



**Department of Science and Technology/
National Research Foundation: Vaccine Preventable Diseases**

**Title: PRE-CLINICAL EVALUATION OF PNEUMOCOCCAL
VACCINE CANDIDATE MOLECULES IN A MOUSE
PNEUMOCOCCAL PNEUMONIA MODEL**

Kgomotso Welheminah Lebogo (336876)

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand,
Johannesburg, in fulfilment of the requirements for the degree of
Doctorate of Philosophy in Medical Microbiology.

DECLARATION

I, Kgomoitso Welheminah Lebogo, hereby declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy (Thesis) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



Signature of candidate

22-July-2016

DEDICATION

"What you still need to know is this: Before a dream is realized, the soul of the world tests everything that was learned along the way. It does this is not because it is evil, but so that we can, in addition to realizing our dreams, master the lessons we've learned as we've moved toward that dream. That's the point at which most people give up. It's the point at which, as we say in the language of the desert, one dies of thirst just when the palm trees have appeared on the horizon'. "Every search begins with beginner's luck. And every search ends with the victor being severely tested."

An excerpt from the Alchemist by Paul Coelho

This work is dedicated to my loving parents, John and the late Nokufa Margaret Lebogo.

Robala ka kagiso ngwetsi ya Lebogo, morwadie Mkhwanazi (1946-2011).

ABSTRACT

Background: *Streptococcus pneumoniae* (pneumococcus) is a major cause of morbidity and mortality in young children. Pneumococcal conjugate vaccines are restricted in their serotype coverage, and hence, focus has been directed towards common protein-based antigens. However, there have been problems associated with testing protein-based antigens in animal models. These include inhumane endpoints, use of large sample sizes, and the inability to detect subtle differences between antigens. This study describes a novel intranasal pneumonia co-inoculation model conducted in mouse and aims to address some of these limitations.

Methods: Eighty female NMRI mice were equally distributed into the vaccinated and placebo groups. The mice were subcutaneously inoculated with recombinant PspC or PpmA or adjuvant (control). Both groups were co-inoculated with 50 μ L of a bacterial suspension containing D39:: *rpsL* (serotype 2) or PJ351:: *rpsL* (serotype 1) wild-type strains and their isogenic knockout strains containing a trimethoprim resistance marker. Mice (n = 5 per group) were terminated at 0, 6, 12 and 24 hours post-inoculation. Bacterial samples were obtained from nasopharyngeal washings, bronchoalveolar lavage fluid (BAL fluid) and homogenised lung tissue. Bacterial counts were obtained from selective media containing either streptomycin or trimethoprim, and the ratios of wild-type and knockout were determined. Serum was collected by cardiac puncture and ELISA was used to monitor the levels of IgG antibodies. To further refine the model, a single time point (T = 24h) in which events were more pronounced was chosen for the rest of the experiments and the number of mice was scaled down to 20 mice per experimental procedure. A group of 20 mice (10 in placebo and 10 in vaccinated mice) were subcutaneously vaccinated with either recombinant PspA, IgA protease and SlrA or adjuvant (control). Both vaccinated and adjuvant groups were co-inoculated with 50 μ L of a bacterial suspension containing D39:: *rpsL* (serotype 2) or PJ351:: *rpsL* (serotype 1) wild-type strains and their isogenic knockout strains. Bacterial samples were collected from mice as described above.

Results: Pneumococcal knockouts of D39 and PJ351 lacking the genes encoding PpmA and PspC were attenuated in their ability to colonise the nasopharynx, BAL fluid and lungs of mice. Knockouts deficient in PspA, IgA bacterial protease and SlrA in the PJ351 background

were attenuated in all sites sampled, whereas knockouts in the D39 background were attenuated only in the nasopharynx. PspC IgG antibodies were able to elicit clearance of PspC producing wild-type D39 and PJ351 strains more effectively compared to PspC deletion knockouts, which were able to persist in the nasopharynx, BAL fluid and lungs. Responses in PpmA vaccinated mice were dependent on the genetic background of the inoculation strains. Clearance of D39 wild-type strains was more noticeable in the nasopharynx than in the BAL fluid and lungs, whereas the wild-type PJ351 was cleared more efficiently than the knockout in the BAL fluid and lungs, compared to the nasopharynx. Responses in IgA bacterial protease vaccinated mice were also dependent on the genetic background of the inoculation strains, with a clearance of the D39 wild-type strain being more noticeable in the nasopharynx. PspA and SlrA IgG antibodies were not protective against infection with both strains of the pneumococcus in the mouse model.

Conclusion: Using PspC, PpmA, PspA, IgA bacterial protease and SlrA as examples, we demonstrated that this mouse co-colonisation model could effectively detect subtle differences in the clearance of wild-type and knockout bacteria in the presence of antibodies against non-essential surface antigens. The model is humanely more acceptable, as it uses far fewer animals than conventional testing, with greater sensitivity. Further, it relies on measurements of bacterial density, rather than fatal disease as endpoints. The model also provides additional benefits in terms of monitoring the virulence characteristics of the target antigens.

ACKNOWLEDGEMENTS

I would like to thank the following people who helped me throughout my research:

To my supervisors, Dr P.V. Adrian and Prof. S. Madhi: **Dr Adrian**, thank you for your willingness to impart some of your knowledge to me. I especially appreciate the fatherly advice you gave me regarding everything in my life. I also appreciate the crash course you gave me for collecting samples from mice. Peter, you went above and beyond the duties of a supervisor and because of that I can safely say, I am a better scientist because of you. **Prof Madhi**, thank you for allowing me to embark on my studies at RMPRU, for your guidance and patience throughout the course of my studies. I appreciate the help you gave me with transport to the Animal Ethics Unit.

To Dr Marta Nunes: I couldn't have constructed knockouts and cloned them without your help. You held my hand and worked me through each detail of cloning. I appreciate your help, immensely.

To Dr Z.S. Mlacha-Kimaro: Thank you for helping with editing and tackling of this thesis into the shape, it is in now. I also appreciate the late nights you spent helping me with my animal experiments.

The Wits Postgraduate Merit Award and NRF/DST South African Research Chair Initiative: Vaccine Preventable Diseases: This research would not have been possible without your funding. Thank you for paying tuition fees and for funding this research.

The staff at the Wits Central Animal Services: Mrs Mary-ann Costello, Mr Patrick Selahle, Ms Lorraine, Ms Amelia, Mrs Khumo Mathikge: Thank you all for imparting your knowledge of handling animals and immunising them. Lorraine, I especially appreciate the sacrifice you took of assisting me at odd hours of the night.

A special thanks to Ms Palesa Morailane for assisting me with animal experiments. I know you are squeamish Mbali, but you bore through it all for me.

To my fellow colleagues and dear friends: Gaurav Kwatra and Dr Mashudu Madzivahandila: Thank you for your understanding and honesty all throughout the years.

Omphile Simani (aka bazala, aka mpintji), thank you for being the sound of reason throughout this whole research. Thank you again for helping me with analysing my ELISA data and being my transport at odd times of the night.

To my family: Parents John and the late Nokufa Lebogo and siblings Mpho Lebogo, Tebogo Lebogo, Mamatea Lebogo and Mamokete Lebogo for their understanding, patience, financial support and for never giving up on me, no matter the urge to do so.

To my husband Ananias Machuence Poopedi: Thank you for being my rock and shelter in the time of need.

Last but not least thank you to God, for His Love without which I would never have made it.

ABBREVIATIONS

ABC	ATP-binding cassette
AOM	Acute otitis media
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AlPO ₄	Aluminium phosphate
BBB	Blood brain barrier
CAS	Central Animal Services
CO ₂	Carbon dioxide
CBPs	Choline binding proteins
CbpA, D, E, G	Choline binding protein A, D, E, G
CD-1 mice	Cesarean Derived 1
CFU	Colony forming unit
Ci	Confidence interval
CI	Competitive index
CTAB	Hexadecyltrimethyl-ammonium bromide
CV	Coefficient of variation
C57BL/6	C57 black 6
DNA	Deoxyribonucleic acid
Dfr	Dihydrofolate reductase
EDTA	Ethylenediaminetetraacetate-2H ₂ O
ELISA	Enzyme-Linked ImmunoSorbent assay
<i>E.coli</i>	<i>Escherichia coli</i>
Erm	Erythromycin
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GEM	Gram-positive Enhancer Matrix
HIV	Human-Immuno Deficiency Virus
His	Histidine
Hic	Factor H-binding inhibitor of complement
IPTG	Isopropyl-thio-[beta]-D-galactoside
IPD	Invasive pneumococcal disease

MOPS	3-Morpholinopropane sulfonic acid
NMRI	Naval medical research institute
ICAM-1	Intercellular Adhesion Molecule 1
IgA1	Immunoglobulin A1 protease
IPD	Invasive Pneumococcal Disease
LM	Phospholipid membrane
LB	Luria Bertani
LTA	Lipoteichoic acids
LytA	Autolysin (N-acetyl muralmoyl-L-alanine amidase)
LytB	Endo-beta-N-acetylglucosaminidase
LytC	1, 4-beta-N-acetylmuramidase
NanA	Neuraminidase A
NICD	National Institute of Communicable Disease
OPA	Opsonophagocytosis assay
PAF	Platelet activating factor receptor
PBS	Phosphate Buffered Saline
PCV	Pneumococcal conjugate vaccine
PCR	Polymerase chain reaction
PG	Peptidoglycan
PavA	Pneumococcal adherence and virulence factor A
PavB	Pneumococcal adherence and virulence factor B
PcpA	Pneumococcal choline binding protein
PcsB	Protein required for cell wall separation of group B streptococcus
6PGD	6-phosphogluconate dehydrogenase
PGK	Phosphoglycerate kinase
PhtA, B, D, E	Pneumococcal histidine triad protein A, B, D, E precursor(s)
PiaA	Pneumococcal iron acquisition
PiuA	Pneumococcal iron uptake
pIgR	Polymeric Ig receptor
Ply	Pneumolysin
PpmA	Putative proteinase maturation protein A
PrtA	Cell wall associated serine proteinase precursor

PspA	Pneumococcal surface protein A
PsaA	Pneumococcal surface antigen A
PCV	Pneumococcal conjugate vaccine
RSV	Respiratory syncytial virus
RMPRU	Respiratory meningeal and pathogens research unit
SlrA	Streptococcal lipoprotein rotamase A
SDS	Sodium dodecyl sulphate
SrtA	Sortase A
StkP	Serine Threonine kinase protein
<i>S. pneumoniae</i>	<i>Streptococcus pneumoniae</i>
TA	Teichoic acid
THY	Todd Hewitt Yeast
Trm	Trimethoprim
SpsA	Surface protein with specific binding to secretory immunoglobulin A and secretory component
SpxB	Pyruvate oxidase
URI	Upper respiratory tract

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CHAPTER 1 INTRODUCTION

1.1 Preface

Invasive pneumococcal disease (IPD) remains the leading cause of mortality among children <5 years of age and in the elderly >65 years. It was estimated that around 935,000 children under 5 years of age died in 2013 from pneumococcal infections (WHO Pneumonia fact sheet 2014). This situation is exacerbated by the emergence of antibiotic-resistant strains of *Streptococcus pneumoniae*, particularly strains resistant to penicillin. This has encouraged the need for the development of other effective means of combating pneumococcal disease.

1.2 Cell structure and virulence factors involved in the pathogenesis of *S. pneumoniae*

The outer surface of the pneumococcus comprises of the cell wall, surface proteins and polysaccharide capsule (as shown in Figure 1.1). The pneumococcal cell wall consists of a peptidoglycan (PG), teichoic acid (TA) and lipoteichoic acids (LTA). Both teichoic acids have identical chain structures but differ in their positioning on the cell wall. Teichoic acid is covalently linked to the peptidoglycan layer of the pneumococcal cell wall whereas LTA is attached to the cytoplasmic membrane by a lipid (Neuhaus & Baddiley 2003). TA and LTA contain phosphorylcholine, which promotes adherence and invasion of the pneumococcus by forming a complex with G-protein-coupled platelet activating factor receptor (PAF receptor) (Cundell *et al.* 1995). Both teichoic acids also serve as an anchor for surface-located choline binding proteins. The pneumococcus also has over 100 surface proteins. Pneumococcal proteins are responsible for the virulence of the pneumococcus by assisting in establishing colonisation and causing invasive disease.

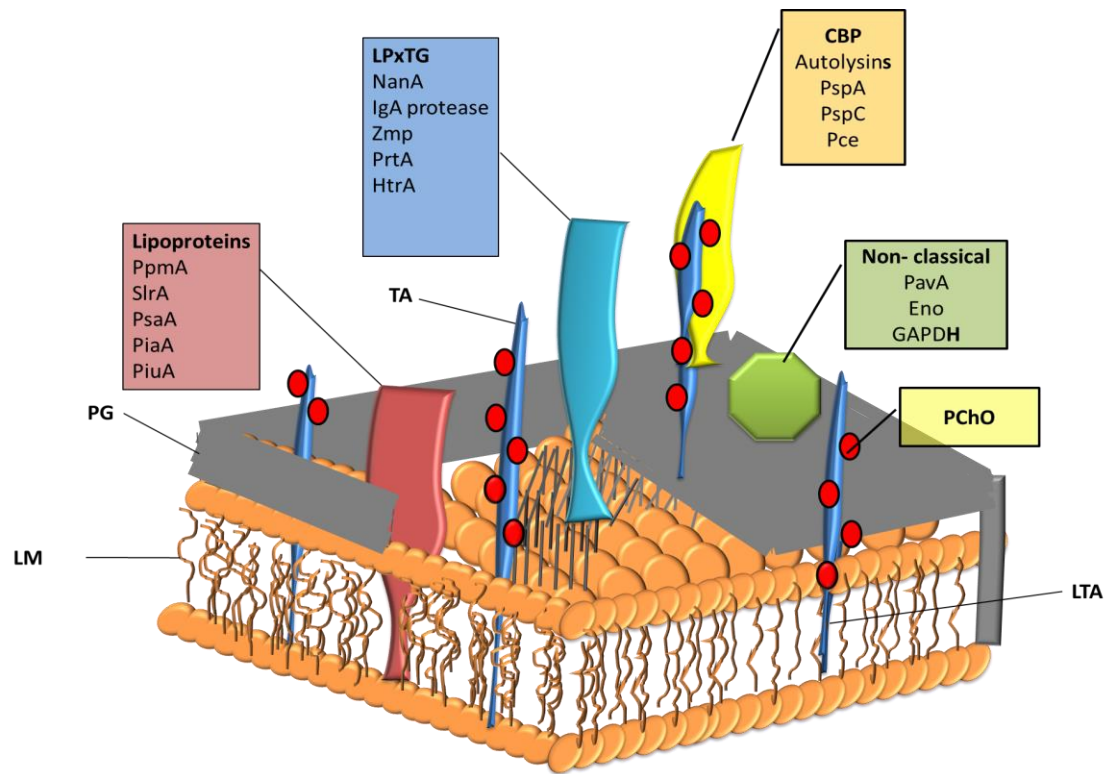


Figure 1.1 Diagram showing pneumococcal cell wall and surface exposed proteins.
 Figure is adapted from (Bergmann & Hammerschmidt 2006).

Covering the external surface of the pneumococcus is an extracellular sugar coating layer mainly composed of high molecular weight polysaccharides (Buttery *et al.* 2002). The capsule was the first virulence factor to be identified in the pneumococcus (Griffith 1928; Avery & Goebel 1931). Early experiments showed that knockout strains lacking the capsule were avirulent in mice compared to the parental strain. At least 93 different capsular types (serotypes) have been identified (Bratcher *et al.* 2011; Park *et al.* 2007; Calix & Nahm 2010). They differ in their chemical structure and are distinguished antigenically by their capsular polysaccharide composition.

The ability of the pneumococcus to regulate the amount of capsule production is important to its survival in the host environment. Depending on its location in the host, the pneumococcus can preferentially switch between a transparent phase and opaque phase. Colonies in the opaque phase have a thick capsule, and are more likely to cause systemic infection. The thick capsule assists the invasive strains to evade opsonophagocytosis. Organisms that express the transparent phenotype are predominantly found in the nasopharynx wherein a thin capsule is produced and the strains are less subjected to opsonophagocytosis than in the blood stream or lower respiratory tract. Transparent organisms have been shown to express higher amounts of teichoic acids, PspC and LytA compared to opaque organisms (Kim & Weiser 1998). These structures are thought to enhance the ability of the pneumococcus to adhere to the epidermal surfaces of the nasopharynx. The role of surface proteins on pneumococcal pathogenesis is discussed in detail in section 1.5.

The requirement of capsule during colonisation is less defined. By employing knockout strains with decreased ability to synthesise capsule, Yother and Magee were able to demonstrate the inefficiency of knockout strains to colonise the nasopharynx of adult mice (Yother & Magee 2001). This was corroborated by Cron *et al.*, who also noticed a reduced ability from one strain that produced low levels of capsule to successfully colonise the nasopharynx of adult CD1 mice (Cron *et al.* 2011). The ability of the pneumococci to establish colonisation was also shown to be related to the number of carbon repeating units in the capsule. The fewer carbons per repeat unit, the heavier encapsulated the serotype and

more likely to persist in the nasopharynx of mice (Weinberger *et al.* 2009). Nelson *et al.*, however, revealed that the capsule inhibited the agglutination of strains to the luminal mucosal layer, ensuring successful colonisation of the epithelial layer (Nelson *et al.* 2007). In fact, the heavy capsule could prevent adhesins such as PspC, SpxB and NanA from gaining access to the epithelial surface, where successful colonisation is established.

What is clear from these studies is that the capsule, to some extent is involved in colonisation by the pneumococcus. The question of whether less or more production of the capsule is enough in order for colonisation to be established remains uncertain. The crucial factor is for the pneumococcus to control the production of the capsule to ensure successful colonisation, without triggering an inflammatory response.

1.3 PROTEIN VIRULENCE FACTORS

1.3.1 Choline binding proteins (CBPs)

Choline binding proteins are anchored to the pneumococcal surface via non-covalent interactions with phosphorylcholine moieties on cell wall teichoic acid and membrane lipoteichoic acid (As shown in Figure 1.1). The examination of the *S. pneumoniae* genome sequences identified 6 and 15 CBPs in the R6 and TIGR4 strains, respectively (Pérez-Dorado *et al.* 2012). The most studied proteins in this family are PspC (Pneumococcal surface protein C) and PspA (Pneumococcal surface protein A) (Yother & White 1994). These ones are also the most highly developed as potential vaccine antigens (See Sections 1.6.2.1 and 1.6.2.2). Other proteins that associate with the cell surface by binding choline include CbpG, CbpD, PcpA and Hydrolases (Gosink *et al.* 2000).

1.3.1.1 Pneumococcal surface protein C (PspC)/Choline binding protein (CbpA)/SpsA)

This surface protein which exists in different allelic forms referred as SpsA, CbpA, PbcA and Hic, is the paralog of PspA because of their similar structural domains involved in cell wall adhesion. PspC proteins are heterogenous in nature, and they are not always recognised to belong to the same group. As such, they can be classified into 11 groups, with PspCs from groups 1-6 attached to the bacterial surface by a phosphocholine moiety. The

remaining groups termed PspC-like proteins are anchored to the bacterial surface via an LPXTG motif (Iannelli *et al.* 2002). PspC is an adhesin that plays a vital role in aiding the pneumococcus in the establishment of nasopharyngeal colonisation. PspC can bind human complement factor H, which then promotes entry of the pneumococcus into epithelial cells through an integrin-mediated pathway. Furthermore, it can bind IgA secretory fragment that assists it to cross the mucosal epithelium thus establishing colonisation (Hammerschmidt *et al.* 1997; Agarwal *et al.* 2010; Zhang *et al.* 2000). In a mouse model of colonisation, PspC was attenuated in its ability to colonise the nasopharynx of mice compared to its isogenic wild-type strain (Balachandran *et al.* 2002).

On the other hand, there are conflicting results regarding the role of PspC in pneumonia. A serotype 19F strain that lacked a *pspC* gene was attenuated in a CBA/N pneumonia mouse model (Balachandran *et al.* 2002). Using an MF1 fatal pneumonia mouse model, strain specificity in virulence of PspC was demonstrated. Serotypes 2, 3 and 19 deficient in PspC behaved similar to their isogenic wild-type strains, whereas a *pspC* knockout in a serotype 4 strain was easily cleared from the system (Kerr *et al.* 2006). A *pspC* knockout in a D39 background was not attenuated in an intraperitoneal CBA/N fatal pneumonia mouse model (Ogunniyi *et al.* 2007).

There are also some conflicting data on the role of PspC in bacteraemia. *In vitro* studies demonstrate that PspC avoids phagocytosis by recruiting functionally active factor H with the Hic surface protein, therefore ensuring its survival in invasive disease (Jarva *et al.* 2004). A *pspC* knockout in a D39 background using BALB/c intraperitoneal model of septicaemia was however not attenuated in its ability to survive in the bloodstream of mice (Rosenow *et al.* 1997). In the BALB/c intravenous bacteraemia model, a *pspC* knockout in a serotype 19F strain background showed an increased ability to grow in the bloodstream of mice (Balachandran *et al.* 2002). These *in vivo* results suggested that PspC does not contribute to the development of septicaemia. Other studies were, however, able to demonstrate the role of PspC in sepsis. The survival time of CBA/Jico and MF1 mice intravenously infected with D39 *pspC*⁻ and A66 *pspC*⁻ knockout strains was significantly delayed (Iannelli *et al.* 2004). This indicated the contribution of PspC in the development of septicaemia.

These studies show that the choice of pneumococcal strain affected the outcome of results in pneumonia and bacteraemia models. The role of PspC in bacteraemia was only demonstrated in mouse models where the intravenous route was used, with the exception of BALB/c mice. This indicates that in addition to the choice of the mouse strain, the route of infection also affected the results in the bacteraemia model. Although the CBA/Jico and MF1 intravenous septicaemia models gave successful results, several studies have shown that pneumococci introduced via the intravenous route are relatively avirulent. A theoretical disadvantage of the intravenous route could be that the smaller inoculum is unable to adapt with respect to phase variation/capsule expression, which enables it to persist in the blood and is thus easily cleared from the mouse system. The disparity in studies showing the role of PspC on bacteraemia makes it difficult to link its role in bacteraemia.

1.3.1.2 Pneumococcal surface protein A (PspA)

Pneumococcal surface protein A is the most extensively studied of the CBPs. PspA N-terminal domain is a highly charged α -helical region, with a coiled-coil structure that extends from the cell wall and protrudes outside of the capsule (Jedrzejewski 2006). Based on the sequence variability of the N-terminal region, PspA proteins have been classified into six classes that can be grouped into 3 families (family 1- clades 1+2, family 2- clades 3, 4+5 and family 3- clade 6) with 95% of isolates belonging to families 1 (clades 1 and 2) or 2 (clades 3, 4 and 5) (Vela Coral *et al.* 2001). Its role is to protect the pneumococcus from host immune defences by interfering with complement fixation. PspA prevents activation of C3b by interfering with complement factor B (Tu *et al.* 1999). Recently a novel mechanism of complement inhibition by PspA which involves phosphocholine (PC) has been described (Gang *et al.* 2012). PspA binds to PC and inhibits the interaction of Complement Reactive Protein (CRP) with PC and, therefore, curbing the classical pathway that leads to the opsonophagocytosis of the pneumococcus. PspA may also promote colonisation of host mucosa by binding lactoferrin and preventing killing by apolactoferrin (Shaper *et al.* 2004).

In vivo studies also confirm the contribution of PspA on pathogenesis of the pneumococcus. A knockout deficient in *pspA* in a D39 background significantly delayed the median survival time of CBA/N (McDaniel *et al.* 1987). Similarly, a serotype 3 *pspA*

knockout was cleared from the bloodstream of CBA/N within 6 hours of infection (Tu *et al.* 1999). A *pspA*⁻ knockout in the D39, WU2 and DBL5 strains extended the survival time of CBA/N mice compared to *pspA*⁺ wild-type strains (Briles *et al.* 1988). Contrary to other published data, virulence of D39 *pspA* knockout strains was similar to that of isogenic wild-type strain in BALB/c mice (Berry & Paton 2000). There are conflicting reports on the *in vivo* role of PspA in colonisation. A strain deficient in *pspA* colonised the nasopharynx of BALB/c and CBA/N mice the same way as the isogenic D39 wild-type strain (Balachandran *et al.* 2002; Orihuela *et al.* 2004). In another study, however, a *pspA* knockout in a D39 background was attenuated in its ability to colonise the nasopharynx of CD1 mice (Ogunniyi *et al.* 2007).

In all these studies in which inbred BALB/c mice were used, the role of PspA on invasive disease and colonisation was not successfully demonstrated. CBA/N mice were appropriate in demonstrating the role of PspA on invasive disease but not colonisation. The strain of pneumococcus did not seem to affect the outcome of the results, as D39 was the most frequently used strain in these studies. Taken together, these results indicate that the choice of mouse strain has a great impact on the outcome of virulence studies.

1.3.2 Lipoproteins

A second family of surface proteins are lipoproteins that contain a lipobox motif LxxC near the N terminus that is cleaved and covalently attached to the palmitic acid in the outer surface of the plasma membrane. Lipoproteins serve a number of important roles such as substrate-binding components of ATP-binding cassette (ABC) transport systems (Khandavilli *et al.* 2008). ABC transporters contribute to many bacterial processes such as acquisition of vital nutrients, stress responses and intracellular signalling, which are vital for bacterial growth and survival *in vivo* and *ex vivo*. Of the 50 lipoproteins, 15 are non-ABC transporters of which their function is not well known (Miyaji *et al.* 2013). Pneumococcal surface antigen A (PsaA) is the most studied protein under ABC transporters and it is one of the candidate protein based pneumococcal vaccine epitopes (Talkington *et al.* 1996). PsaA is a 37-KDa highly conserved protein produced by all pneumococcal strains. PsaA shares homology with the family of metal binding lipoproteins which are involved in manganese

and zinc transport. Initially the role of PsaA as an adhesin was deduced from its clonal relationship with members of this protein family designated, lipoprotein receptor-associated antigen I (LraI). These proteins promote adhesion of the streptococcus genus to oral cavities or mucosal surfaces of the respiratory tract. The class includes fimbrial adhesin (FimA) from *Streptococcus parasanguis*, streptococcal saliva-binding protein (SsaB) from *Streptococcus sanguis*, streptococcal coaggregation adhesin (ScaA) from *Streptococcus gordonii*, and of course, PsaA from *S. pneumoniae* (Burnette-Curley *et al.* 1995; Kolenbrander *et al.* 1998). Work done by Berry and Paton was the first of its kind to demonstrate PsaA as an adhesin. The authors demonstrated the inability of PsaA deficient knockout strains to adhere to pneumocytes (Berry & Paton 1996). Romero-Steiner *et al* expanded on the role of PsaA as an adhesin. In their studies, they demonstrated that PsaA knockout strains were attenuated in their ability to adhere to nasopharyngeal cells. (Romero-Steiner *et al.* 2003). Although there was some doubt as to whether PsaA was a direct adhesin or not (Russell *et al.* 1990), work done by Anderton *et al* showed that PsaA adhered to nasopharyngeal cells through interaction with the human cellular receptor, E-cadherin (Anderton *et al.* 2007). The *in vivo* role of PsaA on pneumococcal pathogenesis was demonstrated by the inability of *psaA* null knockout strains to establish colonisation, cause systemic infection or otitis media (Marra *et al.* 2002; Novak *et al.* 1998). This could be attributed to the hypersensitivity of PsaA knockouts to oxidative stress and impaired growth in Mn_2^+ (McAllister *et al.* 2004). Other proteins studied in this class are the iron uptake lipoproteins PiuA and PiaA. These proteins are highly conserved among the pneumococcal strains and are immunologically cross-reactive. Immunization with a combination of PiuA and PiaA provides a higher degree of protection against systemic disease than with PiuA alone (Brown *et al.* 2001).

