Immunological responses associated with the use of a putative dual vaccine against Hepatitis B Virus and *Streptococcus pneumoniae*

2015 USQ Honours Project – Daniel Smith

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Acknowledgments

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I would like to extend sincere gratitude to all those who have contributed in any way to the project I have embarked upon this year. I firstly thank my supervisor Professor Michael Kotiw for giving me the opportunity to work on this project and providing training and assistance throughout the year. To Stephen Wanyonyi, Hieng Lu and Lisa Seymour, I am indebted to you all for sharing your skills, your intelligence and your kindness.

I have appreciated the help in various aspects of the project from numerous others throughout the year. In particular, I thank Sharyn Carnahan and Wendy Merrin for their instruction in histology and the staff at the Toowoomba Base Hospital for assistance in tissue staining. I also am thankful for John Dearnaley in giving support above and beyond what he needed to.

Lastly, I sincerely thank to my family not only for their support this year, but for all the years previous and I'm sure the years to come.

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Chapter 1 – Abstract

Streptococcus pneumoniae is a significant pathogen globally and a lead cause of invasive disease, pneumonia, sepsis and otitis media. The bacteria's capacity for establishing an infection is aided by virulence factors (especially the presence of a capsule) and a high prevalence of nasopharyngeal carriage (up to 30% in healthy populations). Infections from *S. pneumoniae* account for a significant burden of morbidity and mortality in the elderly and children globally, with substantial morbidity in Indigenous children due to very high otitis media rates. There are at present two vaccines available for protection against *S. pneumoniae*. Pneumococcal Polysaccharide Vaccine (PPSV) conveys protection against 23 of the 90+ serotypes, but does not provide a memory response in children under 2 years old. Pneumococcal Conjugate Vaccine (PCV) only conveys protection against 13 serotypes, but because it utilises protein conjugation it is able to stimulate a memory immune response in children under 2 years old. The focus of this project was on two strong vaccine candidates, the pneumococcal capsular proteins PiaA and PiuA. BALB/c mice were vaccinated with these proteins to elicit a T-cell dependent, humoral and Cell Mediated Immunity (CMI) response. An adjuvant (Advax™) and conjugation of both PiaA and PiuA to Hepatitis B surface antigen (HBsAg) were techniques used to stimulate this response. The efficacy of the vaccine was first measured by determining the immunological response to the vaccine through antibody production. Mice were then challenged with *S. pneumoniae* and their response was analysed to determine if protection had been achieved. The vaccine produced a mostly humoral response, though CMI was higher than for PiuA and PiaA vaccines in previous studies in the literature. The vaccination appeared to prevent invasive disease by significantly lowering numbers of the bacteria in the blood, though signs of infection were present from examination of lung tissue and presence of bacteria in lungs. The HBsAg was able to stimulate a humoral immunological response against HBV, and conjugation to PiaA and PiuA did not inhibit this. Both PiaA and PiuA produced a level of protection against *S. pneumoniae* and can be potentially conjugated to HBsAg to protect against HBV simultaneously.

Chapter 2 Literature Review

2.1 Introduction

Streptococcus pneumoniae is a gram positive, facultative anaerobic diplococcus that often colonizes the nasopharynx in humans. It exists in the upper respiratory tract mainly as a commensal bacterium in healthy carriers and this carriage is conducive to its ongoing prevalence in communities (van der Poll & Opal 2009). There are currently 94 distinct serotypes of *S. pneumoniae* with varying propensities for nasopharyngeal colonisation and inducing invasive disease (Calix et al. 2012). It continues to be a leading bacterial cause of invasive disease, pneumonia and otitis media. Strategies to reduce the global burden of the pathogen involve treatments (dependent on the nature and severity of the infection) and prevention in the form of vaccination. Vaccination attempts against *S. pneumoniae* began in the early 1900s with killed pneumococci acting as the immunogenic agent. These had limited success due mainly to the underestimated variability between serotypes (Malley & Anderson 2012). The range of serotypes continues to be problematic for vaccines today, as well as the establishing of lasting immunity. Much research has been conducted recently attempting to address these two criteria, but relatively little progress has been made. Surface proteins on the capsule of the bacterium have been viewed as a vaccine candidate with high potential, especially if the proteins are shared amongst all serotypes.

2.2 *Streptococcus pneumoniae*

S. pneumoniae is a significant pathogen on a global scale. The mechanisms by which it causes disease, the significance of these diseases and the current strategies aimed at preventing these diseases are discussed in this section.

2.2.2 Virulence Factors

The polysaccharide capsule surrounding the bacterium has been well recognised as the most important virulence factor due to its anti-phagocytic properties (Jonsson et al. 1985; Mitchell & Mitchell 2010). The capsule is effective in evading mucus clearance (Nelson et al. 2007), reducing

exposure to antibiotics (van der Poll & Opal 2009) and in prohibiting antibodies from binding to phagocytes (Musher 1992). The bacterium has a number of other virulence factors and these are summarised in Table 1.

Virulence Factor	Method of Virulence
Phosphorylcholine (ChoP)	Adheres to epithelial surface through binding to the receptor for platelet-activating factor (rPAF) (Bergmann & Hammerschmidt 2006)
Choline-binding protein A (CbpA)	Binds to secretory component on sIgA to inhibit binding of sIgA to bacterium (Hammerschmidt et al. 1997)
Neuraminidase A (NanA), β-galactosidase A (BgaA), β-N-acetylglucosaminidase (StrH)	Molecules act successively in deglycosylating N-linked glycans on host glycoproteins (King, Hippe & Weiser 2006)
Hyaluronate lyase (Hyl)	Breaks down Hyaluronan (a polysaccharide of connective tissues) and promotes adhesion to surface (Jedrzejas et al. 2002)
Pneumococcal adhesion virulence A (PavA)	Binds to the extracellular-matrix component, fibronectin (Holmes et al. 2001)
α-Enolase	Binds to the extracellular-matrix component, plasminogen (Bergmann et al. 2001)
Pneumocin	Antimicrobial peptide which specifically targets similar bacteria to counter competition (Dawid, Roche & Weiser 2007)
IgA1 protease	Cleaves IgA1 of the host (Polissi et al. 1998; Proctor & Manning 1990)
Pneumococcal surface protein A (PspA)	Inhibits binding of the complement component C3 with the surface of the bacterium. Also thought to protect from bactericidal activity of apolactoferrin (Paton et al. 2008)

Pneumolysin	Very important virulence factor responsible for lysing host cells through creating transmembrane pores (Tweten 2005)

Table 1. Virulence factors in *S. pneumoniae* and their method of action

2.2.3 Host Carriage

It is not unusual for carriage to occur in the normal population, yet colonization is clinically important since it is an essential step in the progression of pneumococcal disease (Bogaert, de Groot & Hermans 2004; Simell et al. 2012). Nasopharyngeal carriage rates in healthy population vary between locations and age groups, but they can be up to 30% (Lee et al. 2014) and are found to generally increase in children as they grow (Syrjänen et al. 2001).

2.2.4 Serotypes

S. pneumoniae serotypes are distinguished through their capsular immunochemistry particularities. Serotypes differ in their potential for invasive disease, the location of infection in the host and the severity of the disease (Rodrigo & Lim 2014). Accordingly, specific serotypes have 'invasive capacities' which are determined according to their likelihood of colonisation and causing disease (Sandgren et al. 2004). These invasive capacities are primarily based on the antiphagocytic properties conferred on the polysaccharides that the bacterium has on its capsule, and they are to recognise the importance each serotype has in pneumococcal disease and colonisation (Yildirim et al. 2010).

2.2.4.1 Worldwide Serotype Distribution

Serotype distribution and replacement is constantly changing with the introduction of vaccines at various times and at various rates (Weinberger, Malley & Lipsitch 2011). However it is possible to estimate the geographical distribution of serotypes. One study on the global distribution of serotypes (Hackel et al. 2013) was conducted from 2005 to 2009 and it produced an approximation of the main serotypes responsible for invasive pneumococcal infections globally and for each region. The global incidence of each of the 8 most common serotypes can be seen in Table 2 compared to their incidence in the Asia-Pacific region. In the Middle East, the most common serotypes were 19A

(14.3%) and 1 (10.7%). In Africa, 19F and 6B account for 14.3% each. Serotypes 14 and 1 were most prevalent in South America with 13.2% and 10.4% respectively, while 19A (21%) and 6A (7.3%) were most common in North America.

Serotype	Global Incidence (%)	Asia/Pacific Incidence (%)
19A	15.6	10.3
6A	6.9	6.4
7F	6.5	2.6
3	6.1	10.8
22F	5.4	5.1
19F	4.4	7.7
14	4.3	8.3
6B	3.8	5.8

Table 2. The 8 most significant serotypes of *S. pneumoniae* with comparison to their prevalence in the Asia Pacific region (Hackel et al. 2013)

2.3 Pneumococcal Infections

The nasopharynx is the ecological niche in the host where the organism most commonly colonises asymptomatically (Blasi et al. 2012). *S. pneumoniae* is capable of spreading through the lower and upper respiratory tract, and into major organs. Accordingly *S. pneumoniae* can cause non-invasive infections such as sinusitis, otitis media or pneumonia (without bacteraemia), as well as invasive infections such as bacteraemia, meningitis, or pneumonia (with bacteraemia) (Durando et al. 2013). The incidence of each type of infection will depend on location, age, serotype and other factors in the host (Mehr & Wood 2012). Since carriage is a prerequisite to acquiring infection, a predisposition to carriage of the bacteria is an important factor in susceptibility (Labout 2008). Predisposing factors in the host for acquiring pneumococcal infections include diabetes mellitus, alcoholism, HIV

infection, asplenia or hyposplenia and other immunocompromised conditions (van der Poll & Opal 2009).

2.3.1 Scope of Infections

Invasive Pneumococcal Disease (IPD) rates have dropped substantially since the introduction of routine vaccinations (Greenhow et al. 2014). Of all pneumococcal infections, pneumonia is by far the most common. Globally, the incidence rate of pneumococcal infections in children under 5 is 2331 per 100000. Of this, approximately 2228 are cases of pneumonia, 17 are cases of meningitis and 87 are due to other infections (O'Brien et al. 2009).

2.3.2 Burden of Infections

The burden of all *S. pneumoniae* infections is predominantly borne by the elderly and the very young. In those aged 65 years or older, the risk of acquiring a pneumococcal infection and the mortality of an IPD are much greater than any other age group (Butler & Schuchat 1999). In particular, the risk of pneumococcal pneumonia in this demographic is increased due to other underlying diseases, poor nutrition, immunosuppression and a general reduction in mucociliary clearance (Fung & Monteagudo-Chu 2010). In children, although the morbidity and mortality of pneumococcal disease has been consistently dropping through vaccination efforts, pneumococcal infections still cause an estimated 476,000 deaths of children under 5 years old according to the World Health Organisation (2012).

Even though the burden is global, it is acute in Asia and Africa which account for 66% of pneumococcal cases (O'Brien et al. 2009). IPD is still a threat in Australia and has been a nationally notifiable disease since 2001. Accordingly, data from the National Notifiable Diseases Surveillance System records the trends in IPD over time and in 2013 there were 1543 cases of IPD recorded in Australia (de Kluyver 2014). In 2011 the cost of per patient was estimated at $10,132 and $8666 for meningitis and bacteraemia respectively (Newall et al. 2011).

2.3.3 Otitis Media

Otitis media (OM) is the term given to any infection of the middle ear and can be divided into separate conditions. These are: otitis media with effusion (OME), acute otitis media (AOM) and chronic suppurative otitis media (CSOM) (Morris & Leach 2009). OME is defined as the absence of acute symptoms whilst having fluid behind the eardrum. AOM involves the presence of both fluid behind the eardrum and acute symptoms (such as swelling, fever or ear pain) with or without perforation. CSOM is diagnosed when there is persistent discharge through an eardrum perforation (Mahadevan et al. 2012; Morris et al. 2011).

S. pneumoniae is not the only causative agent for OM. Other bacteria including *Haemophilus influenzae* (especially non typable *H. influenzae*) and *Moraxella catarrhalis* or viruses such as respiratory syncytial virus (RSV), rhinoviruses, adenoviruses and influenza viruses can be the aetiological agents (Massa, Cripps & Lehmann 2009).

OM is a substantial burden to healthcare worldwide. It costs the United States approximately $3-5 billion per year in health care funding (Rovers 2008). Globally, approximately 709 million cases of AOM are reported annually, and 51% of these occur in children below 5 years old. CSOM occurs in 31 million people per year with 22.6% of these occurring in children under 5 (Monasta et al. 2012). These infections can be responsible for varying levels of morbidity and mortality. Hearing loss is often associated with each OM, with degrees of loss dependent on age, type and severity of infection, serotypes and population (Jensen, Koch & Homøe 2013; Yehudai, Most & Luntz 2015). Because of its prevalence in school age children, OM has for a long time been seen as a barrier to education and school performance (Lieu 2013; Manders & Tyberghein 1984), although the level of its impact has been debated (Roberts, Rosenfeld & Zeisel 2004). Additionally, clinical complications associated with OM are responsible for approximately 21,000 deaths worldwide each year (Monasta et al. 2012) and OM infections represent a significant burden on healthcare systems because they are one of the leading causes of antibiotic prescriptions (Qureishi et al. 2014).

2.3.3.1 Prevalence and economic cost of OM in Australia

It was estimated by Taylor et al. (2009) that the number of cases of OM in Australia in 2008 was between 1 and 2.5 million. Because of the uncertainty of the prevalence of OM in Australia combined with the variety of treatment options, it is difficult to obtain an accurate estimate of the economic impact of OM in Australia. Taylor et al. (2009) suggested that treatment costs were $100-400 million annually.

2.3.3.2 High Risk Groups

In Australia, 73% of children in their first year of life are expected to contract a form of OM (Mahadevan et al. 2012). Several studies have shown the highest proportion of OM cases occur in indigenous communities. In one of the most recent studies, 709 young indigenous children from remote communities were assessed and it was found that 91% were affected by OM (Morris et al. 2005). Of all children assessed, 41% had OME, 26% had AOM and 15% had CSOM. This prevalence rate is outstandingly high in comparison to other populations including countries in the southeast Asia region such as China and Thailand in which OM prevalence rates of 10% and 3.25% have been reported respectively (Chayarpham et al. 1996; Chen et al. 2003). These statistics corroborate other studies (Gunasekera et al. 2007) which suggest that Australia's indigenous population has one the highest rates of OM incidence in the world. Since several communities declined to participate in this study because they did not consider OM to be a major concern in their community, the data was potentially skewed and the incidence rate could be higher or lower than reported. Additionally, universal definitions for what constitutes OM do not exist leading to over diagnosis accounting for some of the data. Nevertheless, this study does show the enormous health problem that OM currently poses to the Australian population, especially to indigenous children in remote communities.

Importantly, indigenous children have considerably higher rates of OM compared to their nonindigenous Australian counterparts as well as to children in many parts of the world (Bluestone 1998). This disproportionate occurrence of OM is not only apparent in the proportion of incidences,

but also in the severity. In indigenous children, 8-15% of OM cases have been classified as severe (CSOM or perforated tympanic membrane), compared to 1.7% for nonindigenous children (Gunasekera et al. 2007; Morris et al. 2005).

2.4 Pneumococcal Vaccine

The enormous burden of pneumococcal disease has led to a large body of research on prevention. The main strategies targeting pneumococcal disease include antibiotics and immunisation. Since antibiotics are only helpful after infection, immunisation is the key to reducing mortality and morbidity worldwide. The task of creating an effective vaccine has been going on for a century (Musher, Watson & Dominguez 1990). Currently there are four main vaccines available, three of which are conjugate vaccines and the fourth is based on a polysaccharide (Table 3).

Vaccine type	Brand name	Serotypes included
PCV7	*Prevnar*	4, 6B, 9V, 14, 18C, 19F, 23F
PCV10	*Synflorix*	4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F
PCV13	*Prevnar 13*	4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F, 3, 19A, 6A
PPSV23	*Pneumovax 23*	4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F, 3, 19A, 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, 33F

Table 3. Current Pneumococcal vaccines and their serotypes (Chiu & McIntyre 2013)

2.4.1 Pneumococcal Polysaccharide Vaccine (PPSV)

The function of PPSVs is to promote antibody activation for specific capsular polysaccharides found on the capsules of certain *S. pneumoniae* serotypes. The first approved PPSV produced in 1977 covered 14 serotypes (Austrian 2000) and was replaced in 1983 by a new vaccine including 23 serotypes (Jackson & Janoff 2008).

The initial PPSV14 covered the serotypes that were responsible for approximately 68% of IPD (Broome & Facklam 1981). The more recent PPSV23 at the time of release accounted for 85% of IPD causing serotypes at the time of approval (Robbins et al. 1983).

2.4.1.1 Efficacy in Adults

With the relatively long history of PPSV vaccine, there have been many trials demonstrating the efficacy of PPSV in adults (Shapiro et al. 1991)

A 2013 Cochrane meta-analysis showed an overall protective efficacy in adults of 74% for IPD. However, the efficacy was diminished in immunocompromised and chronically ill adults (Moberley et al. 2008). Since protection from pneumococcal disease is closely associated with levels of circulating antibody (Huo et al. 2004; Musher et al. 2000), it is important to consider the maintenance of antibodies in circulation for ongoing protection (Pichichero 2009).

Numerous studies have shown that levels of antibody are considerably higher in those recently vaccinated compared to those who had received polysaccharide vaccination 3 or more years earlier (Hammitt et al. 2011; Manoff, S. et al. 2010; Manoff, S. B. et al. 2010; Musher et al. 2010). Between studies, there is a contrast in how long the elevated immunity from vaccination lasts. Several studies indicate that antibodies produced from PPSV continue to be elevated above unvaccinated levels for 5-10 years at least (Jackson et al. 1999; Waites et al. 2008). Other studies have recorded a return to regular levels within 3-4 years of vaccination (Sankilampi et al. 1997; Törling et al. 2003). Regardless, there is unanimity that antibody concentration decreases over time and current Australian and United States recommendations include repeat vaccinations for any individuals with increased risk (Chiu & McIntyre 2013; Iyer, Ohtola & Westerink 2015).

2.4.1.2 Efficacy in Children

Soon after the licensing and release of PPSV23, it was noted that the vaccine had little or no effect in children under 2 (Douglas & Miles 1984). The low efficacy of PPSV23 has been associated with the inability of capsular polysaccharide antigens to stimulate an appropriate response in immune-

incompetent individuals including infants. Capsular polysaccharides including PPSV23 belong to a class of T-cell independent (TI) antigens known as TI-2 which are incapable of stimulating B cell proliferation but are able to stimulate antibody secretion in mature B cells (Hodgins & Shewen 2012). TI-2 antigens are thus ineffective for infants with an immature immune system, but they become increasingly effective as the immune system develops (Bondada et al. 2000). One method for overcoming this is to conjugate the capsular polysaccharide to a carrier protein which can induce T cell dependent immune response (Hodgins & Shewen 2012).

2.4.2 Pneumococcal Conjugate Vaccine

2.4.2.1 Prevnar (PCV7)

Prevnar (PCV7) is a heptavalent vaccine that consists of capsular polysaccharides from seven serotypes of *S. pneumoniae* conjugated to CRM_{197} (a diphtheria protein) and was produced by Wyeth in the 1990s. It was effective against the respective pneumococcal serotypes in clinical trials (Black et al. 2000) and subsequently in post licensure (Black et al. 2001).

The serotypes included in the PCV7 vaccine were found to cause 80.5% of IPD in children under 5 in the United States at the time when the vaccine was released (Beall et al. 2006; Zangwill et al. 1996). A large scale study in the United States showed that by 2007, the overall incidence of IPD in this age group was 76% lower, and IPD caused by serotypes covered in PCV7 had dropped by 99% (Pilishvili et al. 2010). The average number of IPD cases across all age groups had shown a drop in the United States by 45%, and IPD caused by vaccine serotypes on average was 94% lower. However, this same study concluded that non-vaccine types were causing 29% more IPD than prior to PCV7 introduction. In the children under 5 years, IPD from non-vaccine serotypes had increased by 51%, and specifically IPD from serotype 19A had increased three fold. This is a form of serotype replacement and it can also occur through a vaccine serotype acquiring the capsule of a non-vaccine type (Coffey et al. 1998).

Since only 7 of the 94 serotypes were accounted for in the vaccine, there was a niche vacated by the vaccine serotypes that invited colonisation by other serotypes. The extent to which the other non-vaccine types would replace vaccine types in causing invasive and non-invasive disease was unknown but was of concern (Hanage et al. 2007). Hence there was a need for a vaccine which could account for more serotypes, specifically those that were replacing the vaccine serotypes. Additionally serotype coverage was predominantly suited to serotypes most prevalent in developed nations, so vaccines accounting for serotypes more prevalent in developing nations were needed (Jefferies et al. 2011; Johnson et al. 2010).

2.4.2.2 Prevnar 13 (PCV13)

PCV13 (Prevnar-13) is an expansion of PCV7 and simply adds 6 new serotypes each conjugated to the same diphtheria protein CRM_{197} (Table 3). These serotypes address the serotype replacement attributed to PCV7 as well as the global burden of pneumococcal disease. Another 10-valent vaccine (called PCV10) was licensed by GSK and obtained WHO prequalification in 2009, but because of the success of PCV13, it had less of an impact (Jefferies et al. 2011). A number of studies confirmed that the seven original serotypes and the 6 additional ones conveyed equivalent safety and immunogenic response as those in PCV7 and none interfered with other immunisations (Bryant et al. 2010; Esposito et al. 2010; Kieninger et al. 2010).

2.5 Immune Response to PCV

2.5.1 Innate Immune Responses

Protection against *S. pneumoniae* is organised on various levels and involves a variety of aspects. The first level is that of the innate response. Part of this response uses mucus clearance to deter initial access of the bacterium to the epithelial surfaces. However, some bacterial virulence factors such as the polysaccharide capsule overcome this barrier (Nelson et al. 2007). After overcoming clearance attempts, the bacterium must adhere to the epithelial surface. A change in gene expression can lead to a ‘transparent variation’ in the capsule of the pneumococcus which will result in a thinner capsule

and other adherence factors (Weiser et al. 1994). The bacterium often initiates the complement system, but through various virulence mechanism the pneumococcus is able to interrupt the complement cascade (Jarva et al. 2002; Quin et al. 2005). Hence, the body requires a specific antibody to prevent or eliminate any pneumococcal strains.

2.5.2 Humoral Immunity

Beyond the innate response, the immune system can also mount a humoral response through antibody release and antigen presentation. However the effectiveness of the humoral response is dependent on the age of the individual since capsular polysaccharides such as PPSV only stimulate mature B cells and are only immunogenic in individuals with a mature immune system. PPSV only produces an immediate response by direct stimulation of B cells which release IgM in response (Pletz et al. 2008).

Once the $CD4^{+}$ cells receive the costimulatory molecules (CD80 and CD86), they begin proliferation and begin releasing inflammatory cytokines. These inflammatory cytokines such as interferon gamma (INFγ), tumour necrosis factor alpha (TNFα) and several interleukin molecules are identifying markers to show that the immune response is T-cell dependent (Pletz et al. 2008).

2.6 Hepatitis B

Hepatitis B is a viral infection that is easily transmissible through bodily fluid from person to person contact. It is a disease of the liver and is caused by hepatitis B virus (HBV). HBV is a circular, 3.2kb, partially double stranded DNA virus (Chang 2007). It involves a large spectrum of symptoms and a large range of severity. Hepatitis B can be acute, meaning that illness is present for up to six months after first exposure to HBV, or chronic, meaning that it is an ongoing illness that remains in the body.

2.6.1 Infection

Hepatitis B largely occurs in children with 90% of cases occurring in infants and up to 70% of these resulting in chronic hepatitis B (Della Corte, Comparcola & Nobili 2012). In contrast, HBV infection in adults with mature immune function only results in chronic infection approximately 5% of the time

(Bauer, Sprinzl & Protzer 2011). Infection can be identified by the detection of Hepatitis B surface antigen (HBsAg), envelope antigen (HBeAg) and core antigen (HBcAg) or through antibody response to these (Bauer, Sprinzl & Protzer 2011; Della Corte, Comparcola & Nobili 2012).

2.6.2 Significance

Globally, over 2 billion people have been infected with HBV and approximately 350 million people have chronic liver infections associated with HBV (Lavanchy 2005). The infection rates vary dramatically around the globe and between different populations. The areas with the highest rates of HBV prevalence are East Asia (apart from Japan), the Amazon Basin, southern Europe and parts of Africa. In Australia there are currently 160,000 people living with chronic hepatitis B (Jimenez et al. 2013) and there has been a recent rise that has been attributed possibly to refugees settling in Australia from HBV highly endemic populations (Donohue & Benson 2007).

2.6.3 HBV Vaccine

The Hepatitis B vaccine is available in Australia under several brand names with varying dosage quantities and number of recommended doses. Each vaccine contains a certain dosage of HBsAg, a protein which will stimulate an immune response.

The efficacy and safety of Infanrix hexa (Curran & Goa 2003), Engerix-B (Keating & Noble 2003), Twinrix (Van Damme & Van Herck 2004) and H-B-VAX II (Lai et al. 1986), the current HBV vaccines available in Australia, have been established. The safety and efficacy of HBV vaccine lends itself to the possible conjugation of HBsAg protein to specific capsular antigens to provide an effective conjugate vaccine.

2.7 Purpose of the Project

The central goal of this project is to test the efficacy of a novel vaccine that could potentially provide immunity from all serotypes of *S. pneumoniae*. This research stems off the back of recent work by Professor Jeremy Brown on the immunogenicity of certain capsular proteins on the surface of the *S. pneumoniae* capsule. These proteins, called PiaA and PiuA, are involved in iron transportation and are found in all known serotypes of *S. pneumoniae* (Sutcliffe & Russell 1995; Tai, Yu & Lee 2003). The

fact that the proteins are found across all serotypes makes them an ideal vaccine candidate. Additionally, due to the importance of iron transportation in in vivo growth (Brown, Gilliland & Holden 2001; Brown & Holden 2002), it would be difficult (though not impossible) for new serotypes to arise without these proteins. Since these antigens are proteins, they overcome the T-cell independent problem faced by current vaccine PPSV, and they do not require conjugation to another protein as in PCV13. Therefore, these proteins are ideal for creating an all serotype encompassing immune response of a T-cell dependent nature. The ability for these proteins to provide this protection upon immunization in BALB/c mice has been documented by Jomaa et al. (2006; 2005), however they only showed a humoral response and full protection requires both humoral immunity and cell mediated immunity (CMI) (Wilson et al. 2014).

If the PiaA or PiuA proteins can be modified in such a way as to elicit CMI in addition to the currently established humoral response, it would show a very high potential for a novel vaccine that can provide protection against *S. pneumoniae* infection. Two possible methods for eliciting a CMI response are investigated in this project.

Firstly, the study by Jomaa et al.(2005) used aluminium hydroxide as an adjuvant to enhance the immunological response. Aluminium hydroxide is a common adjuvant, yet recent studies into the possibility of novel adjuvants have shown a better immunological response. Specifically, in this project the adjuvant Advax™ will be used in place of aluminium hydroxide because it has been known to enhance a CMI response in other vaccines (Honda-Okubo, Saade & Petrovsky 2012; Saade et al. 2013). It is hoped that Advax™ may work similarly with PiaA and PiuA.

Secondly, as discussed in section 2.5.3, vaccination with HBsAg has been very effective in providing protection from HBV in humans for decades. HBsAg is not only helpful in providing protection against HBV, but it is also able to assist in the immunogenicity of other vaccines. Specifically, virus-like particles (VLPs) composed of HBsAg have been successfully used as carriers in stimulating a CMI and humoral response (Kotiw et al. 2012; Nyström et al. 2008; Petrovsky 2006). This project will incorporate the independent conjugation of PiaA and PiuA to a VLP composed of HBsAg to promote a CMI response. The other benefit to incorporating HBsAg into the vaccine is that it may function as a dual vaccine to provide protection against *S. pneumoniae* and HBV. The safety and efficacy of HBV vaccination has been known for decades, yet as discussed in Section 2.5.2, there is still a great need for immunization.

In summation, this project was largely similar to the work conducted by Jomaa et al. (2005), but with additional measures to improve CMI response. BALB/c mice were vaccinated with various combinations of PiaA and PiuA conjugated to VLP (HBsAg). The response was measured by comparison between unvaccinated mice, mice vaccinated with PiaA or PiuA or a combination, mice

vaccinated with PiaA or PiuA or both conjugated to VLP. Because *S. pneumoniae* infections start with nasopharyngeal colonisation, additional mice were used to test whether delivering the vaccine via an intranasal route would increase immunogenicity. The project will centre firstly on confirming whether vaccinating with PiaA and PiuA produces a greater immunological response than no vaccination and secondly on whether a conjugated vaccine produces a greater response than an unconjugated vaccine.

Chapter 3 Methods

3.1 Mice

Seventy two female BALB/c mice aged between 3 and 5 weeks were acquired from the Animal Resource Centre, Canning Vale, West Australia and were housed in temperature and humidity-controlled ventilated cabinets (Techniplast, cat No. 1264C). The mice were allowed ad libitum access to water and food and all procedures performed in adherence to animal ethics guidelines provided by the University of Southern Queensland Animal Ethics Committee (AEC) and in accordance with the NHMRC guidelines (NHMRC 2008), Vaccination procedures commenced when the mice were 6 to 8 weeks old after allowing at least one week of acclimatisation to the housing environment.

3.2 Preparation of the vaccine

The research project involved taking a premade vaccine and testing in mice to determine the immunological response. The process is explained here to provide important background information on the vaccine construction and the method by which conjugation took place since this is fundamental to the results. This information was provided from Dr Stephen Wanyonyi.

