a possible causative role due to a decreased capacity to activate the mannose binding lectin pathway of the complement cascade (Kilpatrick *et al*., 2009).

1.2.8 Adaptive Immune System

The adaptive immune system is a highly specific system required to protect the lungs from organisms which evade phagocytosis by the inate immune system. Where the inate immune system is effective in recognising pathogens, it mainly relies on recognition of surface molecules common to a variety of pathogens. Bacterial evolution dictates that mechanisms have evolved to protect the recognition of the bacterial surface by the inate immune system, for example encapsulation, thereby limiting phagocytosis by resident macrophages. The adaptive immune sytem can overcome this limitation on the innate immue system by recognising and developing memory for an infinite array of antigens. As stated previously cell mediated immunity by T cells forms a vital part of the adaptive response but its role in bronchiectasis is less well defined. There are however well recognised associations between B cell antibody formation, infection and bronchiectasis.

Briefly, the adaptive immue response is typically triggered in response to infection with antigen initially presented to immature T. lymphocytes by macrophages or dendritic cells. T – helper cells activate B cells which produce antibody specific to the antigen whilst cytotoxic T cells destroy the pathogen presented to it. Immature circulating lymphocytes have antigen

receptors of a single specificity with different specificity of each lymphocyte. When an antigen is bound to the specific receptor on the lymphocyte, it is activated to form a lymphoblast and subsequent clonal expansion takes place whereby clone cells differentiate to effector cells which secrete antibody with the same specificity as the surface receptor that was first activated. Antibodies are then secreted from the B cell antigen receptor (Janeway *et al*., 2001). This process takes a number of days to complete. Some effector cells persist after the antigen has been eliminated which forms the basis of immunological memory, whereby at the time of the second exposure to the same antigen the response is more rapid. This secondary immune response produces higher amounts of more specific antibody to antigen to prevent reinfection and forms the basic principle of vaccination therapies.

Three classes of B lymphocytes exist in humans. B-1 lymphocytes and B-2 lymphocytes which are further differentiated to marginal zone (MZ) and follicular (FO) lineages (Hoffman *et al,* 2016). B-1 cells are part of the innate immune system, producing immunoglobulins , recognising self antigens and epitopes such as carbohydrate. B-2 cells are present in secondary lymphoid organs and generally are considered mediators of adaptive immunity (Montecino-Rodriguez and Dorshkind, 2012). All lineages arise initially from progenitor cells derived from haemopoietic stem cells (HCS). B1 progenitor cells in the fetal liver and B-cell progenitor cells found in bone marrow further develop to B1 cells residing in pleural and peritoneal cavities and transitional 2 (T2 B) cells in the spleen which further differentiate to MZ lymphocytes found in the marginal zone of the spleen and FO lymphocytes found in spleen and lymph nodes. B lymphocytes of each lineage have roles in antigen recognition through T dependent and independent pathways, rapid production of IgM antibodies, and the production of IgG forming the basis of immunological memory. Distinct functions of B1 lymphocytes include the production of IgA, which has a role in mucosal

defence, and is discussed in more detail later. MZ B-lymphocytes, in addition to the overlapping roles as described above, transport complement bound opsonins on to FO dendritic cells. In addition to B1 cells, MZ B cells mediate rapid IgM response. IgG antibodies, are derived from FO B cells forming the basis of immunological memory. They are the most abundant of all B cells with its primary role the generation of high-affinity long-lasting IgG antibodies in a T cell dependant role. FO B cells also have overlapping functions with MZ B cells and B1 lympocytes with the production of all immunoglobulin subtypes, including IgM, in a T cell-independent response (Hoffman *et al*, 2016).

Three main antibody groups are found in human serum – immunoglobulin A, M and G. IgD and IgE are also found but constitute a small percentage of total immunoglobulin with a role in allergic response/parasite clearance for E rather than infection and the role for D remains uncertain. The main functions of antibody are to activate the classical and alternative pathways of the complement system. The classical pathway is activated predominantly by IgM followed by IgG with the alternative pathway activated by IgG and IgA (Janeway *et al*.,2001). Opsonisation is a function of IgG and IgM. IgA as discussed previously has a role in mucosal protection. The role of each antibody and subgroups are shown in table 1.3.

Immunoglobulin class	Abundance in serum	Complement fixation	Opsonisation	Function
IgA	15%	No	No	Local mucosal response
IgM	10%	yes	yes	Primary response.
IgG	75%			Secondary response
IgG1	67%	Yes	Yes	Induced in response to protein and polypeptide antigens
IgG2	22%	Yes	Yes	Induced in response to Polysaccharide antigens
IgG3	7%	Yes	Yes	Induced in response to protein antigens
IgG4	4%	no	No	Role not fully established. Some response to polysaccharide antigens.

Table 1.3 Immunoglobulin subclasses found in human serum with the proportion of each contributing to total immunoglobulin. The ability to fix complement, function in opsonisation and function is outlined for each subtype. (Schroeder and Cavacini, 2010)

1.2.9 Immunoglobulin G Structure

Immunoglobulin G is the most abundant antibody in human serum. Its role in secondary immune response and subgroup response to particular antigens make it of particular interest in host, bacterial interactions. Its structure is crucial in its binding to antigens and activating complement. IgG is formed of a 4 polypeptide chain backbone. It contains two heavy class γ chains,(H) and two identical light chains (L) connected by disulphide bonds (-s-s-). These are arranged in a Y structure with two antigen binding sites. The amino acid sequences which form the terminal ends of the peptide chains are referred to variable regions. The light chains consist of one variable domain (VL) and one constant domain (CL). There are two types of light chains referred to as lambda (λ) and kappa (κ) chains. The heavy chains have one variable domain (VH) with three constant domains (CH1, CH2 and CH3).

The variable region binds to antigen whilst the constant domains are involved in complement binding and binding to the cell membrane. Different subclasses of IgG are characterised by differences in the amino acid content of the heavy chains and the ratio of κ to λ light chains. The major structural difference between subgroups occurs in the hinge region. The hinge forms a connection between the two heavy chains whilst providing some flexibility to the IgG enabling the fragment antigen binding portion (Fab) to interact with different sized epitopes (Vidarsson, Dekkers and Rispens, 2014). The Fab region is the region of the antibody which binds to antigens. The Fc region interacts with cell surface Fc receptors and proteins of the complement system thereby activating the immune system (Janeway *et al* 2001; Schroeder and Cavacini, 2010; Vidarsson, Dekkers and Rispens, 2014). The structure is shown in figure 1.3.

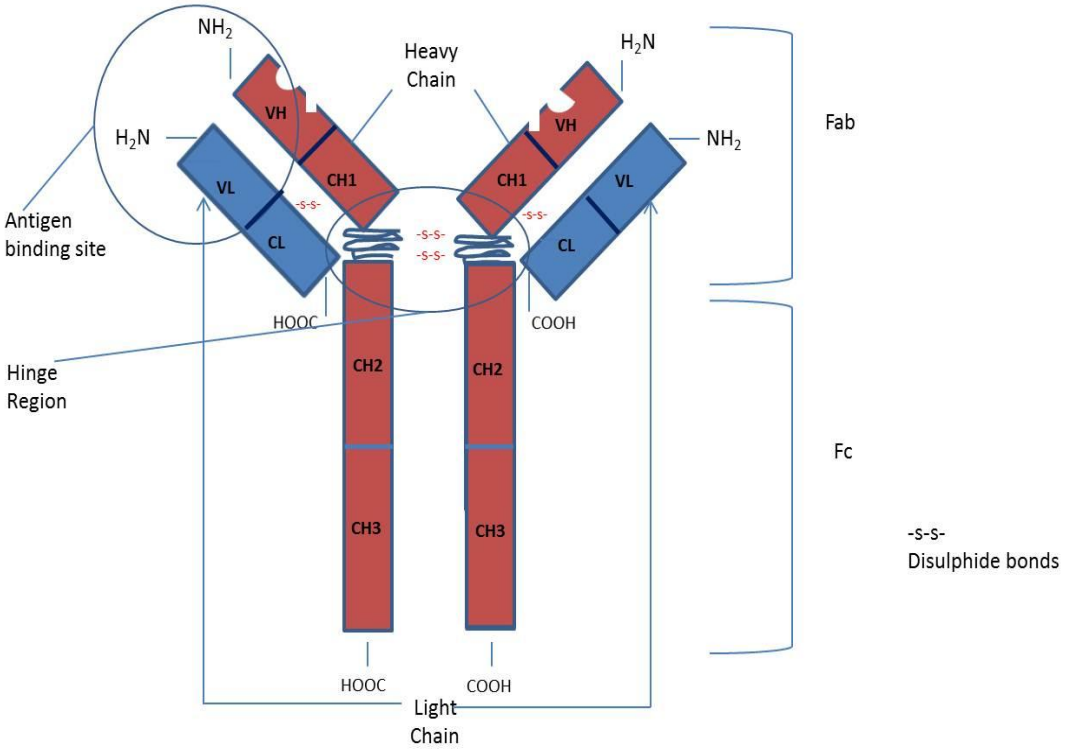


Figure 1.3 Structure of immunoglobulin G showing the 4 polypeptide chain backbone that forms IgG arranged in a Y structure. There are two heavy class γ chains,(H) and two identical light chains (L) connected by disulphide bonds (-s-s-) with two antigen binding sites (circled). The light chains consist of one variable domain (VL) and one constant domain (CL). There are two types of light chains referred to as lambda (λ) and kappa (κ) chains. The heavy chains have one variable domain (VH) with three constant domains (CH1, CH2 and CH3).

1.2.10 Antibody Deficiency Syndromes

Assessment for antibody deficiency is routine in the initial assessment of patients with bronchiectasis. Primary antibody deficiency syndromes (PADS), where there is partial or complete deficiency in one or more immunoglobulin subclass have been associated with bronchiectasis (Verma, Grimbacher and Hurst, 2015). An inherited antibody deficiency may predispose to developing bronchiectasis and the impaired ability to mount and effective response is likely to be a factor in maintenance of the cycle of infection with impaired clearance and subsequent ongoing damage. Up to 8% of adults with bronchiectasis are said to have a primary immune deficiency (Pasteur *et al*., 2000; Stead *et al*., 2002; Li *et al*., 2005; Shoemark, Ozerovitch and Wilson, 2007). The major groups of primary antibody deficiency syndromes are listed in table 1.4.

The effect of Common Variable Immune Deficiency (CVID) is an inability to mount an appropriate and competent antibody response to pathogen epitopes. PADs are rare conditions but there is a consistently high prevalence of bronchiectasis reported among those affected (Kainulainen *et al*., 1999; Quinti *et al*., 2007). Selective IgA deficiency is said to be common in the normal population(>1/800), (Holt, Tandy and Anstee, 1977) but usually patients are symptom free. Despite adequate immunoglobulin replacement Kainulainen *et al* reported progression of bronchiectasis in patients with either CVID or agammaglobulinaemia (Kainulainen *et al*., 1999) suggesting that despite optimal treatment with immunoglobulin, respiratory infections are still a problem in this group, perpetuating ongoing damage to the lung. It may be that other strategies, for example with long term prophylactic antibiotic, enhancing chest clearance and ensuring these patients have dual care with a specialist respiratory team has a specific role here too in these patients.

Primary Antibody Deficiency	Immunoglobulin deficiency
Common Variable Immune Deficiency (CVID)	Low IgG. Low IgA. +/- low IgM. Unable to mount secondary response to vaccine. Diagnosed in infants
X linked aggammaglobulinaemia (XLA)	Low IgA, IgM, IgG. Diagnosed in male infants
IgG subclass deficiencies	Low IgG subgroup 1-4
Specific antibody deficiency	Normal levels of Ig but fail to respond to vaccination.
Selective IgA deficiency	Undetectable IgA in serum or secretions. Often symptom free.
X linked hyper IgM Syndrome	Increased IgM. Low IgG, IgA.

Table 1.4. Primary antibody deficiency syndromes are shown with the immunoglobulin deficiency that define, and are diagnostic of each of these (Whitters *et al*., 2012).

Acquired hypogammaglobulinaeamia asscociated with other medical conditions is also seen in protein losing states, B cell lymphoproliferative disease such as chronic lymphocytic leukaemia (CLL) and multiple myeloma. 25% of newly diagnosed patients with CLL are said to have hypogammaglobulinaemia at the time of diagnosis with a further 25% developing this over the course of the disease (Parikh *et al*., 2015). The incidence of bronchiectasis in this group is not known but may form an important, under recognised group at this time.

1.2.11 The significance of IgG Subclass Deficiencies in Bronchiectasis.

IgG has four subclasses each with different antibody responses. Low levels of isolated IgG subclasses have been identified, with undetectable levels of serum IgG4 present in up to 10% of healthy adults (Buckley, 2002). Studies evaluating IgG subclass deficiency in bronchiectasis have provided conflicting results and as a consequence their role in causation and maintenance of the inflammatory response remains uncertain. Hill *et al* concluded that IgG subclass deficiency was comparatively rare in patients with bronchiectasis compared to age matched healthy individuals (Hill *et al*., 1998). Similar findings have subsequently been reported elsewhere (Stead *et al*., 2002; King *et al*., 2006). Other groups have however found a small number of cases of severe IgG2 deficiency with defective antibody production in bronchiectasis suggesting that it may have a significant role in some individuals (Pasteur *et al*., 2000). The significance of subgroup deficiency therefore remains undetermined. On the other hand patients have been identified with normal levels of immunoglobulin but with a poor functional response to immunisation (Pasteur *et al*., 2000; Vendrell *et al*., 2005) hence the importance of evaluating functional response to immunisation rather than simple measurement of immunoglobulin levels.

Factors that facilitate bacterial colonisation are not confined to changes in host defence but also involve factors and behaviours inherent to bacteria themselves that can evolve to ensure its ability to invade and colonise the lung. This is a particular problem in patients with bronchiectasis colonised by *P. aeruginosa* causing significant morbidity and its interactions with host defence are discussed.

1.3 *Pseudomonas aeruginosa*

P. aeruginosa is an opportunistic, environmental Gram-negative rod. As previously stated it is one of the two most common colonising organisms in bronchiectasis. It is the colonising organism in up to 80% of patients with bronchiectasis related to cystic fibrosis and is responsible for 10-20% of hospital acquired infections (El Solh *et al*., 2008). It is a cause of sepsis in immunosuppressed patients and its resistance to common antibiotics renders it a significant clinical pathogen with a need for alternative therapies. Its sophisticated array of virulence factors have resulted in an organism able to counteract and subvert host defences culminating in a persistent inflammatory response. Most of the studies evaluating chronic infection with *P. aeruginosa* have involved patients with cystic fibrosis and so much of what has been learned about its behaviour in the lung has been established in this group of patients. Although the clinical condition which affects the lungs of cystic fibrosis is bronchiectasis, there are a number of unique characteristics of the CF airway which may dictate bacterial behaviour in that environment is not necessarily replicated in the non CF lung. However it remains likely that there are similarities in the factors governing *P. aeruginosa* colonisation in both conditions.

1.3.1 Virulence Factors

There are multiple features associated with *P. aeruginosa* that enable and enhance its ability to colonise a host. It has several cell-associated and secreted virulence factors. For example it expresses two proteases, Elastase and alkaline protease expressed in both environmental and clinical strains of *P.aeruginosa* (Nicas and Iglewski, 1986).

Purified *P. aeruginosa* elastase has been shown in animal models to increase the permeability of epithelium and this method of tissue destruction could potentially contribute to the ongoing inflammatory response seen in patients chronically colonised with *P. aeruginosa* (Azghani, Bedinghaus, Klein, 2000). It also inactivates Immunoglobulins, complement components and lysozyme by proteolytic cleavage thus reducing the host immune response (Pelletier *et al*., 1989). Exotoxin A, induces apoptosis in host cells and inhibits protein synthesis amongst other actions (Michalska and Wolf, 2015), and phospholipase is reported to suppress neutrophil burst (Terada *et al*., 1999).

Pyocyanin (PCN) is a secondary metabolite produced by *P. aeruginosa.* At blood pH it can easily penetrate biological membranes and gives *P. aeruginosa* its associated green pigmentation. It has a variety of effects upon human cells such as inhibition of cell respiration, disruption of cilia function, inhibition of epidermal cell growth, disruption of calcium homeostasis, induction of apoptosis in neutrophils and inactivation of α1 protease inhibitor (Lau *et al*., 2004a; Lau *et al*,. 2004b). Flagella and pili enable motilility of the cell but can also initiate an inflammatory response, in their own right, during an infection. (Gellatly and Hancock, 2013).

The production of the extracellular polysaccharide, alginate – produced by mucoid strains of *P. aeruginosa* – contributes to the formation of biofilms although it is not essential for

biofilm formation *in vitro* (Stapper *et al*., 2004). It does however play a role in the biofilm structure and may be required for the formation of thicker three dimensional biofilms in vivo (Nivens *et al*., 2001). Patients with CF tend to be colonised with mucoid strains of *P. aeruginosa* and therefore in these patients the production of alginate is likely to be an important virulence factor (Stapper *et al*., 2004).

1.3.2 Biofilm formation and Quorum Sensing

Biofilm formation is a feature of *P. aeruginosa* which forms an impenetrable barrier protecting the bacteria from the immune system defence. Irreversible attachment of the bacteria to a surface initially takes place, followed by rapid multiplication of the bacteria and formation of microcolonies which produce an extracellular polymeric substance (EPS) which forms a matrix and holds these colonies together. As the biofilm matures it appears as mushroom like structures from which cells can detach and bind irreversibly to another surface for the process to begin again (Donlan and Costerton, 2002; Whitters and Stockley, 2012; Gellatly and Hancock, 2013). (Fig 1.4). The phenotype of the colonising strain of *P. aeruginosa* may also have an influence on biofilm formation. Strains isolated from patients with CF and chronic colonisation are frequently of the mucoid phenotype (Schelstraete *et al*., 2013). It is thought that the change of non mucoid to mucoid strains which is seen in CF takes place in response to environmental factors encountered during chronic infection. In vitro this takes place under adverse growth conditions (Govan and Deretik, 1996; Pujana *et al*., 1999). These mucoid strains can influence biofilm formation by overexpression of alginate which lends a tendency to form lumpy uneven biofilms rather than smoother flatter biofilms seen with strains unable to produce alginate (Flemming and Wingender, 2010).

P. aeruginosa also utilise quorum sensing – a highly regulated cell to cell communication system. It is defined as the capacity to detect extracellular, small molecule signals and to alter gene expression in response to bacterial population densities that reach a threshold concentration (Asad et al., 2008). Two quorum sensing systems termed LasR and rhlR have been identified in *P. aeruginosa*. (Bjarnsholt and Givskov, 2006; Popat, Crusz and Diggle 2008). In addition to a role in modulating expression of virulence factors of *P. aeruginosa* (Smith *et al*., 2002 Asad and Opal, 2008), this complex signalling system has been shown to have a role in biofilm production with manipulation of quorum sensing pathways and the creation of mutant strains seen to alter the shape of biofilm formed (Davies *et al*., 1998).

Burr et al have recently demonstrated some success in inhibiting quorum sensing of *P. aeruginosa* using low dose macrolide in patients with non cystic fibrosis suggesting therapies which impede this signalling pathway may have a role in future management of these patients and that quorum sensing is a factor influencing colonisation in bronchiectasis patients (Burr *et al*., 2016).

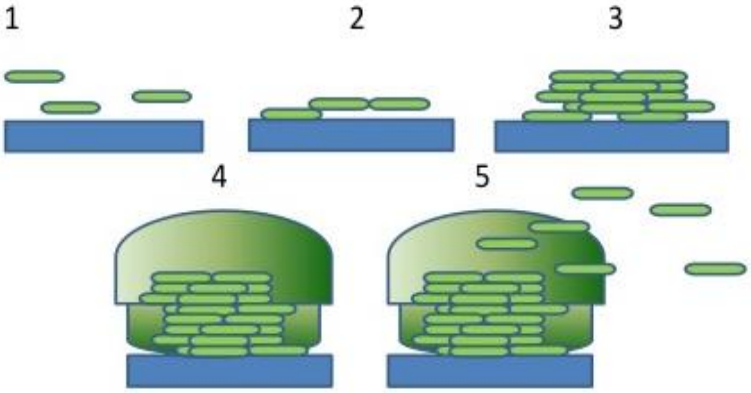


Figure 1.4 Biofilm lifecycle shows bacteria initially attaching to a surface (1). Next there is irreversible attachement (2) followed by the formation of microcolonies (3) which become engulfed in a mushroom shaped EPS structure (4). Finally bacteria are able to disperse from this and recolonize a new surface (5), and the cycle begins again.

1.3.3 Pseudomonas aeruginosa Lipopolysaccharide Structure

P. aeruginosa lipopolysaccharide has an essential role in the structure and function of the outer membrane of *P. aeruginosa* and is crucial in mediating bacterial virulence and host response. Lipopolysaccharide is an integral component of the *P. aeruginosa* cell envelope consisting of three structural domains. Lipid A, core oligosaccharides consisting of an inner and outer core and the distal O- antigen (Fig 1.5).

Lipid A attaches the LPS chain to the outer membrane of *P. aeruginosa.* Lipid A modifications demonstrated in CF *P. aeruginosa* strains have been shown to be unique to CF and result in resistance to antimicrobial peptides suggesting a potential role for lipid A modifications in colonisation in CF (Ernst *et al*., 2003; Ernst *et al*., 2007). The core oligosaccharide is a short chain of sugar residues which is relatively well preserved between strains with little variation in its structure (Lam *et al*., 2011).

O-antigen is the component of LPS which varies in length due to the number of repeating units of sugar in its composition. Most strains of *P. aeruginosa* express two distinct forms of O-antigen which can exist simultaneously. One termed the common polysaccharide elicits a weak antibody response whilst the O specific polysaccharide varies in chain length between strains and it is this which is highly immunogenic. This variation in chain length confers serogroup specificity and is the basis of serotyping (Lam *et al*., 2011; Gellatly and Hancock, 2013). The gene sequences for the proteins involved in O-antigen synthesis and assembly have been identified and the major enzymes responsible are encoded in a cluster of genes that occupy a common genetic locus (Raymond *et al.*, 2002). A *wzz* gene has been identified within every locus and this Wzz protein is responsible for regulating the long O-antigen chain lengths (12 to 16 and 22 to 30 subunits) but not very long chain lengths (40-50 subunits) in

the wild type strain PAO1 (Burrows, Chow and Lam, 1997). Two *wzz* genes have been identified (*wzz1* and *wzz2*). The Wzz protein has been shown to be important for the expression of long chain O-antigen length whilst the Wzz2 protein is important in production of very long chain O-antigen (Kintz et al, 2008).

The terminology of rough or smooth strains is used to describe LPS with or without O-antigen. Rough refers to those without, and smooth to those expressing O-antigen (King *et al*., 2009). The common polysaccharide antigen appears to become the major LPS antigen over time in cystic fibrosis with rough strain being the dominant one found (Hancock *et al*., 1983; Penketh *et al*., 1983). It has been hypothesised that this may be due to selective pressures for loss of the high molecular mass O-antigen, or indeed it may be the unique CF lung environment that contributes to this finding. Longitudinal data is not available to suggest whether or not this occurs in non CF bronchiectasis. Multiple studies do seem to suggest that the expression of O-antigen or smooth form, is required for *P. aeruginosa* to cause systemic infection (Pier and Thomas, 1982; Cryz *et al*., 1984; Goldberg *et al*., 1995; Ohno *et al*., 1995). Animal studies have supported this with failure of rough LPS to spread in mice following introduction into the respiratory tract. However at a significantly high dose it could cause pneumonia and death without significant evidence of systemic spread. This may be indicative of the ability of rough strains at a sufficiently high dose being able to overwhelm the local immune response (Priebe *et al*., 2004). This would indicate that although O-antigen is a requirement for systemic spread of *P. aeruginosa* it is not required to cause infection localised to the lung and that with a high bacterial load can overwhelm the innate immune system. As O-antigen expression appears pivotal in the ability of *P. aeruginosa* to spread systemically, the ability of serum and the adaptive immune system to deal with this then becomes crucial.

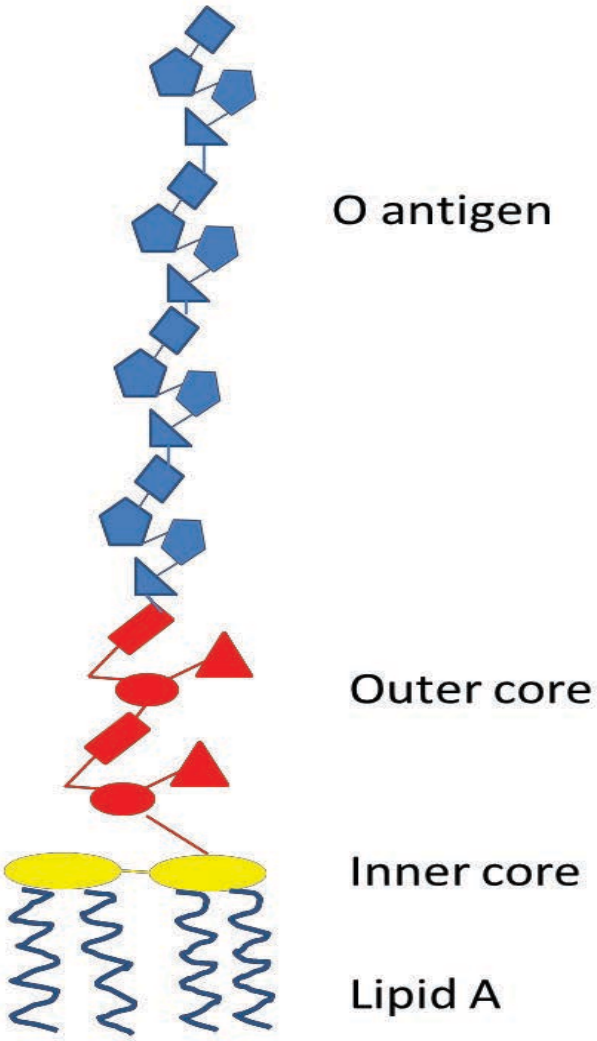


Figure 1.5 *P. aeruginosa* LPS structure. The three components of *P. aeruginosa* LPS with lipid A which attaches to the outer membrane of *P. aeruginosa*, the core oligosaccharides forming the inner and outer core and O- antigen which forms the distal component of LPS and varies in length. This is the highly immunogenic component of LPS.

1.3.4 Serum killing of *P. aeruginosa*

P. aeruginosa strains isolated from patients with cystic fibrosis are generally sensitive in normal healthy serum whilst the presence of O-antigen is a feature of strains which are resistant to killing by normal healthy serum (Hancock *et al*., 1983; Knirel *et al* 2006). Lab studies of mutant strains of *P. aeruginosa* have also shown serum sensitivity in those strains lacking O-antigen as well as those expressing only the common polysaccharide, suggesting that this confers no protection against serum mediated killing (Dasgupta *et al*., 1994). . Serum resistance of clinial and lab strains is not due to an inability to activate complement (Borowski and Schiller, 1983; Pier and Ames, 1984; Schiller and Joiner, 1986; Schiller, 1988). However there have been no studies specifically examining the serum sensitivity of strains isolated from patients with non CF bronchiectasis.

Both rough and smooth strains are able to activate complement although interestingly it has been shown that although the serum resistant strain consumed more C3, C5 and C9, there was significantly less C3 and C9 bound to the outer membrane of the serum resistant strain in comparison to a serum sensitive strain (Schiller and Joiner,1986). This led to the hypothesis that perhaps the presence of long chain O-antigen was protective in inhibiting stable insertion of the membrane attack complex at the cell membrane by forming a barrier. The same study also noted that a much greater proportion of activated C3 was able to bind to the cell surface in serum sensitive strains than in those that were resistant to killing again raising the possibility that long chain O-antigen formed a physical barrier to binding or there was an poor availability of binding sites for C3 (Schiller and Joiner 1986). A similar mechanism of serum resistance has been shown with strains of other gram negative organisms including *Salmonella minnesota* and *Eschericia coli* suggesting that there is possibly a common mechanism of serum resistance in gram negative organisms, with O-antigen protecting from

insertion for the terminal products of the complement cascade to the cell membrane (Joiner *et al*., 1982; Goldman, Joiner and Lieve, 1984; Joiner *et al*., 1984; Taylor and Kroll, 1984). Although studies have generally indicated O-antigen expression is required for serum resistance its expression does not necessarily mean that the strain will be resistant to serum. For example Dasgupta and colleagues observed that one of two lab strains expressing O-antigen was sensitive to serum killing (Dasgupta *et al*., 1994). An ultrastructural analysis of these strains by the same group noted that the distribution of long O-antigen side chains may influence serum sensitivity as the strain noted to be sensitive to killing by healthy control serum had a patchy distribution of LPS over its surface whilst the resistant strain had an extensive coat on the outer leaflet of the outer membrane suggesting that it may not be the presence of long chain O-antigen alone which confers resistance, but the pattern of distribution (Lam *et al*., 1992).

As a consequence of its immunogenic properties *P. aeruginosa* LPS has provided a potential target for vaccination therapy. The surface accessibility makes it an ideal vaccine target although to date no effective vaccine has been developed despite three vaccine trials targeting LPS in CF (see below).

1.3.5 LPS Based Vaccination for *P. aeruginosa*.

Pseudogen® was a heptavalent lipopolysaccharide *Pseudomonas* vaccine evaluated in twelve patients with cystic fibrosis already infected with *P. aeruginosa* and twenty two patients with acute leukaemia. This was associated with significant side effects including fever, pain, and headache – without any evidence of tolerance with progressive injections. There was no evidence of clearance of *P. aeruginosa* from the airway secretions, or improvements in lung

function. The toxic effects of this vaccine were attributed to lipid A which is recognised for its ability as an endotoxin and ability to stimulate the production of pro inflammatory cytokines. It was also considered that it may have increased immune complex formation in infected CF patients causing an inflammatory reaction via recruited neutrophils (Pennington *et al*., 1975; Doring and Pier, 2008).

PEV-1 was an improved LPS based polyvalent vaccine (16 serotypes) to investigate the prophylactic benefit prior to the onset of *P. aeruginosa* infection in CF. It was tested in 17 patients with CF who were investigated prospectively to determine whether this would prevent initial colonisation with *P. aeruginosa*. The acquisition of *P. aeruginosa* was unchanged compared to a matched control group at 3 year follow up. The vaccination group also demonstrated a larger fall in peak flow measurement of lung function and decreased clinical status based on activity, physical examination, nutrition and x-ray findings. This trial was therefore discontinued (Langford and Hiller, 1984).

Auregen® is a octavalent O- polysaccharide conjugate vaccine (conjugated with exotoxin A). Initially efficacy of this vaccine in patients not colonised with *P. aeruginosa* were promising compared to a retrospective control group with 35% of the vaccine group acquiring *P. aeruginosa* compared with 75% of subjects expected to become colonised 6 years after immunisation. High levels of IgG antibodies were induced against all nine antigens which began to decrease rapidly after immunisation but were restored by annual boosters. This regimen ensured a significant mean polysaccharide specific serum IgG level was maintained over a ten year period in the vaccinated uninfected group. A decade after immunisation there were significant reductions in *P. aeruginosa* infection and improved health status in the vaccination group (Zuercher *et al*., 2006).

The success of this unblinded study led to a double blind, randomised placebo controlled large group phase III study which failed to find a benefit and further development was suspended. The full results have not been published and thus the reasons for failure remain unknown (Sharma, Krause and Worgall, 2011).

LPS and O-antigen would seem to be optimal targets for a vaccine in *P. aeruginosa* yet to date human trials have been unsuccessful and in some cases appear to be detrimental. Vaccines to target *P. aeruginosa* antigens of flagellae and outer membrane proteins have shown some promise but are still in early stages of development (Sharma, Krause and Worgall, 2011) and are not discussed here.

1.4 *Pseudomonas aeruginosa* Colonisation in Non Cystic Fibrosis Bronchiectasis.

Bronchiectasis is characterised by the clinical symptoms indicative of an ongoing inflammatory response in the host lungs. The vicious cycle hypothesis of bronchiectasis describes the ongoing interaction of ineffective host defences, neutrophilic inflammation and persistent bacterial colonisation. *P. aeruginosa* is a colonising organism of particular interest in this condition in part due to its association with worsening clinical parameters, but also because its inherent behaviours, adaptability and ability to protect itself from, and interact, with host defences. These data suggest a greater understanding of the interaction with host defences may provide pivotal data to develop targeted therapies for this pathogen which is largely resistant to common antibiotics. Its complexity also suggests that there is an opportunity to explore other features of *P. aeruginosa* that may enhance its ability to colonise the host and perpetuate inflammation.

1.5 Hypothesis

The publication of evidence of dysregulated adaptive immunity in the serum of HIV infected individuals with *Salmonella* Typhimurium (*S.* Typhimurium) suggested a specific interaction of the host immune system with pathogens. Here, there was inhibition of serum killing due to production of excess inhibitory IgG antibodies directed against *S.* Typhimurium LPS. When IgG was removed and added to HIV uninfected serum, normally able to kill *S.* Typhimurium, inhibition of bacterial killing was confirming anti LPS IgG as an inhibitor of *S.* Typhimurium serum killing (MacLennan *et al*.,2010).

A review of the literature, revealed that the concept of inhibitory or blocking antibodies in association with Gram-negative infections and specifically *P. aeruginosa* had been raised in the past without identification of the specificity of the bacterial / host interaction/s. Initially an inhibitory factor in the serum of patients with chronic Gram-negative infections, preventing normal serum killing of these organisms was noted without identification of its nature (Waisbren and Brown, 1966). Blocking IgG was then identified in the serum of a patient with chronic *P. aeruginosa* infection (Guttman and Waisbren, 1975), with inhibition of serum killing by autologous serum also noted in patients with *P. aeruginosa* and CF, compared to healthy serum (HØlby and Olling, 1977).

Hornick and Fick described IgG2 containing immune complexes in the serum of CF patients chronically colonised by *P. aeruginosa* which inhibited phagocytosis and serum killing of *P. aeruginosa* with an associated elevated level of IgG2 in the serum (Hornick and Fick 1990). Finally, an association between CF patients and poorer clinical status was reported with inhibitory sera to their own strains which were sensitive to killing in normal healthy serum,

and therefore indicative of an inhibitory factor in patient serum, (Thomassen and Demko, 1981).

These observations led to the hypothesis that a similar phenomenon may be present in the serum of patients with bronchiectasis and chronic *P. aeruginosa* colonization, providing an explanation to the failure of host defenses to sterilize the airway

1.6 Aims of my MD thesis

The aims of my thesis were to collect serum and sputum samples in a well characterised group of patients with bronchiectasis.

Where *P. aeruginosa* was identified as a colonising organism, experiments would be focussed on the presence of serum sensitivity of these strains using both patient and healthy control serum.

If, in keeping, with previous studies of *P. aeruginosa* in CF, a discrepancy was found between strains sensitive in healthy serum but resistant to killing in autologous serum, further experiments would be undertaken to identify the mechanism.

CHAPTER 2 THESIS METHODS

2.1 Patient Selection and Diagnosis

Patients were recruited from the National Health Service bronchiectasis clinic at the Queen Elizabeth Hospital, Birmingham. Patients were referred both from primary care physicians and from secondary health care providers, mainly respiratory physicians, for specialist management of bronchiectasis. Patients with cystic fibrosis were also recruited for research purposes from the specialist cystic fibrosis clinic at New Cross Hospital, Wolverhampton.
Approval for this study was given by South Birmingham Research Ethics Committee and by University Hospital Birmingham NHS Foundation Trust Research and Development Committee (07/H1207/231). Informed and written consent was obtained from each subject (Study Code:RRK3404). Unselected patients routinely attending this clinic were invited to take part in this study to identify particular groups of patients with non-CF bronchiectasis by proving sputum and blood samples for research purposes at their out patient appointments with no need for extra outpatient visits.

Each patient recruited had a diagnosis of bronchiectasis confirmed by High Resolution Computed Tomography scan (HRCT). A full medical history was obtained from each patient, usually at the time of their initial referral and clinical assessment, to establish, where possible, any underlying cause of their bronchiectasis. This was undertaken as part of their routine NHS care.

Each patient had pulmonary function testing as part of their usual care. Pulmonary function tests were performed by trained respiratory physiologists in the department of lung function

and sleep at the Queen Elizabeth Hospital. All tests were performed according to national guidelines. (British Thoracic Society and Association of Respiratory Technicians and Physiologists). Pre-bronchodilator values of Forced Expiratory Volume exhaled at the end of the first second of forced expiration ($FEV_{1)}$ and Forced Vital Capacity (FVC) were recorded for each patient.

Medical Research Council (MRC) dyspnoea scores were recorded for each patient. This scale is a measurement of the disability experienced as a result of dyspnoea. It is shown in table 2.1. Information regarding MRC dyspnoea scores, was collected using information taken as part of the patients assessment at their routine clinic attendances.

The St Georges Respiratory Questionnaire (SGRQ) was used to assess health related quality of life. This was collected on one occasion at the time of recruitment to the study. The SGRQ (Jones, Quirk and Baveystock, 1991) is a self-administrated, health related quality of life questionnaire designed to measure impact on overall health, daily life and perceived well-being in patients with obstructive airways disease. It consists of 50 items. Each questionnaire response has a unique empirically derived weight. Three component scores are calculated (symptoms, activity and impacts) in addition to a total score which summarises the impact of the disease on overall health status. Scores range from 0 – 100 with higher scores indicating more impairment. Each component score is calculated by dividing the summed weight by the adjusted maximum possible weight for the component and expressed as a percentage. The total score is calculated in the same manner. Although initially designed as a tool for assessing the impact of COPD on quality of life it has also been validated for use in non cystic fibrosis bronchiectasis (Wilson *et al*., 1997).

Grade	Degree of Breathlessness
1	Not troubled by breathlessness except on strenuous exercise.
2	Short of breath when hurrying on a level or when walking up a slight hill.
3	Walks slower than most people on the level, stops after a mile or so, or has to stop for breath walking at own pace.
4	Stops for breath after walking 100yds, or after a few minutes on level ground.
5	Too breathless to leave the house, or breathless when dressing/undressing.

Table 2.1 MRC dyspnoa scores. (Bestall *et al*., 1999)

Patients were also routinely asked about the number of exacerbations they had experienced between each clinic appointments. Most patients attended clinic regularly every 4-6 months and therefore the period of recollection for exacerbation history was short and many recorded this data in a daily symptom diary. Information regarding all hospital attendances related to bronchiectasis was available on electronic hospital records. Where patients had been admitted to hospital other than the Queen Elizabeth University Hospital Birmingham, the information was recorded at their next clinic attendance. Patients recruited to the study had a number of different organisms isolated from sputum. The focus of the work on this thesis is with regards to *P. aeruginosa* and thus much of the discussion focuses on those identified as being colonised with *P. aeruginosa.*

2.1.1 Blood Sample Collection

Each patient had blood samples collected for serum (collected in Z serum clot activator tube), plasma (collected in EDTA tube) and DNA (collected in EDTA tube and stored as whole sample). Venous blood was taken peripherally using the vacutainer® system (Becton Dickson Ltd Oxford UK). The serum and plasma samples were harvested by centrifuging the blood samples at 1500g for 10 minutes at room temperature. Blood was processed within 2 hours of collection and serum and plasma were stored in aliquots at -80ºC until required. Blood was processed in the same manner from healthy control subjects.

2.1.2 Sputum Sample Collection and Processing

Sputum was usually processed by grading and recording purulence using the Bronkotest colour chart, and storage of sol phase and quantitative bacterial culture established within 2

hours of collection at the time of patient's clinic attendance. Where this was not possible arrangements were made for the patient to attend the research unit at a convenient time, to bring a sample of sputum produced on that day. Matched serum/plasma samples were taken at the same time. Repeated samples were taken when the opportunity arose as the patient attended for a usual NHS follow up.

Sputum colour was assessed using a 5 point grading system. This ranges from 1-2 (clear to white) which is classified as mucoid. 3-5 (yellow to green), which is classified as mucopurulent to purulent. This is utilised clinically where patients are encouraged to monitor the colour of their sputum with increasing purulence from their baseline being indicative of an exacerbation. This visual grading of sputum correlates to the degree of activity of the underlying markers of airway inflammation.

Sputum sol phase was obtained by ultracentrifugation of a minimum of 1g of sputum (29,000xg for 90 mins at 4°). The supernatant was removed and stored at -80°C until use. Quantitative bacterial culture to determine the number of viable organisms was performed as follows. A minimum of 1 gram of sputum was removed from the fresh sample and added to an equal volume of dithiothreitol (Sputasol, Oxoid, UK). 1000µL of the homogenised sputum sample was then serially diluted with distilled water creating dilutions from 10^{-1} to $10^{-5.}$ One chocolate agar + PolyVite X (chocolate) plate was inoculated with 5µl of the initial homogenised sputum sample to create a primary inoculum plate using the 4 streak method to ensure viable numbers of bacteria. Then 10µl of dilutions 10^{-3}, 10^{-4}, 10^{-5} were taken and used to inoculate a Chocolate agar and PolyViteX plate and a Columbia Agar +5% Horse blood (blood) plate (BioMerieux) – in the case of 10^{-4} dilution only and spread across the whole of the surface of the plate using a hockey stick. These plates were incubated for 24-48 hours

(37°C, 5% CO_2) and the number of colonies counted from the appropriate plate. The number of colony forming units per ml (cfu/ml) was then calculated based on the dilution.

2.2 Identification of Bacterial Species

Bacterial species were preliminarily identified using the morphological appearance of bacterial growth on the culture plates according to local laboratory guidelines shown in tables 2.2 and 2.3. Further tests including Gram staining, and functional testing were performed to confirm bacterial species. Specific functional tests utilised for species identification are discussed.

2.2.1 Oxidase Reaction

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase (indophenol oxidase). Oxidase positive bacteria contain the enzymes cytochrome oxidase and therefore the oxidase test can be used to differentiate between organisms which possess this enzyme and those which do not. Cytochrome oxidase catalyses the transport of electrons from donor compounds to electron acceptors. The test reagent *N,N,N',N'- tetra – methyl – p – phenylenediamine dihydrochloride* (1% solution) acts as an electron acceptor for the enzyme oxidase. The reagent turns blue, forming indophenol blue, in response to being oxidised (Gov.uk, 2017). Reagent was added to colonies either by touching colonies on a growth plate with filter paper or swab soaked in reagent or scraping colonies from the growth plate on to an impregnated oxidase test strip.

Characteristics	***Haemophilus influenzae***	***Haemophilus parainfluenzae***	***Moraxella catarrhalis***	***Pseudomonas aeruginosa***	***Enterobacteriaceae***	***Pasteurella spp***	***Burkholderia cepacia***	***Xanthomonas maltophilia***
Growth on agar	Chocolate	Chocolate Blood	Chocolate Blood	Chocolate Blood MacConkey	Chocolate Blood MacConkey	Chocolate Blood	Chocolate Blood MacConkey	Chocolate Blood MacConkey
Colony morphology	Small, greyish translucent	Small, greyish whitish, yellowish.	Opaque white-cream, dry texture, 'hockey-puck' movement	Rough to smooth appearance. Mucoid- non mucoid. colourless, haemolysis of blood agar	Variable	Small, greyish metallic/white	Variable	Flat, opaque lavender/green colouration on blood
Colony odour	Moth-balls			Sweet, musty			'Cigarettes'	Ammonia
Cell morphology	Small, coccobacilli	Small coccobacilli	Large diplococci	Bacilli +/- capsule	Bacilli	Bacilli	Bacilli	Bacilli
Oxidase reaction			Positive	Positive	Negative	Positive	Positive	Negative
Glucose fermentation			Negative	Negative	Positive	Negative	Positive	Positive
DNase activity			Positive					
Catalase reaction				Positive	Positive		Positive	Positive
Nitrate reduction			Positive					
Pigments				Pyocyanin, pyomelanine				
Physiological testing	X and V factors api NH	V factor api NH	Tributyrin	api 20NE	api 20E		Cepacia selective media api 20NE	api 20NE

Table 2.2 Guidelines for identification of important respiratory pathogens. Gram Negative Bacteria,

Characteristics	***Streptococcus pneumoniae***	**B-haemolytic streptococci**	***Staphylococcus aureus***
Growth on agar	Blood Columbia CNA	Blood Columbia CNA	Blood Columbia CNA
Colony morphology	Dark green, draughtsman or mucoid colonies	Small and dry, relative large haemolytic zone	Large and often yellowish, relative small haemolytic zone
Cell morphology	Lancet formed diplococci	Cocci in chains	Cocci in clusters
Optochin	Sensitive		
Bile solubility	Positive		
DNase activity			Positive
Catalase reaction		Negative	
Physiological testing			

Table 2.3 Guidelines for identification of important respiratory pathogens. Gram Positive Bacteria.