Under the latter class of lipoproteins, non-ABC transporters, there is Putative proteinase maturation protein A (PpmA) and Streptococcal lipoprotein rotamase A (SlrA). Although they are known to be immunogenic, their potential as vaccine candidates needs to be investigated (Bergmann & Hammerschmidt 2006). Peptidyl-prolyl cis/trans isomerases (PPIases) are universal enzymes that accelerate the process of protein folding by reducing the energy of cis-trans conformational changes of peptidyl proline bonds. PPIases can be classified into three groups: i) Cyclophilins, which have a high binding affinity to

immunosuppressant cyclosporin A ii) Parvulins, and iii) FK506-binding protein, which have a high affinity for the immunosuppressant drug FK506. *S. pneumoniae* expresses two conserved lipoproteins attached to the surface of the pneumococcus that share homology with two families of PPIases namely: the putative maturation proteinase A (PpmA) and streptococcal lipoprotein rotamase A (SlrA) (Hermans *et al.* 2006; Overweg *et al.* 2000). PpmA is a 35 Kda protein which shares identity with the parvulin class; however, PPIase activity could not be demonstrated. Studies by Overweg *et al* demonstrated the contribution of PpmA to pathogenicity of the pneumococcus in a serotype 2 mouse model of pneumonia (Overweg *et al.* 2000). The median survival time of mice infected with a PpmA null strain was significantly longer compared to the mice infected with the wild-type strain. A knockout deficient in PpmA was also shown to be mutated in its ability to colonise the nasopharynx of mice, elucidating the role of ppmA in colonisation (Cron *et al.* 2009). SlrA belongs to a class of cyclophilins that has been demonstrated to possess PPIase activity, and presumably is involved in the correct folding of proteins on the cell surface. D39 *slrA* null knockout strains were attenuated in their ability to colonise the nasopharynx and BAL fluid of mice. It does not, however, have a role in invasive disease as both the knockout and its isogenic wild-type strain were equal in their ability to infect lungs and bloodstream of mice (Hermans *et al.* 2006). To date, the exact role in virulence of both these proteins is unclear; however, their contribution to colonisation in mouse models of disease has been well documented.

1.3.3 Sortase dependent proteins (LPXTG-anchored proteins)

Sortase dependent proteins are an unrelated family of proteins that are characterised by a recognition signal that consists of the motif LPXTG (Leu-Pro-any-Thr-Gly), followed by a highly hydrophobic transmembrane sequence, then a cluster of basic residues such as arginine. Cleavage occurs between the Thr and Gly, with transient attachment through the Thr residue to the active site Cys residue, followed by transpeptidation that attaches the protein covalently to the cell wall. Within this heterogeneous class of proteins, there are proteases; HtrA (high-temperature requirement A) and PrtA (serine protease). There are zinc metalloproteases; immunoglobulin A1 (IgA1), zinc metalloproteases (ZmpB, ZmpC and ZmpD). There is also adhesins, pneumococcal serine-rich protein (PsrP) and Rlr pilus

(consisting of three protein subunits RrgA, RrgB and RrgC) and three sialidases; neuraminidases (NanA, NanB) and beta-galactosidase (BagA) (Pérez-Dorado *et al.* 2012).

1.3.3.1 IgA1 bacterial protease

IgA1 bacterial protease is one of the most extensively studied proteins in this group. IgA1 bacterial protease specifically cleaves IgA1, but not IgA2, at its hinge region releasing the Fc α effector domain from the antigen-binding Fab fragments. These α effector domains are able to adhere to host epithelial cells *in vitro* and inhibit phagocytic killing of pneumococci *in vitro* and *in vivo* (Weiser *et al.* 2003). Moreover, surface-bound specific IgA1-Fab fragments might shield pneumococci from intact antibody molecules and complement proteins, thus maintaining the survival of *S. pneumoniae* within the host (Kilian *et al.* 1988). One of the challenges with working with IgA1 bacterial protease is that the specificity of IgA1 cleavage is limited to human host IgA1, and thus there are no suitable animal models which one can use (Reinholdt & Kilian 1991). Despite this, animal experiments have indicated that it is an important virulence factor as it contributes to some stages of pathogenesis. In mouse models of pneumonia and colonisation, strains deficient in IgA1 bacterial protease were attenuated in their ability to cause infection and establish colonisation (Chiavolini *et al.* 2003; Hava & Camilli 2002). Other studies report that strains deficient in *iga* are attenuated in a septicaemia mouse model (Polissi *et al.* 1998). However, these results are not consistent with its suggested role as an adhesin that promotes colonisation. There is data that suggests that IgA bacterial protease is not a virulence factor as it does not contribute to any stage of pathogenesis (Lau *et al.* 2001). As in other studies, the choice of mouse model appeared to be a critical factor determining the outcome.

1.3.3.2 Neuraminidase A (NanA)

NanA is a well characterised member of the sialidases and the only one with neuraminidase activity. Although its exact role in pathogenesis is not well known, increased NanA expression is associated with colonies in the transparent phase which are associated with nasopharyngeal colonisation (King *et al.* 2004). This is supported by the inability of *nanA* deficient strains to adhere to nasopharyngeal and epithelial cells (Brittan *et al.* 2012).

There are studies that also demonstrate the requirement of *nanA* on long term nasopharyngeal carriage and persistent growth in the middle ear. This was evident by the effective clearance of strains deficient in the *nanA* gene from the nasopharynx of chinchillas two weeks earlier compared to their D39 parent strain. Similarly, the isogenic D39 strain persisted in the middle ear of chinchillas for approximately 20 days, compared to the knockout strain which was cleared in 10 days (Tong *et al.* 2000). NanA thus contributes to the formation of otitis media by revealing receptors on the host cells such as lactoferrin and IgA2, which are important for adherence (King *et al.* 2004). This protein was also essential in biofilm formation (Trappetti *et al.* 2009); with an indication that *nanA* knockout strains had a reduced capacity to form biofilms during *in vitro* biofilm experiments (Parker *et al.* 2009). The involvement of NanA in formation of biofilms thus contributes to prolonged colonisation by the pneumococcus.

1.3.4 Other virulence factors

There are proteins which are located in the cytoplasm, i.e. pneumolysin; and those that are surface exposed but lack classic leader signal peptides and membrane-anchoring motifs, i.e. Serine-threonine kinase P (StkP), pneumococcal cell surface protein A (PavA), pneumococcal cell surface protein B (PavB), Enolase, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), 6-phosphogluconate dehydrogenase (6PGD) and Phosphoglycerate kinase (PGK). These proteins contribute to the virulence of the pneumococcus by promoting systemic infection and colonisation by the pneumococcus. PavA and PavB mediate adherence and invasion of the pneumococcus into epithelial cells through their interaction with host proteins, fibronectin and plasminogen (Jensch *et al.* 2010; Kadioglu *et al.* 2010). Proteins of interest in this group are pneumolysin, which is one of the first proteins to be considered as a potential pneumococcal vaccine; and serine threonine kinase (StkP) which is one of the novel types of pneumococcal vaccine antigens discovered using genomic scale antigenic fingerprinting. Below is a brief description of these proteins.

1.3.4.1 Pneumolysin

Pneumolysin is a thiol-activated haemolysin which is located in the cytoplasm and released when cells undergo autolysis. Pneumolysin uses cholesterol as a receptor for binding to the host's cell membrane, followed by formation of oligomeric transmembrane pores causing lysis. *In vitro* results demonstrated the importance of Ply in colonisation by the pneumococcus by inhibiting the ciliary beat of the human respiratory epithelium (Steinfort *et al.* 1989), therefore allowing it to adhere to the epithelial cells where successful colonisation could be established. In addition, the toxin is also important for disrupting the tight junctions between epithelial cells and allowing a portal of entry for pneumococcal invasion (Thornton & McDaniel 2005). Data from the same study by Thornton & McDaniel showed that *ply* induces transcription of host ICAM-1, an epithelial cell adhesion molecule that plays a role in pneumococcal adherence. In a mouse model of systemic infection, *ply*⁻ knockout strains survived longer than mice infected with wild-type isogenic strains (Berry & Paton 2000), therefore demonstrating the requirement of Ply for survival in the bloodstream. Ply deficient knockout strains were also attenuated in their ability to multiply in lungs of mice compared to their isogenic wild-type strains (Kadioglu *et al.* 2000). Work done by Ogunniyi *et al.*, revealed the requirement of pneumolysin in establishing colonisation, but not for prolonging it (Ogunniyi *et al.* 2007). This was evident by the low bacterial recovery of D39 Ply deficient strains from CBA/N mice at days 1 and 2 of the seven days sampled. On the other hand Richards *et al* successfully indicated that this protein was required for persistent colonisation for at least 14 days in MF1 mice (Richards *et al.* 2010). In two separate studies, however, pneumolysin knockouts in serotype 2 and 14 backgrounds behaved similarly to their wild-type counterparts (Kadioglu *et al.* 2002; Rubins *et al.* 1998).

In general, Ply is required for the development of pneumococcal pneumonia and survival of the pneumococcus in the blood. Although there are results negating the role of Ply on the early stages of disease, there is convincing data indicating its importance in establishing and ensuring prolonged colonisation by the pneumococcus in the nasopharynx. Thus, Ply is important in multiple stages of pathogenesis, colonisation, pneumococcal pneumonia and septicaemia.

1.3.4.2 Serine-threonine kinase P (*StkP*)

StkP has a key role in the regulation and in maintaining the physiology of the pneumococcus. The absence of the *stkP* gene makes the pneumococcus less competent in genetic transformation and more prone to extracellular stress. In addition, it leads to disruption of pneumococcal cell division that translates to slow growth of the pneumococcus *in vitro* (Nováková *et al.* 2010; Giefing *et al.* 2008; Fleurie *et al.* 2012). The role of *stkP* in virulence was demonstrated by the inability of knockout strains to infect the lungs and grow in the bloodstream of mice (Echenique *et al.* 2004).

1.4 NASOPHARYNGEAL COLONISATION

S. pneumoniae is spread from person-to-person by respiratory droplets, meaning that transmission to others generally occurs during coughing or sneezing. Colonisation is transient, dynamic, and most children experience multiple episodes of colonisation by the age of two. Acquisition of the pneumococcus is normally at an earlier age for children in developing countries compared to more developed countries (B M Gray *et al.* 1980; Kwambana *et al.* 2011). The risk of acquiring the pneumococcus is exacerbated by factors such as having siblings less than 10 years of age, overcrowding at homes and attending day care (Kinabo *et al.* 2013). Prevalence of colonisation is strongly associated with age and geographical location. In Gambia, over 80% of babies in the 3rd month are colonised with the pneumococcus and remain colonised as such up until 11 months (Hill *et al.* 2008). This is in contrast to the 9% of Finnish infants who become colonised when they are 2 months old, increasing to 43% by 24 months of age (Syrjanen *et al.* 2001). Whilst the prevalence of nasopharyngeal colonisation declines to 10% and generally remains at that level through adulthood in developed countries (Goldblatt *et al.* 2005; Gray & Dillon 1988), a high prevalence of colonisation may persist in African settings among children (97%) and in adults (85%) in low-income settings (Hill *et al.* 2010).

The initial step in the establishment of pneumococcal infection is adherence of the bacteria to the epithelial cells (see Figure 1.2). The host mucosal system which is greatly glycosylated mainly with glycan structures is the first barrier of protection against bacterial

infection. In order to ensure successful colonisation of the nasopharynx, the pneumococcus must break down the mucosal barrier and evade the host immune responses to ensure close contact with the host receptors. The pneumococcus expresses NanA that compromise the protective barrier by revealing receptors on the host cells important for adherence (King *et al.* 2006). In order to evade the mucosal barrier the pneumococcus produces bacterial IgA1 protease and pneumolysin. These virulence factors are described in detail in Section 1.3.

Once the mucosal barrier is compromised and the receptors are exposed, adhesins, expressed by the pneumococcus, bind to nasopharyngeal cells through interaction with those human cellular receptors. For example, PsaA binds to nasopharyngeal epithelial cells through interaction with E-cadherin; PavB binds fibronectin and plasminogen and PspC binds polymeric Ig receptor (Anderton *et al.* 2007; Papasergi *et al.* 2010; Zhang 2000). In addition, PspC is one protein that will actually ensure a lengthy colonisation by the pneumococcus in the nasopharynx.

Interestingly the pneumococcus can reside in the nasopharynx without causing any symptoms or disease. This can be explained by the fact that some serotypes can generate capsular antibodies that may effectively protect against subsequent colonisation by the homotypic capsular types (Weinberger *et al.* 2008). In a separate study, Richards *et al.*, indicated that mice previously colonised with a pneumolysin negative isogenic mutant (PLN-A) had a reduced duration of subsequent colonisation with a wild-type D39 strain and significantly increased survival following invasive pneumococcal challenge (Richards *et al.* 2010). These studies indicate the immunising effect of prolonged colonisation as one of the protective factors against pneumococcal disease. The question that arises then is how does disease develop? Gray *et al* investigated carriage and disease in 82 infants for 24 months of life. Nasopharyngeal swabs were collected for the first six months of life and at 2-3 months thereafter. Pneumococcal infection which was confirmed in 31 infants with the majority of infection being attributed to acute otitis media (AOM), was mainly associated with a newly acquired serotype (Gray *et al.* 1980). Thus, development of disease is seldom associated with prolonged carriage, but rather from acquisition with a new serotype. Colonisation with *S. pneumoniae* is therefore a pre-requisite for developing pneumococcal disease when the

organism progresses beyond the nasopharynx into the lungs, meninges and blood. It is suggested that pneumococcal vaccine candidate antigens that can prevent or limit colonisation could prevent the development of pneumococcal disease. It is also imperative to develop or improve mouse models of disease that mimic the natural course of disease in children, to understand the dynamics of colonisation in order to combat infections due to the pneumococcus.

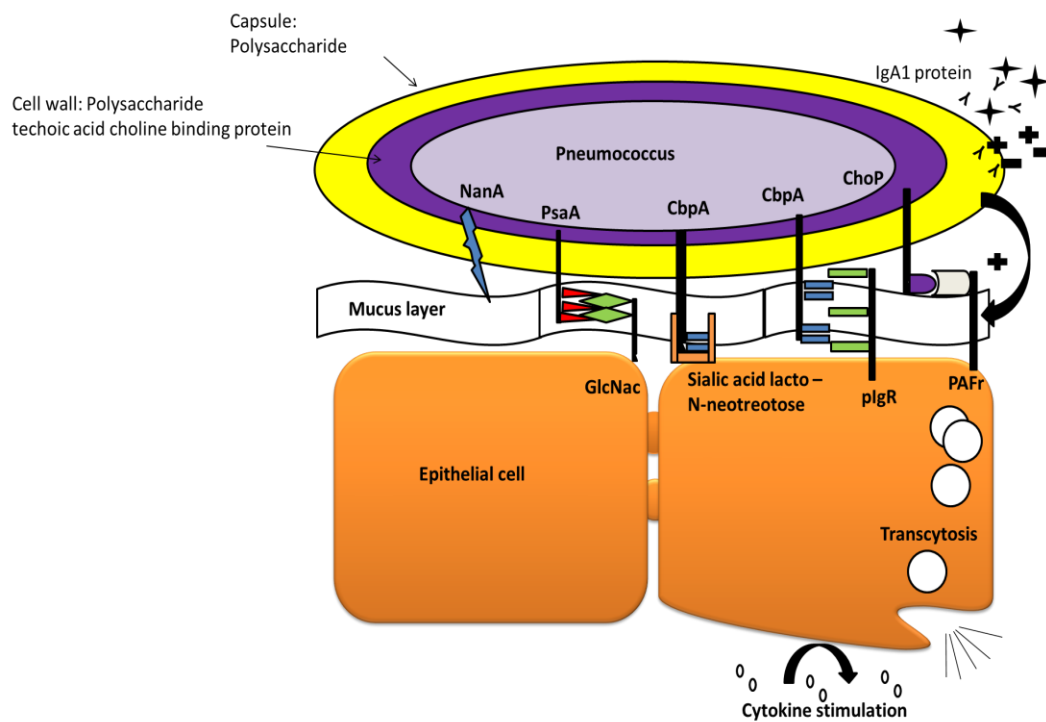


Figure 1.2 Schematic diagram of various virulence factors involved in *S. pneumoniae* adherence to host cells. Adapted from (Bogaert *et al.* 2004).

1.5 PNEUMOCOCCAL PATHOGENESIS

S. pneumoniae can cause bacteraemia, pneumonia, paranasal sinusitis and otitis media, or meningitis, which is usually secondary to one of the former infections. Once in the bloodstream, the pneumococcus can transcend the endothelial layer covering the blood brain barrier (BBB) into the subarachnoid space to cause bacterial meningitis (Henriques-Normark & Tuomanen 2013). Acute otitis media (AOM) is defined by inflammation of the middle ear. The three most common otopathogens associated with AOM and carried asymptotically in the nasopharynx of children are *S. pneumoniae*, nontypeable *Haemophilus influenzae* and *Moraxella catarrhalis*. Viral upper respiratory tract infections (URI) increase the risk of AOM by triggering proliferation of otopathogens causing inflammation of the eustachian tube and nasopharynx. The result of which is infection of the middle ear due to presence of bacteria. The presence of bacteria in the middle ear induces an influx of neutrophils (Bakaletz 2010). The intense inflammation observed in pneumococcal otitis is directed by pneumococcal cell wall components such as pneumolysin and lipopolysaccharides from Gram- positive and –negative bacteria, respectively (Heikkinen & Chonmaitree 2000; Rynnel-Dagöö & Agren 2000).

There are situations when the bacteria in the nasopharynx manage to escape the mucosal defensive barrier and disseminate into the alveolus, thus leading to pneumonia (Henriques-Normark & Tuomanen 2013). Upon encountering the lungs, pneumococci then adhere to pneumocytes via glycoconjugates, GalNac β -3-Gal and GalNac β -4-Gal. The subsequent process of lung epithelial invasion is mediated by the G-protein-coupled platelet activating factor receptor (PAF receptor). PAF receptors binds to phosphorylcholine that forms part of the pneumococcal cell wall and triggers a pro-inflammatory cytokines response, which then upregulates PAF receptor expression (Cundell *et al.* 1995). The pneumococcus thus utilises the interaction between PAF receptors and phosphorylcholine for internalisation through the lung epithelial cells. At this point, LytA plays a major role in the development of pneumonia. A study by Orihuela *et al* indicated a reduced ability of the *lytA* deficient mutant to replicate in the lungs of mice (Orihuela *et al.* 2004). LytA acts by lysing the pneumococcus and in the process, releasing Ply. The presence of both proteins ensures the progression of disease by inducing an inflammatory response that proves detrimental to the host.

Since the PAF receptor is also present on the surface of vascular endothelial cells, the pneumococcus can utilise the biological interaction between the PAF receptor and phosphorylcholine to reach the bloodstream. The pneumococcus can also reach the bloodstream by transcending the nasopharyngeal epithelial cells. PspC binds polymeric Ig receptor which then translocates the pneumococcus across the nasopharyngeal epithelial cells in order to invade the bloodstream (Zhang *et al.* 2000). However, the role of PspC in septicaemia is less defined as reviewed in section 1.3.1.1, thus giving rise to questions about the role of the PspC and pIgR complex in pneumococcal invasion. Another mechanism by which the pneumococcus can cross epithelial cells is by inter or pericellular migration. By binding to plasminogen, pneumococcal proteins such as enolase, CbpE and Gly3Ph enhance the binding of the pneumococcus to endothelial cells. The complex between pneumococcal proteins and plasminogen also assists in compromising the tight junctions between endothelial cells by degrading a cell adhesion receptor, cadherin (Attali *et al.* 2008; Pancholi *et al.* 2003).

Once the pathogen is in the blood it uses its primary virulence factor, the capsule, to thrive. The capsule assists pneumococci to survive in the bloodstream by avoiding phagocytosis (Section 1.2). Key virulent factors like PspA and Ply further increase the survival rate of pneumococci in the blood stream by curbing the classical complement pathway (Sections 1.3.1.2 and 1.3.4.1). Occasionally the pneumococcus can transcend the endothelial layer covering the blood-brain barrier (BBB) into the subarachnoid space to cause bacterial meningitis. The pneumococcus can cross the blood brain barrier by utilising pneumolysin to disrupt the tight junctions of microvascular endothelial cells (Section 1.3.4.1). Unlike the bloodstream that is abundant with complement components and phagocytes, the central nervous system is low on both. Autolysis of the pneumococcus by its main autolytic protein, LytA results in the release of pneumolysin, LTA and peptidoglycans (Koedel *et al.* 2002). The presence of these products activates a proinflammatory response and neuronal damage seen in pneumococcal meningitis.

1.6 VACCINES AGAINST *S. PNEUMONIAE*

1.6.1 Polysaccharide-based vaccines

The polysaccharide vaccine is a polyvalent vaccine based on the formulation of various capsular polysaccharide antigens. The first pneumococcal vaccine licensed was Pneumovax-14. The formulation of Pneumovax-14 was based on the 14 pneumococcal serotypes derived from epidemiologic information from the United States, parts of Europe and South Africa and on limited immunochemical information, (Robbins *et al.* 1983). The 14-valent vaccine was reformulated to cover 23-valent serotypes after the emergence of additional data on the distribution of serotypes causing disease and on the biochemical and immunologic properties of the capsular polysaccharide (Hilleman *et al.* 1981; Riley *et al.* 1977). Pneumovax-23 is recommended to elderly people ≥ 65 years of age and to young adults who are at risk of contracting IPD and pneumococcal pneumonia. Since pneumovax-23 is composed entirely of polysaccharides, the immune response elicited by the vaccine is T-cell independent. Polysaccharides induce B cell activation without recruiting T-cell help. The consequence of this is that memory maturation in these antigens is poor since this feature is dependent on helper T cells (Obukhanych & Nussenzweig 2006). In addition, children below the age of 2 years are unable to elicit a response to this vaccine since children's T-cell independent immune system is immature (Douglas *et al.* 1983; Rijkers *et al.* 1998).

In order to change the immune response from a T-cell independent to a T-cell dependent one, capsular polysaccharides were linked to an immunogenic carrier protein such as tetanus toxoid and a non-toxic knockout of diphtheria toxin (CRM₁₉₇) and were shown to be highly immunogenic in children < 2 years (Lesinski & Westerink 2001). A 7 valent conjugate vaccine, PCV7 (Prenar, Wyeth Pharmaceuticals, Inc., a subsidiary of Pfizer, Inc.) was licensed in the US in 2000. It contains seven serotypes: 4, 6B, 9V, 14, 18C, 19F and 23F, which are the serotypes that most frequently cause invasive disease in young children in the USA. After introduction of PCV7 in the USA, the overall incidence of IPD among children aged < 5 years decreased from 98.7 cases per 100,000 during 1998-1999 to 23.4 cases per 100,000 in 2005. An additional herd effect resulted in a decrease in streptococcal infections in non-vaccinated children and the elderly (Smith *et al.* 2007). In South Africa, the overall incidence of IPD among children aged < 2 years decreased from 54.8 cases per 100,000

during 2005-2008 to 17.0 cases per 100,000 in 2012. Significant reductions were also observed in the incidence of IPD caused by vaccine serotypes among persons 25 to 44 years of age (von Gottberg *et al.* 2014). The 9 valent pneumococcal conjugate vaccine (Wyeth Pharmaceuticals, Inc., a subsidiary of Pfizer, Inc.) was developed in order to address the increasing burden of IPD in children in developing countries, by serotypes 1 and 5. Clinical trials conducted in South Africa using the 9-valent pneumococcal conjugate vaccine (PCV9) indicated an efficacy rate of 65% in HIV-infected children and 83% in HIV-uninfected children against IPD, 20% in HIV-infected children against radiologically confirmed pneumonia and 13% in HIV-uninfected children (Klugman *et al.* 2003). In Gambia, the efficacy of PCV9 was 77% against IPD caused by vaccine serotypes, 50% against disease caused by all serotypes, 15% against all-cause admissions and 16% against mortality (Cutts *et al.* 2005). PCV9 was only for clinical trial purposes and was never licensed as a vaccine, but rather included in the 13-valent PCV formulation.

Despite the initial success of PCV7, the limited serotype coverage has resulted in cases of serotype replacement, whereby the nasopharyngeal ecological niche previously occupied by vaccine serotypes is colonised by non-vaccine serotypes. Increases in non-vaccine serotypes are due to capsule switching, whereby a pneumococcus acquires a new capsule operon by horizontal gene transfer. The result of which is the potential for vaccine efficacy being diminished by an increase in non-vaccine type diseases (Singleton *et al.* 2007; Sabharwal *et al.* 2013). In order to meet the shortcomings of PCV7, two higher valent conjugate vaccines were developed. In 2009, 10-valent (PCV10; Synflorix, GlaxoSmithKline) which includes additional serotypes 1, 5 and 7F above the PCV7 serotypes was licensed for use in children less than 2 years of age. In Kilifi, 2 years after the introduction of PCV10 in 2011, carriage of vaccine serotypes was reduced by two-thirds in both children younger than 5 years and in older individuals (Hammitt *et al.* 2014). In 2010, PCV13 (Prevnar, Wyeth Pharmaceuticals, Inc., a subsidiary of Pfizer, Inc.) which includes serotypes 1, 3, 5, 6A, 7F, and 19A above the PCV7 serotypes, was introduced in some industrialised countries and later in 2011 in South Africa, to extend the limited serotype coverage of PCV7. IPD caused by the six additional serotypes included in PCV13 over and above those in PCV7, declined by 93% in children less > 5 years old in USA by July 2012 to

June 2013. The incidence of IPD among adults caused by the six additional serotypes in PCV13 also declined by 58-72%, depending on age by July 2012 to June 2013 (Moore *et al.* 2015).

What is clear from these studies is that despite the success of PCVs, the replacement of non-vaccine serotypes and the prevalence of antimicrobial resistance should be continuously monitored. Another challenge with PCV is that the conjugation chemistry is technically difficult thus making the process expensive. This translates to unaffordable polysaccharide conjugate vaccines to many low-income countries where the need is greatest.