Full length *S. pneumoniae* iron uptake ABC proteins, PiaA and PiuA were purified as described by Jomaa *et al (2006)* and eluted in phosphate buffered saline (PBS). Recombinant Hepatitis B surface antigen (HBsAg) also referred to in this study as Virus-like Particle (VLP) was purchased from Resolving Images, Preston, Victoria (Cat No. MBS637600). Conjugated VLP-PiaA and VLP-PiuA proteins were provided to the study by Dr Lisa Seymour (Centre for Health Sciences Research, USQ). The conjugated proteins were constructed by chemical cross-linking using the carbodiimide crosslinker chemistry. In brief, carboxyl activation was performed by incubating 300 µg of VLP antigen in activation buffer (0.1M morpholino ethane sulfonic acid (MES), 0.5M NaCl, pH 6.0) containing 0.1mg 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), (Thermofisher, Cat,.No. PIE22980) and 0.15mg N-Hydroxysulfosuccinimide (NHS) for 15 minutes at room temperature. The activation reaction was quenched by adding 0.85 µl mercaptoethanol and

the pH adjusted to 7.0 by adding 20 µl 20X PBS. Subsequently, PiaA and PiuA were individually conjugated to VLP in separate reactions by adding 390 µg of each protein to the activation mix and incubating for 2 hr at room temperature and conjugation stopped by adding hydroxylamine to a final concentration of 10 µM. The conjugated proteins were equilibrated to PBS using 5ml single-use desalting columns (Thermofisher, Cat. No. 89891).

3.3 Vaccination

Vaccinations and mice handling were performed by Dr Stephen Wanyonyi, Dr Lisa Seymour and Heing Lu. The 72 mice were divided into 12 groups of 6 mice each according to the vaccination regimen (Table 4). Mice were vaccinated with 5µg of each conjugate protein three times at 14 day intervals (day 0, 14 and 28) (Table 5). Vaccination was conducted through intranasal and intramuscular routes. Each individual mouse was anaesthetised with 4% isofluorane (Provet QLD, Brisbane) in 100% medical grade oxygen at a flow rate of 1L/min using the isoflurane vaporiser (NorVap, North Yorkshire, UK) before the specific vaccination into the nasal cavity or the hind thigh as specified in Table 6. For each mouse the vaccine was prepared by mixing 5µg of the p-proteins (PiaA or PiuA) with either 1mg of Advax P or 1 µg of Advax M (Vaxine, Bedford Park, South Australia) depending on the vaccination route and the volume adjusted to 50µl per vaccination site. Advax P was used for intramuscular vaccination (IM) while Advax M was used for intranasal (IN) inoculation. Intramuscular vaccinations were performed using 26G needles following sanitisation of the injection site with 70% ethanol.

Group	Vaccination	Vacc. Route	Protein concentration	Vol. of protein (µl)	Vol. of Advax (µl)	Vol. of Saline (µl)	Total Vol. (µl)
1	Saline	IM	0	0	20	30	50
		IN	0	0	0.1	19.9	20
2	Saline	IM	0	0	20	30	50

		IN	0	0	0.1	19.9	20
3	VLP	IM	4250	1.2	20	28.8	50
4	PiaA	IM	251	19.9	20	10.1	50
5	PiuA	IM	280	17.9	20	12.1	50
6	PiaA + PiuA	IM		10 PiaA 9 PiuA	20	11	50
7	VLP: PiaA	IM	344.4	14.5	20	15.5	50
8	VLP: PiuA	IM	383.7	13	20	17	50
9	VLP: PiaA + VLP: PiuA	IM		7.3 VLP: PiaA 6.5 VLP: PiuA	20	16.2	50
10	VLP: PiaA	IM	344.4	14.5	20	15.5	50
		IN		14.5	0.1	5.4	20
11	VLP: PiuA	IM	383.7	13	20	17	50
		IN		13	0.1	6.9	20
12	VLP: PiaA + VLP: PiuA	IM		7.3 VLP: PiaA 6.5 VLP: PiuA	20	16.2	50
		IN		7.3 VLP: PiaA	0.1	6.1	20

Table 4. The vaccination type assigned to each mouse group. Group 1 and 2 are both control groups however only Group 2 was challenged with the bacteria

A summary of the vaccination schedule, tail bleeds, bacterial challenge and euthanasia is shown in Table 5.

Day	Procedure
0	Initial tail bleed and 1st Vaccination
14	Tail bleed and 2nd Vaccination
28	Tail bleed and 3rd Vaccination

38	Final Bleed
42	Bacterial Challenge
44	Euthanasia and Sample Collection

Table 5. The vaccine and euthanasia schedule for each mouse group.

3.4 Blood Collection and preparation of serum

Immediately prior to each vaccination, approximately 50µl of blood sample was collected from the tail vein of each mouse and pooled into a single tube for every vaccination group. This step was conducted by Dr Stephen Wanyonyi, Dr Lisa Seymour and Heing Lu. The blood was stored for up to 16hr at 4°C to allow for clotting following which the blood was centrifuged at 500g for 10 minutes at room temperature. The serum was pipetted into a separate 1.5ml microfuge tube and stored at -20°C until required. Following euthanasia, a terminal bleed was performed through 23g needle puncture of the jugular vein and serum harvested in a similar manner. Terminal serum samples from each mouse were stored individually at -20°C until required.

3.5 Bacterial Challenge

14 days after the final vaccination (day 42) the mice (with the exception of the negative control group) were anaesthetised in the same manner as previously described and were challenged intranasally by Dr Stephen Wanyonyi with approximately 10^6 colony forming units (CFU) of *Streptococcus pneumoniae* strain D39 in 50µl of PBS. All bacterial inoculations were performed in a Biosafety Class II cabinet and mice placed in a cage equipped with a Hepa filter before being placed back into the ventilated Hepa filtered cabinet. Infected mice were monitored for clinical signs of disease at two hour intervals for the first day and twice a day for the second day according to the following schedule of animal clinical signs.

3.6 Euthanasia and tissue collection

48 hours post bacterial challenge the mice were euthanized for sample collection. Mice were individually anesthetised by Professor Michael Kotiw using the method previously described and

then given an intraperitoneal injection of 200µl sodium pentobarbitone (Virbac, Lethabarb) using a 24G needle.

3.6.1 NPA and Blood Cultures

In order to collect Nasopharyngeal Aspirate (NPA), 2ml of Phosphate Buffered Saline (PBS) was injected into the trachea in such a way that it would travel through the nasopharynx and out the nostrils. This was collected and a 100µl aliquot of the NPA was serially diluted and cultured on Columbia Horse Blood Agar (HBA) plates, (Macromedia, cat. No.1085) and the plates incubated at 37°C, 5% CO^{2} for 16hr followed by examination and colony enumeration of *S. pneumoniae*. As described in Section 3.4, a terminal bleed was taken and cultured in the same form as the NPAs. I assisted Professor Michael Kotiw in both of these procedures.

3.7 ELISA

Enzyme Linked Immunosorbent Assays (ELISA's) were performed to measure the level of total IgG, IgG1 and IgG2a induced by vaccination with either PiaA, PiuA or VLP. 96 well plates (NUNC) were coated overnight at 4°C with 2µg/ml of either PiaA or PiuA in 0.1M NaHCO3 (pH8), or 0.5µg/ml VLP in 0.1M NaHCO3 (pH9.5). The plates were then blocked for 1hr with 2% skim milk dissolved in PBS. Serial dilutions (diluted in 1% skim milk from 1/200 to 1/32000000) from the final bleed (day 38) of pooled sera from each group were allowed to bind for 2 hours before washing 3 times using PBS with 0.1% Tween-20 (PBST). Appropriate goat anti-mouse antibody (AbD Serotec) diluted (IgG 1:3000, IgG1 and IgG2a 1:5000) in 1% skim milk was added to wells and incubated for 1 hour at room temperature. After washing 6 times with PBST, 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-Aldrich) was added before stopping colour development with 0.1M Sulfuric Acid. Plates were read at 450nm.

3.8 Histology

3.8.1 Lung Collection

After mice were euthanized and following blood and BALF collection, a lung lobe was collected using sterilised tweezers and scalpel from each mouse and stored in 10% buffered formalin until use.

3.8.2 Slide Preparation

With assistance from Sharyn Carnahan and Wendy Merrin, lung tissue was trimmed (approximately to a 1cm^2 piece) and fixed overnight using a Shandon Pathcentre© Tissue Processor before being embedded in paraffin. Sections 5µm thick were cut and put on slides.

3.8.3 Haematoxylin and Eosin (H&E) staining

H&E staining was conducted for determining pathology of the lung tissue including visible signs of inflammation or tissue damage as described in Gurcan et al. (2009). This step was conducted by Queensland Health Staff at the Toowoomba Base Hospital. For H&E staining, slides were added to xylene 3 times for 2 minutes each for dewaxing. The slides were then placed in decreasing concentrations of ethanol for 2 minutes each (100% x2, 90%, 70%) and then placed under running water for 2 minutes. The slides were placed in haematoxylin stain for 6 minutes then placed back into running water for another 2 minutes. 70% ethanol was applied to the slides for 2 minutes before being placed in Eosin Yellowish counterstain for 6 minutes. The slides were then placed in increasing ethanol concentrations and then fixed in xylene in exactly the reverse of the steps at the beginning of this process. Slides were examined by Professor Kotiw using an Olympus BX61 digital imaging microscope and images taken using x40 and x20 magnification.

3.8.4 Gram Stain

A Gram stain was conducted on the slides to establish the presence of bacteria in the lung sections following the procedure described by Beveridge (2001). This procedure was also conducted by the staff at the Toowoomba Base Hospital. In brief, after dewaxing (as detailed in the H&E method) each

slide was flooded with Crystal Violet for 1 minute before rinsing with distilled water and applying Gram's Iodine for 1 minute. The water rinse was repeated and the sections were decolourised with acetone before applying 95% ethanol as a decolourising agent for up to 5 seconds before another rinse. Then the safranin stain was applied for 30 seconds before rinsing a final time and leaving to dry. Slides were examined and images taken at x100 magnification by Professor Kotiw under oil emersion using an Olympus BX61 digital imaging microscope.

Chapter 4 Results

4.1 ELISA

In order to determine the immunologic response to vaccination with *Streptococcus pneumonia* outer membrane proteins PiaA and PiuA, ELISA's were performed on the final bleed sera (Day 38) to measure PiaA and PiuA-specific total IgG, IgG1 and IgG2a titres. Serial dilutions were performed on sera starting at 1/200 until the dilution which yielded an optical density (O.D) reading lower than a predetermined cut-off (negative control O.D). The cut-off point is for differentiating a positive reading from background absorbance and was calculated as the mean of O.D readings from 24 control wells plus their Standard Deviation (SD). Using statistical analysis software (GraphPad Prism): Mean = 0.05563; SD = 0.01272. Therefore the cut-off value was 0.06835.

The ELISA results are categorised by antibody sub-type (total IgG, IgG1 and IgG2a), and these sections are divided into the particular protein that the antibody is specific for PiaA, PiuA and the VLP. Most of the vaccination was conducted through the intramuscular route. Three mouse groups were given intranasal and intramuscular vaccination and these are denoted by "(IM+IN)".

The serial dilutions are presented on the x-axis in the graphs in figures 1,3,5,7,9,11,13,15 and 17 where the dotted line represents the cut-off point (0.06835). The raw data has been aggregated and placed in the appendix for reference.

4.1.1 Total IgG Response

4.1.1.1 Anti-PiaA Total IgG

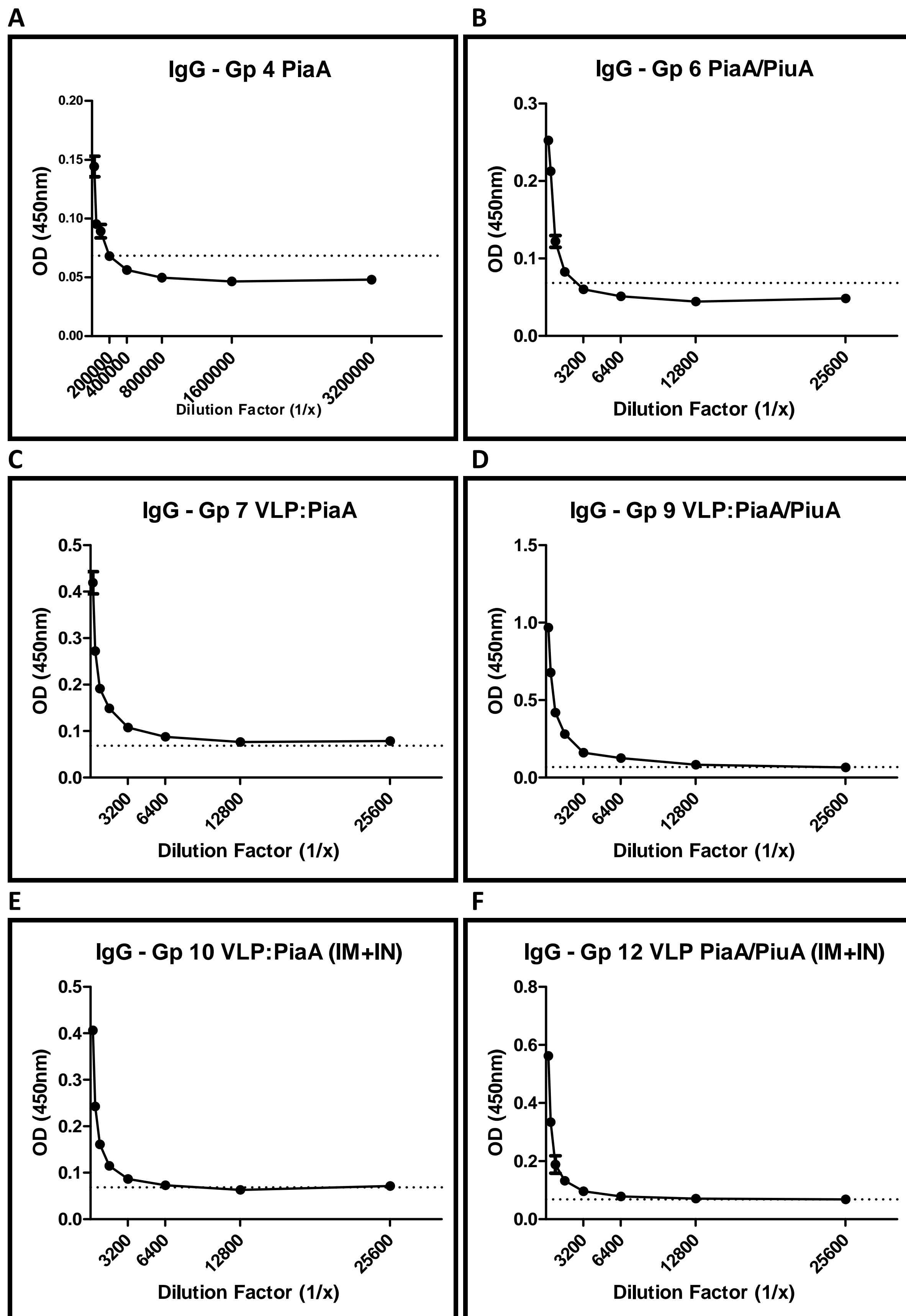


Figure 1. Total anti-PiaA IgG antibody titres in serum from mice vaccinated with PiaA and PiuA. Values were measured in triplicate. Error bars are present for dilutions with significant variation. A) anti-PiaA IgG antibody titre in mice vaccinated with PiaA. The serum was serially diluted and the titre determined to be

the dilution factor corresponding to the O.D reading immediately before the cut-off (shown with a dotted line). B) anti-PiaA IgG antibody titre in mice vaccinated with PiaA and PiuA. C) anti-PiaA IgG antibody titre in mice vaccinated with PiaA conjugated to VLP. D) anti-PiaA IgG antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates E) anti-PiaA IgG antibody titre in mice vaccinated with VLP-PiaA conjugate through intramuscular and intranasal routes. F) anti-PiaA IgG antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates through intramuscular and intranasal routes

The titre points determined from Figure 1 have been collated and compared in Figure 2. Notably, all groups vaccinated with the protein elicited an IgG response. Titres of the VLP:PiaA and VLP:PiaA/PiuA vaccinates were relatively similar, with only 2 dilutions apart between them. Those vaccinated with PiaA alone had a titre 6 times higher than when it was combined with the PiuA protein.

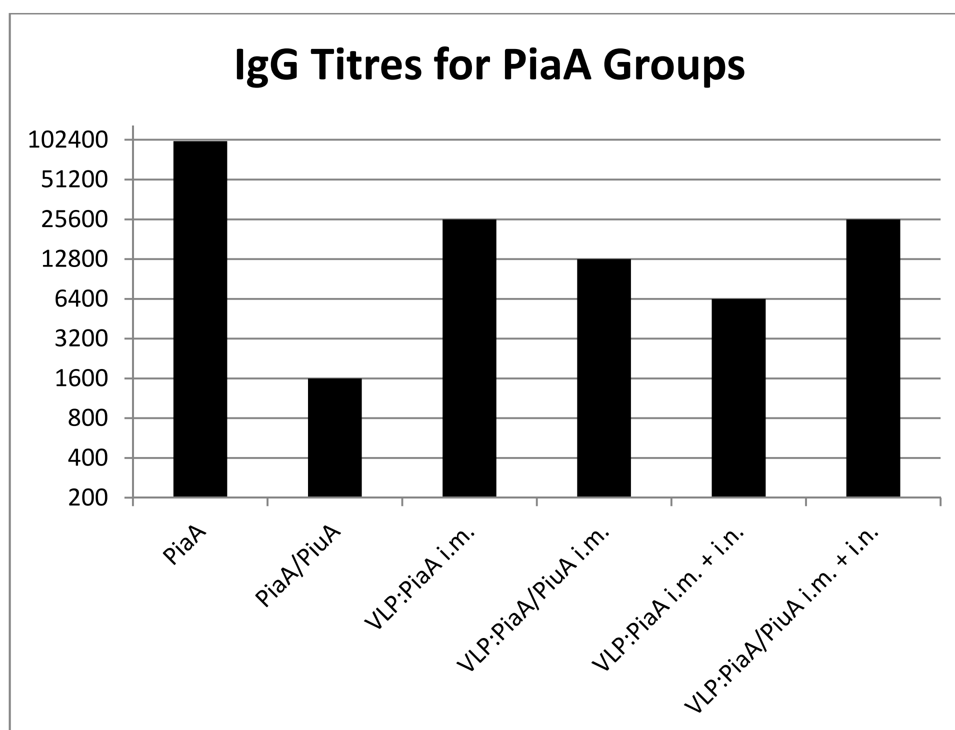


Figure 2. Collated titres of Anti-PiaA total IgG for mouse groups vaccinated with the PiaA protein. Some mouse groups were vaccinated with a combination of both PiaA and PiuA. In these cases, it is only the Anti-PiaA IgG that is measured. Anti-PiuA IgG is measured in the PiuA section (4.1.1.2).

4.1.1.2 Anti-PiuA Total IgG

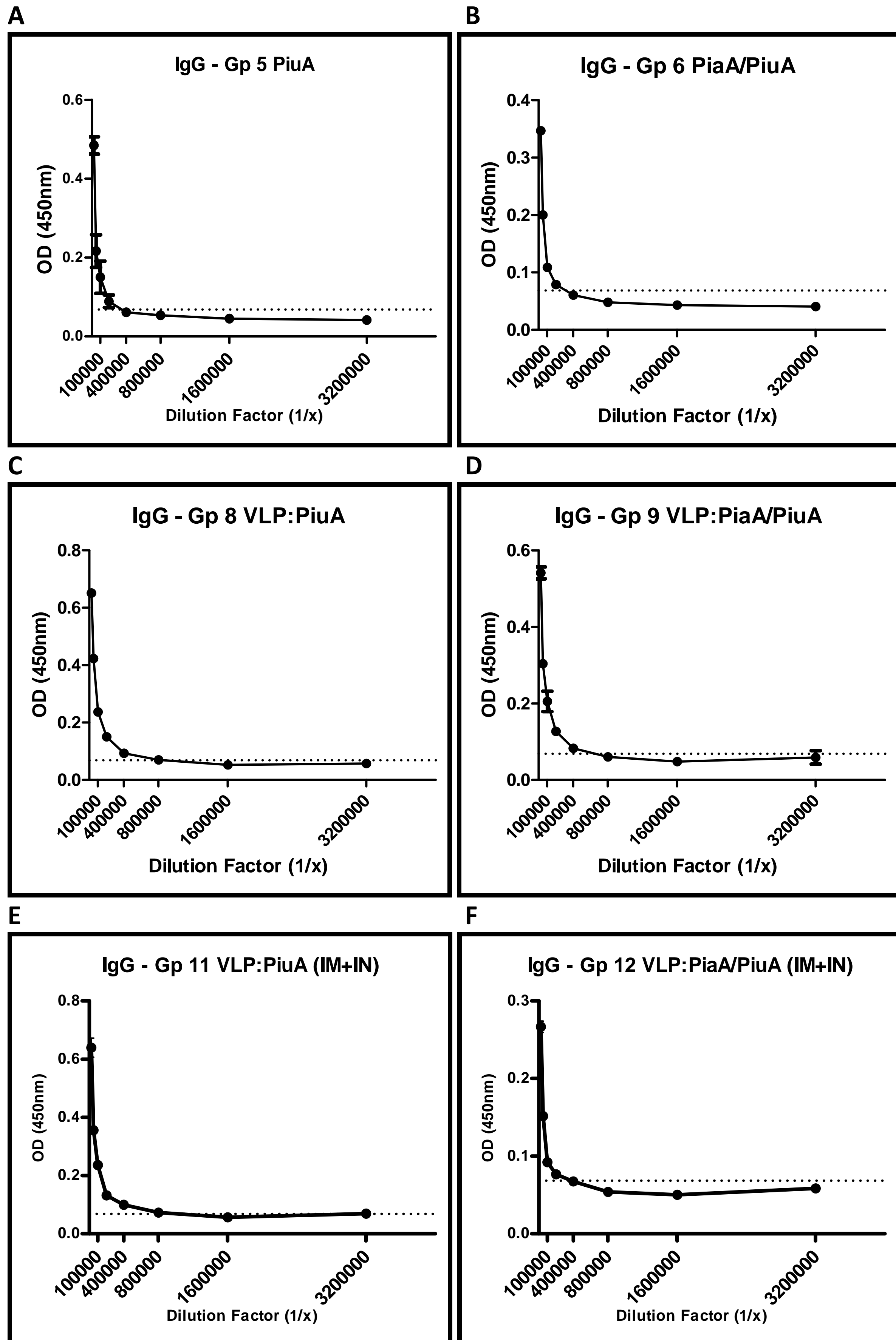


Figure 3. Total anti-PiuA IgG antibody titres in serum from mice vaccinated with PiaA and PiuA. A) anti-PiuA IgG antibody titre in mice vaccinated with PiuA. B) anti-PiuA IgG antibody titre in mice vaccinated with PiaA

and PiuA. C) anti-PiuA IgG antibody titre in mice vaccinated with PiuA conjugated to VLP. D) anti-PiuA IgG antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates E) anti-PiuA IgG antibody titre in mice vaccinated with VLP-PiuA conjugate through intramuscular and intranasal routes. F) anti-PiuA IgG antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates through intramuscular and intranasal routes.

There was no difference in total IgG titre between mice vaccinated with PiuA alone and a combination of PiuA and PiaA. Except for mice vaccinated with a combination of VLP:PiaA and VLP:PiuA through both the intramuscular and intranasal routes the conjugation of PiuA to VLP induced a greater total IgG titre than the unconjugated vaccines.

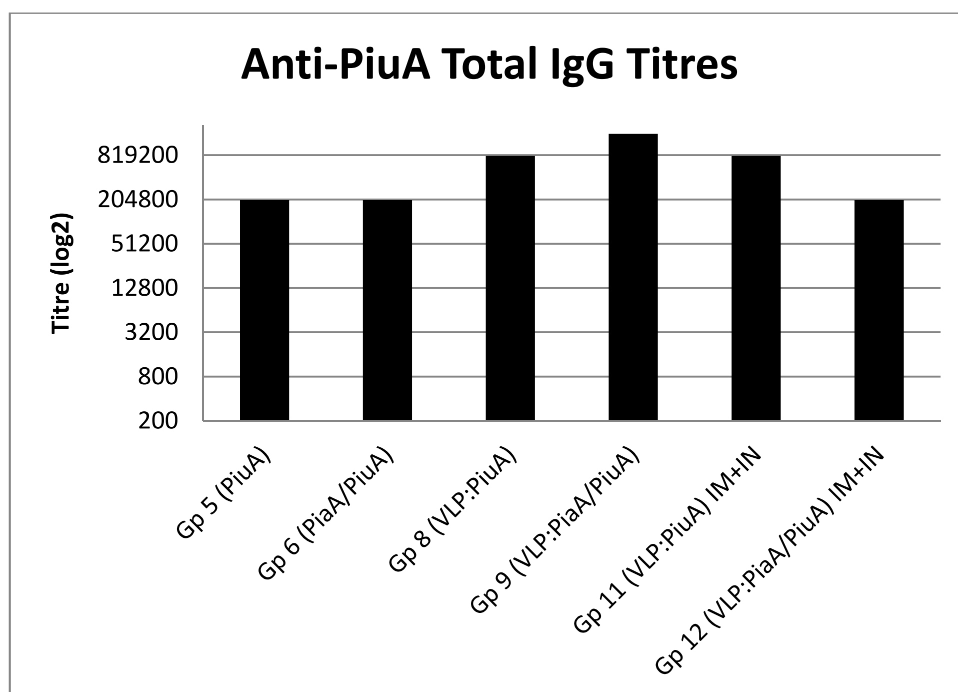
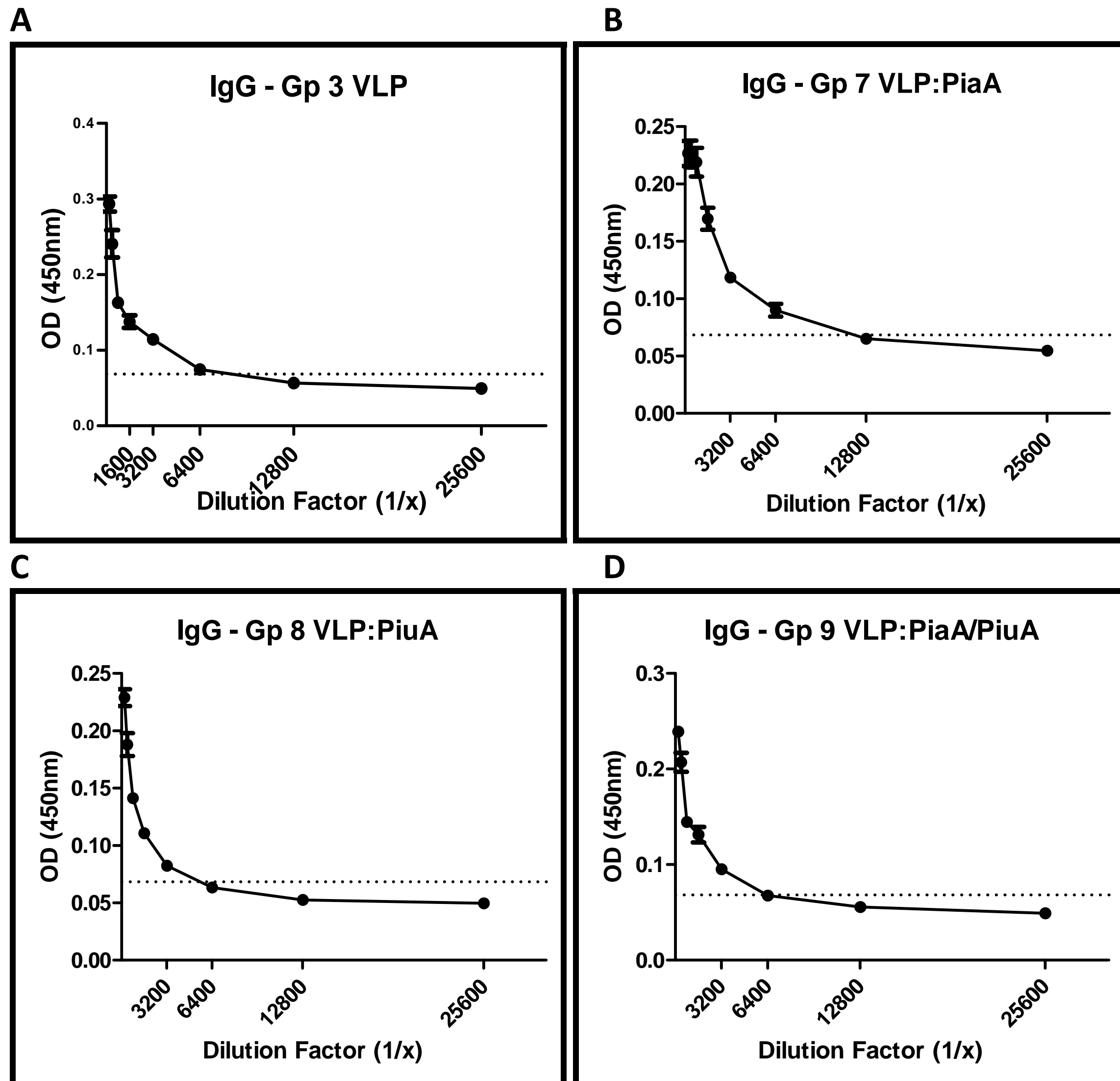


Figure 4. Collated titres of Anti-PiuA total IgG for mouse groups vaccinated with the PiuA protein.

In comparing the total IgG titres between PiaA and PiuA, PiuA induced a significantly higher titre than PiaA. The lowest PiuA titre (204800) was greater than the highest PiaA titre (102400).

4.1.1.2 Anti-VLP Total IgG

Within the experiment, there were 6 groups which had either PiaA or PiuA conjugated to the virus like particle (VLP) composed of hepatitis B surface antigen (HBsAg). VLP-specific IgG was examined to establish the immunogenicity of the VLP and determine the potential of a dual pneumococcus and HBV vaccine.