2.2.2 Catalase Test

The catalase test detects this enzyme in bacteria and thereby differentiates between those which are catalase positive and those which are negative. The catalase enzyme serves to neutralise the bactericidal effects of hydrogen peroxide by facilitating the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen. The reaction is identified by the formation of bubbles (UK Standard for Microbiological Investigations. Gov.uk, 2017).

Bacterial colonies were added to 3% H_2O_2 (Sigma-Aldrich) either in a test tube or on a microscope slide and observed for the rapid bubble formation.

2.2.3 Optochin Susceptibility Test

The optochin susceptibility test is used to differentiate *S. pneumoniae* from other haemolytic *Streptococci.* Colonies were streaked onto blood agar medium and a filter paper disc impregnated with ethylhydrocupreine hydrochloride - optochin (Oxoid UK) was placed in this area and the plate incubated for 24 hours. As *pneumococci* are lysed by optochin an inhibition zone is created around the disc if the colonies are *pneumococci.* A zone of inhibition >5mm is indicative of *S.pneumoniae* . Less than 5mm requires further investigation with Slidex pneumo kit (BioMerieux). Here, several colonies of suspected α- haemolytic species are emulsified in sterile saline before 2 drops are placed on a slide. A drop of reagent R1 (latex *S. pneumonia*) and R2 (latex negative control) are added to each smear and mixed. A positive reaction shows distinct agglutination within 2 minutes whilst a negative reaction remains as a homogenous suspension or with only slight agglutination which does not intensify. (UK Standard for Microbiological Investigations. Gov.uk, 2017).

2.2.4 Tributyrin Hydrolysis Test

The tributyrin hydrolysis test is a chromographic test used to differentiate between *Moraxella catarrhalis* and *Neisseria sp* and relies on the production of the enzyme butyrate esterase by *M. catarrhalis.* (Perez *et al*., 1990) 2-3 similar colonies were emulsified in sterile saline before the addition of a tributyrin tablet (Rosco). This suspension was incubated at room temperature for 2- 4 hours. A positive test was indicated by a yellow/yellow orange colour change indicating the presence of butyrate esterase. Negative tests were red.

2.2.5 Physiological Tests of *Haemophilus influenzae* and *Haemophilus species*

It may not be possible to differentiate between *H. influenzae* and other *Haemophilus sp.* such as *H. parainfluenzae, H. haemolyticus. H. parahaemolyticus* based on morphological appearances on growth media alone. Instead, the fastidious growth requirements of these strains was used to differentiate between the species.

H. influenzae requires supplementation with both hemin (X factor) and Nicotinamide Adenine Dinucleotide - NAD (V factor) for growth, while *Haemophilus parainfluenzae* requires only NAD (V) supplementation. Both factors are normally present in blood. Therefore testing was performed by placing discs containing either factor V or both factor V and X (Thermo Scientific) onto nutrient agar (BioMerieux 33606) with the colony to be tested. As both factors are present in blood it was crucial that blood containing agar is not transferred from the original plate when the colony to be tested was picked.

Inoculated plates were incubated at 37ºC for 18-48 hours in 5% CO_2 and observed for growth around each disc margin. If X and V factors were both required, as in the case of *H. influenzae,* growth was only observed around that disc margin.

Where *H. influenzae* was identified it was stored in 800µl supplemented Brain Heart infusion broth +200 µl glycerol at -80°C until required.

2.2.6 *Pseudomonas aeruginosa* Isolation

The presence of *P. aeruginosa* was further confirmed following functional testing by inoculating *Pseudomonas* Selective Agar (BioMerieux) with a colony of *Pseudomonas* taken from the original chocolate agar plate. Where confirmed, each species identified was then stored in glycerol stock (800µl culture in Luria Bertani Broth (LBB) + 200µl glycerol at -80ºC until required.

2.2.7 Analytical Profile Index (API) Identification of Pseudomonas Species

The Analytical Profile Index (API) is a miniaturized panel of biochemical tests compiled for identification of groups of closely related bacteria. (BioMerieux)

The API-20NE test kit was used to identify the precise strain of *Pseudomonas* where it was isolated from sputum using the methods described previously. This standardised system uses an API 20 NE strip with 20 microtubules containing dehydrated substrates which, when inoculated with a distilled water bacterial suspension (3-4 colonies emulsified in 4mls of distilled water), reconstitutes the media during 24-48 hours incubation (according to the manufacturers instructions) if the bacteria are capable of growth by utilising the respective

substrate. These changes are evidenced by colour changes either spontaneously or by the addition of reagents.

The reactions were converted to a seven digit code. This numerical profile was then entered into the manufacturer's database (https://apiweb.biomerieux.com) or correlated with codes in the database book, to identify the precise genus and species of *Pseudomonas*.

2.3 Bacterial Cultures in Liquid Media

P. aeruginosa was grown in LB (Lysogeny) broth (LBB) by placing a colony in 5-10mls of media and incubating overnight with shaking at 37ºC.

H. influenzae was grown in supplemented brain heart infusion broth (BHI) by placing a colony in 5mls BHI + 5µl β-nicotinamide adenine dinucleotide (β NAD) (Sigma) + 100µl haemin (Sigma). This was incubated at 37°C for 4 hours.

2.3.1. Growth Curves (*Pseudomonas*)

Growth curves for individual strains of *P. aeruginosa* were performed at 37ºC. Growth curves were performed by placing a colony of each strain into LBB and incubating at 37ºC. After 2 hours of incubation the optical density at an OD:600 of each strain was measured at regular 30 minute intervals until stationary phase was reached. Figure 2.1 shows the normal phases of growth of bacteria. This is not specific for *P. aeruginosa* and is a visual representation of the growth phases of bacteria generally.

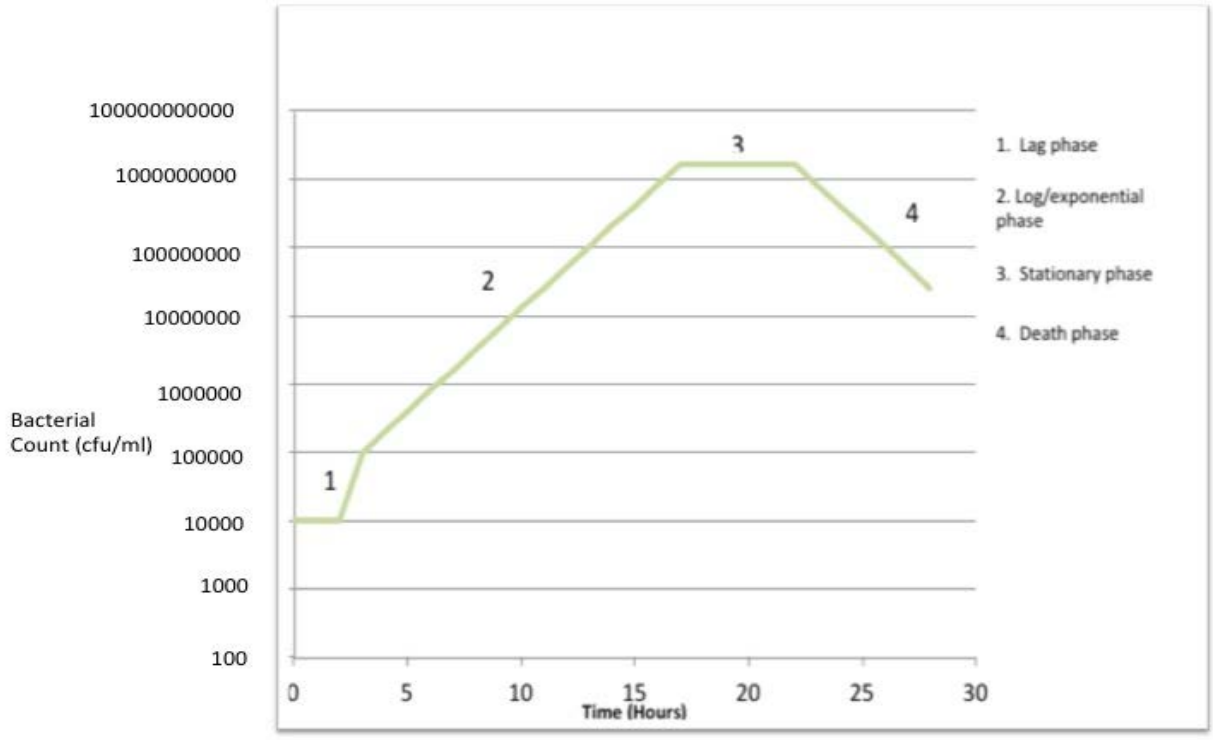


Figure 2.1 shows an example of the phases of a bacterial growth curve. During lag phase bacterial cells are maturing and are not yet able to divide. During Log phase the number of cells doubles at a constant rate. During stationary phase the population levels off as cell death equals cell doubling and death phase sees the death of bacterial populations due to lack of nutrients, or an adverse environment or temperature for continued growth.

2.4 Bactericidal Assays

2.4.1 *Pseudomonas aeruginosa* Bactericidal Assays

This protocol was adapted from that used by MacLennan *et al* (MacLennan *et al*., 2010), Briefly, stationary phase bacterial cultures were prepared by adding a colony of *P. aeruginosa* to 5 mls of LBB. These were vortexed and incubated overnight at 37ºC.

The following day optical density (OD) at 600nm (OD_{600}) was measured for each strain and the volume of culture media to be used standardised to 1.5mls OD_{600}= 0.6. This was optimised to ensure a standard number (10^8) bacteria were present in the inoculum cultures. *Pseudomonas* isolates were washed by spinning at 10,000 rcf for 5 minutes. The supernatant was removed and bacterial pellets resuspended in 1ml of PBS. This was repeated and bacterial pellets resuspended in 900 µl PBS. One hundred µl of this stock inoculum was then added to 900 µl PBS making a 10^{-1} dilution. Ten µl of dilution 1 was added to 90 µl serum , and incubated at 37ºC on a rocker set at 180 rpm for 180 minutes with samples taken at T1 (45 minutes), T2 (90 minutes) and T3 (180 minutes).

To determine inoculum concentration 10µl of the stock inoculum was serially diluted using a multichannel pipette along 8 wells of a 96-wellplate filled with 90 µl PBS in each well. A Miles and Misra plate (Fig 2.2) was prepared for the inoculum and incubated overnight at 37ºC. At each time point (45, 90 and 180 minutes) 10 µl of serum was taken from each sample and added to 90 µl PBS in a 96-wellplate and serially diluted along 4 wells of the plate. For each sample in a row of wells, a Miles and Misra plate was prepared which was also incubated overnight as above. Three x 10 µl spots of each dilution were pipetted on to the corresponding wedge on the Miles and Misra plate. The following day the colonies that grew in a wedge (where the average number was equal to ten or greater) were counted and the colony counts plotted on excel to observe the serum killing trend.

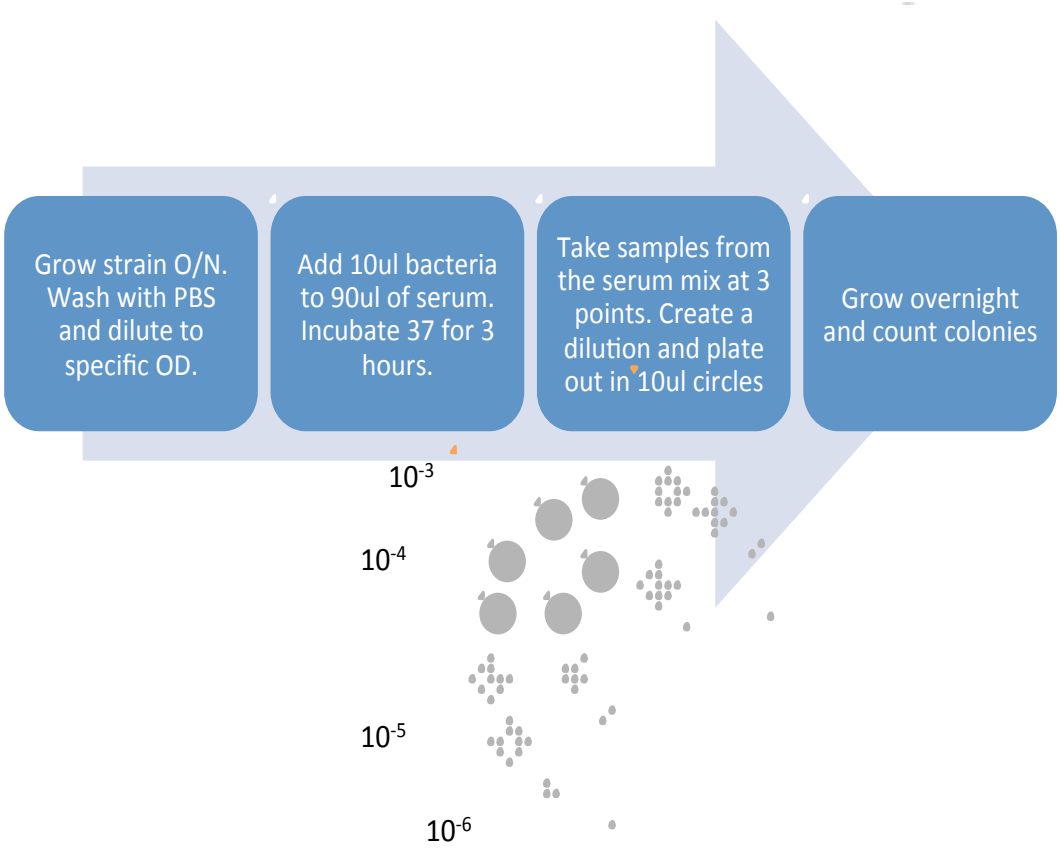


Figure 2.2 depicts the method used for performing bactericidal assays of *P. aeruginosa.* Following incubation of stationary phase cultures with both patient and healthy control sera, a Miles and Misra plate was prepared at each time point (45, 90 and 180 minutes). The following day colonies were counted following overnight incubation.

2.5 Preparation of Serum

2.5.1 Size Fractionation

Fractionation of serum was performed using size exclusion columns (Vivaspin – GE Healthcare) with either 30, 100 or 300 kDa size-exclusion membranes enabling broad separation based on molecular weight. Initially 1ml of serum (from either healthy control samples or patient serum) was run through the 300kDa exclusion column via centrifugation which retained ~200ul of the serum fraction with weight over 300kDa. The flow through fraction containing proteins with molecular weight <300kDa was then standardised back to 1ml with PBS and the procedure repeated consecutively with 100kDa and 30kDa size exclusion columns. All fractions (>300kDa, 100-300kDa, 30-100kDa and <30kDa) were standardised to 1ml by the addition of PBS. Bactericidal assays were then performed with these preparations of differing molecular weight.

2.5.2 IgG Isolation and Extraction

IgG was removed from serum using Protein A-sepharose 4B (Sigma P9424) and Protein G-sepharose (GE Healthcare). Protein A and protein G are both isolated from the bacterial cell wall of *Staphylococcus aureus* and group G *Streptococci* (Bjorck *et al*., 1984). Both have a high affinity for the Fc regions of IgG molecules but have different affinity for the subtypes. Immobilisation of protein A creates an affinity resin that can be used to isolate IgG fractions from serum whilst Protein G-sepharose, though similar, does not bind IgG3. The respective binding capacity of protein A and protein G to all immunoglobulin classes in human serum are shown in table 2.4.

Immunoglobulin	Binding Protein A	Binding Protein G
IgG1	High	High
IgG2	High	High
IgG3	No affinity	High
IgG4	High	High
IgA	Moderate/slight affinity	No affinity----
IgM	Moderate/slight affinity	No affinity.

Table 2.4 Binding affinities to Human IgG subclasses of Protein A and Protein G. (Abcam.com, 2017)

The human serum used in the following experiments had total immunoglobulin levels and subclass levels measured by the routine immunology laboratory at the Queen Elizabeth hospital. (IgG 6.00-16.00g/L, IgA 0.80-4.00g/L, IgM 0.50-2.00g/L).

2.5.3 IgG isolation Using Protein A-sepharose 4B

The binding capacity of protein A- sepharose was approximately 35mg/ml human IgG according to manufacturers guidelines. Antibody was isolated following manufacturers (Sigma) guidelines. Briefly, 1.2 ml of resin was added to 1 ml of buffer A (0.02M NaH_2PO_4 + 0.15M NaCl). This was poured into the column and washed with 20 mls of Buffer A. 1 ml of serum was then added with aliquots of flow through collected in 250 µl aliquots. The resin was further washed with 10 ml of buffer A. Elution was then carried out by adding 3 ml of buffer B (0.2 M Na_2HPO_4 + 0.1M $C_6H_8O_7$) pH 3. The eluted IgG was then neutralised with 1M Tris (tris(hydroxymethyl)aminomethane $HOCH_2)_3CNH_2$). Bactericidal assays were then performed using the collected aliquots of serum and eluted IgG.

2.5.4 IgG isolation Using Protein G-sepharose

The binding capacity of protein G- sepharose 4B was > 20mg/ml human IgG according to manufacturers guidelines. The resin was prepared as per manufacturers instructions. Briefly, 1 ml of serum sample was loaded on to the column lined with the resin. This was washed with 3 mls of starting buffer and the flow through recovered in 500 µl aliquots. The column was then washed with a further 8 mls of starting buffer. IgG was eluted with 4 mls of 100 mM glycine as specified and brought to a neutral pH with 1M tris. Bactericidal assays were performed with the recovered serum aliquots and the eluted IgG.

2.5.5 LPS Specific Antibody Extraction

Anti-LPS antibodies were removed from serum by first binding patient bacterial lipopolysaccharide (LPS) to polymyxin-B agarose (Sigma, P1411) overnight. Polymyxin B has an affinity for bacterial cell membranes and, when immobilised on agarose, is used to remove endotoxins from solutions. The polymyxin B agarose has a binding capacity of 500 µg/ml so should be saturated with *Pseudomonas* LPS. This resin lined the column similarly to that described above and was extensively washed with 10ml of 0.1M ammonium bicarbonate buffer (pH8.0). One ml serum was then passed over the column and aliquots collected. The column was washed with 10 column volumes of wash buffer. Bound antibody was eluted with a pH gradient using a citric acid buffer and the eluted protein was then buffer exchanged into PBS.

2.5.6 Buffer Exchange

Where necessary column fractions were buffer exchanged into PBS using 30kDa MW viva-spin columns. Samples were added to the top of the column and spun. Flow through was discarded and the retained fraction washed/ diluted in 10x the original elution volume of PBS. The sample was re-spun and the retained fraction resuspended in PBS to the original elution volume.

Protein concentration of serum/ flow through fractions was determined with the Bio-Rad Protein Assay kit following manufacturer's instructions. The varying colour change in response to differing protein concentrations when measured at 595 nm with a microplate reader, provides a relative measurement of protein concentration in comparison to a standard

curve. The standard used for determining approximate protein concentrations was Bovine Serum Albumin (BSA). (Bio-rad.com)

2.5.7 Positive Selection of IgG2 Fraction of Serum.

IgG was first purified from serum using Protein A –agarose (IgGBind^{TM} resin) as previously described. This serum fraction was buffer exchanged into PBS using ultrafiltration columns (Vivascience). Following this, the purified IgG antibody in PBS was run through an anti-human IgG2 monoclonal HP6200-sepharose column with the aim of isolating the IgG2 subgroup of IgG by binding to the specific antibody. The retained IgG2 was then eluted with 0.1M citrate to a pH of 2.8 and then 2.3. It was equilibrated in PBS before finally being neutralised with TRIS (pH 9.1) (Jefferis *et al*, 1992). Flow through was collected and both the flow through fractions and elute were retained for use in bactericidal assays.

2.5.8 Complement Inactivation

Inactivation of serum complement was achieved by heating serum to 56ºC in a waterbath. This was then immediately used in for bactericidal assays with the protocol described above.

2.6 Antibody – Binding Assay (*Pseudomonas*)

2.6.1 Preparation of Stock Inoculum

Initially stock inoculum was prepared using stationary phase cultures prepared as previously described. Log phase inoculums were then generated by transferring 100µl of overnight culture to 10ml of LB broth and incubating at 37ºC to an OD600 :0.6 on a rocker plate. One

point five mls of bacteria was then pelleted by spinning for five mins at 10,000rcf. Supernatant was aspirated and the pellet resuspended in 1ml of PBS. This was then spun in a 1.5ml microcentrifuge tube at 6000rpm for 5 minutes to pellet the bacteria and the supernatant was removed before adding 1ml of PBS and repeating x1. A final volume of 1ml PBS was added and vortexed to resuspend (stock inoculum) giving a final concentration of $>2x10^9$/ml bacteria.

This phase was later adapted to use stationary phase cultures.

Stationary phase cultures were prepared as described. The volume of culture to be processed was determined by measuring the optical density (OD600) of each strain to standardise the concentration of bacteria used with all strains as before and resuspended in 1ml PBS. The volume used was calculated as $1000/OD_{600}$ for each strain.

At this stage a Miles Misra plate was prepared as previously described using serial dilutions of 10µl stock innoculum. This was incubated overnight and the stock inoculum concentration determined by counting colonies the following day.

Paraformaldehyde (PFA 300µl 4%) was then added to the stock inoculum to fix the bacteria.

2.6.2 Determining Levels of Antibody-Binding Through Fluorescent-Activated Cell Sorting

A 1:10 dilution of thawed serum was prepared in 1xPBS and 5 µl of 4% PFA fixed stock was added to 45µl of serum in a microcentrifuge tube. Forty-five µL of PBS was used as the negative control.

These tubes with serum and PBS were then vortexed and incubated for 20 minutes before adding 1ml PBS to each tube and then centrifuging at 6000 rcf, pouring off supernatant, racking the tubes and then adding 750 µl PBS and mixing by inversion. This was repeated

once more without the 750 µl of PBS and 50µl of supernatant remained in the microcentrifuge tube.

Two µl anti-Human IgG1/IgG2/IgG3/IgG4/IgA/IgM – FITC (Fluorescein isothiocyanate) antibody was then added to each tube as appropriate and mixed by pipetting.

This preparation was incubated in the dark for 20 minutes and then 1 ml of PBS was added and the bacteria pelleted at 6000rpm for 5 minutes. Supernatant was poured off and the tubes were racked before adding 1ml of PBS and mixing by inversion. This was repeated and 200µl 4% PFA was added to each tube. These were then vortexed and transferred to FACS tubes before running samples on the flow cytometer.

2.6.3 Fluorescent-Activated Cell Sorting (FACS) Protocol

Bacterial cells labelled as above, were resuspended in 0.5 ml PBS and analysed by flow cytometry using FACSAria II (BD Biosciences) equipped with 13 mW 488 nm solid state laser. The flow rate was usually adjusted to analyse 1000-2000 events/sec and 10^4 events per sample were recorded. The ND filter used was 1.0 and typically, the voltages used were: FITC (green fluorescence) – 450, FSC (forward scatter) and SSC (side scatter) – 280-300. A discriminator of 1000 was used on FSC to reduce electronic and small particle noise. Green fluorescence from FITC-labelled secondary antibody was collected using standard FITC filter set (502LB, 530/30BP). Data acquisition and analysis were undertaken using FACSDiva software (BD Biosciences). The labelled negative control sample was used to establish the baseline FITC fluorescence level. In the test samples the fluorescing and non-fluorescing cell populations were gated, quantified (percentage of parent population) and relevant parameters (FITC mean and median) were calculated using FASCDiva software.

2.6.4 Complement Deposition Assay

Preparation of stock inoculum was as documented for measuring antibody deposition.

Two µl anti-Human C1q/C3-FITC/anti-Human C5b-9 antibody (DAKO) was added to the microcentrifuge tubes containing a mixture of stock inoculum and dilute serum as appropriate. These were processed as previously described in section 2.6.2.

C1q/C3 samples – supernatant was poured off before racking the tubes and adding 200µl PBS with 1% formaldehyde to each tube.

C5b-9 samples – Supernatant was aspirated leaving 50µl which was then resuspended by pipetting up and down.

Two µl rabbit-anti-mouse-FITC antibody (DAKO) was added to each tube and mixed by pipetting. Samples were incubated in the dark for 20 minutes and then treated as previously described for measuring antibody deposition.

Monoclonal Mouse Anti-Human C5b-9 (M0777) was used to label the C9 component in the C5b-9 complex – Membrane Attack Complex (MAC), one of the terminal products of the complement cascade.

Anti- Human C3 antibody (F0201) reacts with isolated human C3c complement and the C3c part of C3 and C3b. The cleavage of C3 to C3a and C3b is a crucial step in the complement cascade where all three pathways merge.

Anti Human C1q Complement (F0254) was used to bind to C1q , the first component of the classical pathway and part of the C1 complex which links the adaptive humoral immune response to the complement system by binding to antibodies complexed with antigens. In addition C1q can also bind directly to the surface of certain pathogens and therefore initiate the complement response in he absence of antibody

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

Figure 2.3 illustrates the principle of ELISA. The principles of preparation of ELISA using LPS antigen specific to *Pseudomonas aeruginosa* strain P1A are shown below.

2.7.1 Coating of ELISA Plates with Lipopolysaccharide Antigen

LPS Antigen from B4 *Pseudomonas* strain was prepared in coating buffer (Na_2CO_3 (0.015M) + $NaHCO_3$ (0.035M) pH 9.6) to roughly a concentration of 100ug/ml and 100µl was added to each well of a 96 well microtitre plate as previously described. The plate was placed in a humid chamber and incubated overnight at 4ºC (overnight coat) or alternatively for 1 hour at 37ºC.

2.7.2 Blocking of ELISA Plates with Bovine Serum Antigen (BSA)

The overnight coat was shaken off and plates washed three times with wash buffer (0.1M PBS, pH 6.8 + 0.05%Tween 20) by immersing the plates in buffer and dry knocking on the bench onto a paper towel.

Blocking buffer (0.1M PBS,pH6.8 +1% BSA) was added (200µl/well) and plates were placed in a humid chamber and incubated for 60-90minutes at 37ºC.

2.7.3 Binding of Test Serum Antibodies to LPS

The microtitre plate was washed with wash buffer (x3) and the required volume of dilution buffer (0.1M PBS pH6.8+0.05% Tween 20+1% BSA) was added to each plate and the

required volume of test serum was added to rows 1-7 of the 96 wells. A dilution series was performed down 6 rows of the microtitre plate ensuring mixing by pipetting up and down, with 50µl discarded from the final well leaving a total volume of 100µl per well. The plate outlay is shown below (Fig 2.4). Plates were then placed in a humid chamber and incubated for one hour at 37ºC.

2.7.4 Secondary Antibody Binding to Test Serum Antibodies

Secondary antibodies were prepared in a 1:2000 dilution with dilution buffer (Southern biotech Goat Anti-human IgG (2047-04)/IgA(2050-04)/IgM(2020-04-AP as appropriate. The microtitre plate was washed with wash buffer (x3) before adding 100µl of 1:2000 secondary antibody/well to the appropriate plate and then placed in a humid chamber for incubation for 1 hour.

2.7.5 Determination of Test Serum Antibody Concentration Through Measurement of Signal

The microtitre plate was washed in three times in wash buffer before antibody concentrations were determined by measurement of signal using SIGMAFAST p-Nitrophenyl phosphate substrate (1x TRIS buffer and 1xPNPP tablet/20ml d H_2O) substrate.

One hundred µl substrate was added per well and the OD was measured at 5 minute intervals using the ELISA reader.

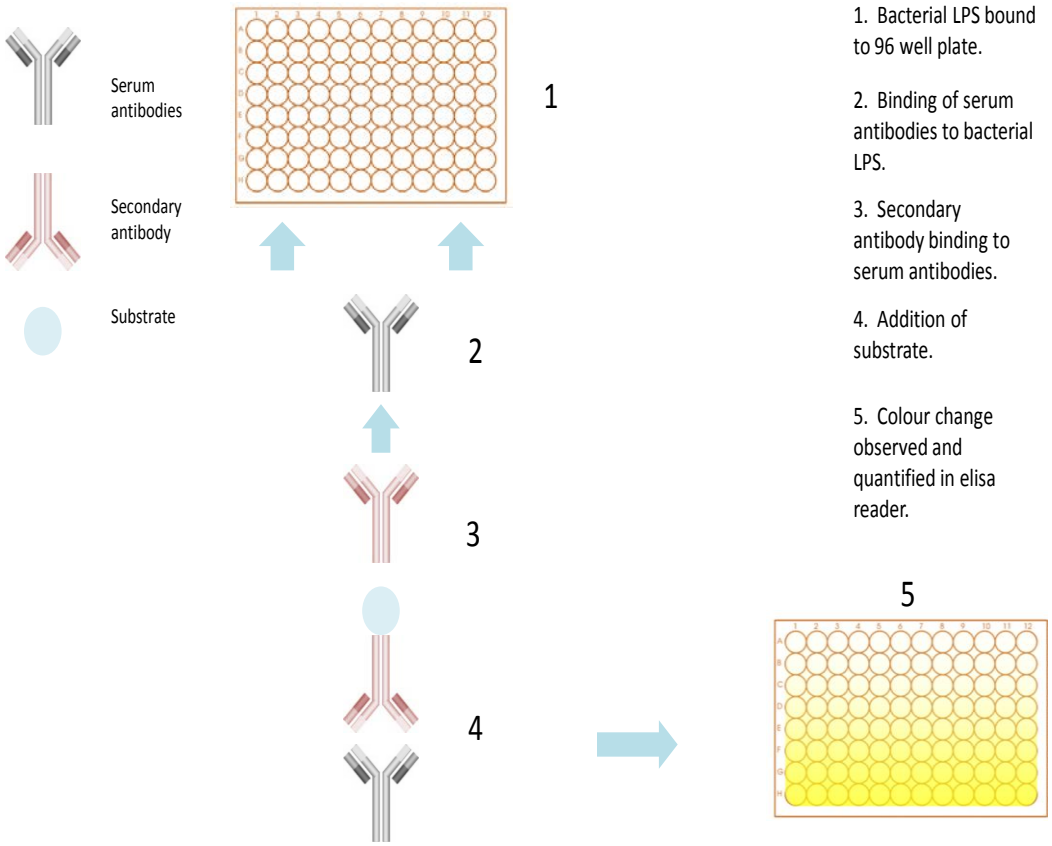


Figure 2.3 depicts ELISA using bacterial LPS bound to a 96 well plate and utilising test serum (patient or healthy control sera) as primary antibody with secondary antibodies anti human IgA, IgG or IgM bound to test serum antibodies. The level of binding of test serum antibody to LPS was determined by colour change observed in ELISA reader.

Sample	1	2	3	4	5	6	7	8
1	1:20	7.5µl 142.5µl		Serum PBS/BSA/Tween		↓ 50µl		Control 1:20
2	1:60	100µl		PBS/BSA/Tween		↓ 50µl		Control 1:60
3	1:180	100µl		PBS/BSA/Tween		↓ 50µl		Control 1:180
4	1:540	100µl		PBS/BSA/Tween		↓ 50µl		Control 1:540
5	1:1620	100µl		PBS/BSA/Tween		↓ 50µl		Control 1:1620
6	1:4860	100µl		PBS/BSA/Tween		→ 50µl		Control 1:4860
Sample	8	9	10	11	12	13	14	
7	1:20	7.5µl 142.5µl		Serum PBS/BSA/Tween		↓ 50µl		Control 1:20
8	1:60	100µl		PBS/BSA/Tween		↓ 50µl		Control 1:60
9	1:180	100µl		PBS/BSA/Tween		↓ 50µl		Control 1:180
10	1:540	100µl		PBS/BSA/Tween		↓ 50µl		Control 1:540
11	1:1620	100µl		PBS/BSA/Tween		↓ 50µl		Control 1:1620
12	1:4860	100µl		PBS/BSA/Tween		→ 50µl		Control 1:4860

Figure 2.4 shows the serial dilution of test serum binding to LPS bound to a 96 well plate.

2.8 LPS and Outer Membrane Preparations

2.8.1 Isolation of LPS from *Pseudomonas* strains.

A 5ml overnight culture of bacteria was grown in LB broth at 37ºC. The OD_{600} was measured for each strain to ensure a standard number of viable bacteria were used and the volume spun at 18000rcf for 10 minutes was determined by $1000/OD_{600.}$ The supernatant was discarded and the remaining pellet resuspended in 100µl of SDS cracking buffer. (0.1 M Tris (pH 5.8), 0.2 M β-mercaptoethanol, 20% glycerol and 2% SDS). The suspension was boiled for four minutes on a heat block before immediately freezing at -80ºC for 5 minutes and then boiling again for a further 4 minutes. These three steps were to ensure full cracking of the bacterial cells. The boiled suspension was spun in a microcentrifuge at 140000g for 2 minutes before removing 80µl of the supernatant and adding 5µl 5mg/ml Proteinase K (Qiagen). Proteinase K cleaves peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids and is used for general digestion of protein in biological samples. This ensured that LPS containing samples alone were generated. This 85µl sample was then heated at 60ºC for one hour before heating at 95ºC for a further 5 minutes. Larger amounts of LPS were prepared in a similar manner simply by proportionally scaling up volumes of bacterial culture used.

2.8.2 Outer Membrane Protein Isolation

Overnight bacterial cultures were prepared as above and cells harvested in the same way. The recovered bacterial pellet was resuspended in 20ml of 10mM Tris buffer ph7.4 and centrifuged at 10000g, 4ºC for 10 minutes before resuspending in 20ml 10mM Tris Buffer pH7.4 containing 2mM PMSF.

This was sonicated 5x30 seconds before centrifuging to remove unbroken cells (6000G, 4ºC 10mins) followed by centrifugation (30000G, 4ºC 90min) to separate the envelope and cytoplasmic fractions.

The pelleted envelopes were resuspended in 10ml 10mM Tris buffer ph 7.4-2% (v/v) TritonX-100 and incubated at 25ºC for 15 min to allow solubilisation of the inner membrane.

The TritonX-100 extracted envelopes were harvested by centrifugation (30000g, 4ºC, 90 min), before washing in 1ml 10mM Tris x 3 and finally resuspending in 1ml 10mM Tris buffer pH 7.4. This pelleted material contained predominantly outer membrane protein. This was aliquoted into 250µl lots and frozen at -80ºC before use.

2.9 Silver staining

LPS samples were prepared from individual bacterial strains as described.

Samples were resolved by SDS-PAGE and visualised using a SilverQuest Silver Staining Kit (Invitrogen) which provides a rapid and sensitive method for detection of protein and polysaccharide products.

SDS-PAGE relies on a discontinuous buffer system. Two ions differing in electrophoretic mobility form a moving boundary when voltage is applied. The intermediate mobility of proteins causes them to concentrate, into a narrow zone at the beginning of electrophoresis. As that zone moves through the gel, the sieving effect of the polyacrylamide gel matrix causes proteins of different molecular weights to move at different rates. The moving boundary concentrates the proteins into sharp bands prior to separation (www.bio-rad.com).

Ten µl of LPS prepared from individual bacterial strains (as described above) were loaded onto a precast gel. (Bis-tris precast NuPAGE gel). Once resolved the gel was fixed to eliminate interfering substances and restrict the movement of protein out of the gel matrix. It

was then sensitised with sensitising solution (Ethanol 30ml, Sensitizer 10ml, Ultrapure water 60ml) to increase the sensitivity and the contrast before being washed with ultrapure water. The silver staining agent (Stainer 1ml Ultrapure water 99ml) was then added and binds silver ions to the protein before further washing to remove excess stainer. The image was developed with a dilute formaldehyde solution (Developer 10ml, Developer enhancer 1 drop, Ultrapure water 90ml) which reduces silver ions to metallic silver on the protein bands resulting in visualisation. The reaction was stopped with stopping solution to prevent overdevelopment before washing the gel in ultrapure water. All solutions were prepared as per SilverQuest™ silver staining fast staining protocol version F (Invitrogen). (Rabilloud *et al*., 1994)

2.10 Western Blot

Silver staining gels of bacterial LPS and outer membrane proteins were prepared as previously described.

2.10.1 Western Transfer

Western transfer was performed using the iBlot® Dry Blotting Transfer System (Invitrogen). This is a self contained blotting unit used for fast, dry blotting of proteins allowing protein transfer in 7 minutes with high detection sensitivity and evenness to a PVDF membrane. The ready availability of ions in the gel matrix enable rapid protein transfers.

2.10.2 Western Blotting

Western blotting was performed using a 1:200 dilution of patient serum and a 1:5000 dilution of either anti-human IgG, IgM, IgA conjugated to alkaline phosphatase (Sigma) before detection with NBP-BCIP (nitroblue tetrazolium chloride–5-bromo-4-chloro-3′-indolylphosphate) as the substrate.

The membranes produced were incubated in Blotto (5% skim milk powder in 1xTBS buffer) for 30 minutes with shaking. Membranes were then incubated in primary antibody (serum) for one hour and then extensively washed with TBST buffer (0.1% Tween 20 in 1x TBS buffer) before incubating in secondary antibody (anti-human IgG, IgM, IgA –AP) for one hour with shaking. Membranes were developed in the dark with NBP-BCIP.

2.11 Immunofluorescence Microscopy

Overnight cultures of *Pseudomonas aeruginosa* strains were prepared as previously described. One ml of solution was spun at 5000g for 4 minutes and then 750µl of supernatant was removed before the pellet was washed with 750µl of 1x PBS and centrifuged once more at 5000g for 4 minutes. Seven hundred and fifty µl of supernatant was again removed before the addition of 750µl fixative (4% PFA). This was vortexed briefly and placed on ice for 20 minutes before washing extensively and then resuspending the pellet in 1x PBS.

Slides were washed in cleaning solution (73ml 96% EtOH + 27ml H_2O + 1ml 37% HCl), H_2O and coating solution (0.01 poly-L-lysin) consecutively for 5-10 minutes each. Slides were then dried at 60ºC for 1 hour.

2.12 Fluorescein Labelling of Cells

Twenty µl fixed cells were placed on the poly-L-lysin coated slides and warmed at 42ºC on a heating block for 10 minutes. Slides were washed (3x10minutes) with PBS + 0.5% Tween 20 in a Coplin jar and then 16µl 1:200 dilution of serum (PBS + 0.5% Tween20), primary antibody, was placed on the slide and incubated for 1 hr in a moisture chamber. The slide was then washed extensively (6X10 minutes) as before and then 16 µl secondary antibody (anti-human IgG, IgA, IgM, C1, C3 or C5b-9 conjugated to FITC) diluted 1:1000 was pipetted onto the slide and incubated for one hour in darkness in a moisture chamber. The slides were again washed extensively before air drying in darkness. Fluorescence was detected for all samples with identical gain and exposure time (0.84 sec). Visualisation was undertaken with a Leica DMRE fluorescence microscope-DC200 digital camera system and captured images were selected randomly from fields of view.

2.13 Statistical Methods

All experiments were performed a minimum of 3 times unless stated otherwise. Statistical significance between patient groups was determined by Student's t test. Error bars represent the mean +/- SD for all bacterial assays. For aanlysis of patient demographic data statistical analysis was performed using SPSS Version 18 IBM New York. Student's *t* test was used with p value <0.05 considered significant.

CHAPTER 3: ASSESSMENT OF BACTERICIDAL ACTIVITY OF PATIENT'S SERUM

3.1. Sputum Bacterial Strains

P. aeruginosa 'strains' were isolated from patient's sputum as described in methods 2.1.2. Quantitative cultures of sputum were prepared as describe in methods 2.1.2. In all patients more than one morphologically different strain of *P. aeruginosa* were visually identified. Other organisms in addition to *P. aeruginosa* were identified from the sputum of P2 and P7. *H. influenza* and *Corynebacterium* were grown on a horse blood agar plate prepared at the same time as quantitative culture of *P. aeruginosa*. Where morphologically different *P. aeruginosa* colonies were identified, a colony was picked of each, and inoculated on *Pseudomonas* Selective Agar (BioMerieux), confirming growth of *P. aeruginosa* and further confirming the different morphology. Each of these strains was then stored in glycerol stock as described in methods 2.2.6. No genotyping was undertaken to confirm genotypically different strains corresponding to differing morphological appearances. Each individual strain isolated was allocated a number (1-11) corresponding to the patient from whom the sputum was isolated and a letter (A-D) corresponding to the morphologically different strain. The quantitative culture (cfu/ml) from each sample was recorded. The total bacterial load isolated from each patient is as listed in table 3.1.

To determine the serum resistance profile, each isolate was exposed individually to autologous serum and healthy control serum (HCS) in triplicate. Bacteria were resuspended in PBS to a final concentration of 10^7 CFU/ml; 10 µl was then mixed with 90 µl of undiluted human serum at 37°C with shaking (180rpm) and viable counts determined by evaluating the number of colony forming units/ml following bactericidal assays as described in methods section 2.4

Patient Number	*Pseudomonas aeruginosa* total bacterial load.	Number of morphologically different strains isolated.	Other Bacterial Strains.
P1	$9.63x10^8$	3 (A-C)	
P2	$2x10^5$	2 (A-B)	*H. influenzae* $1x10^7$
P3	$2.29x10^8$	4 (A-D)	
P4	$9.1x10^7$	3 (A-C)	
P5	$2x10^5$	2 (A-B)	
P6	$1.995x10^8$	3 (A-C)	
P7	$1.016x10^7$	2 (A-B)	*Corynebacterium* $1x10^7$
P8	$2.765x10^8$	4 (A-D)	
P9	$1.315x10^9$	4 (A-D)	
P10	$1.11x10^7$	3 (A-C)	
P11	$1.244x10^7$	4 (A-D)	

Table 3.1 shows the total bacterial load of the sputum samples from the patients included in this study with the number of different morphological strains of *P. aeruginosa* isolated from the sputum sample also documented. Morphologically different strains are labelled A, B, C and D. Any other significant pathogens isolated are also shown.

The results were plotted on Microsoft excel with an average bacterial count calculated at each time point. Graphs were plotted using the numbers of colony forming units present at each time point. For each strain these were standardised to $\log^{10}$ change in colony forming units. Each strain is represented numerically. P1A represents bacterial strain A from Patient 1, P1B bacterial strain B from patient 1 and S1 represents serum from patient 1, S2 from patient 2 etc. The demographics of P1-P11 are shown in table 3.2. Significant comorbidities were considered to be any coexisting cardiorespiratory disease, autoimmune disease or disease requiring the use of regular immunosuppressant therapy. Long term antibiotics where taken are shown. From the total of eleven patients two were male with nine female patients. The average age was 64.3 years with the age range between 48 and 87 years of age. Nine patients were receiving long term prophylactic antibiotic at the time of sample collection. One patient was on long term oral steroid therapy for rheumatoid arthritis.

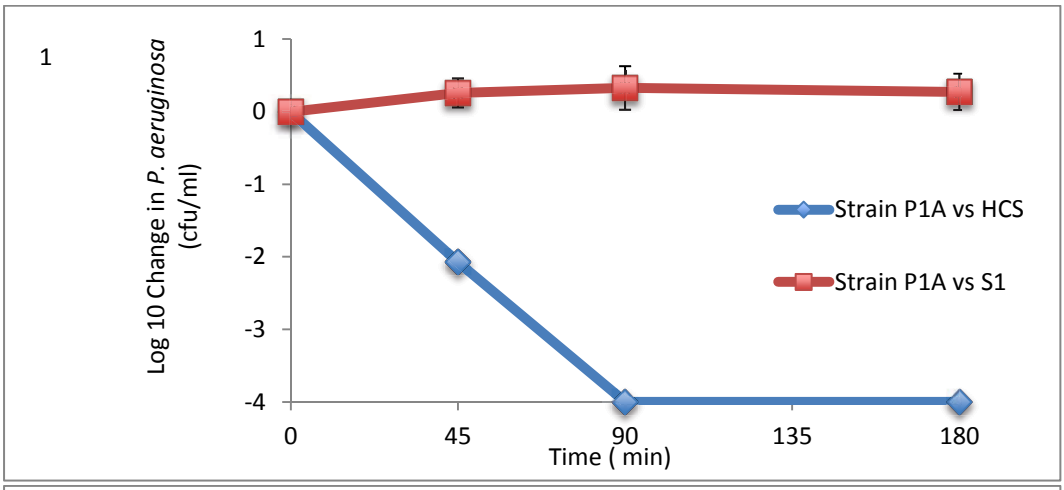
3.2 *P. aeruginosa* Bactericidal Assay Results

The results of bactericidal assays for each bacterial strain isolated from P1-P11 are represented in figure 3.1 1-33. The vast majority of the strains were sensitive both to patient and healthy control serum killing. Three of the 33 strains (P6A, P6B and P7A) were resistant in all serum tested indicating these strains are innately serum resistant. Interestingly, P1, P2 and P3 all had strains that, although sensitive in healthy control serum, were not killed by their own serum (Table 3.3).