1.6.2 Protein based pneumococcal vaccines

Due to the limitations and disadvantages surrounding PCVs, attention has been focused on other surface structures of pneumococci, such as pneumococcal proteins as vaccine candidates. Vaccines made from recombinant proteins are likely to be inexpensive to produce, offer broader serotype coverage, and are able to illicit T-cell dependent responses in young children. Below is a description of some of the prominent proteins that are at advanced stages of development. Table 1.1 summarises on-going trials on promising protein candidate vaccines.

1.6.2.1 PspC

The *pspC* gene is present in about 75% of pneumococcal strains but the lower estimate is at least partly due to the fact that the PspC proteins are heterogeneous in nature, and they are not always recognized to belong to the same group (Brooks-walter *et al.* 1999). Therefore, PspC may not seem like an ideal vaccine candidate due to its polymorphic heterogenous regions. Early studies, however, revealed the protective potential of choline binding proteins, with PspC being the dominant protein, against nasopharyngeal carriage and pneumococcal pneumonia (Rosenow *et al.* 1997). Further studies using PspC recombinant protein, demonstrated its protective efficacy in a mouse model of colonisation (Balachandran *et al.* 2002). Vaccination of mice with the entire length of PspC was shown to protect against a strain that did not produce the protein (Iannelli *et al.* 2002). This effect can be explained by

the fact that the choline binding domain of PspC is cross reactive to other choline binding proteins such as PspA, thus making it an ideal vaccine candidate. These results are in agreement with human colonisation studies conducted by McCool *et al*, demonstrating the increase in IgG titres of PspC at the onset of colonisation with the pneumococcus (McCool *et al*. 2003). Now, a PspC based vaccine that is at the preclinical stages is a pneumolysoid/ PspC peptide recombinant fusion protein that has been successfully demonstrated to protect mice in models of nasopharyngeal carriage, otitis media, pneumonia and sepsis (Mann *et al*. 2014). The suggestion is to include PspC in a protein based vaccine since its production correlates with onset of colonisation.

1.6.2.2 PspA

Early studies on PspA revealed its indirect role in protecting mice against infection due to the pneumococcus, despite the lack of its purified form. McDaniel *et al* and Briles *et al* revealed that two monoclonal antibodies that bind to PspA were demonstrated to be protective against fatal pneumococcal infection by several strains (McDaniel *et al*. 1984; Briles *et al*. 1989). McDaniel and co-workers went further to prove that PspA⁺ pneumococci and not PspA⁻ pneumococci were protective against a lethal infection with pneumococci in Rx mice (McDaniel *et al*. 1987). These studies form a basis for much of research and advances made on this protein. Talkington *et al*, used PspA in its purified form and demonstrated its protective efficacy in a fatal pneumococcal model against 4 pneumococcal strains tested (Talkington *et al*. 1991). Ogunniyi *et al*, investigated the efficacy of recombinant PspA in a mouse model of pneumococcal invasive disease with results indicating the median survival time for vaccinated mice was significantly longer than those for unvaccinated mice (Ogunniyi *et al*. 2007).

Protection against colonisation with the pneumococcus in mice vaccinated with recombinant PspA seems to depend on the length of PspA used. For example, mice vaccinated with full length recombinant PspA were protected against carriage with the pneumococcus and also had significantly higher PspA specific mucosal IgA antibody levels (Wu *et al*. 1997; Arulanandam *et al*. 2001; Palaniappan *et al*. 2005). However, a truncated PspA recombinant protein elicited less protection against pneumococcal carriage (Briles *et al*.

2003). In spite of its amino acid sequence heterogeneity, PspA can elicit antibodies that are cross protective and cross reactive. PspA clade4 and clade5 induce antibodies with a high degree of cross reactivity that protects mice against lethal challenge with the pneumococcus (Moreno *et al.* 2010). A combination of PspA clade 3 and clade 2 elicited protective and cross reactive antibodies against pneumococcal challenge with D39 (clade 2), WU2 (clade 2), TIGR4 (clade 3) and BG9739 (clade 1), EF5668 (clade 4) and ATCC 6303 (clade 5) (Piao *et al.* 2014). These results can be translated to human studies where sera from adults vaccinated with a PspA clade 2 cross reacted with 37 strains with different PspA clades (1-5) (Nabors *et al.* 2000). The importance of these studies emphasises the fact that a PspA based vaccine should contain at least one fragment from each of the two major families to enable broad coverage against diverse pneumococcal strains. Despite this protein being one the forerunners of protein based vaccine candidates, there are concerns about its potential to cross react with the human protein myosin, which could lead to induction of autoimmune cardiac disorders. This was, however, not shown to be the case by Perciani *et al.*, who reported that the presence of cross-reactive antibodies does not represent a risk to the development of an autoimmune response. Human trials conducted should be designed to exclude PspA regions that cross reacts to the human protein, myosin (Perciani *et al.* 2012). Table 1.1 shows PspA based vaccines in the pre-clinical phase, indicating that these vaccines will be highly protective against colonisation as well as in invasive disease.

1.6.2.3 PpmA, SlrA and IgA bacterial protease

In vitro studies have demonstrated the protective potential of PpmA protein with its ability to opsonise several pneumococcal strains (Overweg *et al.* 2000). However, PpmA antibodies were not protective against fatal infection with a serotype 3 strain (Gor *et al.* 2005). The combination of the three proteins in a Gram-positive Enhancer Matrix (GEM) based trivalent vaccine was protective against pneumococcal pneumonia (Audouy *et al.* 2007). The presence of PpmA, SlrA and IgA bacterial protease antibodies in children induced by the presence of the pneumococcus, suggest that these proteins are immunogenic (Shoma *et al.* 2011). However the presence of PpmA, SlrA and IgA bacterial protease antibodies does not correlate with a decrease in colonisation and were not protective against subsequent colonisation (Adrian *et al.* 2004). The presence of antibodies to PpmA, SlrA and IgA

bacterial protease, however, partially protected against respiratory tract infection (RTI) such as pneumonia and otitis media (Bogaert *et al.* 2006; Lebon *et al.* 2011).

Table 1.1 On-going trials of promising protein vaccines antigens

Antigens	Vaccine based combinations	Current status and comments	References
CbpA/PspC	A.Active CbpA peptides fused with L460D pneumolysoid.	• Pre-clinical phase. • Broadly immunogenic against pneumococcal infection (colonisation, otitis media, pneumonia, bacteraemia, meningitis, and meningococcal sepsis). • Induced functional antibodies.	(Brooks-walter <i>et al.</i> 1999; Mann <i>et al.</i> 2014)
PspA	A. Attenuated <i>Salmonella typhi</i> vectors, family 1, clade 2 PspA vaccine.	• Phase I complete. • Non-immunogenic PspA antibodies passed safety tests.	(Frey <i>et al.</i> 2013)
	B. Nanogel based, family1, clade 2 PspA vaccine.	• Pre-clinical phase. • Induces high levels of anti-PspA serum IgG and nasal and bronchial IgA antibodies. • No functionality assay performed.	(Kong <i>et al.</i> 2013)
	C.PspA5+diphthera and tetanus toxoid + adjuvant; <i>Bordetella pertussis</i> lipopolysaccharide vaccine	• Pre-clinical phase. • Induces high levels of systemic IgG and protects against lethal challenge in mice. • No functionality assay performed.	(Lima <i>et al.</i> 2013)
	D.Conjugate serotype 6B with PspA clade1 as a carrier.	• Pre-clinical phase. • Induces functional anti-rPspA and anti-PS6B antibodies. • No functionality assay performed.	(Perciani <i>et al.</i> 2013)
	E.PspA-Pd fusion proteins.	• Pre-clinical phase. • Induces high levels of systemic IgG and protects against lethal challenge in mice. • Induced functional antibodies.	(Goulart <i>et al.</i> 2011)
Stkp+PsaA+PcsB	A.IC47 vaccine.	• Early stage clinical evaluation. • Protects against nasopharyngeal colonisation, pneumonia, and septicaemia. • No functionality assay.	(Schmid <i>et al.</i> 2011)
SlrA+PpmA+IgA1 bacterial protease	A.GEM-based trivalent vaccine.	• Pre-clinical phase. • Confers significant protection against fatal pneumococcal pneumonia. • No functionality assay performed.	(Audouy <i>et al.</i> 2007)
Pneumolysin(Pdb)	A.Pneumolysoid PID1.	• Phase 1 complete. • Immunogenic PlyD1 IgG antibodies with toxin neutralising activity. • Induced functional antibodies.	(Bologa <i>et al.</i> 2012)
	B.Trivalent vaccine [Ply+PhtD+ non-typeable <i>H. influenzae</i> (NTH1) protein D (PD)].	• Phase 1 complete. • Increased levels of antibodies in serum. • Transient activation of cell-mediated immune response. • No functionality assay performed.	(Berglund <i>et al.</i> 2014)
	C. ΔA146Ply fused with heat shock protein, DNA J.	• Pre-clinical phase. • Protects mice against challenge with lethal dose of pneumococcus. Fusion protein induces significantly higher levels of anti-DnaJ IgG in serum and secretory IgA (sIgA). • No functionality assay performed.	(Liu <i>et al.</i> 2014)
PcpA	A. Bivalent PcpA-pneumococcal histidine triad protein D vaccine.	• Phase I trial. • Induces significant systemic IgG antibodies. • No functionality assay performed.	(Khan <i>et al.</i> 2012)

Antigens	Vaccine based combinations	• Current status and comments	References
	B.Monovalent PcpA vaccine.	• Phase I trial. • Immunogenic PcpA IgG antibodies. • No functionality assay performed.	(Bologa <i>et al.</i> 2012)
PhtD	A. Monovalent PhtD vaccine.	• Phase I/Phase II complete. • PhtD adjuvanted with AS02v increases PhtD-specific cell-mediated immune responses in young and older adults. • No functionality assay performed.	(Leroux-Roels <i>et al.</i> 2013; Plumptre <i>et al.</i> 2013)
	B.Truncated derivatives of PhtD.	• Pre-clinical phase. • Protects mice against septicaemia and highly immunogenic over the full-length derivative. • No functionality assay performed.	(Josée Hamel <i>et al.</i> 2004)

1.6.2.4 *PLY*

Pneumolysin has potential as a candidate vaccine because of its amino acid sequence conservation and antigenicity among clinical isolates. However, due to its cytolytic activity, *PLY* is not a suitable vaccine in human studies. Removing or mutating certain amino acids in *PLY* toxoid (Pdb) reduces its toxicity considerably, and this has been successfully used in human studies (Lock *et al.* 1996). In humans, pneumolysin antibodies are stimulated upon infection with the pneumococcus (Kamtchoua *et al.* 2013). Presence of pneumolysin IgG antibodies does not, however, correlate with a decrease in pneumococcal colonisation (McCool *et al.* 2003). This observation is in agreement with animal models of disease that fail to show any effect of Pdb antibodies against colonisation (Briles *et al.* 2000). Pdb antibodies are, however, protective against fulminant pneumonia and significantly increases survival time of mice infected with the pneumococcus (Briles *et al.* 2003). More importantly is that Pdb antibodies protects mice against 9 serotypes of the pneumococcus, displaying its broad spectrum capabilities (Janet E Alexander *et al.* 1994). It is presumed that vaccination with Pdb toxoid protects mice against infection by neutralising the biological functions of the toxoid rather than by inducing antibodies that promote bacterial opsonophagocytosis (Paton & Boslego 2008). Based on that fact, it is suggested to combine pneumolysin toxoid with other bacterial proteins that have the capability of eliciting opsonic antibodies. A phase I trial on a trivalent vaccine *PLY D1/PhtD/PD* developed by GSK was completed and results revealed it induced a cell mediated immune response and was safe in adults (Berglund *et al.* 2014). In addition, there is the broadly protective pneumolysin toxoid-CbpA peptide recombinant fusion protein, which is in the pre-clinical stages of evaluation (Mann *et al.* 2014). In all these studies, immunogenicity of Pdb is clear but whether antibodies are protective is yet to be determined.

1.6.2.5 *StkP*

StkP was protective against serotype 1- induced bacteraemia in neonatal and adult mice and against strains of serotypes 3- and 19F induced pneumonia in adult mice. Interestingly, the protective efficacy of recombinant *StkP* in a pneumonia model induced with a strain of serotype 19F was comparable to that of PCV7 (Giefing *et al.* 2008; Olafsdottir *et al.* 2012). *StkP* formed part of the study, together with *PsaA* and *PcsB* that demonstrated the immunogenicity of pneumococcal proteins from adults previously exposed to the pneumococcus (Schmid *et al.* 2011). The combination of these three proteins, make up a

candidate protein vaccine, IC47. Safety and immunogenicity testing has been completed on this candidate protein vaccine (Ginsburg *et al.* 2012). The IC47 might be beneficial for protection against pneumococcal colonisation because it activates the Th17 response. The role of Th17 cells in mucosal protection against the pneumococcus is well documented (Moffitt *et al.* 2011; Olafsdottir *et al.* 2012).

1.7 Challenges with pneumococcal protein vaccines

Given the wealth of information and extensive research on pneumococcal proteins, there is still no licenced protein based vaccine. There are a number of factors hindering progress in this area of research. Firstly, it is important to note that whilst an effective pneumococcus vaccine is available, it will be unethical to conduct placebo-based control trials. In such a situation, non-inferiority trials would have to be carried out including PCV being given to all participants. The mechanism of protection of protein based vaccines and PCVs will differ, hence not being able to use current correlates of protection criteria for the licensure of protein-based vaccines, but rather evaluation against the residual remaining clinical disease (Paton & Boslego 2008). This will require a large number of subjects and would be expensive. One way of working around the issue of ethics and costs in an environment when there is already an effective vaccine, is to increase the serotype coverage of already existing pneumococcal vaccines by combining them with pneumococcal proteins. One such candidate is being developed by GSK-biologicals to broaden the spectrum of PCV10 coverage by combining PCV10 with PhtD (pneumococcal histidine triad protein D) and PLYD1. The vaccine has completed phase II trials in children aged 12-23 months old in The Gambia and Czech Republic, and healthy adults from Sweden aged 18-40 (Leroux-Roels *et al.* 2013; Prymula *et al.* 2014).

Another challenge with the development of pneumococcal protein vaccines centres on the choice of an appropriate functional assay for antibodies to pneumococcal proteins. Currently, the common method of assessing the protective ability of antibodies is by way of ELISA (Enzyme-Linked ImmunoSorbent Assay). This assay however cannot determine the functionality of antibodies compared to an Opsonophagocytosis assay (OPA) (Nahm & Romero-Steiner 2008). However, to date, OPA has not been well established for antibodies generated against the pneumococcal surface proteins. As a consequence, there is no correlate of protection that has been standardised for protein antigens (Genschmer *et al.* 2013). The

difficulty in standardising the correlates of protection for protein antigens lies in the protective mechanism of the pneumococcus driven by the capsular polysaccharide. (Harfouche *et al.* 2012). Based on these reasons, the pathway for further development of protein based vaccines continues to be developed.

1.8 ANIMAL MODELS OF COLONISATION AND PNEUMONIA

The choice of animal models in this study stems from the intrinsic limitations in the use of human experimental models. Much of the research in protein vaccine candidates is conducted in mice. This enables easier studies on the pathophysiological process of disease. Mice are easy to manipulate, cheap to acquire and easy to breed in comparison to other animal models (Mizgerd & Skerrett 2008). Rats have been used in pneumococcal colonisation studies but one study demonstrated that density of colonisation fluctuated until it was stabilised by administration of ampicillin (Iizawa *et al.* 1996). That observation casts doubt on the quality of data achieved on other rat models of pneumococcal colonisation that did not take measures on stabilising the density of colonisation. An intrathoracic pneumococcal model of low inoculum has been described in rats (Saladino *et al.* 2004). However, the route of infection does not mimic the natural route of infection observed in humans. In an attempt to find suitable animal models that are physiologically related to humans, Phillip and others demonstrated the use of the rhesus macaques (Philipp *et al.* 2012). Colonisation was maintained for two weeks, giving time to assess the natural course of disease as observed in humans. The use of larger animals, however, proved to be expensive and hence the decision to continue using mice (Philipp *et al.* 2012).

Wu and his co-workers laid the foundation for establishing carriage models in mice. In order to induce long term carriage without the development of disease, 10 μ L of different serotypes 4, 6B, 14, and 23F at 10⁷ CFU was intranasally administered to BALB/c, C57BL/6 and CBA/N mice (Wu *et al.* 1997). The robustness of the model was demonstrated by using different inbred mouse strains. Many researchers in demonstrating the role of pneumococcal proteins in pathogenesis adapted this model of colonisation. The role of PspA on the early stages of colonisation was not demonstrated in BALB/c and CBA/N mice models of colonisation (reviewed in section 1.3.1.1). These results are inconsistent with its indirect role on colonisation by the pneumococcus by binding lactoferrin and preventing killing by

apaloctoferrin. Inbred strains like the BALB/c strain are relatively resistant to infection with the pneumococcus (Gingles *et al.* 2001). In addition, duration and density of colonisation of BALB/c, C57BL/6 and CBA/N inbred strains has been shown to differ, as demonstrated by their response to the serotype 23F strain. BALB/c mice were colonised with a mean density of 7.1×10^5 CFU/mL at day 3 post inoculation and by day 65, no pneumococci were detected. C57BL/6 mice were colonised at a mean density of 6.6×10^4 CFU/mL at 3 days post inoculation and had no pneumococci detected by day 28. CBA/J mice were colonised with a mean density of 2.5×10^3 CFU/mL at day 14 post inoculation and had no detectable pneumococci by day 42 (McCool & Weiser 2004).

From the results of studies in inbred mice, (Wu *et al.* 1997; Gingles *et al.* 2001), it is clear that there is great diversity in the way that each strain responds to different pneumococcal serotypes which makes it difficult to choose a representative mouse strain for further experiments. On the other hand, outbred strains of mice such as MF-1, CD-1 and NMRI have been shown to produce consistent results following inoculation with pneumococcal strains such as D39 and WU2 (Gingles *et al.* 2001; Kerr *et al.* 2004; Cohen *et al.* 2012; Bergeron *et al.* 1998; Blue *et al.* 2003; Hamel *et al.* 2004). Further, outbred mice have been reported to produce similar and in some cases lower SDs with respect to survival times and bacterial outgrowth to inbred mice (Gingles *et al.* 2001). In comparison to inbred strains, outbred strains are heterogeneous and are representative of the human population, making them suitable choice for this study.

There are three methods of inducing pneumonia in animals, the intranasal, aerosol and intratracheal route. Of all these methods, the intranasal route is the ideal method of inducing pneumonia since it follows the natural course of disease in humans, in that disease stems from the nasopharynx. The drawback with the aerosol method is that effective inoculation cannot be obtained and some pathogens do not survive well in aerosolised conditions (Bakker-Woudenberg 2003). The intratracheal route through the tracheal wall is an invasive method of inducing pneumococcal pneumonia and there are chances of pathogens other than *S. pneumoniae* being introduced (Candiani *et al.* 1997). The intratracheal inoculation via the oral route requires surgical skill, a lack of which can compromise the success of the experiment (Bakker-Woudenberg 2003).

Vaccine efficacy in these models can be assessed through recovery of bacteria from the nasopharynx or lungs. Vaccination studies using a number of these pneumococcal surface proteins have been shown to protect mice from disease (Ogunniyi *et al.* 2001; Godfroid *et al.* 2011; Mann *et al.* 2014; Plumptre *et al.* 2013). However, they have limitations in that the vaccine efficacy is tested by having wild-type inoculated into a limited sample size of vaccinated and unvaccinated mice. In addition, survival can be scored when the animals reach the moribund state and have to be euthanized. What is evident from the literature are contradictions with respect to which pneumococcal protein antigens are protective and not protective against challenge with the pneumococcus. Using a whole genome approach (Wizemann *et al.* 2001) identified 108 antigens, but the approach did not pick PspC and PpmA despite the fact that these antigens have been shown to be protective in other studies (Rosenow *et al.* 1997; Overweg *et al.* 2000). In addition, other evidence includes the fact that many of the antigens are unable to protect mice on their own, such as PpmA and PspC, but in combination with other antigens there is a protective effect (Audouy *et al.* 2007; Ogunniyi *et al.* 2007). These contradictions suggest that many of the approaches used to screen pneumococcal protein antigens as potential vaccine epitopes in mice are not sensitive enough.

1.9 BACKGROUND AND STUDY RATIONALE

Vaccines containing polysaccharide antigens are commercially available in the prevention of pneumococcal disease but are restricted in their serotype coverage, and hence focus has been directed towards common protein-based antigens. The pneumococcus is estimated to express over 100 surface proteins which could be explored as possible vaccine candidates. Vaccination studies using a number of these pneumococcal surface proteins have been shown to protect mice from disease (Ogunniyi *et al.* 2001; Godfroid *et al.* 2011; Mann *et al.* 2014; Plumptre *et al.* 2013). However, current animal models of disease for evaluating pneumococcal surface proteins as potential vaccine candidates have limitations. They lack sensitivity in that protective potential is often difficult to demonstrate for some of the most promising candidates in experiments with a limited sample size. Another limitation with some of the current models is that they depend on inhumane endpoints such as bacteraemia, sepsis and mortality. These models therefore may not be appropriate for the evaluation of vaccines for the prevention of non-bacteraemic or mucosal pneumococcal disease. Considering these limitations, it is probable that the potential of some protein antigens might have been overlooked in previous studies by testing their efficacy in an inappropriate model.

In the quest to develop a refined model that is capable of detecting subtle vaccine effects, a lot can be learned from the approach taken by (Kerr *et al.* 2004; Hermans *et al.* 2006) that was effective in demonstrating subtle roles in virulence of pneumococcal surface proteins. This approach inoculated mice with both wild-type and knockout strains expressing different selectable markers and subsequently measuring output with quantitative culture-based end-points. The ratio of the co-inoculated strains in each individual mouse is the measureable effect. These ratios are not susceptible to variation in the inoculation dose, or efficiency of sample collection, and thus increase the precision with which measurements can be made. The consequence of this is that the sample size required for experiments gets reduced. The intranasal co-infection model has been employed by other groups to assess the role of pneumococcal proteins on colonisation and pneumococcal pneumonia (Kerr *et al.* 2004; Cron *et al.* 2011; Ogunniyi *et al.* 2012; McAllister *et al.* 2012). In this thesis, we applied this model for the evaluation of five well characterised pneumococcal candidate antigens: PspC, PpmA, SlrA, IgA bacterial protease and PspA. In addition, we included a vaccinated cohort of mice that allowed us to measure the impact of opsonising antibodies on the clearance of the wild-type in comparison to an isogenic knockout. These data are of value in that they provides a rationale for applying the model in the selection of the most appropriate pneumococcal protein antigens to be used in future vaccines.

1.9.1 Aims and Objectives

The overall aim of this study was to evaluate pneumococcal surface proteins for their potential to protect against pneumococcal infection in a mouse co-infection model:

The specific aims were to:

- i) Establish and validate an intranasal pneumonia mouse co-infection model with vaccinated and unvaccinated arms using PspC and PpmA candidate antigens,
- ii) Examine the contribution of PspC, PpmA, SlrA, IgA bacterial protease and StkP to pneumococcal virulence by constructing knockout mutants and testing them in the co-infection model,

ii) Use this model to determine the protective potentials of SlrA, IgA bacterial protease and PspA candidate antigens.

CHAPTER 2 MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

S. pneumoniae was cultured on blood agar plates at 37 °C in 5% CO₂, or in Todd-Hewitt broth (Becton, Dickinson and Company, UK) supplemented with 0.5% yeast extract (THY). Knockout strains were grown on blood agar plates in the presence of appropriate antibiotic (25 µg mL⁻¹ trimethoprim or 0.25 µg mL⁻¹ erythromycin). Broth culture growth was monitored by measuring optical density (OD) at 660 nm (OD 660). *S. pneumoniae* strains were grown to an OD of between 0.1-0.2 and aliquots were stored at -70 °C in 15% glycerol. Pneumococcal strains used in this study are listed in Table 2.1. *Escherichia coli* (*E. coli*) strains DH5α, Novablue competent cells (Novagen) were grown in Luria-Bertani (LB) medium at 37 °C. Liquid cultures of *E. coli* were grown with continuous shaking at 200 rpm, 37 °C (Kuhner AG, Switzerland) and colonies were grown on LB agar plates. Plasmid pBluescript was selected using ampicillin and the TOPO plasmid was selected using appropriate antibiotics according to manufacturer's instructions. For long term storage, strains were cultured overnight in LB broth and stored at -70 °C in 15% glycerol. When required the LB medium was supplemented with 100 µg/mL ampicillin and the Todd Hewitt yeast (THY) medium was supplemented with 100 µg/mL streptomycin.

2.2 Viable counting

Using a round-bottomed 96 well microtitre plate, serial dilutions (10⁻¹-10⁻⁶) of pneumococci from broth were performed by adding 20 µL of the bacterial suspension to 180 µL of Phosphate Buffered Saline (PBS), in duplicate. Three 20 µL volumes of each dilution were dispensed onto blood agar plates and incubated as previously described. After 24 hours, viable colonies were counted in sections where between 5 and 50 colonies could be observed, and bacterial numbers were expressed as colony forming units per mL.

CFU/mL = Total number of colonies counted in sector ÷ 3 x dilution factor x 50.

2.3 Growth curves of *S. pneumoniae*

In order to culture the bacteria, bacterial stocks stored at -80 °C were streaked out on blood agar plates and incubated for 24 hours at 37 °C with 5% CO₂. An overnight culture was prepared with 1 colony in 5 mL of THY broth. The tubes were incubated at 37 °C in 5% CO₂ atmosphere for 9 hours. One thousand microliters of the overnight culture were subcultured

in 50 mL of THY broth and the OD₆₀₀ nm was measured every hour. For each strain, the growth curve experiments were performed in triplicate

2.4 BACTERIAL TRANSFORMATION

2.4.1 Preparation of *E. coli* competent cells

A 50 mL overnight culture was diluted one hundred fold in LB and left to grow to an OD₆₀₀ of 0.6-0.7. The cells were then incubated on ice for 15 min, centrifuged at 835 x g for 5 min at 4 °C and the supernatant discarded. The pellet was then re-suspended in 1/3 of the original volume with Transformation buffer-1 (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, 15% glycerol (v/v), pH 5.8) and incubated for 15 min on ice. The cells were then centrifuged, re-suspended in Transformation buffer-2 (10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15% glycerol (v/v), pH 6.5) at 1/12 of the original culture volume and left on ice for 15 min. Cells were then aliquoted into ice-cold microcentrifuge tubes, flash-frozen in dry ice-ethanol and stored at -70 °C.

2.4.1.1 Transformation

Transformation of *E. coli* was performed by mixing 5 µL of ligation reaction with 100 µL of competent *E. coli* DH5α cell. Competent cells were first thawed on ice and 100 µL of cells were added to prechilled ligation mixture in a microcentrifuge tube followed by incubation on ice for 30 min. The cells were heat shocked at 42 °C for 30 s followed by the addition of 300 µL of 2X TY rescue media to the cells before incubation for an hour at 37 °C to allow for the expression of genes encoding for antibiotic resistance. Cells were then plated on YT agar plates supplemented with the relevant antibiotic and incubated overnight at 37 °C. Correct inserts in the plasmids from the transformants were confirmed by colony PCR, restriction digestion and / or sequencing.