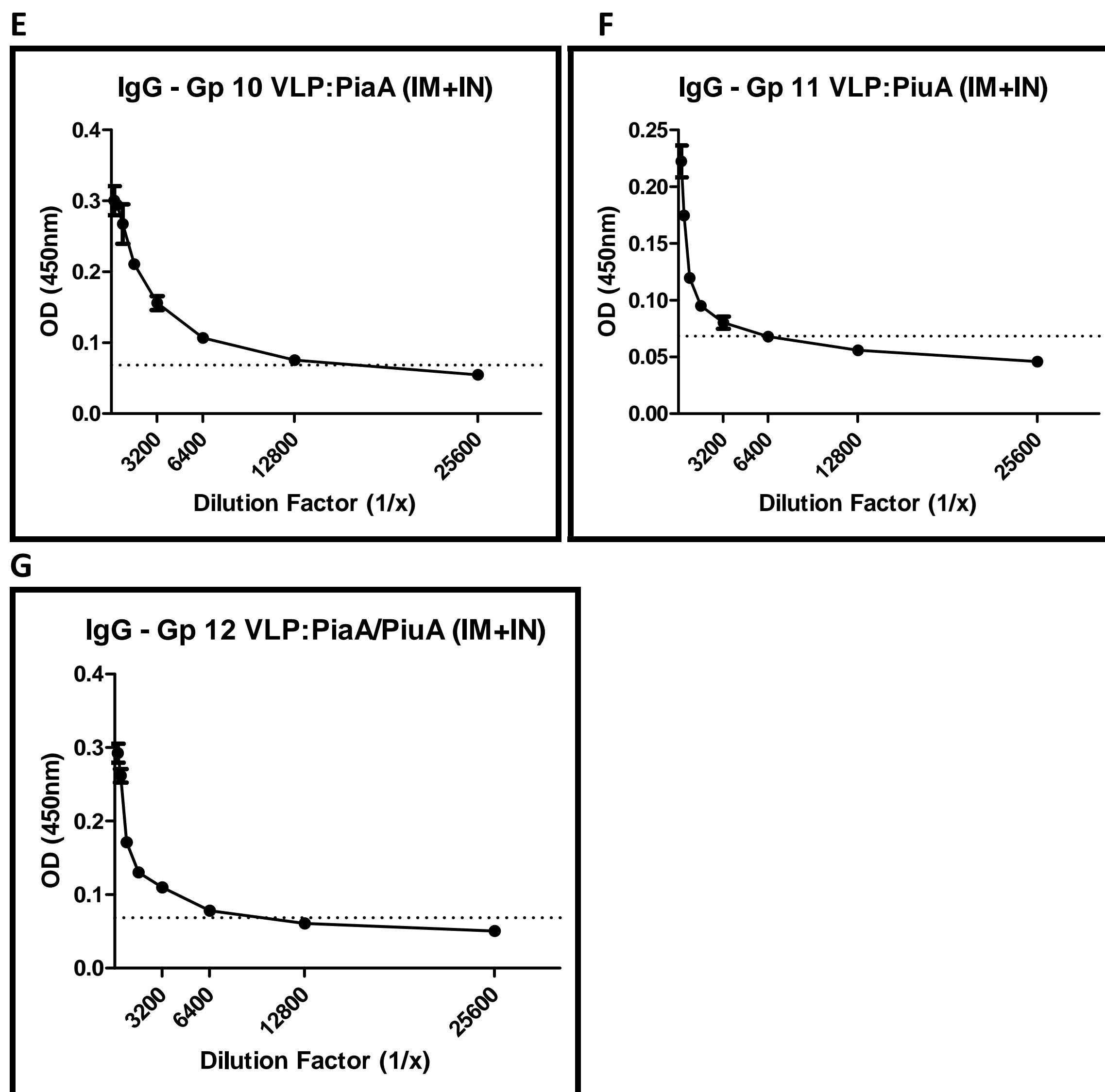


Figure 5. Total anti-VLP IgG antibody titres in serum from mice vaccinated with HBsAg, PiaA and PiuA. A) anti-VLP IgG antibody titre in mice vaccinated with VLP. B) anti-VLP IgG antibody titre in mice vaccinated with VLP-PiuA conjugate. C) anti-VLP IgG antibody titre in mice vaccinated with PiuA conjugated to VLP. D) anti-VLP IgG antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates E) anti-VLP IgG antibody titre in mice vaccinated with VLP-PiaA conjugate through intramuscular and intranasal routes. F) anti-VLP IgG antibody titre in mice vaccinated with VLP-PiuA through intramuscular and intranasal routes. G) anti-VLP IgG antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA through intramuscular and intranasal routes

The VLP control vaccine produced a titre of 6400 which was within one dilution from all the vaccinations involving VLP conjugation. This suggests that the conjugation of VLP to either PiaA or PiuA does not diminish the overall immune response from VLP-alone vaccination.

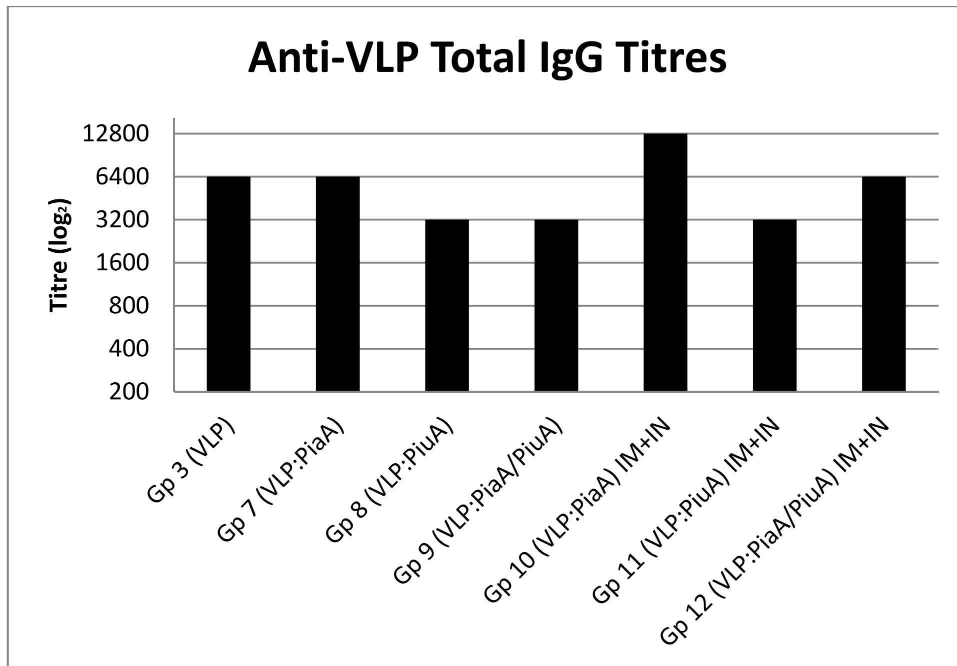
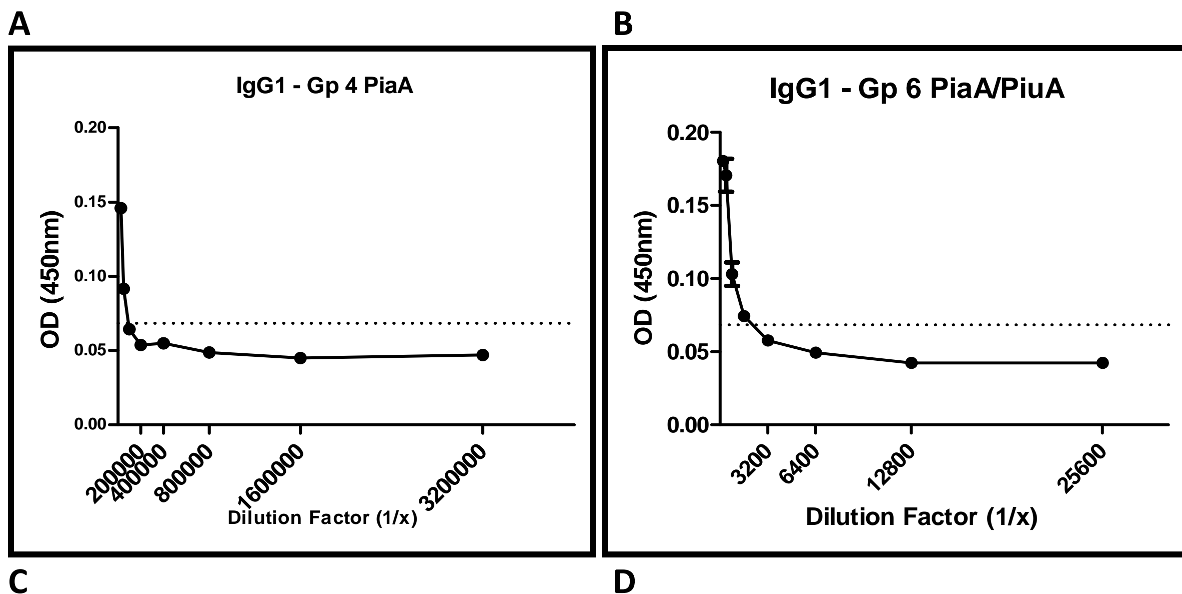


Figure 6. Collated titres of VLP total IgG for mouse groups vaccinated with the VLP.

4.1.2 IgG1 Response

The IgG1 response provides insight into the nature of the immunological response. Specifically, a high IgG1 titre represents a humoral response to the specific antigen used in the vaccination. Accordingly the titres of PiaA, PiuA and VLP specific-IgG1 were measured and are presented in figures 7-12.

4.1.2.1 Anti-PiaA IgG1



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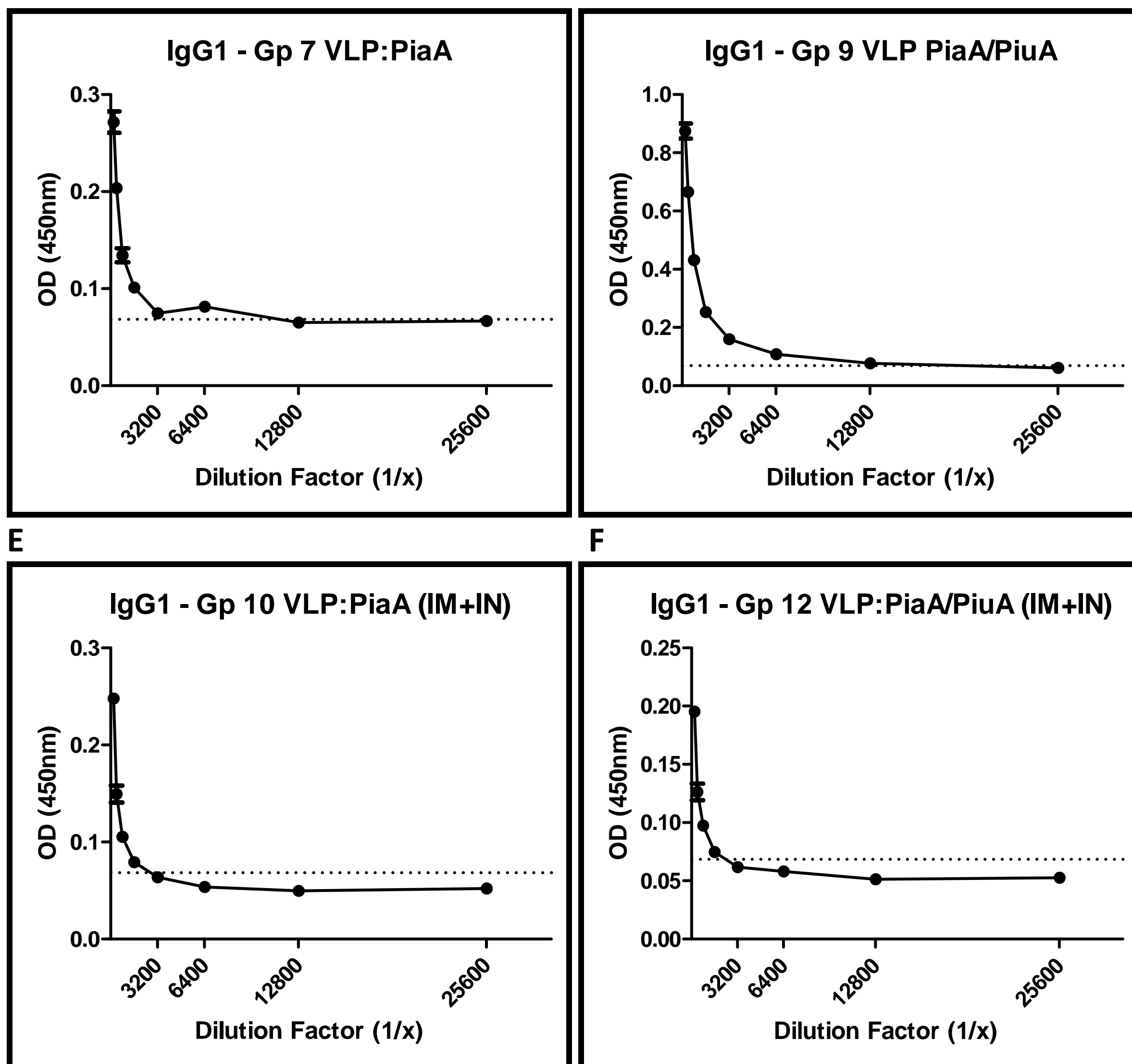


Figure 7. Anti-PiaA IgG1 antibody titres in serum from mice vaccinated with PiaA and PiuA. A) anti-PiaA IgG1 antibody titre in mice vaccinated with PiaA. B) anti-PiaA IgG1 antibody titre in mice vaccinated with PiaA and PiuA. C) anti-PiaA IgG1 antibody titre in mice vaccinated with PiaA conjugated to VLP. D) anti-PiaA IgG1 antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates E) anti-PiaA IgG1 antibody titre in mice vaccinated with VLP-PiaA conjugate through intramuscular and intranasal routes. F) anti-PiaA IgG1 antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates through intramuscular and intranasal routes.

PiaA alone (Group 4) induced a higher anti-PiaA titre than when it was combined with PiuA (Group 6). The IgG1 titre was almost identical to the total IgG titres for vaccination groups.

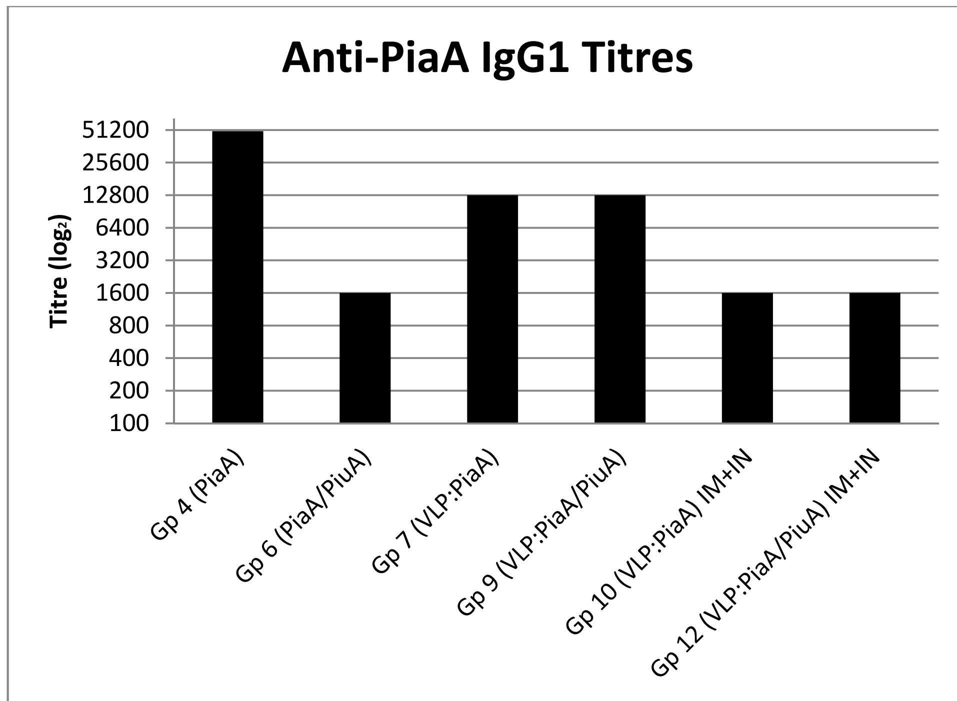
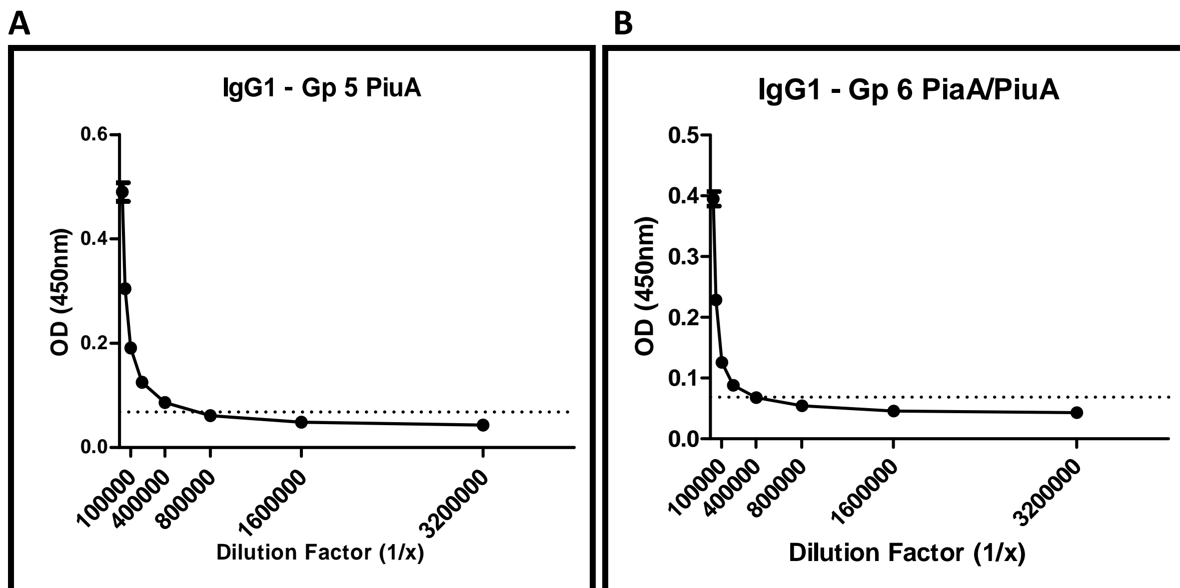


Figure 8. Collated titres of PiaA specific IgG1 for mouse groups vaccinated with PiaA.

4.1.2.2 Anti-PiuA IgG1



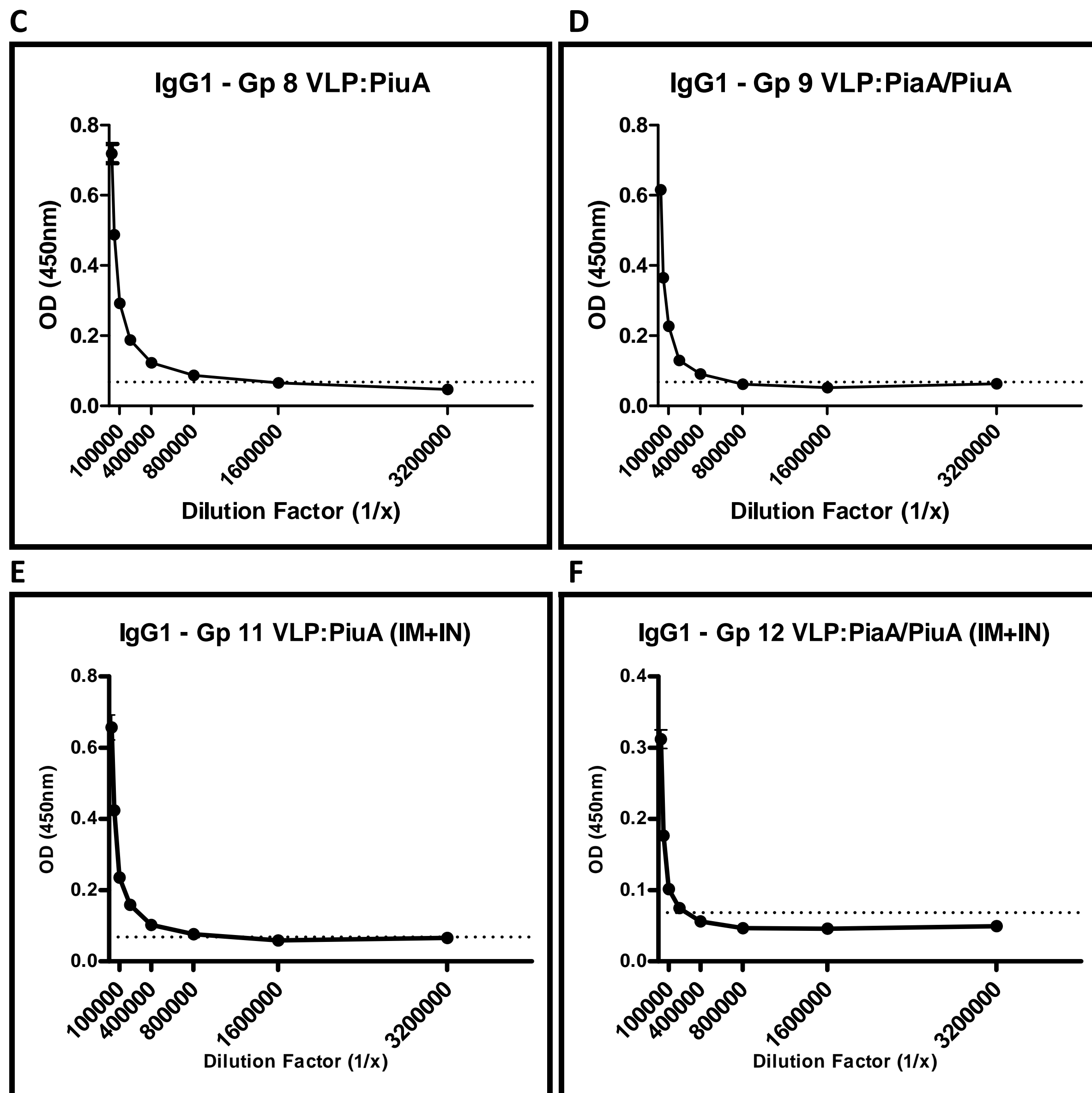


Figure 9. Anti-PiuA IgG1 antibody titres in serum from mice vaccinated with PiaA and PiuA. A) anti-PiuA IgG1 antibody titre in mice vaccinated with PiuA. B) anti-PiuA IgG1 antibody titre in mice vaccinated with PiaA and PiuA. C) anti-PiuA IgG1 antibody titre in mice vaccinated with PiuA conjugated to VLP. D) anti-PiuA IgG1 antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates E) anti-PiuA IgG1 antibody titre in mice vaccinated with VLP-PiuA conjugate through intramuscular and intranasal routes. F) anti-PiuA IgG1 antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates through intramuscular and intranasal routes.

The PiuA-specific IgG1 titres aligned with the trend observed for anti-PiuA total IgG titres. Specifically, highest titres were observed in all VLP-conjugated vaccines with the exception of the intramuscular and intranasal VLP:PiaA/PiuA group (Group 12).

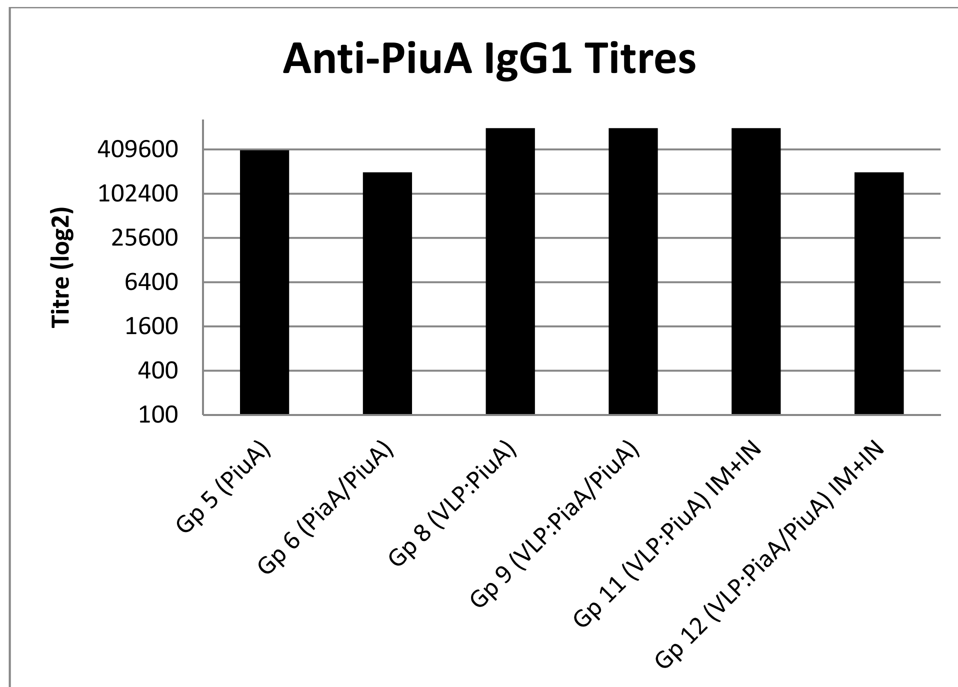
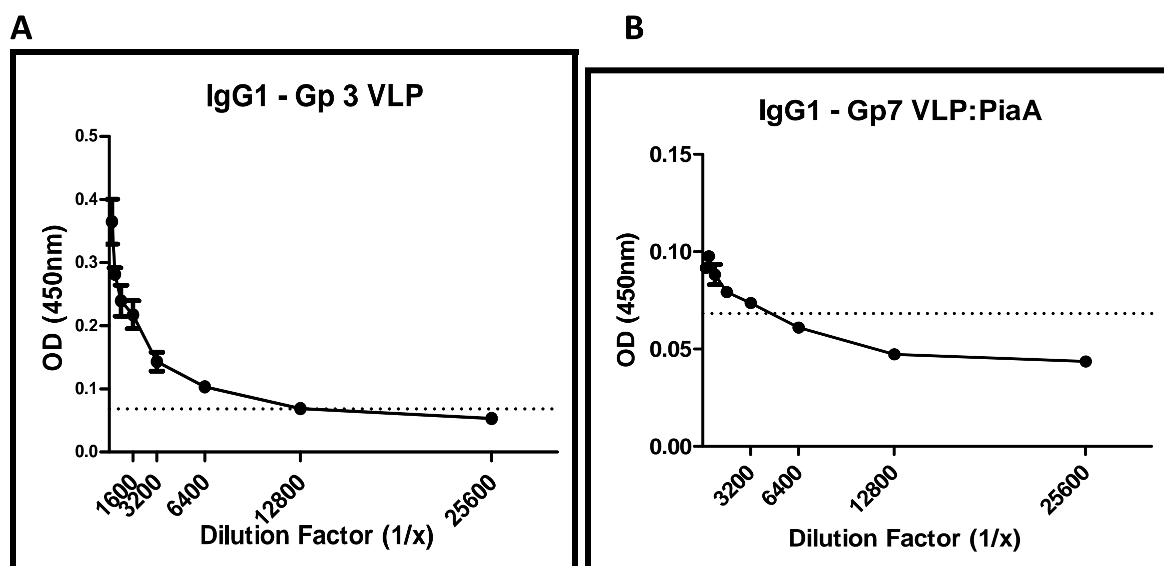


Figure 10. Collated titres of PiuA specific IgG1 for mouse groups vaccinated with PiuA.

It is notable that overall the IgG1 titres for PiuA were considerably greater than anti-PiaA titres as was observed for total IgG.

4.1.2.3 Anti-VLP IgG1



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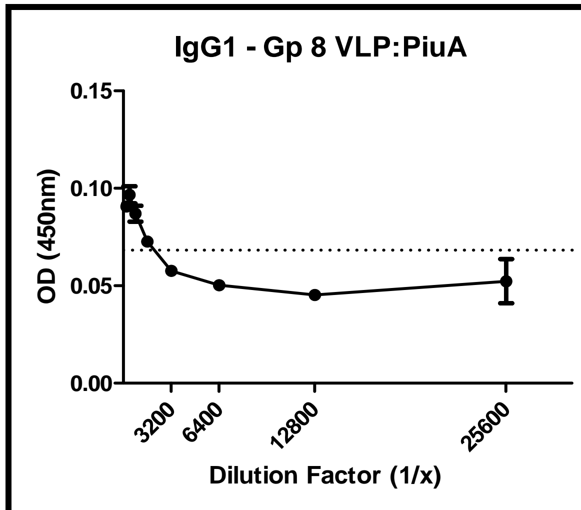

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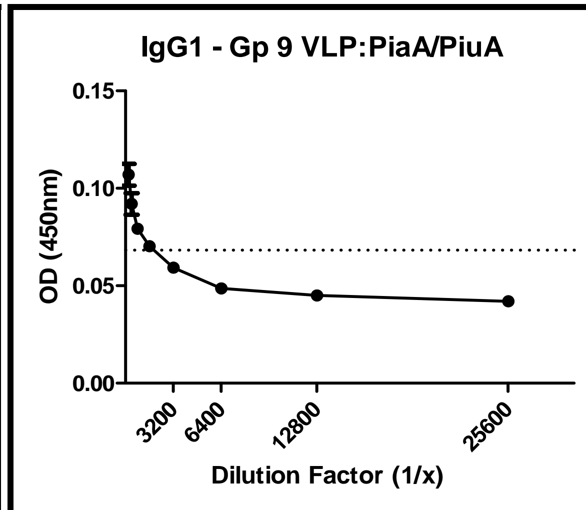

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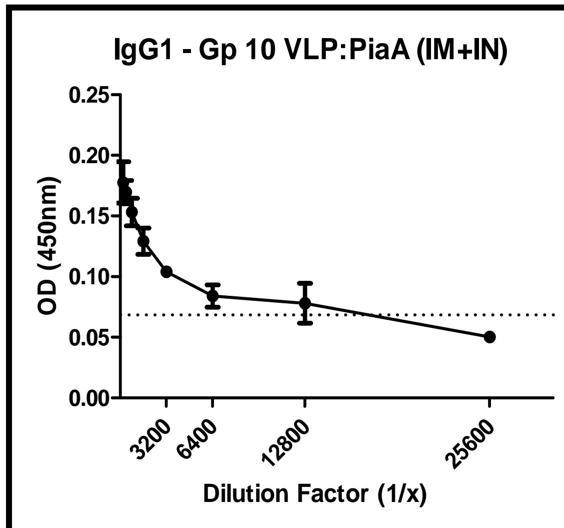

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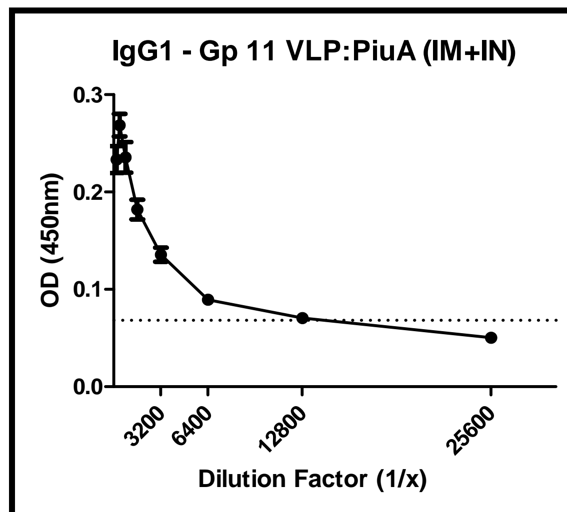

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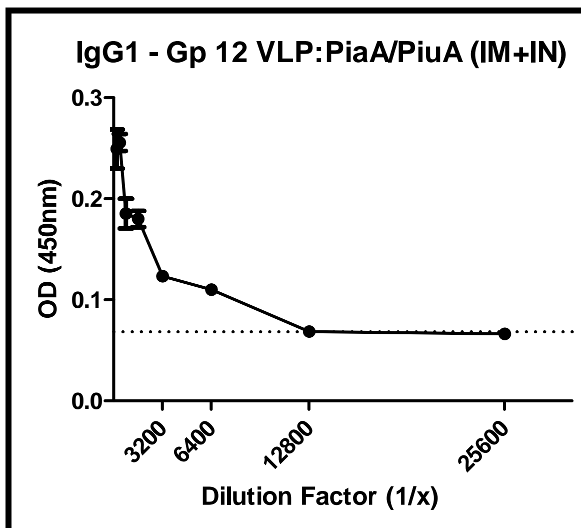


Figure 11. Anti-VLP IgG1 antibody titres in serum from mice vaccinated with VLP, PiaA and PiuA. A) anti-VLP IgG1 antibody titre in mice vaccinated with VLP. B) anti-VLP IgG1 antibody titre in mice vaccinated with VLP-

PiaA conjugate. C) anti-VLP IgG1 antibody titre in mice vaccinated with PiuA conjugated to VLP. D) anti-VLP IgG1 antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates. E) anti-VLP IgG1 antibody titre in mice vaccinated with VLP-PiaA conjugate through intramuscular and intranasal routes. F) anti-VLP IgG1 antibody titre in mice vaccinated with VLP-PiuA through intramuscular and intranasal routes. G) anti-VLP IgG1 antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA through intramuscular and intranasal routes.