Patient	Gender	Age	Long term antibiotic	Inhaled corticosteroid	Immunosuppressant therapy.	Significant Comorbidities.
P1	Male	48	Nebulised colomycin	Yes	No	No
P2	Female	59	Nebulised colomycin	No	No	No
P3	Male	68	Nebulised colomycin	Yes	No	COPD
P4	Female	60	Nebulised colomycin	No	No	No
P5	Female	63	Nebulised cefuroxime	Yes	No	COPD
P6	Female	55	Nebulised colomycin	No	No	No
P7	Female	68	Nil	No	No	No
P8	Female	60	Oral azithromycin (3x weekly)	No	No	No
P9	Female	64	Nil	No	Yes (Oral corticosteroid)	Rheumatoid arthritis
P10	Female	87	Nil	Yes	No	Ischaemic heart disease
P11	Female	76	Nebulised colomycin	No	No	Ischaemic heart disease.

Table 3.2 Patient demographics. The characteristics of the patients whose serum and sputum was utilised in this study are shown, including details of those who were treated with long term oral or inhaled antibiotic in addition to other relevant illness or treatments they were receiving.

1


Log 10 Change in P. aeruginosa (cfu/ml)
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-4
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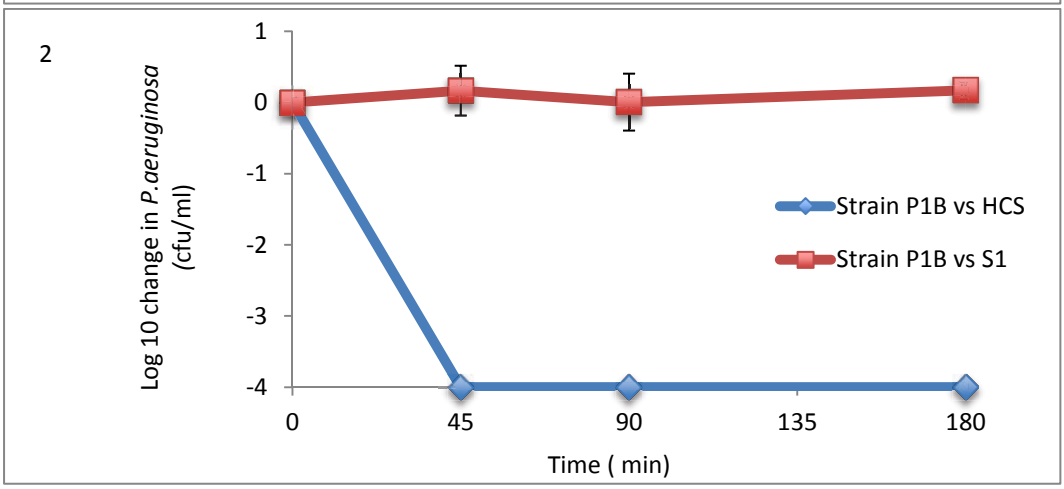
45
90
135
180
Time (min)
Strain P1A vs HCS
Strain P1A vs S1

2


Log 10 change in P.aeruginosa (cfu/ml)
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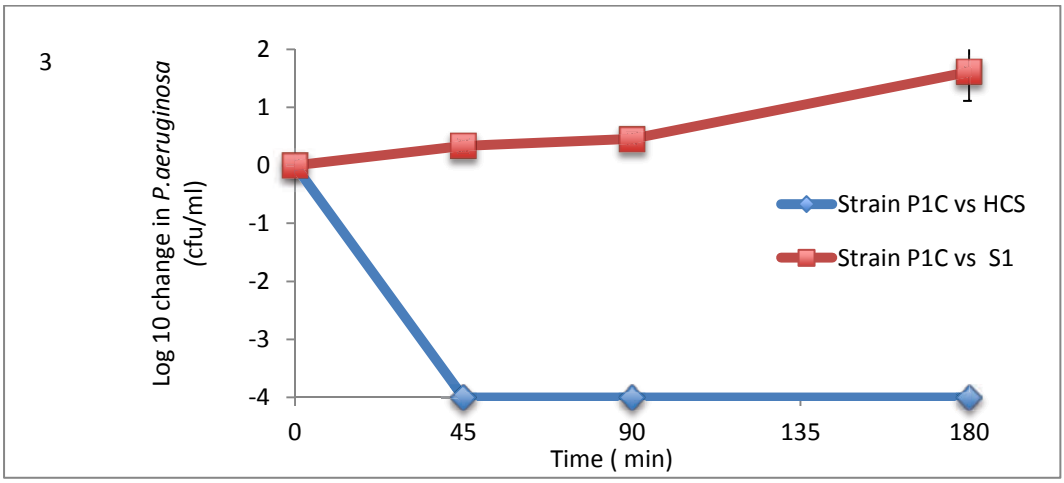
-1
-2
-3
-4
0
45
90
135
180
Time (min)
Strain P1B vs HCS
Strain P1B vs S1

3


Log 10 change in P.aeruginosa (cfu/ml)
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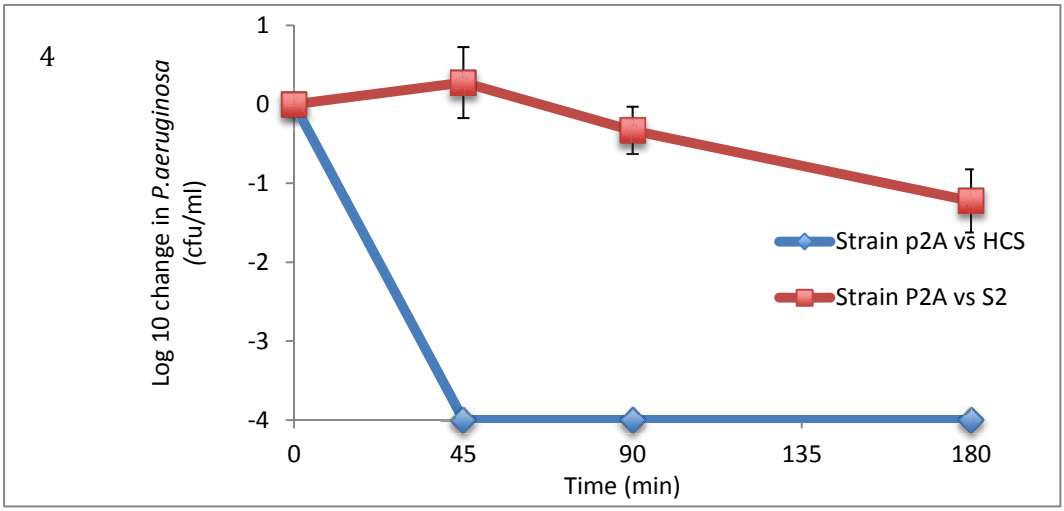
1
0
-1
-2
-3
-4
0
45
90
135
180
Time (min)
Strain P1C vs HCS
Strain P1C vs S1

4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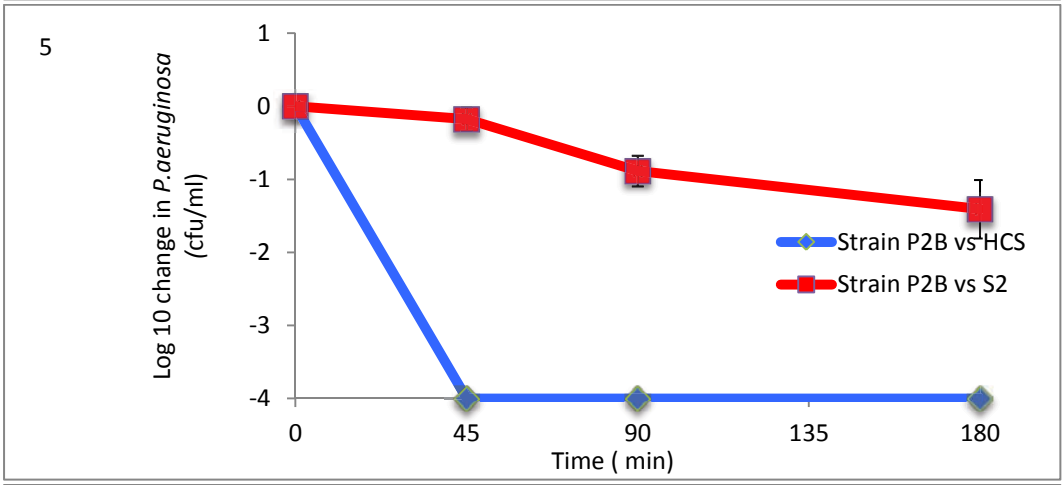
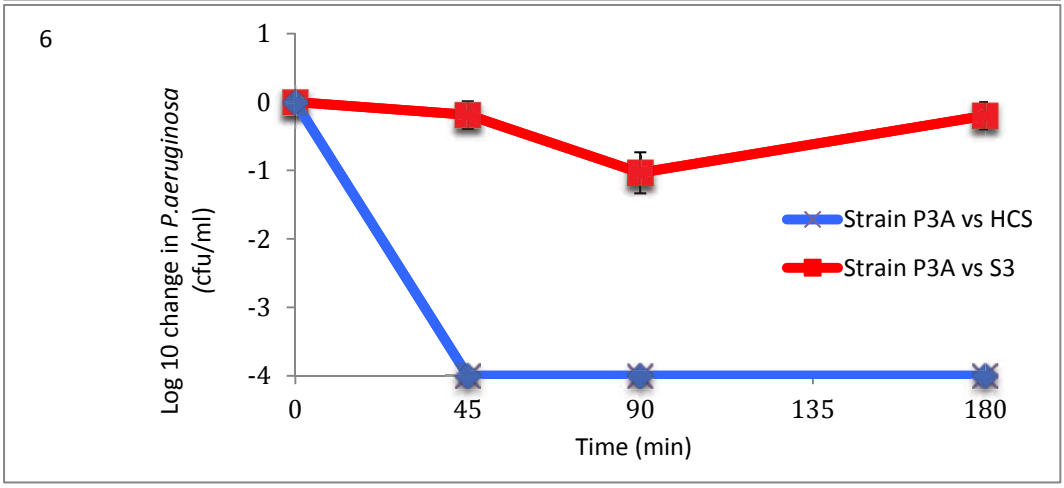
-2
-3
-4
0
45
90
135
180
Time (min)
Strain p2A vs HCS
Strain P2A vs S2

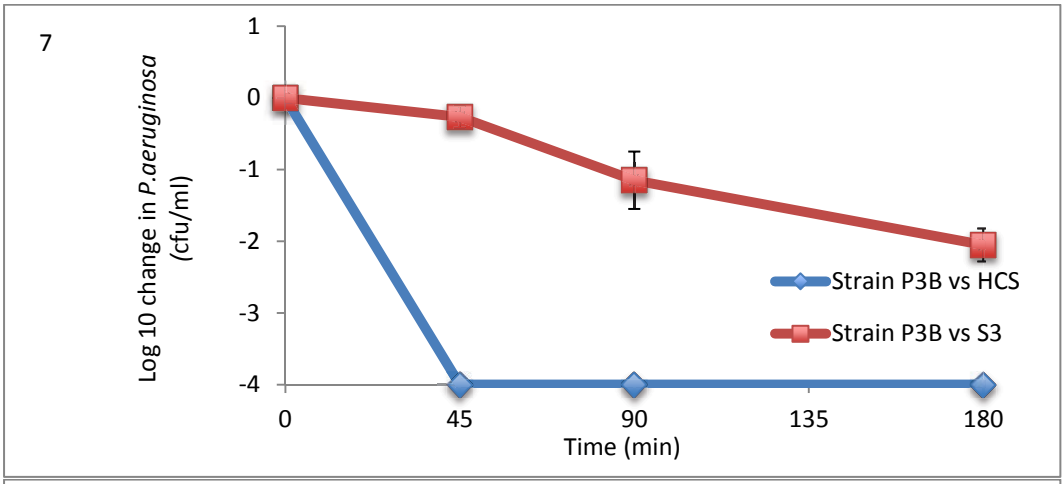
5


Log 10 change in P.aeruginosa (cfu/ml)
1
0
-1
-2
-3
-4
0
45
90
135
180
Time (min)
Strain P2B vs HCS
Strain P2B vs S2

6


Log 10 change in P.aeruginosa (cfu/ml)
1
0
-1
-2
-3
-4
0
45
90
135
180
Time (min)
Strain P3A vs HCS
Strain P3A vs S3

7


Log 10 change in P.aeruginosa (cfu/ml)
1
0
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0
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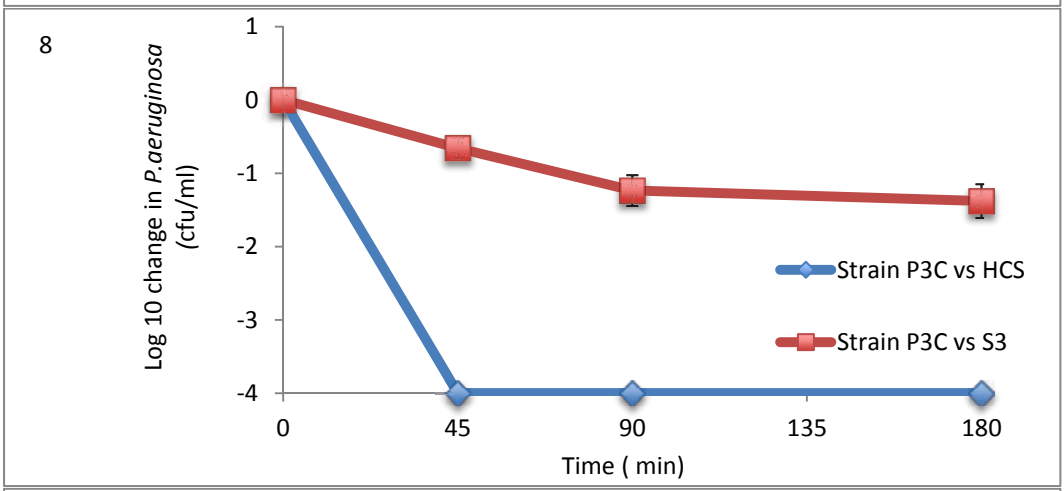
90
135
180
Time (min)
Strain P3B vs HCS
Strain P3B vs S3

8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-3
-4
0
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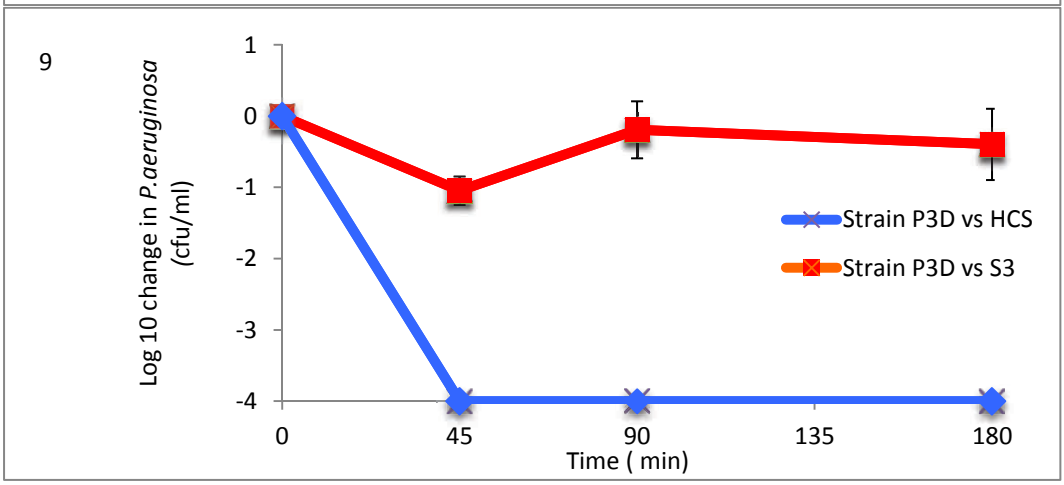
90
135
180
Time (min)
Strain P3C vs HCS
Strain P3C vs S3

9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-2
-3
-4
0
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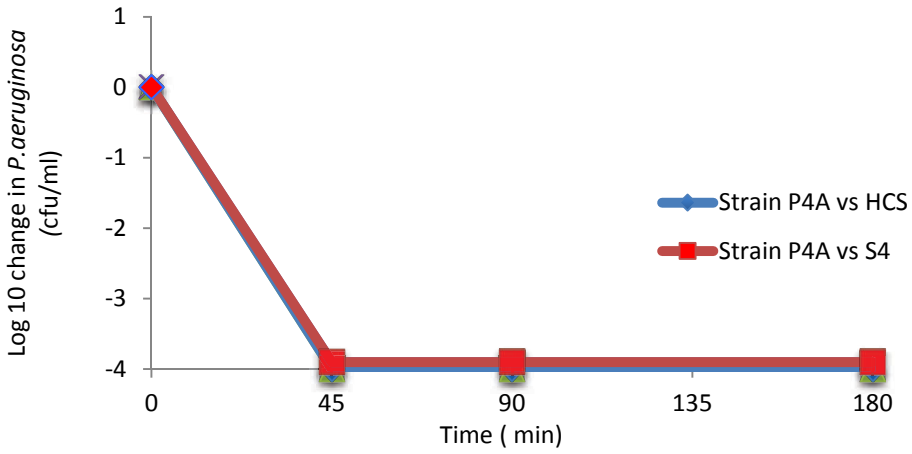
90
135
180
Time (min)
Strain P3D vs HCS
Strain P3D vs S3

10


Log 10 change in P.aeruginosa (cfu/ml)
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0
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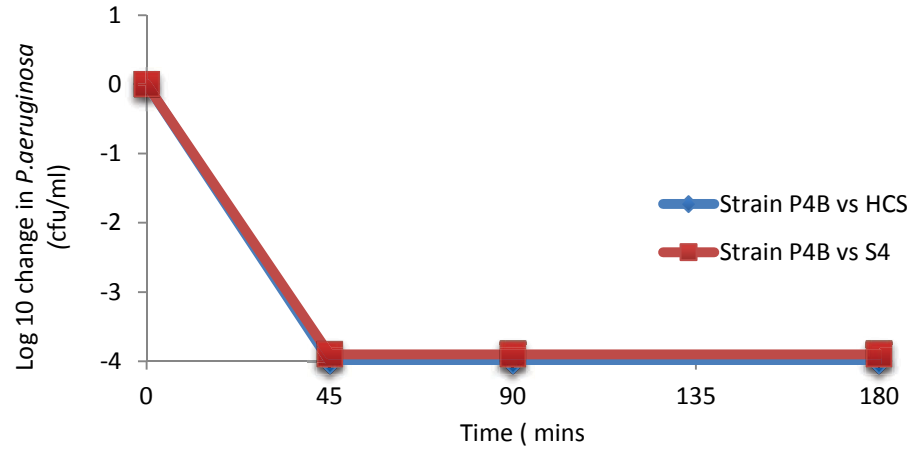
-2
-3
-4
0
45
90
135
180
Time (min)
Strain P4A vs HCS
Strain P4A vs S4

11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-2
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-4
0
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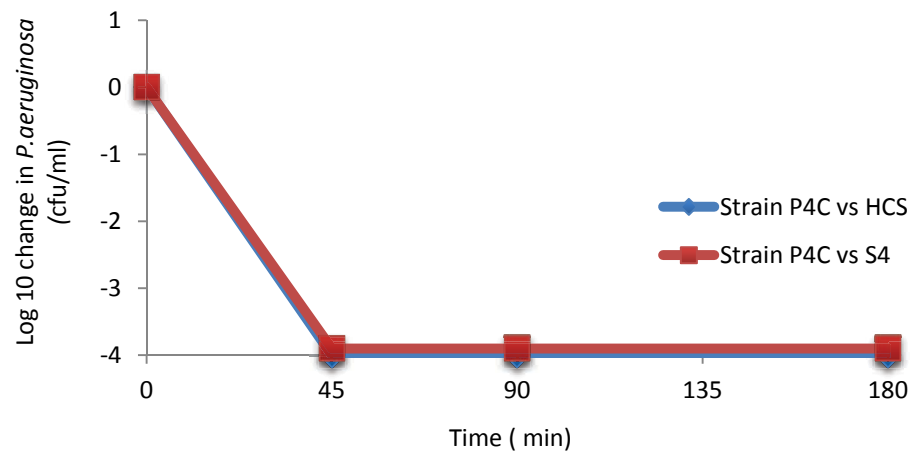
90
135
180
Time (mins
Strain P4B vs HCS
Strain P4B vs S4

12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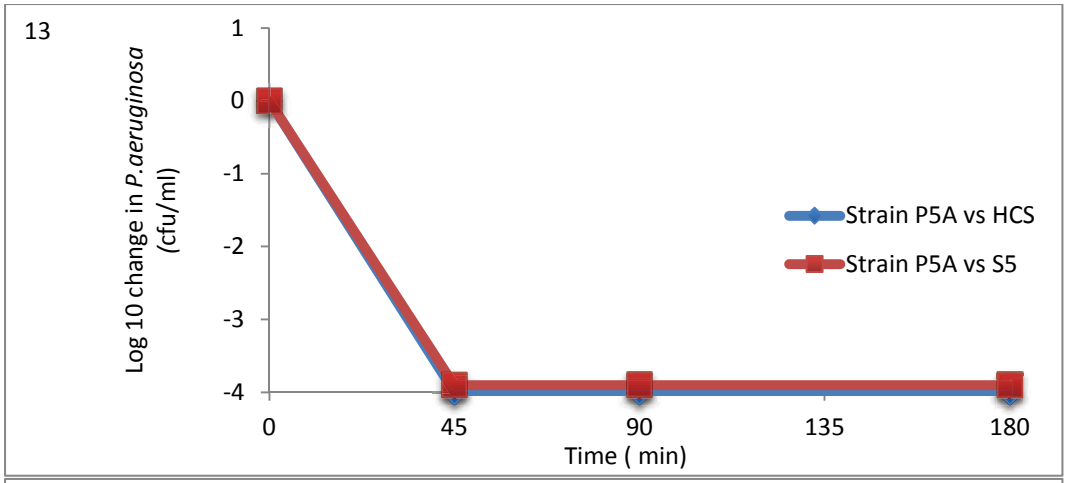
0
45
90
135
180
Time (min)
Strain P4C vs HCS
Strain P4C vs S4

13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0
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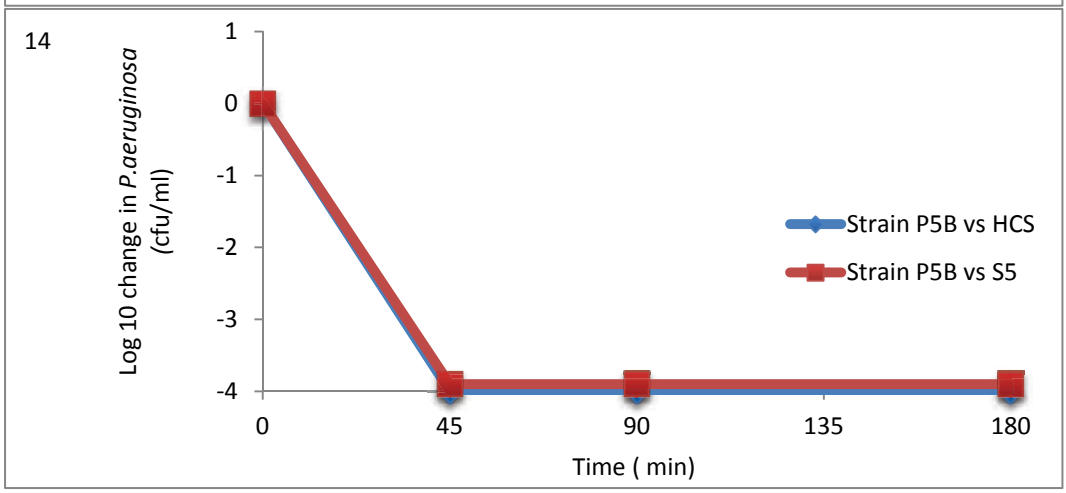
90
135
180
Time (min)
Strain P5A vs HCS
Strain P5A vs S5

14


Log 10 change in P.aeruginosa (cfu/ml)
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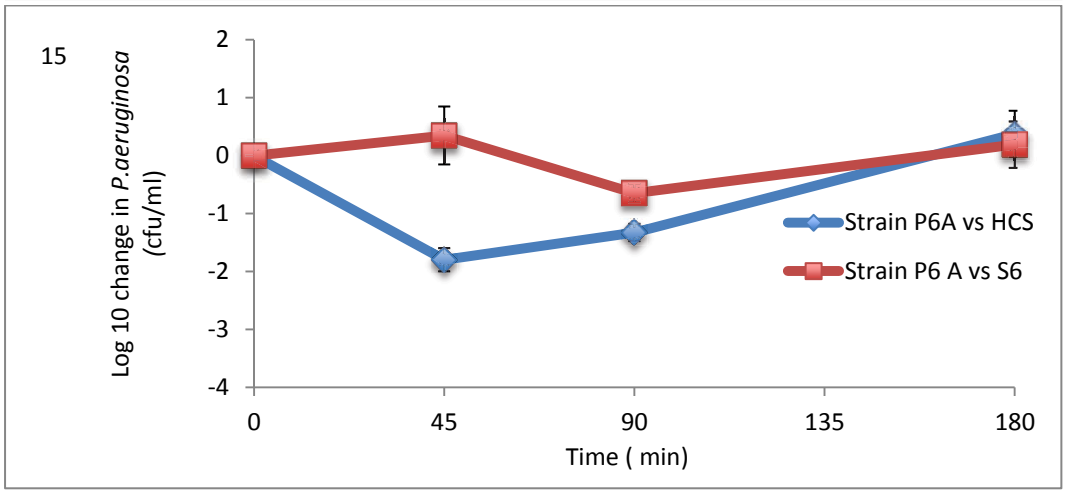
90
135
180
Time (min)
Strain P5B vs HCS
Strain P5B vs S5

15


Log 10 change in P.aeruginosa (cfu/ml)
2
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0
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-2
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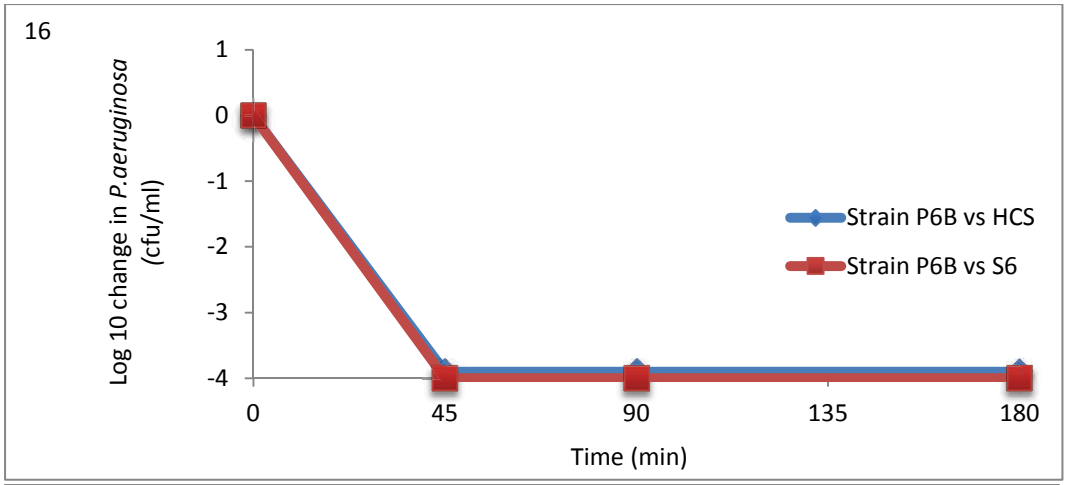
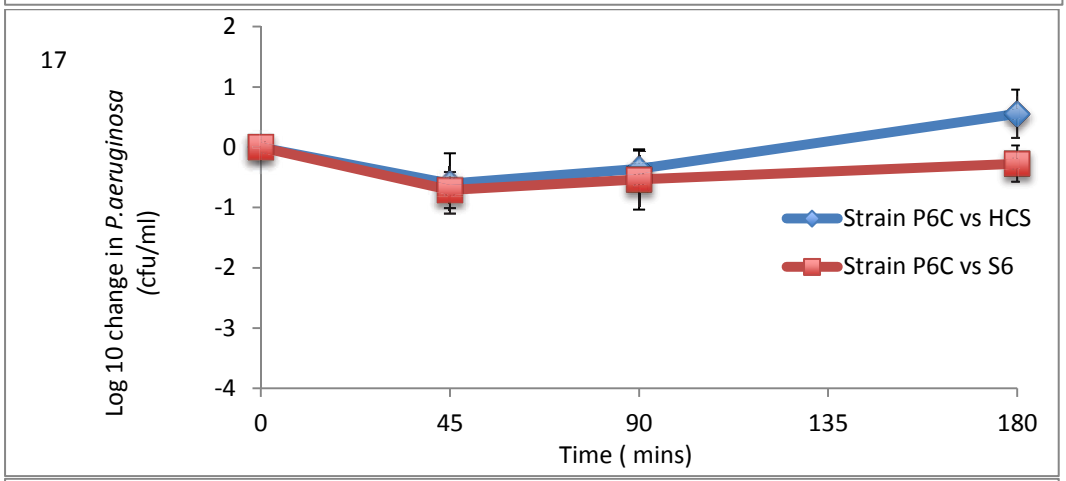
-4
0
45
90
135
180
Time (min)
Strain P6A vs HCS
Strain P6 A vs S6

16


Log 10 change in P.aeruginosa (cfu/ml)
1
0
-1
-2
-3
-4
0
45
90
135
180
Time (min)
Strain P6B vs HCS
Strain P6B vs S6

17


Log 10 change in P.aeruginosa (cfu/ml)
2
1
0
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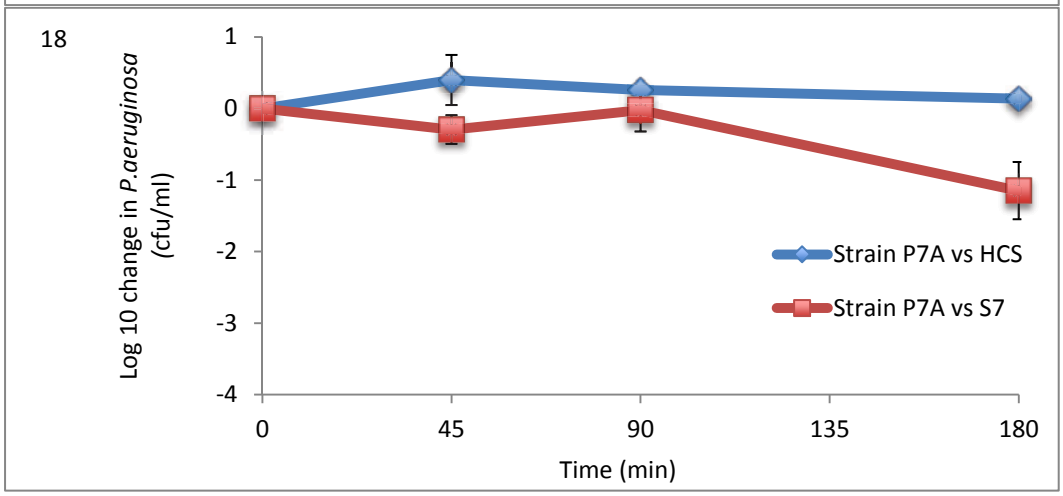
-2
-3
-4
0
45
90
135
180
Time (mins)
Strain P6C vs HCS
Strain P6C vs S6

18


Log 10 change in P.aeruginosa (cfu/ml)
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0
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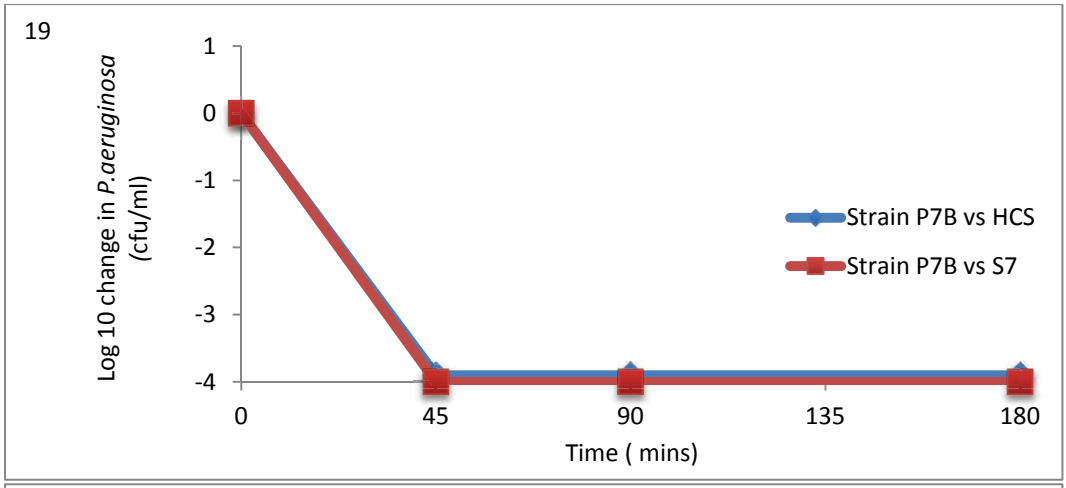
-2
-3
-4
0
45
90
135
180
Time (min)
Strain P7A vs HCS
Strain P7A vs S7

19


Log 10 change in P.aeruginosa (cfu/ml)
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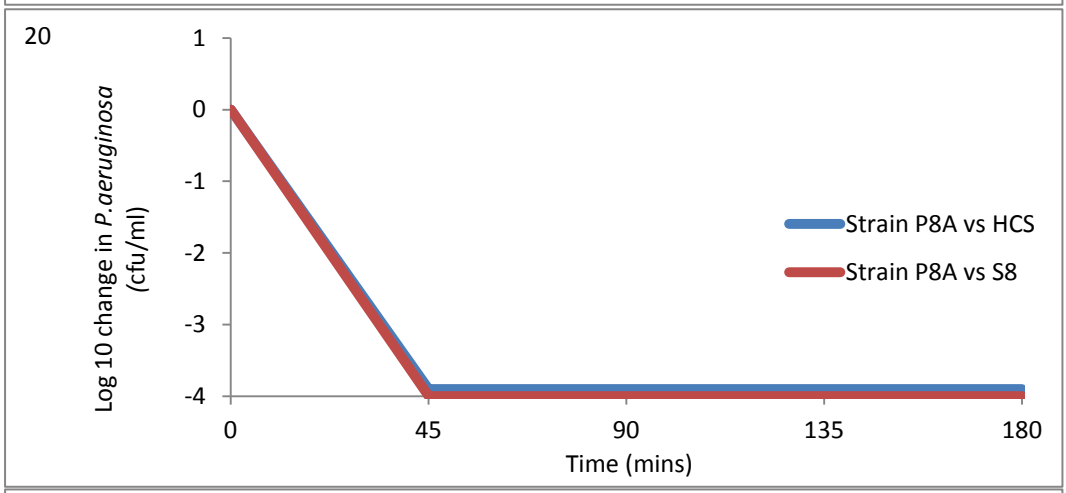
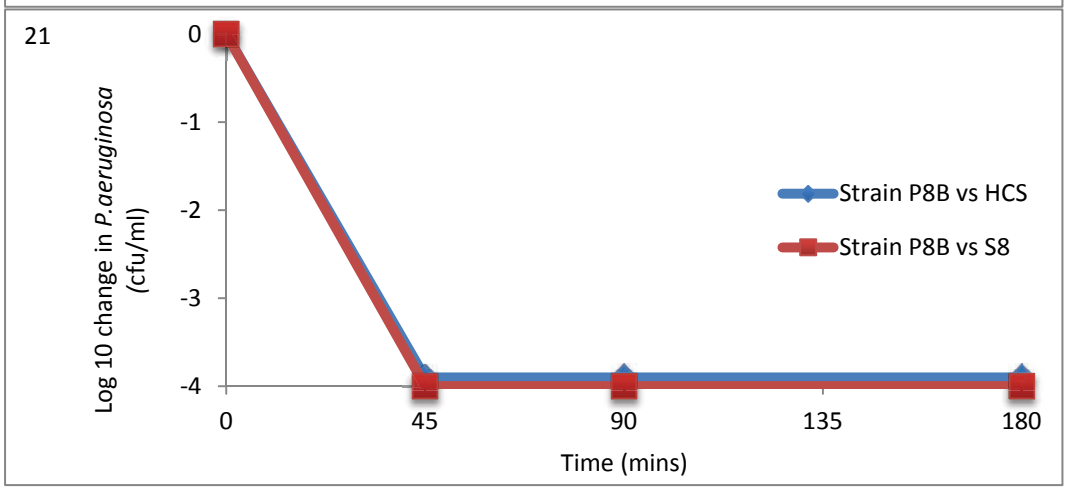
0
-1
-2
-3
-4
0
45
90
135
180
Time (mins)
Strain P7B vs HCS
Strain P7B vs S7

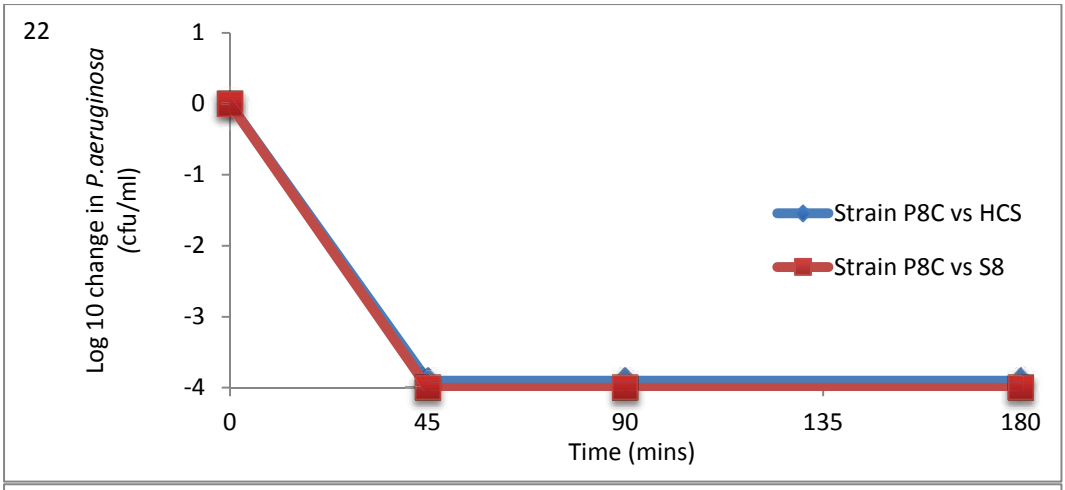
20


Log 10 change in P.aeruginosa (cfu/ml)
1
0
-1
-2
-3
-4
0
45
90
135
180
Time (mins)
Strain P8A vs HCS
Strain P8A vs S8

21


Log 10 change in P.aeruginosa (cfu/ml)
0
-1
-2
-3
-4
0
45
90
135
180
Time (mins)
Strain P8B vs HCS
Strain P8B vs S8

22


Log 10 change in P.aeruginosa (cfu/ml)
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0
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-2
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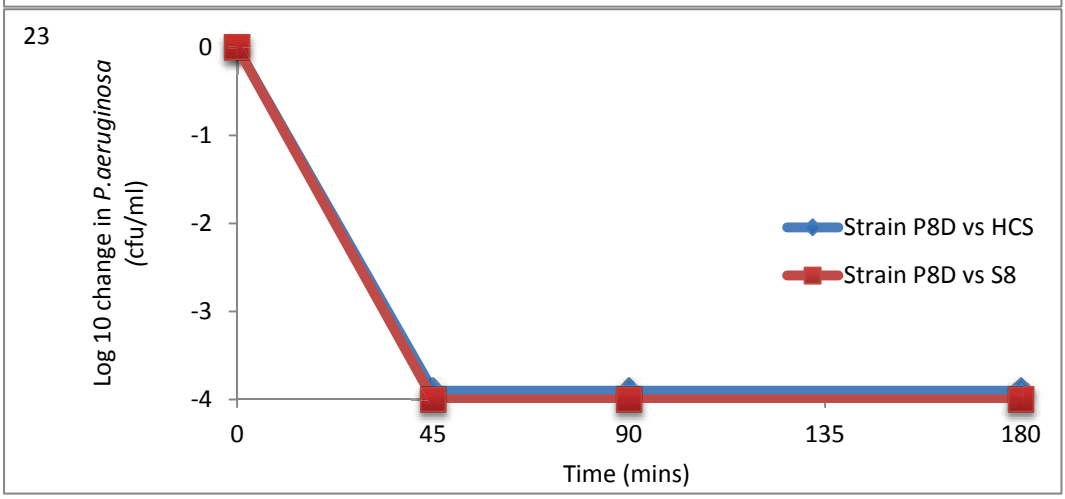
-4
0
45
90
135
180
Time (mins)
Strain P8C vs HCS
Strain P8C vs S8

23


Log 10 change in P.aeruginosa (cfu/ml)
0
-1
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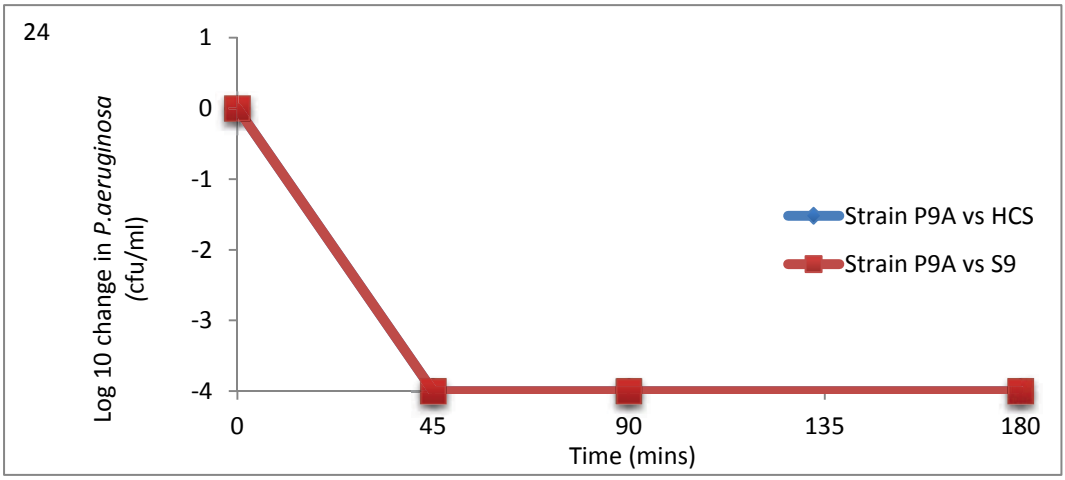
-3
-4
0
45
90
135
180
Time (mins)
Strain P8D vs HCS
Strain P8D vs S8

24


Log 10 change in P.aeruginosa (cfu/ml)
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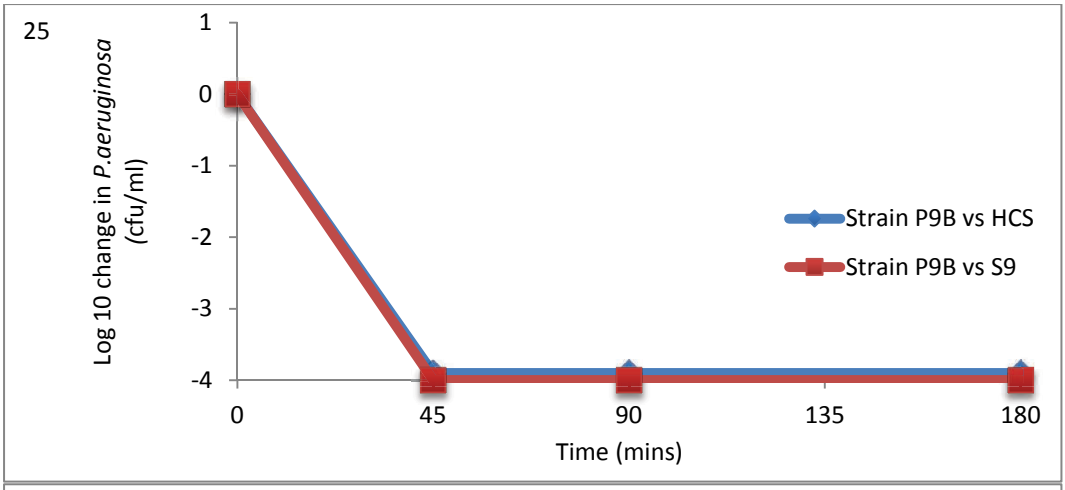
-2
-3
-4
0
45
90
135
180
Time (mins)
Strain P9A vs HCS
Strain P9A vs S9