2.4.2 Preparation of *S. pneumoniae* competent cells

An overnight culture of *S. pneumoniae* D39 was inoculated into 50 mL of pre-warmed Todd Hewitt Broth (THY) containing 0.5% yeast extract, 10% serum (FBS) and 1 mM CaCl₂. *S. pneumoniae* PJ351 was inoculated into 50 mL of pre-warmed Todd Hewitt Broth (THY) containing 0.2% glucose, 0.2% BSA and 0.02% CaCl₂ (pH 7.2-7.4). Pneumococci were made competent by growing a culture of *S. pneumoniae* to a density of 0.05 (D39) and 0.4 (PJ351) at 600 nm and adding 100 ng/mL of CSP1 (D39) and 500 ng/mL of CSP2 (PJ351). Aliquots (1 mL) were made and stored in 15% glycerol at -70 °C until used.

2.4.2.1 Transformation

Frozen cultures (1 mL) were thawed at 37 °C and added to 1 µg/mL of DNA at the same temperature. The cells were pre-incubated at 30 °C for an hour and further incubated at 37 °C for 2 hours in the presence of 5% CO₂ to allow for phenotypic expression. Viable counting was performed as described in section 2.2, except plating was done on antibiotic containing plates. The identity of successful transformants was confirmed by PCR and / or sequencing.

2.5 DNA EXTRACTION AND PURIFICATION OF BACTERIA

2.5.1 Small scale extraction of plasmid DNA from *E. coli*

Isolation of plasmid DNA was performed with either the QIAquick plasmid extraction kit according to manufacturer's instructions (Qiagen, South Africa) or by a manual alkali lysis method. Briefly, overnight cultures of *E. coli* grown in 1 mL of LB were transferred into 1.5 mL microcentrifuge tubes, centrifuged in an Eppendorf 5415D microcentrifuge at 15682 x g for 30 s at room temperature and the supernatant discarded. The cells were then re-suspended in 100 µL Solution I (0.5 M Glucose, 50 mM Tris.HCl pH 8.0, 10 mM EDTA), and lysed with the addition of 200 µL Solution II (0.2 M NaOH, 1% (w/v) SDS) with gentle mixing. The lysates were incubated for 5 min at room temperature. Thereafter, 150 µL of Solution III (3 M potassium acetate, pH 5.5) was added to neutralise the solution and this was followed by centrifugation at 15682 x g for 5 min. The supernatants were then transferred to new microcentrifuge tubes; RNase A was added to a final concentration of 50 µg/mL and incubated at 42 °C for 30 min. To precipitate DNA, 350 µL of isopropanol was added followed by incubation at room temperature for 5 min and then centrifuged for 10 min at 15682 x g at room temperature. The supernatants were discarded and the pellets washed once

with 1 mL of ice-cold 70 % ethanol and air dried. The DNA was then resuspended in 50-150 μ L sterile distilled water.

2.5.2 Bulk plasmid DNA preparation from *E. coli*

Overnight cultures in 100 mL LB were harvested by centrifugation for 10 min at 2320 x g at 4 °C. Bulk plasmid DNA preparation and purification was done using the QIAquick plasmid extraction kit according to manufacturer's instructions (Qiagen, South Africa).

2.5.3 Extraction of genomic DNA from *S. pneumoniae*

A few bacterial cells were scraped from plates using a plastic loop and re-suspended in 500 μ L of distilled water. Lysozyme at a concentration of 10 μ g/mL was added to the bacterial cells and subsequently incubated for an hour or overnight at 37 °C. Genomic DNA was then extracted with the QIAquick DNA extraction kit (Qiagen) according to the manufacturer's instructions. The bacterial cells were incubated as above and then incubated for 10 min at 100 °C. The DNA containing aqueous phase was then directly used as a template for PCR. To remove excess salt or inhibitors from prepared DNA samples, the volume of DNA containing solution was made up to 300 μ L with TE. Thereafter, 1/3 of the volume of TE-saturated phenol (10 g phenol, 10 mL TE) was added, followed by mixing and centrifugation for 5 min at 15682 x g at room temperature. The aqueous phase was then transferred to a fresh sterile microcentrifuge tube and a 1/3 volume of chloroform/isoamyl alcohol (24:1 v/v) was added followed by room temperature centrifugation for 30 s at 15682 x g. The aqueous phase was transferred to new tubes and DNA precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.3) and 2.5 volumes of 100 % ice-cold ethanol. After incubation for 30 min at -20°C precipitated DNA was harvested by centrifugation at 15682 x g at 4°C, washed with ice-cold 70 % ethanol, air dried and suspended in sterile distilled water.

2.5.4 Purification of DNA fragments from agarose gels

Purification of DNA fragments from agarose gels was performed with the QIAquick Gel Extraction kit according to the manufacturer's instructions (Qiagen). Briefly, DNA fragments were cut from low-melting agarose gels and binding DNA buffer was added to the fragments in a 1:1 ratio. DNA fragments were subsequently melted at 50 °C for 10 min. Once

dissolved, 3X the volume of isopropanol was added to the sample to ensure precipitation of salts. The sample was then bound to silica membrane tubes and eluted with 50 μ L of distilled water.

2.5.5 Purification of PCR products by Qiagen QIA quick columns

Restriction digests or PCR fragments were purified with Qiagen QIA quick columns. Briefly, 5X the volume of PB buffer (Qiagen) was added to the reaction product, mixed and transferred to QiagenQIAquick columns and centrifuged at 15682 x g for 1 min. The membrane bound DNA was washed 2 times with 750 μ L of PE buffer (Qiagen) and centrifuged for 2 min to remove traces of PE buffer, then eluted in 30 μ L of 10 mM Tris-Cl pH 8.0 (Qiagen).

2.6 DNA MANIPULATIONS

2.6.1 Agarose gel electrophoresis

One percent agarose gels were prepared by boiling 1 g of agarose (Molecular Biology Grade, Melford) in 100 mL of Tris-acetic-EDTA (TAE) buffer (pH 8.0). The gel was allowed to cool down to about 60 °C before adding 1 mg/mL of ethidium bromide and cast into a gel mould. The gel was allowed to set for at least 45 min after which the gel was submerged into the gel tank containing TAE buffer. Three microliters of the PCR product was mixed with 1 μ L of the tracking dye and loaded into the wells of the gel. The gel was run at 100 V with an Elpho power supply (Life sciences, Austria) for 60 min. The PCR product was visualised by illuminating the gel with the ultraviolet (UV) light on a transilluminator (UVP, USA). The relative size of the PCR products was determined by comparing them to a positive control band and the 1kb DNA ladder (Gene Ruler, Fermentas, Life Sciences: # SM0311).

2.6.2 Restriction digests Dephosphorylation and ligation of DNA fragments

Restriction enzymes used in this study were obtained from Roche Biochemicals (Germany) or Inqaba biotech (South Africa, Pretoria). Plasmid preparations (4 ug) were digested with 4 U of restriction enzymes at 37 °C. For ligations, the digested plasmid products were dephosphorylated by the addition of 1 U of alkaline phosphatase obtained from Invitrogen (United Kingdom) (20 U/ μ L) and incubating for up to 60 min at 37 °C. Once

digested and dephosphorylated, plasmids were cleaned with a Qiagen spin kit (see above) and eluted in 50 µL of distilled water. Ligations were performed in a total volume of 30 µL of 1X ligation buffer (660 mM TrisCl pH 7.6, 50 mM MgCl₂, 50 mM DTT, 10 mM ATP, pH 7.5) with 1 U of T4 DNA ligase (Roche Biochemicals). Ligation reactions were performed overnight at 4 °C with variable ratios of prepared plasmid DNA and insert DNA (1:1 to 1:3). Negative controls included plasmids ligated without insert DNA and reactions without T4 DNA ligase. A quarter of the total reaction volume was used to transform 100 µL of *E. coli* competent cells.

2.7 Polymerase chain reaction (PCR)

Standard PCR reactions were performed with the Econo Taq master mix kit (Fermentas, South Africa) as per manufacturer's instructions. Amplification of fragments for use in homologous recombination was with the aid of either Expand High Fidelity PCR System (Roche Biochemicals, Germany) or High Fidelity PCR Enzyme Mix (Fermentas, Lithuania). Amplifications from plasmid and genomic DNA were performed with 0.1-15 ng and 50-100 ng of template DNA, respectively, in 25 µL reaction volumes. Standard thermal cycler settings were: denaturation at 94 °C for 5 min followed by 35 cycles with each cycle consisting of denaturation at 94 °C for 30 s, annealing at 50-65 °C for 60 s, extension at 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. Long template thermal cycler settings were: denaturation at 94 °C for 2 min followed by 35 cycles with each cycle consisting of denaturation at 94 °C for 10 s, annealing at 50-65°C for 30 s, extension at 68 °C for 2 min, followed by a final extension at 72 °C for 7 min. All PCR reactions were performed with an Eppendorf Mastercycler gradient (Eppendorf, Germany).

2.8 Construction of pneumococcal knockouts

Construction of pneumococcal knockouts were performed as previously described with some modifications (Mowa *et al.* 2009; Hermans *et al.* 2006). To obtain the streptomycin-resistant *S. pneumoniae* D39 and PJ351 strains, the *rpsL* gene encoding a streptomycin-resistant mutant of the ribosomal protein S12 was amplified from a streptomycin resistant *S. pneumoniae* clinical strain, S303 with primer pair *rpsLFWD* (TCGTCCCAAGTCCATAGTCC) and *rpsLRV* (ATATGAGCTGGAACGGGATG). This PCR product was introduced into D39 and PJ351 by CSP-1- and CSP-2-induced

transformation, respectively, and selected for streptomycin resistance. *PspC*, *ppmA*, *pspA*, *igA1* and *slrA* gene fragments were deleted from the parental strain D39 by in-frame insertion deletion mutagenesis with the resistance cassette *dfr13*, encoding resistance to trimethoprim. About a 1000 bp of upstream (5' UTR) and downstream (3' UTR) flanking sequences, excluding the coding sequences, were amplified from chromosomal DNA from the D39 strain. The PCR products were cloned into pBlue- Script KS and transformed into *E. coli* DH5 α . To avoid inadvertent mutations, all PCR amplicons for deletion mutagenesis were first cloned into pCR2.1-TOPO (Table 2.2) and sequenced before cloning into pbluescript. All primers used for targeted gene knockout and confirmation of final pneumococcal constructs are outlined in Table 2.3. For a blue-white selection of both *E. coli* strains, solid media was supplemented with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) to a final concentration of 40 μ g/mL. These amplicons were ligated with the PCR-amplified *dfr13* and transformed to *E. coli* DH5 α (Invitrogen). The primers covering the 5'-flanking sequence of genes were designed to include *PstI* restriction sites that overlap the start codons of these genes. Similarly, *dfr13* was amplified with *PstI* sites at the start codon, to ensure that resistance cassettes were cloned in the correct orientation, and were transcribed from the host promoters, starting from the ATG start codon. The trimethoprim-resistant knock-out constructs were used to delete the genes in strains D39 and PJ351 by CSP-1 and CSP-2 induced recombination. The fragments that were removed were of equal length (500 bp) to the inserted cassettes to eliminate the possibility of any polar effects. Deletion of genes in resulting transformants was confirmed by colony PCR.

2.9 DNA sequencing and bioinformatics software applied

Sequencing was performed by Inqaba Biotech (South Africa) with a Spectrumedix 2410 Capillary Electrophoresis automated DNA sequencer and Big Dye Terminator V3.1 software from ABI for sequence analysis. The genomic DNA sequences of *S. pneumoniae* strains PJ351 and D39 of serotypes- 1 and 2, respectively, were obtained using the *S. pneumoniae* Sybil software (www.sybilpneumo.com). Blast searches and alignments of the available complete and incomplete bacterial nucleotide and protein databases were performed using the NCBI website ([http:// www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)).

Table 2.1 Strains used in this study

Strains	Relevant characteristics	Reference
PJ351	Wild-type (Serotype 1)	ATCC BAA-
D39	Wild-type (Serotype 2)	NCTC 7466
$\Delta pspC$	<i>pspC</i> deletion in strains PJ351 and D39	This study
$\Delta ppmA$	<i>ppmA</i> deletion in strains PJ351 and D39	This study
$\Delta pspA$	<i>pspA</i> deletion in strains PJ351 and D39	This study
$\Delta igA1$	<i>igA1</i> deletion in strains PJ351 and D39	This study
$\Delta slrA$	<i>slrA</i> deletion in strains PJ351 and D39	This study

Str^r, streptomycin resistance; Trim^r, trimethoprim resistance; *pspC*, pneumococcal surface protein; *ppmA*, putative proteinase maturation protein

Table 2.2 Plasmids used in this study

Plasmid or target	Relevant characteristics	Reference source
Plasmids		
pKOb	Donor plasmid for Tm ^r cassette	This study
pCR2.1	Cloning vector (Amp ^r , Km ^r)	Invitrogen
pBluescript (KS+)	Cloning vector (Amp ^r)	Stratagene
pBS $\Delta pspC::dfr13$	Knockout vector for creating a <i>dfr13</i> marked deletion in <i>pspC</i> of <i>S. pneumoniae</i> .	This study
pBS $\Delta ppmA::dfr13$	Knockout vector for creating a <i>dfr13</i> marked deletion in <i>ppmA</i> of <i>S. pneumoniae</i> .	This study
pBS $\Delta pspA::dfr13$	Knockout vector for creating a <i>dfr13</i> marked deletion in <i>pspA</i> of <i>S. pneumoniae</i> .	This study
pBS $\Delta igA1::dfr13$	Knockout vector for creating a <i>dfr13</i> marked deletion in <i>igA1</i> of <i>S. pneumoniae</i> .	This study
pBS $\Delta slrA::dfr13$	Knockout vector for creating a <i>dfr13</i> marked deletion in <i>slrA</i> of <i>S. pneumoniae</i> .	This study

Table 2.3 Oligonucleotides used for constructing *S. pneumoniae* knockouts

Primers	Relevant characteristics or nucleotide sequence (5'to 3')	Amplicon size
<i>pspC</i> F1	ATGTG <i>gagctc</i> TAAGTGACCAAGAACTAGAA	888 bp
<i>pspC</i> R1	ATGCAAA <i>ctgcag</i> TATTTCTTCTATATTTTTCTTTAACCTTACTATAT	888 bp
<i>pspC</i> F2	ACTAGTAA <i>aagctt</i> AAGCTAAAGAATCTCGAAACGAGG	1736 bp
<i>pspC</i> R2	CTCCGT <i>ggtacc</i> ATTTTCGCAAGCTAGCACCCCTAG	1736 bp
<i>pspA</i> F1	CCTCTTTT <i>ctgcag</i> GTCATTTTCAGACTATACTTATATTAAGTATAG	1736 bp
<i>pspA</i> R1	GGTGACAT <i>ggtacc</i> ATAGTAGTAAGCACACTATGTGGCTT	1736 bp
<i>pspA</i> F2	CAAACAAA <i>aagctt</i> ATGCTGAAGAAGTCGCTCCTCAAGCTA	900 bp
<i>pspA</i> F2	CGGCT <i>ggtacc</i> CATTACCATTTGGCA	900 bp
<i>stkP</i> F1	TTTGAT <i>ctgcag</i> GCATCCTCCTCGTTCATAGAAACAA	927 bp
<i>stkP</i> R1	TGTAAG <i>gagctc</i> ATGAAAGACGGCTGTATC	927 bp
<i>stkP</i> F2	CAGTTCA <i>aagctt</i> GTCACCAGAGCAGGCGCGT	996 bp
<i>stkP</i> R2	AATATT <i>ggtacc</i> TTCAATAGAGAAAAAGATAGC	996 bp
<i>igA1</i> F1	GGCAATA <i>aagctt</i> GCGCCTGAGAGAGACTACCAGCTTTT	1000 bp
<i>igA1</i> R1	TCACCAAAAATAC <i>ggtacc</i> ATTATTCCTCCTTGAAATAAAATTTATATATGT TACAAAGAC	1000 bp
<i>igA1</i> F1	TGTTTCTT <i>gagctc</i> AGCCGACAGAAGTTCAAGTAGTTGAAAAAC	970 bp
<i>igA1</i> F2	TATATT <i>ctgcag</i> ATGGGGCTACCTGCTCTGGTTCA	970 bp
<i>ppmA</i> F1	TTTTCTT <i>ctgcag</i> TACTCCTTTGAGATAAGTGTTACTCTATCTA	1000 bp
<i>ppmA</i> F2	AAGACCGT <i>gagctc</i> TATATCGACTACCTCAAGGC	1000 bp
<i>ppmA</i> F1	TCAAAA <i>aagctt</i> CTTTCGCTTTAGATGTGGATGGTGTTT	940 bp
<i>ppmA</i> F2	CTACGAG <i>ggtacc</i> ATTCTCCATAGTGACCAA	940 bp
<i>slrA</i> F1	TTCATT <i>ctgcag</i> CTCTCAAAAATTCATT	1000 bp
<i>slrA</i> F2	CCTTC <i>agagctc</i> CTGAACGAGAGTTGGATA	1000 bp
<i>slrA</i> F1	GCCTACG <i>gtacc</i> CAGCAGAAAGAGATT	1000 bp
<i>slrA</i> F2	AAAGATA <i>aagctt</i> CTAAACTCCCTACAAGCAAGTATC	1000 bp

***Restriction sites within the primer sequences utilised for subsequent cloning of PCR fragments are italicized, F1/F2 represents a forward primer, R1/R2 represents a reverse primer.**

Table 2.4 Oligonucleotides used for confirming the presence of knockouts constructs

Primers	Relevant characteristics or nucleotide sequence (5'to 3')	Amplicon size
<i>pspC F1</i>	ATGTG <i>gagctc</i> TAAGTGACCAGGAAGTAGAA	3600 bp
<i>pspC R2</i>	CTCCGT <i>ggtacc</i> ATTTGCAAGCTAGCACCTAG	3600 bp
<i>pspA F1</i>	CCTCCTTTT <i>ctgcag</i> GTCATTTTCAGACTATACTTATATTAAGTATAG	2815 bp
<i>pspA R2</i>	CGGCT <i>ggtacc</i> CATTCACCATTTGGCA	2815 bp
<i>stkP F1</i>	TGTAAG <i>gagctc</i> ATGAAAGACGGCTGTATC	2984 bp
<i>stkP R2</i>	AATATT <i>ggtacc</i> TTCAATAGAGAAAAAGATAGC	2984 bp
<i>igA1 F1</i>	GGCAATA <i>agctt</i> GCGCCTGAGAGAGACTACCAGCTTTT	2713 bp
<i>igA1 R2</i>	TATATT <i>ctgcag</i> ATGGGGCTACCTGCTCTGGTTCA	2713 bp
<i>ppmA F1</i>	TTCATT <i>ctgcag</i> CTCTCAAAAATTCATT	2928 bp
<i>ppmA R2</i>	AAAGATA <i>agctt</i> CTAAACTCCCTACAAGCAAGTATC	2928 bp
<i>slrA F1</i>	TTCATT <i>ctgcag</i> CTCAAAAATTCATT	2800 bp
<i>slrA R2</i>	AAAGATA <i>aagctt</i> CTAAACTCCCTACAAGCAAGTATC	2800 bp

***Restriction sites within the primer sequences utilised for subsequent cloning of PCR fragments are italicized, F1/F2 represents a forward primer, R1/R2 represents a reverse primer.**

2.10 IN VIVO METHODS

2.10.1 Ethics statement

All animal experiments were performed with the approval of the University of the Witwatersrand Animal Ethics Committee (Ethics number 2012/05/2B).

2.10.2 Mouse strain

Five-week-old female NMRI outbred mice were purchased from NICD (National Institute of Communicable disease in South Africa) and housed under standard conditions at the Central Animal Service (CAS) situated at the University of the Witwatersrand. Mice weighed between 20 and 25 grams at the commencement of experiments. Mice were acclimatised for a week prior to the experiments.

2.10.3 Pneumococcal passaging

Pneumococcal passaging were performed as previously reported (Alexander *et al.* 1994). Five-week-old female NMRI mice were injected intraperitoneally with different concentrations (10^5 - 10^7 CFU) of serotype 1 (PJ351/1), serotype 2 (D39), serotype 3 (WU2), serotype 4 (TIGR4), serotype 5 (ATCC 6305) and serotype 6B (ATCC 6326) of *S. pneumoniae* at a dose of 100 μ L. Mice were monitored for signs of disease such as conjunctivitis, lethargy, hunched back, spiked fur and a moribund state until the animals were deemed moribund. Blood samples were collected under anaesthesia by cardiac puncture and plated out on blood agar plates. The plates were incubated at 37 °C, for 18 hours. Three single colonies were then picked from blood agar plates, grown in Todd-Hewitt broth until OD = 0.1-0.2 and properly stored at -70 °C.

2.10.4 Preparation of inoculum

The number of viable organisms in the frozen THY stocks of wild-type and knockout strains was determined from a thawed aliquot from each batch, and the number of CFU/mL was used to calculate the dilution factor for each frozen stock. The frozen stocks were thawed, diluted and then centrifuged to remove traces of THY and glycerol. The cell pellet was resuspended in sterile PBS. Colony counts of the diluted working stock were performed on plain blood agar and media containing the appropriate antibiotic to confirm the wild-type

and knockout bacterial CFU. All mice in the same experiment were infected using the same aliquot to ensure consistency.

2.10.5 Immunization of mice with pneumococcal antigens

Six-week-old mice were immunised subcutaneously with pneumococcal antigens PspC, PspA, SlrA, IgA1 bacterial protease or PpmA. Recombinant proteins were purified and expressed by Dr Peter V. Adrian from our laboratory. Each mouse received three doses of 50 µg of each antigen diluted with PBS and adjuvanted with 100 µg of saponin, at a 14 day interval. The unvaccinated control mice were given a placebo consisting of PBS plus the saponin adjuvant. Mice were subsequently challenged intranasally (I.N) with the wild-type PJ351 (serotype 1) or D39 (serotype 2) and their isogenic knockouts (10^6 CFU total) 2 weeks after the last immunisation and sampled at 0, 6, 12 and 24 hours post-infection as described below. The time point of 0 hours was chosen as it was the true time zero, taking into account any inoculation inaccuracies.

2.10.6 Mouse model: co-infection

Co-infection of mice was performed as previously described with slight modifications (Hermans *et al.* 2006). Mice were lightly anaesthetized with 2.5 % (v/v) isoflurane over oxygen ($1.5 \text{ litre min}^{-1}$), administered with a calibrated vapouriser and the effect of anaesthesia was confirmed by observing no pinch reflex action. Once anaesthetized, the animals were scruffed with the nose held upright and 50 µL of a bacterial suspension containing 1:1 ratio (approximately 10^6 CFU) of streptomycin-resistant *S. pneumoniae* wild-type and pneumococcal knockout was administered intranasally by adding a series of small droplets of the inoculum into the nostrils of the mice. After inoculation, mice were held upright for 20 s and then laid on their backs until recovery. At 0, 6, 12, and 24 hours post inoculation, groups of five mice were euthanised by intraperitoneal injection (I.P) of 0.2 mL of sodium pentobarbital (Euthapent, Kyron Laboratories (Pty) Ltd. South Africa) and 0.5-1 mL of blood was drawn from each mouse by cardiac puncture. Serum was separated and stored at -80 °C until used for immunoassays. The nasopharynx was washed using a method of Wu *et al.* (1997). Briefly, the trachea was exposed and clamped and 2 mL of sterile PBS was passed through the nasopharynx via a 16-gauge non-pyrogenic angiocath. BAL and lung were performed as previously described (Kerr *et al.* 2002). BAL was performed by slowly

delivering 1.5 mL of PBS (pH 7.3), through the tracheal tube via a 16 gauge non-pyrogenic angiocath. The tracheal tube was exposed and the fluid was slowly withdrawn by gentle suction and plated onto blood agar plates. Both lungs were removed and homogenised in 2 mL sterile PBS. Viable counts were determined by serial 10-fold dilutions plated on BA plates. Bacterial loads were determined by plating out 10-fold dilutions onto blood agar plates containing 100 µg/mL of streptomycin or 25 µg/mL of trimethoprim. Competitive indices (CI) were calculated as the ratio of knockout to wild-type bacteria recovered from each animal and the ratio of knockout to wild-type bacteria that were inoculated into each animal. In this setup, a value equal to 1 indicates an equal proliferation of both strains. A value below one is indicative of severe attenuation of the knockout relative to the wild-type strain.

2.11 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA assays for all proteins were performed as previously reported (Quataert *et al.* 2001) with some modifications. IgG titres were determined by enzyme-linked immunosorbent assay (ELISA). Microtitre plates (NUNC maxi sorb) were coated overnight at 4 °C with 0.625 µg/mL of PspC and SlrA. PspA, IgA1 bacterial protease and PpmA were coated overnight with coating concentrations of 2.5 µg/mL dissolved in PBS. Antigen coating concentrations were determined as explained in section 2.11.1. Plates were washed with 100 L of PBS containing 0.05% Tween (PBS-T) buffer and blocked with PBS buffer containing 10% foetal bovine serum (PBS-F) pH 7.3, for an hour, at 37 °C. Pre-dilutions of the reference, controls, and unknown sera were made in PBS-F. Samples were then serially diluted and 100 µL/well was added to the pre-coated microtiter plates. Following 2 hours incubation at 37 °C, plates were washed three times with PBS-T and alkaline phosphatase conjugated anti-mouse IgG (Sigma-Aldrich, Cat # A3188, Munich, Germany) was added to all wells and incubated for 2 hours at 37 °C. After incubation, the plate was washed three times with PBS-T and the colour was developed for an hour in the dark using p-nitrophenyl phosphate substrate (Sigma-Aldrich, Cat # 71768, Munich, Germany). Plates were read at 405 nm with the RS 232C Labsystem Multiskan RC plate reader (Labsystems, Helsinki, Finland). Two internal controls were included on each plate: a medium and low control, consisting of a pool of in-house medium-titred and low-titred mouse sera, respectively. To avoid bias, serum samples from the different groups were always assayed on the same plate.

2.11.1 Determination of optimal antigen concentration

The optimum antigen concentration for coating ELISA plates was determined as follows: PspC, PspA, PpmA, SlrA and IgA1 bacterial protease were diluted in increasing concentrations (0.01-10 µg/mL). One hundred microliters of each concentration were pipetted into 4 wells each of a microtiter plate and incubated overnight at 4 °C. After coating, the plates were washed and a standard ELISA protocol was followed with sera of high and low concentrations. The antigen concentration that produced the highest dynamic range, i.e. the strongest signal with low background was selected for further analysis (World Health Organization Pneumococcal Serology Reference Laboratories 2004). The optimum coating concentration for PspA, PpmA, and IgA1 bacterial protease was 2.5 µg/mL, and for PspC and SlrA, it was 0.625 µg/mL (Figure 1.1 A in appendix 1).