The anti-VLP IgG1 titre for the VLP control group gave an identical titre to those in the conjugated vaccines delivered both intranasally and intramuscularly. The conjugated vaccines that were only given intramuscularly all had lower titres, though it is important to note that these were vaccinated with a lower overall dose of VLP. All Anti-VLP IgG1 titres are summarised in Figure 12.

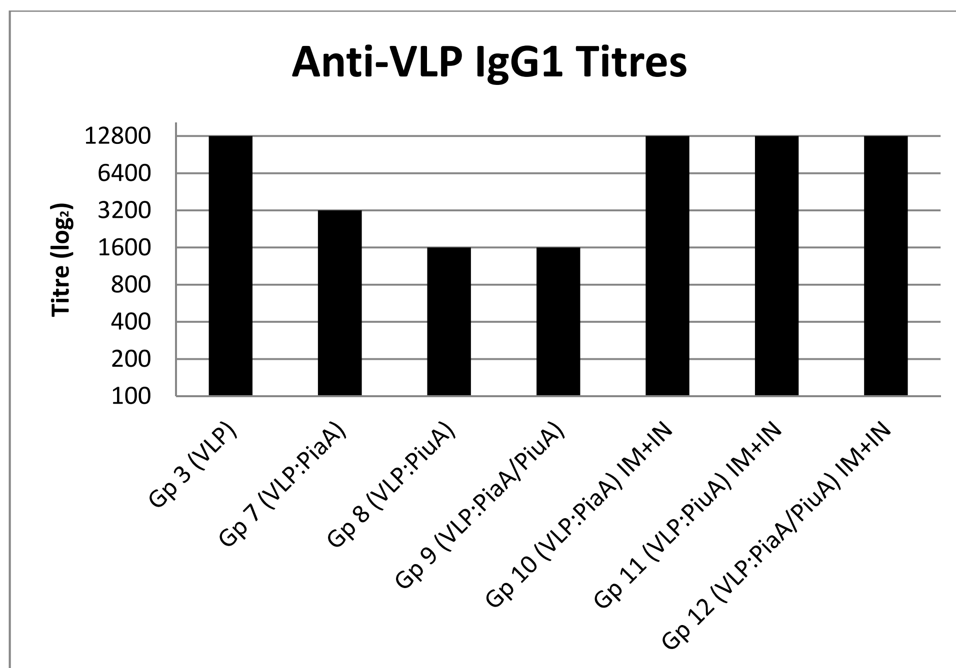


Figure 12. Collated titres of VLP specific IgG1 for mouse groups vaccinated with VLP.

4.1.3 IgG2a Response

4.1.3.1 Anti-PiaA IgG2a

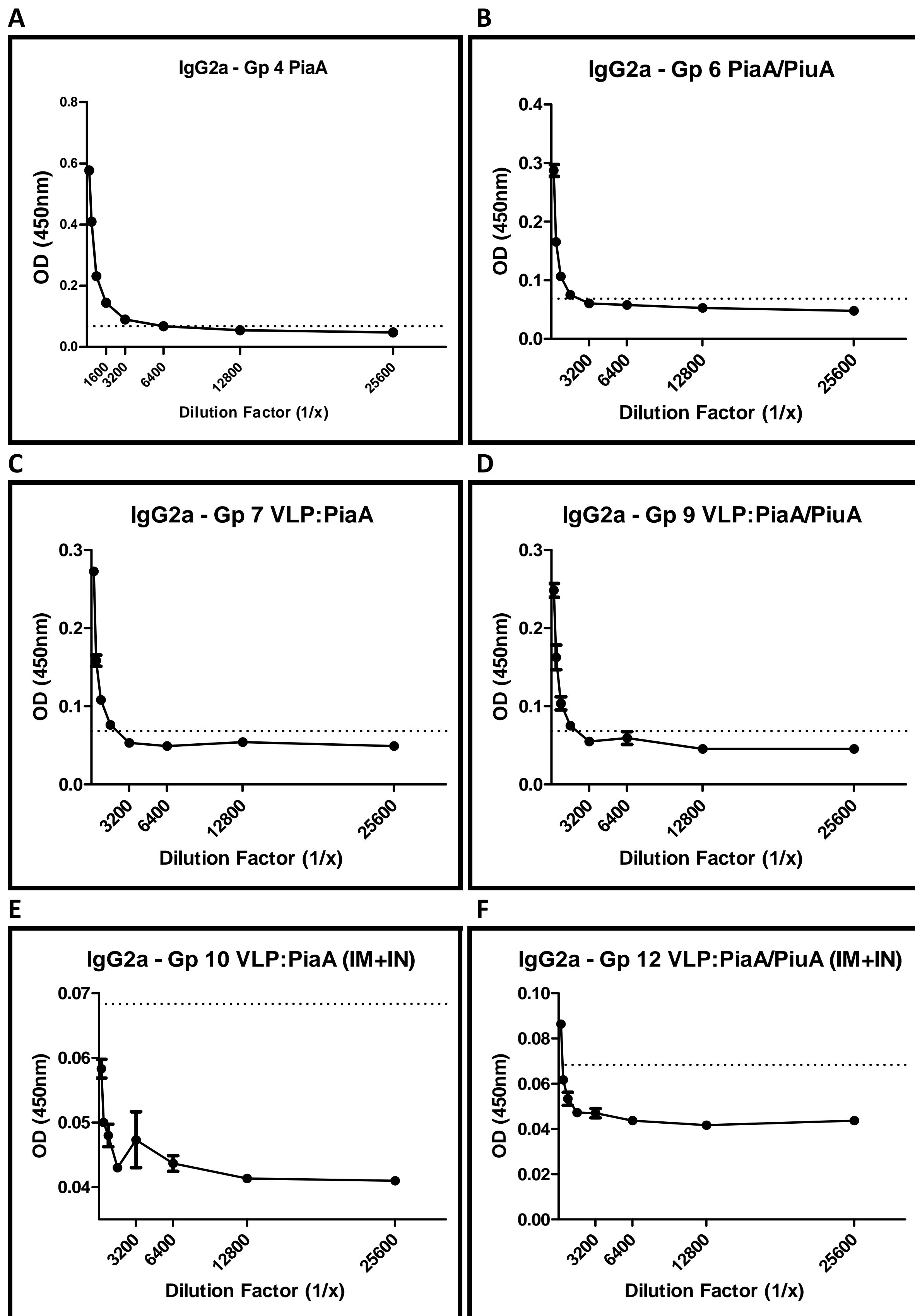


Figure 13. Anti-PiaA IgG2a antibody titres in serum from mice vaccinated with PiaA and PiuA. A) anti-PiaA IgG2a antibody titre in mice vaccinated with PiaA. B) anti-PiaA IgG2a antibody titre in mice vaccinated with PiaA and PiuA. C) anti-PiaA IgG2a antibody titre in mice vaccinated with PiaA conjugated to VLP. D) anti-

PiaA IgG2a antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates E) anti-PiaA IgG2a antibody titre in mice vaccinated with VLP-PiaA conjugate through intramuscular and intranasal routes. F) anti-PiaA IgG2a antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates through intramuscular and intranasal routes.

The IgG2a response to PiaA showed a much smaller difference between the PiaA alone vaccine (Group 4) and the combined PiaA/PiuA vaccine (Group 6) than in the total IgG and IgG1. A higher IgG2a titre was recorded in the conjugated vaccines given intramuscularly rather than through both intramuscular and intranasal routes.

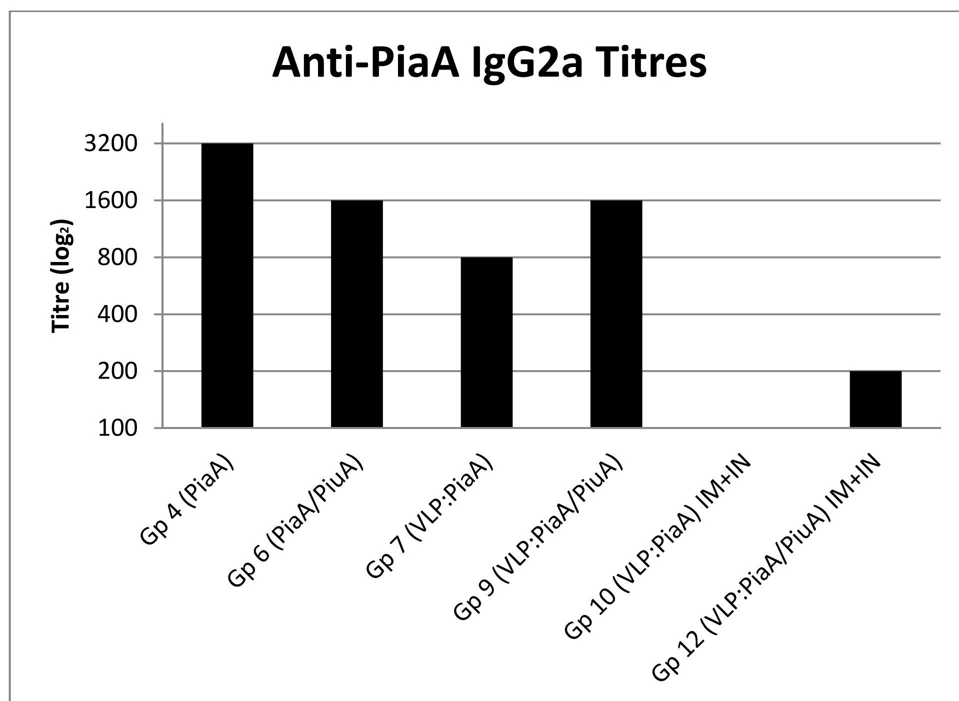


Figure 14. Collated titres of PiaA specific IgG2a for mouse groups vaccinated with PiaA

4.1.3.2 Anti-PiuA IgG2a

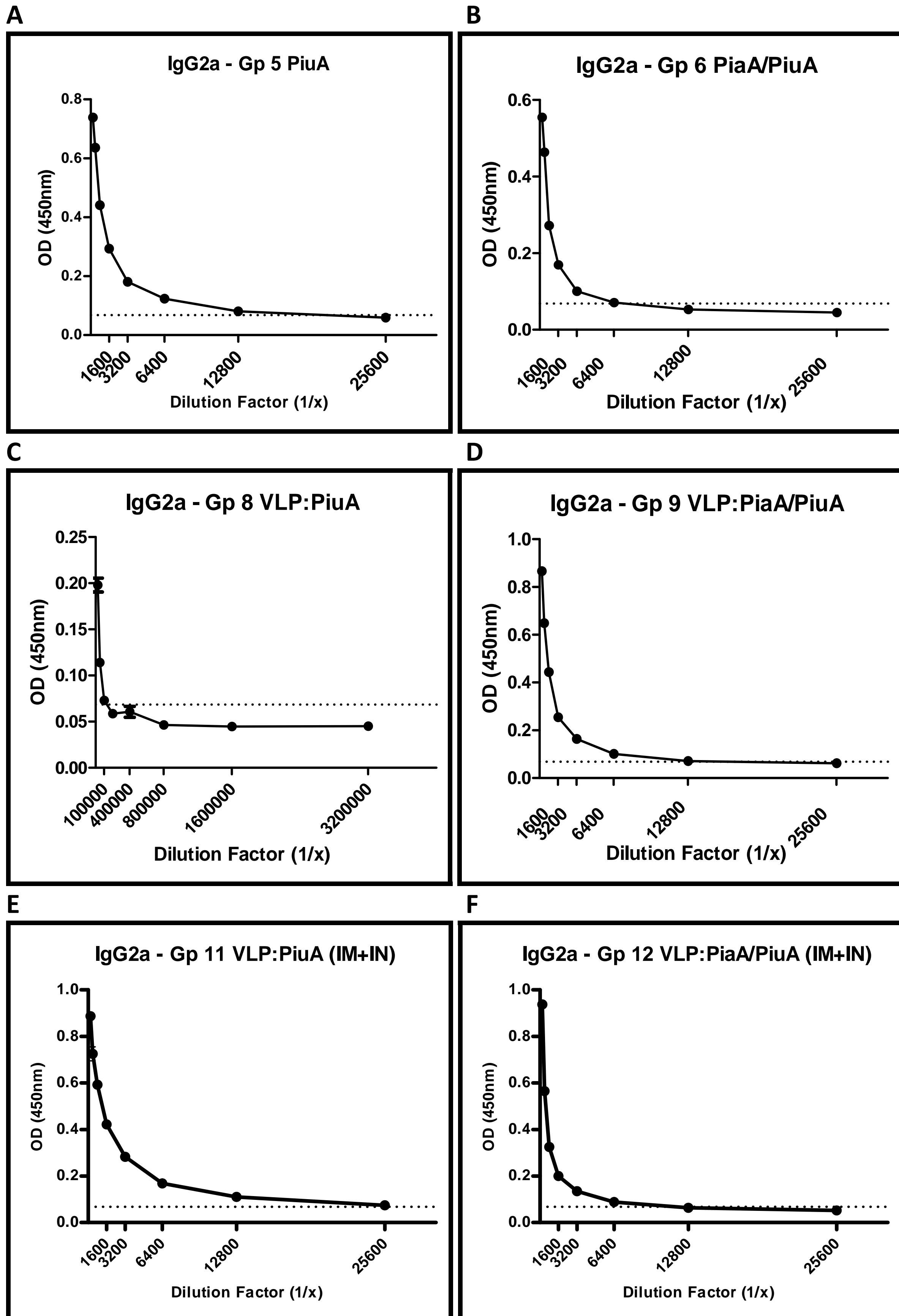


Figure 15. Anti-PiuA IgG2a antibody titres in serum from mice vaccinated with PiaA and PiuA. A) anti-PiuA IgG2a antibody titre in mice vaccinated with PiuA. B) anti-PiuA IgG2a antibody titre in mice vaccinated with

PiaA and PiuA. C) anti-PiuA IgG2a antibody titre in mice vaccinated with PiuA conjugated to VLP. D) anti-PiuA IgG2a antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates E) anti-PiuA IgG2a antibody titre in mice vaccinated with VLP-PiuA conjugate through intramuscular and intranasal routes. F) anti-PiuA IgG2a antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates through intramuscular and intranasal routes.

As seen in Figure 16, all vaccine types (whether conjugated or not) produced a similar anti-PiuA IgG2a titre with the exception of the VLP:PiuA intramuscular vaccination which yielded a considerably higher titre.

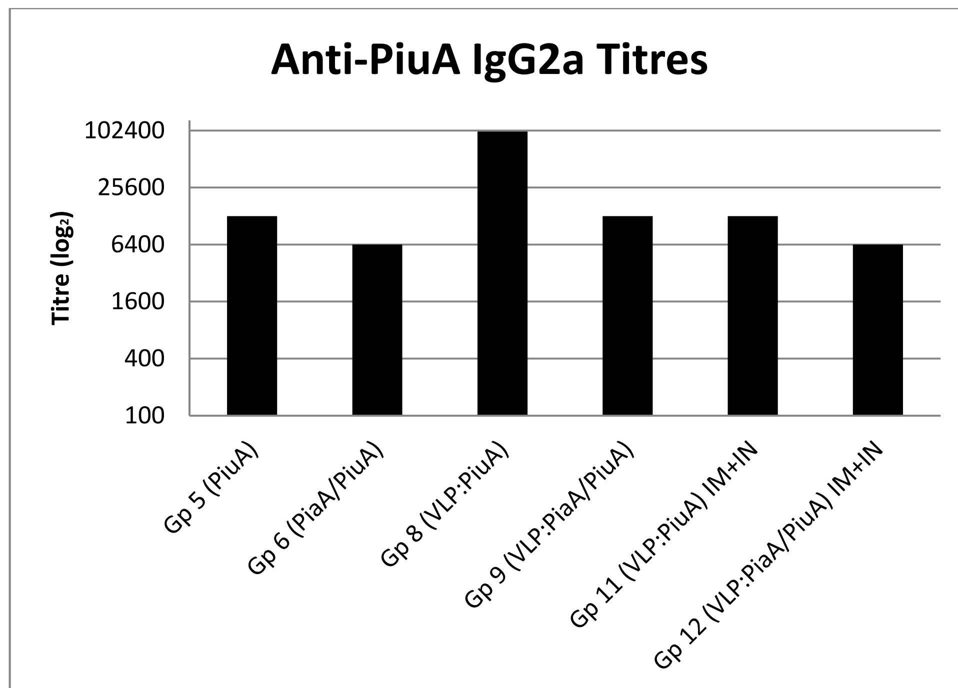
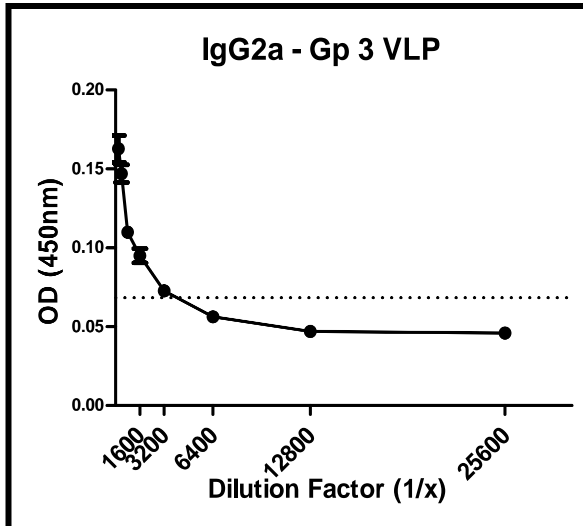


Figure 16. Collated titres of PiuA specific IgG2a for mouse groups vaccinated with PiuA

4.1.3.3 Anti-VLP IgG2a

A



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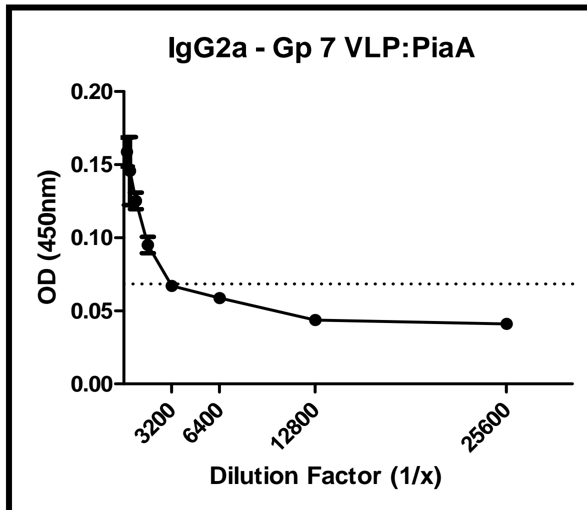

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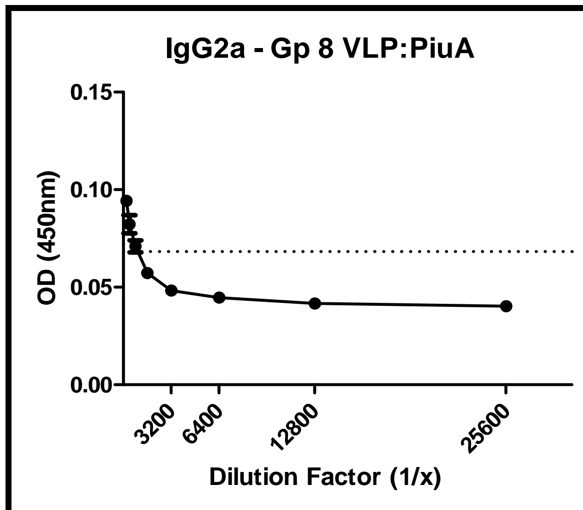

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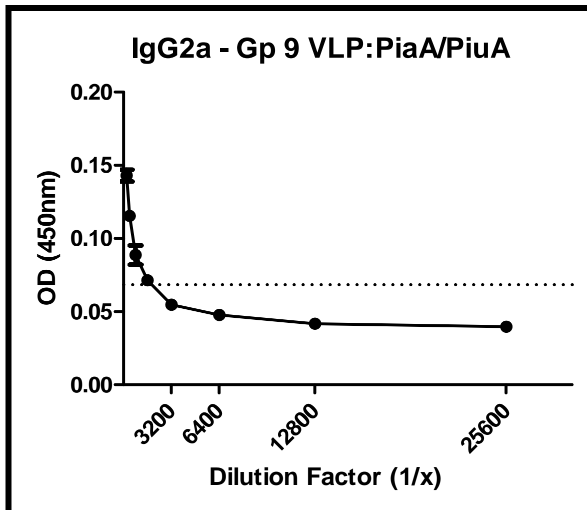

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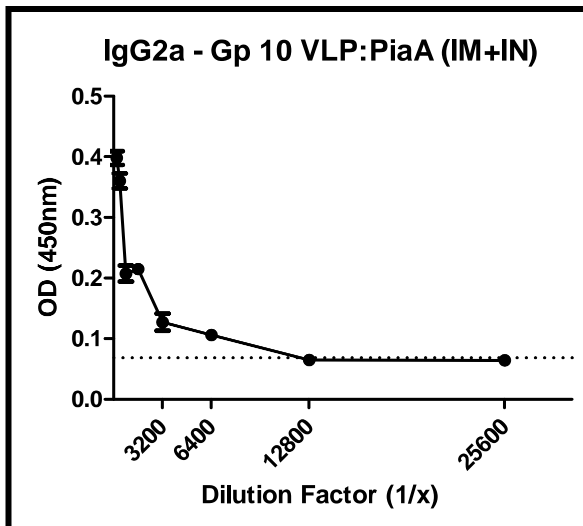

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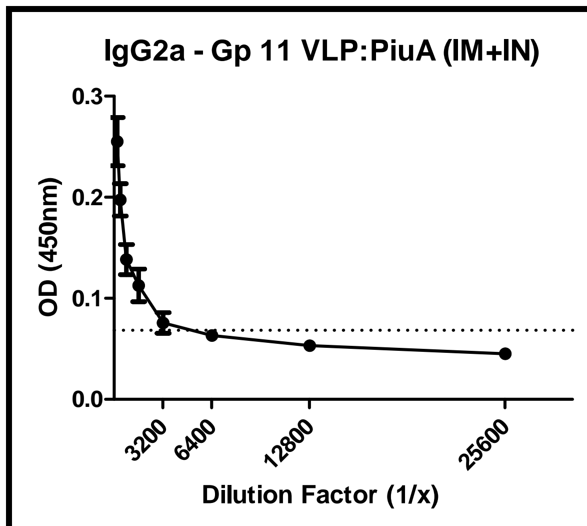

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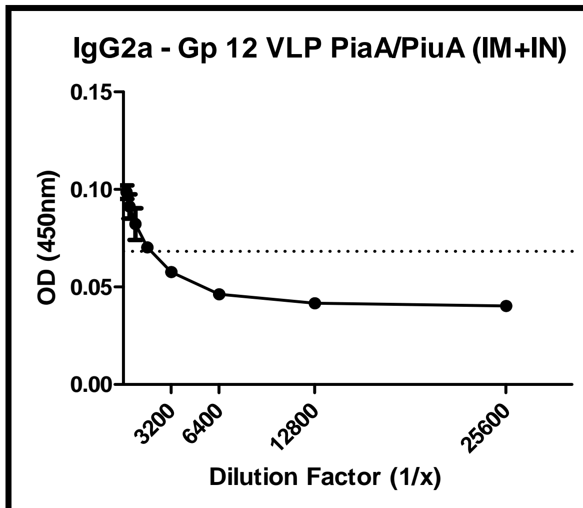


Figure 17. Anti-VLP IgG2a antibody titres in serum from mice vaccinated with VLP, PiaA and PiuA. A) anti-VLP IgG2a antibody titre in mice vaccinated with VLP. B) anti-VLP IgG1 antibody titre in mice vaccinated with VLP-PiaA conjugate. C) anti-VLP IgG2a antibody titre in mice vaccinated with PiuA conjugated to VLP. D) anti-VLP IgG2a antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA conjugates. E) anti-VLP IgG2a antibody titre in mice vaccinated with VLP-PiaA conjugate through intramuscular and intranasal routes. F) anti-VLP IgG2a antibody titre in mice vaccinated with VLP-PiuA through intramuscular and intranasal routes. G) anti-VLP IgG2a antibody titre in mice vaccinated with a combination of VLP-PiaA and VLP-PiuA through intramuscular and intranasal routes.

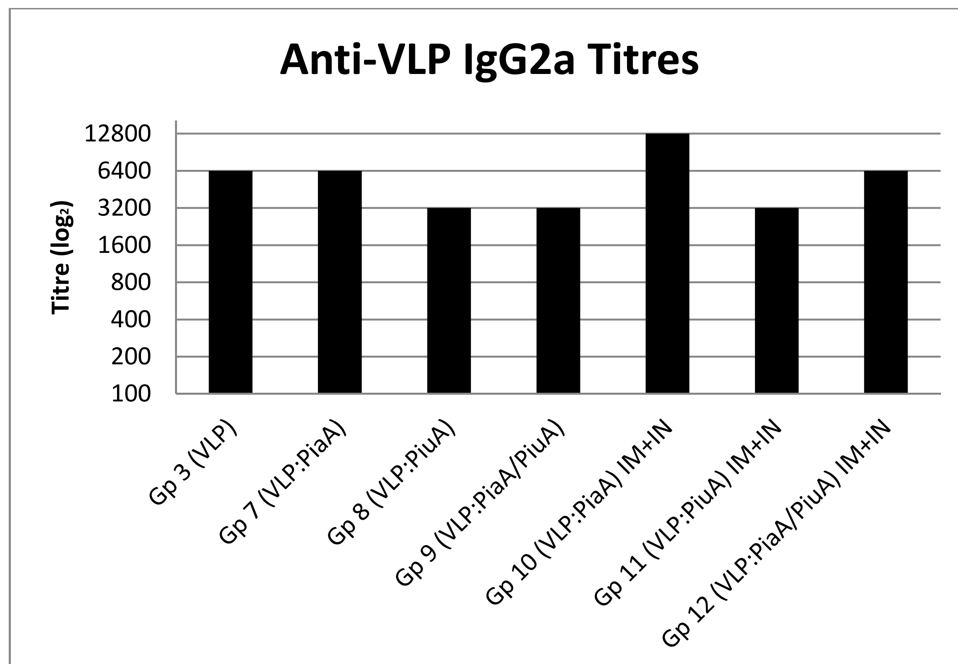


Figure 18. Collated titres of VLP specific IgG2a for mouse groups vaccinated with VLP

4.2 Bacterial Load

In order to estimate the bacterial load in the blood and the lung, blood and NPA samples were collected following euthanasia (48 hours after bacterial challenge), the bacterial load was estimated by spreading blood and NPA samples on HBA plates and counting haemolytic colonies. The raw data for bacterial load in each mouse has been collated and added to the appendix for reference. The raw data was collected by Lisa Seymour and was provided to me for analysis for the purpose of assisting interpretation of serological antibody data.

4.2.1 *S. pneumoniae* Cultured from Blood

Figure 19 shows the blood *S. pneumoniae* load average for each vaccine type.

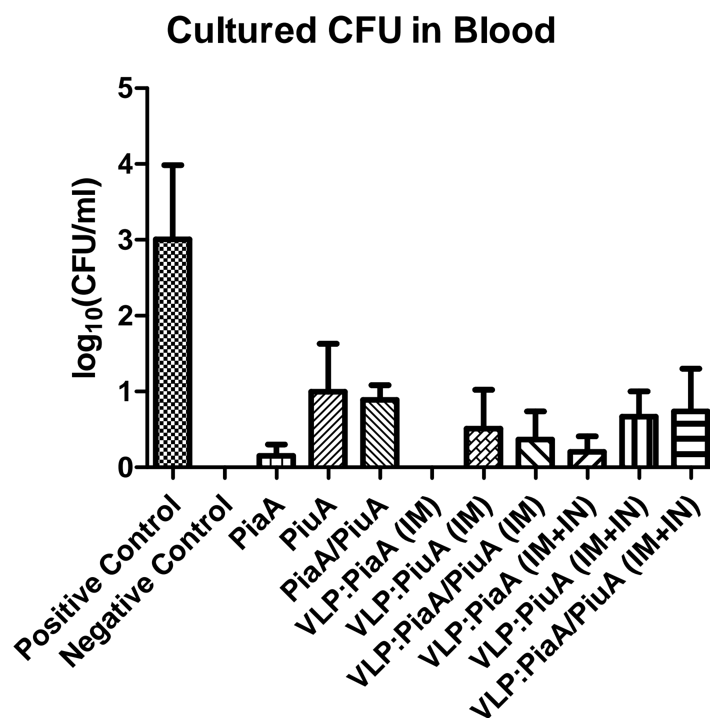


Figure 19. Blood *S. pneumoniae* loads for each vaccinated group. Positive controls were challenged but unvaccinated, negative controls were unchallenged and unvaccinated.