25


Log 10 change in P.aeruginosa (cfu/ml)
1
0
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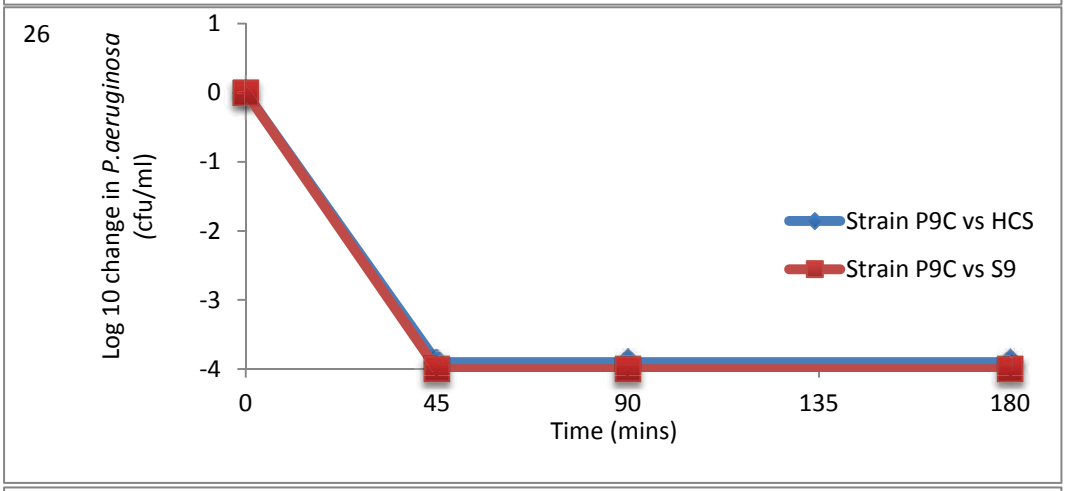
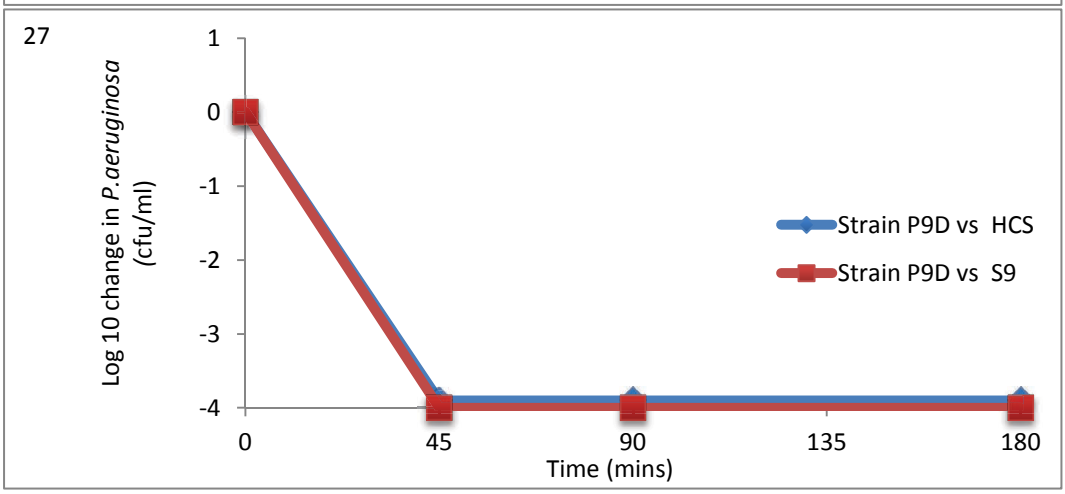
-2
-3
-4
0
45
90
135
180
Time (mins)
Strain P9B vs HCS
Strain P9B vs S9

26


Log 10 change in P.aeruginosa (cfu/ml)
1
0
-1
-2
-3
-4
0
45
90
135
180
Time (mins)
Strain P9C vs HCS
Strain P9C vs S9

27


Log 10 change in P.aeruginosa (cfu/ml)
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0
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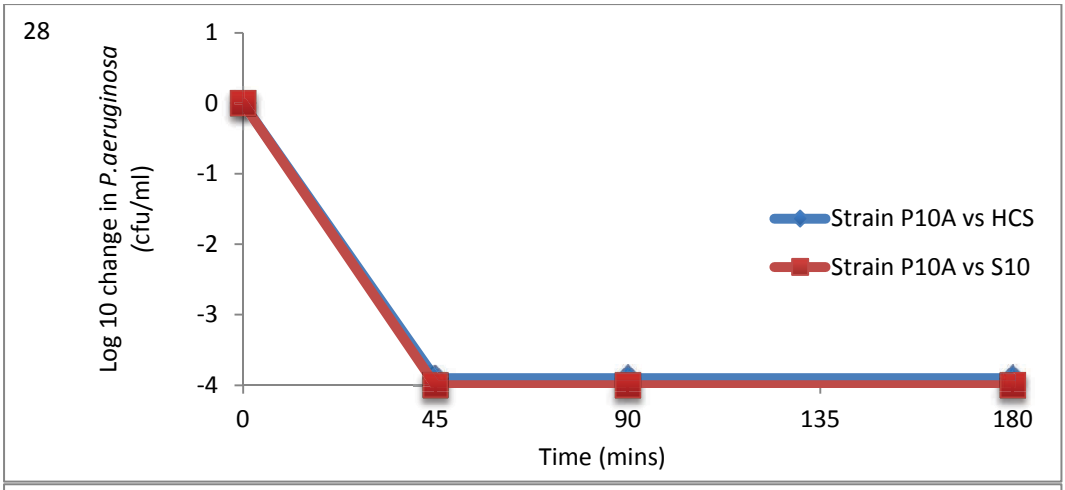
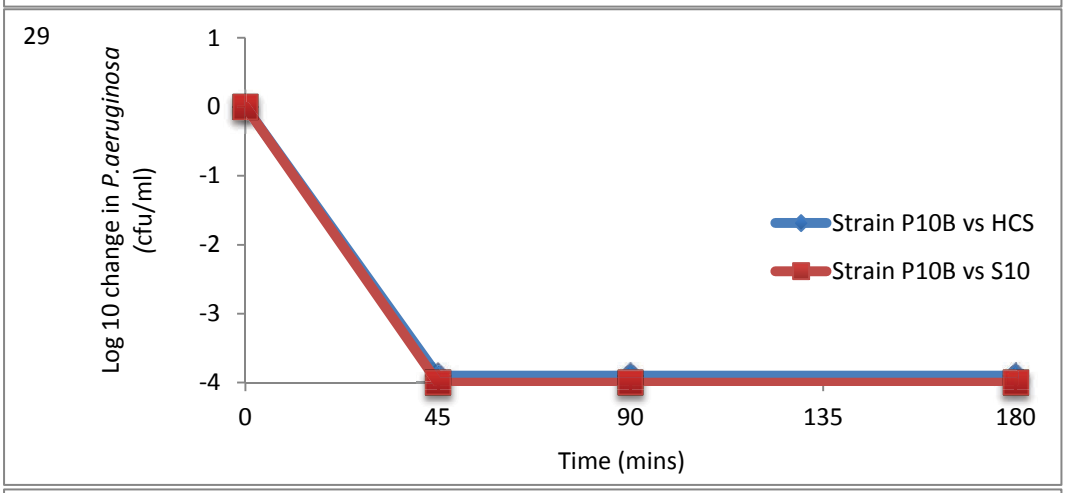
-2
-3
-4
0
45
90
135
180
Time (mins)
Strain P9D vs HCS
Strain P9D vs S9

28


Log 10 change in P.aeruginosa (cfu/ml)
1
0
-1
-2
-3
-4
0
45
90
135
180
Time (mins)
Strain P10A vs HCS
Strain P10A vs S10

29


Log 10 change in P.aeruginosa (cfu/ml)
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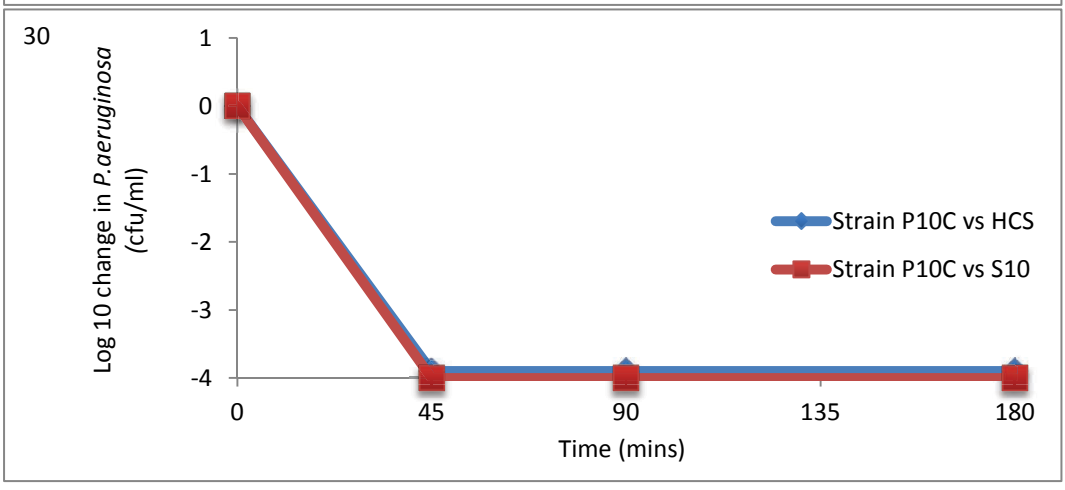
-2
-3
-4
0
45
90
135
180
Time (mins)
Strain P10B vs HCS
Strain P10B vs S10

30


Log 10 change in P.aeruginosa (cfu/ml)
1
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-1
-2
-3
-4
0
45
90
135
180
Time (mins)
Strain P10C vs HCS
Strain P10C vs S10

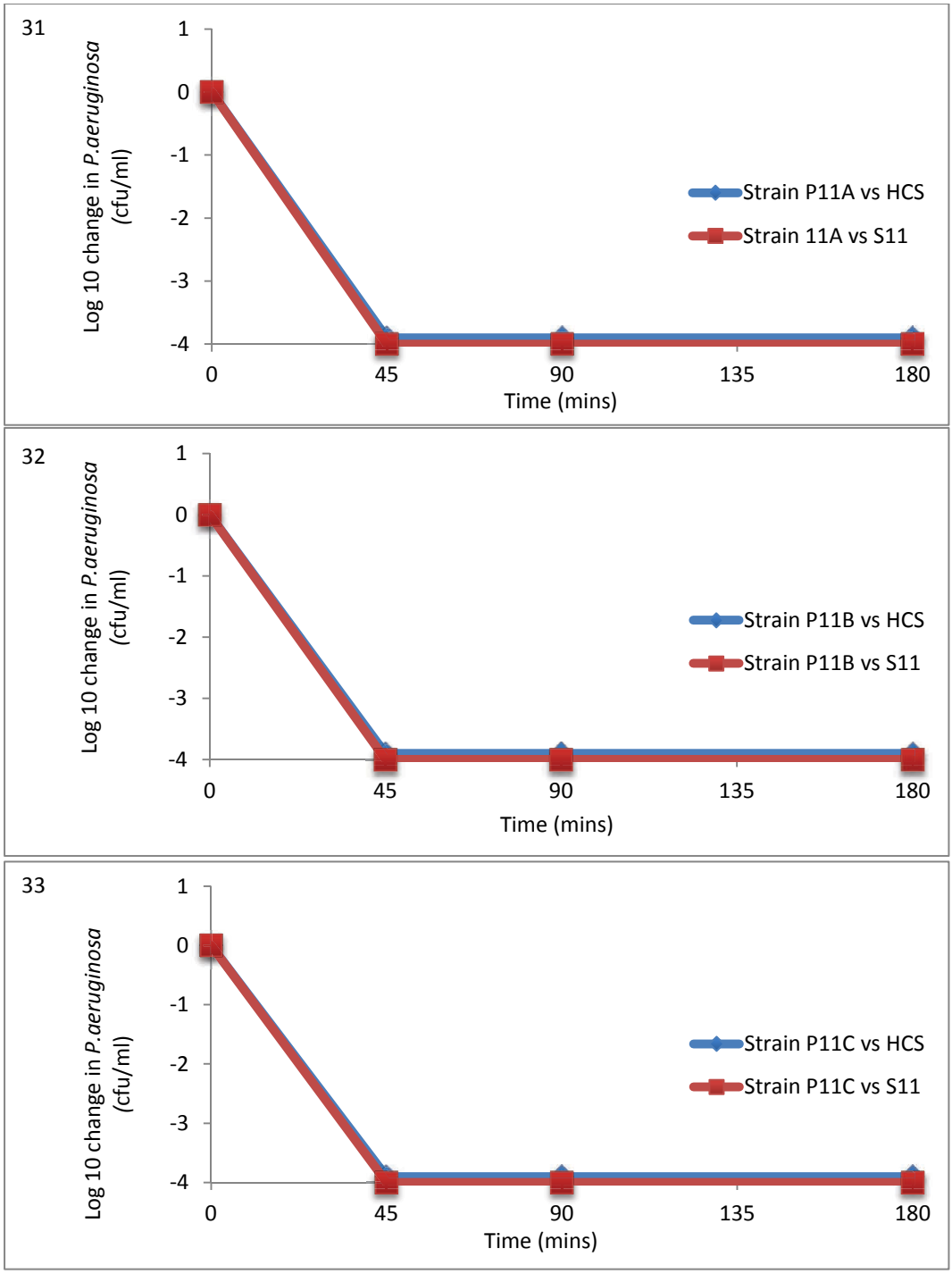


Figure 3.1 (1-33) Killing curves of 33 morphologically different strains from 11 patients with bronchiectasis were subject to bactericidal killing assays with autologous serum and healthy control serum. Counts of *P. aeruginosa* colony forming units (cfu) were taken at 45, 90 and 180 minutes. Negative values correspond with a decrease in viable *P. aeruginosa* compared with initial concentration. Strains isolated from 3 patients (P1, P2, and P3) were resistant to serum killing by the patients own serum but fully sensitive to killing by healthy control sera. P6 and P7 both isolated strains which were resistant to serum killing both with autologous and healthy control sera (P6A, P6C and P7A). All other strains were sensitive to killing by both autologous and healthy control sera. For all data, assays were performed in triplicate and an average colony count taken at each time point. Error bars represent the mean +/- SD for each assay.

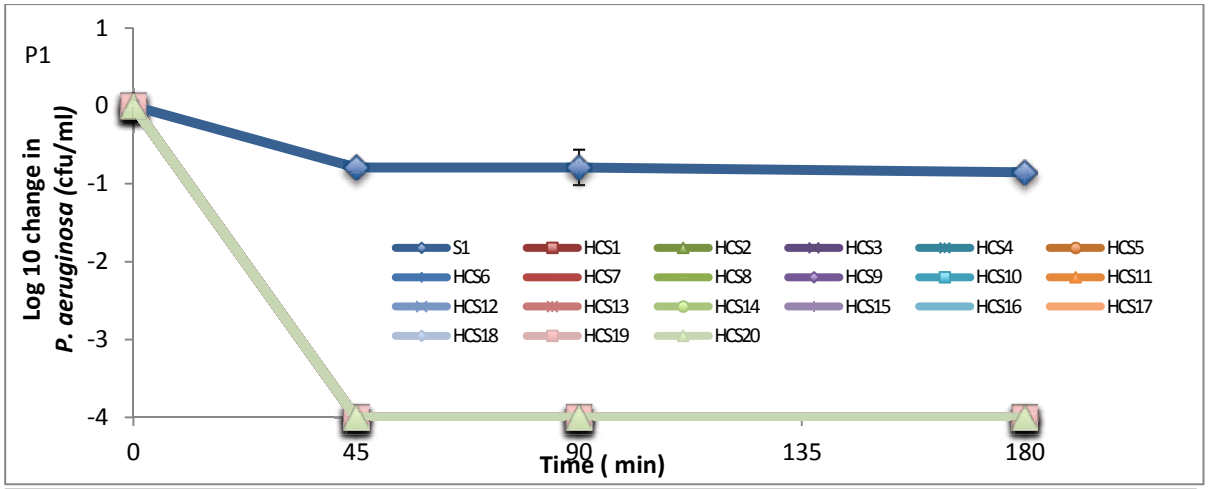
Patient	Total number of morphologically different strains	Serum resistant strains in HCS and patient sera.	Serum sensitive strains in both HCS and patient sera	Strains sensitive in HCS. Resistant in patient serum.
P1	3	0	0	**3**
P2	2	0	0	**2**
P3	4	0	0	**4**
P4	3	0	3	0
P5	2	0	2	0
P6	3	2	1	0
P7	2	1	1	0
P8	4	0	4	0
P9	4	0	4	0
P10	3	0	3	0
P11	3	0	3	0

Table 3.3. Summary of serum resistant strains from all 11 patients showing that P1-P3 had strains resistant to killing by autologous sera whilst sensitive in HCS.

Three patients (P1-P3) were shown to grow strains of *P. aeruginosa* which were sensitive to killing in healthy control sera, indicating that they were serum sensitive strains, but not sensitive to killing in autologous serum. To confirm that these strains were sensitive to serum killing these strains P1A, P2A and P3A were exposed to the sera of twenty separate healthy control subjects with the same results. Bactericidal assays were carried out as described in methods section 2.1.1. The results are shown in figure 3.2. All three strains were shown to be serum sensitive after 45 minutes of incubation whilst again confirming resistance to killing by autologous serum. After 180 minutes of incubation there was a significant difference in cfu/ml for all three strains in autologuous serum when compared to HCS ($p<0.005$).

The finding that strains P1A, P2A and P3A were fully sensitive to killing in healthy control sera but not in autologous sera indicated that these were not serum resistant strains and that the lack of serum killing was an inherent feature of the serum from these patients.

A



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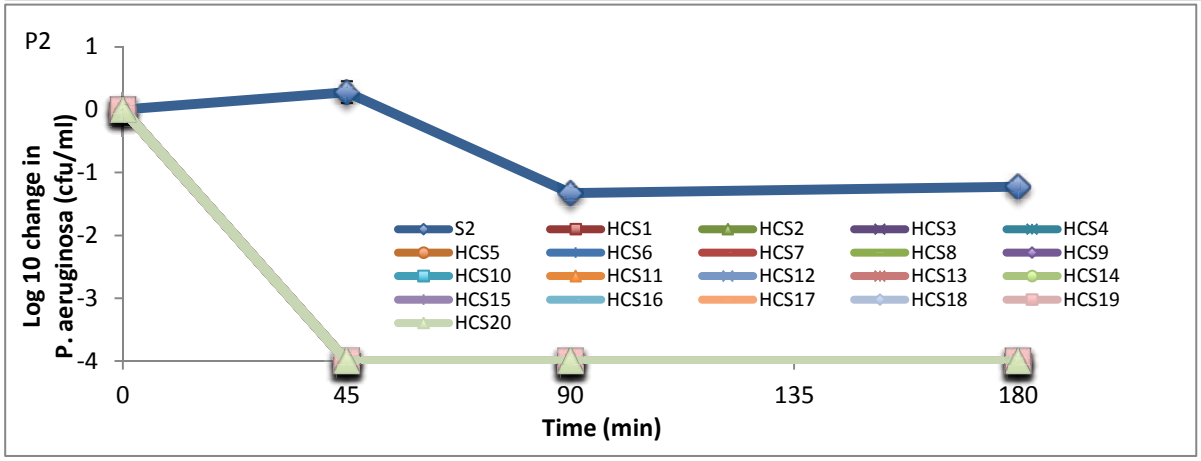

C

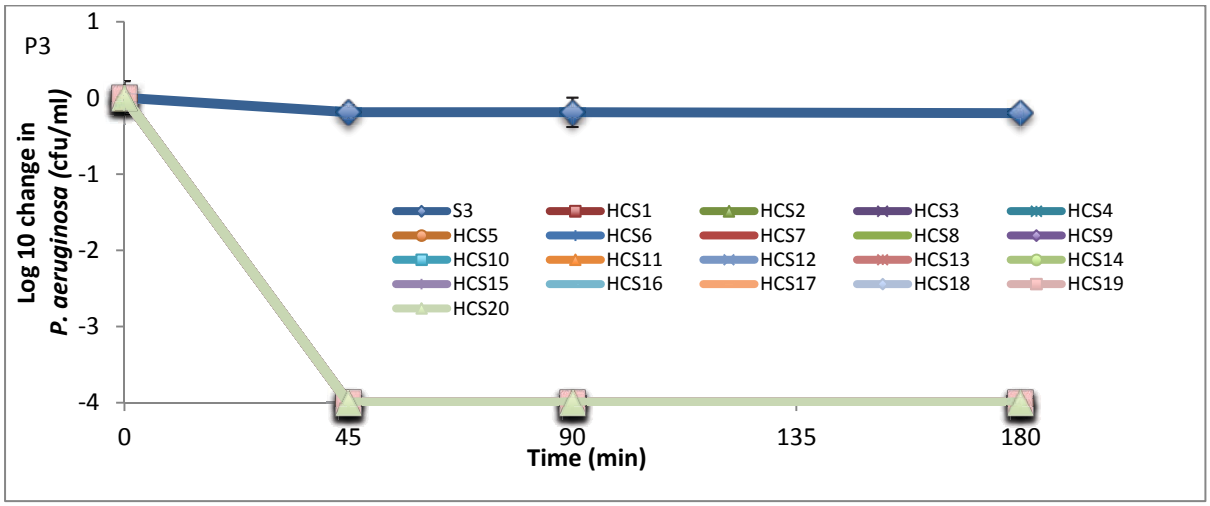

Figure 3.2: Killing curves of *P.aeruginosa* strains identified from patients P1, P2 and P3 in autologous serum and sera from 20 healthy control volunteers. Negative values correspond with a decrease in viable *P. aeruginosa* compared with initial concentration. *P. aeruginosa* strains P1A, P2A and P3A were shown to be resistant to killing in autologous serum and fully sensitive to killing in serum from 20 healthy control subjects. All strains were killed in all HCS after 45 minutes incubation. There was a clear difference in cfu/ml for all HCS vs autologous serum at 180 minutes incubation ($p<0.005$ by Students t test) for all three strains. The curves depicting bacterial killing by healthy control sera and autologous sera are overlaid for simplicity. For all data, assays were performed in triplicate and an average colony count taken at each time point. Error bars represent the mean +/- SD for each assay.

3.3 Serum Total Immunoglobulins

Serum total immunoglobulins were checked as part of routine clinical care in these patients. Total antibody deficiency was not the reason for the lack of serum killing (table 3.4). All patients had normal total levels of immunoglobulins, Immunoglobulin G subclasses were also in the normal range for all three patients with serum unable to kill autologous strains indicating that hypogammaglobulinaemia or IgG subclass deficiency was not the reason. IgG subclasses were mainly collected at a different time point from that of total IgG, which explains why the combined total of IgG subclass does not always equal the total IgG level. The identification of 3 patients who had strains of *P. aeruginosa,* sensitive to killing by HCS but resistant to killing by autologous serum, with normal immunoglobulin levels suggested either the absence of another serum component essential for bacterial killing or the presence of an extra factor in the serum that blocks bacterial killing. The following experiments were designed to establish whether the absence of bactericidal activity in the serum of these patients was due to the absence of a serum component essential for bactericidal activity or the presence of an inhibitory factor.

3.4 Identification of Serum Factors Inhibiting Bacterial Killing

First, the bactericidal ability of serum from patients P4-11 against strain P1A was investigated with the aim of establishing whether or not this strain, shown to be resistant to killing in autologous serum, was also resistant to killing in the serum from other patients similarly colonised with *P. aeruginosa*. The ability of serum from patients 1-3 to kill strains from patients P4-11 was also investigated with the aim of establishing whether the serum from these patients was capable of killing other *P. aeruginosa* strains. The results of these

Antibody subclasses and normal ranges. (g/L)	**P1**	**P2**	**P3**
IgA (0.80-4.00)	2.09	2.17	4.94
IgM (0.50-2.00)	0.85	0.82	1.05
IgG (6.00-16.00)	10.66	12.66	10.69
IgG1 (3.20-10.20)	4.34	5.56	4.13
IgG2 (1.20-6.60)	4.34	7.29	3,31
IgG3 (0.20-1.90)	2.09	1.15	1.67
IgG4 (0.00-1.30)	<0.01	0.77	0.13

Table 3.4 shows the total immunoglobulin levels present in the sera of patients P1-P3. None were found to be deficient in IgA, IgG or IgM. IgG subgroups were also within the normal range as measured by the immunology laboratory for all patients.

assays was indicative of the factors mediating resistance to serum killing being common to strains P1–3 and sera S1–3 but absent from the other strains and sera of patients similarly colonised with *P. aeruginosa*. The serum of all 11 patients was tested and serum S4-S11 were all able to kill P1A (Fig 3.3) whereas S1,S2 and S3 were unable to effect killing with a significant difference in cfu/ml at 180 minutes between S1-3 and S4-11 ($p<0.005$). The serum sensitive strain P4A was also tested against the serum of patients 4-11 (Fig 3.4) all of which were able to effect killing against this strain. Although S1 was unable to kill its own strain it could kill the *P. aeruginosa* isolates from P4-P11 suggesting that it did not lack the necessary factors to kill *P. aeruginosa* strains. Interestingly S1 was also unable to kill P2A and P3A (Fig 3.5) and S1-S3 were all able to effect killing of P4A but none could kill P1A (Fig 3.6). These results suggest that the inability of sera S1-S3 to kill strain P1A –P3A is both serum and strain specific. To assess the immune factors mediating bacterial killing, the role of complement in killing *P. aeruginosa* and measurement of specific anti *P. aeruginosa* antibody levels in the sera of P1-P3 was assessed.

3.5 Complement Inactivation of Serum

To demonstrate that complement mediated killing is crucial in the ability of serum to kill *P. aeruginosa,* assays were performed assessing the ability of healthy control serum and S4 to kill strains P1A and P4A with following complement inactivation. P1A and P4A were both sensitive to killing in HCS and S4 at 37°C. Vials with 90 µL of serum were heated on a heating block to 56ºC for 20 min prior to mixing with bacterial strains. Bactericidal assays were performed as previously described in methods 2.4.

This results of these assays demonstrates that sera, including HCS, that can kill strains of *P. aeruginosa* at physiological temperatures can no longer kill these strains when complement is inactivated confirming the crucial role of complement in bacterial killing ($p<0.005$).

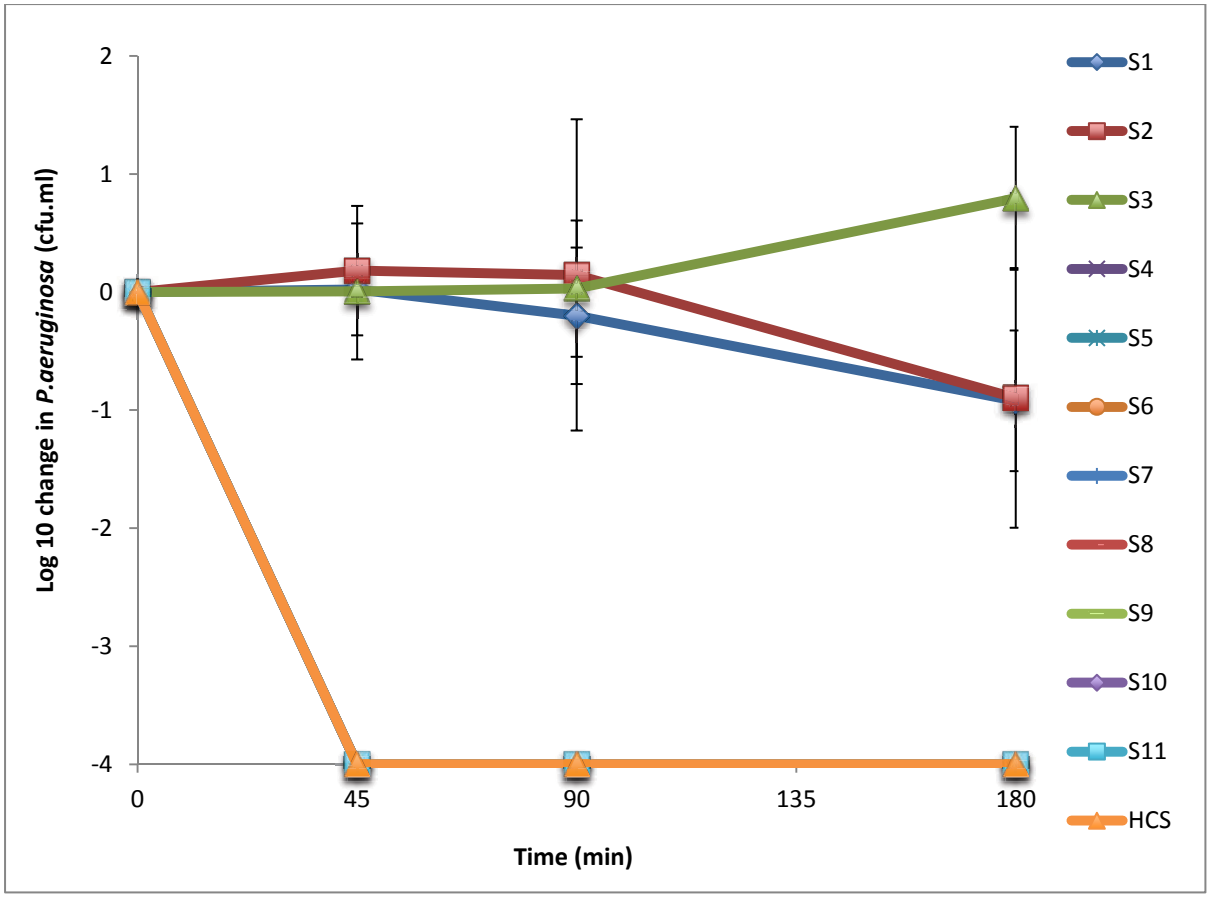

Figure 3.3 shows killing curves of strain P1A vs S1-S11. Negative values correspond with a decrease in viable *P. aeruginosa* as compared with initial concentration. P1A was resistant in autologous serum as previously demonstrated but also in S2 and S3 whilst P1A was fully sensitive to killing in S4-S11 ($p < 0.005$ by Student's ttest for colony counts of P1A in S1, S2 and S3, compared with S4 – S11 and HCS after 180 minutes incubation). Curves of HCS and S4-S11 overlaid for simplicity . Assays performed in triplicate. Error bars represent the mean +/- SD for each assay.

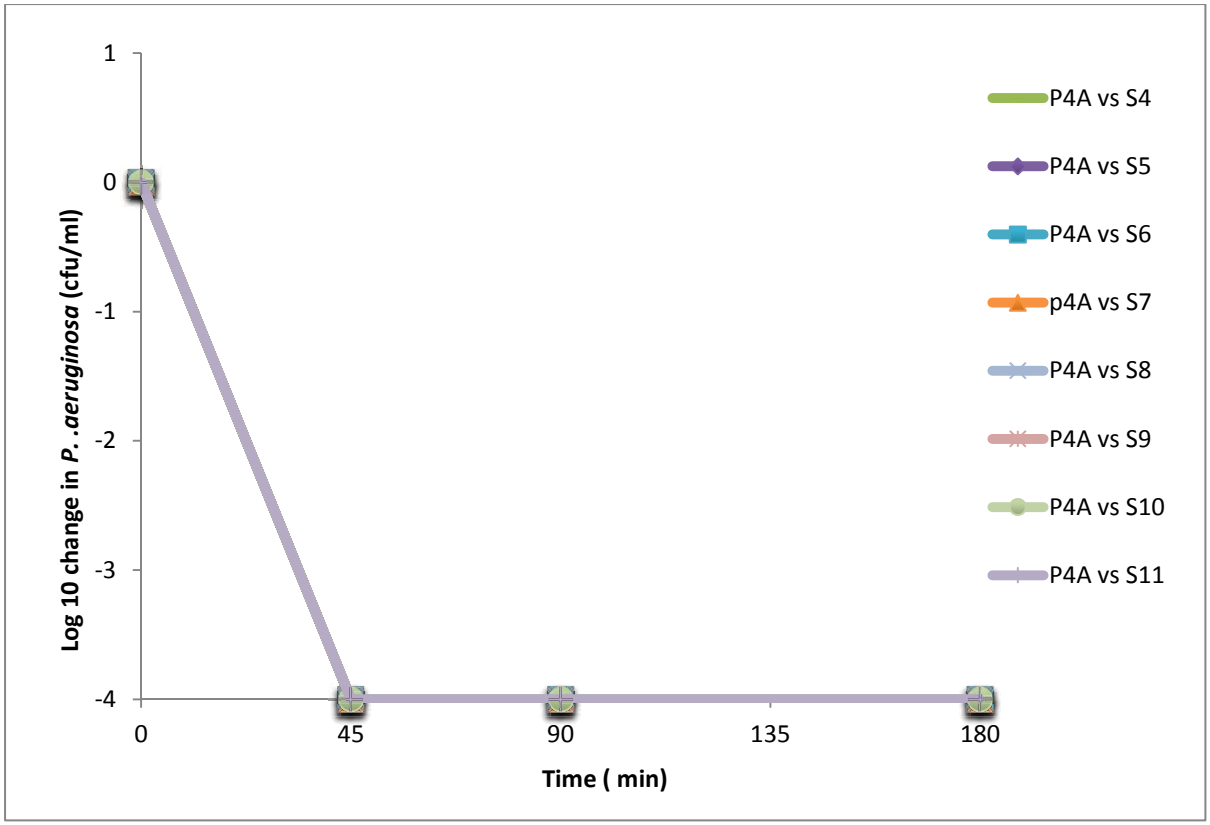

Figure 3.4 shows killing curves of strain P4A (a strain sensitive to killing by autologous serum) vs S4-S11. Sera from those patients colonised with *P. aeruginosa* sensitive to killing by autologous sera were previously shown to be able to kill P1A as shown in Figure 3.3. This sera was also able to kill P4A, a strain shown to be sensitive to killing by autologous sera. Strains of P4A were dead after 45 minutes incubation with serum from patients 4-11. Curves overlaid for simplicity.

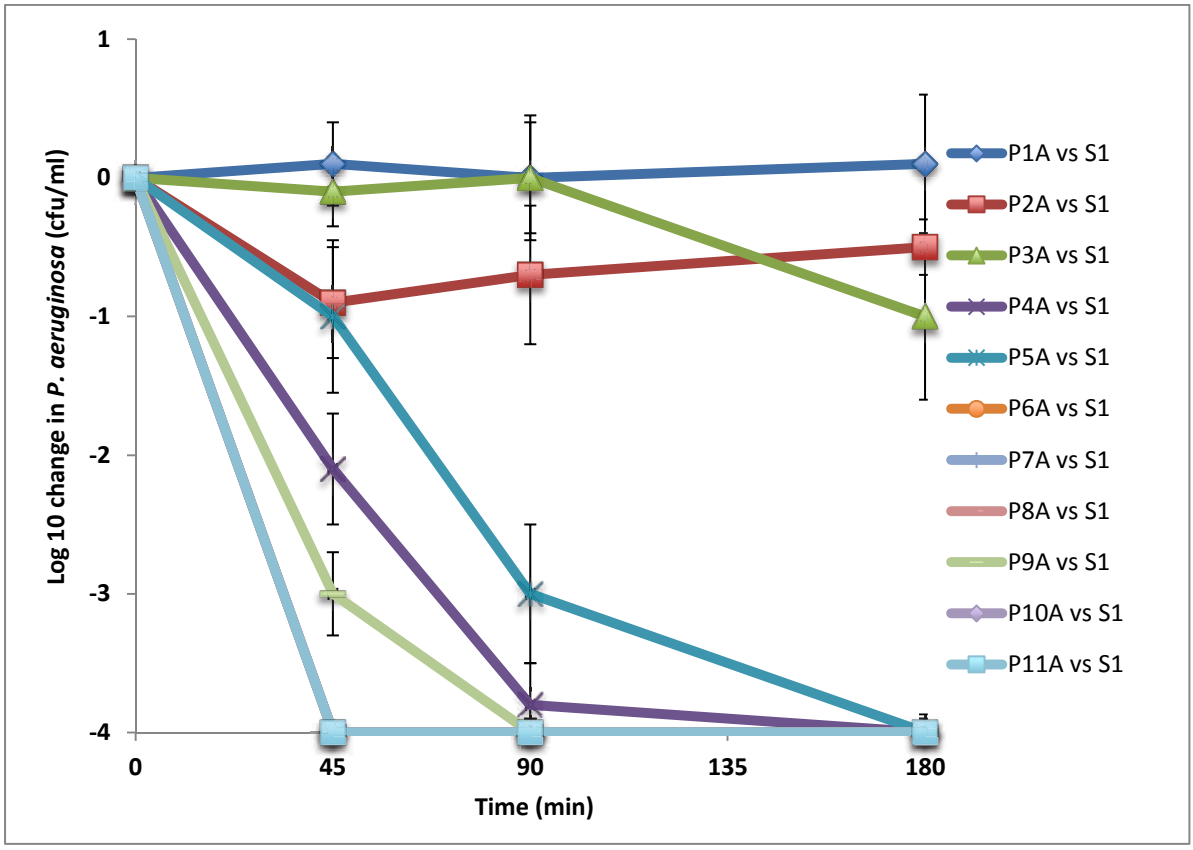

Figure 3.5. Killing curves of strains P1-P11 vs S1. Strains P1-3 are resistant to killing in S1 whilst all other strains are serum sensitive after 180 minutes incubation.. Strains P6A, P7A, P8A, P10A and P11A were all dead at 45 minutes incubation time and therefore the curves are overlaid for simplicity. There was a significant difference in cfu/ml between P1A, P2A and P3A compared with P4A-P11A in S1 after 180 minutes incubation ($p<0.005$ by Student's t test) This is representative of 3 independent assays. Error bars represent the mean +/- SD for each assay.

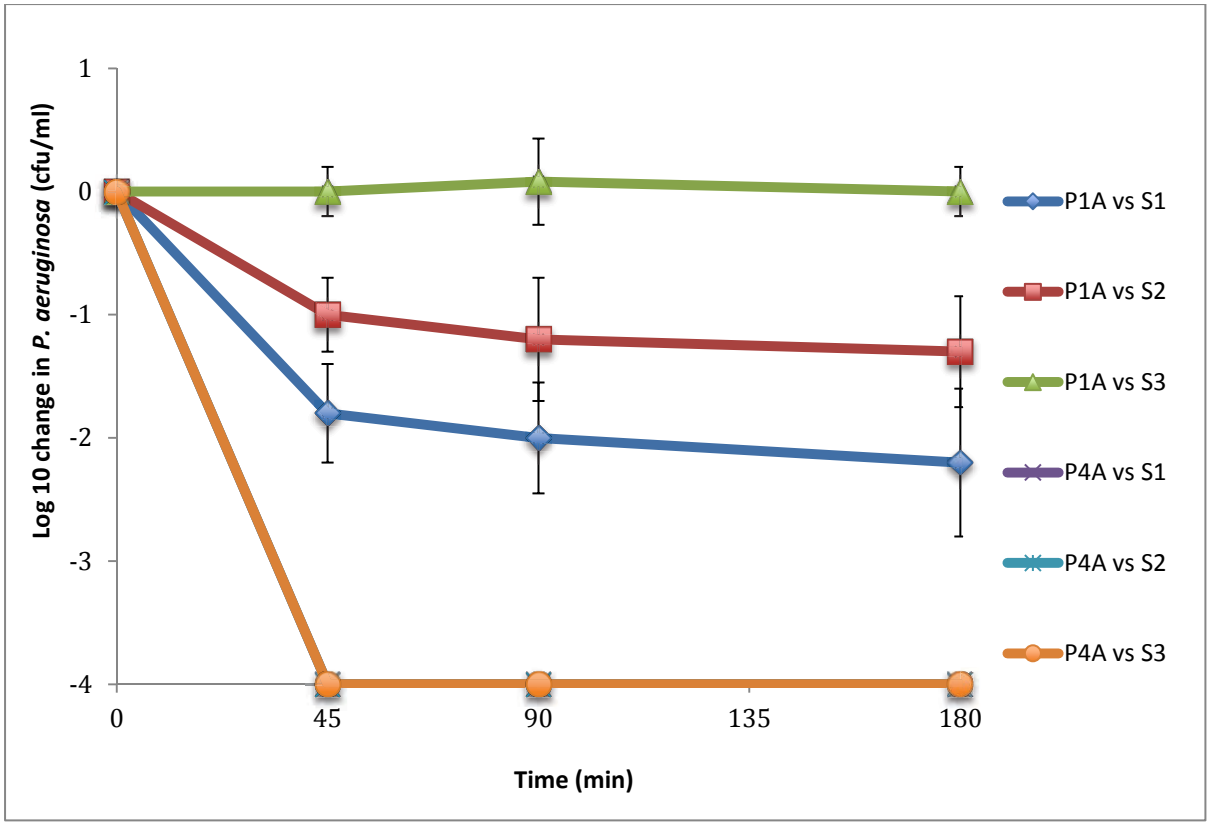

Figure 3.6 Killing curves of strains P1A and P4A vs S1, S2 and S3. P4A was serum sensitive in S1-S3 (curves overlaid for simplicity) but S1-3 could not kill P1A at 180 minutes incubation. This is representative of 3 independent assays. Error bars represent the mean +/- SD for each assay.

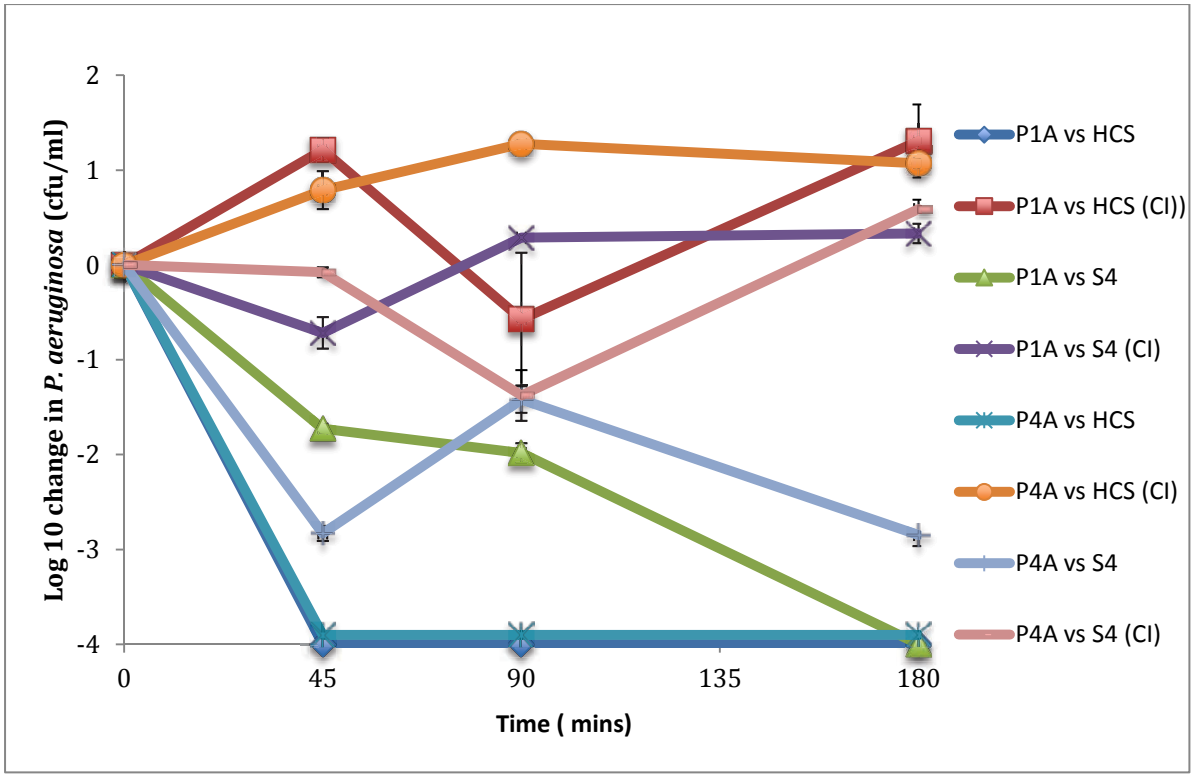

Figure 3.7 Killing curves of strains P1A and P4A by HCS and S4 and HCS and S4 heated to 56°C to inactivate complement. Complement inactivation is depicted as CI. Complement inactivation of HCS and S4 inhibited killing of both strains which were fully sensitive to unheated serum killing at 45 minutes in HCS and S4 (curves overlaid for simplicity) indicating that complement mediated killing is crucial in serum bacterial killing of *P. aeruginosa*. P1A was shown, as before, to be sensitive to killing by HCS. However when complement was inactivated in HCS there was a significant change in cfu/ml at 180 minutes incubation ($p<0.00001$ by Student's t test). For all data, assays were performed in triplicate and an average colony count taken at each time point. Error bars represent the mean +/- SD for each assay.