2.11.2 Determination of alkaline phosphatase conjugated anti-mouse IgG

Plates were coated with antigen, and washed with 100 µL of PBS containing 0.05% Tween (PBS-T) buffer and blocked with PBS buffer containing 10% foetal bovine serum (PBS-F) pH 7.3, for an hour, at 37 °C. A reference serum was serially diluted (100 µL/well) across the pre-coated microtiter plates to produce a maximum and minimum signal. Following 2 hours incubation at 37°C, plates were washed three times with PBS-T and alkaline phosphatase conjugated anti-mouse IgG was added (100 µL/well). The conjugate concentrations were tested in the range diluted from 1:1000-1:8000, and incubated at 37 °C for 2 hours. Following incubation at 37°C, ELISA was completed using the normal steps for ELISA. For each ELISA, the optimum conjugate concentration was selected as the dilution which produced the highest dynamic range with the lowest background (World Health Organization Pneumococcal Serology Reference Laboratories 2004). A maximum signal (>3.0) was produced at conjugate dilutions of 1:2000 and above. However, since a concentration of 1:1000 produced unacceptable background, a concentration of 1:2000 was selected for further experiments (Figure 1.1 B in appendix 1).

2.11.3 Competitive inhibition studies

To determine the specificity of ELISA assay, inhibition studies were performed as previously described with slight modifications (Pickering *et al.* 2002). A serum with known high concentrations of IgG antibodies directed against PspA, PspC, SlrA, PpmA and IgA1 bacterial protease was diluted 1:500 times. Each aliquot was incubated with an equal volume of one of the four purified antigens in a concentration of 6 µg/mL for 1 hour at room temperature. A 1:1000 dilution of the serum was used as a control. The absorbed and unabsorbed sera were then assayed by ELISA, as described in section 2.11. The results were reported as percent change in concentration in response to the addition of individual antigens to the serum. The addition of the homologous protein to the serum at saturated concentrations (6 µg/mL) resulted in a marked inhibition of the signal; 96% for PspC, 98% for PpmA, 95% for SlrA, 93% for PspA and 97% for IgA1 bacterial protease compared to 9.15%, 4.47%, 9.34%, 7.50% and 8.20% in the control sample. This data suggests that the assay was highly specific for the antigens tested.

2.11.4 Assay sensitivity

For the determination of the sensitivity of the assay, the reference serum was diluted in eight steps of 3-fold dilutions. The standard deviation of the blank was calculated for each protein. The lower limit of quantification (LLOQ) was interpolated from the standard curve of the reference serum and was calculated by adding 3 SD to twenty blanks and reported in arbitrary units per millilitre (U/mL). Estimation of the upper limit of quantification (ULOQ) was based on the cut-off value of 3.5 optical densities for the ELISA method. The ULOQ was reported in U/mL for the ELISA. The calculated LLOQ was 0.1 U/mL for all antigens. All samples with an OD of 3.5 were re-diluted to fall within the measurable range of the standard curve of the reference serum (Pickering *et al.* 2002).

2.11.5 Assay reproducibility

Reproducibility of the assay was assessed by determining the variation between intra- and interassays of replicate ELISA results. Intra-assay variation was determined from testing sera assayed on different wells within one plate. Inter-assay variation was assessed by testing samples in different assays and time points, and then calculating the percentage of variation (CV%) between results from each assay (World Health Organization Pneumococcal Serology

Reference Laboratories 2004). The method was reproducible; the percentage for the CV of the high and low control samples was less than 20% in both intra- and inter-assay comparisons. The CVs were 6% and 4% for the high QC and low QC, respectively. ELISA was optimised to meet several other performance requirements mentioned in Table 1.1 found in appendix 1.

2.11.6 Statistical analysis

All data were analysed using GraphPad Prism version 5.0. For analysis of bacterial load in unvaccinated animals, a paired two-sample t-test was used to calculate statistical significance between the wild-type and knockout strain co-inoculated in each animal. The Mann-Whitney test was used for comparison of the mean bacterial load in NP, BAL fluid and lung homogenate between the placebo and vaccinated group. Differences in antibody titres between vaccinated and unvaccinated mice were analysed by the Mann-Whitney U-test and a P value of <0.05 was considered significantly significant. Data was mathematically corrected to account for the differences in bacterial count of the initial inoculum.

CHAPTER 3 RESULTS

3.1 DEVELOPMENT OF AN INTRANASAL PNEUMONIA CO-INOCULATION MODEL

3.1.1 Infection kinetics of PJ351 (serotype 1), D39 (serotype 2) and TIGR4 (serotype 4) in the nasopharynx and lungs of NMRI mice

In order to identify pneumococcal strains with suitable virulence characteristics, strains were passaged through mice to augment their virulence. Mice were intraperitoneally injected with 100 μ L of different concentrations (1×10^5 to 5×10^7) of pneumococci (PJ351, D39, WU2, ATCC 6305 and ATCC 6326) to determine the concentrations that were able to elicit invasive pneumococcal disease within 24 hours. Mice were closely monitored for signs of disease until the mice were deemed moribund.

Strains were considered virulent if they caused disease in mice within a period of 24 hours. Any strain that fell outside of this classification was excluded from further experiments. ATCC 6305 (serotype 5) and ATCC 6326 (serotype 6B) did not cause disease in mice within 24 hours of inoculation and were excluded from further analysis. WU2 caused conjunctivitis 18 hours after intraperitoneal injection. Even though WU2 (serotype 3) caused disease in mice within 18 hours of infection, it was excluded from further analysis because its mucoid phenotype made the counting of distinct colonies challenging. D39 was the most virulent, and induced a moribund state within 12 hours of inoculation with a concentration of 10^5 CFU. Initial infection of mice with 6×10^6 CFU of PJ351 resulted in the presence of bacteria in the bloodstream following 30 hours of infection without an observation of the classical signs of disease already mentioned in materials and methods. A single colony was isolated from the collected blood and passaged at a higher concentration. Following 12 hours from challenge with PJ351 at a concentration of 2×10^7 CFU, the mice showed typical signs of disease. Infection with 10^7 CFU of the TIGR4 strain successfully caused septicaemia in mice within 21 hours of infection.

Since PJ351, D39 and TIGR4 met the criteria of causing disease in mice within 24 hours after I.P challenge, their virulence after intranasal challenge was studied. Five mice per group were challenged intranasally with 50 μ L of 10^6 CFU of PJ351, D39 or TIGR4 strains. At 0, 6, 12 and 24 hour time points, mice were euthanised and lung homogenates,

nasopharyngeal wash, BAL fluid, and blood were subsequently harvested and cultured as described in the materials and methods section.

There was successful colonisation of the nasopharynx by all strains, yielding counts of between 10^3 CFU and 10^5 CFU at all-time points. For all strains, there were no statistically significant differences in the concentration of bacteria recovered from the nasopharynx at the various time points, or between pneumococcal strains. This suggests that nasal colonisation is stable within the 24 hour monitoring period following inoculation in this model. The specific data points for each mouse are illustrated in (Figure 3.1 A).

The recovery of strains in the bronchoalveolar tract indicated variability in their growth over time. All three strains grew out consistently by 1 log in the first 6 hours, but thereafter, responses differed between strains at the 12 and 24 hour time points. A concentration of 6.29×10^4 CFU of the PJ351 strain was recovered in the bronchoalveolar tract of mice at 0 hours. That count significantly increased to 2.07×10^5 CFU ($p = 0.0079$) at 6 hours and reached 7.30×10^4 CFU at 12 hours to ($p = 1.000$). Pneumococci persisted in the bronchoalveolar tract up to 24 hours (1.32×10^5 CFU, $p = 0.5476$). The initial bacterial load of the D39 strain at 0 hours was 7.03×10^4 CFU and it steadily increased to 2.94×10^5 CFU ($p = 0.0079$) at 6 hours and reached 3.08×10^5 CFU at 12 hours ($p = 0.0556$). D39 declined to a bacterial count of 2.23×10^5 CFU at 24 hours ($p = 0.6905$). Initial bacterial load in the bronchoalveolar tract of mice at 0 hours was found to be 3.99×10^4 CFU of TIGR4. There were no significant differences in the bacterial load recovered at 6 hours (2.01×10^5 CFU, $p = 0.2222$). Significantly lower bacterial counts of 3.60×10^3 CFU were found at 12 hours ($p = 0.0079$) and continued to significantly decline to 5.10×10^3 CFU ($p = 0.0079$) at 24 hours (Figure 3.1 B).

The different bacterial strains demonstrated different kinetics in terms of their outgrowth in the lungs. The bacterial counts of PJ351 rose steadily from 5.39×10^4 CFU at 0 hours to 8.41×10^4 CFU ($p = 0.4206$) at 6 hours, 1.53×10^5 CFU ($p = 0.3095$) at 12 hours and 4.53×10^5 CFU ($p = 0.0079$) at 24 hours. D39 showed similar kinetics with 6.37×10^4 CFU at 0 hours to 1.28×10^5 CFU ($p = 0.0952$) at 6 hours, 2.76×10^5 CFU ($p = 0.0079$) at 12 hours and 3.62×10^5 CFU ($p = 0.3095$) at 24 hours. In contrast, the TIGR4 strain showed different kinetics to the other strains. The initial increase from 4.96×10^4 CFU at 0 hours to

7×10^4 CFU ($p = 0.5476$) at 6 hours, was not sustainable over 12 and 24 hours, with the mean CFU dropping by a log to 1.29×10^3 CFU ($p = 0.0937$) at 12 hours and maintaining the growth at 1.42×10^3 CFU ($p = 0.1425$) at 24 hours. Further, some mice in the group were able to reduce the bacterial counts of TIGR4 to below that of the detection limit (Figure 3.1 C). The recovery of 10^4 CFU/mL of lung tissue immediately after inoculation suggests that the intranasal mode of inoculation is an effective route of introducing bacteria into the lungs in order to cause pneumonia. Results for PJ351 and D39 indicated little variation at all-time points. On the other hand, highly variable data that corresponded with the larger spread in bacterial numbers was obtained for the TIGR4 strain at all time points. These results suggest that the TIGR4 strain was highly variable in its ability to infect the lungs, compared to both strains.

Further, the coefficient of variation (CV%) between PJ351, D39 and TIGR4 at different time points was calculated in order to determine the ability of all strains to colonise the nasopharynx and infect the bronchoalveolar tract and lungs. In this thesis, the confidence interval was abbreviated as Ci to differentiate it from the abbreviation for competitive index (CI), mentioned later on in the text. Coefficient of variation for the PJ351 strain from the nasopharynx of mice at 0, 6, 12 and 24 hour time points suggest a relatively high variability for the PJ351 strain as indicated by high CVs at earlier time points compared to D39 and TIGR4 strains. Overall, CVs for D39 and TIGR4 indicate that these two strains were more able to colonise the nasopharynx of mice than the PJ351 strain (Table 3.1). Results for PJ351 from the BAL fluid suggests that there was low variation in the bacterial dispersion at all time points except at the 12 hours that was marked by a relatively high CV of 99%. D39 indicated low variation at all time points compared to both strains. TIGR4 strain, however, indicated relatively high variability at all time points sampled. Overall, CVs suggest that PJ351 and D39 strains were probably more able to infect the bronchoalveolar tract compared to the TIGR4 strain (Table 3.2). Results for PJ351 and D39 from the lung tissue indicated little variation at all-time points. On the other hand, highly variable data that corresponded with the larger spread in bacterial numbers was obtained for the TIGR4 strain at all time points. These results suggest that the TIGR4 strain was highly variable in its ability to infect the lungs, compared to both strains (Table 3.3).

Markers of invasive disease as determined by the recovery of viable pneumococci from the blood stream, could not be used in this model. No pneumococci of any serotype were recovered from the blood of any of the mice at any of the time points. Further, no clinical symptoms were used, as by 24 hours, none of the observable clinical symptoms of severe invasive disease, such as conjunctivitis, hunched back, lethargy and spiked fur were observed.

In conclusion, two pneumococcal strains, PJ351 and D39 were shown to be suitable to use in this mouse model, in that they could be easily passaged to enhance their virulence, and that they were able to effectively colonise the upper and lower respiratory tract and lung tissue consistently over the observed 24 hour period.

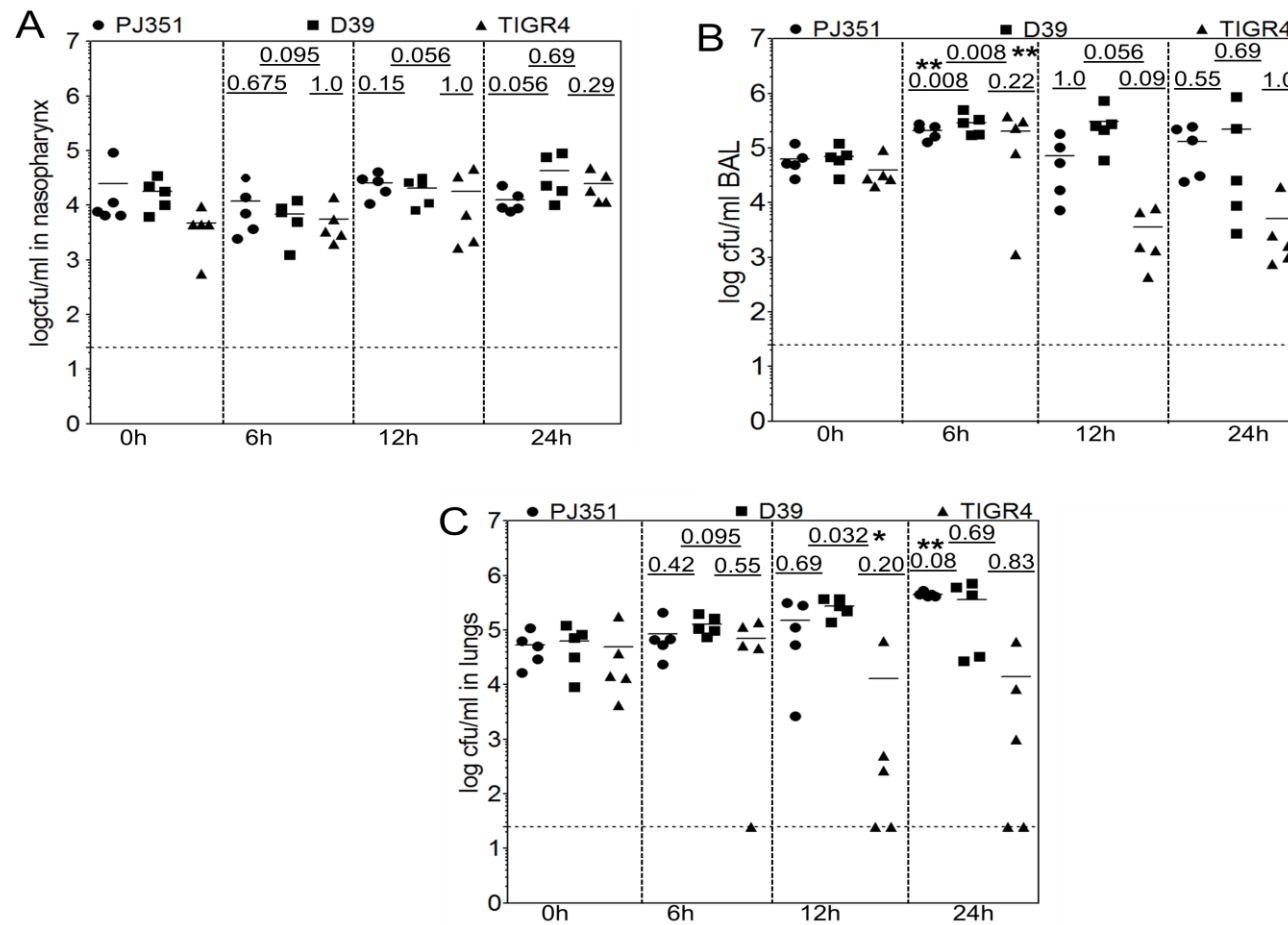


Figure 3.1 Infection kinetics within NMRI mice after intranasal infection with PJ351, D39 and TIGR4 strains. Groups of 5-week old female mice (n = 5/group) were infected with PJ351, D39 or TIGR4 strains. Pneumococci were recovered at 0, 6, 12 and 24 hours post infection from (A) Nasopharynx, (B) Bronchoalveolar tract and (C) Lungs. Each symbol represents a single mouse. Mean values are represented by horizontal bars. The dotted line shows the limit of detection. Data represent average CFUs from a single experiment. The calculated P-values are presented, with values below 0.05 considered significant. * $p \leq 0.05$, ** $p \leq 0.001$.

Table 3.1 Standard deviations and coefficients of variations based on the mean bacterial counts of PJ351, D39 and TIGR4 strains in the nasopharynx.

A. NASOPHARYNX												
	PJ351				D39				TIGR4			
	0 h	6 h	12 h	24 h	0 h	6 h	12 h	24 h	0 h	6 h	12 h	24 h
Mean	24697	11908	25431	12723	18164	6976	20322	43287	4713	5567	18013	25033
Std. Deviation	37451	12187	11463	6400	10980	4175	10262	36295	3172	4901	20465	16142
Lower 95% Ci of mean	-21805	-3225	11197	4776	4531	1792	7580	-1780	775	-519	-7397	4991
Upper 95% Ci of mean	71199	27041	39665	20670	31797	12160	33064	88354	8652	11652	43424	450
Coefficient of variation (CV)	152%	102%	45%	50%	60%	60%	50%	84%	67%	88%	114%	64%

Table 3.2 Standard deviations and coefficients of variations based on the mean bacterial counts of PJ351, D39 and TIGR4 strains in the BAL fluid.

A. BAL FLUID												
	PJ351				D39				TIGR4			
	0 h	6 h	12 h	24 h	0 h	6 h	12 h	24 h	0 h	6 h	12 h	24 h
Mean	62868	207242	72953	132097	294200	294200	308250	77870	39870	200896	3596	5101
Std. Deviation	35201	59631	72429	103572	133915	133915	253464	86580	30203	158145	3477	8133
Lower 95% Ci of mean	19160	133200	-16980	3495	127921	127921	-6468	-29633	2368	4532	-720.5	-4997
Upper 95% Ci of mean	106576	281284	162886	260699	460479	460479	622968	185373	77372	397261	7913	15200

B. BAL FLUID												
	PJ351				D39				TIGR4			
	0 h	6 h	12 h	24 h	0 h	6 h	12 h	24 h	0 h	6 h	12 h	24 h
Coefficient of variation (CV)	56%	29%	99%	78%	49%	46%	82%	111%	76%	79%	97%	159%

Table 3.3 Standard deviations and coefficients of variations based on the mean bacterial counts and of PJ351, D39 and TIGR4 strains in the lungs.

A. LUNGS												
	PJ351				D39				TIGR4			
	0 h	6 h	12 h	24 h	0 h	6 h	12 h	24 h	0 h	6 h	12 h	24 h
Mean	53970	84170	152789	453630	362393	275700	275700	362393	49615	70072	12830	14210
Std. Deviation	36263	71723	138232	46579	318907	100713	100713	318907	72998	55498	28233	26756
Lower 95% Ci of mean	8943	-4886	-18850	395794	-33585	150648	150648	-33585	-41024	1162	-22226	-19013
Upper 95% Ci of mean	98997	173226	324428	511466	758371	400753	400753	758371	140255	138982	47886	47433
Coefficient of variation (CV)	67%	85%	90%	10%	70%	41%	37%	88%	147%	79%	220%	188%

3.1.2 Construction of pneumococcal gene deletion knockouts in PJ351 and D39 strains

To demonstrate the usefulness of the model to study pneumococcal virulence, *pspC*, *pspA*, *slrA*, *igA1*, *stkP* and *ppmA* knockout strains were constructed by in-frame insertion deletion mutagenesis (IDM). The genes encoding the above-mentioned genes were partially deleted from the parental strains PJ351 and D39 using the resistance cassette *dfr13*, encoding resistance to trimethoprim (Figure 3.2A-Figure 3.7D). These proteins were chosen due to their known involvement in virulence.

The left and right flanks were successfully amplified using the primer pairs P_{1Fwd} + P_{2Rev} and P_{3Fwd} + P_{4Rev}. In order to partially delete the gene of interest, the left and right flanks were ligated to the *dfr13* resistance marker cassette. The fragments of genes that were deleted were of equal length (500 bp) to the trimethoprim inserted cassette to eliminate the possibility of any polar effects. The *dfr13* was introduced in such a way that it would be under the control of the promoter from the deleted gene. The ligated product was subsequently inserted into the pbluescript vector. To ensure that the orientation of the ligated product was maintained and to prevent self-ligation of the plasmid, two different enzymes digested the pbluescript vector. To demonstrate that the correct flanks were ligated to the *dfr13* resistance marker cassette, PCR was performed using the primer pairs P_{1Fwd} + P_{2Rev} for the left flank + *dfr13* and primer pairs P_{3Fwd} + P_{4Rev} for the right flank + *dfr13* (Figure 3.2A-Figure 3.7D). Further, the plasmid constructs were sequenced, and it was found that the knockout constructs were as intended and that the DNA sequence was intact. The deletion constructs were then transformed into the PJ351 and D39 strains of *S. pneumoniae*. Deletion knockout strains were successfully obtained, and the deletion constructs were confirmed using the genomic DNA of the knockout strains as a template to amplify the junctions of the left and right flanks surrounding the deleted target genes (Figure 3.2E-Figure 3.7E). PCR across all genes using P_{1Fwd} and P_{2Rev} primers generated similar sized bands in the wild-type and knockout strains because the *dfr13* gene and the deleted genes of interest were of similar size (Figure 3.2F-Figure 3.7F).

To obtain the streptomycin-resistant *S. pneumoniae* PJ351 and D39 strains, the *rpsL* gene encoding a streptomycin-resistant knockout of the ribosomal protein S12 was amplified from a streptomycin resistant *S. pneumoniae* with primer pair *rpsLFWD* and *rpsLRV* (refer to materials and methods). This PCR product was introduced into PJ351 and D39 by CSP-1- and

CSP-2-induced transformation, respectively, and selected for streptomycin resistance. Confirmation of the point mutation was confirmed by DNA sequencing.

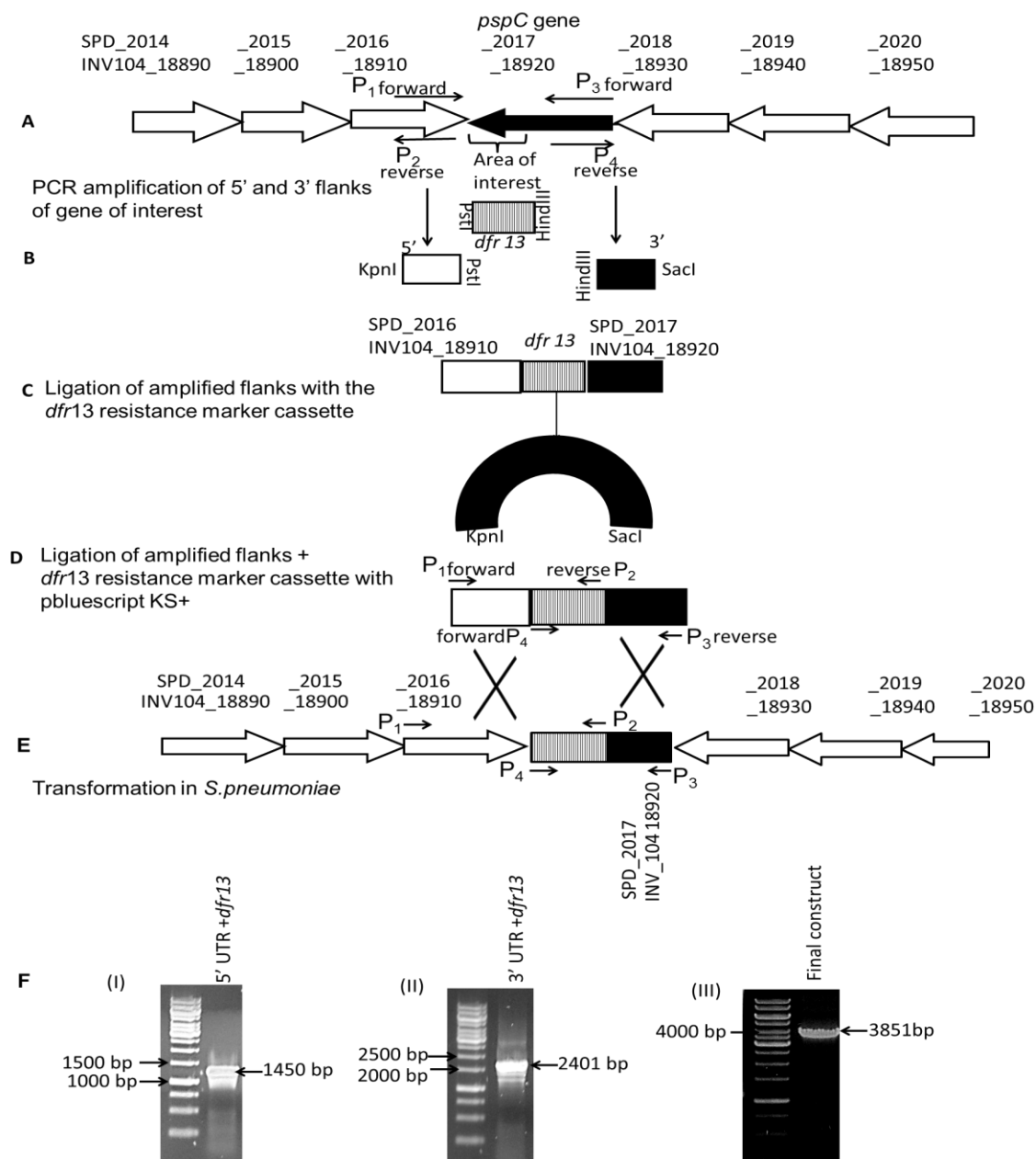


Figure 3.2 Schematic diagram of the deletion of *pspC* in PJ351 and D39 by in-frame insertion deletion mutagenesis. (A, B) The SPD_2017/INV104_18920 gene, shaded in black, was chosen for deletion. (SPD_2017 and INV104_18920 are different annotations for the same gene in different strains). Small black arrows indicate the forward and reverse primers used for IDM to obtain the SPD_2017/INV104_18920 deletion constructs by replacing part of the SPD_2017/INV104_18920 genes with the trimethoprim gene (*dfr13*). (C) IDM to obtain the SPD_2017/INV104_18920 deletion constructs by replacing part of SPD_2017/INV104_18920 with the trimethoprim genes (*dfr13*). (D) Successful ligation of the PCR product into pbluescript was confirmed by PCR with primers P₁+P₂ and P₃+P₄. (E) Representation of the product of transformation of the Δ SPD_2017/INV104_18920 constructs into the D39 and PJ351 *S. pneumoniae* strain. (F) **Gel electrophoresis images.** Analysis of amplicons used to construct SPD_2017/INV104_18920 deficient strains. (I) 5'UTR+*dfr13* (upstream flank+*dfr13*) (II) 3'UTR+*dfr13* (downstream flank+*dfr13*) (III) Final construct (upstream +downstream flank+*dfr13*). Expected sizes for the amplicons were (I) 1382 bp (II) 2230 bp and (III) 3600 bp.