A one-way ANOVA established that there was a statistically significant difference in the CFUs in the blood between vaccination types, $F(11,60) = 3.354$, $p = 0.0012$. Specifically, t-tests between the positive control and the individual vaccine types showed significant variation in the PiaA vaccine

(p=0.0164), the VLP:PiaA (IM) vaccine (p=0.0119), the VLP:PiuA (IM) vaccine (p=0.0477), the VLP:PiaA/PiuA (IM) vaccine (p=0.0306), the VLP:PiaA (IM+IN) vaccine (p=0.0189) and the VLP: PiuA (IM+IN) vaccine (p=0.0475).

4.2.2 *S. pneumoniae* Cultured from Nasopharyngeal Aspirate (NPA)

Figure 20 shows the NPA bacterial load average for each group.

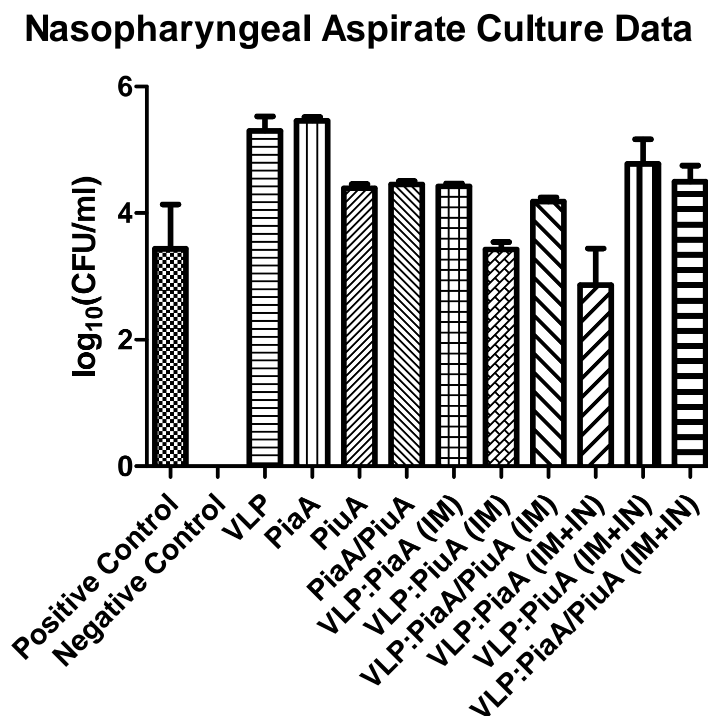


Figure 20. NPA cultures for each vaccination type. Positive controls were challenged but unvaccinated, negative controls were unchallenged and unvaccinated.

A one-way ANOVA showed that there was statistically significant differences in the CFUs in NPA between different vaccine groups, F (11,60) = 22.40, p = 0.0001. Further t-tests comparing vaccine groups showed that only the VLP vaccine (0.0302) and the PiaA vaccine (0.0165) had statistically significant results when compared to the unvaccinated, challenged mice. Counterintuitively, these values were higher than the positive control, which suggests the possibility of a technical error in the challenge in these two groups.

4.3 Histology

4.3.1 H&E Stain

4.3.1.1 Lung Sections of Positive Control – Challenged, Unvaccinated

The positive control mice show clear signs of pathology that are expected 48 hours post challenge. Specifically, the abundance of neutrophils, the increased number of macrophages, congested capillaries and a thickening of alveolar walls.

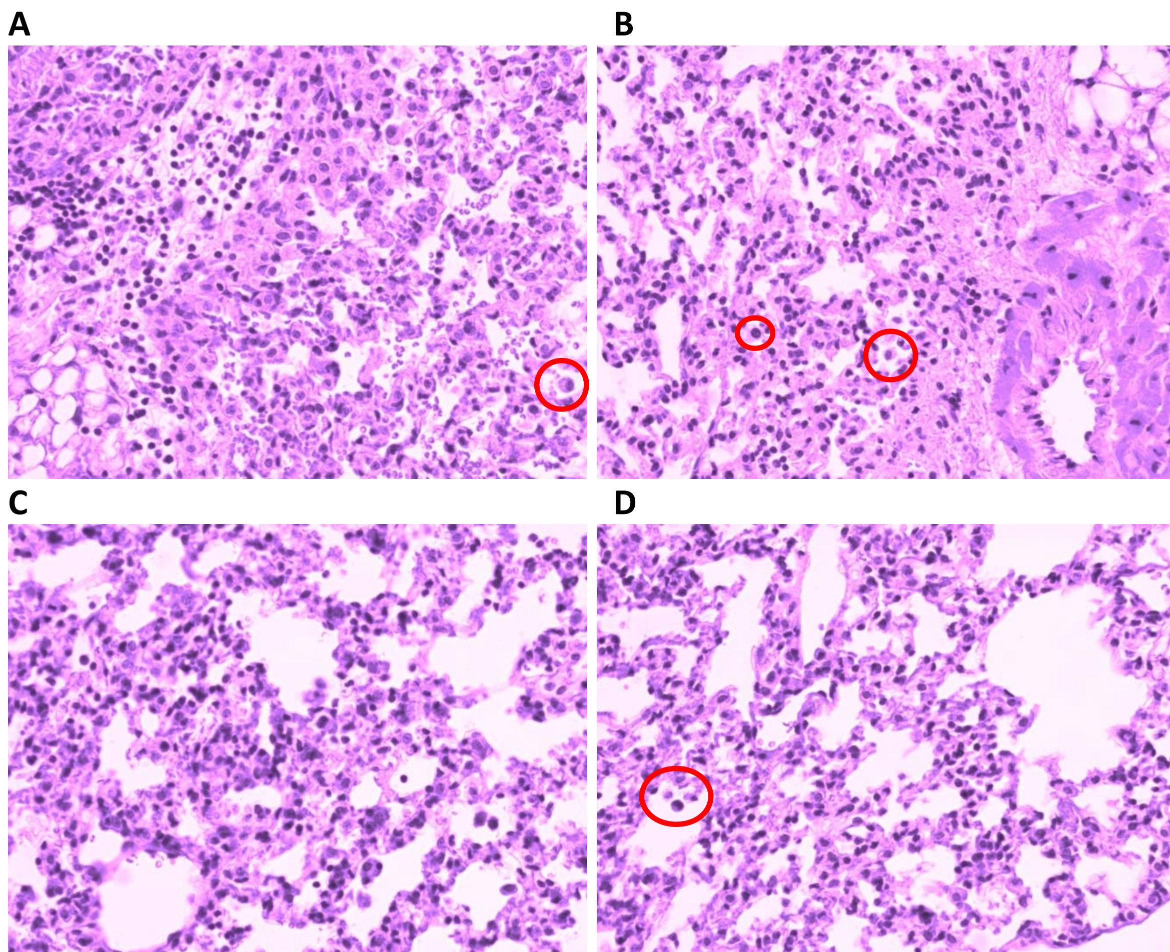


Figure 21. H&E Stained lung tissue at x20 magnification from the unvaccinated *S. pneumoniae* challenged positive control group (Group 1). A) Mouse 2. B) Mouse 3. C). Mouse 4. D) Mouse 5. Macrophages are circled in red.

4.3.1.2 Lung Sections of Negative Control – Unchallenged, Unvaccinated

Mice that were unvaccinated and unchallenged showed healthy lung tissue (Figure 22). Alveoli walls were thinner and the cells were considerably less congested than those in the positive control (Figure 21). Importantly, macrophages were still present, though not as common as in the positive control.

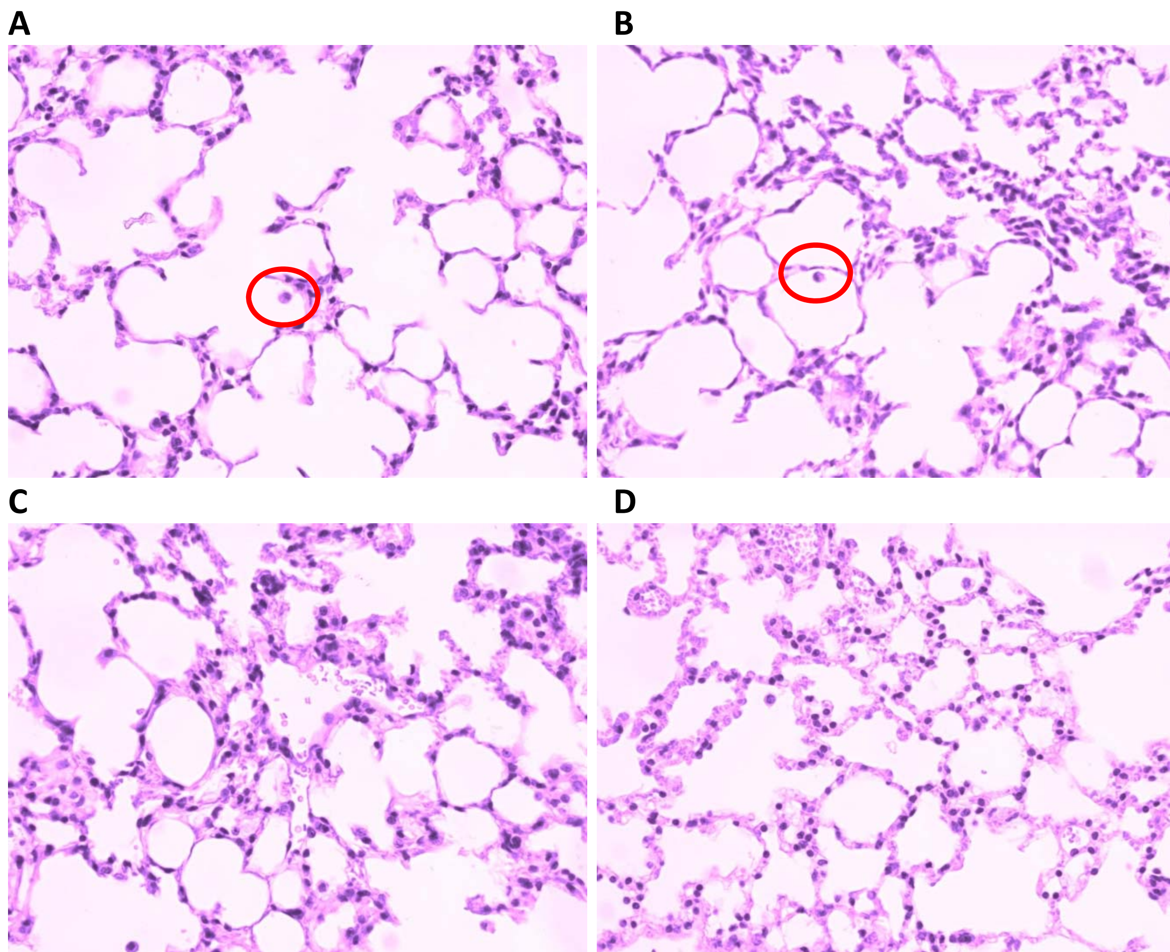


Figure 22. H&E stained lung tissue at x20 magnification from the unvaccinated, unchallenged negative control group (Group 2). A) Mouse 1 – macrophage is circled in red. B) Mouse 2 – macrophage is circled in red. C). Mouse 3. D) Mouse 4.

4.3.1.3 Lung Sections of PiaA Vaccinates

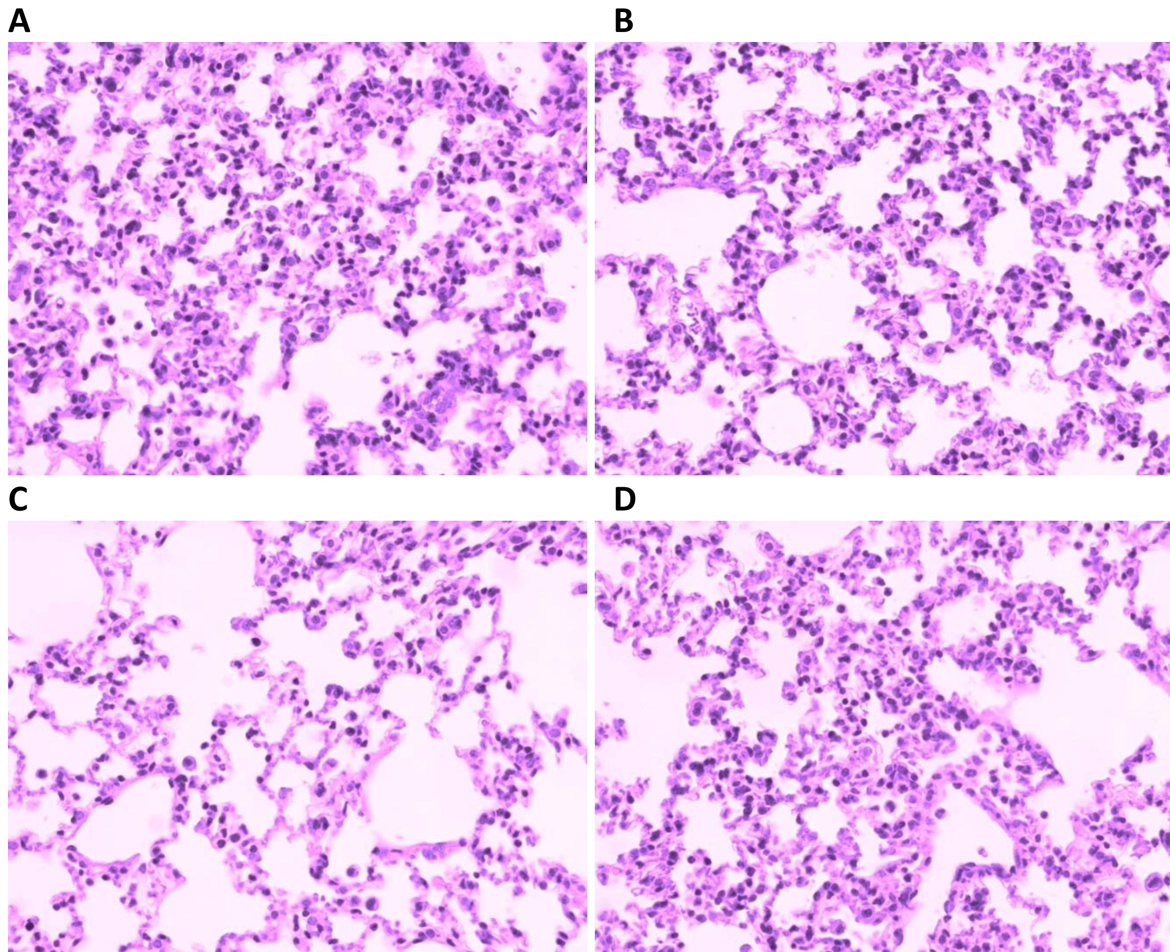


Figure 23. H&E stained lung tissue at x20 magnification from mice vaccinated with the PiaA protein. A) Mouse 2. B) Mouse 3. C). Mouse 4. D) Mouse 5.

4.3.1.4 Lung Sections of PiuA Vaccinates

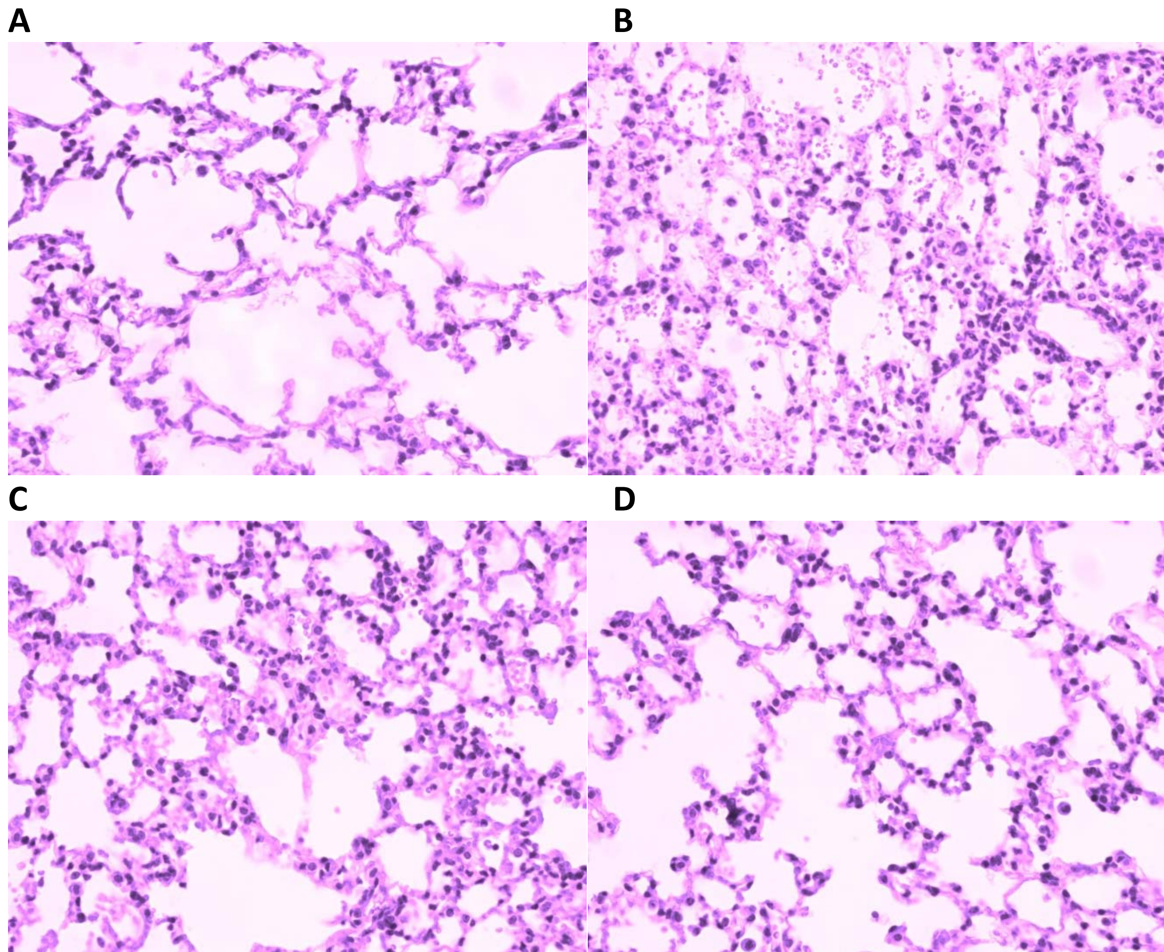


Figure 24. H&E stained lung tissue at x20 magnification from mice vaccinated with the PiuA protein. A) Mouse 2. B) Mouse 3. C). Mouse 4. D) Mouse 5.

4.3.1.5 Lung Sections of PiaA/PiuA Vaccinates

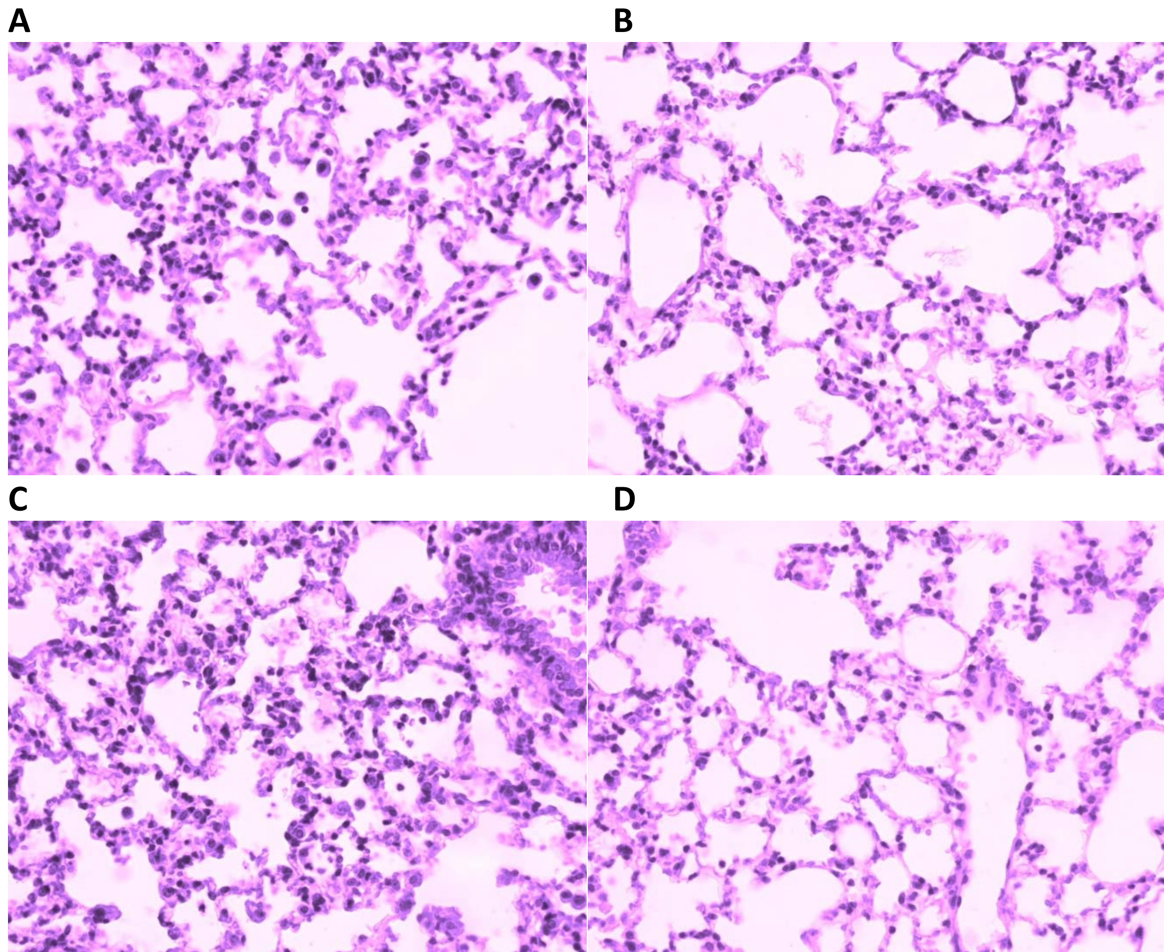


Figure 25. H&E stained lung tissue at x20 magnification from mice vaccinated with the PiaA/PiuA protein. A) Mouse 3. B) Mouse 4. C). Mouse 5. D) Mouse 6.

4.3.1.6 Lung Sections of VLP:PiaA Vaccinates

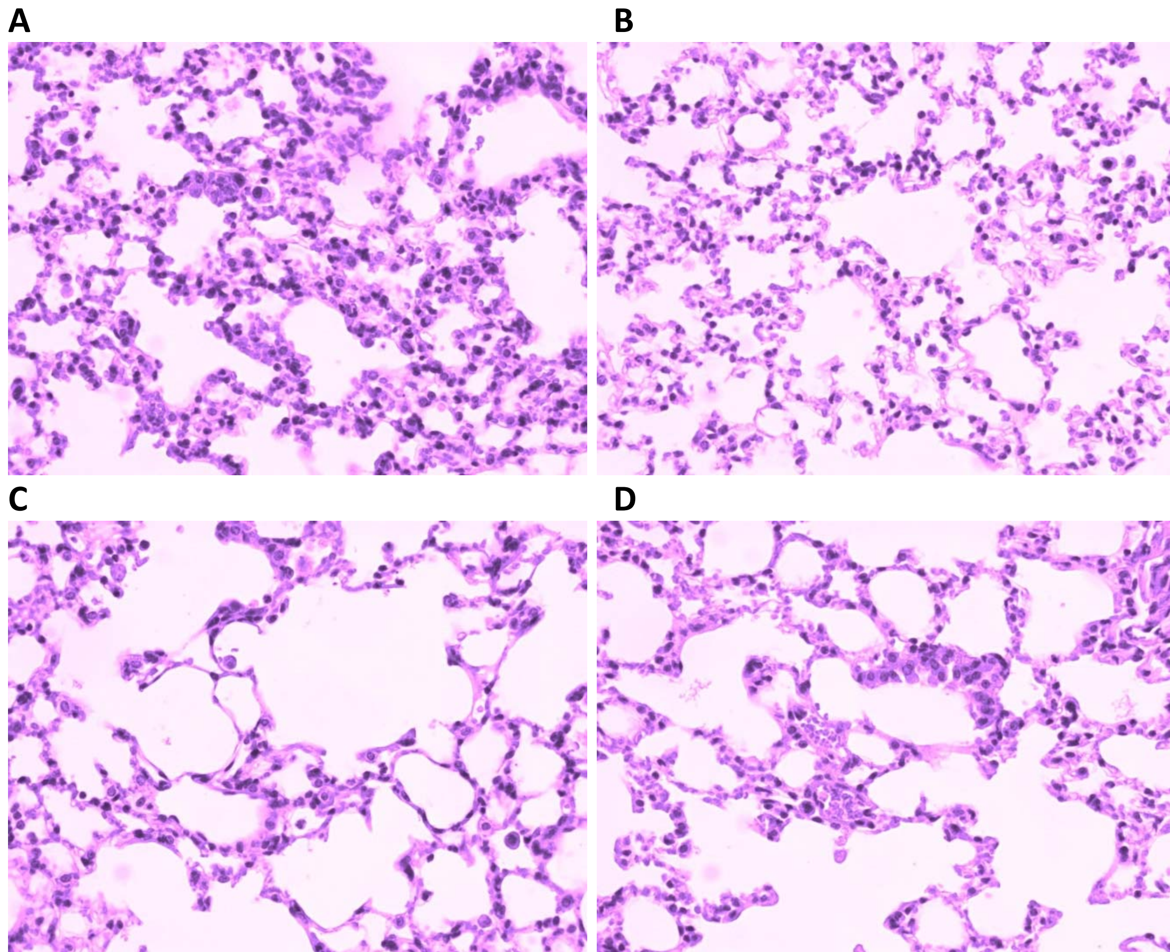


Figure 26. H&E stained lung tissue at x20 magnification from mice challenged with *S. pneumoniae* and vaccinated with i.m. with VLP:PiaA. A) Mouse 1. B) Mouse 2. C). Mouse 3. D) Mouse 4.

4.3.1.7 Lung Sections of VLP:PiuA Vaccinates

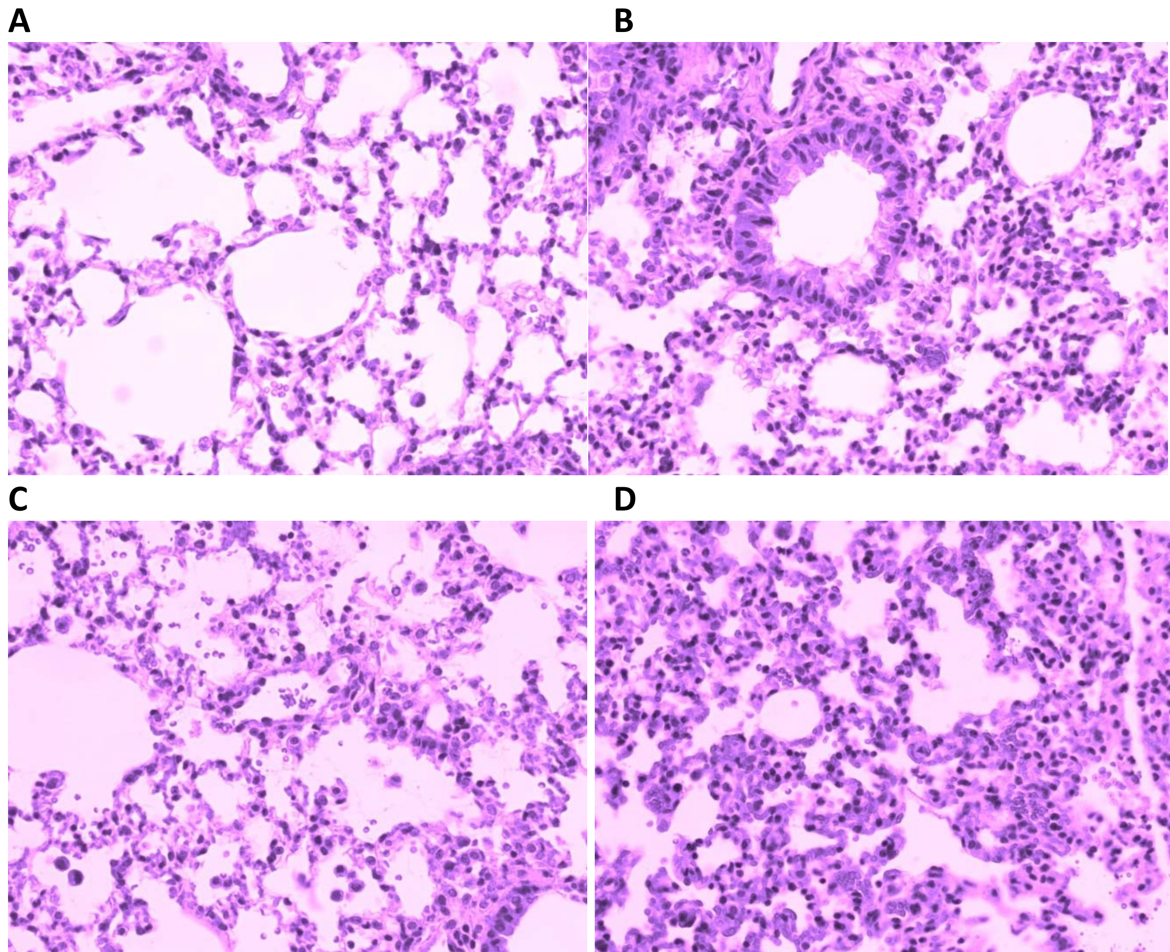


Figure 27. H&E stained lung tissue at x20 magnification from mice challenged with *S. pneumoniae* and vaccinated i.m. with VLP:PiuA. A) Mouse 1. B) Mouse 3. C). Mouse 5. D) Mouse 6.