3.6 Measurement of Specific Anti *P. aeruginosa* Antibodies

Having demonstrated that the serum from P1-P3 had normal levels of total antibody (IgA, IgG and IgM), and that complement was crucial in bacterial killing, we next sought to determine if antibody and complement proteins were binding to the bacterial surface. The levels of binding of serum IgG, IgA, IgM, C1, C3 and C5b-9 to patient's isolated strains was measured using flow cytometric analysis. Samples were prepared as described in methods 2.6.2. Data acquisition and analysis were done using FACSDiva software (BD Biosciences). The labelled negative control sample (PBS) was used to establish the baseline FITC fluorescence level. In the test samples the fluorescing and non-fluorescing cell populations were gated, quantified (percentage of parent population) and relevant statistical parameters (FITC mean and median) were calculated using FASCDiva software. The geometric mean was then used to plot level of FITC binding on bar charts as shown. Histograms representative of immunoglobulin deposition are shown in appendix 1.

It was shown that patient sera had specific binding of all proteins tested in levels similar to, or greater than that in HCS which was able to kill strains P1-P3 (Fig 3.8) Furthermore, IgG and complement components C1q, C3, and the C5b-9 membrane attack complex (MAC) were shown to be deposited on all strains (Fig 3.9)

Impaired bacterial killing of strains P1A –P3A in autologous serum was therefore concluded not to be due a lack of complement or antibody binding as complement components were deposited on all strains. Therefore, I assessed the possibility that the lack of bacterial killing was due to an inhibitory factor present in the serum of patients 1-3.

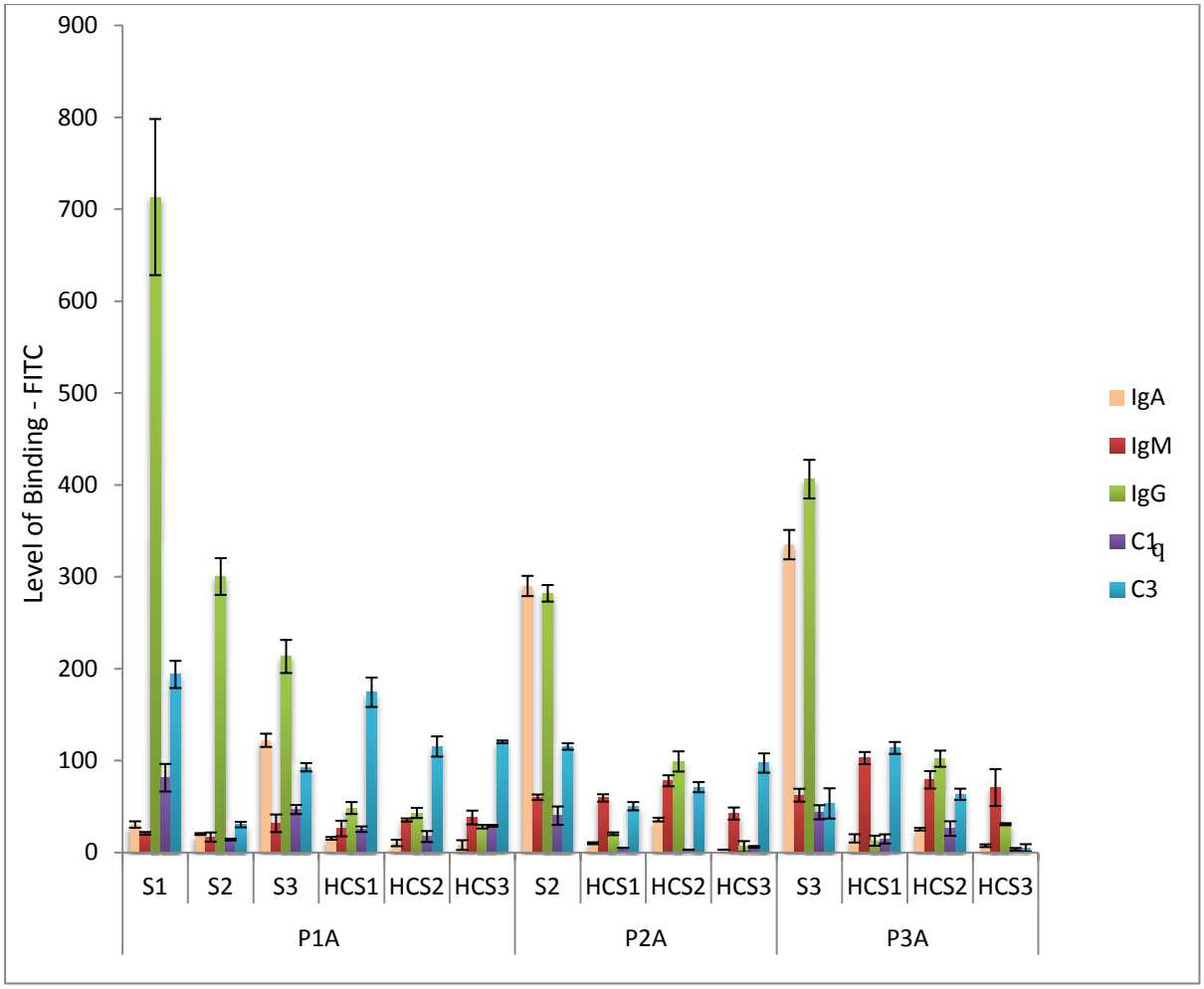

Figure 3.8. Binding of specific IgG, IgM, IgA antibodies and C1q and C3 complement factors from indicated patient serum and HCS to P1A, P2A, and P3A. Each strain was tested with autologous serum and at least three separate healthy controls. Data are representative of three independent experiments. P1A was also tested with S2 and S3 serum. Anti *P. aeruginosa* IgG adherence was noted to be higher than that in HCS in S1-S3.

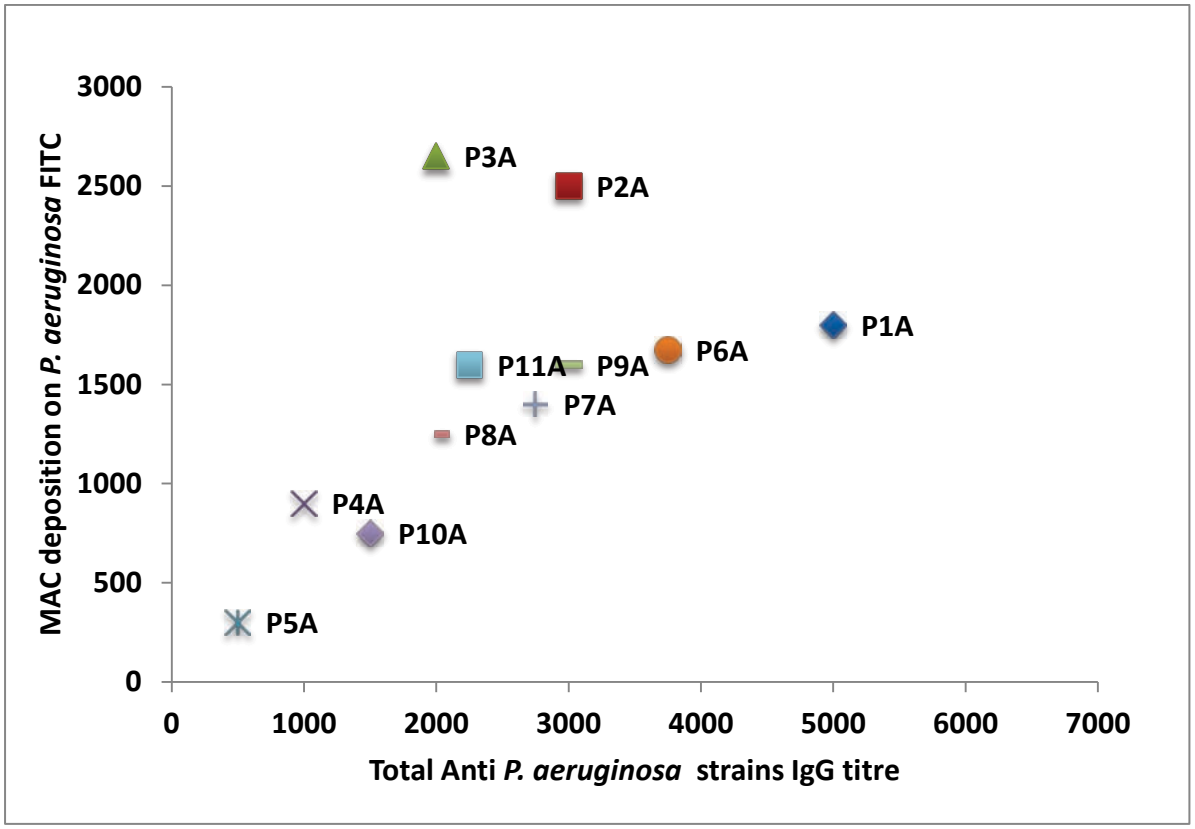

Figure 3.9 Serum titres of *P. aeruginosa* specific IgG compared with C5b-9 MAC deposition on autologous strains. MAC deposition was demonstrated on each of the strains resistant to killing in autologous serum. The trend was towards increased MAC deposition with increasing total anti *P. aeruginosa* IgG titre for the strains sensitive to killing in autologous serum with P2A and P3A demonstrating the highest levels of MAC deposition with moderate IgG titres.

3.7 Mixing of Patient Serum and Healthy Control Serum

As it was shown that patient serum was not lacking a factor to effect killing of *P. aeruginosa* next explored was whether this serum had an extra factor that was blocking complement killing. Each strain P1A –P3A was exposed to autologous serum mixed with healthy control serum in differing concentrations to establish whether this would restore bacterial killing of autologous serum. Bactericidal assays were performed with these serum mixes as described in chapter 2. First HCS was added to S1, S2 or S3 (50:50 ratio). HCS similarly diluted with PBS readily killed *P. aeruginosa.* In contrast, HCS mixed with patient sera instigated little to no killing of bacterial isolates supporting the presence of a blocking factor (Fig 3.10).

As bacterial killing could be inhibited in HCS with the addition of patient serum in a 50:50 mixture, HCS was added to patient serum in steadily increasing amounts and assays carried out at each increment, to establish the ratio at which the action of HCS could overcome the inhibition. HCS was unable to effect killing of P1A even when it constituted 90% of the serum mix (Fig 3.11). A killing curve equivalent to HCS alone could only be achieved when HCS constituted 97% of the serum mix (Fig 3.12). This is shown for strains P1A (Figs 3.11 and 3.12). This demonstrated the presence of a potent factor inhibiting bacterial killing present in the serum of P1. The same assays were carried out with the other strains which had demonstrated resistance to killing by autologous serum with similar results. P2A in S2 (Fig 3.13 and P3A in S3 (Fig 3.14)

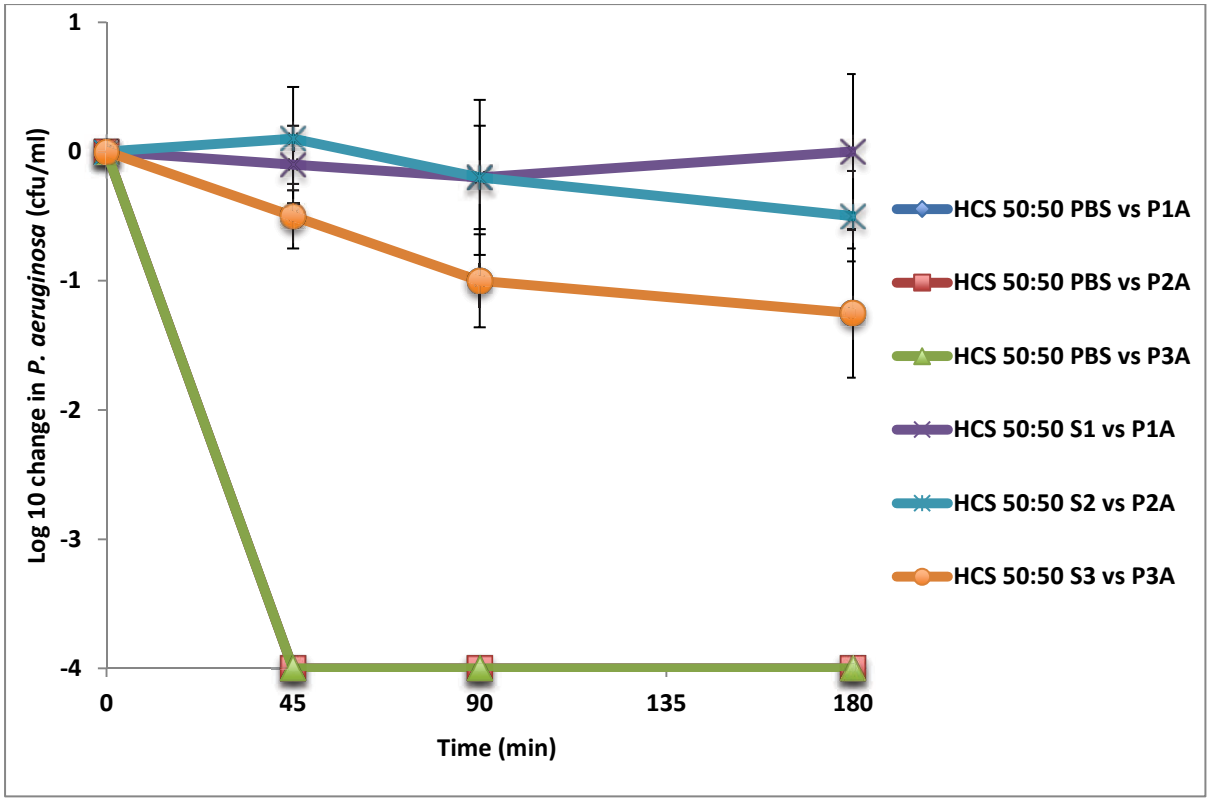

Figure 3.10. Bactericidal assays with mixes of HCS and phosphate buffered saline mixed in a 50%50% ratio showed that HCS retained the ability to kill strains P1A-P3A (curves overlaid for simplicity). When patient serum was mixed 50%/50% with HCS these mixtures were unable to kill autologous strains P1A-P3A. There was a significant change in cfu/ml at 180 minutes incubation for all three strains when HCS mixed with PBS is compared with HCS and autologous strain ($p<0.005$ by Students t test) for all three strains. For all data,assays were performed in triplicate and an average colony count taken at each time point. Error bars represent the mean +/- SD for each assay.

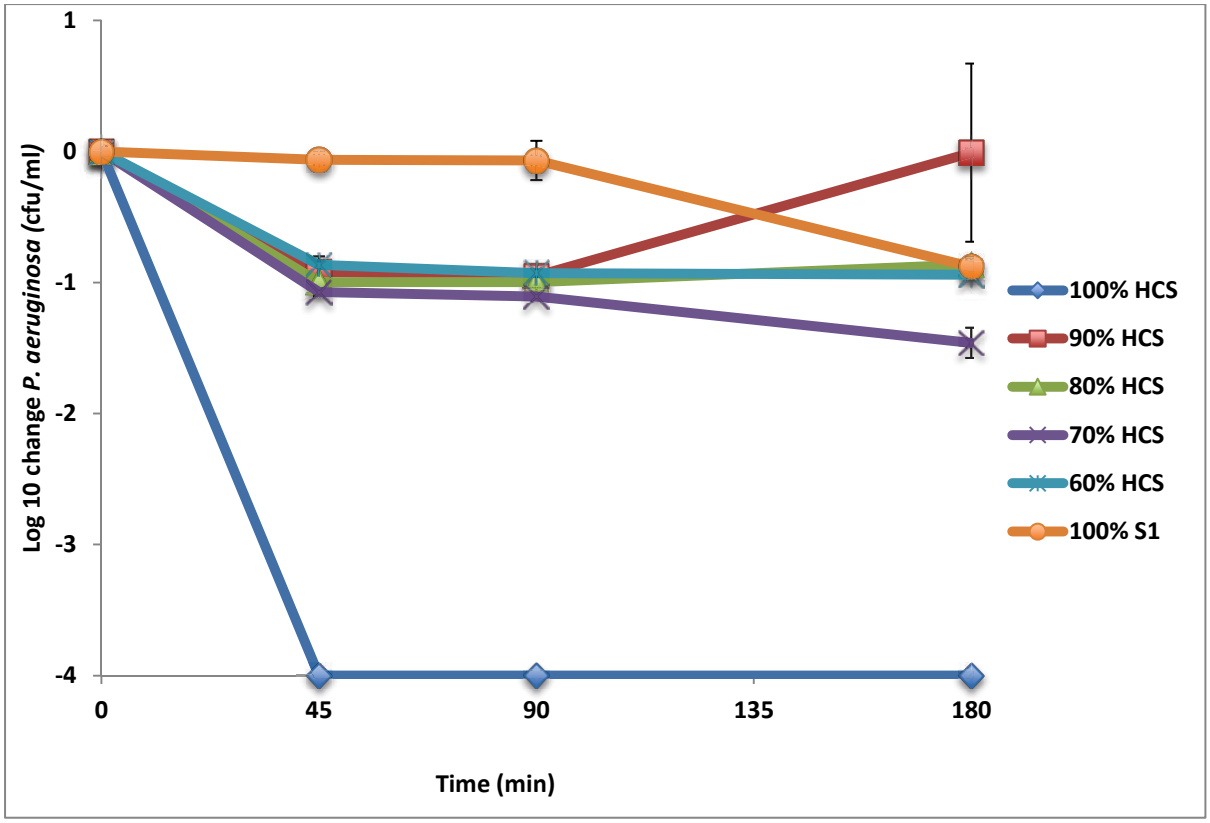

Figure 3.11 demonstrates that with increased concentrations of HCS of up to 90% mixed with autologous serum, serum mediated killing of P1A was still inhibited. Killing was greatest at 180 minutes incubation where the mixture contained 70% HCS but even this constituted only -1 log 10 change from inoculum. For all data, assays were performed in triplicate with average colony count taken at each time point. Error bars represent the mean +/- SD for each assay.

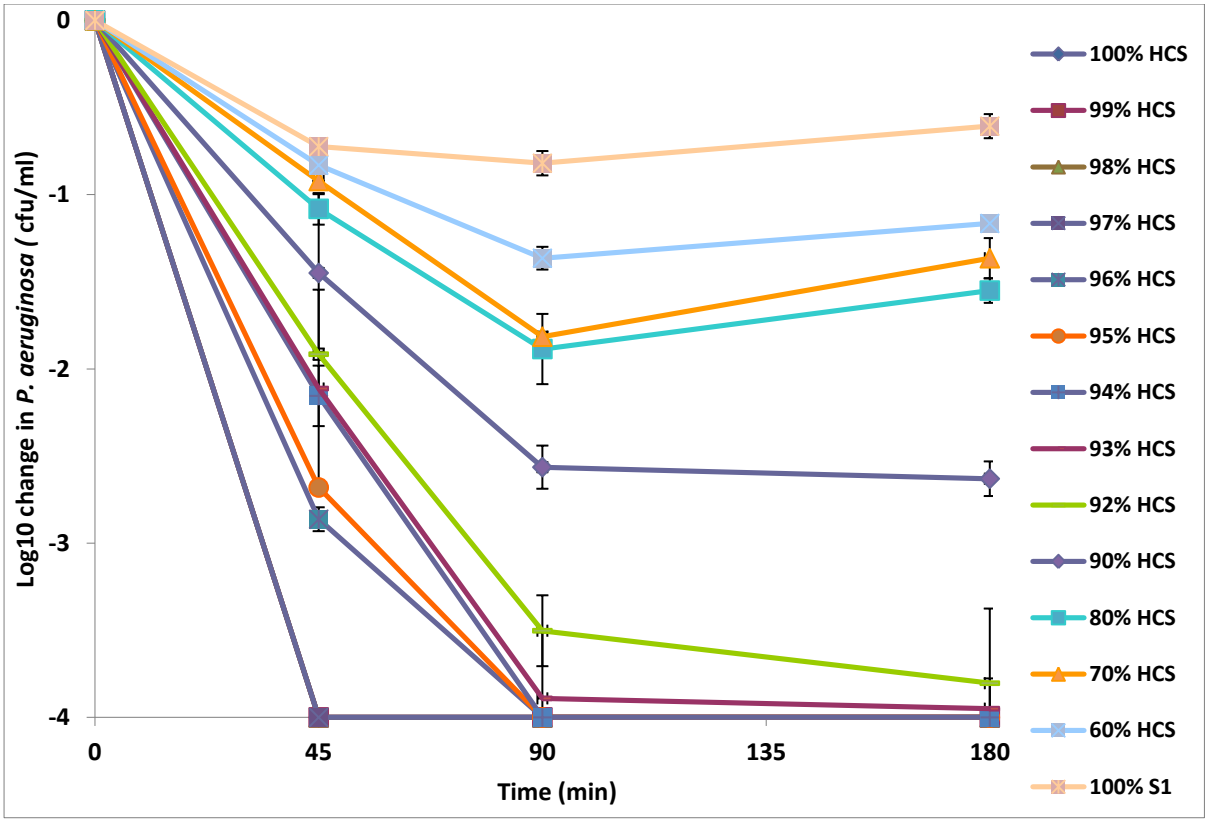

Figure 3.12 demonstrates that killing of P1A in mixes of S1 and HCS (90-100%) showed a steady reduction in cfu/ml at 180 minutes incubation with increasing concentrations of HCS but that complete killing of bacteria at 90 minutes incubation was not restored until the mixture contained 93% HCS and 7% S1. For all data, assays were performed in triplicate with average colony count taken at each time point. Error bars represent the mean +/- SD for each assay.

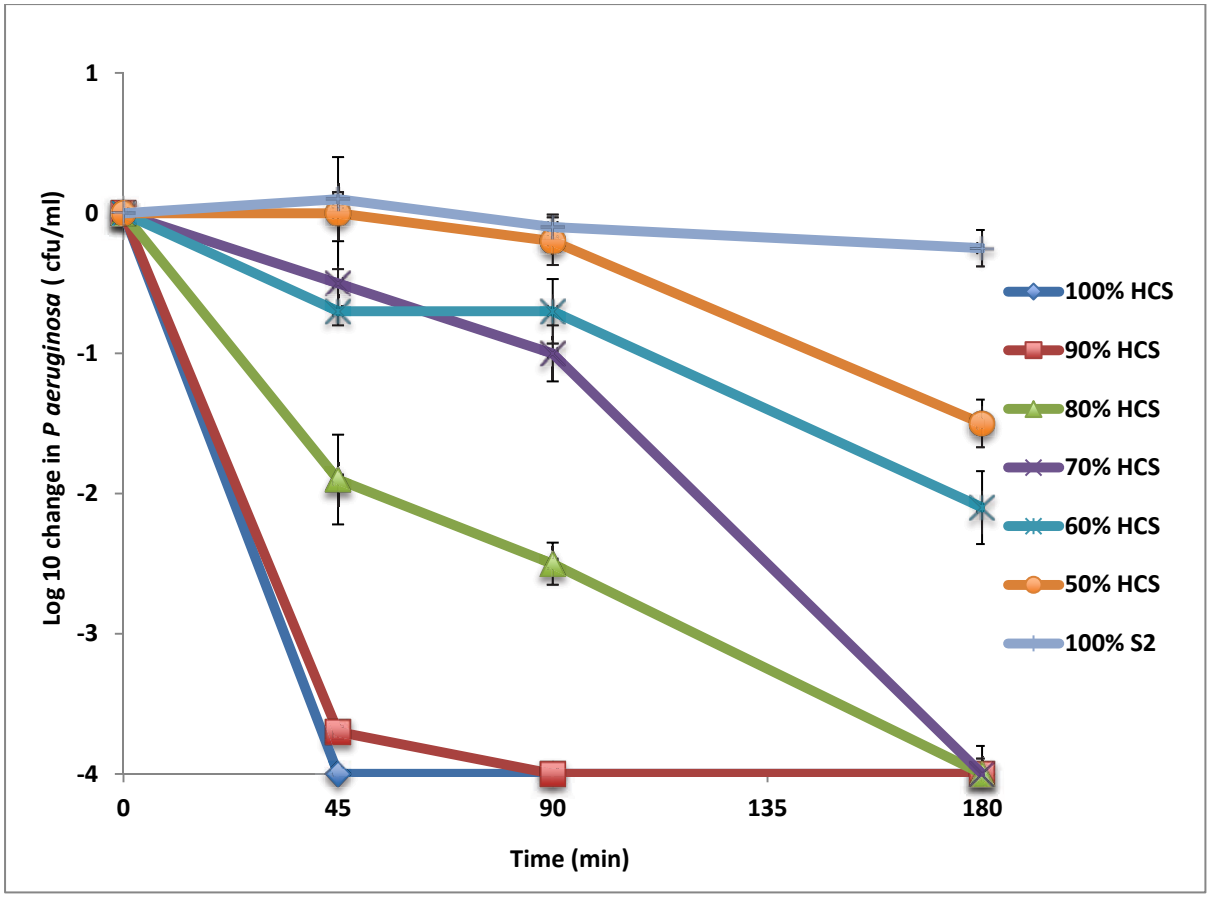

Figure 3.13 S2 in mixes with healthy control sera was able to inhibit serum mediated bacterial killing of P2A until the sera contained 70% HCS at which concentration complete bacterial killing was observed at 180 minutes. For all data assays were performed in triplicate with an average colony count calculated at each time point. Assays were performed in triplicate. Error bars represent the mean +/- SD for each assay.

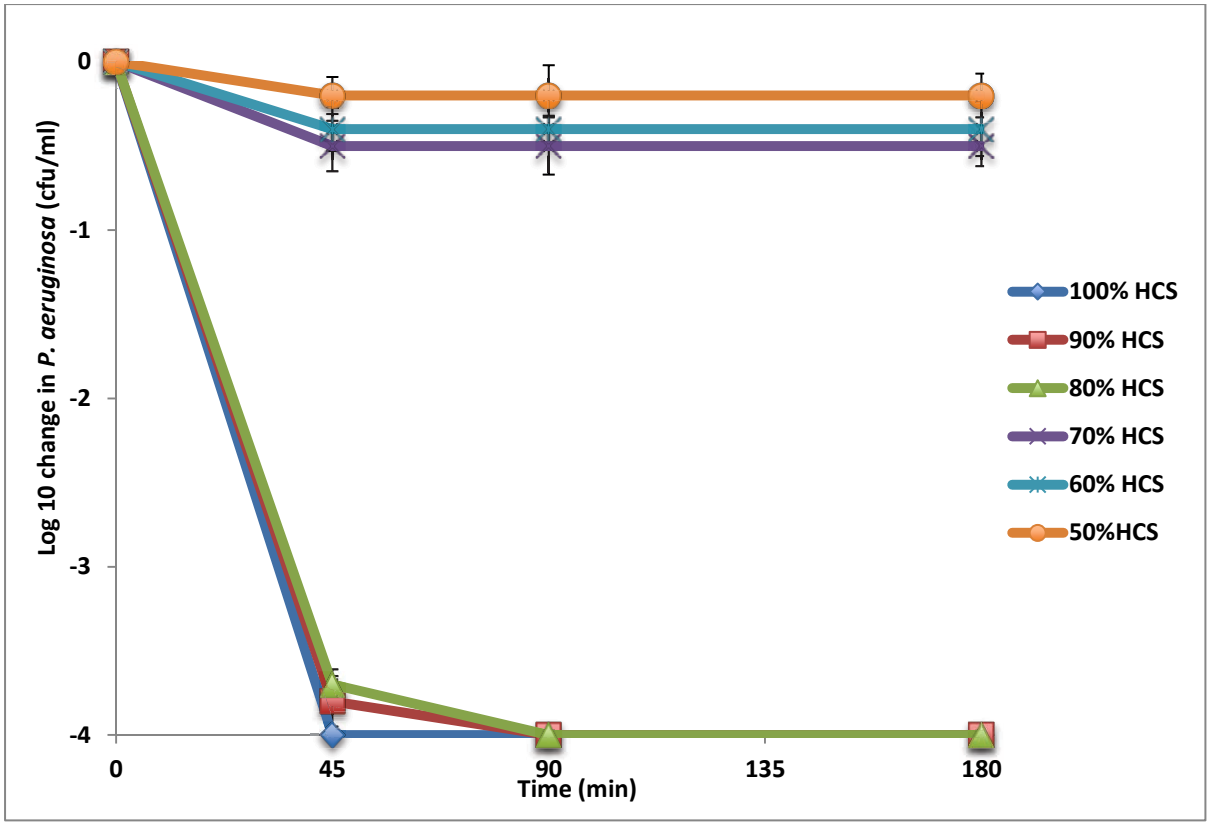

Figure 3.14 S3 in mixes with HCS was able to inhibit serum mediated bacterial killing of P3A until the serum contained 80% HCS at which level complete bacterial killing was observed at 90 minutes. For all data assays were performed in triplicate with an average colony count calculated at each time point. Assays were performed in triplicate. Error bars represent the mean +/- SD for each assay.

The results of bactericidal assays with serum mixes of HCS and patient serum confirmed the presence of an inhibitory factor in the serum of patients P1-P3 which inhibited killing of autologous bacterial strains. Complete killing by S1–3 was only restored when HCS represented 93, 70, and 80%, respectively, of the mixed sera indicating a potent capacity to inhibit bacterial killing.

3.8 Conclusion

Analysis of the serum and sputum samples from a well characterised group of patients with non CF bronchiectasis demonstrated the presence of a potent inhibitory factor in the serum of 3 of these patients from a cohort of 11 patients chronically colonised with *P. aeruginosa.* Detailed analysis of the serum and sputum from these patients demonstrated that this factor modulating serum killing is common to S1-3 and P1-3 but absent from the other sera and *P. aeruginosa* strains isolated from the remaining 8 patients. The patients all had normal serum immunoglobulin levels but specific anti *P. aeruginosa* IgG levels were higher than seen in HCS. High levels of MAC deposition were also demonstrated for autologous serum on these strains again suggesting that immune deficiency was not the underlying reason for the inability of S1-S3 to kill autologous strains. Strains P1-P3 were also shown to be serum sensitive strains both in HCS and in the sera of other patients colonized with *P. aeruginosa* confirming the capacity for bacterial killing was present but the defect resided in the patient's plasma but was only relevant to their autologous strains.

The next step was to identify the nature of the factor inhibiting serum bacterial killing.

CHAPTER 4 IDENTIFICATION OF SERUM INHIBITORY FACTOR

The aim of the experiments described in this chapter were to isolate and identify the inhibitory factor/s present in patient serum responsible for the blocking of bactericidal activity. Unless stated otherwise serum from patient P1 and strain P1A were used in the following experiments. This serum had previously shown potent ability to block bacterial killing in healthy control serum.

4.1 Fractionation of P1 Serum

To determine the molecular weight of the inhibitory factor, S1 was fractionated as previously described in chapter 2.5.1 together with HCS. The serum was fractionated into ranges of molecular weights >300kDa, 100-300kDa, 30-100kDa and <30kDa and standardised to 1ml with the addition of PBS, as previously described.

Bactericidal assays were then performed as previously described using each recovered fraction of S1 and HCS with P1A as the target organism to determine which fraction contained the blocking factor. 45 μL of each fraction recovered from S1 and HCS was mixed 50:50 with a further whole sample of HCS and utilised in this assay, and 45 μL of PBS was added to 45 μL of HCS as a control.

HCS was able to kill P1A bacteria when mixed with all fractions except for 100-300 kDa of S1 ($p=0.000$) (Fig 4.1). This demonstrated that the inhibitory factor found in S1 has a molecular weight between 100 kDa and 300 kDa.

IgG has a molecular weight of 160kDa (2 light chains weigh 23-25kDa each and 2 heavy chains weigh 53kDa each). IgA similarly has a molecular weight of approximately 160kDa, whilst IgM weighs approximately 970kDa (Janeway *et al*., 2001). It was therefore hypothesised that the blocking activity in S1 serum was IgG mediated. Previously, it was demonstrated that there were high titres of IgG expressed by S1, S2 and S3 against

autologous bacterial strains suggesting a potential role of IgG in serum blocking activity. Therefore, we aimed to determine if patient IgG could inhibit HCS bactericidal killing.

4.2 Purification of Immunoglobulin G (IgG) From Serum

To determine if IgG was responsible for the inhibition of serum killing of *P. aeruginosa* we extracted the antibody from patient serum to determine if IgG-depleted sera could also inhibit HCS killing. Furthermore, the purified patient-IgG could be added to HCS to determine if it was sufficient alone to inhibit bacterial killing. IgG purification was first carried out using Protein A Sepharose 4B column as described in Methods 2.5.3. IgG was also purified by affinity chromatography using protein G immobilised on agarose as described in methods 2.5.4.

Bactericidal assays were carried out as previously described. Recovered fractions of S1 serum deplete of IgG was added incrementally to HCS to a total volume of 90μL. Antibody depleted S1 from the protein A column mixed with HCS did not inhibit the bactericidal activity of HCS supporting the role of IgG in inhibiting killing of P1A in autologous serum. However 90 μL of S1 depleted of IgG alone still inhibited killing of P1A suggesting incomplete removal of IgG by the protein A column. (Fig 4.2) IgG depletion was therefore also performed using a protein G column with Protein G having a high affinity for all subclasses of human IgG. (Table 2.4). Assays using recovered flow through of S1 depleted of IgG using protein G extraction showed that 90 μL S1 depleted of IgG was able to kill P1A at 180 minutes incubation. (Fig 4.3)

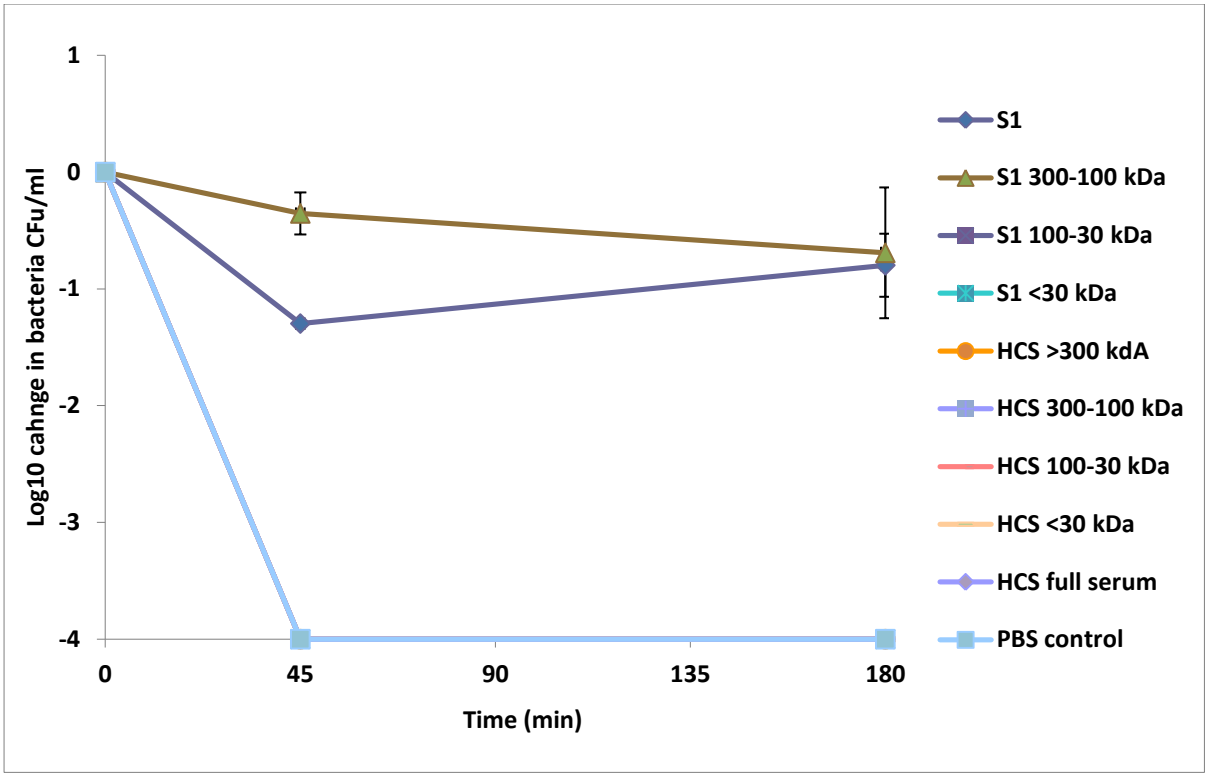

Figure 4.1 Serum assays of fractionated S1 and HCS. Each fraction (45µL) was added to 45µL of HCS for each assay. Killing of strain P1A was inhibited in S1 as previously shown. It was also inhibited by S1 fractions containing serum components with weight 100-300KDa. Bacterial killing after 180 minutes incubation was significantly different in serum containing this S1 fraction when compared to all fractions of HCS (p=0.0009 by Student's t test). Removal of these fractions from S1 serum restored killing of P1A after 45 minutes incubation in assays containing S1 fractions with molecular weight < 100kDa. P1A was sensitive to serum killing with assays containing all fractions of HCS. Curves overlaid for simplicity. Assays were performed in triplicate. Error bars represent the mean +/- SD for each assay.

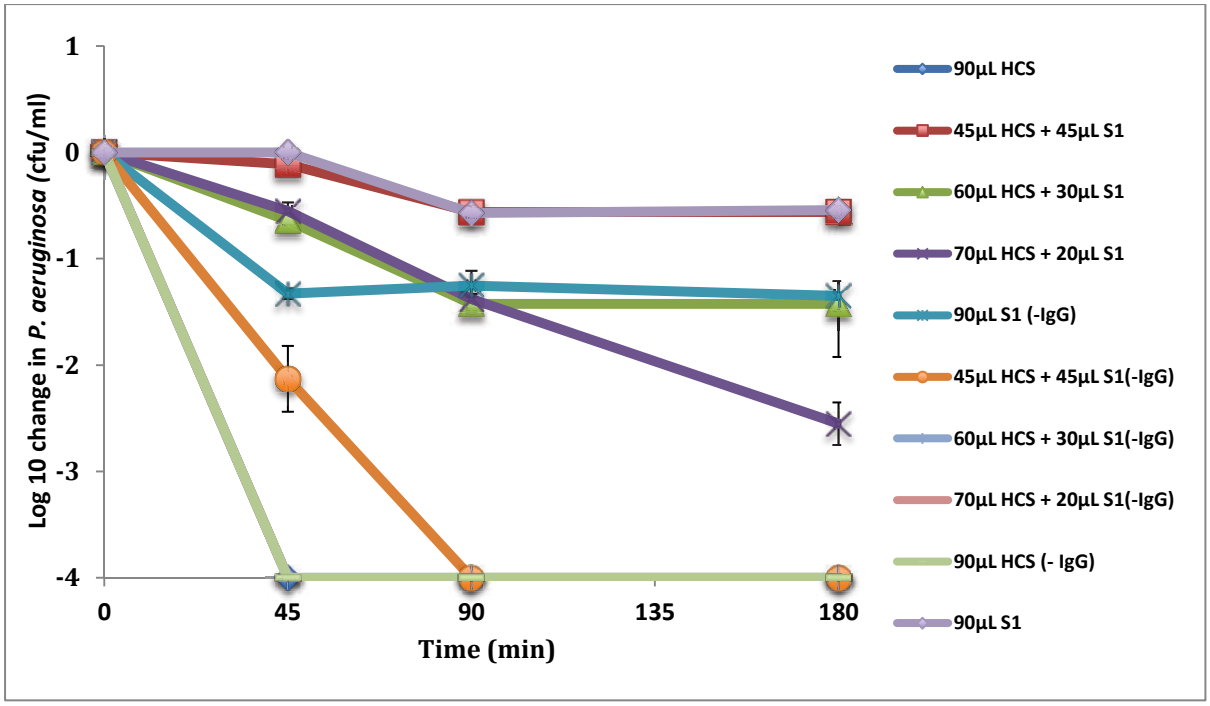

Figure 4.2 Serum assays of IgG deplete serum from protein A column vs P1A. Bacterial killing was inhibited when S1 was mixed with HCS confirming the presence of the inhibitory factor prior to IgG depletion. Where HCS was added to IgG deplete S1 serum bacterial killing was present at 45 minutes incubation. (Lines representing 60µL HCS + 30µL S1(-IgG), 70µL HCS+20µL S1(-IgG) and 90µL HCS (-IgG) overlap with all strains dead at 45minutes incubation). Inhibition of killing was seen where 90µL S1 (-IgG) was utilised. This is thought to be due to incomplete removal of IgG by protein A and possibly depletion of other serum factors needed to effect killing. There was a significant difference in bacterial killing between 45µL HCS mixed with 45µL of S1 with and without IgG at 180 minutes incubation ($p= 0.0001$ measured by Student's t test). Similarly with mixes of 60 µLof HCS and S1 with and without IgG there was a significant difference in bacterial killing at 180 minutes incubation ($p= 0.0009$ measured by Student's t test). This is representative of 3 independent assays. Error bars represent the mean +/- SD for each assay.

4.3 Bactericidal Assays Using IgG Depletion with Protein G

Bactericidal assays were repeated using 4 recovered fractions of S1 following IgG extraction with protein G and then mixed with HCS. When HCS was added to IgG deplete fractions of S1 following protein G depletion, bacterial killing was restored at 45 minutes incubation for fractions 2,3 and 4 and 90 minutes for fraction 1. Where 90µL of S1 deplete of IgG was utilised killing was restored at 180 minutes (Fig 4.3). The next step was to establish whether addition of the S1 eluate to HCS would inhibit bactericidal activity.

4.4 Addition of S1 Eluate to HCS

Bactericidal assays were performed as described previously. Using 45µL of IgG eluate added to 45µL of HCS with a HCS/PBS control. This mixture was able to inhibit killing of P1A compared to HCS/PBS control, killing P1A after 45 minutes incubation demonstrating that the addition of IgG directly to HCS was sufficient to inhibit bacterial killing (Fig 4.4). To establish the potency of IgG required to introduce inhibition of bacterial killing to HCS, purified S1 IgG (eluted from protein G column) was added to HCS in varying amounts and bactericidal assays were carried out as before. It was established that only 4% of S1 IgG was needed to inhibit all killing of bacteria up to 180 mins incubation. Bacterial killing was restored in HCS when the concentration of IgG eluate was 1/64 after 90 minutes incubation. This data demonstrated that bactericidal killing was inhibited at IgG proportion of 6.25% and only restored at 90 minutes incubation with a proportion of IgG equating to 3.125%. (Fig 4.5) This confirmed previous findings that the potent inhibitory factor present in S1 is IgG. The aim of the following experiments was to establish which subclass of IgG was responsible for this inhibitory behaviour.