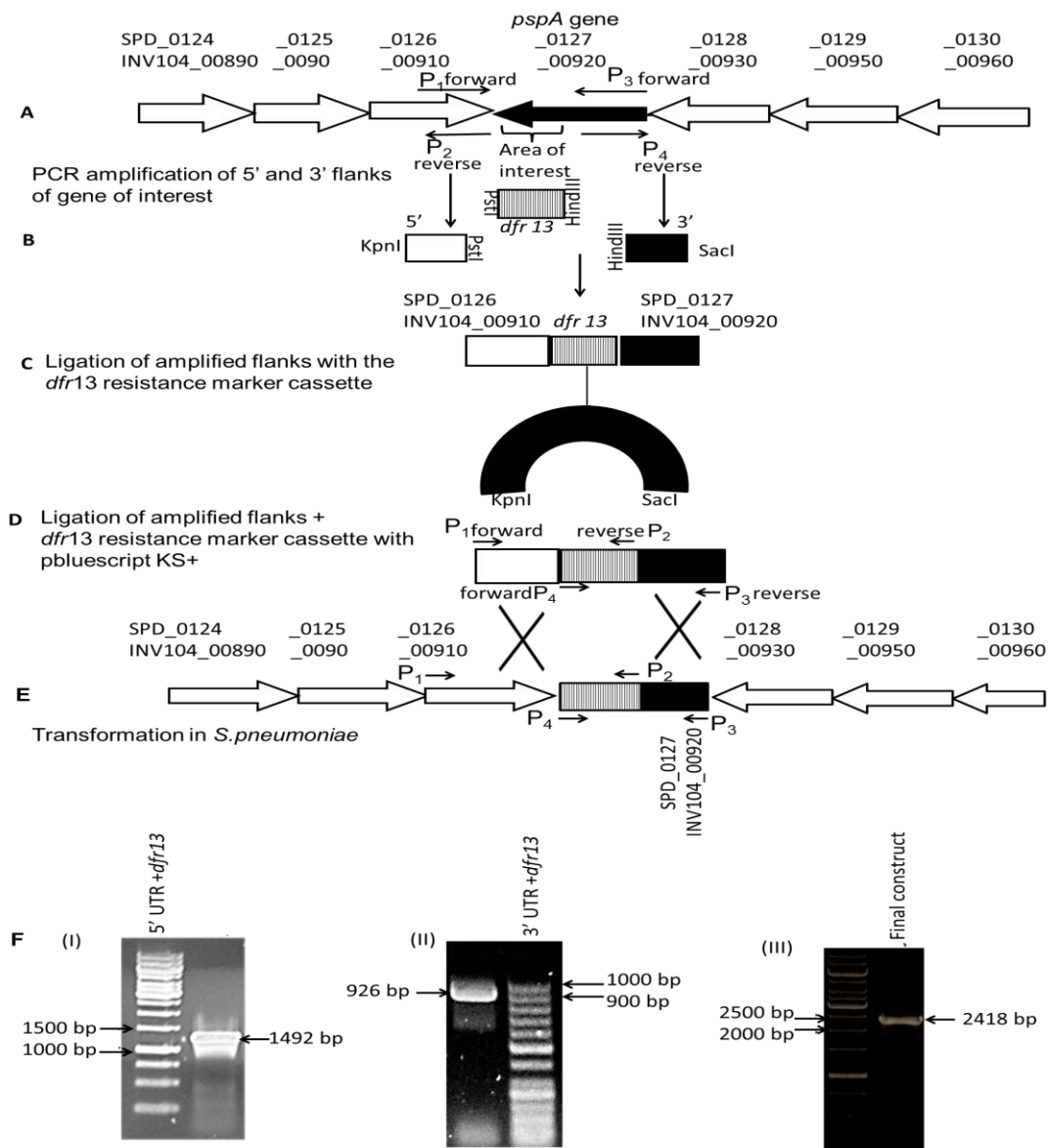


Figure 3.3 Schematic diagram of the deletion of *pspA* in PJ351 and D39 by in-frame insertion deletion mutagenesis. (A, B) The SPD_0127/INV104_00920 gene, shaded in black, was chosen for deletion. (SPD_0127 and INV104_00920 are different annotations for the same gene in different strains). Small black arrows indicate the forward and reverse primers used for IDM to obtain the SPD_0127/INV104_00920 deletion constructs by replacing part of the SPD_0127/INV104_00920 genes with the trimethoprim gene (*dfr13*). (C) IDM to obtain the SPD_0127/INV104_00920 deletion constructs by replacing part of SPD_0127/INV104_00920 with the trimethoprim gene (*dfr13*). (D) Successful ligation of the PCR product into pbluescript was confirmed by PCR with primers P₁+P₂ and P₃+P₄. (E) Representation of the product of transformation of the ΔSPD_0127/INV104_00920 constructs into the D39 and PJ351 *S. pneumoniae* strains. (F) **Gel electrophoresis images.** Analysis of amplicons used to construct SPD_0127/INV104_00920 deficient strains. (I) 5' UTR+*dfr13* (upstream flank +*dfr13*), (II) 3'UTR+*dfr13* (downstream flank+*dfr13*) (III) Final construct (upstream+downstream flank+*dfr13*). Expected sizes for the amplicons were (I) 1382 bp (II) 2230 bp and (III) 3600bp.

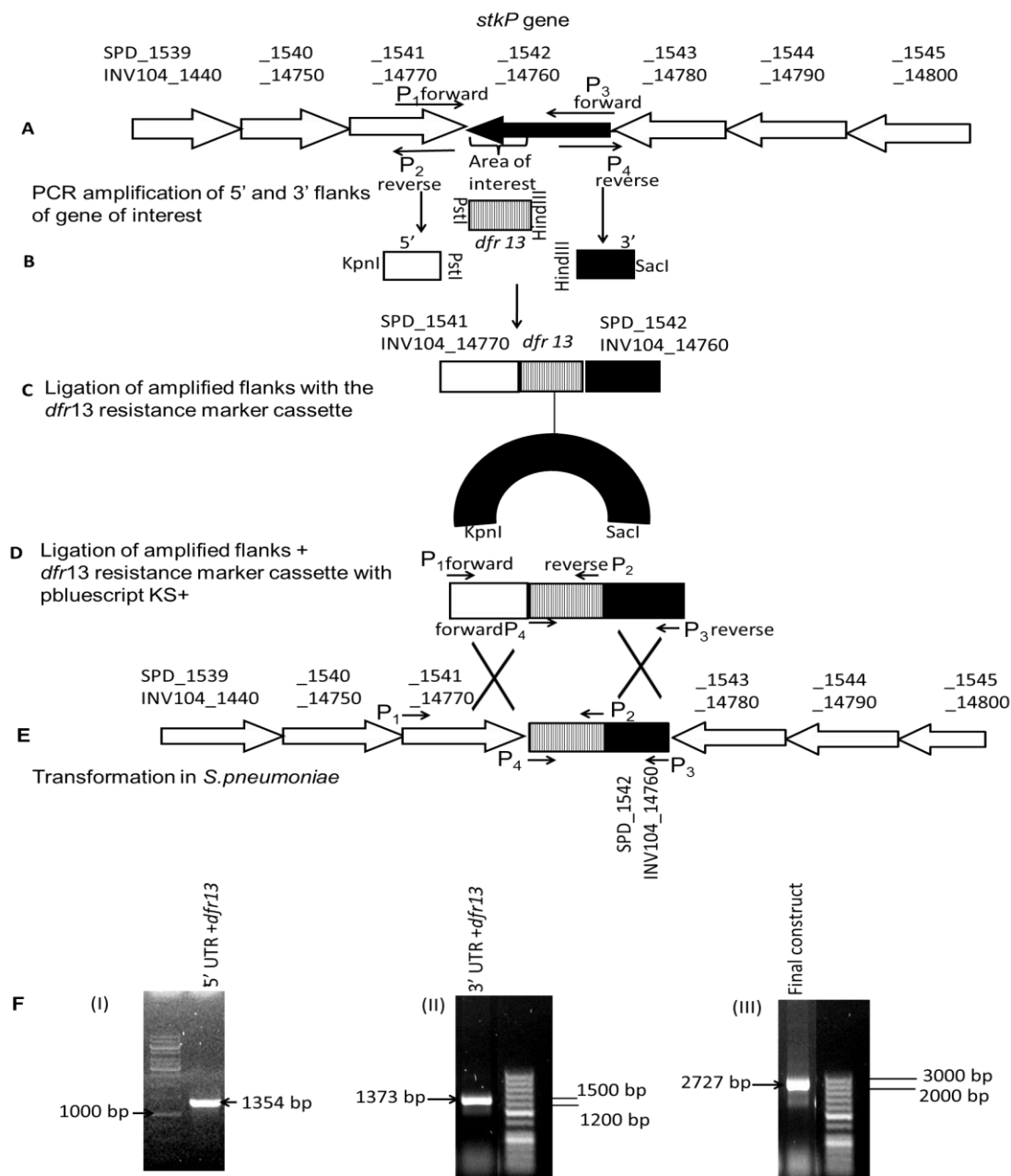


Figure 3.4 Schematic diagram of the deletion of *stkP* in PJ351 and D39 by in-frame insertion deletion mutagenesis. (A, B) The SPD_1542/INV104_14760 gene, shaded in black, was chosen for deletion. (SPD_1542 and INV104_14760 are different annotations for the same gene in different strains). Small black arrows indicate the forward and reverse primers used for IDM to obtain the SPD_1542/INV104_14760 deletion constructs by replacing part of the SPD_1542/INV104_14760 genes with the trimethoprim gene (*dfr13*). (C) IDM to obtain the SPD_1542/INV104_14760 deletion constructs by replacing part of SPD_1542/INV104_14760 with the trimethoprim gene (*dfr13*). (D, E) Successful ligation of the PCR product into pbluescript was confirmed by PCR with primers P₁+P₂ and P₃+P₄ (E) Representation of the products of transformation of the ΔSPD_1542/INV104_14760 constructs into the D39 and PJ351 *S. pneumoniae* strains. (F) **Gel electrophoresis images.** Analysis of amplicons used to construct SPD_1542/INV104_14760 deficient strains. (I) 5'UTR+*dfr13* (upstream flank+*dfr13*) (II) 3'UTR+*dfr13* (downstream flank+*dfr13*) (III) Final construct (upstream+downstream flanks+*dfr13*). Expected sizes for the amplicons were (I) 1394 bp, (II) 1421 bp and (III) 2815 bp.

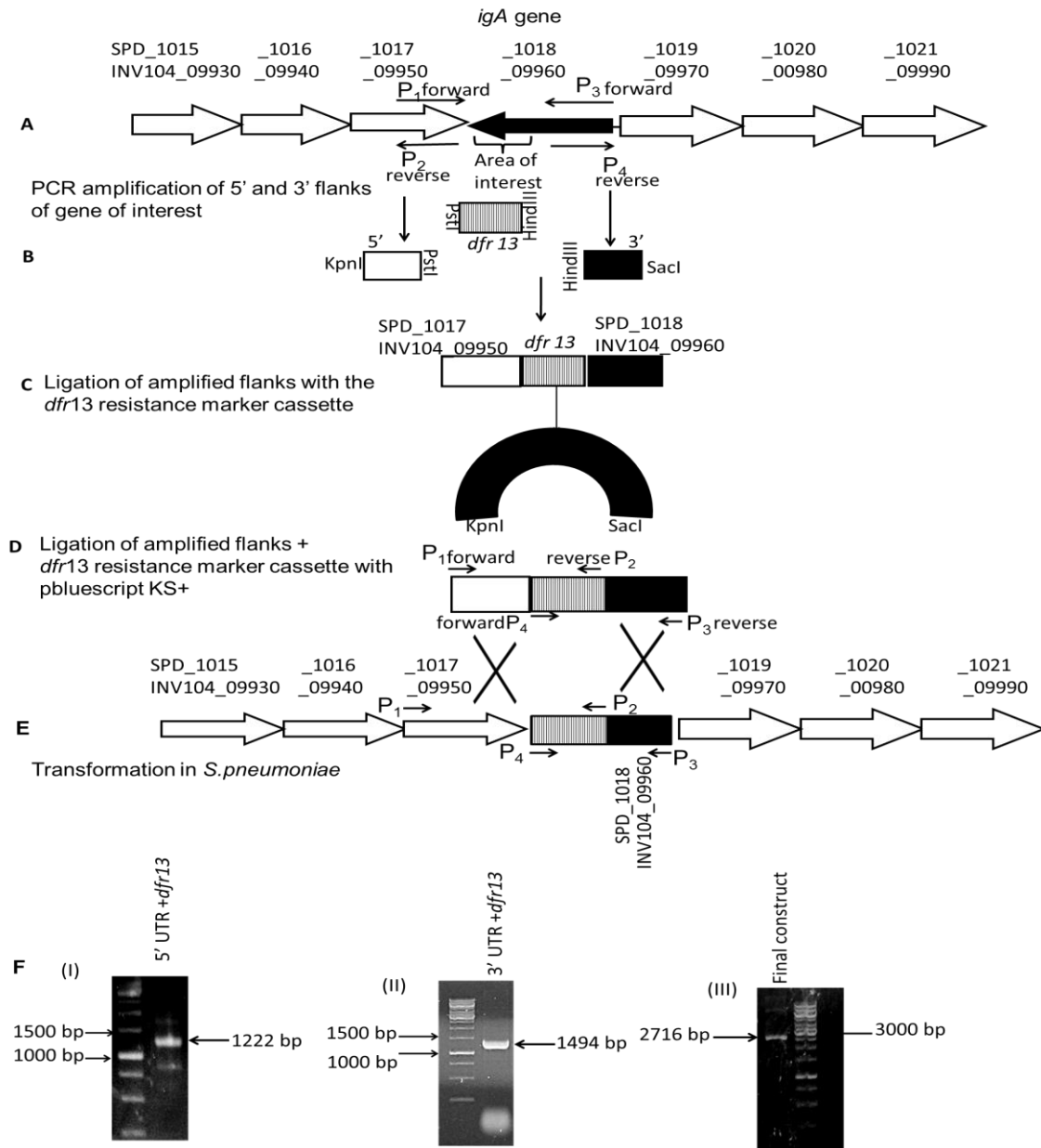


Figure 3.5 Schematic diagram of the deletion of *igA1* in PJ351 and D39 by in-frame insertion deletion mutagenesis. (A, B) The SPD_1018/INV104_09960 gene, shaded in black, was chosen for deletion. (SPD_1018 and INV104_09960 are different annotations for the same gene in different strains). Small black arrows indicate the forward and reverse primers used for IDM to obtain the SPD_1018/INV104_09960 deletion constructs by replacing part of the SPD_1018/INV104_09960 genes with the trimethoprim gene (*dfr13*). (C) IDM to obtain the SPD_1018/INV104_09960 deletion construct by replacing part of SPD_1018/INV104_09960 with the trimethoprim gene (*dfr13*). (D) Successful ligation of the PCR product into pbluescript was confirmed by PCR with primers P₁+P₂ and P₃+P₄. (E) Representation of the product of transformation of the ΔSPD_1018/INV104_09960 construct into the D39 and PJ351 *S. pneumoniae* strain. (F) **Gel electrophoresis images.** Analysis of amplicons used to construct SPD_1018/INV104_09960 deficient strains. (I) 5' UTR + *dfr13* (upstream flank + *dfr13*), (II) 3' UTR + *dfr13* (downstream flank + *dfr13*), (III) Final construct (upstream + downstream flanks + *dfr13*) Expected sizes for the amplicons were (I) 1490 bp, (II) 1494 bp and (III) 2984 bp.

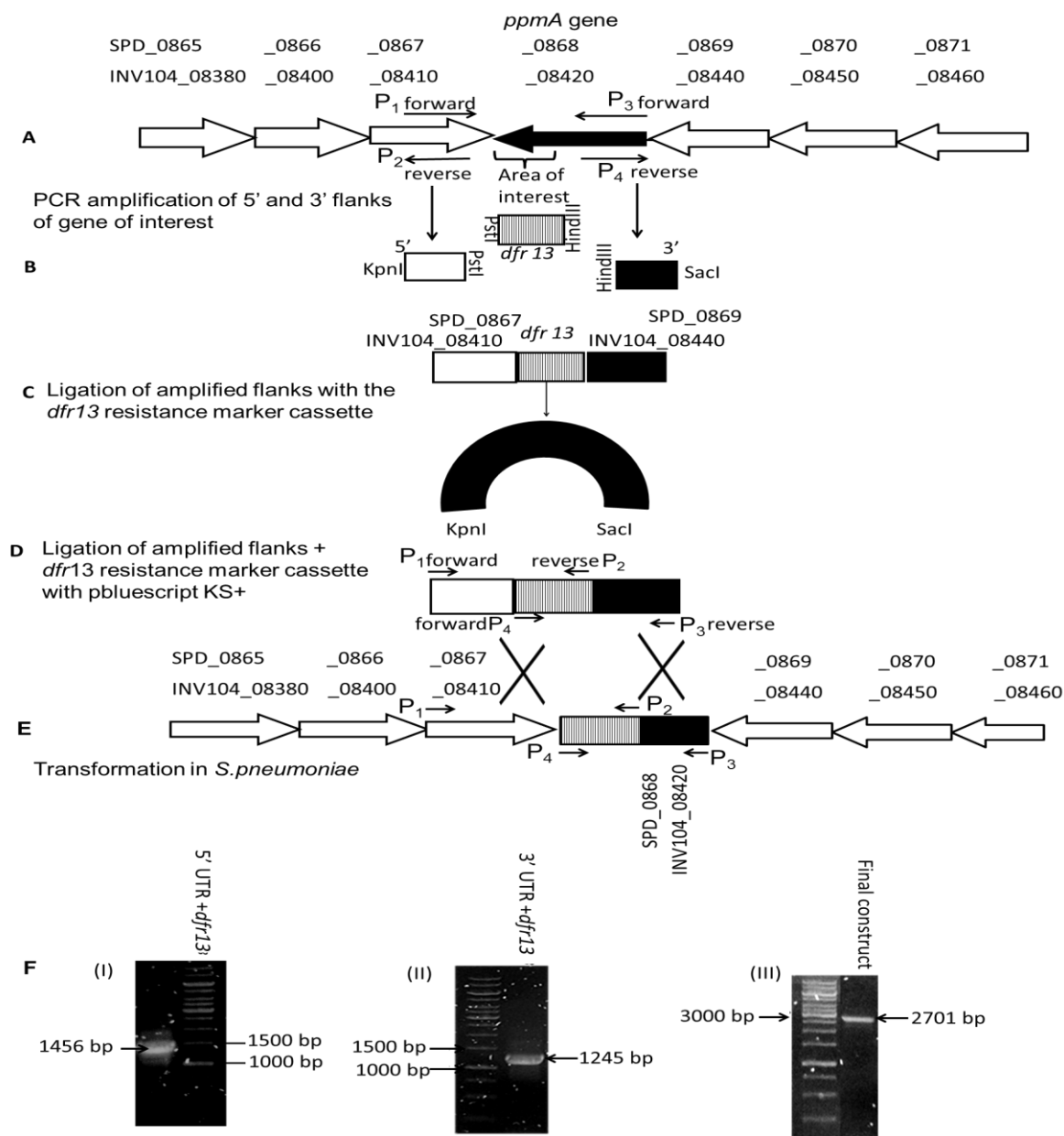


Figure 3.6 Schematic diagram of the deletion of *ppmA* in PJ351 and D39 by in-frame insertion deletion mutagenesis. (A, B) The SPD_0868/INV104_08420 gene, shaded in black, was chosen for deletion. (SPD_0868 and INV104_08420 are different annotations for the same gene in different strains). Small black arrows indicate the forward and reverse primers used for IDM to obtain the SPD_0868/INV104_08420 deletion constructs by replacing part of the SPD_0868/INV104_08420 genes with the trimethoprim gene (*dfr13*). (C) IDM to obtain the SPD_0868/INV104_08420 deletion constructs by replacing part of SPD_0868/INV104_08420 with the trimethoprim gene (*dfr13*). (D) Successful ligation of the PCR product with pbluescript was confirmed by PCR with primers P₁+P₂ and P₃+P₄ (E) Representation of the product of transformation of the Δ SPD_0868/INV104_08420 construct into the D39 and PJ351 *S. pneumoniae* strain. (F) **Gel electrophoresis images.** Analysis of amplicons used to construct SPD_0868/INV104_08420 deficient strains. (I) 5' UTR+*dfr13* (upstream flank+*dfr13*) (II) 3' UTR+*dfr13* (downstream flank+*dfr13*) (III) Final construct (upstream +downstream+*dfr13*). Expected sizes for the amplicons were (I) 1464 bp (II) 1249 bp and (III) 2713 bp.

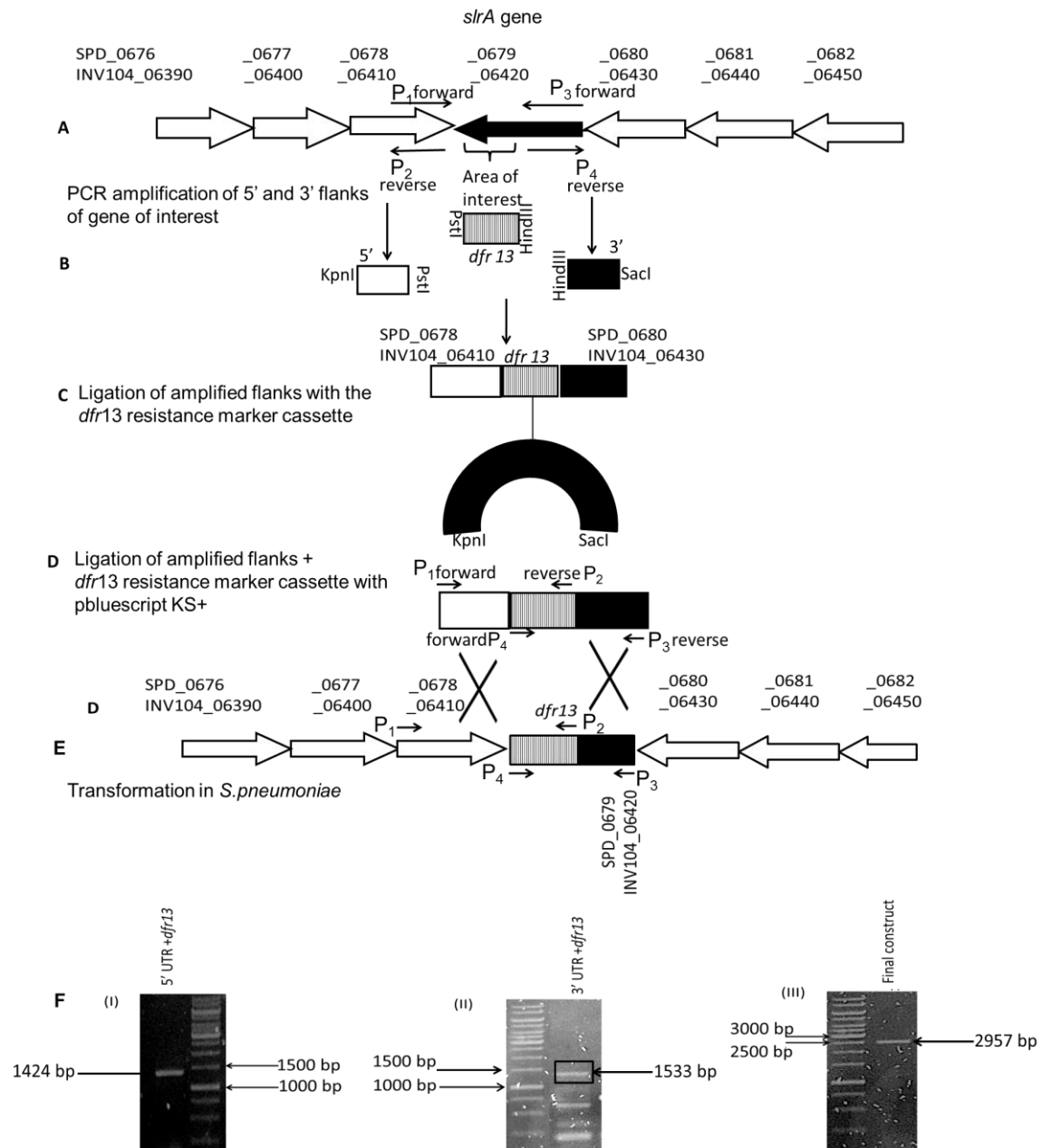


Figure 3.7 Schematic diagram of the deletion of *slrA* in PJ351 and D39 by in-frame insertion deletion mutagenesis. (A, B) The SPD_0679/INV104_06420 gene, shaded in black, was chosen for deletion. (SPD_0679 and INV104_06420 are different annotations for the same gene in different strains). Small black arrows indicate the forward and reverse primers used for IDM to obtain the SPD_0679/INV104_06420 and deletion constructs by replacing part of the gene with the trimethoprim gene (*dfr13*). (C) IDM to obtain SPD_0679 /INV104_06420 deletion constructs by replacing part of the gene with the trimethoprim gene (*dfr13*). (D) Successful ligation of the PCR product with pbluescript was confirmed by PCR with primers P₁+P₂ and P₃+P₄ (E) Representation of the product of transformation of the ΔSPD_0679/INV104_06420 constructs into the D39 and PJ351 *S. pneumoniae* strain. (F) **Gel electrophoresis images.** Analysis of amplicons used to construct SPD_0679/INV104_06420 deficient strains. (I) 5' UTR + *dfr13* (upstream flank+*dfr13*), (II) 3 UTR+ *dfr13* (downstream flank+*dfr13*), (III) Final construct (upstream +downstream+*dfr13*). Expected sizes for the amplicons were (I) 1434 bp, (II) 1494 bp and (III) 2928 bp.

All the knockout strains, with the exception of the *stkP* knockout, exhibited growth in THY broth that was similar to the wild-type strain (Figure 3.8-Figure 3.9). Since this study involves the co-inoculation of strains into a single mouse and the output was based on the ratio of the two strains, the *stkP* gene was excluded from further analysis because it was attenuated in growth rate compared to the wild-type strain *in vitro*. Furthermore, this attenuation was very severe *in vivo*, and by 24 hours, this strain was not detectable at all sites that were tested within the mouse. All the isogenic knockouts mentioned above were checked for the stability of the mutation by culturing the knockout strains in THY broth in the absence of antibiotic (and therefore with no selective pressure for the mutation) for two 8 hour growth cycles followed by plating onto antibiotic-free and antibiotic-supplemented plates. *S. pneumoniae* knockout strains had a similar number of colonies on the antibiotic-free and antibiotic-supplemented plates confirming the mutation was stable over these time periods.