4.3.1.8 Lung Sections of VLP:PiaA + VLP:PiuA Vaccinates

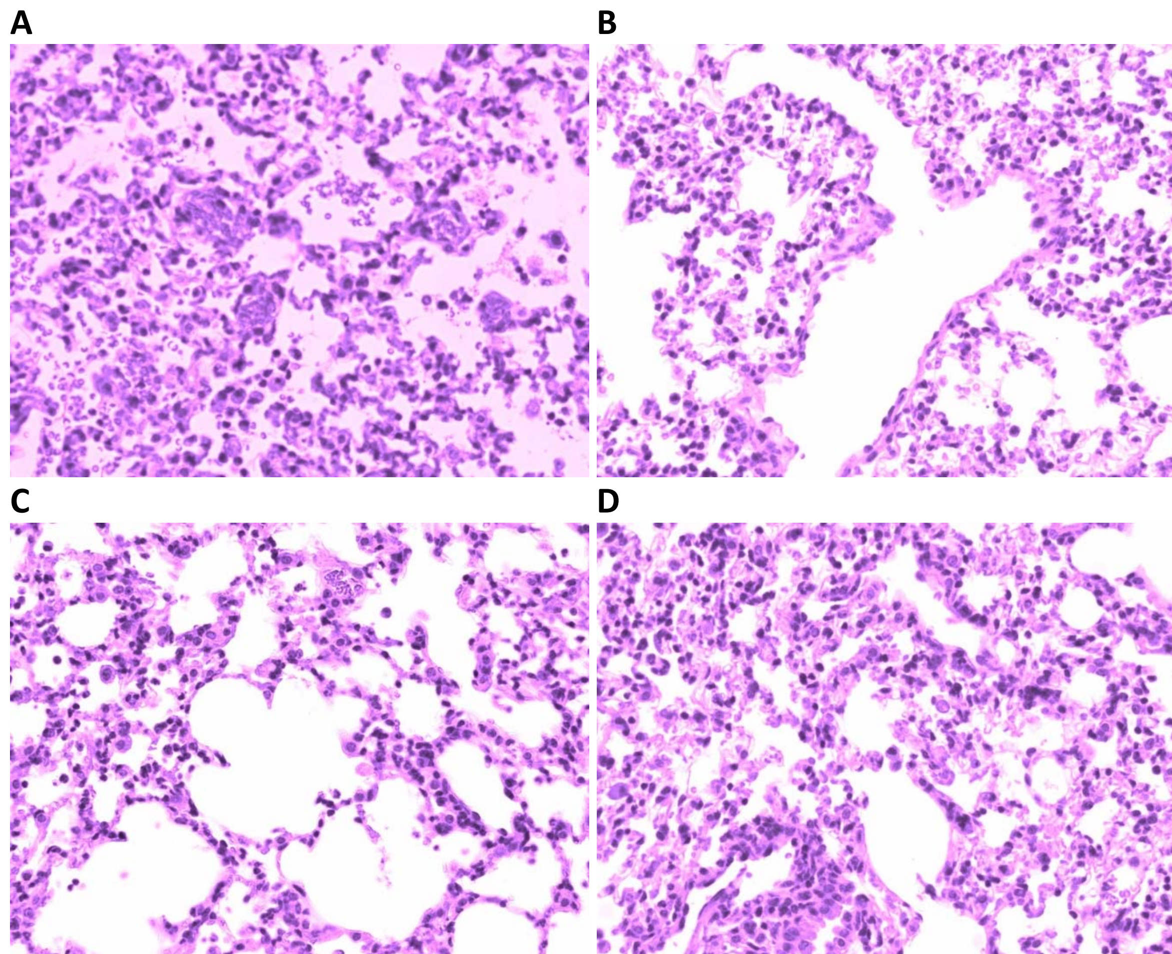


Figure 28. H&E stained lung tissue from mice challenged with *S. pneumoniae* and vaccinated i.m. with VLP:PiuA. A) Mouse 1 (x20). B) Mouse 2 (x20). C). Mouse 3 (x40). D) Mouse 4 (x40).

4.3.2 Gram Stained Lung Sections

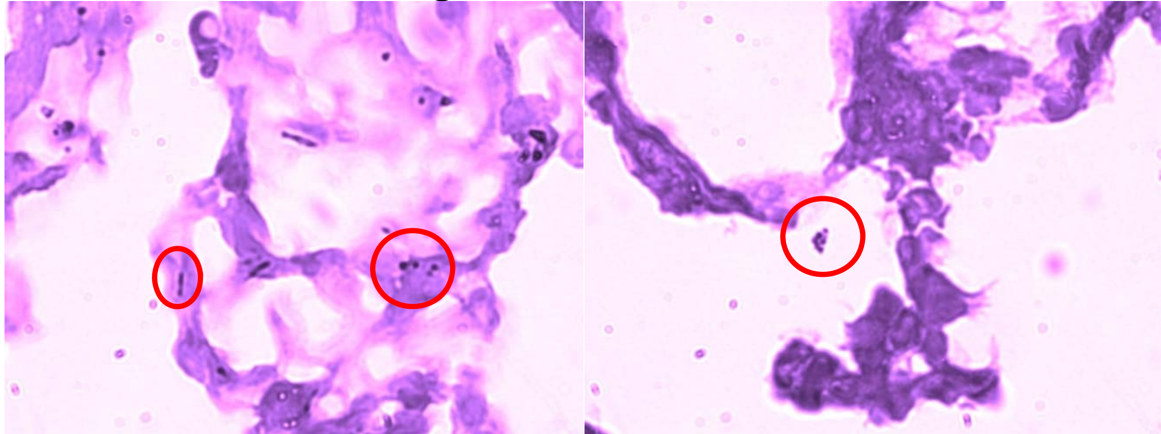


Figure 29. Unvaccinated mice challenged with *S. pneumoniae* show bacteria clusters in the lung tissue as observed at 100x magnification.

4.3.2.1 Lung Sections of PiaA Vaccinates

Mice vaccinated with the PiaA protein alone showed a high IgG titre and a statistically significant decrease of culture in the blood. Mice from this group showed the presence of *S. pneumoniae* in the lung tissue (Figure 30).

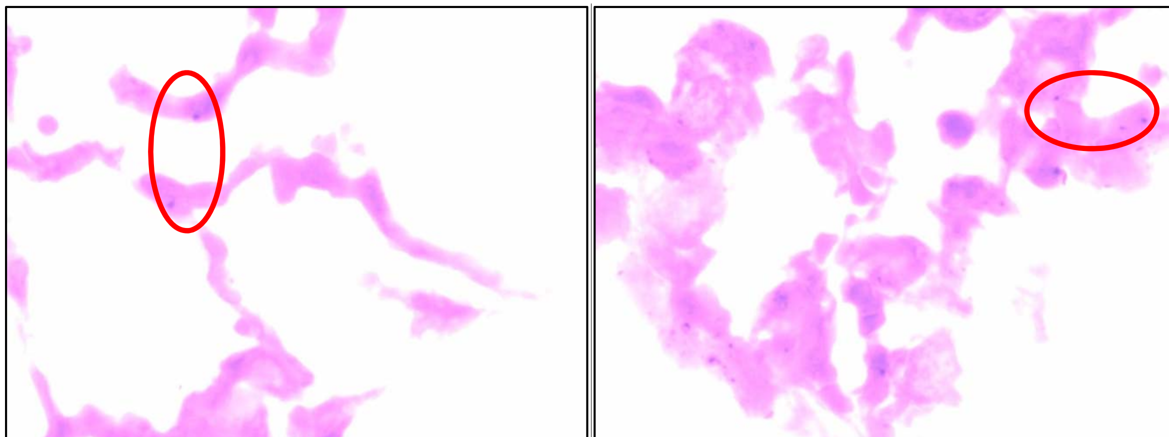


Figure 30. Gram Stain of mice vaccinated with PiaA showing very sparse *S. pneumoniae* in lung tissue at 100x magnification.

4.3.2.2 Lung Sections of PiuA Vaccinates

PiuA vaccination produced a significant IgG titre, though the blood culture had no statistically significant difference than that of the positive control. These mice showed colonies of *S. pneumoniae* that were in chains (Figure 31).

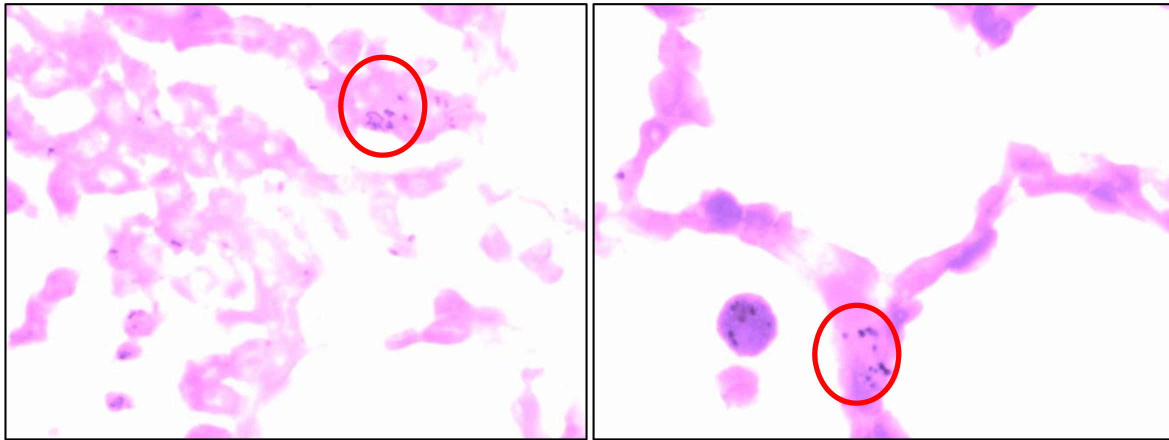


Figure 31. Gram Stain of mice vaccinated with PiaA showing very sparse *S. pneumoniae* in lung tissue at 100x magnification.

4.3.2.3 Lung Sections of PiaA/PiuA Vaccinates

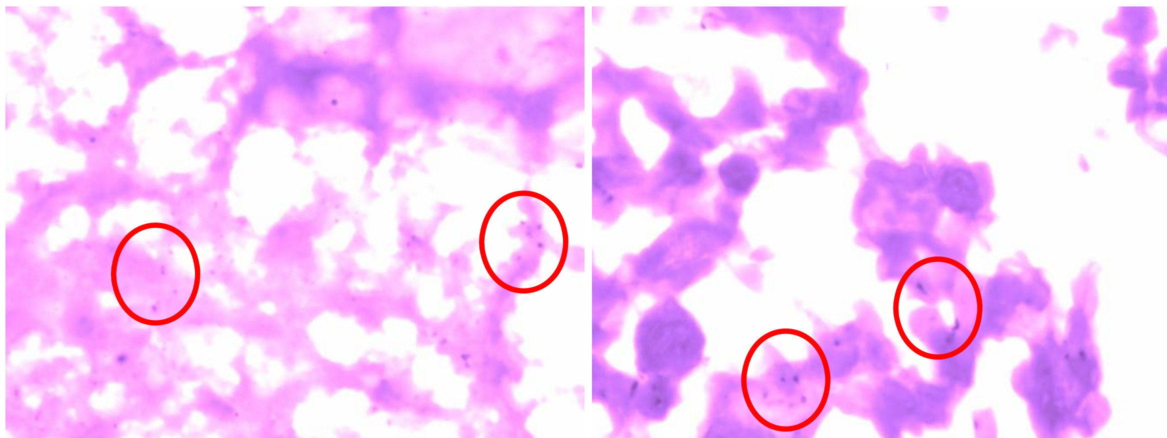


Figure 32. Gram Stain of mice vaccinated with PiaA/PiuA showing *S. pneumoniae* in lung tissue of PiaA/PiuA vaccinates at 100x magnification.

4.3.2.4 Lung Sections of VLP:PiaA Vaccinates

The conjugated VLP:PiaA vaccine (whether given IM or IN+IM) had high IgG titres coupled with significantly decreased blood culture. The H&E stains showed that alveoli had thin walls and a low number of neutrophils and macrophages visible (Figure 26). Overall, the gram stained sections showed very few pneumococci present with the few that were observed appearing singularly and not in chains. It was noted earlier that mice vaccinated with VLP:PiaA through both intramuscular and intranasal routes has a noticeably smaller IgG1 and IgG2a titre than when the vaccine was

delivered intramuscularly only. The mouse lung sections from the intramuscular and intranasal vaccine had *S. pneumoniae* just as all other challenged groups had.

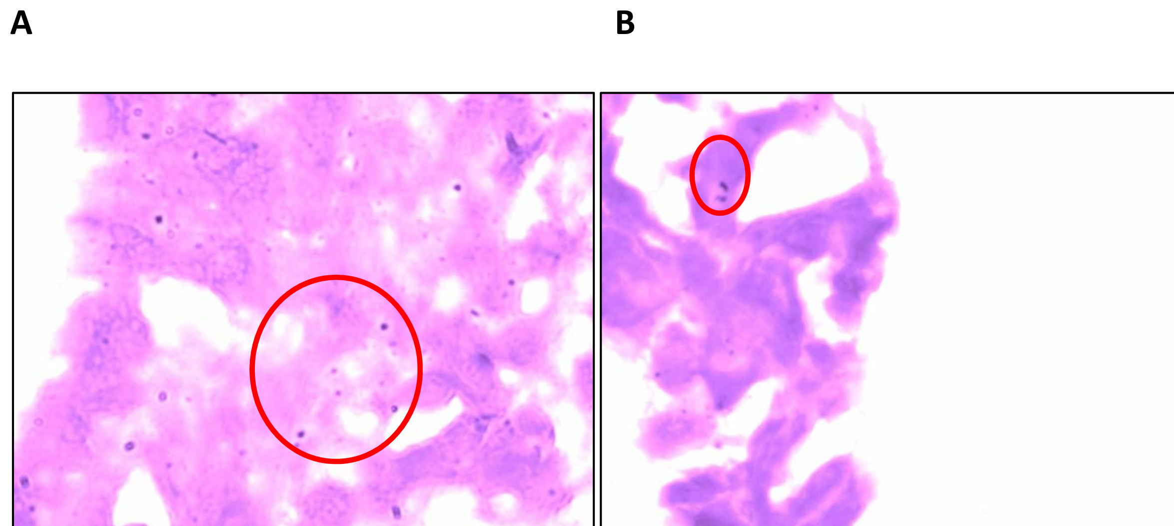


Figure 33. Gram Stain of mice vaccinated with VLP:PiaA showing small numbers of *S. pneumoniae* in lung tissue at 100x magnification. A) Mouse lung from VLP:PiaA vaccine delivered IM+IN. B) Mouse lung from VLP:PiaA vaccine delivered IN.

4.3.2.5 Lung Sections of VLP:PiuA Vaccinates

For VLP:PiuA vaccinated mice, high titres were measured for total IgG and IgG1. In IgG2a, a very high titre was recorded in the intramuscular vaccinates, but a much lower titre was determined for those vaccinated with intramuscular and intranasal VLP:PiuA. The H&E stains showed a thin alveoli wall in some sections, yet still there was notable congestion and a number of macrophages and neutrophils. Gram staining only showed the small presence of pneumococci (Figure 34).

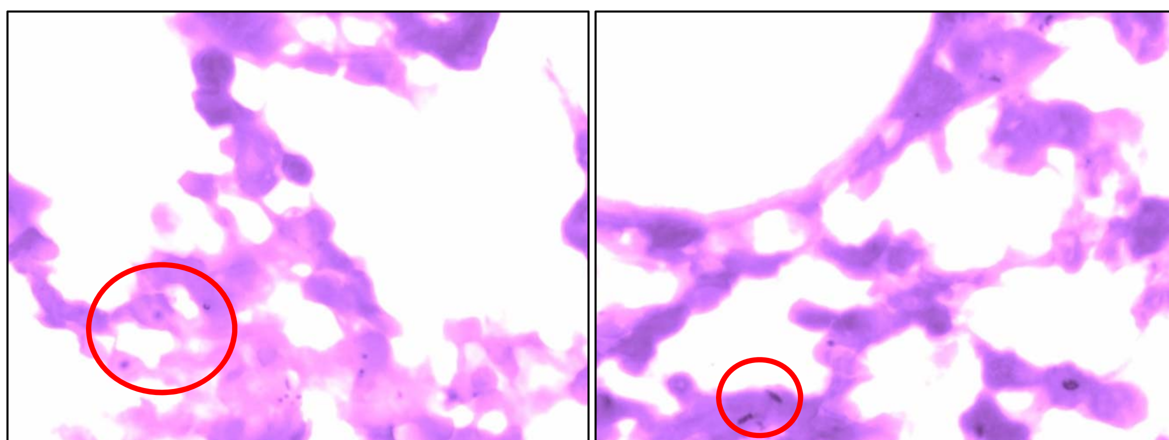
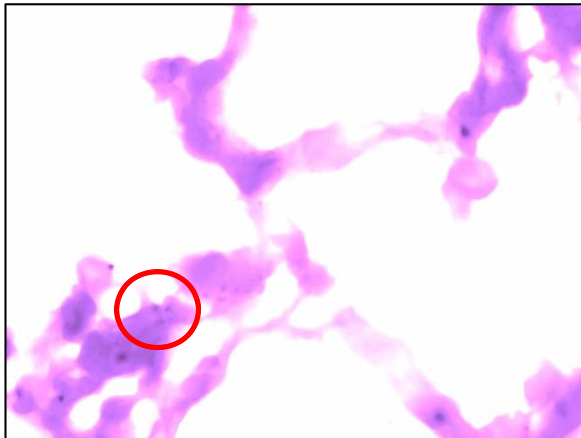


Figure 34. Gram Stain of mice vaccinated with VLP:PiuA showing small numbers of *S. pneumoniae* in lung tissue at 100x magnification.

4.3.2.6 Lung Sections of VLP:PiaA/PiuA Vaccinates

Mice vaccinated with both VLP:PiaA and VLP:PiuA showed very high total IgG titres. The IgG1 and IgG2a responses were high in the IN vaccinates, but less so when the vaccine was delivered through IN+IM. Similarly in blood culture, the vaccine significantly decreases the CFUs in the IM delivery, but there was no significant decrease when it was administered IN+IM. H&E staining shows some level of congestion in alveoli walls, but this was more pronounced in the IN+IM vaccinates than the IM alone. Similarly, the gram staining of lung from these groups shows that while a number of *S. pneumoniae* were present, fewer were shown in the IM vaccinates.

A **B**



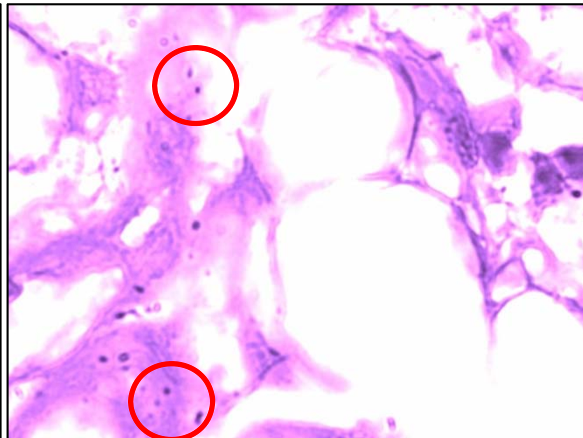


Figure 35. Gram Stain of mice vaccinated with VLP:PiaA/PiuA showing *S. pneumoniae* in lung tissue at 100x magnification. A) VLP:PiaA/PiuA mouse lung from IM vaccinate. B) VLP:PiaA/PiuA mouse lung from IM+IN vaccinate.

Chapter 5 Discussion

Antibody titres are quantitative measurements of the size and nature of the immunological response. Total IgG is a measurement of all IgG subclasses (including IgG1, IgG2a, IgG2b and IgG3 in mice). IgG is the major antibody in sera and is a standard indication of the overall immunological response that is occurring. IgG1 is associated with a T_h2 cell response which is humoral, whereas IgG2a is associated with a T_h1 response which is cell mediated. The total IgG value will indicate the presence and strength of an immune response. The measurement of IgG1 and IgG2a in each mouse group indicates the form of immunity generated.

5.1 HBV

HBV already has a safe and effective vaccine that has been available for decades. HbsAg is in itself not immunogenic, and is therefore coupled with an adjuvant (usually aluminium hydroxide) to stimulate an immune response. Recently it was found that Advax™ has the potential to replace aluminium hydroxide since it confers an improved response.

The addition of Advax™ as an adjuvant to HBsAg has been shown to increase the total IgG response predominantly through IgG1 and to a lesser extent IgG2a in BALB/c mice (Saade et al. 2013).

In our study, mice vaccinated with the VLP(HBsAg)/Advax™ mixture exhibited a medium IgG and IgG1 response and a lowered IgG2a response as expected.

5.1.1 Antibody Response in Conjugate Vaccines

VLP-specific IgG, IgG1 and IgG2a were measured in all conjugate vaccine types. The total IgG titres from the different conjugate vaccines were all within one dilution of the VLP-alone vaccine response, showing that conjugating the VLP to the pneumococcal proteins did nothing to diminish the overall immune response towards VLP.

5.1.1.1 Comparing Vaccination Routes (i.m. vs i.n. + i.m.)

The nature of the immune response was shown through IgG1 and IgG2a. The VLP-alone vaccination gave an IgG1 titre of 12800, and this was identical to the IgG1 titres for all conjugate vaccines given intranasally and intramuscularly (VLP:PiaA, VLP:PiuA and VLP:PiaA/PiuA). However, IgG1 titres in conjugate vaccines delivered only intramuscularly delivered a lower titre (1600-3200). This difference is likely due to the fact that mice given i.m. and i.n. vaccination were injected with more protein than i.m. alone. The i.n. route has shown to be an effective vaccination method for VLP (Jaganathan & Vyas 2006). Similar results were observed in IgG2a, with IgG2a titres in the i.n. and i.m. vaccinates being higher or equivalent to i.m. alone.

5.1.1.2 IgG1/IgG2 Ratio

By determining the ratio of IgG1 to IgG2, it can be established whether the immune response was CMI or humoral. Figure 36 compares the IgG1 titre to the IgG2a titre obtained from each vaccination type. High IgG1 titres resulted in IgG1 being the majority of the response in almost every case, as was expected (Saade et al. 2013).

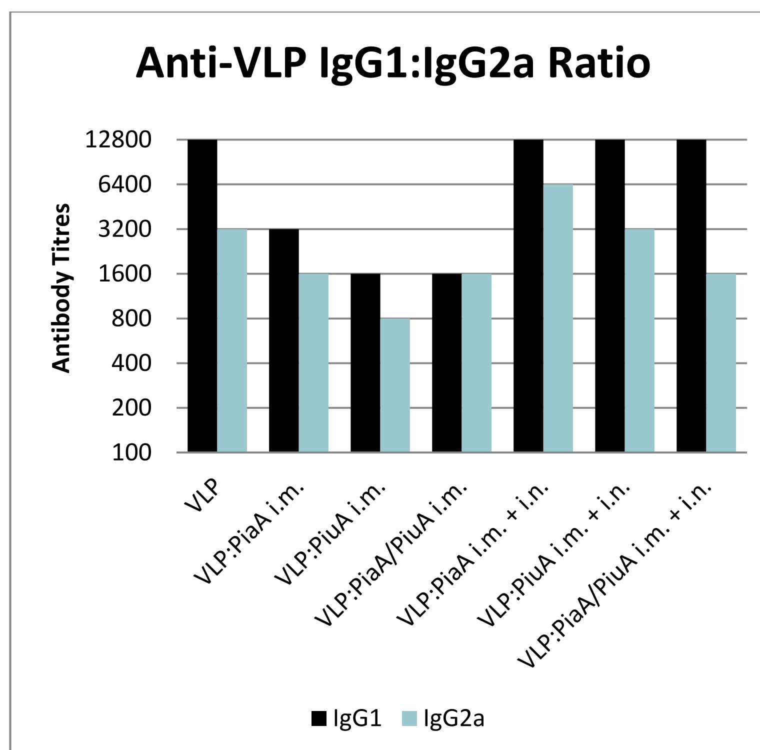


Figure 36. The Anti-VLP IgG1 and IgG2a titres for each vaccine type are compared. IgG1 makes up the majority of the response with the exception of VLP:PiaA i.m.

5.1.2 Potential as a Dual Vaccine

Comparing the VLP-specific IgG, IgG1 and IgG2a titres of the VLP-alone vaccinated mice with the conjugate-vaccinated mice has shown that the conjugation of pneumococcal capsular proteins have not diminished the immunological response. This lends to the possibility of VLP being used as an effective vaccine against HBV and not only to stimulate an IgG2a response.

5.2 Pneumococcal Proteins

The pneumococcal proteins, PiaA and PiuA were selected as vaccine candidates because of their potential in giving immunity to all serotypes of *S. pneumoniae*. In addition to being an antigen relevant to all serotypes, it also is able to provide a T-cell dependent response (required for lasting immunity). These proteins have been investigated for their potential as a vaccine, yet they fail to give an adequate humoral and cell mediated response (Jomaa et al. 2005) which is necessary to confer protection. By conjugating the VLP, which is known to produce both a humoral and a CMI response, to the pneumococcal proteins, it is hoped that a T-cell dependent CMI and humoral response will be generated (Kotiw et al. 2012). Therefore, the antibody titres will determine whether the vaccine has developed an antibody response and if so, the nature of that response. Then, additional results will determine the effectiveness of these antibody titres in actually preventing infection.

5.2.1 PiaA Response

5.2.1.1 Response in PiaA vaccine and PiaA/PiuA

The vaccinations of the pneumococcal proteins alone gave a standard by which we could compare the conjugated vaccines. With the PiaA protein alone, vaccination produced a very high IgG titre (102400) compared to the PiaA/PiuA combined vaccination (1600). The lower titre in the combined vaccine could be potentially due to the fact that only half the amount of PiaA protein was in the combined vaccination compared to the PiaA alone vaccine (10µl vs 19.9µl). This explanation is not

supported by Jomaa et al. (2005), who found that a 5µl vaccination of PiaA produced an identical response to a 10µl or 20µl vaccination.

The PiaA alone vaccine had a statistically significant clearance of *S. pneumoniae* CFUs in the blood, whereas in the PiaA/PiuA vaccine there was no significant difference from the positive (unvaccinated) control (Figure 19). In the lung, H&E staining indicated that there were varying levels of alveoli congestion in both vaccination groups, though a very high number of macrophages and neutrophils are clear in the PiaA/PiuA combination (see Figure 25, A & C). From this, there is good evidence that the PiaA/PiuA vaccine was less successful than the PiaA alone vaccine. This is counter to the Jomaa et al. (2005) study which proposed that there was no noticeable difference whether the proteins were combined or not.

5.2.1.2 Determining Precision

Due to sera being pooled upon collection (so that sufficient collection for ELISAs could take place), the antibody titre for each vaccination type comes as one number with no error bars, standard deviation, or any way of knowing the precision of the results. This method has proven to be difficult with regards to determining statistical significance in titre concentrations, since outliers are included and can impact the overall titre significantly.

5.2.1.3 Determining IgG1/IgG2a ratio

However, since the aim of this project is not to measure how high the titres are, but rather to produce a humoral and CMI response to the proteins, the efficacy of the project fundamentally rests on the IgG1 to IgG2a ratio in each vaccination type. The comparative titres for anti-PiaA IgG1 to IgG2a in each group vaccinated with PiaA is seen in Figure 37.

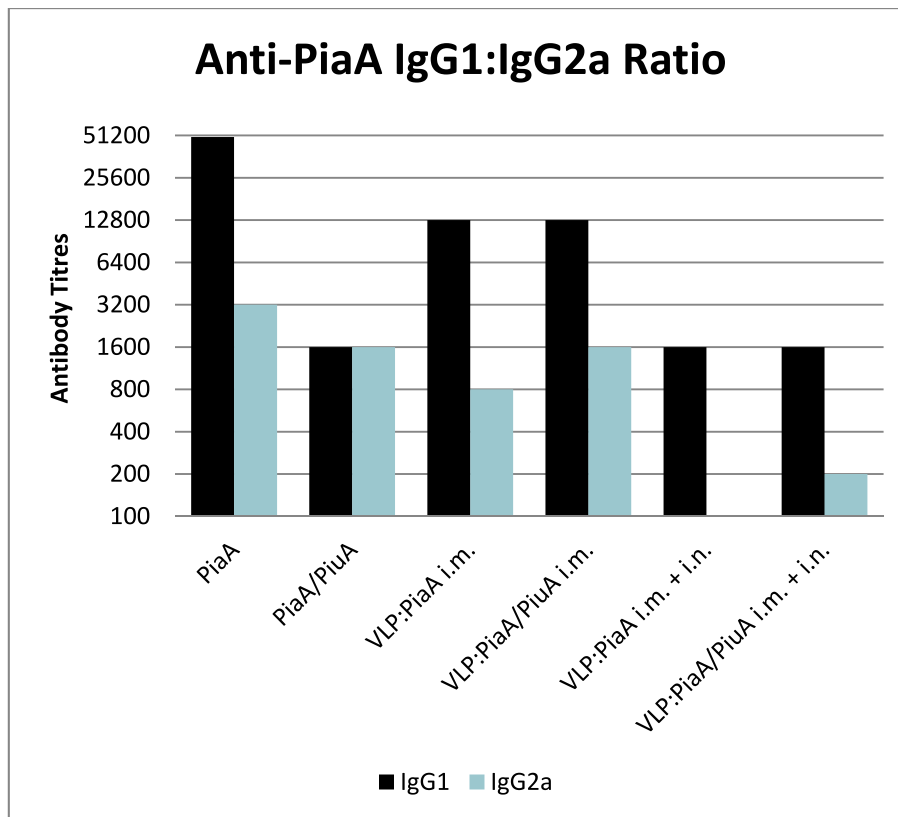


Figure 37. The Anti-PiaA IgG1 and IgG2a titres for each vaccine type are compared. IgG1 makes up the majority of the response with the exception of PiaA/PiuA.