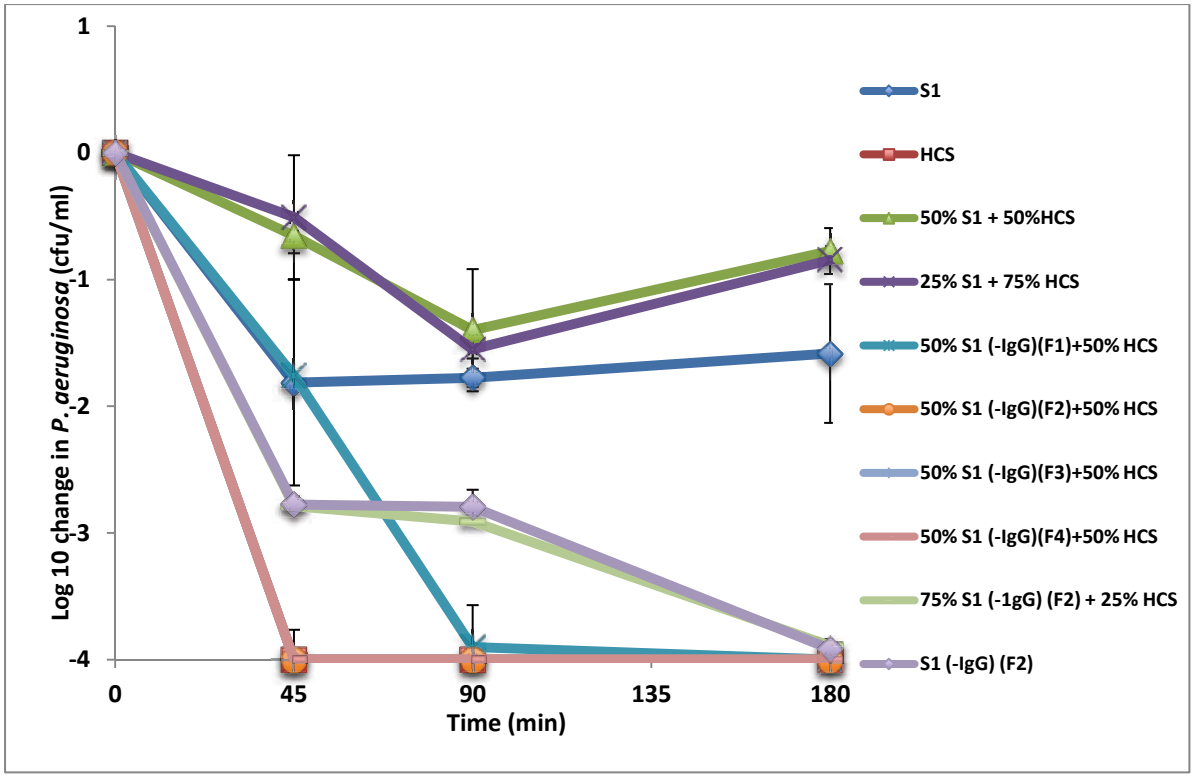

Figure 4.3 Bactericidal assays of recovered fractions of IgG depleted S1 using protein G column against P1A. Inhibition of bacterial killing of P1A was found with S1 added to HCS. When HCS was added to IgG deplete fractions of S1 bactericidal killing was restored at 45 minutes incubation (fractions 2, 3 and 4 – curves overlaid for simplicity). There was a significant log 10 difference in cfu/ml between 50%S1 and fractions 2,3, an 4 mixed with HCS at 45 minutes incubation ($p=0.006$ by Student's t test). Bactericidal killing was restored at 90 minutes for fraction 1. Where the assay was performed using larger amounts of IgG depleted S1 (75% and 100%) killing was restored at 180 minutes incubation (curves overlaid for simplicity). This is representative of 3 independent assays. Error bars represent the mean +/- SD for each assay.

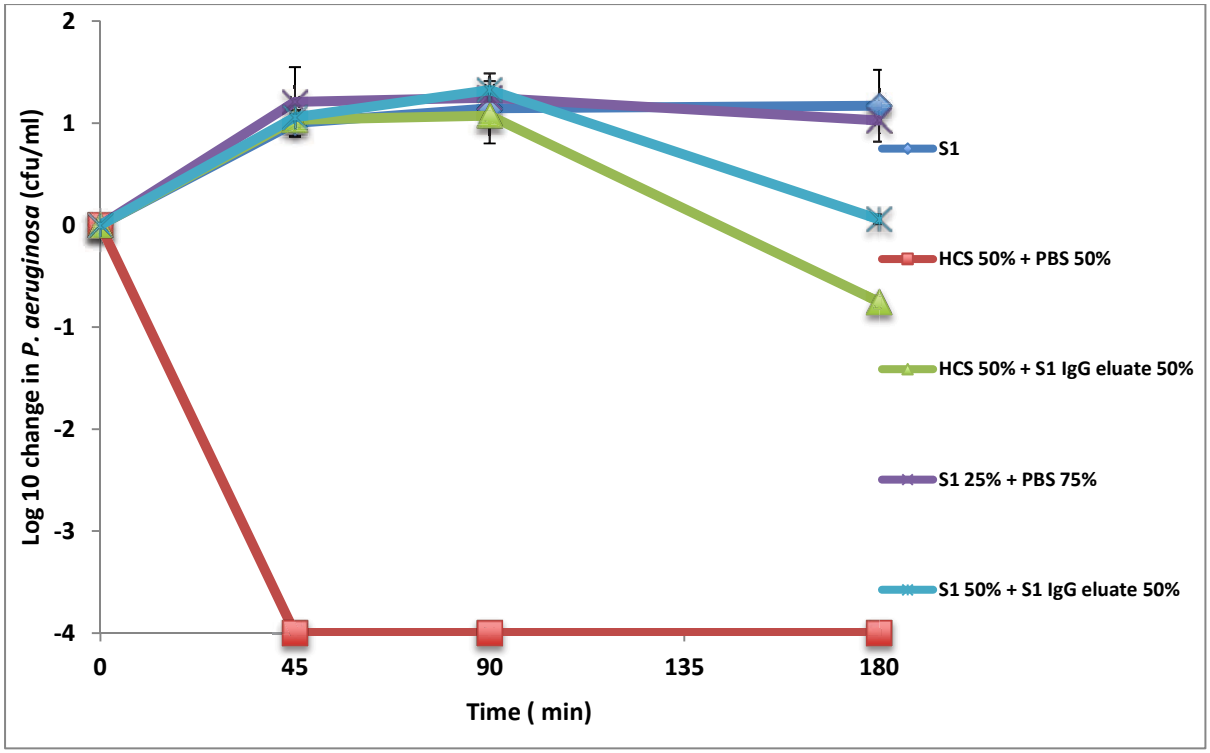

Figure 4.4 depicts bactericidal assays with S1 IgG eluate added to HCS vs P1A. The addition of IgG eluate to HCS inhibited the bactericidal activity of HCS to P1A. HCS retained bactericidal activity with the addition of PBS. The inhibitory activity of S1 serum was maintained with the addition of PBS to S1. There was a significant difference in bacterial killing after 180 minutes incubation when HCS was mixed with 50% PBS or mixed with 50% IgG eluate ($p=0.0003$ by Student's t test). This is representative of 3 independent assays. Error bars represent the mean +/- SD for each assay.

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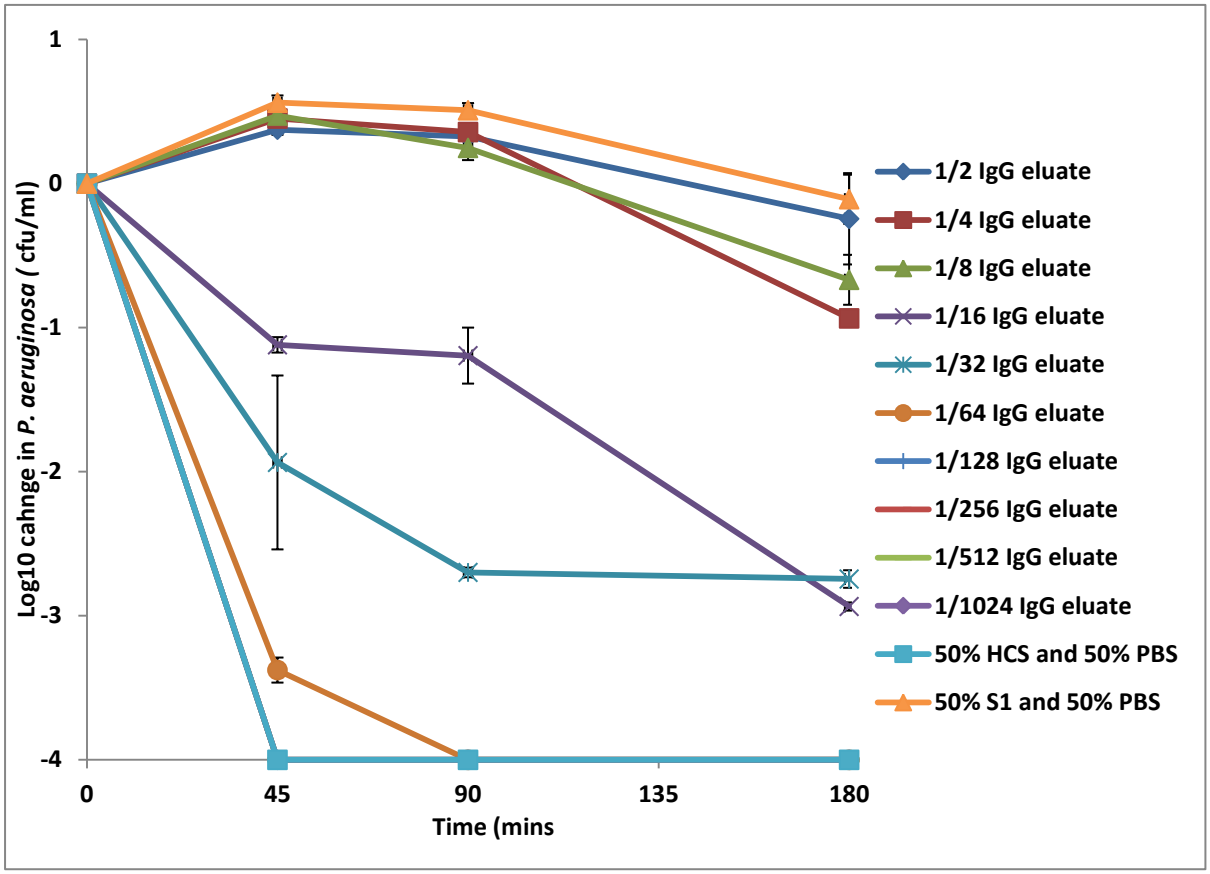

Figure 4.5. Bactericidal assays with serial dilutions of S1 IgG eluate vs P1A. Bacterial killing was inhibited in HCS with dilutions of 1/2 to 1/32 IgG eluate. Bactericidal killing was restored in HCS at 90 minutes incubation with concentrations of 1/64 IgG eluate. Higher numbers of colony forming units were present with increased concentrations of IgG eluate in HCS. Data are representative of 3 individual assays. Error bars represent the mean +/- SD for each assay.

4.5. Identification of IgG Subclass Responsible for Inhibition of Bacterial Killing

If it was a specific IgG subtype leading to the inhibition of serum killing, it should bind to the relevant patient strains more than patients without inhibition. To determine the levels of IgG1,2,3 and 4 binding to each patient isolate we used flow cytometry as described in methods. Patients 1-3 did not have statistically different levels of IgG1, 3 or 4 binding to their cognate strains compared to patients 4-11. In contrast, these three patients had much higher IgG2 binding to their cognate strains than any of the other patients. HCS also did not have significant IgG2 binding to these three strains. (Fig 4.6)

The high titres of IgG2 seen in the blocking sera suggested that IgG2 was the responsible specific inhibitory factor in the serum of patients S1-S3. This experiment was also replicated measuring IgG3 and IgG4 titres against autologuous strains where no significant differences were seen in IgG3 and IgG4 subtype titres between blocking and non blocking sera.. (Fig 4.7)

This finding was not unexpected as both IgG3 and IgG4 contribute only 11% of total IgG in human serum. IgG3 provides the immune response against protein and polypeptide antigen but the role of IgG4 is not fully established. The results of both figure 4.6 and 4.7 suggested that IgG2 was likely to be the factor inhibiting killing of bacterial strains in patients P1-P3.

To determine the role of IgG2 further, immunofluorescent labeling of P1A was performed using first IgG depleted S1 serum (S1-IgG) as the primary antibody. Immunofluorescence labelling of P1A cells with either FITC labelled anti- human-IgG1 or anti human-IgG2 specific secondary antibodies was also performed. S1 serum and HCS were used as primary

antibodies (methods 2.11). The aim was to visualise antibody subgroup binding to bacteria and determine any differences between blocking sera and HCS.

Immunofluorescence labelling of P1A cells showed that there were marked differences in visualised IgG2 binding between samples when HCS and S1 serum were used as primary antibodies showing a major increase with S1. This adds further support to the findings of flow cytometry. Immunofluorescence also confirmed that S1 serum with IgG removed by the protein A column had little detectable IgG1 or IgG2. (Fig 4.8)

Flow cytometry and immunofluorescence therefore suggested that IgG2 was likely to play a role in inhibiting bacterial killing. To determine further if IgG2 was responsible for the serum-inhibition, the effect of removing IgG2 from serum was examined. IgG2 was removed and purified from S1 by passing the serum over an affinity column coated with a monoclonal antibody against human IgG2 (Jefferis *et al*, 1992) followed by elution as described in methods 2.5.7. Bactericidal assays were performed as previously described in methods. HCS was mixed with IgG2 deplete S1 and purified IgG2 in a 50/50 ratio. Killing was inhibited in in HCS when mixed with purified IgG2 whilst P1A was killed after 180 minutes incubation when mixed with S1 serum depleted of IgG2. This demonstrated the central role of IgG2 in inhibiting bacterial killing. IgG2 deplete flow- through lost its inhibitory ability whilst IgG2 subsequently eluted from the column blocked the serum bactericidal activity of HCS.

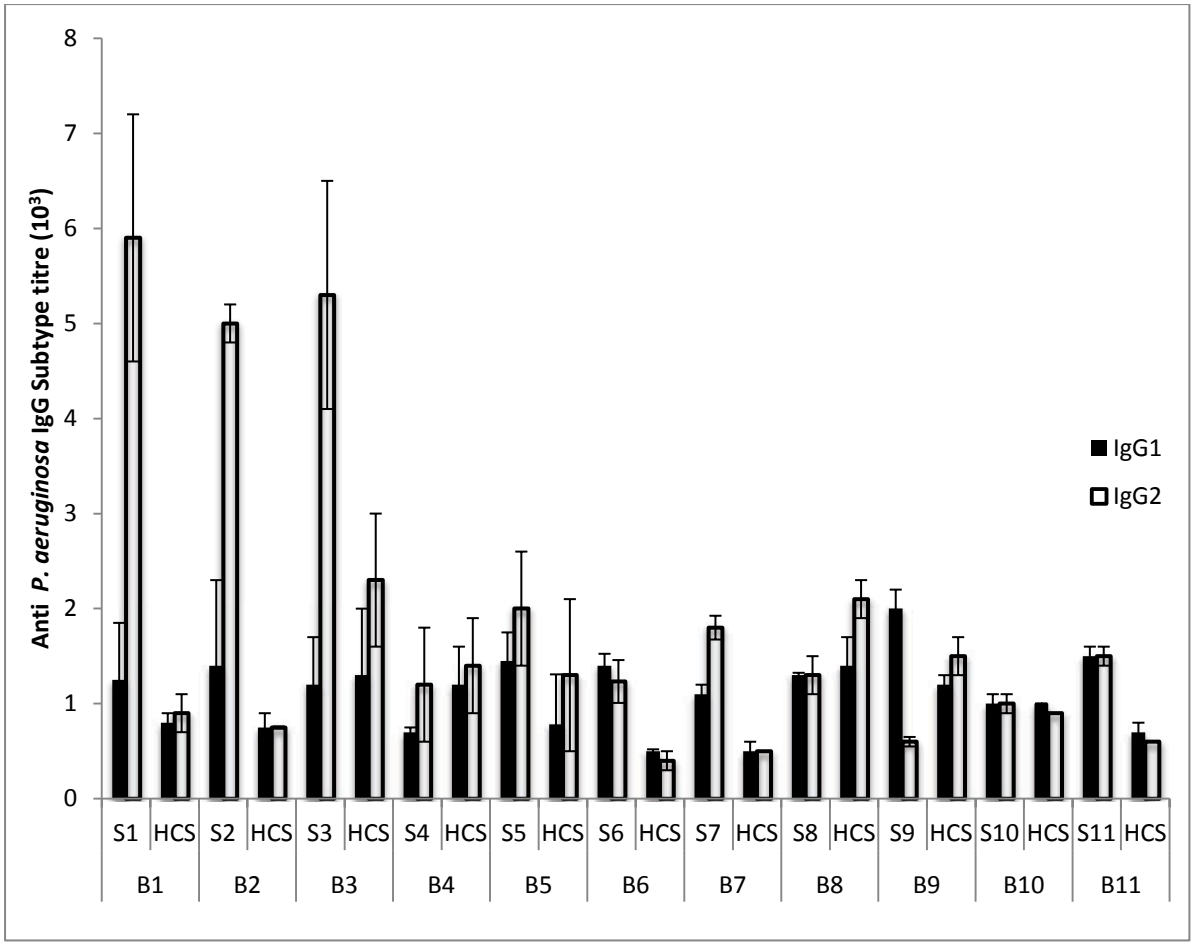

Figure 4.6 Anti *P. aeruginosa* titres of IgG1 and IgG2 isotypes in HCS and patient sera specific to autologous strains as determined by FACS. IgG1 titres were not statistically different between the blocking sera (S1-S3) and those without blocking activity (S4-S11). S1 – S3 demonstrated statistically higher ($p<0.001$) IgG2 titres compared with S4-S11.

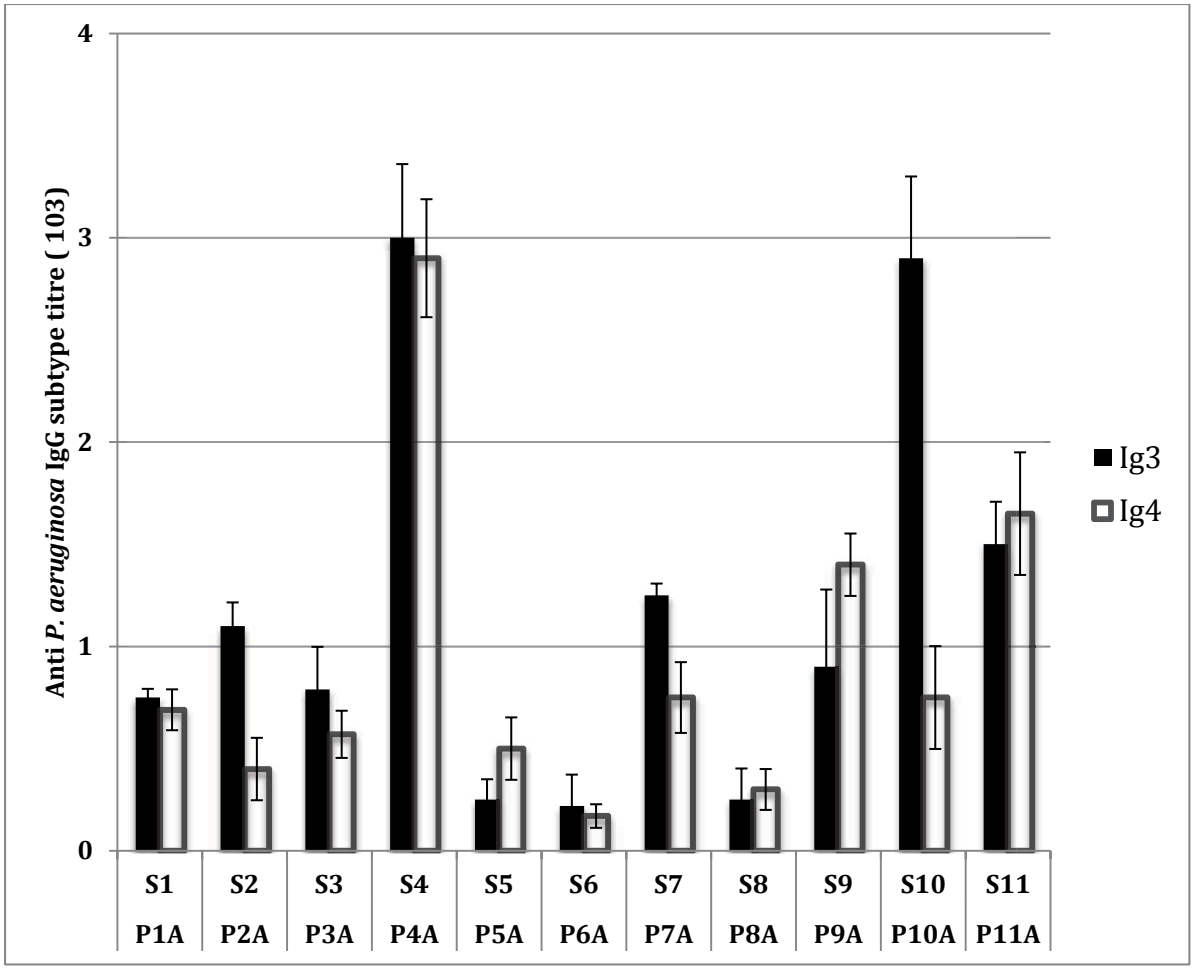

Figure 4.7 Titres of IgG3 and IgG4 isotypes in patient sera specific to autologous strains. No significant differences were found between the blocking sera (S1-S3) and non blocking sera (S4-S11).

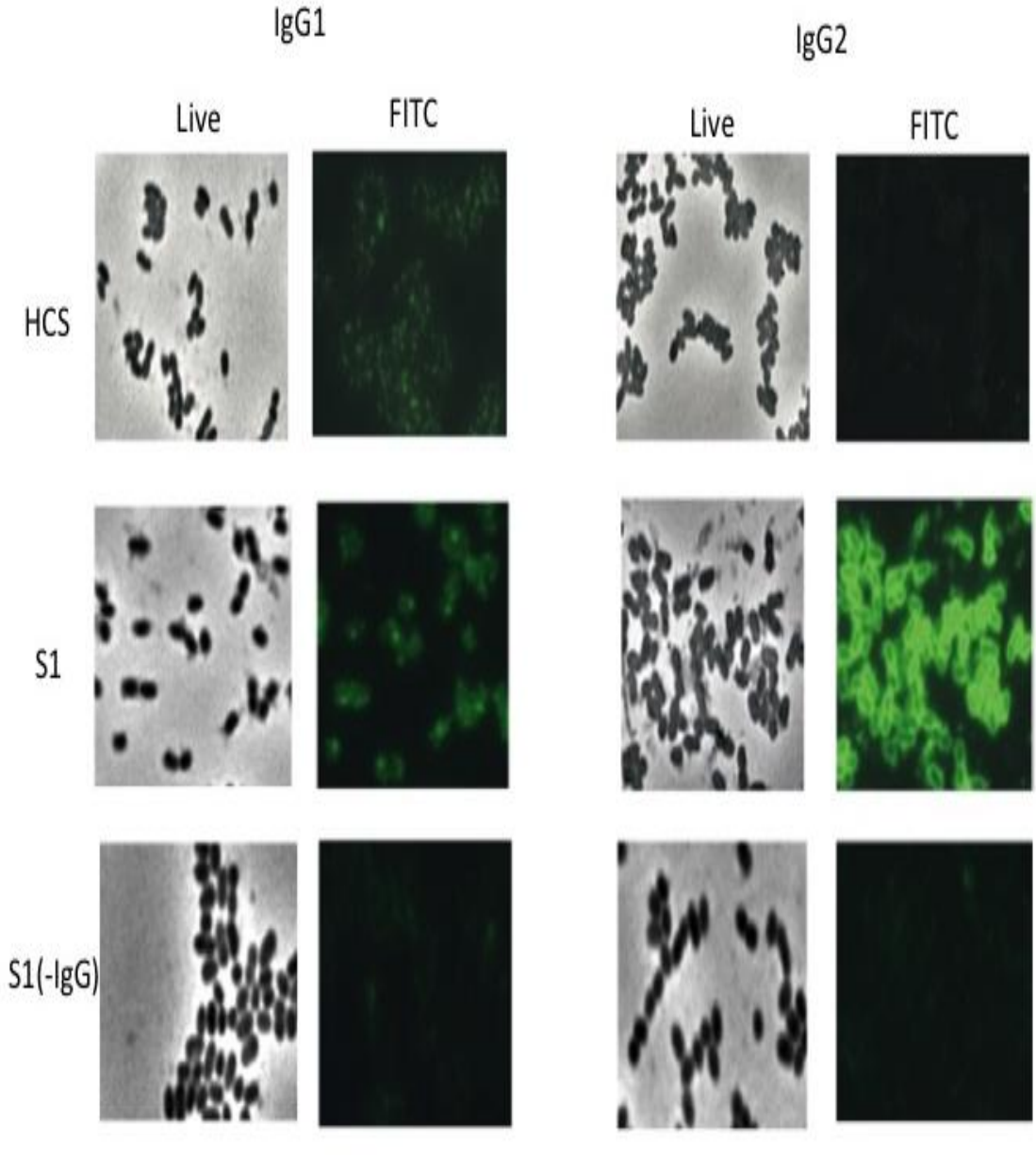

Figure 4.8 Immunofluorescence microscopy of P1A in S1 and HCS labeled with antihuman IgG1 and IgG2 shows a clear difference in observable IgG2 binding with S1 compared to HCS. Immunofluorescence of S1 which had been passed through a protein A column to deplete IgG has little detectable IgG1 or 2 binding suggesting that these had been successfully removed from serum.

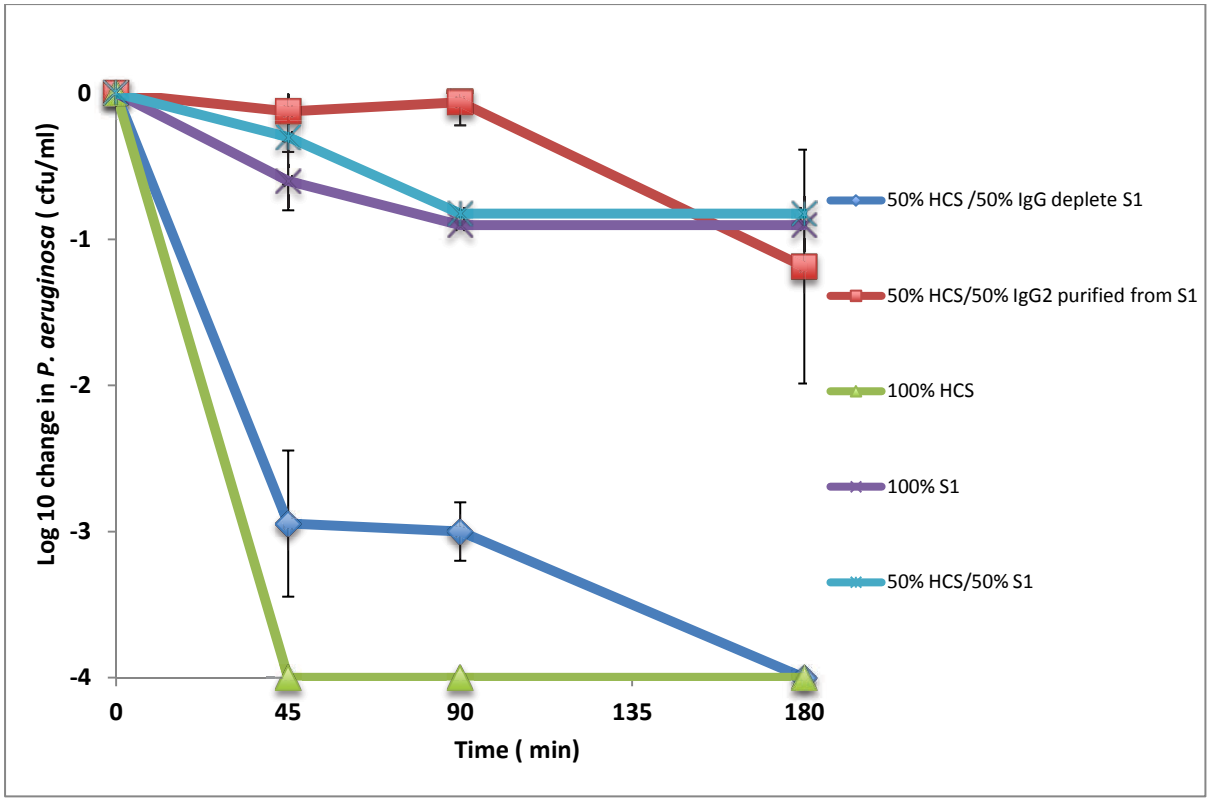

Figure 4.9 depicts bactericidal assays against P1A using 1gG2 purified from S1 mixed with HCS, and S1 depleted of IgG2 mixed with HCS in a 50/50 ratio. Purified IgG2 inhibited bacterial killing when added to HCS whereas the addition of IgG2 depleted S1 to HCS restored bacterial killing of P1A at 180 minutes incubation with a significant difference in cfu/ml after 180 minutes incubation ($p=0.003$ by Student's t test). As before P1A was resistant to killing by S1 and fully sensitive in HCS. Bacterial killing was also inhibited in HCS mixed 50% with S1. Data are representative of 3 individual assays. Error bars represent the mean +/- SD for each assay.

4.6 Conclusion

IgG2 was demonstrated to be the inhibitory factor present in inhibitory serum. Initially it was shown that removal of total IgG from inhibitory serum restored bactericidal activity. This was shown to be a potent inhibitor of bacterial killing with eluted IgG from inhibitory serum able to inhibit killing by HCS even when present in small quantities. FACS and immuofluorescence microscopy demonstrated that the IgG subgroup likely to be responsible for the inhibitory actions of S1 serum was IgG2 as it demonstrated avid binding to P1A visualised by immunofluorescence and was present in significantly higher titres in blocking sera compared to non blocking sera. Removal of IgG2 restored the ability of S1 serum to kill autologous strains of *P.aeruginosa*, whilst addition of the recovered IgG2 eluate to HCS inhibited the ability of HCS to kill these strains.

CHAPTER 5 IDENTIFYING BACTERIAL TARGET FOR INHIBITORY IgG2

5.1 Analysis of Bacterial Strains

Having established that an inappropriate IgG2 response in the serum of patients P1-P3 was responsible for inhibition of bacterial killing of *P. aeruginosa* strains isolated from these patients, the next step was to characterise the bacterial strains isolated from these patients. IgG2 forms the prevalent immune response to polysaccharide antigens in humans, therefore the following experiments were specifically targeted to examining the properties of the lipopolysaccharide (LPS) component of *P. aeruginosa.* In particular, the O-antigen side chain was studied given its immunogenic properties (Joiner *et al*., 1984; Knirel *et al*., 2006; Doring and Pier, 2008; King *et al*., 2009).

The initial aim was to determine whether or not there were any features of the *P. aeruginosa* strains isolated from the sputum of patients P1-P3 which were different from the strains isolated from other patients P4-P11. LPS preparations were made from P1A, as described in methods section 2.8.1.

5.2 Silver Stained Lipopolysaccharide Gels

Bacterial LPS was purified from all strains separated on a SDS-Page gel and visualised using SilverQuest Kit (Invitrogen), as described in methods chapter 2.9. Silver staining of LPS preparations revealed that the strains P1A, P2A, and P3A produced significant amounts of long-chain O-antigen in contrast to the isolates from other patients, which had no detectable O-antigen. The best representation of silver stained LPS gel is shown in Figure 5.1.

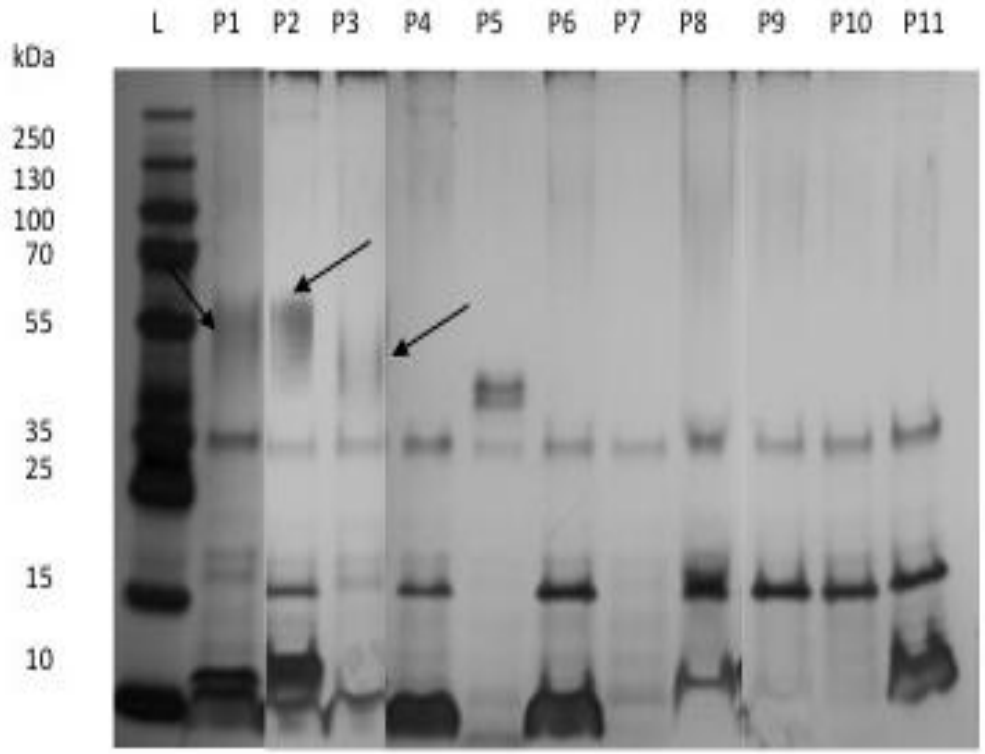

Figure 5.1 A pattern of banding was seen on analysis of P1A, P2A and P3A strains visualised by silver stain of polysaccharide preparations of *P. aeruginosa*, between 35 -55 kDa. This banding was not seen in the other strains analysed. This corresponds to the long chain O-antigen of LPS and was expressed by the 3 strains, which were resistant to killing in autologous sera.. Silver staining gels were repeated 4 times in total with consistent results. The images shown indicate the results for several patient strains and the LPS bands are indicated by arrows.

Having identified this feature that was common to the *P. aeruginosa* strains P1A, P2A and P3A, Western immunoblotting was performed to assess whether inhibitory IgG2 targeted a specific component of bacterial structure. Western blotting of outer membrane protein (OMP) preparations, (Fig 5.2) and LPS preparations were performed with patient serum and subsequent anti-human IgG labelling as described in methods 2.10. S1 was shown to recognise the outer membrane proteins of each strain with no clear difference between the response to P1A-P3A compared to the response to P4A-P11A (Fig 5.2). S1 was also shown to recognise the O-antigen chains of LPS from P1A, P2A and P3A with no detectable response to the LPS preparations from the remaining eight strains P4A-P11 (Fig 5.3). S2 and S3 similarly recognised O-antigen of P1A, P2A and P3A. In contrast HCS had no detectable IgG response to the O-antigen from P1A, P2A or P3A (Fig 5.4 and 5.5). This indicated that the target of inhibitory IgG was LPS and more specifically O-antigen expressed by strains P1A, P2A and P3A.

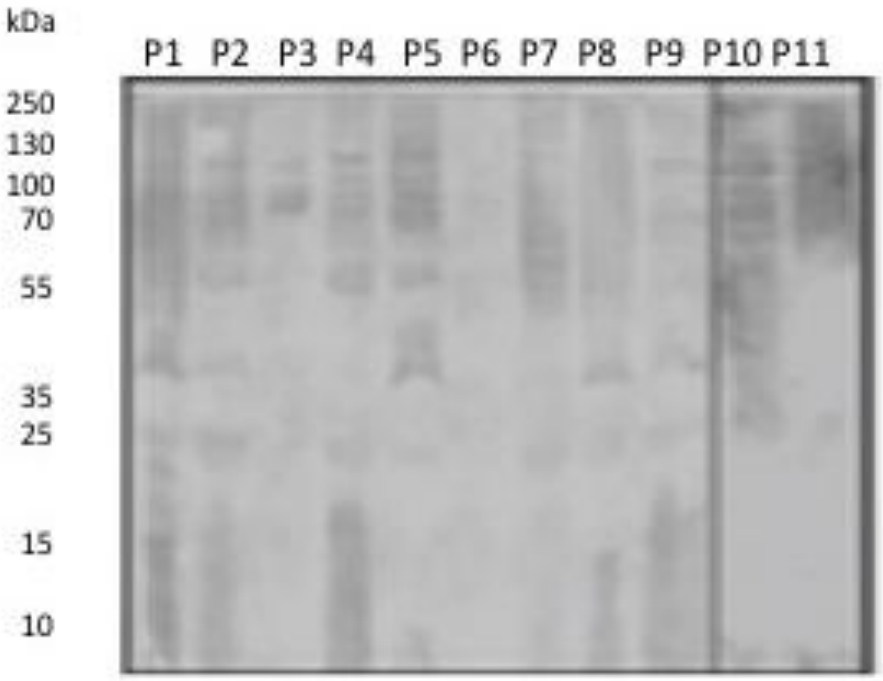

Figure 5.2 Western immunoblotting of outer membrane proteins preparations of all *P. aeruginosa* strains with S1 as the primary antibody source and anti human IgG as the secondary antibody. The gel demonstrated that S1 bound to several outer membrane proteins from all 11 patient strains with not visible difference between strains resistant to killing by S1.

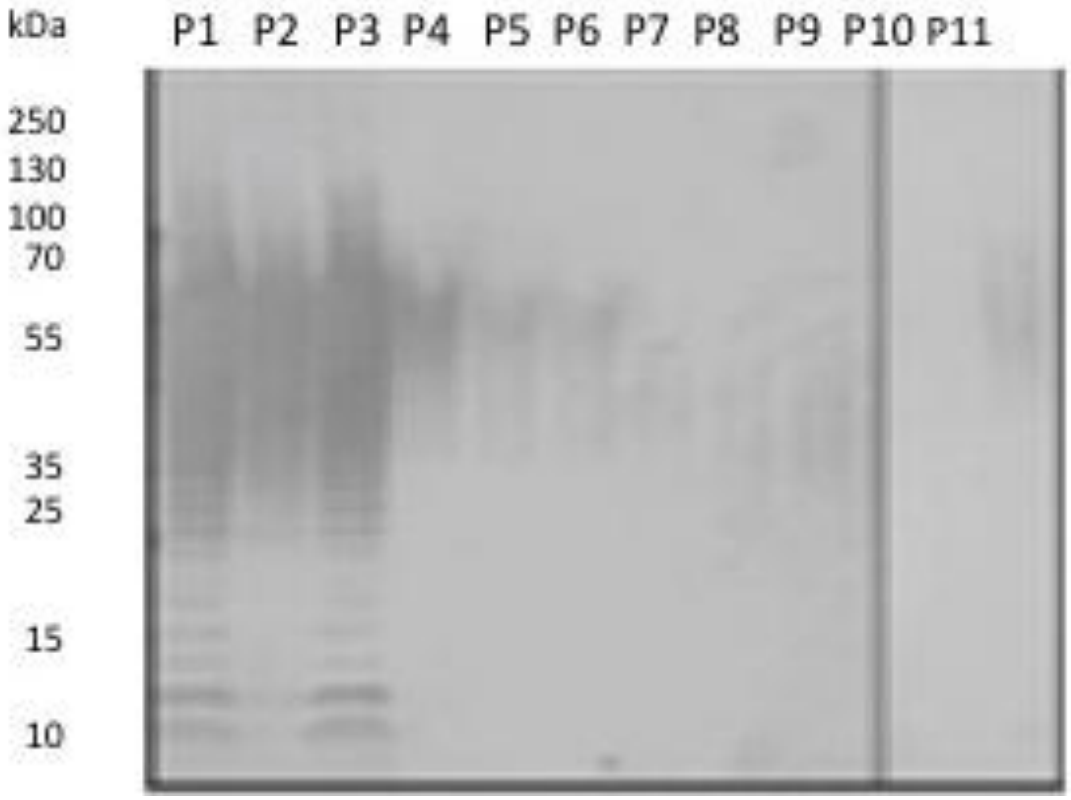

Figure 5.3. Western immunoblotting of LPS preparations of all *P. aeruginosa* strains with S1 as antibody source and anti human IgG as secondary antibody showed that S1 bound to the O-antigen of P1A, P2A and P3A with no or minimal binding to the other strains (P4-11A).

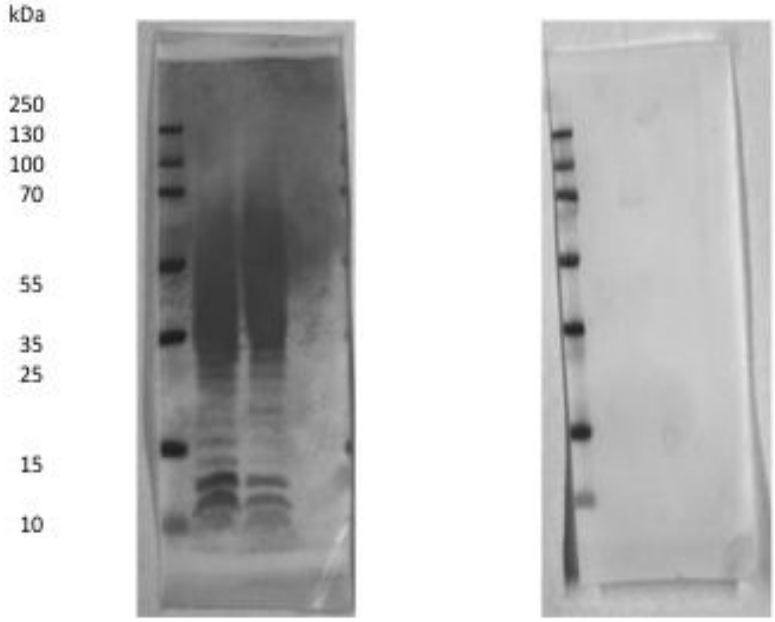

Figure 5.4 shows a comparison of Western Immunoblotting with S1 and HCS as the primary antibody source. LPS preparations of P1A were separated on SDS-Page and the presence of O antigen was detected using S1 sera (image on the Left), or HCS (image on the Right) as the primary antibody, and anti-human IgG as the secondary antibody. No anti LPS IgG was detected in HCS in contrast to S1.

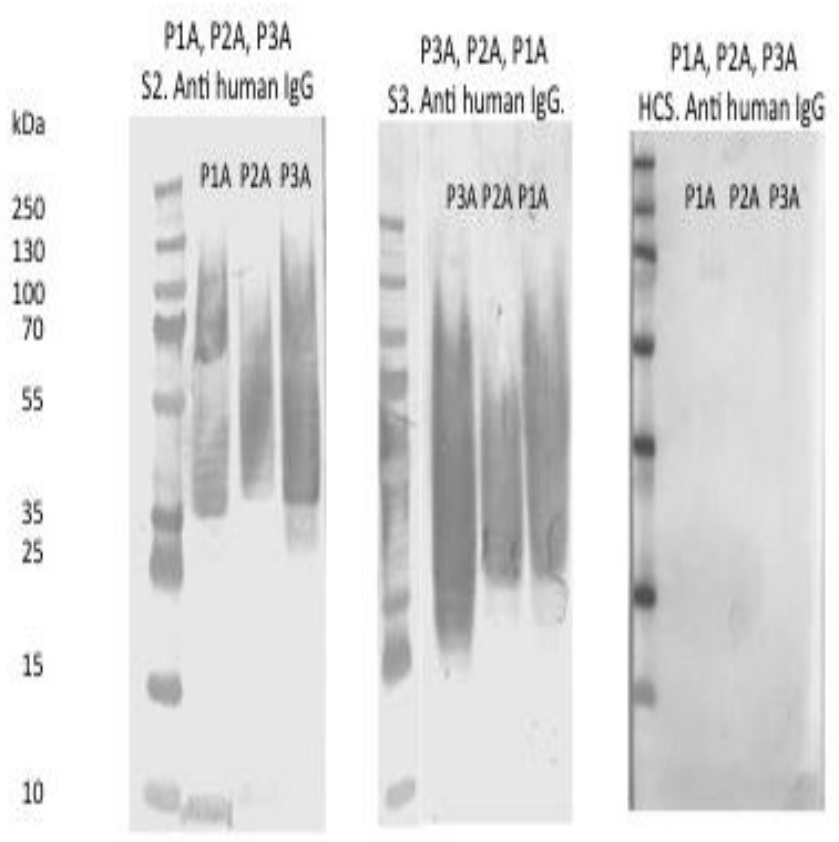

Figure 5.5 shows Western immunoblotting of LPS preparations of P1A, P2A and P3A. In each case S2, S3 or HCS were the primary antibody source, with anti-human IgG as secondary antibody.. There was a high LPS specific IgG binding detected from both S2 and S3 to all three strains whereas no such binding was found with HCS.

5.3 The Role of anti LPS Antibodies in Inhibiting Killing of *P. aeruginosa*

We next sought to investigate whether inhibitory IgG2, found in the serum of patients P1, P2 and P3 bound long chain O-antigen of LPS from strains of *P. aeruginosa* found in the sputum of these patients to block bacterial killing.

To determine this link, LPS specific antibodies were removed from S1 serum by immobilising LPS purified from P1A on a polymyxin-B agarose column and passing S1 through the column to remove any specific LPS antibody (Methods 2.5.5). The recovered flow through fractions were then utilised in bactericidal assays to determine whether the removal of specific antibody bound to LPS would restore the ability of S1 serum to kill P1A. Similarly, the eluate was examined in bactericidal assays to assess whether the addition of this purified anti LPS antibody to HCS would inhibit bacterial killing. Bactericidal assays were performed as previously described in methods 2.4.