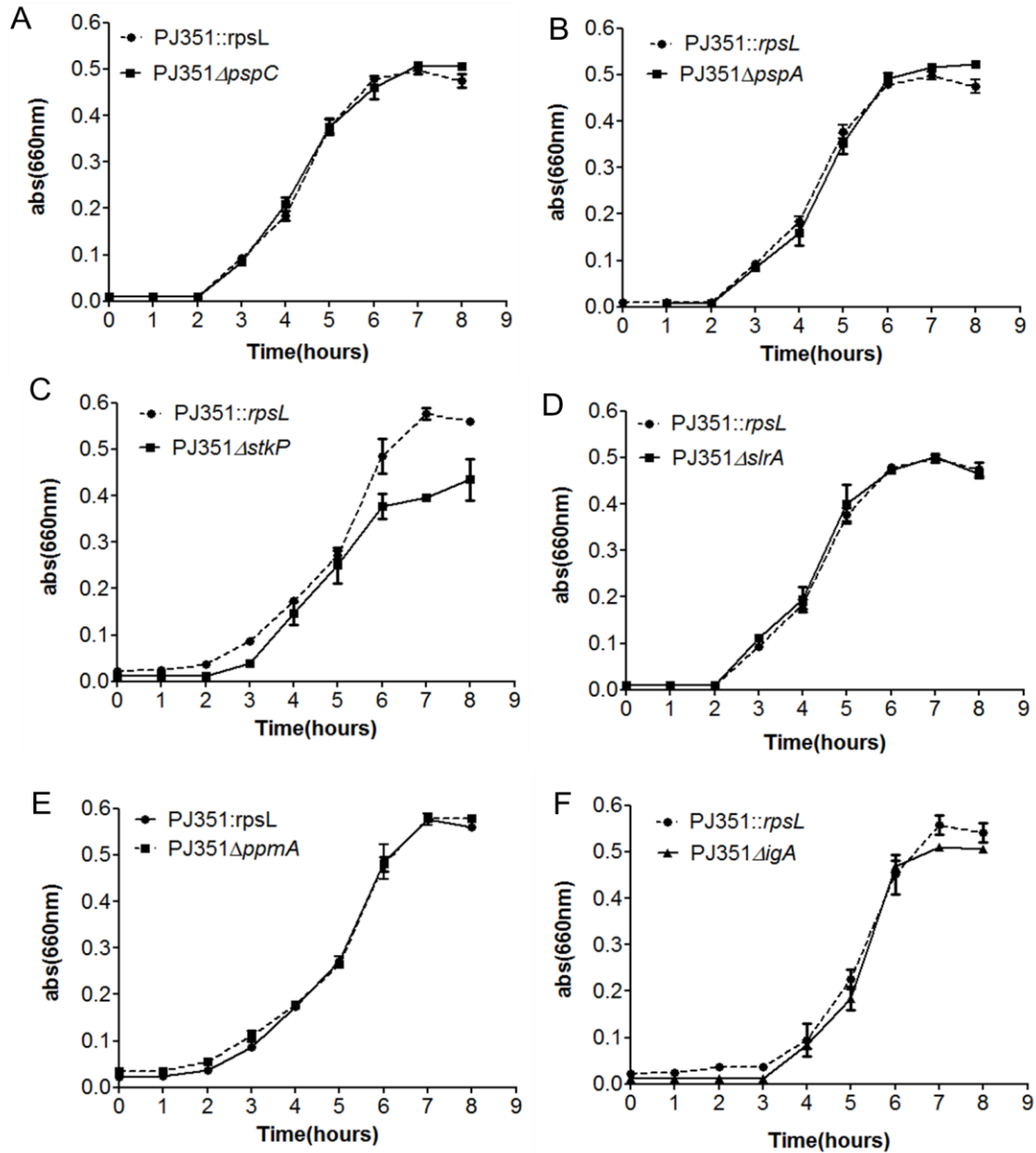


Figure 3.8 Growth curves of PJ351 pneumococcal knockout strains in comparison with their PJ351 isogenic wild-type strain under standard *in vitro* growth conditions. An overnight culture was prepared with 1 colony in 5 mL of THY broth. The tubes were incubated at 37 °C in 5% CO₂ atmosphere for 10 hours. A 1 mL aliquot of the overnight culture was subcultured in 50 mL of THY broth and the OD₆₀₀ nm was measured every hour. Results presented are the mean ODs of triplicate samples for each strain and error bars indicate standard deviations between the samples.

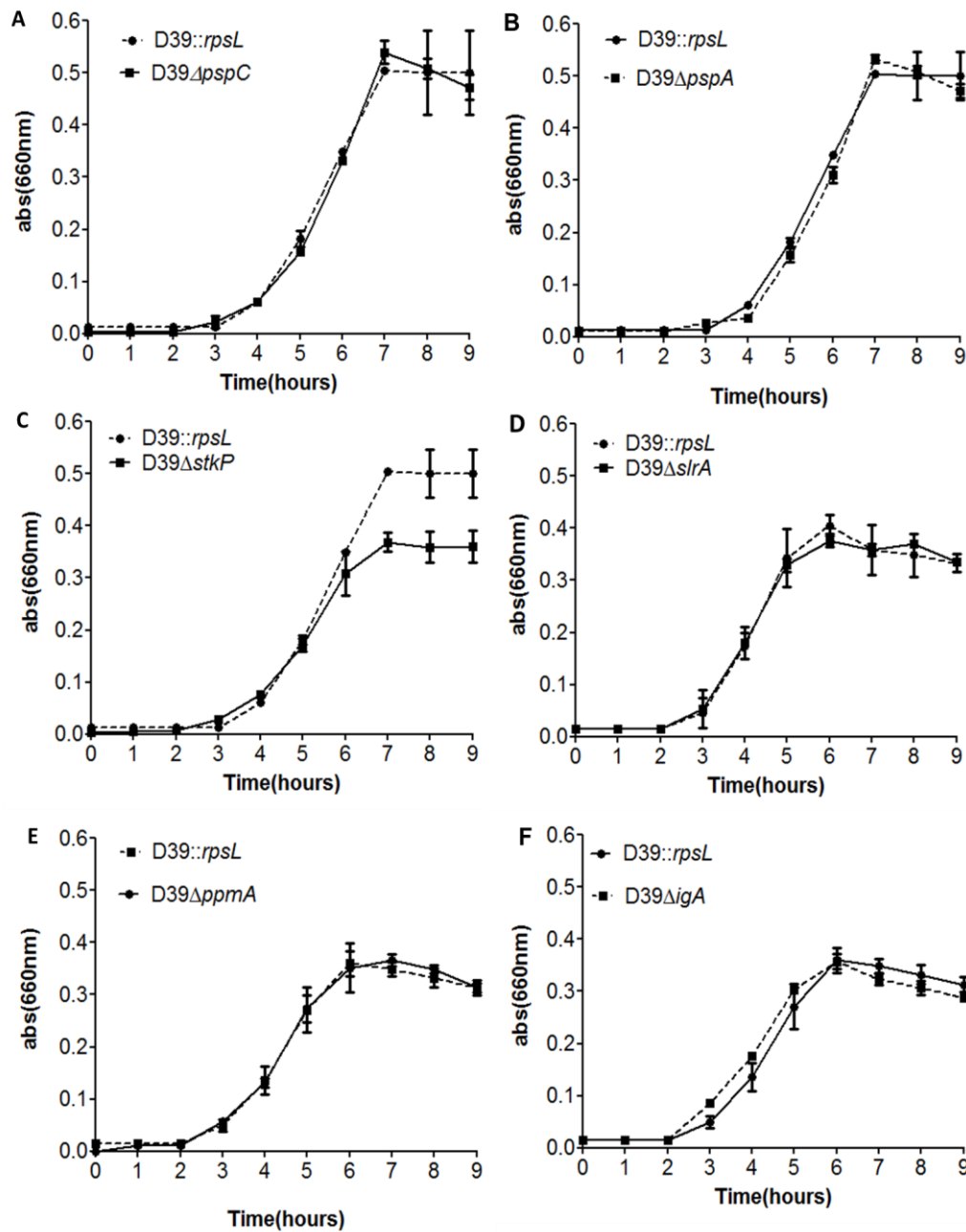


Figure 3.9 Growth curves of D39 pneumococcal knockout strains in comparison with their D39 isogenic wild-type strain under standard *in vitro* growth conditions. An overnight culture was prepared with 1 colony in 5 mL of THY broth. The tubes were incubated at 37 °C in 5% CO₂ atmosphere for 10 hours. A 1 mL aliquot of the overnight culture was subcultured in 50 mL of THY broth and the OD₆₀₀ nm was measured every hour. Results presented are the mean ODs of triplicate samples for each strain and error bars indicate standard deviations between the samples.

3.1.3 Validation of an intranasal pneumonia co-inoculation model in the presence of circulating antibodies

In order to demonstrate the proof of concept of using the co-inoculation model to evaluate pneumococcal proteins as potential vaccine antigens, we investigated the dynamics of *S. pneumoniae* presence or survival within a host. The underpinning theory being, that a knockout strain in an unvaccinated mouse should have identical growth characteristics to a knockout strain growing in a vaccinated mouse, where the antibodies present will have no impact in the absence of the antibody target. Should the growth characteristics of the knockout be identical in the vaccinated and unvaccinated host backgrounds, the knockout will serve as an internal calibrator with which to monitor the impact of opsonising antibodies on a co-inoculated wild-type strain which has the homotypic antigen present. In this setup, a ratio of 1 or closer to 1 indicated an equal recovery of knockout bacterial load in placebo and vaccinated mice. Wild-type strains in unvaccinated and vaccinated mice were also monitored for growth; wherein the former group could outcompete or grow at a similar rate to the latter group, depending on the impact that the presence of the antibodies will have on the antigen. A ratio of higher than 1 indicated an unequal recovery of bacterial load in both groups.

NMRI mice (n = 5 per time point) were subcutaneously vaccinated with pneumococcal proteins PpmA, PspC or adjuvant (control), once every two weeks for a period of four weeks. Mice were subsequently challenged intranasally (10^6 CFU total), with the wild-type PJ351 (serotype 1) or D39 (serotype 2) and their isogenic PpmA or PspC knockouts 2 weeks after the last immunisation. Mice were sacrificed at 0, 6, 12 and 24 hours post-inoculation. Colony counts of wild-type and isogenic knockouts were performed from the nasopharyngeal wash, BAL fluid and lung tissue samples as described in materials and methods, on selective media. A ratio was calculated as the total bacterial load of knockout strains in the placebo group divided with that of the vaccinated group.

The number of PJ351 and D39 pneumococcal cells per sample from the nasopharynx following inoculation with 10^6 CFU of pneumococci was around 10^4 CFU. PJ351 and D39 bacterial counts were maintained at 10^5 CFU in BAL fluid and lungs for the duration of the experiment. There were no statistically significant differences in bacterial numbers of PJ351 knockout bacteria from the nasopharynx, BAL fluid and lungs of placebo and recombinant PspC or PpmA vaccinated mice at all time points observed. The ratio between the two groups

was closely maintained at 1 or close to 1 at all-time points shown. On the other hand, there were statistically significant differences in bacterial numbers of PJ351 wild-type bacteria at all sites sampled from the placebo and recombinant PspC and PpmA vaccinated mice, at most time points. The ratio between the two groups was higher than 1 at most time points, with ratios ranging from 2.3:2.3 – 97:97 (Figures 3.10 and 3.11).

Results for the D39 knockout bacteria in placebo and recombinant PspC or PpmA vaccinated mice showed no significant differences between the two groups. Ratios between the two groups was closely maintained at 1 or close to 1 at all-time points observed. Bacterial counts for D39 wild-type bacteria recovered from the nasopharyngeal lavage, BAL fluid and lungs of placebo and recombinant PspC vaccinated mice indicated a non-significant difference at 24 hours. The difference in D39 bacterial counts for placebo and recombinant PpmA vaccinated mice was observed in the nasopharynx. Ratios higher than 1 were observed for wild-type strains between placebo and PspC or PpmA vaccinated mice (Figure 3.12 and Figure 3.13).

We have successfully validated the use of this mouse model in the analysis of vaccinated mice data, by demonstrating that the quantitative count of the knockout strain remained the same in both the placebo and vaccinated mice. This was indicated by the ratio of 1 or closer to 1 in all sites sampled, between knockout strains in the placebo and vaccinated mice. Ratios between wild-type strains in the placebo and vaccinated mice were higher than 1 indicating that the wild-type strain was outcompeted in vaccinated mice compared to the wild-type strain in unvaccinated mice. This indicates that results observed in vaccinated mice are due to changes to the wild-type in the presence of circulating antibodies and this shift is unlikely to be influenced by the presence of the knockout strain.

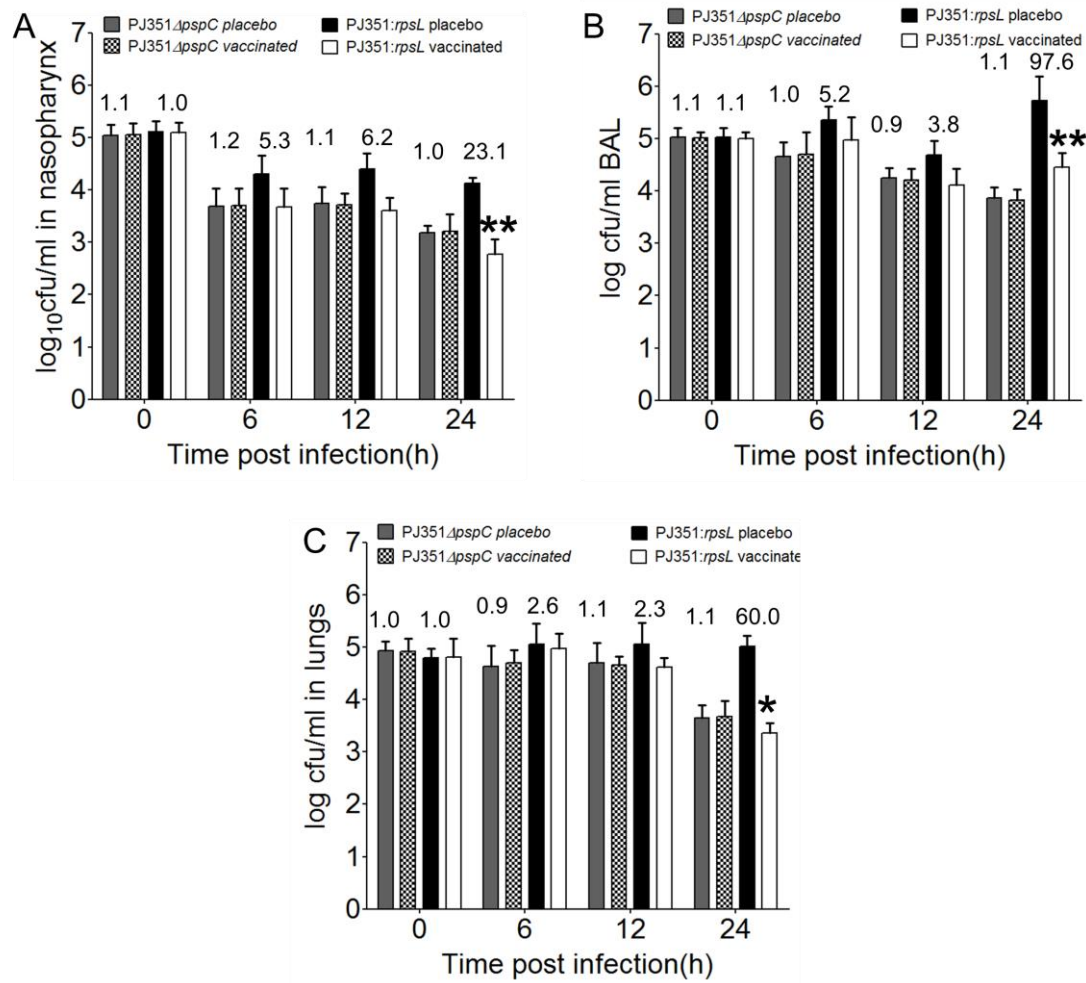


Figure 3.10. Ratio analysis of the PJ351ΔpspC knockout and wild-type strains in placebo and vaccinated groups. Female NMRI mice were infected intranasally with an equal amount (10^6 CFU) of PJ351 streptomycin-resistant wild-type and PJ351ΔpspC strains per 50 μ L dose. Five animals per group were terminated at 6 hour time intervals for 24 hours. Samples from the (A) Nasopharynx (B) BAL fluid and (C) Lungs were plated on appropriate selective blood agar plates. Data are presented as mean CFU with standard deviation from five animals. The ratios given are the mean bacterial load of knockout strains in the placebo group divided with that in the vaccinated group. Data represent average CFUs from a single experiment and error bars indicate standard deviations from five animals. * $p \leq 0.05$ **, $p \leq 0.001$.

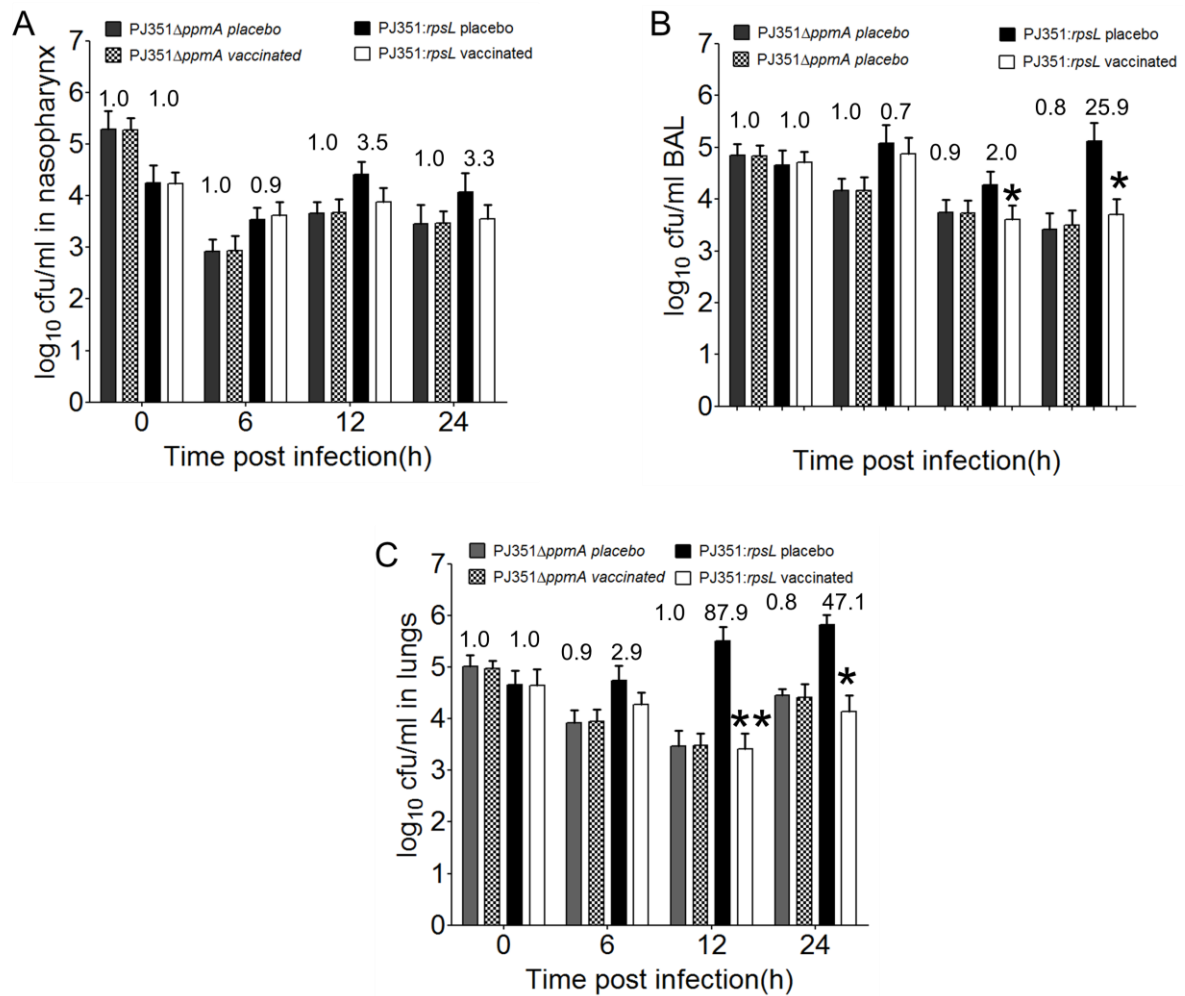


Figure 3.11. Ratio analysis of the PJ351ΔppmA knockout and wild-type strains in placebo and vaccinated groups. Female NMRI mice were infected intranasally with an equal amount (10^6 CFU) of PJ351 streptomycin-resistant wild-type and PJ351ΔppmA strains per 50 μ L dose. Five animals per group were terminated at 6 hour time intervals for 24 hours. Samples from the (A) Nasopharynx (B) BAL fluid and (C) Lungs were plated on appropriate selective blood agar plates. Data are presented as mean CFU with standard deviation from five animals. The ratios given are the mean bacterial load of knockout strains in the placebo group divided with that in the vaccinated group. Data represent average CFUs from a single experiment and error bars indicate standard deviations from five animals. * $p \leq 0.05$, ** $p \leq 0.001$.

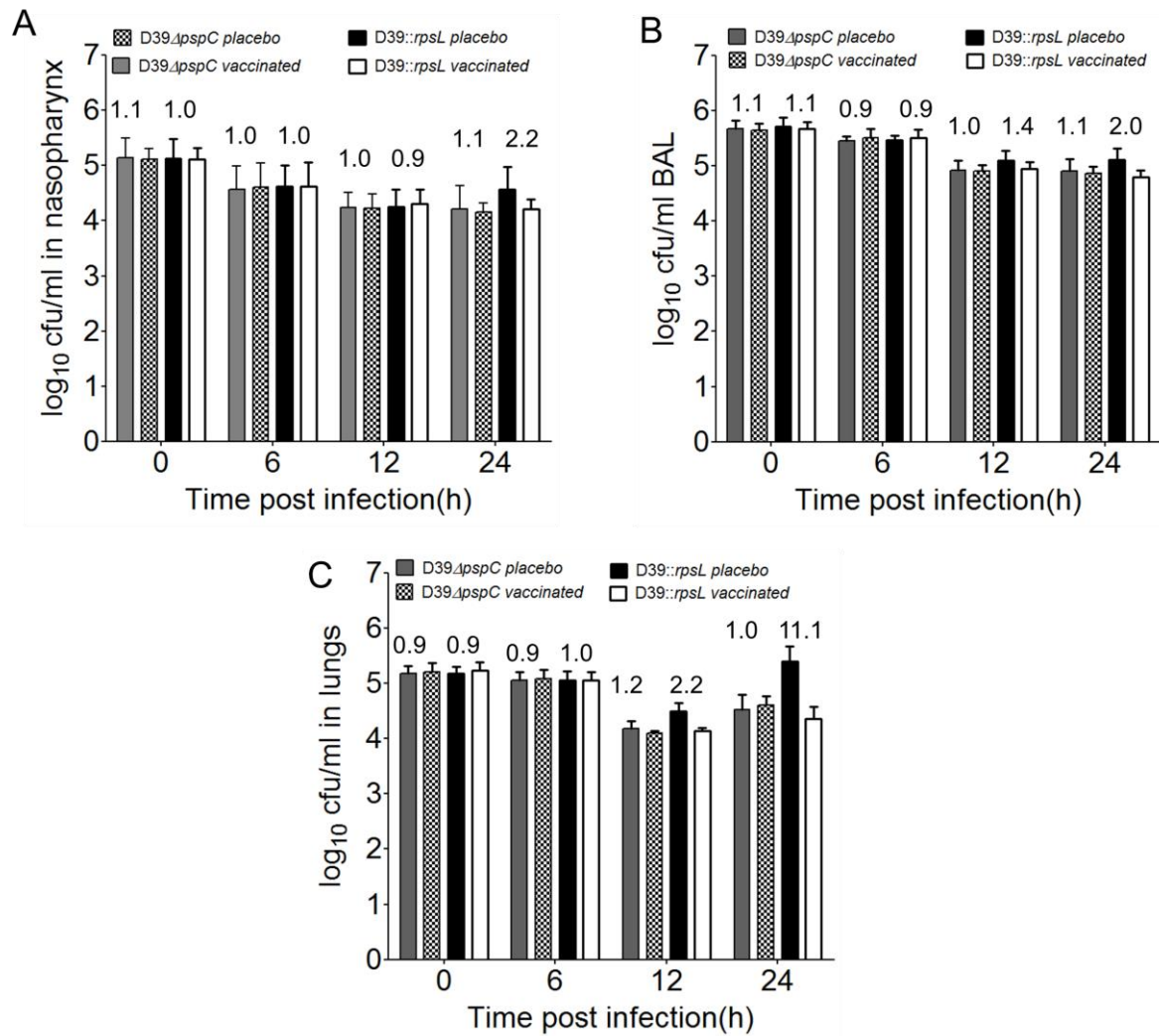


Figure 3.12. Ratio analysis of the D39ΔpspC knockout and wild-type strains in placebo and vaccinated groups. Female NMRI mice were infected intranasally with an equal amount (10^6 CFU) of D39 streptomycin-resistant wild-type and D39ΔpspC strains per 50 μ L dose. Five animals per group were terminated at 6 hour time intervals for 24 hours. Samples from the (A) Nasopharynx (B) BAL fluid and (C) Lungs were plated on appropriate selective blood agar plates. Data are presented as mean CFU with standard deviation from five animals. The ratios given are the mean bacterial load of knockout strains in the placebo group divided with that in the vaccinated group. Data represent average CFUs from a single experiment and error bars indicate standard deviations from five animals.