Of all the PiaA vaccinations, the one with the most promising IgG1:IgG2a ratio was the PiaA/PiuA unconjugated vaccine (with an equal IgG1 to IgG2a response). However, as discussed previously, this group failed to clear pneumococcus from the blood, had bacteria in the gram stains and produced congested lung tissue in the H&E stains. Since the PiaA/PiuA vaccination yielded very low Anti-PiaA response, yet the Anti-PiuA response with the same vaccination yielded a high response, it is likely that a technical error occurred in either the construct or the delivery of the vaccine resulting in an inability to produce the expected antibody response.

The PiaA alone vaccine produced an IgG2a titre of 3200, which was a very high response compared to PiaA vaccinated mice in the study by Jomaa et al. (2005) which yielded a titre close to zero. The difference between the mice in that study was the use of aluminium hydroxide as opposed to the use of Advax™. Advax™ is known to stimulate both a humoral and CMI response, which suggests the likelihood that Advax™ is the agent responsible for the IgG2a titre.

The conjugated PiaA vaccinations produced clear IgG1 biased responses. Despite the response being largely IgG1 based (and subsequently humoral based), the titres for IgG2a are still indicative that some cell mediated immunity was generated. It is unclear whether this response is the result of the Advax™ adjuvant or the VLP conjugation, though since equivalently high titres were obtained in the unconjugated vaccinations, the former option is more probable. This component of the experimentation could have been more effective if there had been another vaccination group with PiaA and aluminium hydroxide to compare the impact that Advax™ was contributing as an adjuvant.

5.2.2 PiuA Response

In the two previous studies regarding PiaA and PiuA immunogenicity in mice, PiuA was seen to produce a greater antibody titre, though this was not necessarily coupled with increased actual protection (Jomaa et al. 2006; Jomaa et al. 2005).

5.2.2.1 Response in PiuA vaccine and PiaA/PiuA vaccine

Total IgG in the PiuA and PiaA/PiuA vaccines gave identical high titres (204800). The IgG1 values for each vaccine type gave similar titres (409600 and 204800 respectively). IgG2a titres were considerably lower than IgG1 responses (12800 and 6400 respectively). However, as with PiaA, the PiuA vaccination was not expected to produce a high IgG2a titre on its own (based on previous studies), and the likely explanation for the high titre is the use of Advax™ as an adjuvant.

5.2.2.2 Determining IgG1/IgG2a Ratio

The comparative titres of IgG1 and IgG2a are summarised in Figure 38.

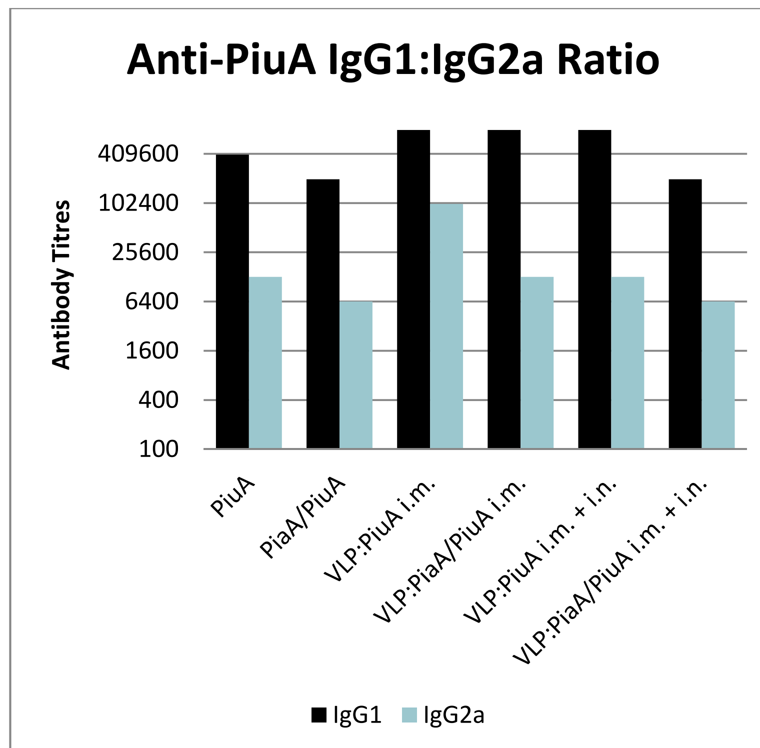


Figure 38. The Anti-PiaA IgG1 and IgG2a titres for each vaccine type are compared. IgG1 is again the dominant response type,

The comparison between different vaccination combinations and routes shows relatively minor differences both in IgG1 and IgG2a titres. The conjugated vaccines were not consistently higher or lower in IgG1 or IgG2a. All vaccination types produced a high IgG1 titre and a medium to high IgG2a titre. The conjugation to VLP and the delivery routes (i.m. or i.n. and i.m.) seem to not produce any significant impact on the titres or the nature of the response.

5.3 Impact of VLP on Immune Response

The conjugation of VLP to either PiaA or PiuA produced no clear shift in the nature of the immune response. In retrospect, the experiment would have produced a clearer result if there had been a control group for PiaA and for PiuA using aluminium hydroxide as the adjuvant. Previous studies had shown that using either PiaA or PiuA with aluminium hydroxide had produced an IgG2a titre only just above zero. In this experiment, two variables were changed which would both potentially affect

the type of immune response. Ideally, the study would have been able to measure the individual effects that Advax™ and conjugation had on IgG1/IgG2a titres. Instead, the results indicated (in an unquantifiable manner) that indeed a greater IgG2a response was being produced than previous studies, though a causal relationship was difficult to establish because there were at least two main variables to account for this. There is good reason to believe that VLP conjugation is not the major cause of this higher IgG2a response due to insignificant differences between conjugated and unconjugated groups. Therefore, it would be wise to pursue further investigation of Advax™ as a possible IgG2a promoter.

The reason VLP conjugation was focused on in this experiment was due to the recent success it has had in eliciting a CMI response with other antigens (Kotiw et al. 2012; Nyström et al. 2008; Petrovsky 2006). Since the concept of manipulating the immune response through the use of a VLP is a relatively recent concept (Schirmbeck, Böhm & Reimann 1996), the exact mechanisms of VLP induced CMI responses are uncertain. The concept of VLP conjugation not eliciting a CMI response is certainly possible due to improper conjugation, conjugation of proteins at positions which hindered immunogenicity (both of VLP and the pneumococcal proteins) or a number of other possibilities relating to protein size, structure, stability, immunogenicity and more.

5.4 Clearing Infections

Antibody titres are beneficial as long as they lead to protection in the form of stopping infection. The histology of the lung tissue showed that some level of protection was being conveyed into each of the vaccinated groups. It was clear upon examining the challenged, unvaccinated controls that there was an improvement on congestion. Though there were also clear signs of infection (including macrophages, neutrophils and occasionally congested alveoli), vaccination with any combination of PiaA and PiuA made an impact in reducing signs of pathology. There was also statistically significant reductions in blood culture CFUs in all vaccinates with the exception of the PiuA vaccinates, the PiaA/PiuA vaccinates and the VLP:PiaA/PiuA i.n. and i.m. vaccinates. Although, there are explanations as to why these groups did not have the same reduction in blood culture. The mouse

group vaccinated with PiuA also had a statistically significantly higher number of CFUs in the NPAs than the positive control, suggesting that this group was actually challenged with a larger number of *S. pneumoniae* (Section 4.2.2). The PiaA/PiuA group produced peculiar antibody titres, suggesting an error in the vaccine construct or delivery (Section 5.2.1.1). The VLP:PiaA/ PiuA i.n. and i.m. vaccinates also produced a peculiarly low anti-PiaA and anti-PiuA titres in IgG1 and IgG2a compared to the other conjugated vaccine types. In summation, the majority of the data regarding the efficacy of the vaccinated mice in clearing the infection showed a significant improvement from the unvaccinated control mouse group. The fact that mice were able to mount a response to the infection indicates that a CMI response was present since protection from *S. pneumoniae* lung infection requires a humoral and CMI response (Wilson et al. 2014).

5.4 Significance

The discovery of an effective and safe vaccine available to all age groups against all known serotypes of *S. pneumoniae* would be a major breakthrough in the global fight against pneumococcal diseases. It is difficult to establish just how much research is currently being conducted into novel pneumococcal vaccines, though recent reviews on new vaccine candidates have shown the variety of antigens each presenting a number of problems (Malley & Anderson 2012). Overall, the potential seen in the PiaA and PiuA proteins is very impressive. They fulfil so many of the criteria in an ideal candidate, with the only exception at this stage being their inability to produce a significant CMI response. This project has been an advancement upon previous studies involving PiaA and PiuA and will hopefully lead to a further discovery which can transition this novel vaccine from being a possibility to being a breakthrough in healthcare worldwide.

5.5 Future Directions

The potential discovery of a novel all-serotype encompassing, effective vaccine for conferring protection in the host towards *Streptococcus pneumoniae* would be a drastic improvement for healthcare universally. As noted earlier, the deficiencies in current vaccines are due to insufficient coverage (not effective in all serotypes) and a non-lasting immune response. Several avenues are

currently being investigated to discover a vaccine capable of delivering this protection and the approach used in this experiment has high potential. The use of PiaA and PiuA are critical since they are present in all serotypes that have been discovered. Additionally, the use of those proteins is important in producing the desired (T-cell dependent) response. The clear next step in the pathway to this being a successful vaccine is being able to produce a humoral and CMI response. The PiaA and PiuA proteins naturally elicit a humoral response. An adequate mechanism for a significant CMI response has remained elusive in this study, though the inclusion of Advax™ has been advantageous to this cause. The VLP conjugation concept could be improved upon by conjugating to specific immunogenic regions on VLPs. The purpose of this search for a CMI response is to provide protection from infection, so upon finding a mechanism for providing a high CMI response the next step is to make sure that this does translate directly into protection. If proper protection is indeed given by the vaccine, the establishment of the safety of the vaccine is of primary concern.

Another important facet to vaccine research is an ability to predict what challenges may be faced following the introduction of a new vaccine to the market. An effective vaccine strategy is to judge what impact a vaccination will have on global health and be two steps ahead of any foreseeable problems. Challenges of serotype replacement can be lessened if these are expected and addressed before they actually pose a health threat. Due to the high recombinogenic nature of *S. pneumoniae*, if a single antigen is used for cross-serotype vaccination it is expected that recombination will quickly result in a new serotype without that specific antigen. Croucher et al. (Croucher et al. 2011) described the high recombination in *S. pneumoniae* and mentioned surface proteins that were potential vaccine candidates until recent discovery of new serotypes that do not express these proteins. Therefore, if PiaA and PiuA continue to be promising vaccine candidates, a future direction will be discovering the next surface protein to replace these when recombination will likely occur. A number of other surface proteins are being studied currently which could serve this purpose (Malley & Anderson 2012).

Chapter 6 Conclusion

The results of this study indicate that PiaA and PiuA coupled with Advax™ has a potential for providing real protection against all serotypes of *Streptococcus pneumoniae*. VLP conjugation provided very little evidence of being able to elicit a CMI response. Though the responses in all vaccines trialled in this experiment were mainly of a humoral nature, there was a noticeable CMI response which was likely due to the use of Advax™ as an adjuvant. This produced a level of protection in the mice that could be observed through a significant reduction of CFUs in blood culture and decreased pathology in lung tissue. Although VLP conjugation did not stimulate the CMI response as expected, there are other conjugation techniques that could potentially elicit this response. Additional research would be worthwhile in identifying how VLP can be utilized to improve the desired CMI response and lead to a vaccine providing full protection from *S. pneumoniae*.

Chapter 7 Appendix

Antibody Titres

Antibody titres were recorded in triplicate in a serial dilution. Titres are recorded below grouped by the

7.1 Anti-PiaA

7.1.1 Total IgG

Vaccination: PiaA			
Dilution	Optical Density Readings (450nm)		
1/50000	0.09	0.102	0.099
1/100000	0.098	0.087	0.074
1/200000	0.072	0.071	0.057
1/400000	0.054	0.056	0.061
1/800000	0.048	0.052	0.051
1/1600000	0.046	0.048	0.046
1/3200000	0.049	0.046	0.048

Vaccination: PiaA/PiuA			
Dilution	Optical Density Readings (450nm)		
1/200	0.242	0.259	0.256
1/400	0.209	0.208	0.221
1/800	0.117	0.137	0.112
1/1600	0.084	0.084	0.08
1/3200	0.056	0.063	0.061
1/6400	0.05	0.053	0.05
1/12800	0.045	0.044	0.044

Vaccination: VLP:PiaA			
Dilution	Optical Density Readings (450nm)		
1/200	0.372	0.449	0.437
1/400	0.254	0.284	0.279
1/800	0.201	0.175	0.198
1/1600	0.161	0.137	0.149
1/3200	0.102	0.103	0.118
1/6400	0.085	0.094	0.084
1/12800	0.076	0.076	0.078

Vaccination: VLP:PiaA/PiuA			
Dilution	Optical Density Readings (450nm)		
1/200	0.946	0.984	0.974
1/400	0.704	0.69	0.641
1/800	0.423	0.424	0.411
1/1600	0.298	0.269	0.274
1/3200	0.16	0.16	0.163
1/6400	0.11	0.137	0.128
1/12800	0.08	0.08	0.089

Vaccination: VLP:PiaA (IM+IN)			
Dilution	Optical Density Readings (450nm)		
1/200	0.408	0.405	0.406
1/400	0.244	0.245	0.239
1/800	0.163	0.157	0.163
1/1600	0.12	0.114	0.11
1/3200	0.088	0.09	0.082
1/6400	0.074	0.073	0.073
1/12800	0.064	0.061	0.064

Vaccination: VLP:PiuA (IM+IN)			
Dilution	Optical Density Readings (450nm)		
1/200	0.547	0.592	0.549
1/400	0.329	0.34	0.333
1/800	0.128	0.222	0.215
1/1600	0.128	0.143	0.125
1/3200	0.095	0.095	0.099
1/6400	0.077	0.074	0.083
1/12800	0.075	0.073	0.066

7.1.2 IgG1

Vaccination: PiaA			
Dilution	**Optical Density Readings (450nm)**		
1/50000	*0.095*	*0.091*	*0.089*
1/100000	*0.063*	*0.064*	*0.066*
1/200000	*0.055*	*0.052*	*0.054*
1/400000	*0.051*	*0.052*	*0.062*
1/800000	*0.049*	*0.05*	*0.047*
1/1600000	*0.045*	*0.044*	*0.046*
1/3200000	*0.048*	*0.048*	*0.045*

Vaccination: PiaA/PiuA			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.174	0.185	0.182
1/400	0.19	0.171	0.151
1/800	0.095	0.119	0.095
1/1600	0.073	0.08	0.07
1/3200	0.052	0.063	0.058
1/6400	0.046	0.055	0.047
1/12800	0.043	0.042	0.042

Vaccination: VLP:PiaA			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.256	0.293	0.266
1/400	0.193	0.206	0.212
1/800	0.147	0.134	0.122
1/1600	0.097	0.099	0.107
1/3200	0.072	0.078	0.074
1/6400	0.085	0.086	0.073
1/12800	0.233	0.069	0.061

Vaccination: VLP:PiaA/PiuA			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.917	0.828	0.878
1/400	0.673	0.648	0.674
1/800	0.441	0.422	0.43
1/1600	0.252	0.252	0.256
1/3200	0.17	0.15	0.16
1/6400	0.102	0.116	0.105
1/12800	0.079	0.074	0.079

Vaccination: VLP:PiaA (IM+IN)			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.247	0.247	0.25
1/400	0.156	0.132	0.16
1/800	0.112	0.103	0.101
1/1600	0.086	0.078	0.073
1/3200	0.063	0.059	0.069
1/6400	0.055	0.053	0.053
1/12800	0.051	0.049	0.049

Vaccination: VLP:PiaA/PiuA (IM+IN)			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.203	0.191	0.192
1/400	0.137	0.129	0.113
1/800	0.1	0.1	0.092
1/1600	0.072	0.076	0.076
1/3200	0.059	0.064	0.062
1/6400	0.056	0.062	0.056
1/12800	0.054	0.052	0.048

7.1.3 IgG2a

Vaccination: PiaA			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.565	0.562	0.604
1/400	0.403	0.414	0.411
1/800	0.23	0.235	0.228
1/1600	0.14	0.147	0.146
1/3200	0.091	0.089	0.09
1/6400	0.068	0.065	0.069
1/12800	0.056	0.053	0.055

Vaccination: PiaA/PiuA			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.285	0.271	0.306
1/400	0.168	0.153	0.175
1/800	0.11	0.105	0.104
1/1600	0.077	0.073	0.075
1/3200	0.06	0.059	0.062
1/6400	0.056	0.063	0.054
1/12800	0.049	0.059	0.05

Vaccination: VLP:PiaA			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.266	0.272	0.28
1/400	0.165	0.166	0.144
1/800	0.111	0.113	0.1
1/1600	0.073	0.083	0.072
1/3200	0.047	0.055	0.057
1/6400	0.05	0.048	0.049
1/12800	0.051	0.057	0.054

Vaccination: VLP:PiaA/PiuA			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.249	0.233	0.263
1/400	0.194	0.145	0.149
1/800	0.119	0.09	0.102
1/1600	0.08	0.071	0.074
1/3200	0.053	0.053	0.059
1/6400	0.051	0.076	0.051
1/12800	0.045	0.045	0.046

Vaccination: VLP:PiaA (IM+IN)			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.056	0.061	0.058
1/400	0.049	0.051	0.05
1/800	0.051	0.048	0.045
1/1600	0.043	0.043	0.043
1/3200	0.056	0.043	0.043
1/6400	0.043	0.046	0.042
1/12800	0.041	0.041	0.042

Vaccination: VLP:PiaA/PiuA (IM+IN)			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.087	0.086	0.086
1/400	0.064	0.063	0.058
1/800	0.059	0.05	0.051
1/1600	0.047	0.048	0.047
1/3200	0.05	0.043	0.048
1/6400	0.044	0.044	0.043
1/12800	0.042	0.041	0.042

7.2 Anti-PiuA

7.2.1 Total IgG

Vaccination: PiuA			
Dilution	Optical Density Readings (450nm)		
1/25000	*0.503*	*0.509*	*0.441*
1/50000	*0.042*	*0.175*	*0.258*
1/100000	*0.07*	*0.109*	*0.191*
1/200000	*0.04*	*0.073*	*0.105*
1/400000	*0.039*	*0.053*	*0.069*
1/800000	*0.038*	*0.049*	*0.058*
1/1600000	*0.036*	*0.042*	*0.048*

Vaccination: PiaA/PiuA			
Dilution	Optical Density Readings (450nm)		
1/25000	*0.34*	*0.348*	*0.352*
1/50000	*0.194*	*0.205*	*0.201*
1/100000	*0.112*	*0.103*	*0.111*
1/200000	*0.078*	*0.078*	*0.08*
1/400000	*0.058*	*0.06*	*0.063*
1/800000	*0.048*	*0.048*	*0.048*
1/1600000	*0.043*	*0.043*	*0.043*

Vaccination: VLP:PiuA			
Dilution	Optical Density Readings (450nm)		
1/25000	*0.649*	*0.657*	*0.647*
1/50000	*0.429*	*0.416*	*0.424*
1/100000	*0.234*	*0.248*	*0.229*
1/200000	*0.149*	*0.146*	*0.156*
1/400000	*0.096*	*0.087*	*0.096*
1/800000	*0.067*	*0.074*	*0.069*
1/1600000	*0.054*	*0.05*	*0.053*

Vaccination: VLP:PiuA			
Dilution	Optical Density Readings (450nm)		
1/200	0.547	0.592	0.549
1/400	0.329	0.34	0.333
1/800	0.128	0.222	0.215
1/1600	0.128	0.143	0.125
1/3200	0.095	0.095	0.099
1/6400	0.077	0.074	0.083
1/12800	0.075	0.073	0.066

Vaccination: VLP:PiuA (IM+IN)			
Dilution	Optical Density Readings (450nm)		
1/25000	*0.674*	*0.67*	*0.574*
1/50000	*0.365*	*0.365*	*0.336*
1/100000	*0.251*	*0.245*	*0.212*
1/200000	*0.125*	*0.137*	*0.133*
1/400000	*0.102*	*0.101*	*0.096*
1/800000	*0.077*	*0.071*	*0.07*
1/1600000	*0.068*	*0.05*	*0.052*

Vaccination: VLP:PiaA/PiuA (IM+IN)			
Dilution	Optical Density Readings (450nm)		
1/25000	*0.252*	*0.275*	*0.273*
1/50000	*0.146*	*0.148*	*0.161*
1/100000	*0.095*	*0.088*	*0.094*
1/200000	*0.083*	*0.077*	*0.07*
1/400000	*0.069*	*0.077*	*0.056*
1/800000	*0.054*	*0.06*	*0.048*
1/1600000	*0.049*	*0.057*	*0.045*

7.1.2 IgG1

Vaccination: PiuA			
Dilution	Optical Density Readings (450nm)		
1/25000	*0.516*	*0.498*	*0.456*
1/50000	*0.307*	*0.304*	*0.303*
1/100000	*0.181*	*0.204*	*0.187*
1/200000	*0.123*	*0.133*	*0.119*
1/400000	*0.087*	*0.09*	*0.082*
1/800000	*0.062*	*0.062*	*0.059*
1/1600000	*0.048*	*0.049*	*0.049*

Vaccination: PiaA/PiuA			
Dilution	Optical Density Readings (450nm)		
1/25000	*0.411*	*0.372*	*0.402*
1/50000	*0.233*	*0.223*	*0.23*
1/100000	*0.125*	*0.122*	*0.13*
1/200000	*0.088*	*0.086*	*0.09*
1/400000	*0.068*	*0.07*	*0.065*
1/800000	*0.056*	*0.054*	*0.053*
1/1600000	*0.046*	*0.042*	*0.049*

Vaccination: VLP:PiuA			
Dilution	Optical Density Readings (450nm)		
1/25000	*0.668*	*0.76*	*0.729*
1/50000	*0.49*	*0.511*	*0.462*
1/100000	*0.296*	*0.272*	*0.308*
1/200000	*0.187*	*0.173*	*0.202*
1/400000	*0.11*	*0.138*	*0.121*
1/800000	*0.077*	*0.076*	*0.108*
1/1600000	*0.056*	*0.074*	*0.068*

Vaccination: VLP:PiaA/PiuA			
Dilution	Optical Density Readings (450nm)		
1/25000	*0.607*	*0.639*	*0.6*
1/50000	*0.37*	*0.385*	*0.341*
1/100000	*0.227*	*0.23*	*0.223*
1/200000	*0.136*	*0.128*	*0.126*
1/400000	*0.091*	*0.087*	*0.094*
1/800000	*0.061*	*0.066*	*0.058*
1/1600000	*0.056*	*0.05*	*0.051*

Vaccination: VLP:PiuA (IM+IN)			
Dilution	Optical Density Readings (450nm)		
1/25000	*0.725*	*0.608*	*0.637*
1/50000	*0.407*	*0.444*	*0.421*
1/100000	*0.237*	*0.234*	*0.235*
1/200000	*0.163*	*0.149*	*0.163*
1/400000	*0.104*	*0.1*	*0.104*
1/800000	*0.071*	*0.07*	*0.089*
1/1600000	*0.056*	*0.069*	*0.052*

Vaccination: VLP:PiaA/PiuA (IM+IN)			
Dilution	Optical Density Readings (450nm)		
1/25000	*0.313*	*0.334*	*0.289*
1/50000	*0.179*	*0.181*	*0.169*
1/100000	*0.104*	*0.093*	*0.107*
1/200000	*0.08*	*0.073*	*0.071*
1/400000	*0.058*	*0.056*	*0.054*
1/800000	*0.047*	*0.047*	*0.046*
1/1600000	*0.044*	*0.051*	*0.043*

7.1.3 IgG2a

Vaccination: PiuA			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.712	0.761	0.742
1/400	0.646	0.635	0.627
1/800	0.461	0.45	0.411
1/1600	0.284	0.293	0.304
1/3200	0.184	0.174	0.184
1/6400	0.12	0.122	0.129
1/12800	0.079	0.081	0.082

Vaccination: PiaA/PiuA			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.54	0.551	0.572
1/400	0.483	0.462	0.444
1/800	0.27	0.286	0.259
1/1600	0.172	0.174	0.162
1/3200	0.104	0.098	0.099
1/6400	0.071	0.07	0.071
1/12800	0.049	0.054	0.055

Vaccination: VLP:PiuA			
Dilution	**Optical Density Readings (450nm)**		
1/25000	*0.183*	*0.205*	*0.206*
1/50000	*0.111*	*0.108*	*0.123*
1/100000	*0.074*	*0.072*	*0.073*
1/200000	*0.062*	*0.054*	*0.06*
1/400000	*0.072*	*0.052*	*0.058*
1/800000	*0.044*	*0.045*	*0.05*
1/1600000	*0.046*	*0.044*	*0.044*

Vaccination: VLP:PiaA/PiuA			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.842	0.883	0.874
1/400	0.639	0.655	0.652
1/800	0.439	0.452	0.441
1/1600	0.257	0.239	0.267
1/3200	0.174	0.157	0.161
1/6400	0.105	0.096	0.102
1/12800	0.074	0.069	0.07

Vaccination: VLP:PiuA (IM+IN)			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.869	0.924	0.869
1/400	0.679	0.781	0.716
1/800	0.593	0.572	0.613
1/1600	0.424	0.43	0.41
1/3200	0.279	0.282	0.287
1/6400	0.176	0.163	0.166
1/12800	0.111	0.113	0.107

Vaccination: VLP:PiaA/PiuA (IM+IN)			
Dilution	**Optical Density Readings (450nm)**		
1/200	0.933	0.921	0.957
1/400	0.592	0.556	0.547
1/800	0.332	0.315	0.329
1/1600	0.203	0.194	0.204
1/3200	0.141	0.127	0.137
1/6400	0.089	0.088	0.087
1/12800	0.064	0.062	0.065

Chapter 8 References

Austrian, R 2000, 'Pneumococcal otitis media and pneumococcal vaccines, a historical perspective', *Vaccine*, vol. 19, , no. 1, pp. 71-7.

Bauer, T, Sprinzl, M & Protzer, U 2011, 'Immune control of hepatitis B virus', *Digestive diseases (Basel)*, vol. 29, no. 4, p. 423.

Beall, B, McEllistrem, MC, Gertz, RE, Wedel, S, Boxrud, DJ, Gonzalez, AL, Medina, M-J, Pai, R, Thompson, TA, Harrison, LH, McGee, L & Whitney, CG 2006, 'Pre- and Postvaccination Clonal Compositions of Invasive Pneumococcal Serotypes for Isolates Collected in the United States in 1999, 2001, and 2002', *Journal of Clinical Microbiology*, vol. 44, no. 3, pp. 999-1017, PMC.

Bergmann, S & Hammerschmidt, S 2006, 'Versatility of pneumococcal surface proteins', *Microbiology*, vol. 152, no. 2, pp. 295-303.

Bergmann, S, Rohde, M, Chhatwal, GS & Hammerschmidt, S 2001, 'α-Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface', *Molecular microbiology*, vol. 40, no. 6, pp. 1273-87.

Beveridge, TJ 2001, 'Use of the Gram stain in microbiology', *Biotechnic & Histochemistry*, vol. 76, no. 3, pp. 111-8.

Black, S, Shinefield, H, Fireman, B, Lewis, E, Ray, P, Hansen, JR, Elvin, L, Ensor, KM, Hackell, J, Siber, G, Malinoski, F, Madore, D, Chang, I, Kohberger, R, Watson, W, Austrian, R & Edwards, K 2000, 'Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group', *The Pediatric infectious disease journal*, vol. 19, no. 3, p. 187.

Black, SB, Shinefield, HR, Hansen, J, Elvin, L, Laufer, D & Malinoski, F 2001, 'Postlicensure evaluation of the effectiveness of seven valent pneumococcal conjugate vaccine', *The Pediatric infectious disease journal*, vol. 20, no. 12, pp. 1105-7.

Blasi, F, Mantero, M, Santus, P & Tarsia, P 2012, 'Understanding the burden of pneumococcal disease in adults', *Clinical Microbiology and Infection*, vol. 18, no. 5, pp. 7-14.

Bluestone, CD 1998, 'Epidemiology and pathogenesis of chronic suppurative otitis media: implications for prevention and treatment1', *Int J Pediatr Otorhinolaryngol*, vol. 42, no. 3, pp. 207-23.

Bogaert, D, de Groot, R & Hermans, PWM 2004, '*Streptococcus pneumoniae* colonisation: the key to pneumococcal disease', *The Lancet Infectious Diseases*, vol. 4, no. 3, pp. 144-54.

Bondada, S, Wu, H-J, Robertson, DA & Chelvarajan, RL 2000, 'Accessory cell defect in unresponsiveness of neonates and aged to polysaccharide vaccines', *Vaccine*, vol. 19, no. 4–5, pp. 557-65.

Broome, CV & Facklam, RR 1981, 'Epidemiology of Clinically Significant Isolates of *Streptococcus pneumoniae* in the United States [with Discussion]', *Reviews of Infectious Diseases*, vol. 3, no. 2, pp. 277-81.

Brown, JS & Holden, DW 2002, 'Iron acquisition by Gram-positive bacterial pathogens', *Microbes and infection*, vol. 4, no. 11, pp. 1149-56.

Brown, JS, Gilliland, SM & Holden, DW 2001, 'A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence', *Molecular microbiology*, vol. 40, no. 3, pp. 572-85.

Bryant, KA, Block, SL, Baker, SA, Gruber, WC & Scott, DA 2010, 'Safety and Immunogenicity of a 13-Valent Pneumococcal Conjugate Vaccine', *Pediatrics*, vol. 125, no. 5, pp. 866-75.

Butler, JC & Schuchat, A 1999, 'Epidemiology of pneumococcal infections in the elderly', *Drugs & Aging*, vol. 15, pp. 11-9.

Calix, JJ, Porambo, RJ, Brady, AM, Larson, TR, Yother, J, Abeygunwardana, C & Nahm, MH 2012, 'Biochemical, Genetic, and Serological Characterization of Two Capsule Subtypes among *Streptococcus pneumoniae* Serotype 20 Strains: Discovery Of A New Pneumococcal Serotype', *The Journal of Biological Chemistry*, vol. 287, no. 33, pp. 27885-94, PMC.