Ninety microlitres of four fractions of S1 deplete of LPS antibody were collected as flow through from the column. Bactericidal assays using these fractions showed that although viable numbers of P1A were still detected after 180 minutes incubation, the number of colony forming units was fewer than in buffer control or S1, suggesting that removal of anti LPS antibody did restore some capacity for killing by S1 serum ($p<0.0001$) (Fig 5.6). As the column extraction may have deactivated much of the complement, eluate containing anti LPS antibody was added to HCS in a ratio of 50% and was able to reduce killing of P1A by HCS after 45 minutes incubation. Again, this assay demonstrated a significant $\log^{10}$ change in cfu where S1 minus anti LPS antibody was used compared with S1 mixed with HCS ($p<0.0001$) (Fig 5.7).

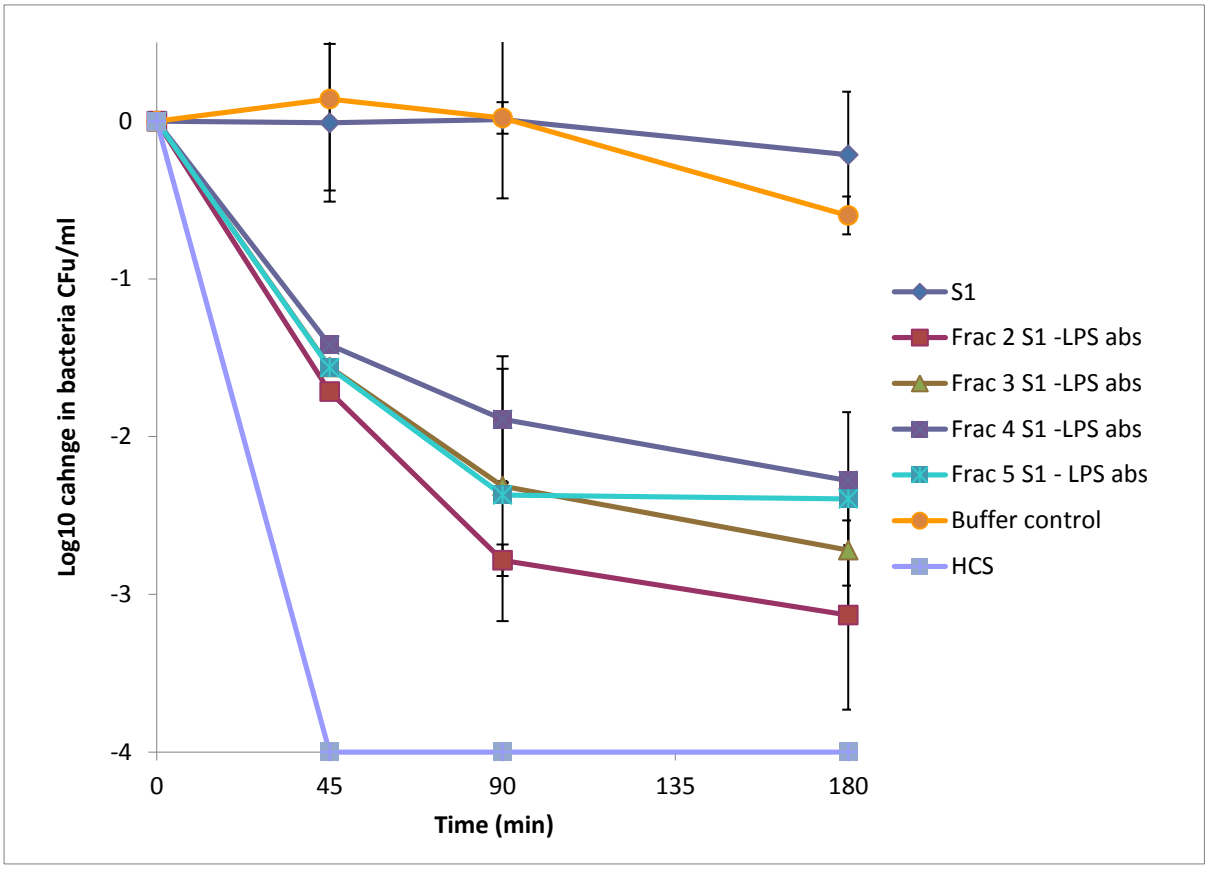

Figure 5.6 shows that P1A was fully sensitive to killing by HCS while killing was inhibited by S1. Killing was improved by the recovered fractions of S1 from the LPS column though still less and slower than for HCS. Inhibition of killing of S1 was significantly different to that of fraction 2 S1 deplete of LPS antibodies at 180 minutes incubation ($p<0.0001$ by Student's t test.) Each assay was performed in triplicate with an average count of colony forming units calculated at each time point. This is representative of 3 independent assays. Error bars represent the mean +/- SD for each assay.

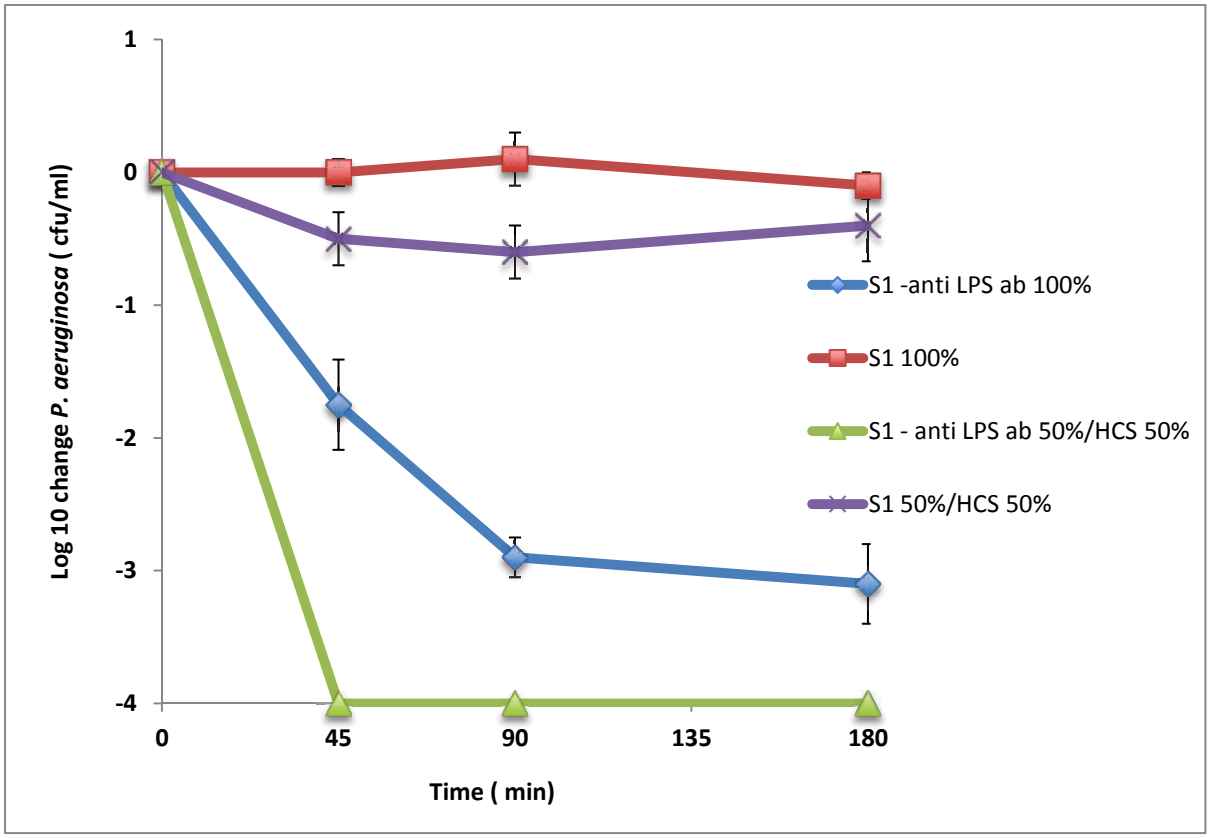

Figure 5.7 depicts serum killing of P1A by HCS mixed with S1 deplete of anti LPS antibody. S1 depleted of anti LPS antibody added to HCS in a 50%/50% mixture in bactericidal assay vs P1A. This was able to kill P1A after 45 minutes incubation. S1 depleted of LPS antibody alone also demonstrated a significant log change in bacterial numbers after 180 minutes incubation compared to the killing curves for undepleted S1 sera or mixes of undepleted S1 sera and HCS (50%/50%) after 180 minutes incubation. ($p<0.0001$ by Student's t-test). Each assay was performed in triplicate with an average count of colony forming units calculated at each time point. Error bars represent the mean +/- SD for each assay.

Having demonstrated that the addition of purified anti LPS antibody to HCS could inhibit bacterial killing of P1A, a dose response with HCS was undertaken. The aim was to establish whether the titre of anti LPS antibody was critical in inhibiting bacterial killing rather than its presence alone. Bacterial assays were carried out as previously described in methods chapter 2. Increasing proportion of purified anti LPS antibody mixed with HCS showed delayed and reduced bacterial killing after 180 minutes incubation when mixed in a ratio of 33% HCS to 67% anti LPS antibody but was less reduced at a 67%/33% ratio (p=0.1) (Fig 5.8). These data confirmed both potency and a proportion dependant effect.

5.4 Immunofluorescence Microscopy of S1 Depleted of anti LPS Antibody

Having established the role for the patient derived anti-LPS antibody to inhibit killing of P1A, immunofluorescence microscopy was performed as described in methods chapter 2 on flow through fractions of S1 following extraction of anti LPS antibody via the polymyxin –B agarose column. Briefly, as before, FITC anti human IgG1 and IgG2 was used to detect IgG 1 and IgG2 subclasses. For comparison, immunofluorescence microscopy of P1A with S1 and HCS and FITC anti human IGg1 and IgG2 is shown in addition to immunofluorescence of recovered fractions of S1 serum from protein G column run through to remove all IgG in the sample.

Immunofluorescence microscopy showed that the flow through fractions of S1, depleted of anti LPS antibody, did have detectable *P. aeruginosa* IgG1 binding, but not IgG2. When compared to the images of P1A with S1 and FITC anti human IgG2 there was a marked difference in visualised IgG2 binding. When compared to images of P1A with S1 depleted of IgG following protein G column extraction there was a similar absence of detectable IgG2

binding. However, there was detectable IgG1 activity suggesting that the anti LPS antibody is of the IgG2 subtype. This was removed by binding to a purified P1A LPS column confirming that this was the target of inhibitory IgG2 (Fig 5.9.).

Previously I had shown that the addition of purified anti LPS antibody from S1 to HCS inhibited killing of P1A in a dose-dependent manner indicating that the potency and amount of anti-LPS antibody is critical for inhibition (Fig 5.7). This was further evaluated as a titre with Enzyme-linked immunosorbent assay (ELISA) described in methods chapter 2. Briefly, P1A LPS was attached to a 96 well plate. Dilutions of serum from patients 1-11 were added in addition to dilutions of HCS from 2 controls. Anti-human IgG conjugated to alkaline phosphatase followed this, with results derived 30 minutes after the addition of developer. The patients with impaired serum killing of P1A were shown to have much higher titres of anti LPS IgG compared to the serum of patients not only able to kill autologous *P.aeruginosa* strains but also able to kill the strain P1A. This added further support that the presence of anti LPS IgG was pivotal in inhibition of *P. aeruginosa* killing in these patients but that the titre was significantly higher in patients 1, 2 and 3 (whose colonising strains were also high LPS producing) indicating that the strain driven antibody response and titre of the subsequent anti LPS IgG was also critical (Fig 5.10).

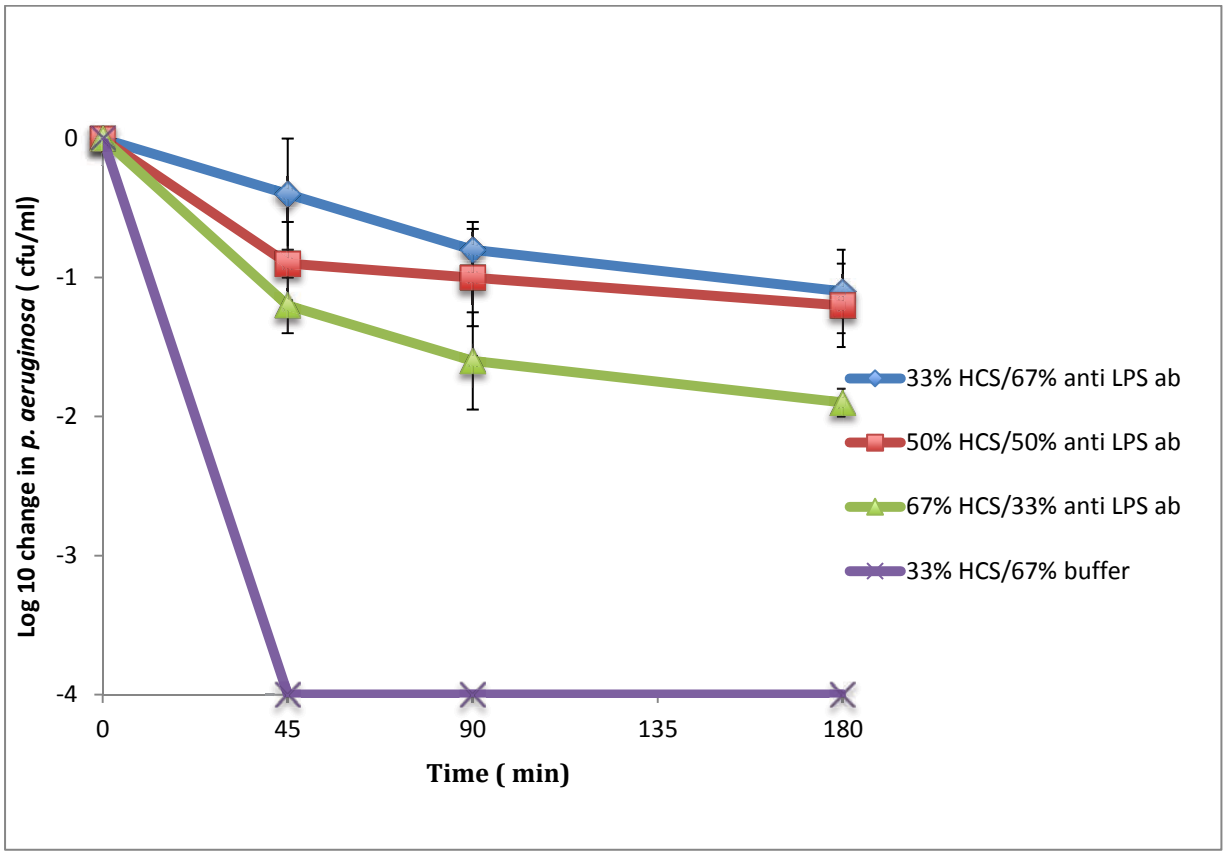

Figure 5.8 depicts results of a bactericidal assay with the addition of purified anti LPS antibody from S1 to HCS in increasing proportions directed against P1A. The addition of anti LPS antibody even in a ratio of 33% to HCS was shown to inhibit bacterial killing of P1A. This effect was greater at 50/50 at 180 minutes incubation, ($p=0.1$ by Student's t-test) but no further inhibition was seen at a mixture of 67% anti LPS antibody and 33% HCS..Each assay was performed in triplicate with an average count of colony forming units calculated at each time point. Error bars represent the mean +/- SD of 3 assays.

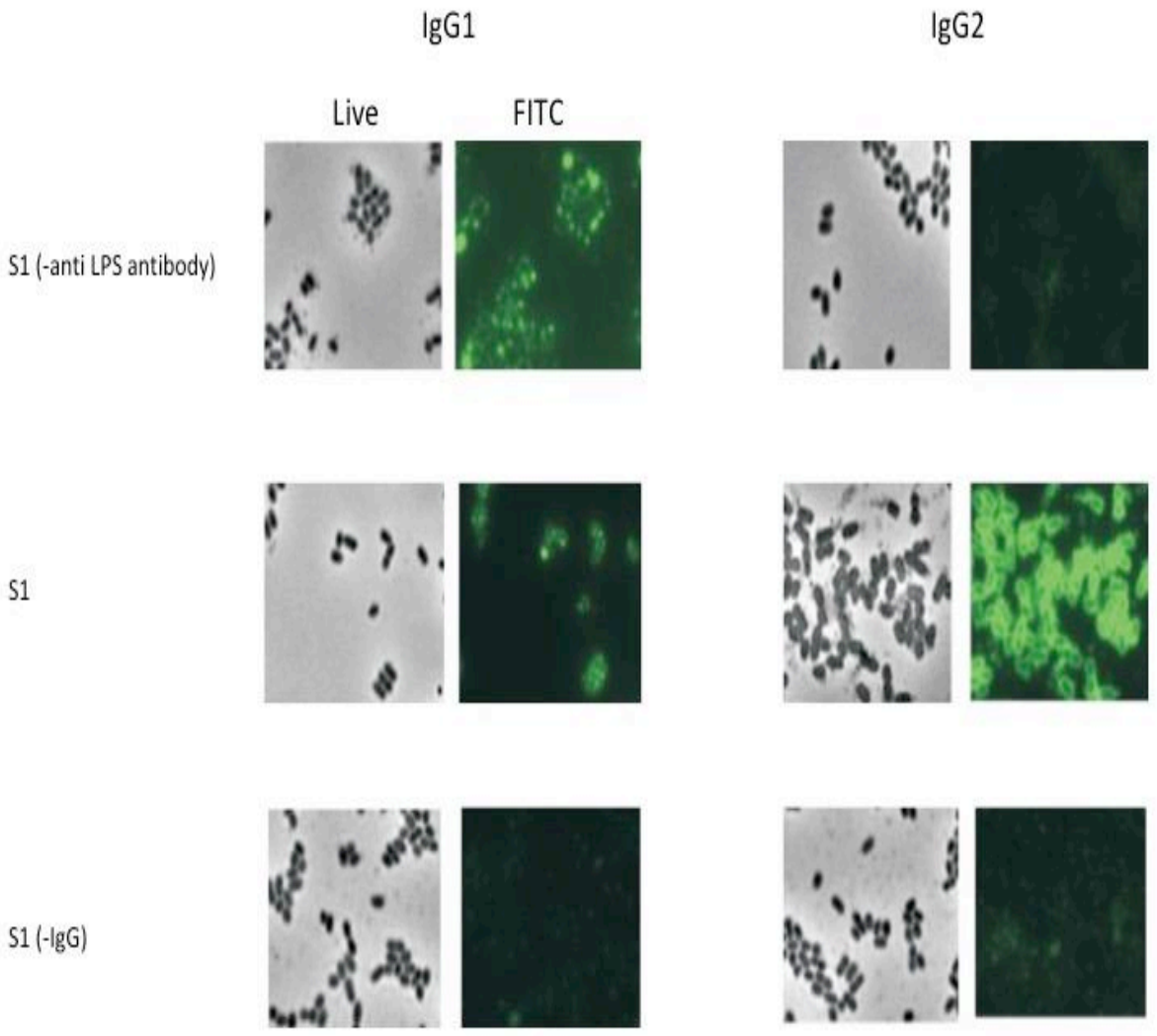

Figure 5.9 Immunofluorescence microscopy of sputum smear P1A in S1 deplete of anti LPS antibody, in neat S1 and following IgG extraction from S1. In all cases FITC labelled anti human IgG1 and IgG2 were utilised to detect IgG1 and IgG2 binding to bacteria. Immunofluorescence of P1A with S1 following anti LPS antibody removal demonstrated detectable anti-*P. aeruginosa* IgG1 but not anti *P. aeruginosa* IgG2.

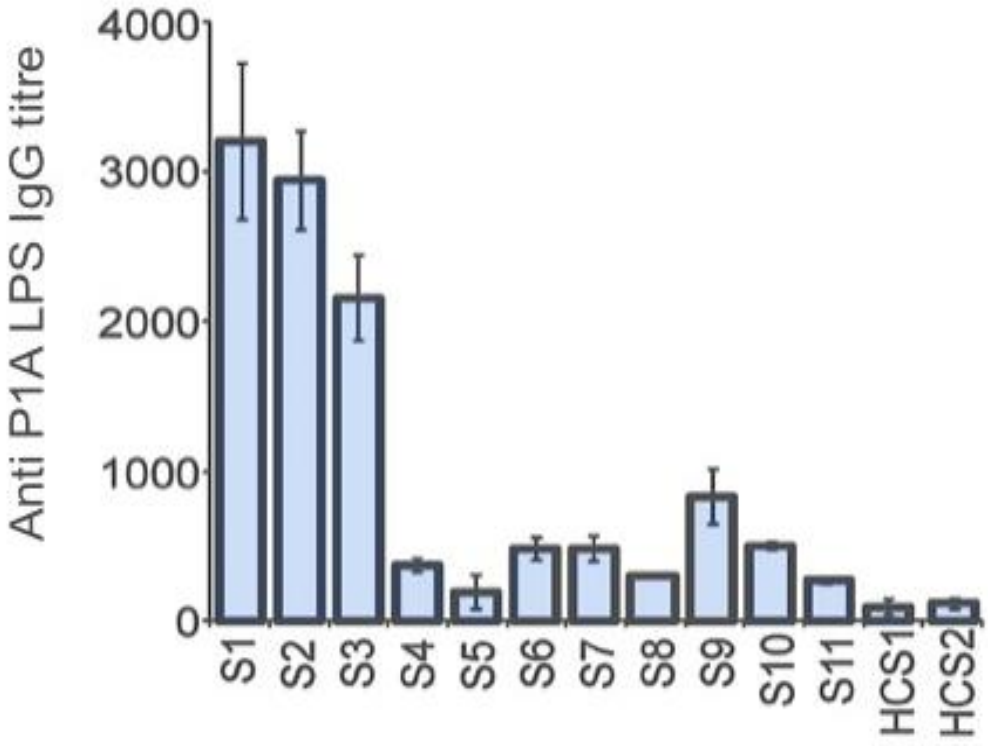

Figure 5.10 Using ELISA S1, S2 and S3 demonstrated high antibody titres to P1A LPS in comparison to HCS and patient sera able kill P1A. Results were derived 30 minutes after addition of developer on plate reader (OD^{405}).

5.5 Confirmation of Role of Anti LPS IgG Titre.

To confirm that this inhibition was not only due to the presence of anti LPS IgG but rather the titre, anti LPS antibodies were purified from S4 – a serum with normal bactericidal killing and shown to be able to kill P1A. This was achieved by running S4 through a column lined with purified P1A LPS as previously described. The eluted anti LPS antibodies were concentrated 10 fold and utilised in bactericidal assays with P1A. The addition of this purified S4 anti LPS antibody to HCS was once more shown to inhibit killing of P1A in a dose dependent manner (Fig 5.11) as previously shown using autologous serum anti LPS antibody (Fig 5.8), but now demonstrated using purified, concentrated anti LPS antibody from a serum previously shown to be able to kill P1A.

The strains P1A, P2A and P3A were all shown to express high amounts of O-antigen, the immunogenic portion of LPS. This common feature indicated that this component of LPS was therefore likely to be the specific target for inhibitory antibody. To test this further, S1 was depleted of antibody to lipid A and core oligosaccharide constituents of LPS by passing it over a polymyxin B column with immobilised purified LPS from P4A which is a strain lacking O-antigen expression and therefore it was considered that the flow through would still contain anti LPS antibodies recognising O-antigen whilst the antibody eluted from the column would recognise lipid A and core oligosaccharide. Simultaneously, S1 was passed over a polymyxin B column on which LPS isolated from P1A was immobilised. As this strain expresses O-antigen the flow through would be depleted of S1 anti LPS antibody whilst the eluate would contain high levels.

ELISA was performed as previously described by binding LPS isolated from P1A to a 96 well plate with the flow through and eluate fractions from each column utilised as primary

antibody and anti human IgG-AP as secondary antibody, to establish comparative titres of anti LPS IgG (Fig 5.12). Bactericidal assays were performed with mixes of HCS and eluate and the flow through fractions from the column lined with purified P4A LPS (Fig 5.13).
ELISA showed that titres of anti LPS IgG in the eluate from S1 in the column lined with P1A LPS and the flow through from S1 run through the column lined with P4A LPS were comparable with S1 alone. The titres were approximately 100 times greater than that from the flow through of S1 from a column lined with P1A LPS and approximately 10 times greater than that from the eluate of S1 from the P4A LPS column (Fig 5.12).

Bactericidal assays were performed using the S1 eluate from the P4A column (S1 anti lipid A/core antibody) and the flow through from the same column (S1 deplete of anti-lipid A/ core antibody). Both were mixed in ratios of 50% with HCS and bactericidal assays performed as previously described in methods. The addition of S1 deplete of lipid A/core antibody to HCS was able to inhibit killing by HCS. Conversely there was a 3 $\log^{10}$ cfu/ml difference at 90 minutes incubation when the eluate containing anti lipid A/core antibody was added to HCS indicative of significant bacterial killing (Fig 5.13) (P=0.0001). The absence of complete killing of P1A at ninety minutes can be explained by the presence of some anti LPS IgG present in this eluate albeit in a lower titre compared with native S1 (Fig 5.12) and its recognised potency. Nevertheless, this indicated that even following removal of antibody specific only to lipid A and other constituents of LPS from S1 serum, the inhibitory antibody was retained in S1 serum. Table 5.1 summarises the findings of assays following extraction of IgG, specific IgG2, O- antigen and lipid A/ core specific antibodies.

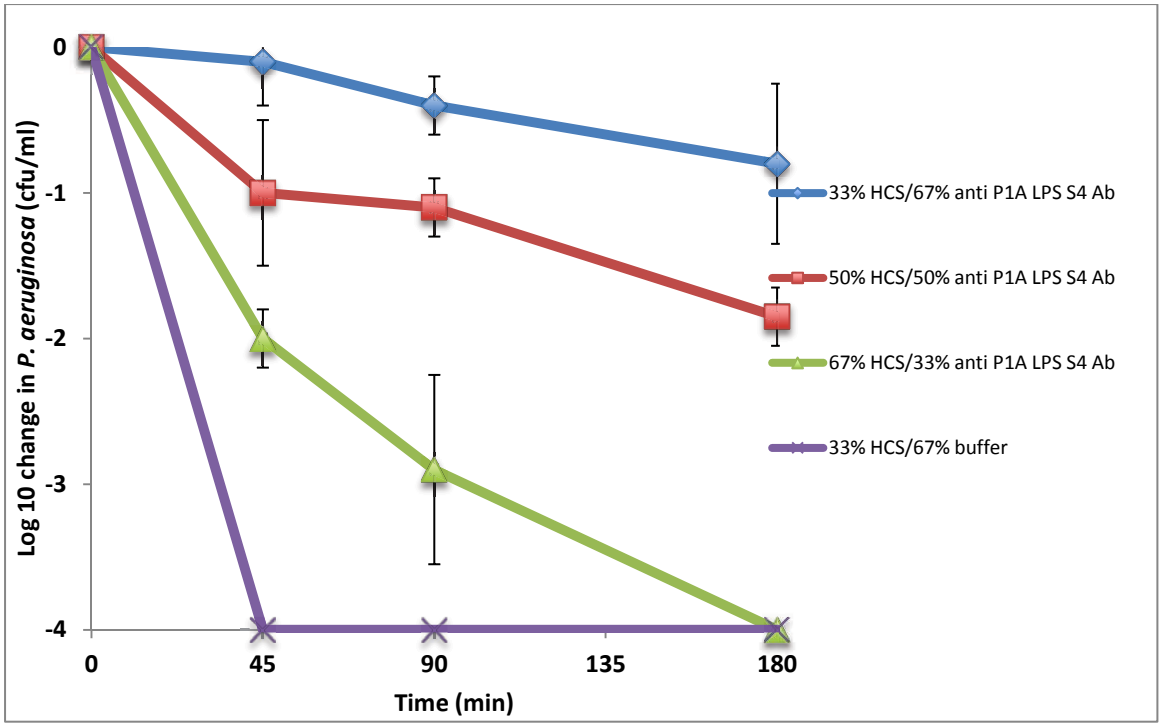

Figure 5.11 depicts bactericidal assay with purified anti LPS antibody from S4 mixed with HCS against P1A. HCS was mixed with buffer as control. Killing of P1A was inhibited in a ratio dependent manner. Full killing of P1A was established after 180 minutes incubation with 33% anti LPS antibody from S4 mixed with HCS. This established that killing of P1A could be inhibited using purified, concentrated anti LPS antibody from serum previously shown to kill both its autologous strain and P1A. There was a $\log^{10}$ cfu/ml increase in bacterial killing when comparing serum mixes containing 67% anti P1A LPS antibody vs 50% ($p=0.1$ by Student's t test). This is representative of 3 independent assays. Error bars represent the mean +/- SE for these assays.

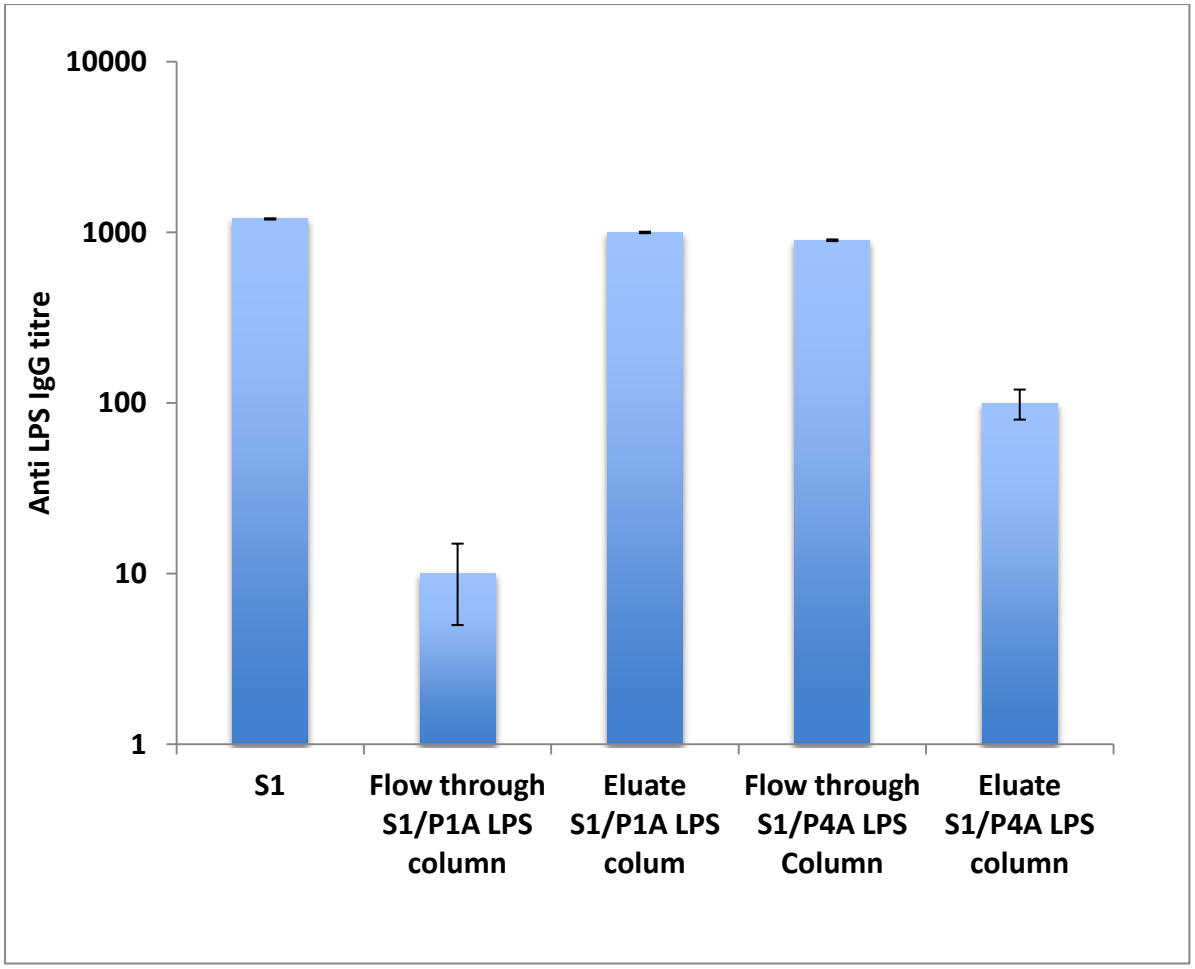

Figure 5.12 shows comparative titres of anti LPS IgG following specific antibody removal as measured by ELISA. Titres of anti-LPS antibodies purified from S1 after passage through a column containing purified LPS with a long chain O-antigen (P1A) or a column containing LPS lacking long chain O-antigen (P4A) were measured using ELISA. Titres of anti LPS IgG in the eluate from S1 following extraction of antibody specific to P1A LPS, and the flow through from S1 run through the column lined with P4A LPS, were similar to the titres from S1. The titres were approximately 100 times greater than that from the flow through of S1 from a column lined with P1A LPS and approximately 10 times greater than that from the eluate of S1 from the P4A LPS column. It is noted that there is still anti LPS antibody present in this eluate therefore suggesting there is some binding of anti LPS antibody to the P4A LPS preparation. Error bars represent the mean +/- SD of three ELISAs.

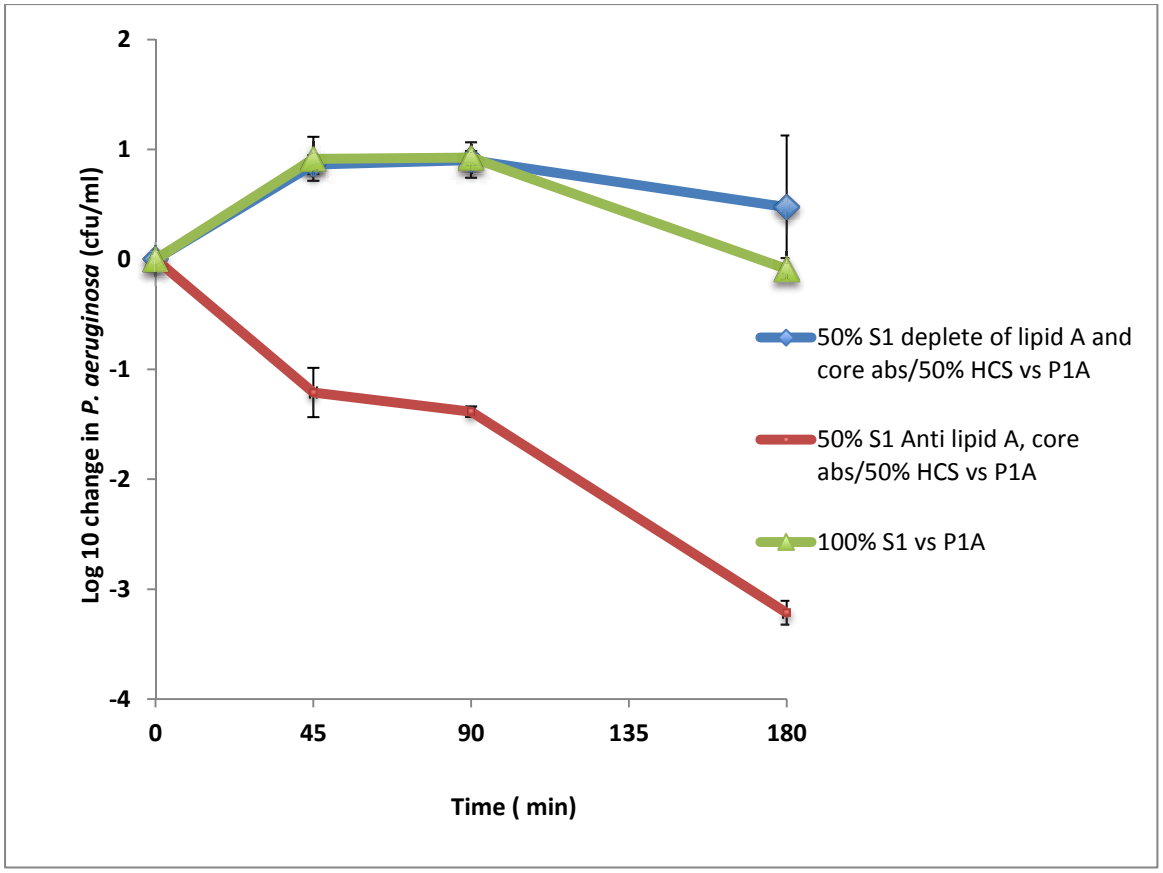

Figure 5.13 Bacterial assays of S1 depleted of antibody specific to LPS constituents including lipid A/ core antibody and S1 anti lipid A/core antibody mixed with HCS vs P1A. Complete inhibition of killing was seen with S1 and S1 deplete of lipid A and core abs. There was a significant decrease in *P.aeruginosa* cfu/ml seen when anti lipid A/core abs eluate was added to HCS suggesting that this was not the factor contributing to the inhibition of bacterial killing by S1. When this was compared to HCS mixed with S1 deplete of lipid A and core abs there was a significant difference in bacterial killing ($p=0.0001$ by Student's t test) at 180 minutes. Assays performed in triplicate. Error bars represent the mean +/- SD of each assay.

Column	**Protein A**		**Protein G**		**Monoclonal IgG2**		**O antigen LPS (P1A) (polymixin B)**		**Lipid A and core LPS (P4A) (polymixin B)**	
Action	• High affinity binding to human IgG1, IgG2, IgG4. • Moderate affinity to human IGA, IgM, IgE		• High affinity binding to human IgG1, IgG2, IgG3,IgG4. • No affinity to human IgA, IgM, IgE		• Binds human IgG2		• Binds O-antigen, core and lipid A specific antibodies		• Binds Lipid A and core specific antibodies.	
Serum Fraction added to 50% HCS	Flow through	Eluate	Flow through	Eluate	Flow through	Eluate	Flow through	Eluate	Flow through	Eluate
Outcome on bacteria after 180 minutes incubation.	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Alive	Dead

Table 5.1 summarises the outcome of bactericidal assays against P1A following specific antibody extraction from S1 using protein A or protein G, monoclonal IgG2, O-antigen LPS or lipid A and core LPS preparations from P1A.

5.6 Patient Demographics

Patients had demographic data recorded as described in methods chapter 2. Having identified three patients from this cohort with high titres of anti LPS IgG2 in their serum and an inability of serum to kill strains of *P. aeruginosa* isolated from their sputum an analysis was made to identify any significant differences between the two groups of patients. The demographic data is recorded in table 5.2.

Statistical analysis was performed using SPSS Version 18 IBM New York. Student's *t* test was used with p value <0.05 considered significant. In the cohort of patients studied, total average SGRQ score was 50.18 (+/- 15 SD), for patients who did not demonstrate any inhibitory activity in their serum, compared to 60.99 (+/- 13.7 SD) for those with inhibitory activity although this did not reach statistical significance which likely reflects the small sample size. In the individual domains (symptoms, activity and impacts), there were no differences between the two groups.

The FEV_1 was lower on average in the patients with inhibitory serum compared to those without (P=0.026). The average FEV_1 in the group with inhibitory serum was 38% predicted (+/- 20 SD) whilst in the group with normal killing the average FEV_1 was 74.6% predicted (+/- 17.5 SD). The data is summarised in table 5.2.

Furthermore, the group with inhibitory serum had significantly more hospital admissions in the year preceding sample collection than those without inhibitory activity. (p = 0.034.) 3 hospital admissions were recorded in total in the blocking group and only one in the group with normal serum killing. No significant difference was observed in MRC dyspnoea scores between the 2 groups

Patient	Age	MRC score	Annual exacerbation rate	Hospital admissions	SGRQ total	Symptoms	Activity	Impacts	FEV_1 (% predicted)
P1	48	4	3	2	67.0	68.8	85.7	55.7	21
P2	59	2	1	0	42.0	45.9	47.0	37.8	67
P3	68	3	4	1	74.0	88.2	77.8	67.5	26
P4	60	2	6	0	43.4	82.5	49.2	27.8	105
P5	63	2	0	0	72.7	88.9	93.3	55.8	78
P6	55	2	3	0	39.1	74.8	35.3	30.1	80
P7	68	3	4	0	68.9	85.9	85.9	54.0	48
P8	60	2	2	0	31.9	66.4	29.3	22.7	59
P9	64	3	2	1	64.3	71.9	79.1	53.4	61
P10	87	3	1	0	45.9	67.0	66.3	27.7	93
P11	76	2	4	0	35.2	56.1	60.2	14.4	73

Table 5.2 Summary of the clinical data of patients with inhibitory serum antibody vs those without.

5.7 Conclusion

IgG2 has been shown to be the factor in serum of patients P1, P2 and P3 which inhibits the killing of autologous strains of *P.aeruginosa* isolated from sputum, which are otherwise fully sensitive in HCS.

Analysis of these *P. aeruginosa* strains has shown the common feature is expression of long chain O antigen which is not seen with strains that are fully sensitive in autologous serum, HCS as well as S1, S2 and S3. This indicated that O-antigen may be the target of these inhibitory antibodies. Further analysis demonstrated that IgG2 is specific to LPS, binding to bacteria with the inhibitory serum from patients 1,2 and 3. Removal of LPS specific antibodies restores patient serum killing whilst addition of O-antigen specific antibody eluate to HCS inhibits killing of that serum.

Bactericidal assays using purified anti LPS from S1 and S4 has demonstrated that not only is the presence of anti LPS antibody required to inhibit killing of these strains but that the titre of anti LPS IgG is also crucial. ELISA has demonstrated a high titre of anti LPS IgG in the serum of patients with impaired serum killing compared to those with normal serum killing of *P. aeruginosa.* Bactericidal assays analysing constituents of LPS where lipid A and core specific antibodies are removed from S1 have shown that the inhibition of bacterial killing is retained whilst the addition of anti lipid A and core antibodies to HCS does not inhibit killing. Where preparations of LPS with long chain O -antigen are purified and used to bind serum antibodies, bactericidal assays using this bound antibody retain the ability to inhibit serum killing.

This, in combination with the expression of long chain O-antigen by strains P1A, P2A and P3A confirms that immunogenic O-antigen is the target of inhibitory IgG2.

There is furthermore the suggestion that this finding is associated with poorer health status as assessed by lung function and hospital admissions in the group of patients. The average FEV1 predicted was lower in the group with inhibitory antibody in comparison to those with normal killing, with more hospital admissions in this group too. As discussed previously the isolation of *P. aeruginosa* in patients with bronchiectasis is associated with poorer quality of life. Whether *P. aeruginosa* worsens lung function and quality of life, or indeed those with poorer health status and lung function have an increased tendency to colonisation with *P. aeruginosa,* is unclear. Nonetheless this association is well established. The finding that markers of health status are more adversely affected in the group with inhibitory antibodies suggests that this may be a further, independent risk factor in patients with bronchiectasis and *P. aeruginosa* colonisation. Similar to the association of worsening health status and *P. aeruginosa* colonisation, it is unclear whether the presence of inhibitory antibodies is a causative factor in poorer health status, or indeed whether those with poorer health status have a predisposition or tendency to develop inhibitory antibodies to *P. aeruginosa.*

CHAPTER 6 DISCUSSION

Non cystic fibrosis bronchiectasis is a condition characterised by cough and chronic sputum production, with a heightened propensity to recurrent lower respiratory tract infections culminating for many patients in an ongoing sequence of chronic infection and inflammation leading to ongoing lung damage. The theory of the vicious cycle of bronchiectasis (Cole and Wilson, 1989) elegantly illustrates this ongoing interplay beginning with an initial insult to the lung and subsequent failure of host defences leading, in some cases, to ongoing damage, failure of mucociliary clearance, ongoing inflammation and bacterial colonisation.

P. aeruginosa is a common colonising organism isolated in the "stable clinical state" in patients with bronchiectasis (Angrill *et al*., 2002; Pasteur *et al*., 2010). Its association with greater impairment of lung function (Wilson *et al*,. 1997; Martinez-Garcia., 2007; Goeminne *et al*., 2012; Rogers *et al*., 2013; Guan *et al*., 2014; McDonnell *et al*., 2015) increased frequency of exacerbations (Rogers *et al*., 2014) and worsened quality of life (Wilson *et al*., 1997), render it one of the most critical pathogens in the sputum of these patients. In addition, the antibiotic resistance patterns of *P. aeruginosa* strains often limit the choice of antibiotic therapies for these patients in clinical practice making management increasingly difficult.