Chang, M-H 2007, 'Hepatitis B virus infection', *Seminars in Fetal and Neonatal Medicine*, vol. 12, no. 3, pp. 160-7.

Chayarpham, S, Stuart, J, Chongsuvivatwong, V, Chinpairoj, S & Lim, A 1996, 'A Study Of The Prevalence Of And Risk Factors For Ear Diseases And Hearing Loss In Primary School Children In Hat Yai, Thailand', *Journal of the Medical Association of Thailand* vol. 79, no. 7, pp. 468-72, PubMed.

Chen, CH, Lin, CJ, Hwang, YH & Ku, CJ 2003, 'Epidemiology Of Otitis Media In Chinese Children', *Clinical Otolaryngology & Allied Sciences*, vol. 28, no. 5, p. 442.

Chiu, C & McIntyre, P 2013, 'Pneumococcal Vaccines: Past, Present And Future', *Australian Prescriber*, vol. 36, no. 3, pp. 88-93.

Coffey, TJ, Enright, MC, Daniels, M, Morona, JK, Morona, R, Hryniewicz, W, Paton, JC & Spratt, BG 1998, 'Recombinational Exchanges At The Capsular Polysaccharide Biosynthetic Locus Lead To Frequent Serotype Changes Among Natural Isolates Of *Streptococcus Pneumoniae*', *Molecular microbiology*, vol. 27, no. 1, p. 73.

Croucher, NJ, Harris, SR, Fraser, C, Quail, MA, Burton, J, van der Linden, M, McGee, L, von Gottberg, A, Song, JH & Ko, KS 2011, 'Rapid pneumococcal evolution in response to clinical interventions', *Science*, vol. 331, no. 6016, pp. 430-4.

Curran, M & Goa, K 2003, 'DTPa-HBV-IPV/Hib Vaccine (Infanrix hexa™)', *Drugs*, vol. 63, no. 7, pp. 673-82.

Dawid, S, Roche, AM & Weiser, JN 2007, 'The blp Bacteriocins of *Streptococcus pneumoniae* Mediate Intraspecies Competition both In Vitro and In Vivo', *Infection and immunity*, vol. 75, no. 1, pp. 443-51.

de Kluyver, R 2014, 'Invasive Pneumococcal Disease Surveillance Australia, 1 October To 31 December 2013', *Communicable Diseases Intelligence Quarterly Report*, vol. 38, no. 1, p. E88.

Della Corte, C, Comparcola, D & Nobili, V 2012, 'Hepatitis B virus infection in children', *Clinics and research in hepatology and gastroenterology*, vol. 36, no. 3, pp. 291-3.

Donohue, W & Benson, J 2007, 'Hepatitis In Refugees Who Settle In Australia', *Australian Family Physician*, vol. 36, no. 9, pp. 719-7.

Douglas, RM & Miles, HB 1984, 'Vaccination against *Streptococcus pneumoniae* in Childhood: Lack of Demonstrable Benefit in Young Australian Children', *The Journal of Infectious Diseases*, vol. 149, no. 6, pp. 861-9.

Durando, P, Faust, SN, Fletcher, M, Krizova, P, Torres, A & Welte, T 2013, 'Experience With Pneumococcal Polysaccharide Conjugate Vaccine (Conjugated To Crm197 Carrier Protein) In Children And Adults', *Clinical Microbiology and Infection*, vol. 19, no. 1, pp. 1-9.

Esposito, S, Tansey, S, Thompson, A, Razmpour, A, Liang, J, Jones, TR, Ferrera, G, Maida, A, Bona, G, Sabatini, C, Pugni, L, Emini, EA, Gruber, WC, Scott, DA & Principi, N 2010, 'Safety and Immunogenicity of a 13-Valent Pneumococcal Conjugate Vaccine Compared to Those of a 7-Valent Pneumococcal Conjugate Vaccine Given as a Three-Dose Series with Routine Vaccines in Healthy Infants and Toddlers', *Clinical and Vaccine Immunology*, vol. 17, no. 6, pp. 1017-26.

Fung, HB & Monteagudo-Chu, MO 2010, 'Community-Acquired Pneumonia In The Elderly', *The American Journal of Geriatric Pharmacotherapy*, vol. 8, no. 1, pp. 47-62.

Greenhow, TL, Hung, Y-Y, Herz, AM, Losada, E & Pantell, RH 2014, 'The Changing Epidemiology Of Serious Bacterial Infections In Young Infants', *The Pediatric infectious disease journal*, vol. 33, no. 6, pp. 595-9.

Gunasekera, H, Knox, S, Morris, P, Britt, H, McIntyre, P & Craig, JC 2007, 'The Spectrum And Management Of Otitis Media In Australian Indigenous And Nonindigenous Children: A National Study', *Pediatric Infectious Disease Journal*, vol. 26, no. 8, pp. 689-92.

Gurcan, MN, Boucheron, L, Can, A, Madabhushi, A, Rajpoot, N & Yener, B 2009, 'Histopathological Image Analysis: A Review', *IEEE reviews in biomedical engineering*, vol. 2, pp. 147-71, Pmc, item: PMC2910932.

Hackel, M, Lascols, C, Bouchillon, S, Hilton, B, Morgenstern, D & Purdy, J 2013, 'Serotype Prevalence And Antibiotic Resistance In *Streptococcus pneumoniae* Clinical Isolates Among Global Populations', *Vaccine*, vol. 31, no. 42, pp. 4881-7.

Hammerschmidt, S, Talay, SR, Brandtzaeg, P & Chhatwal, GS 1997, 'Spsa, A Novel Pneumococcal Surface Protein With Specific Binding To Secretory Immunoglobulin A And Secretory Component', *Molecular microbiology*, vol. 25, no. 6, pp. 1113-24.

Hammitt, LL, Bulkow, LR, Singleton, RJ, Nuorti, JP, Hummel, KB, Miernyk, KM, Zanis, C, Whaley, M, Romero-Steiner, S, Butler, JC, Rudolph, K & Hennessy, TW 2011, 'Repeat Revaccination With 23-Valent Pneumococcal Polysaccharide Vaccine Among Adults Aged 55–74 Years Living In Alaska: No Evidence Of Hyporesponsiveness', *Vaccine*, vol. 29, no. 12, pp. 2287-95.

Hanage, WP, Huang, SS, Lipsitch, M, Bishop, CJ, Godoy, D, Pelton, SI, Goldstein, R, Huot, H & Finkelstein, JA 2007, 'Diversity and Antibiotic Resistance among Nonvaccine Serotypes of *Streptococcus pneumoniae* Carriage Isolates in the Post-Heptavalent Conjugate Vaccine Era', *The Journal of Infectious Diseases*, vol. 195, no. 3, pp. 347-52.

Hodgins, DC & Shewen, PE 2012, 'Vaccination of Neonates: Problem and issues', *Vaccine*, vol. 30, no. 9, pp. 1541-59.

Holmes, AR, McNab, R, Millsap, KW, Rohde, M, Hammerschmidt, S, Mawdsley, JL & Jenkinson, HF 2001, 'The pavA Gene Of *Streptococcus pneumoniae* Encodes A Fibronectin-Binding Protein That Is Essential For Virulence', *Molecular microbiology*, vol. 41, no. 6, pp. 1395-408.

Honda-Okubo, Y, Saade, F & Petrovsky, N 2012, 'Advax™, a polysaccharide adjuvant derived from delta inulin, provides improved influenza vaccine protection through broad-based enhancement of adaptive immune responses', *Vaccine*, vol. 30, no. 36, pp. 5373-81.

Huo, Z, Spencer, O, Miles, J, Johnson, J, Holliman, R, Sheldon, J & Riches, P 2004, 'Antibody Response To Pneumolysin And To Pneumococcal Capsular Polysaccharide In Healthy Individuals And *Streptococcus pneumoniae* Infected Patients', *Vaccine*, vol. 22, no. 9–10, pp. 1157-61.

Iyer, AS, Ohtola, JA & Westerink, MAJ 2015, 'Age-Related Immune Response To Pneumococcal Polysaccharide Vaccination: Lessons For The Clinic', *Expert Review of Vaccines*, vol. 14, no. 1, pp. 85-97.

Jackson, LA & Janoff, EN 2008, 'Pneumococcal Vaccination of Elderly Adults: New Paradigms for Protection', *Clinical Infectious Diseases*, vol. 47, no. 10, pp. 1328-38.

Jackson, LA, Holder, P, Lezhava, T, Williams, WW, Benson, P, Sneller, V-P, Butler, JC, Thompson, RS, Chen, RT, Lewis, LS, Carlone, G & DeStefano, F 1999, 'Safety Of Revaccination With Pneumococcal Polysaccharide Vaccine', *Journal of the American Medical Association*, vol. 281, no. 3, pp. 243-8.

Jaganathan, K & Vyas, SP 2006, 'Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant hepatitis B antigen administered intranasally', *Vaccine*, vol. 24, no. 19, pp. 4201-11.

Jarva, H, Janulczyk, R, Hellwage, J, Zipfel, PF, Bjorck, L, Meri, S, Department of Experimental Medical, S, Lunds, u, Medicin, Faculty of, M, Lund, U & Institutionen för experimentell medicinsk vetenskap, L 2002, '*Streptococcus pneumoniae* Evades Complement Attack and Opsonophagocytosis by Expressing the pspC Locus-Encoded Hic Protein That Binds to Short Consensus Repeats 8-11 of Factor H', *The Journal of Immunology*, vol. 168, no. 4, p. 1886.

Jedrzejas, MJ, Mello, LV, de Groot, BL & Li, S 2002, 'Mechanism Of Hyaluronan Degradation By *Streptococcus pneumoniae* Hyaluronate Lyase. Structures Of Complexes With The Substrate', *The Journal of Biological Chemistry*, vol. 277, no. 31, pp. 28287-97.

Jefferies, JM, Macdonald, E, Faust, SN & Clarke, SC 2011, '13-Valent Pneumococcal Conjugate Vaccine (PCV13)', *Hum Vaccin*, vol. 7, no. 10, pp. 1012-8.

Jensen, RG, Koch, A & Homøe, P 2013, 'The Risk Of Hearing Loss In A Population With A High Prevalence Of Chronic Suppurative Otitis Media', *Int J Pediatr Otorhinolaryngol*, vol. 77, no. 9, pp. 1530-5.

Jimenez, G, Alex, G, Paxton, G & Connell, TG 2013, 'B Alert: Hepatitis B Virus Infection In Children In Victoria', *Journal of paediatrics and child health*, vol. 49, no. 3, pp. E213-E6.

Johnson, HL, Deloria-Knoll, M, Levine, OS, Stoszek, SK, Freimanis Hance, L, Reithinger, R, Muenz, LR & O'Brien, KL 2010, 'Systematic Evaluation Of Serotypes Causing Invasive Pneumococcal Disease Among Children Under Five: The Pneumococcal Global Serotype Project', *PLoS Med*, vol. 7, no. 10.

Jomaa, M, Yuste, J, Paton, JC, Jones, C, Dougan, G & Brown, JS 2005, 'Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of *Streptococcus pneumoniae*', *Infection and immunity*, vol. 73, no. 10, pp. 6852-9.

Jomaa, M, Terry S Fau - Hale, C, Hale C Fau - Jones, C, Jones C Fau - Dougan, G, Dougan G Fau - Brown, J & Brown, J 2006, 'Immunization with the iron uptake ABC transporter proteins PiaA and PiuA prevents respiratory infection with *Streptococcus pneumoniae*', no. 0264-410X (Print), 2006 Jun 12.

Jonsson, S, Musher, DM, Chapman, A, Goree, A & Lawrence, EC 1985, 'Phagocytosis and Killing of Common Bacterial Pathogens of the Lung by Human Alveolar Macrophages', *Journal of Infectious Diseases*, vol. 152, no. 1, pp. 4-13.

Keating, G & Noble, S 2003, 'Recombinant Hepatitis B Vaccine (Engerix-B®)', *Drugs*, vol. 63, no. 10, pp. 1021-51.

Kieninger, DM, Kueper, K, Steul, K, Juergens, C, Ahlers, N, Baker, S, Jansen, KU, Devlin, C, Gruber, WC, Emini, EA, Scott, DA & study, g 2010, 'Safety, Tolerability, And Immunologic Noninferiority Of A 13-Valent Pneumococcal Conjugate Vaccine Compared To A 7-Valent Pneumococcal Conjugate Vaccine Given With Routine Pediatric Vaccinations In Germany', *Vaccine*, vol. 28, no. 25, pp. 4192-203.

King, SJ, Hippe, KR & Weiser, JN 2006, 'Deglycosylation Of Human Glycoconjugates By The Sequential Activities Of Exoglycosidases Expressed By *Streptococcus pneumoniae*', *Molecular microbiology*, vol. 59, no. 3, pp. 961-74, item: 16420364.

Kotiw, M, Johnson, M, Pandey, M, Fry, S, Hazell, SL, Netter, HJ, Good, MF & Olive, C 2012, 'Immunological response to parenteral vaccination with recombinant hepatitis B virus surface antigen virus-like particles expressing Helicobacter pylori KatA epitopes in a murine H. pylori challenge model', *Clinical and Vaccine Immunology*, vol. 19, no. 2, pp. 268-76.

Labout, JA 2008, 'Factors associated with pneumococcal carriage in healthy Dutch infants: the generation R study', *Journal of Pediatrics*, vol. 153, no. 6, pp. 771-6.e1.

Lai, CL, Yeoh, EK, Chang, WK, Lo, VWL & Ng, LNK 1986, 'Use Of The Hepatitis B Recombinant DNA Yeast Vaccine (H-B-VAX II) In Children: Two Doses Vs. Three Doses of 5 µg Regime; An Interim Report', *Journal of Infection*, vol. 13, Supplement A, no. 0, pp. 19-25.

Lavanchy, D 2005, 'Worldwide Epidemiology Of Hbv Infection, Disease Burden, And Vaccine Prevention', *Journal of Clinical Virology*, vol. 34, pp. S1-S3.

Lee, GM, Kleinman, K, Pelton, SI, Hanage, W, Huang, SS, Lakoma, M, Dutta-Linn, M, Croucher, NJ, Stevenson, A & Finkelstein, JA 2014, 'Impact Of 13-Valent Pneumococcal Conjugate Vaccination On *Streptococcus pneumoniae* Carriage In Young Children In Massachusetts', *Journal of the Pediatric Infectious Diseases Society*, vol. 3, no. 1, pp. 23-32.

Lieu, JEC 2013, 'Unilateral Hearing Loss In Children: Speech-Language And School Performance', *B-ENT*, vol. 1, no. 21, pp. 107-15, Scopus.

Mahadevan, M, Navarro-Locsin, G, Tan, HKK, Yamanaka, N, Sonsuwan, N, Wang, P-C, Dung, NTN, Restuti, RD, Hashim, SSM & Vijayasekaran, S 2012, 'A Review Of The Burden Of Disease Due To Otitis Media In The Asia-Pacific', *Int J Pediatr Otorhinolaryngol*, vol. 76, no. 5, pp. 623-35.

Malley, R & Anderson, PW 2012, 'Serotype-independent pneumococcal experimental vaccines that induce cellular as well as humoral immunity', *Proceedings of the National Academy of Sciences*, vol. 109, no. 10, pp. 3623-7.

Manders, E & Tyberghein, J 1984, 'The Possible Effects Of Conductive Hearing Loss On Language Development', *Acta Otorhinolaryngologica Belgica*, vol. 38, no. 3, pp. 255-60, Nlm, item: 6393690.

Manoff, S, McFetridge, R, Liss, C, Marchese, R, Rabb, J, Rueda, A & Musher, D 2010, 'Antibody Persistence 10 Years After 1st And 2nd Doses Of 23-Valent Pneumococcal Polysaccharide Vaccine (Pn23), And Immunogenicity And Safety Of 2nd And 3rd Doses In Older Adults', *International Journal of Infectious Diseases*, vol. 14, pp. e454-e.

Manoff, SB, Liss, C, Caulfield, MJ, Marchese, RD, Silber, J, Boslego, J, Romero-Steiner, S, Rajam, G, Glass, NE, Whitney, CG & Carlone, GM 2010, 'Revaccination with a 23-Valent Pneumococcal Polysaccharide Vaccine Induces Elevated and Persistent Functional Antibody Responses in Adults Aged ⩾65 Years', *Journal of Infectious Diseases*, vol. 201, no. 4, pp. 525-33.

Massa, HM, Cripps, AW & Lehmann, D 2009, 'Otitis Media: Viruses, Bacteria, Biofilms And Vaccines', *Medical Journal of Australia*, vol. 191, no. 9, p. S44.

Mehr, S & Wood, N 2012, '*Streptococcus pneumoniae* - A Review Of Carriage, Infection, Serotype Replacement And Vaccination', *Paediatric Respiratory Reviews*, vol. 13, no. 4, pp. 258-64.

Mitchell, AM & Mitchell, TJ 2010, '*Streptococcus* Pneumoniae: Virulence Factors And Variation', *Clinical Microbiology and Infection*, vol. 16, no. 5, pp. 411-8.

Moberley, SA, Holden, J, Tatham, DP & Andrews, RM 2008, 'Vaccines For Preventing Pneumococcal Infection In Adults', *Cochrane database of systematic reviews (Online)*, no. 1.

Monasta, L, Ronfani, L, Marchetti, F, Montico, M, Vecchi Brumatti, L, Bavcar, A, Grasso, D, Barbiero, C & Tamburlini, G 2012, 'Burden of Disease Caused by Otitis Media: Systematic Review and Global Estimates', *PLoS One*, vol. 7, no. 4.

Morris, P, Leach, A, Shah, P, Nelson, S, Anand, A, Daby, J, Allnutt, R, Bainbridge, D, Edwards, K & Patel, H 2011, 'Recommendations for Clinical Care Guidelines on the Management of Otitis Media in Aboriginal and Torres Strait Islander Populations (Updated 2010)', *Canberra: Commonwealth of Australia*.

Morris, PS & Leach, AJ 2009, 'Acute and Chronic Otitis Media', *Pediatric Clinics of North America*, vol. 56, no. 6, pp. 1383-99.

Morris, PS, Leach, AJ, Silberberg, P, Mellon, G, Wilson, C, Hamilton, E & Beissbarth, J 2005, 'Otitis media in young Aboriginal children from remote communities in Northern and Central Australia: a cross-sectional survey', *BMC pediatrics*, vol. 5, p. 27.

Musher, DM 1992, 'Infections Caused by *Streptococcus pneumoniae*: Clinical Spectrum, Pathogenesis, Immunity, and Treatment', *Clinical Infectious Diseases*, vol. 14, no. 4, pp. 801-7.

Musher, DM, Watson, DA & Dominguez, EA 1990, 'Pneumococcal Vaccination: Work to Date and Future Prospects', *The American Journal of the Medical Sciences*, vol. 300, no. 1, pp. 45-52.

Musher, DM, Phan, HM, Watson, DA & Baughn, RE 2000, 'Antibody to Capsular Polysaccharide of *Streptococcus pneumoniae* at the Time of Hospital Admission for Pneumococcal Pneumonia', *Journal of Infectious Diseases*, vol. 182, no. 1, pp. 158-67.

Musher, DM, Manoff, SB, Liss, C, McFetridge, RD, Marchese, RD, Bushnell, B, Alvarez, F, Painter, C, Blum, MD & Silber, JL 2010, 'Safety and Antibody Response, Including Antibody Persistence for 5 Years, after Primary Vaccination or Revaccination with Pneumococcal Polysaccharide Vaccine in Middle-Aged and Older Adults', *Journal of Infectious Diseases*, vol. 201, no. 4, pp. 516-24.

Nelson, AL, Roche, AM, Gould, JM, Chim, K, Ratner, AJ & Weiser, JN 2007, 'Capsule Enhances Pneumococcal Colonization by Limiting Mucus-Mediated Clearance', *Infection and immunity*, vol. 75, no. 1, pp. 83-90.

Newall, AT, Creighton, P, Philp, DJ, Wood, JG & MacIntyre, CR 2011, 'The Potential Cost-Effectiveness Of Infant Pneumococcal Vaccines In Australia', *Vaccine*, vol. 29, no. 45, pp. 8077-85.

NHMRC, *Guidelines to promote the wellbeing of animals used for scientific purposes: The assessment and alleviation of pain and distress in research animals,* 2008, Canberra, Australia.

Nyström, J, Cardell, K, Björnsdottir, TB, Fryden, A, Hultgren, C & Sällberg, M 2008, 'Improved cell mediated immune responses after successful re-vaccination of non-responders to the hepatitis B virus surface antigen (HBsAg) vaccine using the combined hepatitis A and B vaccine', *Vaccine*, vol. 26, no. 47, pp. 5967-72.

O'Brien, KL, Wolfson, LJ, Watt, JP, Henkle, E, Deloria-Knoll, M, McCall, N, Lee, E, Mulholland, K, Levine, OS & Cherian, T 2009, 'Burden Of Disease Caused By *Streptococcus pneumoniae* In Children Younger Than 5 Years: Global Estimates', *The Lancet*, vol. 374, no. 9693, pp. 893-902.

Paton, JC, Kadioglu, A, Andrew, PW & Weiser, JN 2008, 'The Role Of *Streptococcus pneumoniae* Virulence Factors In Host Respiratory Colonization And Disease', *Nature Reviews Microbiology*, vol. 6, no. 4, pp. 288-301.

Petrovsky, N 2006, 'Novel human polysaccharide adjuvants with dual Th1 and Th2 potentiating activity', *Vaccine*, vol. 24, pp. S26-S9.

Pichichero, ME 2009, 'Booster Vaccinations: Can Immunologic Memory Outpace Disease Pathogenesis?', *Pediatrics*, vol. 124, no. 6, pp. 1633-41.

Pilishvili, T, Lexau, C, Farley, MM, Hadler, J, Harrison, LH, Bennett, NM, Reingold, A, Thomas, A, Schaffner, W, Craig, AS, Smith, PJ, Beall, BW, Whitney, CG & Moore, MR 2010, 'Sustained Reductions in Invasive Pneumococcal Disease in the Era of Conjugate Vaccine', *Journal of Infectious Diseases*, vol. 201, no. 1, pp. 32-41.

Pletz, MW, Maus, U, Krug, N, Welte, T & Lode, H 2008, 'Pneumococcal Vaccines: Mechanism Of Action, Impact On Epidemiology And Adaption Of The Species', *International Journal of Antimicrobial Agents*, vol. 32, no. 3, pp. 199-206.

Polissi, A, Pontiggia, A, Feger, G, Altieri, M, Mottl, H, Ferrari, L & Simon, D 1998, 'Large-Scale Identification of Virulence Genes from *Streptococcus pneumoniae*', *Infection and immunity*, vol. 66, no. 12, pp. 5620-9.

Proctor, M & Manning, PJ 1990, 'Production Of Immunoglobulin A Protease by *Streptococcus pneumoniae* From Animals', *Infection and immunity*, vol. 58, no. 9, pp. 2733-7.

Quin, LR, Carmicle, S, Dave, S, Pangburn, MK, Evenhuis, JP & McDaniel, LS 2005, 'In Vivo Binding of Complement Regulator Factor H by *Streptococcus pneumoniae*', *The Journal of Infectious Diseases*, vol. 192, no. 11, pp. 1996-2003.

Qureishi, A, Lee, Y, Belfield, K, Birchall, JP & Daniel, M 2014, 'Update On Otitis Media - Prevention And Treatment', *Infection and Drug Resistance*, vol. 7, pp. 15-24.

Robbins, JB, Austrian, R, Lee, CJ, Rastogi, SC, Schiffman, G, Henrichsen, J, Mäkelä, PH, Broome, CV, Facklam, RR, Tiesjema, RH & Jr, JCP 1983, 'Considerations for Formulating the Second-Generation Pneumococcal Capsular Polysaccharide Vaccine with Emphasis on the Cross-Reactive Types within Groups', *The Journal of Infectious Diseases*, vol. 148, no. 6, pp. 1136-59.

Roberts, JE, Rosenfeld, RM & Zeisel, SA 2004, 'Otitis Media and Speech and Language: A Meta-analysis of Prospective Studies', *Pediatrics*, vol. 113, no. 3, pp. 238-48.

Rodrigo, C & Lim, WS 2014, 'The Relevance Of Pneumococcal Serotypes', *Curr Infect Dis Rep*, vol. 16, no. 4, p. 403.

Rovers, MM 2008, 'The Burden Of Otitis Media', *Vaccine*, vol. 26, no. 7, pp. 2-4.

Saade, F, Honda-Okubo, Y, Trec, S & Petrovsky, N 2013, 'A novel hepatitis B vaccine containing Advax™, a polysaccharide adjuvant derived from delta inulin, induces robust humoral and cellular immunity with minimal reactogenicity in preclinical testing', *Vaccine*, vol. 31, no. 15, pp. 1999-2007.

Sandgren, A, Sjöström, K, Liljequist, BO, Christensson, B, Samuelsson, A, Kronvall, G & Normark, BH 2004, 'Effect of Clonal and Serotype-Specific Properties on the Invasive Capacity of *Streptococcus pneumoniae*', *Journal of Infectious Diseases*, vol. 189, no. 5, pp. 785-96.

Sankilampi, U, Honkanen, PO, Bloigu, A & Leinonen, M 1997, 'Persistence of Antibodies to Pneumococcal Capsular Polysaccharide Vaccine in the Elderly', *The Journal of Infectious Diseases*, vol. 176, no. 4, pp. 1100-4.

Schirmbeck, R, Böhm, W & Reimann, J 1996, 'Virus-like particles induce MHC class I-restricted T-cell responses', *Intervirology*, vol. 39, no. 1-2, pp. 111-9.

Shapiro, ED, Berg, AT, Austrian, R, Schroeder, D, Parcells, V, Margolis, A, Adair, RK & Clemens, JD 1991, 'The Protective Efficacy of Polyvalent Pneumococcal Polysaccharide Vaccine', *New England Journal of Medicine*, vol. 325, no. 21, pp. 1453-60.

Simell, B, Auranen, K, Käyhty, H, Goldblatt, D, Dagan, R & O'Brien, KL 2012, 'The Fundamental Link Between Pneumococcal Carriage And Disease', *Expert Review of Vaccines*, vol. 11, no. 7, pp. 841-55, Scopus.

Sutcliffe, IC & Russell, R 1995, 'Lipoproteins of gram-positive bacteria', *Journal of bacteriology*, vol. 177, no. 5, p. 1123.

Syrjänen, RK, Kilpi, TM, Kaijalainen, TH, Herva, EE & Takala, AK 2001, 'Nasopharyngeal Carriage of *Streptococcus pneumoniae* in Finnish Children Younger than 2 Years Old', *The Journal of Infectious Diseases*, vol. 184, no. 4, pp. 451-9.

Tai, SS, Yu, C & Lee, JK 2003, 'A solute binding protein of *Streptococcus pneumoniae* iron transport', *FEMS microbiology letters*, vol. 220, no. 2, pp. 303-8.

Taylor, PS, Faeth, I, Marks, MK, Del Mar, CB, Skull, SA, Pezzullo, ML, Havyatt, SM & Coates, HL 2009, 'Cost Of Treating Otitis Media In Australia', *Expert Review of Pharmacoeconomics and Outcomes Research*, vol. 9, no. 2, pp. 133-41.

Törling, J, Hedlund, J, Konradsen, HB & Örtqvist, Å 2003, 'Revaccination With The 23-Valent Pneumococcal Polysaccharide Vaccine In Middle-Aged And Elderly Persons Previously Treated For Pneumonia', *Vaccine*, vol. 22, no. 1, pp. 96-103.

Tweten, RK 2005, 'Cholesterol-Dependent Cytolysins, a Family of Versatile Pore-Forming Toxins', *Infection and immunity*, vol. 73, no. 10, pp. 6199-209.

Van Damme, P & Van Herck, K 2004, 'A Review Of The Efficacy, Immunogenicity And Tolerability Of A Combined Hepatitis A And B Vaccine', *Expert Review of Vaccines*, vol. 3, no. 3, pp. 249-67.

van der Poll, T & Opal, SM 2009, 'Pathogenesis, Treatment, And Prevention Of Pneumococcal Pneumonia', *The Lancet*, vol. 374, no. 9700, pp. 1543-56.

Waites, KB, Canupp, KC, Chen, Y-y, DeVivo, MJ & Nahm, MH 2008, 'Revaccination of Adults With Spinal Cord Injury Using the 23-Valent Pneumococcal Polysaccharide Vaccine', *The Journal of Spinal Cord Medicine*, vol. 31, no. 1, pp. 53-9, PMC.

Weinberger, DM, Malley, R & Lipsitch, M 2011, 'Serotype Replacement In Disease After Pneumococcal Vaccination', *The Lancet*, vol. 378, no. 9807, pp. 1962-73.

Weiser, JN, Austrian, R, Sreenivasan, PK & Masure, HR 1994, 'Phase Variation In Pneumococcal Opacity: Relationship Between Colonial Morphology And Nasopharyngeal Colonization', *Infection and immunity*, vol. 62, no. 6, pp. 2582-9.

WHO 2012, 'Pneumococcal vaccines WHO position paper - 2012', *Weekly epidemiological record*, vol. 87, no. 14, pp. 129-44.

Wilson, R, Cohen, J, Jose, R, de Vogel, C, Baxendale, H & Brown, J 2014, 'Protection against *Streptococcus pneumoniae* lung infection after nasopharyngeal colonization requires both humoral and cellular immune responses', *Mucosal immunology*.

Yehudai, N, Most, T & Luntz, M 2015, 'Risk Factors For Sensorineural Hearing Loss In Pediatric Chronic Otitis Media', *Int J Pediatr Otorhinolaryngol*, vol. 79, no. 1, pp. 26-30.

Yildirim, I, Hanage, WP, Lipsitch, M, Shea, KM, Stevenson, A, Finkelstein, J, Huang, SS, Lee, GM, Kleinman, K & Pelton, SI 2010, 'Serotype Specific Invasive Capacity And Persistent Reduction In Invasive Pneumococcal Disease', *Vaccine*, vol. 29, no. 2, pp. 283-8.

Zangwill, KM, Vadheim, CM, Vannier, AM, Hemenway, LS, Greenberg, DP & Ward, JI 1996, 'Epidemiology of Invasive Pneumococcal Disease in Southern California: Implications for the Design and Conduct of a Pneumococcal Conjugate Vaccine Efficacy Trial', *The Journal of Infectious Diseases*, vol. 174, no. 4, pp. 752-9.