The patients in the current study were well characterised with radiologically confirmed bronchiectasis and persistent growth of *P. aeruginosa* in repeated sputum samples in the absence of an acute episode of deterioration to suggest an exacerbation. All patients had at least 3 sputum samples positive for growth of *P. aeruginosa* in the year preceding entry to the study (including the sample taken at the

time of consent).

Inhibition of bactericidal activity in the serum of patients with chronic Gram-negative infection was first described in the 1960s with the suggestion of the presence of an inhibitory factor which rather than promoting serum killing did the opposite (Waisbren *et al*., 1966). Later studies in the 1970s described the presence of " blocking " antibodies in the serum of patients with *P. aeruginosa* (Guttman *et al*., 1975). Subsequent studies in cystic fibrosis with *P. aeruginosa* colonisation suggested that IgG2 inhibited bacterial killing by formation of IgG2 containing immune complexes in the serum (Hornick *et al*., 1990). In addition, patients with cystic fibrosis and *P. aeruginosa* who had strains resistant to killing by autologous serum had poorer clinical health. However, this concept of an inhibitory factor in the serum, although appearing clinically important, was not explored further (Thomassen *et al*., 1981)

More recently, similar inhibitory activity of serum against killing of *S.* Typhimurium has been described in serum of HIV infected individuals due to anti LPS IgG (MacLennan *et al*., 2010). This publication led to the hypothesis that a similar process may be present in patients with non cystic fibrosis bronchiectasis and *P. aeruginosa* colonisation and formed the basis of the experiments described in this thesis.

6.1 Anti LPS IgG2 is the Inhibitor of *P. aeruginosa* Killing by Serum.

Initial analysis of the serum and sputum isolated from eleven patients with *P. aeruginosa* colonisation was undertaken using a bactericidal assay modified from that described by MacLennan *et al* to demonstrate serum resistance of *S.* Typhimurium

(MacLennan *et al*., 2010). It was consistently demonstrated that 3 of the 11 patients had *P. aeruginosa* strains in sputum, which although sensitive to serum killing in healthy control serum were resistant when mixed with autologous serum. Interestingly however the serum from these 3 patients could effect killing of *P. aeruginosa* from the other patients in the cohort suggesting that the effect was strain specific. Each of the 3 patients had normal serum levels of immunoglobulin and had no recognised immune deficiency to explain the lack of serum killing. The sensitivity of the *P. aeruginosa* strains from these 3 patients to killing by healthy control serum confirmed that they were not inherently serum resistant.

Having concluded that the results likely reflected a patient and strain specific inhibitory factor was present in the serum of the 3 patients, further work was carried out, with careful analysis of serum establishing that the inhibitory factor was IgG and of the IgG2 subclass. This was confirmed following removal of IgG and the addition of purified IgG2 from inhibitory serum to HCS and repeating the bactericidal assays. Patient serum depleted of IgG became able to kill *P. aeruginosa* strains previously resistant to killing by this serum and addition of the IgG2 from the blocking serum to HCS rendered it no longer able to kill the "resistant" strain even at very low concentrations. Analysis of "blocking" serum from the 3 patients showed markedly higher levels of anti *P. aeruginosa* IgG2 than in healthy control serum and serum from other patients within the cohort. Immunofluorescence microscopy of *P. aeruginosa* cultures adds additional support to the role of IgG2 by showing excessive binding of IgG2 to the bacteria present.

In view of these supporting data, further bacterial assays were undertaken with serum

depleted of IgG2 which became able to effect killing of the "resistant" *P. aeruginosa* strains. The addition of IgG2 purified from the defective serum to HCS inhibited previously effective *P. aeruginosa* killing.

Having concluded that both serum and bacterial factors specific to these 3 patients were central to prevention of *P. aeruginosa* killing, and that IgG2 was pivotal, analysis of the *P. aeruginosa* strains by silver staining gels demonstrated that these autologous strains expressed large quantities of the long chain O-antigen, suggesting this feature of LPS might explain the results. It would be consistent with the blocking by autologous serum but the effective killing of the same strain by serum from other patients colonised with strains not expressing the O-antigen and hence not developing a specific immune response.

Western immunoblotting, ELISA and immunofluorescence microscopy all provided further supporting evidence that specific anti LPS antibodies were responsible for the inhibition of killing of *P. aeruginosa.* Removal of the antibodies and specifically IgG2 from blocking serum restored the ability to kill autologous strains, whilst the addition of purified anti LPS antibody to effective HCS caused blocking of *P. aeruginosa* killing. Not only was the presence of anti LPS antibody shown to be crucial in the inhibition of serum killing, but the titre was also shown to have an influence. Anti LPS antibodies from serum normally able to effect killing of the "resistant" strains, was also able to inhibit killing when concentrated and added to HCS.

Removal of antibodies specific to the O-antigen and antibodies specific to the other core constituents of LPS enabled the specific antigen target of the LPS molecule to be

confirmed. Antibodies that bound to lipid A and core constituents of LPS had no effect on the ability of HCS to kill *P. aeruginosa.* On the other hand addition of antibodies to O- antigen to HCS inhibited killing. The results confirmed that high IgG2 titres, targeted against the O- antigen constituent of *P. aeruginosa* LPS was able to block serum killing of *P. aeruginosa* strains expressing the long chain O-antigen.

6.2 Proposed Mechanism of Inhibition of Serum Killing.

Antibodies are a physiological response to foreign material and especially associated with protection against invasive infection. Normally, the specific recognition of antigen by antibody leads to its binding and, in the case of bacteria, destruction through the complement cascade as described in chapter 1 ultimately ending in MAC insertion into the bacterial cell membrane leading to cell lysis. Human IgG1 forms the main response to protein antigens and IgG2 is the main antibody subgroup response to polysaccharide antigen (Siber *et al.*, 1980). *P. aeruginosa* and other bacteria express both protein antigens on the cell surface and varying degrees of polysaccharide as part of the LPS complex. Thus, both a specific IgG1 and IgG2 response would be expected.

The proposed mechanism responsible for the blocking of complement activated *P. aeruginosa* killing seen here is that high titres of anti O-antigen IgG2 inhibits killing of *P. aeruginosa* by binding to the LPS long chain O-antigen, and hence depositing complement distal to the cell surface membrane and sterically impeding MAC insertion at the cell membrane where it would ordinarily compromise the integrity of the cell membrane leading to lysis. This mechanism may also block access of cell

membrane protein IgG1 to the cell membrane where it should activate complement and MAC insertion (MacLennan *et al*., 2010; Brown, Joiner and Frank, 1983; Moffitt and Frank, 1984; Wells *et al*., 2014). (Fig 6.1)

The mechanism described also accounts for the fact that inhibitory serum was still able to effectively kill other strains of *P. aeruginosa* where O- antigen was not highly expressed, as both the inhibiting antibody and the specific LPS antigen on the bacteria need to be present to block the process.

P. aeruginosa strains resistant to complement mediated killing were previously noted to activate complement normally and in a similar fashion to serum sensitive strains, but were thought to be resistant to serum killing due to an inability of MAC to insert stably into the cell membrane of a *P. aeruginosa* strain expressing long chain O-antigen. (Schiller and Joiner, 1986). It was noted that this apparent bacterial self protective mechanism was common among gram-negative organisms with long LPS O side chains however, no pathway leading to instability of MAC insertion was defined (Joiner *et al*., 1982; Schiller and Joiner, 1986), but it may likely be that proposed in the current thesis. Similarly, I have shown that the *P. aeruginosa* strains P1A, P2A and P3A could activate complement and therefore did not account for the ineffective killing nature of some serum to these strains.

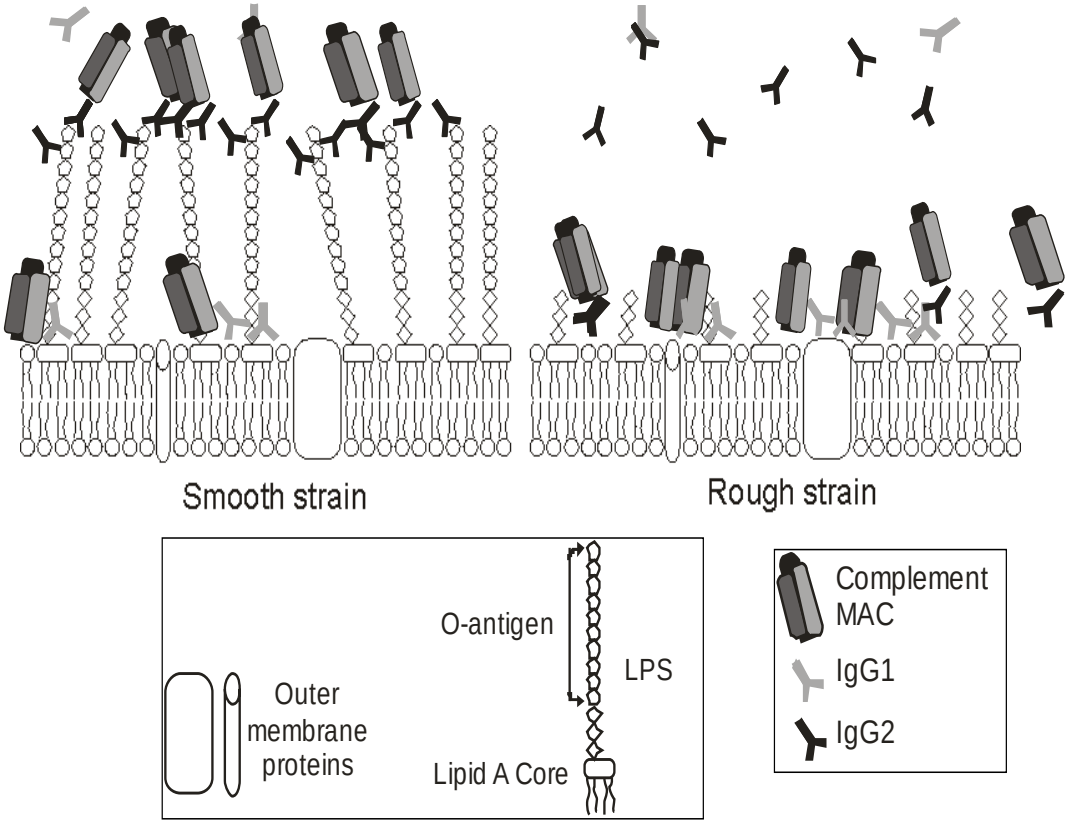

Figure 6.1 Proposed mechanisms for inhibition of serum-mediated killing. High titres of O-antigen-specific IgG2 bind *P. aeruginosa* O-antigen and impede access of complement or protective IgG1 antibody to the *P. aeruginosa* surface. Low levels of LPS-specific IgG2 cannot completely block access of complement-depositing antibodies to the bacterial membrane. *P. aeruginosa* strains with no O-antigen do not bind specific anti-LPS IgG2 distal from the surface and thus complement is able to reach bacterial membrane and effect killing. (Figure 6.1 reproduced with the kind permission of Dr Timothy Wells. Unpublished data.)

6.3 Implications for Patients

Patients who suffer from bronchiectasis experience daily symptoms as previously described causing significant morbidity. *P. aeruginosa* isolation is associated with poorer quality of life and worse lung function with more frequent exacerbations in this patient group. (Wilson *et al*., 1997; Martinez-Garcia., 2007; Goeminne *et al*., 2012; Rogers *et al*., 2013; Guan *et al*., 2014; Rogers *et al*., 2014; McDonnell *et al*., 2015). This means that it is an organism of particular impact in bronchiectasis. Antibiotic therapies are typically limited to ciprofloxacin as the only effective oral antibiotic available, or intravenous therapies with meropenem, piperacillin and tazobactam, ceftazadime, aztreonam, tobramycin, colistin, and gentamicin. For patients this often requires admission to hospital for lengthy courses of intravenous antibiotic when they exacerbate, or home administration of long-term nebulised antibiotics in some cases. Therefore, not only does infection with *P. aeruginosa* have an effect on patients health but also has wider cost implications with hospital admissions, time absent from work and long term therapy.

Demographic data was collected on this cohort of patients as described in methods chapter and presented in chapter 5. The lung function as measured by FEV_1 predicted was significantly worse in the group with inhibitory serum with more hospital admissions. There was a trend towards poorer quality of life as measured by the SGRQ but because of the low numbers it was not possible to undertake multivariate analysis to determine whether the increased hospital admissions were as a consequence of the reduction in bacterial killing alone. It is however, worthy of note that within a group of patients already known to suffer poor quality of life and poor lung function that there may be a further immunological factor affecting some patients that is associated with poorer lung function, increased hospital

admission and hence may be a target for alternative therapies. Indeed, a recent report by Wells *et al* suggesting that plasmaphoresis in patients with IgG2 blocking antibodies has a significant (though temporary) effect on exacerbations and hospital admissions (Wells *et al*., 2017). This report is later discussed in more detail.

Work published since the completion of the thesis, has studied a second well characterised cohort of patients with bronchiectasis and identified that a similar proportion of patients colonised with *P. aeruginosa* have IgG2 mediated inhibition of serum killing (Newcastle UK). From a total of 41 patients with bronchiectasis (including the 11 included in this thesis), 28 were found to be colonised with *P. aeruginosa*. Of this 28, 6 demonstrated IgG2 mediated inhibition of serum killing. These 6 patients had poorer lung function (as measured by FEV_1 % predicted) compared to those without inhibition ($p<0.002$) and the 13 who were not colonised with *P. aeruginosa* ($p<0.05$). The average FEV_1 of the group with inhibited serum killing was 34% predicted compared with 67% for those with *P. aeruginosa* and normal killing and 52% for those colonised with other organisms (Wells *et al*., 2014) The results from this larger cohort of patients continues to suggest poorer lung function in those with inhibitory IgG2 compared to those with normal killing and supports the clinical impact for such patients. As discussed previously it is unclear whether poorer lung function predisposes to, or is in part caused by, development of inhibitory IgG2. It is also not known why only a proportion of patients with *P. aeruginosa* have inhibitory IgG2. This may relate to the length of time that a patient has been colonised for, or indeed whether they are colonised with mucoid or non-mucoid strains. In the cohort of patients studied here it is not possible to accurately determine the time point at which they first isolated *P. aeruginosa* in sputum to establish whether the length of time they have been colonised for is relevant, and this is often difficult to determine in patients with non CF bronchiectasis. It may be practical to determine this in a cohort of patients with cystic fibrosis where normal clinical care

encompasses regular and frequent sputum sampling, where there is emphasis on the first isolation of *P. aeruginosa* due to the need to attempt eradication when first isolated.

6.4 Implications for Potential *P. aeruginosa* Vaccine Therapies.

Vaccination against *P. aeruginosa* infection is considered to be an aim in the management of colonised patients but to date this strategy has failed. Theoretically a vaccination would be considered to have a role in attempting to prevent initial *P. aeruginosa* infection in those deemed most at risk of pulmonary infection/colonisation, such as patients with cystic fibrosis, bronchiectasis and other chronic lung disease where infective exacerbations with this organism are common, but also those considered at risk of hospital acquired infections including patients in intensive care units and burns patients (Church *et al*., 2006). Multi drug resistant *P. aeruginosa* infection is associated with a high mortality in cases of ventilator associated pneumonia (Rello *et al*., 1997; Sievert *et al*., 2013) and thus vaccination may also be considered useful in this group of patients. More recently *P. aeruginosa* has been identified as a major source of combat related wound infections (Peterson *et al.*, 2007; Murray *et al*., 2011) suggesting that a vaccine against this pathogen would potentially have a role in protecting at risk individuals, hence having a broad range of indications. No trials have taken place in patients in non-CF bronchiectasis.

Most studies of potential vaccine targets for *P. aeruginosa* have focused on LPS as the immunogenic target due to its accessibility on the bacterial cell surface. However, to date studies have either demonstrated no benefit, some detrimental effects or have been suspended (Doring and Pier, 2008; Sharma, Krause and Worgall, 2011). A full overview of these studies

is given in the introduction in chapter 1. A recent Cochrane review concluded that vaccination for *P. aeruginosa* could not be recommended in light of the failure of these trials (Johansen and Gotzsche, 2015). The failure to develop an effective vaccine against *P. aeruginosa* is thought, in part, to be due to the difficulties in enrolling patients susceptible to *P. aeruginosa* infection or colonisation in trials to generate sufficient statistically power to prove efficacy (Doring and Pier, 2008; Worgall., 2012). The nature of colonisation of *P. aeruginosa* in the most widely studied group of patients with CF, has inherent difficulties in recruiting to clinical trials as ideally patients would be vaccinated before they have *P. aeruginosa* isolated in sputum but colonisation often takes place in childhood (Worgall., 2012). This creates some problems to introduce long lasting immunity by vaccination in young children in addition to the difficulties in recruiting to clinical trials.

When non-mucoid strains are identified in the sputum of patients with CF, aggressive eradication can be undertaken to prevent, or delay, colonisation with the mucoid phenotype with evidence to indicate eradication at this stage (Hansen, Pressler and Holby, 2008; Schelstraete *et al*., 2013). Colonisation with the mucoid phenotype predicts shorter survival in CF and cannot readily be eradicated hence the logical need to identify and eradicate *P. aeruginosa* where possible (Henry, Mellis, and Petrovic, 1992; Govan and Deretik, 1996; Nixon *et al*., 2001). Although previously thought impossible to eradicate mucoid *P. aeruginosa,* there is evidence to suggest this is not impossible and attempts at eradication should be attempted (Douglas *et al*., 2009; Troxler *et al*., 2011) Conversion to the mucoid phenotype is associated not only with alginate production but these strains often lack O-antigen (Doring and Pier, 2008).

Conversely the virulent non mucoid strains which initially infect the lungs of CF patients are

usually of the smooth type – expressing O-antigen, and these are the strains more commonly isolated from the sputum of patients with bronchiectasis with mucoid strains isolated much less commonly (Rivera and Nicotra, 1982; Hancock *et al*., 1983; Hatano, Goldberg and Pier, 1995; Nicotra *et al*., 1995; Hart and Borowitz, 2004; Doring and Pier, 2008). Interestingly these mucoid phenotypes often convert to non mucoid in lab cultures indicating that they are phenotypically flexible (Bergan and Holby, 1975; Cetin, Toreci and Ang, 1965).

This pattern of colonisation with *P. aeruginosa,* although complex, does not fully explain the lack of an effective vaccine as although strains are able mutate from non mucoid to mucoid phenotypes in response to the CF airway, with variation in LPS O- antigen, altered lipid A (Ernst *et al*., 2006; Ernst *et al*., 2007), other antigens such as outer membrane proteins are highly conserved therefore possibly providing a suitable target for vaccine development (Doring and Pier, 2008). In light of these findings, vaccine therapy targeting other antigens rather than LPS may be prudent. Additionally, the significance of colonisation with *P. aeruginosa*, particularly in CF, emphasises the need for an effective vaccine.

The findings of anti-LPS antibody inhibiting killing of *P. aeruginosa* presented in this thesis raises a caution and a possible mechanism for the failure of vaccine where *P. aeruginosa* LPS is the antigenic target. The finding that strains of *P. aeruginosa* expressing long chain O-antigen are associated with the production of antibody that inhibits killing by serum, suggests that there is potentially a risk that O-antigen polysaccharide based vaccines may induce a similar response with induction of an inappropriate IgG2 antibody response making vaccination ineffective especially for subsequent strains with high O-antigen expression.

6.5 Future Therapies

The identification of anti LPS IgG2 in the serum of patients with bronchiectasis has offered

an opportunity to explore other potential targets for treatment in patients colonised by *P. aeruginosa* other than antibiotic therapy which has formed the mainstay of treatment thus far. Increasing antimicrobial resistance has made the need for alternative therapies even greater with antibiotic resistance identified by the World Health Organisation as one of the biggest threats to global health. (World Health Organisation., 2015).

Therapies targeting an inappropriate antigen or generating inappropriate immune reactions are already well established in other medical conditions. In particular, autoimmune or neurological conditions such as myasthenia gravis and Guillain-Barré syndrome. In Myasthenia Gravis circulating antibodies against the acetylcholine receptor (AChR) block or destroy receptors for acetylcholine at the neuromuscular junction and treatment with IV immunoglobulin replacement from healthy donors and plasmaphoresis have become established treatments. Plasmaphoresis is utilised to remove harmful circulating antibodies directed against the acetylcholine receptor with recognized short term benefits (Dau, 1982).

The potential for utilising a similar strategy in bronchiectasis patients identified as having blocking anti LPS antibody identified in their serum has been explored as mentioned above. Two such patients with severe symptoms, recurrent exacerbations and poor lung function were identified who were failing to respond to standard therapies and ethical approval was obtained for plasmaphoresis followed by administration of IV immunoglobulin. The results suggested an overall improvement with a significant reduction in days in hospital and subjective improvements in quality of life. There were statistically significant improvements in days spent in hospital, IV antibiotic use and inflammatory markers measured in sera. One patient had an improvement in lung function in the months following therapy. There was an initial reduction in anti LPS IgG titres as would be expected, which increased over the following 3 months to a level that inhibited bacterial and coincided with the re-emergence of

P. aeruginosa colonisation (Wells *et al*., 2017).

These preliminary results suggest symptomatic and clinical benefit in patients identified with anti LPS IgG2 in their serum but clearly larger and longer studies are required to determine the real cost / risk benefit. The wider use of this treatment strategy for treating *P. aeruginosa* colonisation would also require a simple method to identify those with inhibitory antibodies such as the Ps IgG2 ELISA generated here.

The results in this thesis and the further cohort of patients (Wells *et al*., 2014), would indicate that this inhibition of serum killing affects around 21% of patients with *P. aeruginosa* colonisation in bronchiectasis and specific identification of suitable patients is necessary. The preliminary results, however, show that removal of inhibitory anti LPS IgG2 both in vitro and in vivo by plasmaphoresis does restore bacterial killing and has identifiable therapeutic benefit.

6.6 Relevance of Inhibitory Antibody in the Lung.

Patients with bronchiectasis and *P. aeruginosa* suffer morbidity associated with their condition and as outlined previously there is a poorer quality of life in those with colonisation (Wilson *et al,*. 1997; Martinez-Garcia., 2007; Goeminne *et al*., 2012; Rogers *et al*., 2013; Guan *et al*., 2014; Rogers *et al*., 2014; McDonnell *et al*., 2015).

The findings reported in this thesis indicate a worse lung function and quality of life in patients with inhibitory anti LPS IgG2. This mechanism however describes a systemic serum antibody response to *P. aeruginosa,* yet bacteraemia and systemic infection with *P. aeruginosa* is rare in such patients and a literature search failed to yield any results concerning prevalence. *P. aeruginosa* bacteraemia is considered a problem in

immunocompromised patients with an increased prevalence associated with haematological malignancies (Fick., 1993). Retrospective studies of patients with identified *P. aeruginosa* bacteraemia demonstrated that 79% - 88% of these were attributed to hospital-acquired infection with the majority of patients being immunocompromised (Aliaga, Mediavilla and Cobo, 2002; Kang *et al*., 2003).

High levels of antibody have been shown to be present in the lungs of patients with bronchiectasis both as a result of diffusion from plasma and that locally produced within the lung (Hill *et al*., 1998). Whether or not a similar inhibitory mechanism to that described in serum is present when antibody is isolated from lung is not known. However, it would seem unlikely that local immune responses would not also be influenced by the presenting antigens and certainly in the presence of active lung inflammation the predominant source of airways antibody would be that derived from the serum.

A study published after the completion of the work for this thesis demonstrated higher levels of IgG1 and IgG2 in the sol phase of sputum from one patient with impaired serum killing compared with a patient with normal serum killing of their *P. aeruginosa* strain and the *P aeruginosa* was shown to be bound to IgG2. When this opsonised bacterium was mixed with HCS, the strains isolated from the patient with impaired serum killing and opsonised with sol phase from this same patient, demonstrated no bacterial killing after 180 minutes incubation. When a resistant *P. aeruginosa* strain from a different patient was opsonised with the same sol phase and exposed to HCS bacterial killing was also reduced. However when opsonised with sol phase from a patient colonised with *P. aeruginosa* but with normal serum killing and exposed to HCS bacterial killing was complete after 45 minutes. This indicated that inhibitory antibodies have a similar effect when isolated from sputum as from serum. Strains from patients with normal serum killing and inhibitory serum killing opsonised with HCS

were both killed rapidly by washed peripheral blood cells. However when the strain from the patient with impaired serum killing was opsonised with his/her own inhibitory sera and incubated with washed peripheral cells it was not killed (Wells *et al*., 2014). This also suggests a role of inhibitory antibody in preventing killing of bacteria within the lung from immune cell mediated clearance.

Recognising that *P. aeruginosa* often exists within a biofilm in the lung it was also shown that incubating the biofilm of the strain from a patient with impaired serum killing with HCS or serum from a patient with normal serum killing reduced the amount of biofilm over a 2hr period, whilst incubation with autologous serum had no effect (Wells *et al*., 2014). This indicated that in addition to preventing direct antibody mediated killing of bacteria, cellular mediated killing via opsonisation is also impaired.

6.7 Future work

Future work would benefit from a continued study of anti LPS IgG2 mediated inhibition of *P. aeruginosa* killing in a larger cohort of patients with colonisation. I have identified trends towards poorer clinical status in the patients with inhibition of serum killing but analysis of a larger group of patients would increase the power to determine the clinical significance on progression of lung disease and the effect on quality of life and symptoms. The presence of impaired serum killing described here should also be explored in other groups of patients susceptible to *P. aeruginosa* colonisation, specifically those with cystic fibrosis. As described previously the strains of *P. aeruginosa* isolated from the CF lung tend to be of the mucoid phenotype compared to non-mucoid in bronchiectasis. These strains are reported to produce less O-antigen compared with non mucoid strains (Hancock *et al*.,1983; Doring and Pier, 2008). Whether this would affect the prevalence of anti O-antigen IgG2 in serum remains to be determined.

There are other structural and immunological differences in the CF lung compared with non CF bronchiectasis. The CFTR mutation results in a dehydrated and thickened airway surface liquid with impaired mucociliary clearance and impaired local immune response as a consequence (Gellatly and Hancock, 2013). Additionally, lung damage tends to occur in a much more generalised pattern of distribution in CF where it can remain localised to a specific lung or lobe in bronchiectasis – the aetiology of bronchiectasis may have some influence. Cystic fibrosis also affects a younger age group than non CF bronchiectasis. CF patients with *P. aeruginosa* colonisation are also managed differently in clinical practice to those with non CF bronchiectasis regularly inhaling anti pseudomonal antibiotics, or attending hospital regularly for administration of prolonged courses of intravenous antibiotics. Therefore, it is likely that any sputum or serum samples collected from patients with CF will be taken at a time when they are receiving antibiotic/s, either systemically or by the inhaled route in contrast to the non CF bronchiectasis patients who provide samples outside these constraints. Non CF bronchiectasis patients more likely take oral or intravenous antibiotic only at the time of an exacerbation in contrast to patients with CF. Therefore it is important to recognise that these differences could potentially affect outcome studies and impact of anti O-antigen IgG2 in patients with CF.

6.8. Conclusion.

A proportion of patients with bronchiectasis and *P. aeruginosa* colonisation, have anti LPS IgG2 in their serum that impairs killing of autologous bacterial strains. These strains have been shown to express long chain O-antigen that is recognized by the inhibitory antibodies. This may partially explain the persistence of *P. aeruginosa* colonisation in these patients although it is recognised that a number of other factors can also affect the virulence of *P. aeruginosa.* Here, I have described an antibody dependent mechanism capable of enhancing

bacterial colonisation. There is an association with poorer lung function and increased hospital admissions in this small group of affected patients.

Future therapeutic strategies could be influenced by these data and the recent report demonstrating some clinical success of removing the inhibitory antibody from serum. The finding of ineffective anti-LPS IgG2 may also have implications for future vaccine design. Future work evaluating the presence and prevalence of this inhibition of bacterial killing by serum, and its presence and role in sputum as a direct factor in bacterial killing and the opsonophagocytic pathway in larger cohorts of patients both with non CF bronchiectasis and CF may allow further understanding of the impact and mechanisms of *P. aeruginosa* colonisation.

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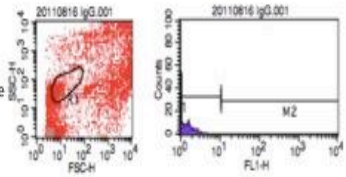
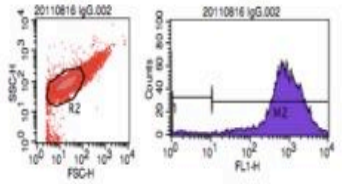
APPENDIX 1

Negative



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	949	100	1.22
M1	1	11	949	100	1.22
M2	11	9910	0	0	**

P1A/S1



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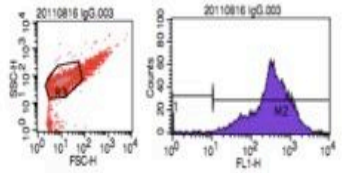
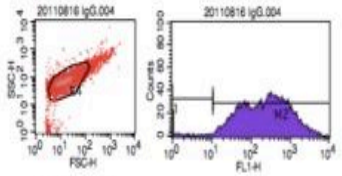
All	1	9910	11001	100	717.62
M1	1	11	190	1.73	1.80
M2	11	9910	10612	96.28	796.95

P1A/S2



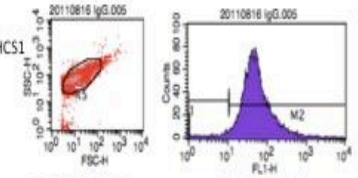
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10408	100	310.12
M1	1	11	89	0.86	2.26
M2	11	9910	10320	99.15	323.5

P1A/S3



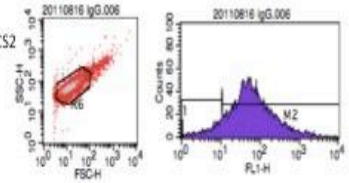
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10650	100	211.86
M1	1	11	107	1.00	1.6
M2	11	9910	10543	99.00	222.5

P1A/HCS1



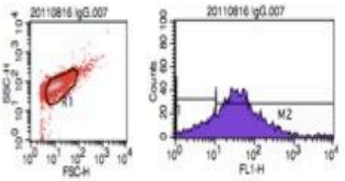
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10512	100	48.6
M1	1	11	229	2.18	4.0
M2	11	9910	10283	97.82	51.4

P1A/HCS2



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10331	100	54.50
M1	1	11	840	8.13	5.61
M2	11	9910	9505	92.00	66.57

P1A/HCS3



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	9439	100	32.03
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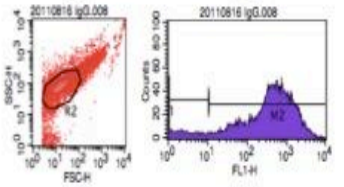
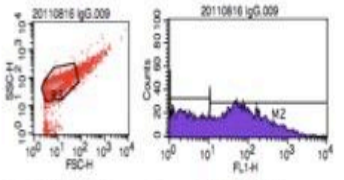
M2	11	9910	7698	81.56	49.99

P2A/S2



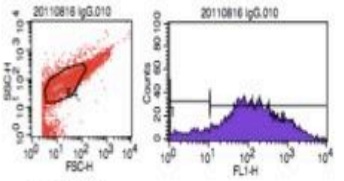
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	11248	100	267.4
M1	1	11	909	8.08	1.8
M2	11	9910	10340	91.93	414.07

P2A/HCS1



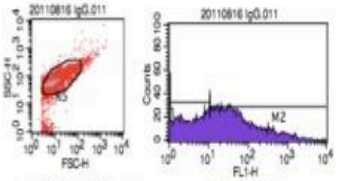
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10825	100	19.15
M1	1	11	4460	41.2	2.41
M2	11	9910	6373	58.87	81.63

P2A/HCS2



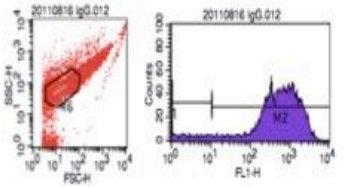
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10838	100	98.87
M1	1	11	1134	10.46	3.0
M2	11	9910	9708	89.57	148.5

P2A/HCS3



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10794	100	14.3
M1	1	11	4713	43.66	2.6
M2	11	9910	6110	56.61	51.9

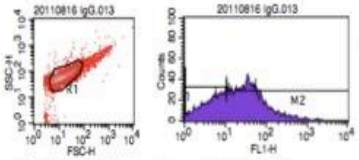
P3A/S3



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	11005	100	406.61
M1	1	11	694	6.31	2.14
M2	11	9910	10312	93.7	578.56

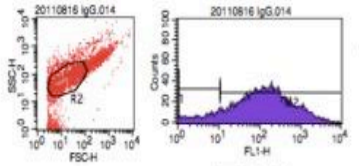
Anti Human IgG- FITC

P3A/HCS1



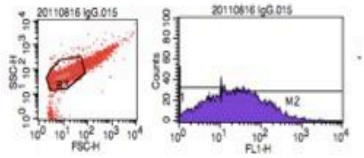
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10007	100	18.45
M1	1	11	3409	34.07	4.29
M2	11	9910	6615	66.10	39.09

P3A/HCS2



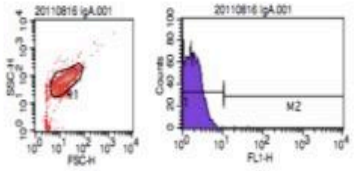
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10902	100	95.5
M1	1	11	1201	11.02	3.39
M2	11	9910	9713	89.09	143.99

P3A/HCS3



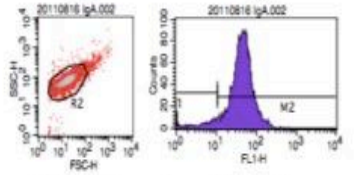
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10482	100	20.22
M1	1	11	3582	34.17	3.84
M2	11	9910	6922	66.04	47.64

Negative



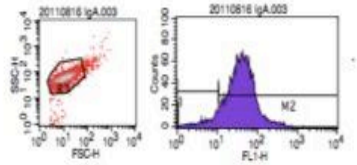
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10021	100	1.65
M1	1	11	10019	99.98	1.63
M2	11	9910	2	0.02	28.52

P1A/S1



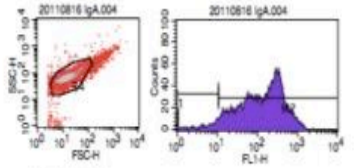
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10359	100	39.7
M1	1	11	470	4.54	5.12
M2	11	9910	9893	95.50	43.8

P1A/S2



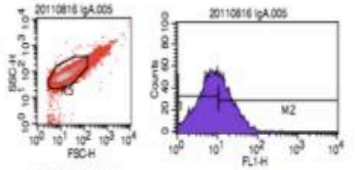
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10337	100	35.29
M1	1	11	703	6.8	5.48
M2	11	9910	9645	93.31	40.36

P1A/S3



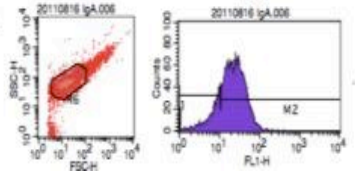
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10742	100	114.5
M1	1	11	395	3.68	4.9
M2	11	9910	10353	96.38	128.9

P1A/HCS1



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	11135	100	8.33
M1	1	11	6849	61.51	4.8
M2	11	9910	4329	38.88	19.7

P1A/HCS2



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10521	100	19.38
M1	1	11	1995	18.96	6.46
M2	11	9910	8563	81.39	24.97

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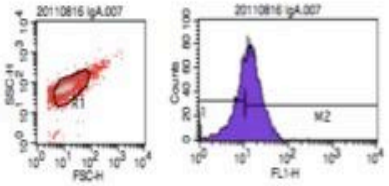
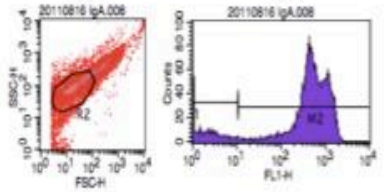
P1A/HCS3



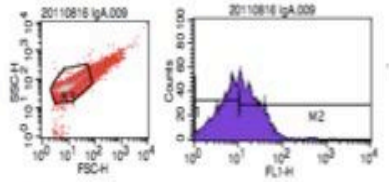
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	9801	100	12.75
M1	1	11	3524	35.96	6.89
M2	11	9910	6324	64.52	17.95

P2A/S2



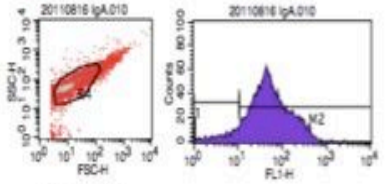
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	11838	100	282.34
M1	1	11	1289	10.89	1.84
M2	11	9910	10550	89.12	522.10

P2A/HCS1



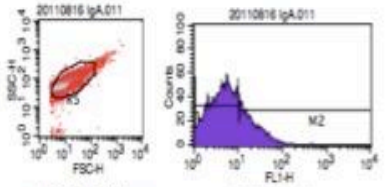
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10645	100	10.29
M1	1	11	5455	51.24	4.97
M2	11	9910	5228	49.11	21.99

P2A/HCS2



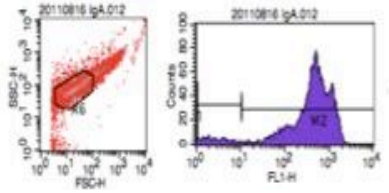
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10760	100	47.56
M1	1	11	726	6.75	5.0
M2	11	9910	10046	93.36	55.8

P2A/HCS3



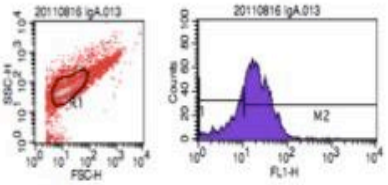
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10881	100	5.53
M1	1	11	8324	76.5	3.76
M2	11	9910	2581	23.72	20.3

P3A/S3



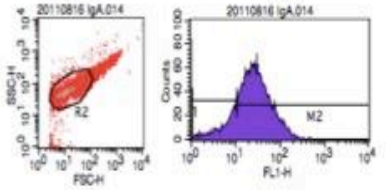
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10901	100	334.91
M1	1	11	554	5.08	2.03
M2	11	9910	10347	94.92	440.23

P3A/HCS1



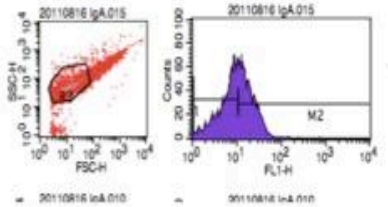
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10278	100	16.48
M1	1	11	2583	25.13	5.46
M2	11	9910	7735	75.26	23.78

P3A/HCS2



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10800	100	22.93
M1	1	11	1724	15.96	5.53
M2	11	9910	9103	84.29	29.9

P3A/HCS3



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10382	100	9.52
M1	1	11	5564	53.59	5.46
M2	11	9910	4885	47.05	17.96

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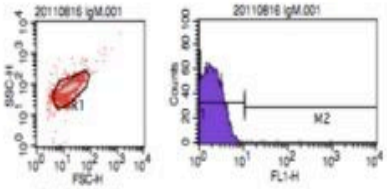
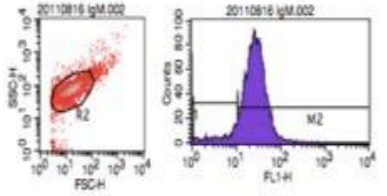
Negative



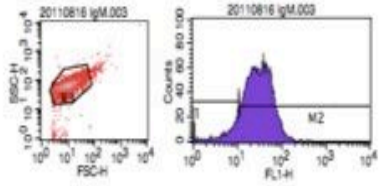
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10005	100	1.72
M1	1	11	9998	99.93	1.71
M2	11	9910	7	0.07	52.87

P1A/S1



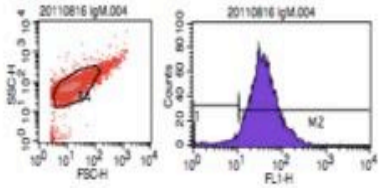
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10379	100	23.3
M1	1	11	791	7.62	4.6
M2	11	9910	9600	92.49	26.69

P1A/S2



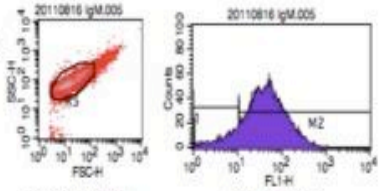
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10516	100	27.12
M1	1	11	946	9.00	6.54
M2	11	9910	9585	91.15	31.16

P1A/S3



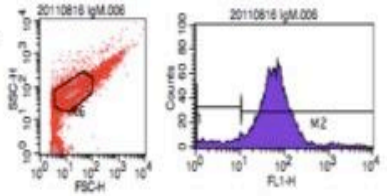
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10494	100	36.9
M1	1	11	313	2.98	4.7
M2	11	9910	10187	97.07	39.3

P1A/HCS1



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	11146	100	38.1
M1	1	11	1145	10.27	5.1
M2	11	9910	10016	89.86	47.6

P1A/HCS2



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10634	100	49.02
M1	1	11	771	7.25	2.91
M2	11	9910	9868	92.80	61.08

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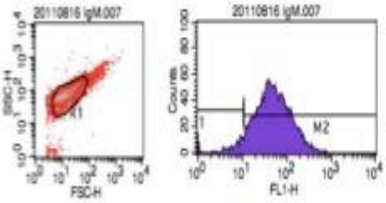
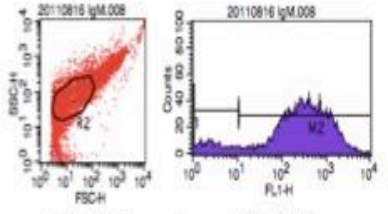
P1A/HCS3



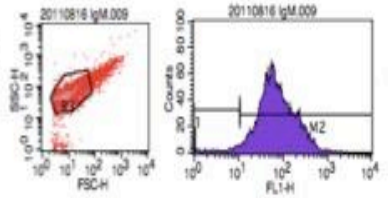
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	9687	100	45.89
M1	1	11	422	4.36	6.73
M2	11	9910	9272	95.72	50.73

P2A/S2



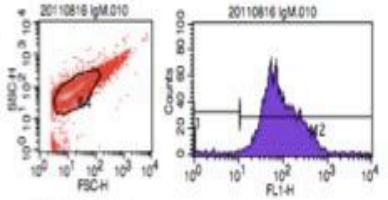
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	11602	100	180.91
M1	1	11	1385	11.94	2.0
M2	11	9910	10220	88.09	332.1

P2A/HCS1



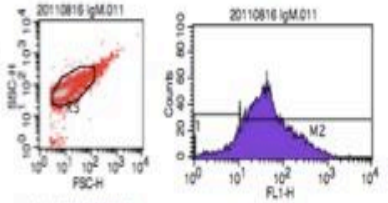
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10802	100	68.54
M1	1	11	222	2.06	3.63
M2	11	9910	10580	97.94	72.95

P2A/HCS2



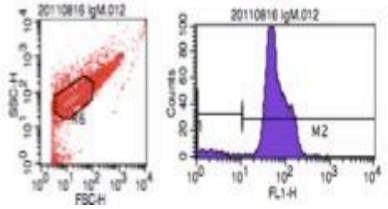
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10821	100	83.4
M1	1	11	176	1.63	2.3
M2	11	9910	10647	98.39	88.43

P2A/HCS3



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10904	100	41.21
M1	1	11	973	8.92	6.0
M2	11	9910	9944	91.2	49.7

P3A/S3



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10789	100	53.08
M1	1	11	448	4.15	1.77
M2	11	9910	10341	95.85	61.5

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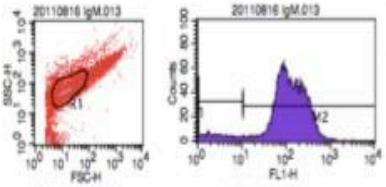
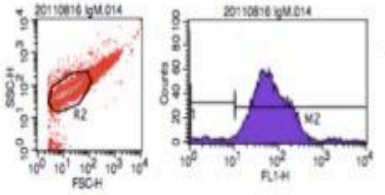
P3A/HCS1



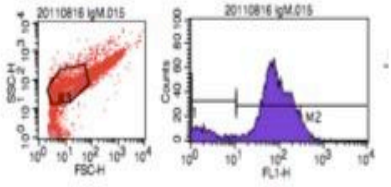
Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10476	100	96.46
M1	1	11	641	6.12	2.24
M2	11	9910	9836	93.89	123.23

P3A/HCS2



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	11008	100	61.6
M1	1	11	366	3.32	2.36
M2	11	9910	10643	96.68	68.93

P3A/HCS3



Marker	Left	Right	Events	% Gated	Geo Mean
All	1	9910	10796	100	54.57
M1	1	11	1327	12.29	1.69
M2	11	9910	9471	87.73	88.79