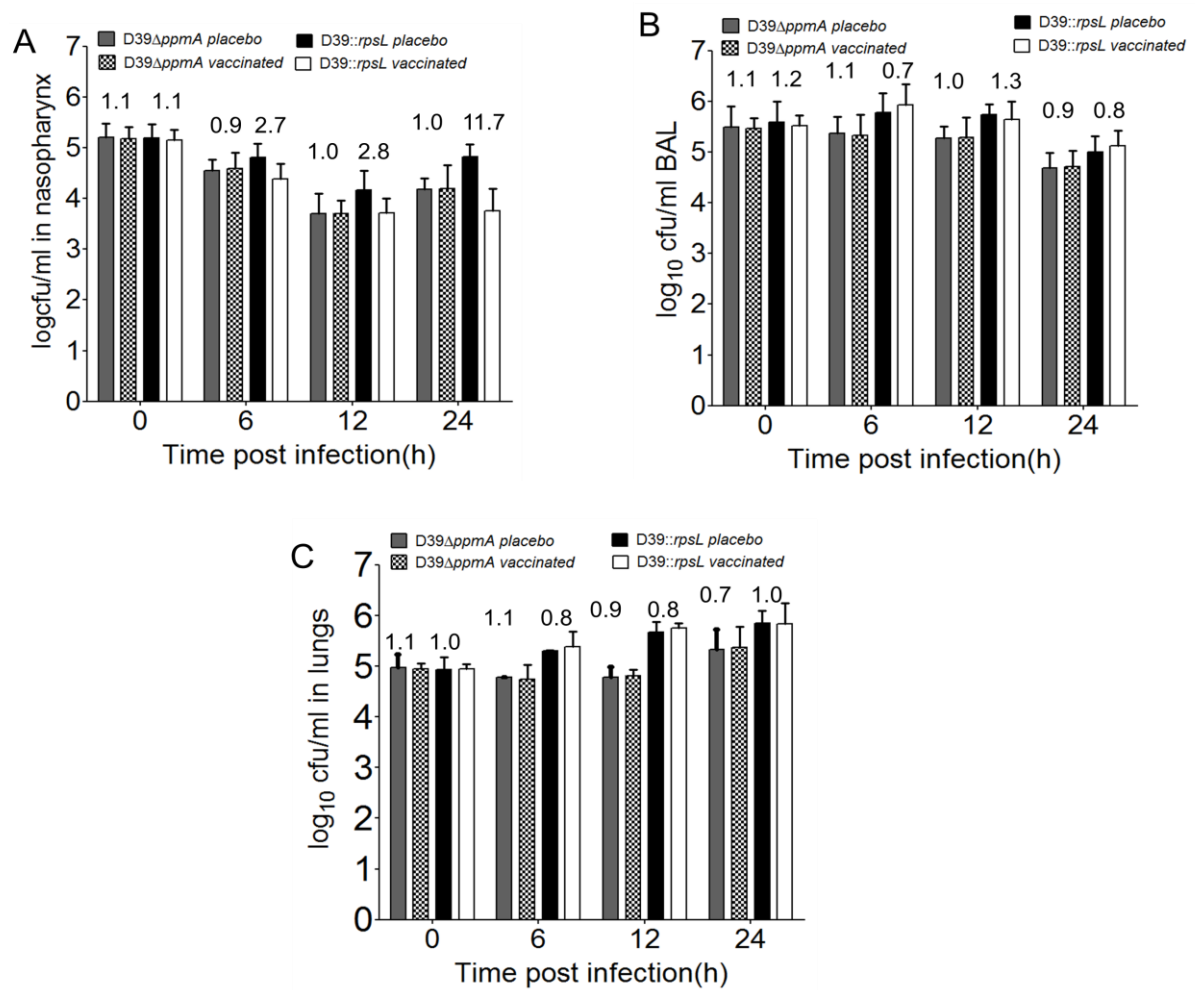


Figure 3.13. Ratio analysis of the D39ΔppmA knockout and wild-type strains in placebo and vaccinated groups. Female NMRI mice were infected intranasally with an equal amount (10^6 CFU) of D39 streptomycin-resistant wild-type and D39ΔppmA strains per 50 μ L dose. Five animals per group were terminated at 6 hour time intervals for 24 hours. Samples from the (A) Nasopharynx (B) BAL fluid and (C) Lungs were plated on appropriate selective blood agar plates. Data are presented as mean CFU with standard deviation from five animals. The ratios given are the mean bacterial load of knockout strains in the placebo group divided with that in the vaccinated group. Data represent average CFUs from a single experiment and error bars indicate standard deviations from five animals.

3.2 GROWTH CHARACTERISTICS OF PNEUMOCOCCAL KNOCKOUTS RELATIVE TO WILD-TYPE STRAINS IN A CO-INOCULATION MODEL IN UNVACCINATED MICE AT MULTIPLE TIME POINTS

Unvaccinated mice were co-inoculated with equal proportions of a PJ351 or D39 wild-type and an isogenic knockout of either *PspC* or *PpmA*. The results were expressed as bacterial counts of the knockout and wild-type bacteria recovered from the nasopharynx, BAL fluid and lungs at multiple time points in each individual mouse.

3.2.1 *PspC* and *ppmA* knockouts in relation to the wild-type PJ351 strain

Throughout the course of the experiment, the PJ351 Δ *pspC* knockout was less able to colonise the nasopharynx of mice compared to the parent strain at 6 hours (4-fold reduction, $p = 0.0002$), 12 hours (5-fold reduction, $p < 0.0001$), and 24 hours (9-fold reduction, $p = 0.0001$) (Figure 3.14 A). A significant difference between the wild-type and the *pspC* knockout from the BAL fluid was seen at 6 hours (5-fold reduction, $p = 0.0001$), 12 hours (4-fold reduction, $p < 0.0001$) and 24 hours (17-fold reduction, $p < 0.0001$) (Figure 3.14 B). Similarly, PJ351 Δ *pspC* was significantly attenuated in its ability to infect the lungs compared to its isogenic wild-type strain starting at 6 hours (3-fold reduction, $p = 0.0002$), 12 hours (2-fold reduction, $p = 0.0010$) and 24 hours (18-fold reduction, $p < 0.0001$) (Figure 3.14 C).

The PJ351 Δ *ppmA* knockout was less able to colonise the nasopharynx of mice compared to the parent strain starting at 6 hours (4-fold reduction, $p < 0.0001$), 12 hours (6-fold reduction, $p = 0.0042$) and 24 hours (4-fold reduction, $p = 0.0035$) (Figure 3.15 A). Deletion of *ppmA* resulted in a significant difference in the ability of the knockout to grow out in the bronchoalveolar tract compared to the wild-type at 6 hours (8-fold reduction, $p = 0.0295$), 12 hours (3-fold reduction, $p = 0.0079$) and 24 hours (91-fold reduction, $p = 0.0017$) (Figure 3.15 B). Similarly in the lungs, the Δ *ppmA* knockout was attenuated compared to the wild-type strain over time at 6 hours (7-fold reduction, $p = 0.0003$), 12 hours (80-fold reduction, $p = 0.0005$) and 24 hours (31-fold reduction, $p < 0.0001$) (Figure 3.15 C).

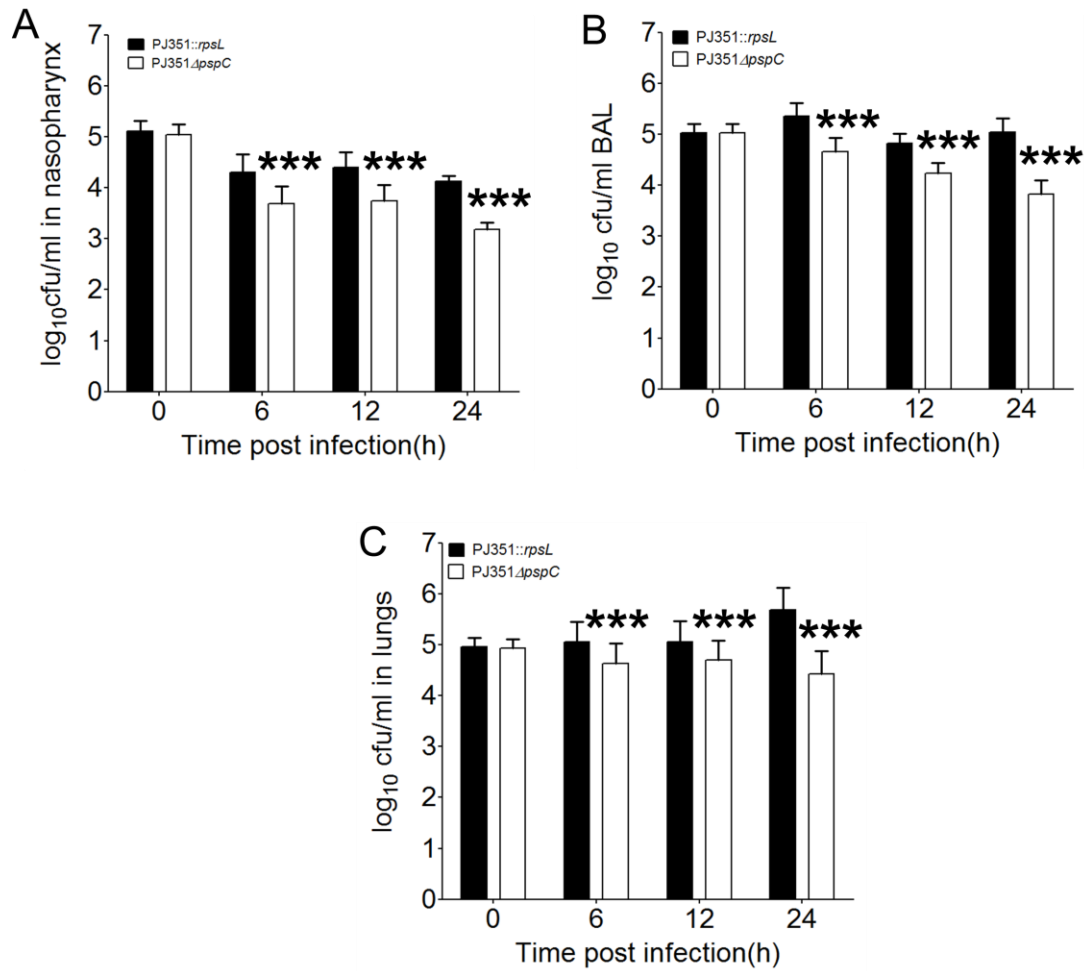


Figure 3.14. Intranasal challenge of unvaccinated mice with streptomycin-resistant pneumococcal strain (PJ351:: *rpsL*) and knockout type strain (PJ351Δ*pspC*). Female NMRI mice were infected intranasally with an equal amount of 10^6 CFU of PJ351 streptomycin-resistant wild-type and PJ351Δ*pspC* strains per 50 μ L dose. Five animals per group were terminated at a 6 hour time interval for 24 hours. Samples from the (A) Nasopharynx (B) BAL fluid and (C) Lungs were plated on appropriate selective blood agar plates. Statistical significance of changes in CFU between the wild-type and knockout strain were analysed with a paired-t- test. Data represent average CFUs from a single experiment and error bars indicate standard deviations from five animals. *** $p \leq 0.0001$.

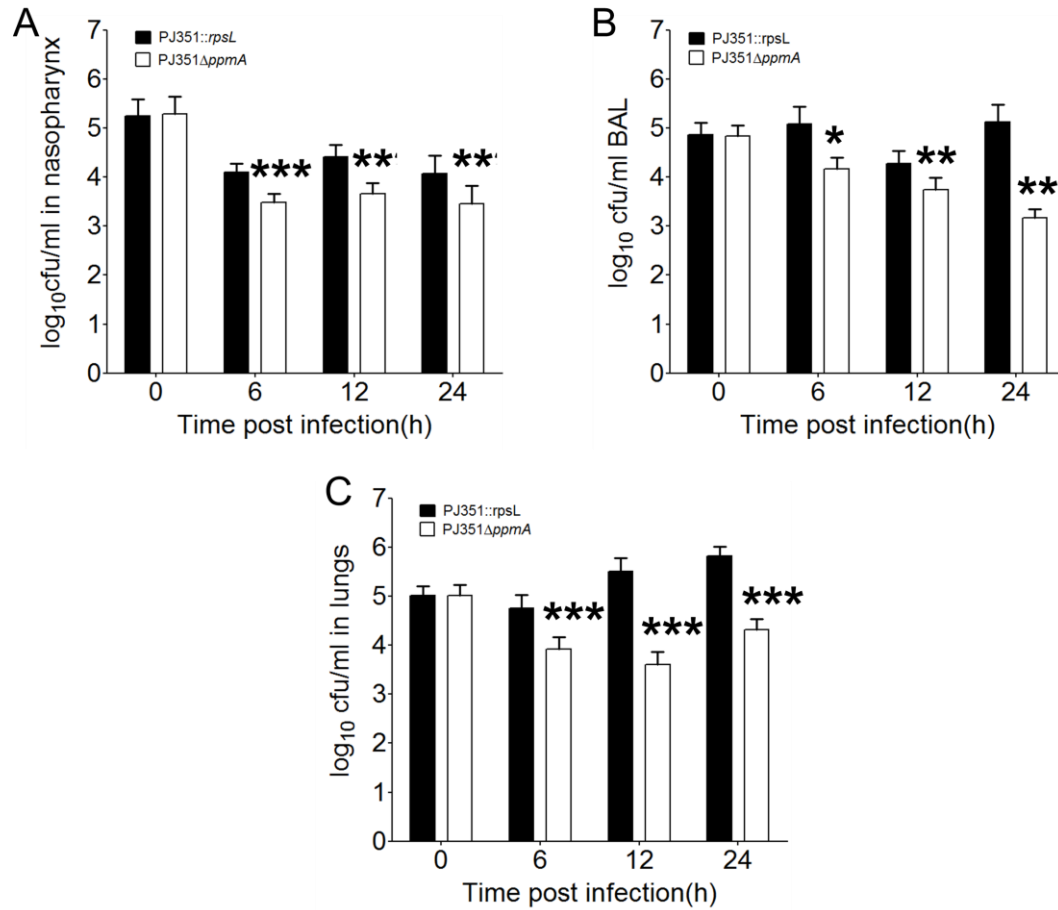


Figure 3.15. Intranasal challenge of unvaccinated mice with streptomycin-resistant pneumococcal strain (PJ351:: *rpsL*) and knockout type strain (PJ351Δ*ppmA*). Female NMRI mice were infected intranasally with an equal amount of 10^6 CFU of PJ351 streptomycin-resistant wild-type and PJ351Δ*ppmA* strains per 50 μ L dose. Five animals per group were terminated at a 6 hour time interval for 24 hours. Samples from the (A) Nasopharynx (B) BAL fluid and (C) Lungs were plated on appropriate selective blood agar plates. Statistical significance of changes in CFU between the wild-type and knockout strain were analysed with a paired-t- test. Data represent average CFUs from a single experiment and error bars indicate standard deviations from five animals * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$.

3.2.2 *pspC* and *ppmA* knockouts in relation to the wild-type D39 strain

In our model, there was a significant decrease in nasopharyngeal colonisation at 24 hours following intranasal inoculation, with D39 Δ *pspC* (2-fold reduction; $p < 0.0001$) compared to the isogenic wild-type strain (Figure 3.16 A). In the BAL fluid, the *pspC* knockout was attenuated relative to the isogenic wild-type strain at 12 hours (2 fold reduction, $p < 0.0001$) and 24 hours (2-fold reduction, $p = 0.0001$) (Figure 3.16 B). Similarly, in lungs there was a significant reduction in the bacterial load between the wild-type and knockout; at 12 hours (2-fold reduction, $p < 0.0001$) and 24 hours (7-fold reduction, $p < 0.0001$) (Figure 3.16 C).

In the nasopharynx of mice, the *ppmA* knockout strain showed significant attenuation in virulence compared to its isogenic parent strain at 12 hours (3-fold reduction, $p = 0.0277$) and 24 hours (5-fold reduction; $p = 0.0001$) (Figure 3.17 A). In the BAL fluid, the attenuation of the *ppmA* knockout relative to the isogenic wild-type strain was observed as early as six hours and continued through to 24 hours (6 hours, 3-fold reduction, $p = 0.0346$; 12 hours, 3-fold reduction, $p = 0.0031$ and 24 hours, 2-fold reduction, $p = 0.0048$) (Figure 3.17 B). In the lungs, bacterial loads of D39 Δ *ppmA* were significantly lower compared to the D39 wild-type strain at 6 hours (3 fold reduction, $p = 0.0009$), 12 hours (2 fold, $p = 0.0016$) and 24 hours (4-fold reduction, $p = 0.0003$) (Figure 3.17 C).

Despite there being evidence that the *pspC* and *ppmA* knockout strains in the D39 background are attenuated at the later time points, it is clear that the knockouts are still virulent and are still able to persist in the nasopharynx, bronchoalveolar tract and lungs. For all-time points in the D39 background, the difference was less than 1 log. Similarly, in the JP351 genetic background, the difference in colony counts at most time points was less than 1 log, with a few points differing by up to 2 logs. At all-time points, the colony counts were always greater than 10^3 providing statistically robust counts with a sufficient dynamic range to support the application of the knockout strain as a useful calibrator against possible changes that may occur in the wild-type strains due to pressure applied to the target antigen by the host immune system.

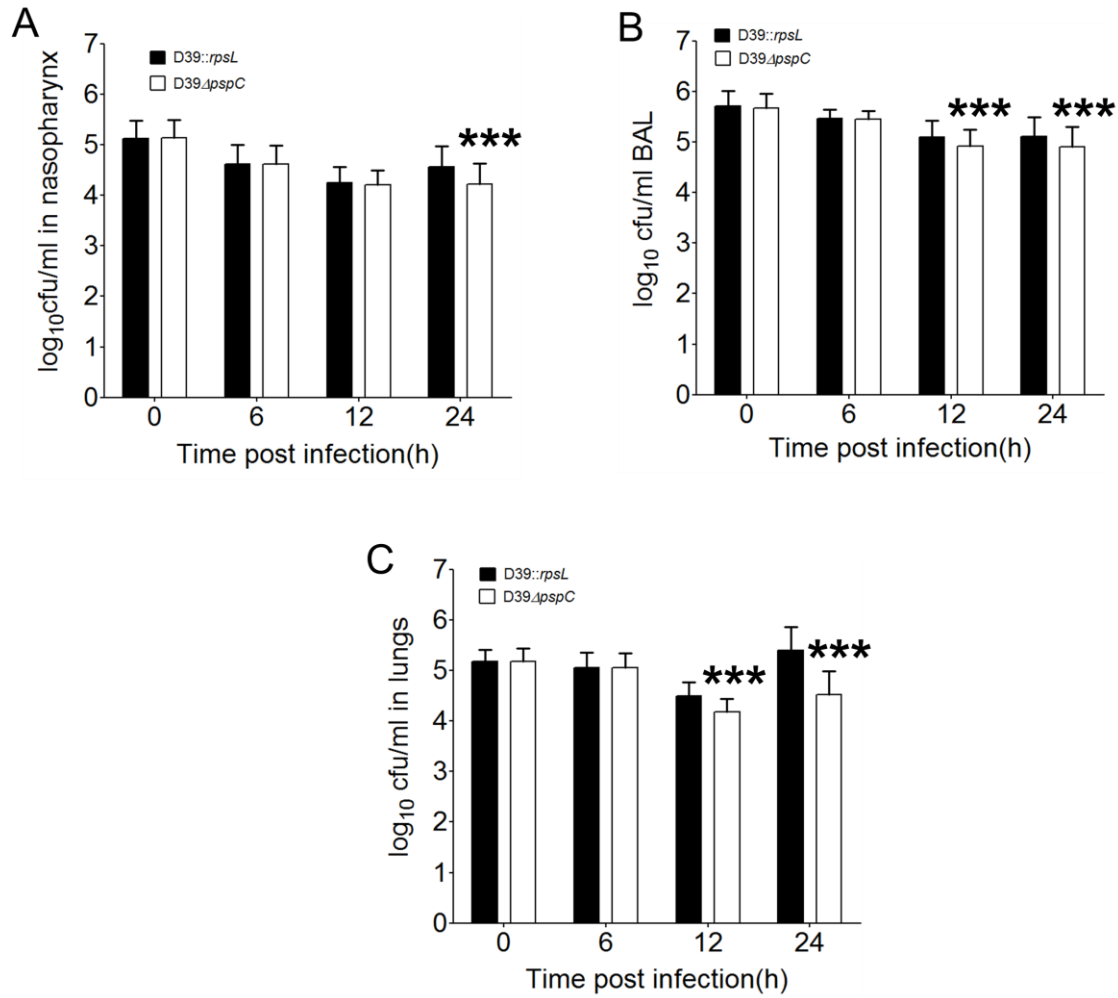


Figure 3.16. Intranasal challenge of unvaccinated mice with streptomycin-resistant pneumococcal strain (D39:: *rpsL*) and knockout strain (D39Δ*pspC*). Female NMRI mice were infected intranasally with an equal amount of 10^6 CFU of D39 streptomycin-resistant wild-type and D39Δ*pspC* strains per 50 μ L dose. Five animals per group were terminated at a 6 hour time interval for 24 hours. Samples from the (A) Nasopharynx (B) BAL fluid and (C) Lungs were plated on appropriate selective blood agar plates. Statistical significance of changes in CFU between the wild-type and knockout strain were analysed with a paired-t- test. Data represent average CFUs from a single experiment and error bars indicate standard deviations from five animals *** $p \leq 0.0001$.

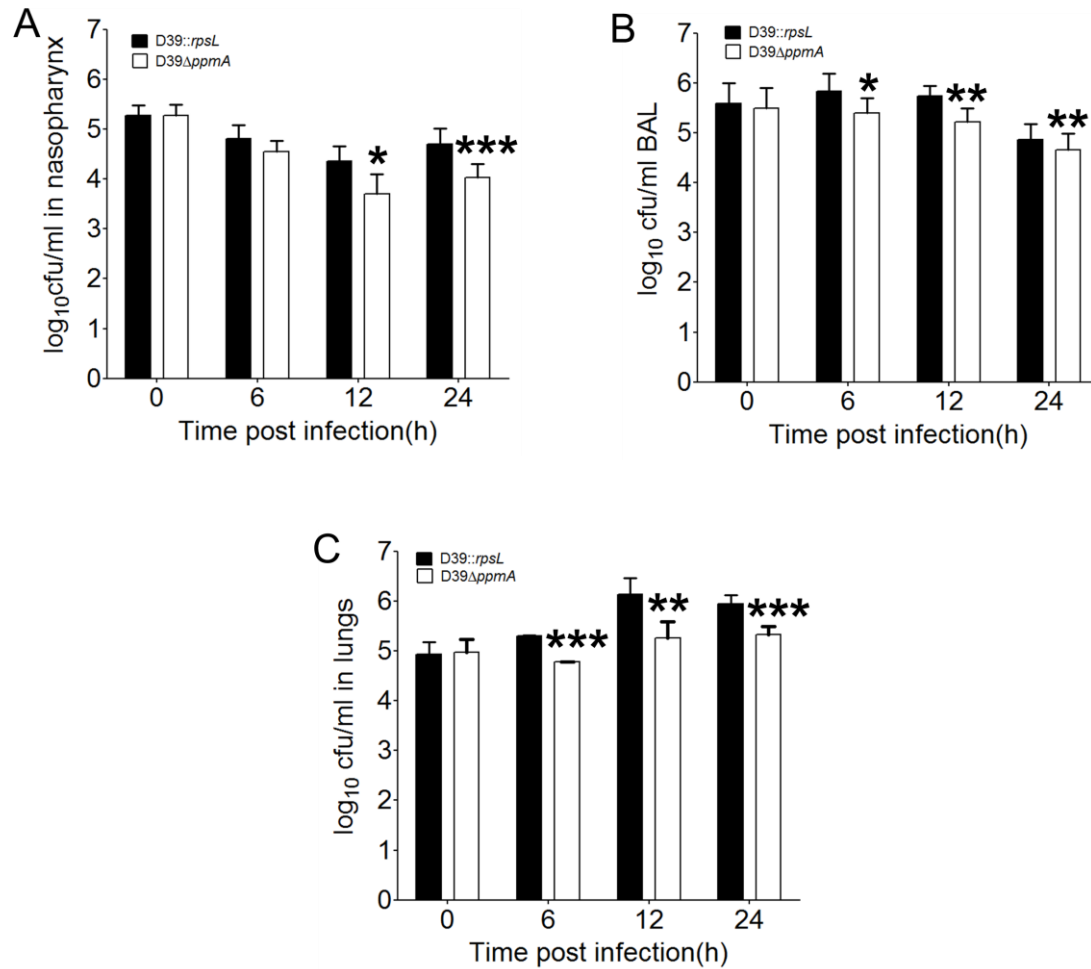


Figure 3.17. Intranasal challenge of unvaccinated mice with streptomycin-resistant pneumococcal strain (D39:: *rpsL*) and knockout type strain (D39Δ*ppmA*). Female NMRI mice were infected intranasally with an equal amount of 10^6 CFU of D39 streptomycin-resistant wild-type and D39Δ*ppmA* strains per 50 μ L dose. Five animals per group were terminated at a 6 hour time interval for 24 hours. Samples from the (A) Nasopharynx (B) BAL fluid and (C) Lungs were plated on appropriate selective blood agar plates. Statistical significance of changes in CFU between the wild-type and knockout strain were analysed with a paired-t- test. Data represent average CFUs from a single experiment and error bars indicate standard deviations from five animals * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.3 IMPACT OF VACCINATION ON THE OUTCOMPETITION OF WILD-TYPE PNEUMOCOCCI IN RELATION TO KNOCKOUTS IN CO-INOCULATED VACCINATED MICE

To demonstrate the value of the co-inoculation model to evaluate the immune protective potential of pneumococcal antigens against colonisation and pneumonia, mice were subcutaneously injected with or recombinant PspC and PpmA proteins and challenged with either the *pspC* or *ppmA* deletion knockout in a 1:1 ratio with their isogenic wild-type strain. The *in vivo* competitive index (CI) was calculated by dividing the ratio of knockout to wild-type bacteria recovered from the nasopharynx, BAL fluid and lungs at each time point. Any impact of vaccination will be indicated by an increase in the CI value in the vaccinated mice in relation to the CI value in the unvaccinated mice. In vaccinated mice, a value equal to 1 indicated that impact of the host immune response to the vaccine target present in the wild-type strain is equivalent to the attenuation caused by the knockout. CI values above 1 in the vaccinated mice indicated that the target antigen in the wild-type is recognised by the immune system and is capable of neutralising the wild-type pneumococci to a greater effect than neutralisation of the target virulence factor.

3.3.1 *PspC* and *ppmA* knockouts in relation to the wild-type PJ351 strain

At 0 hours, there was an equal ability of both strains to colonise the the nasopharynx of both placebo and vaccinated mice. A significant reduction in the CI of placebo mice compared to an increase in CI of PspC vaccinated mice was observed from 6 hours and onwards in the nasopharynx (6 hours, $p = 0.0117$; 12 hours, $p = 0.0117$ and 24 hours, $p = 0.0119$) (Figure 3.18 A). Likewise in the BAL fluid, a significant reduction in the CI of placebo mice compared to an increase in CI of vaccinated mice was observed from 6 hours until 24 hours (6 hours, $p = 0.0119$, 12 hours, $p = 0.0079$ and 24 hours, $p = 0.0112$) (Figure 3.18 B). In the lungs, a significant difference between competitive indices of placebo and vaccinated mice was observed at 12 and 24 hours (12 hours, $p = 0.0079$ and 24 hours, $p = 0.0079$) (Figure 3.18 C).

There were no changes observed in the CIs of knockout to wild-type strains in the nasopharynx of placebo and PpmA vaccinated mice at 0 hours. There were no significant differences ($p = 0.2873$) at 6 hours in CI scores of placebo and vaccinated mice. At 12 hours and 24 hours there were significant reductions in the CI score of placebo mice compared to

an increase in the CI score of vaccinated mice (12 hours, $p = 0.0019$ and 24 hours $p = 0.0079$) (Figure 3.19 A). The CIs in both placebo and vaccinated mice from the BAL fluid was equal at 0 hours. Statistical comparison of CIs in the placebo and vaccinated mice at 6 hours indicated no significant difference ($p = 0.1150$). A significant reduction in competitive indices was observed at 12 and 24 hours (12 hours, $p = 0.0119$ and 24 hours, $p = 0.0109$). Competitive indices of placebo and vaccinated mice in the lung tissues indicated a significant reduction in CIs starting at 6 hours and onwards (6 hours, $p = 0.0119$; 12 hours, $p = 0.0119$ and 24 hours, $p = 0.0119$) (Figure 3.19 C).

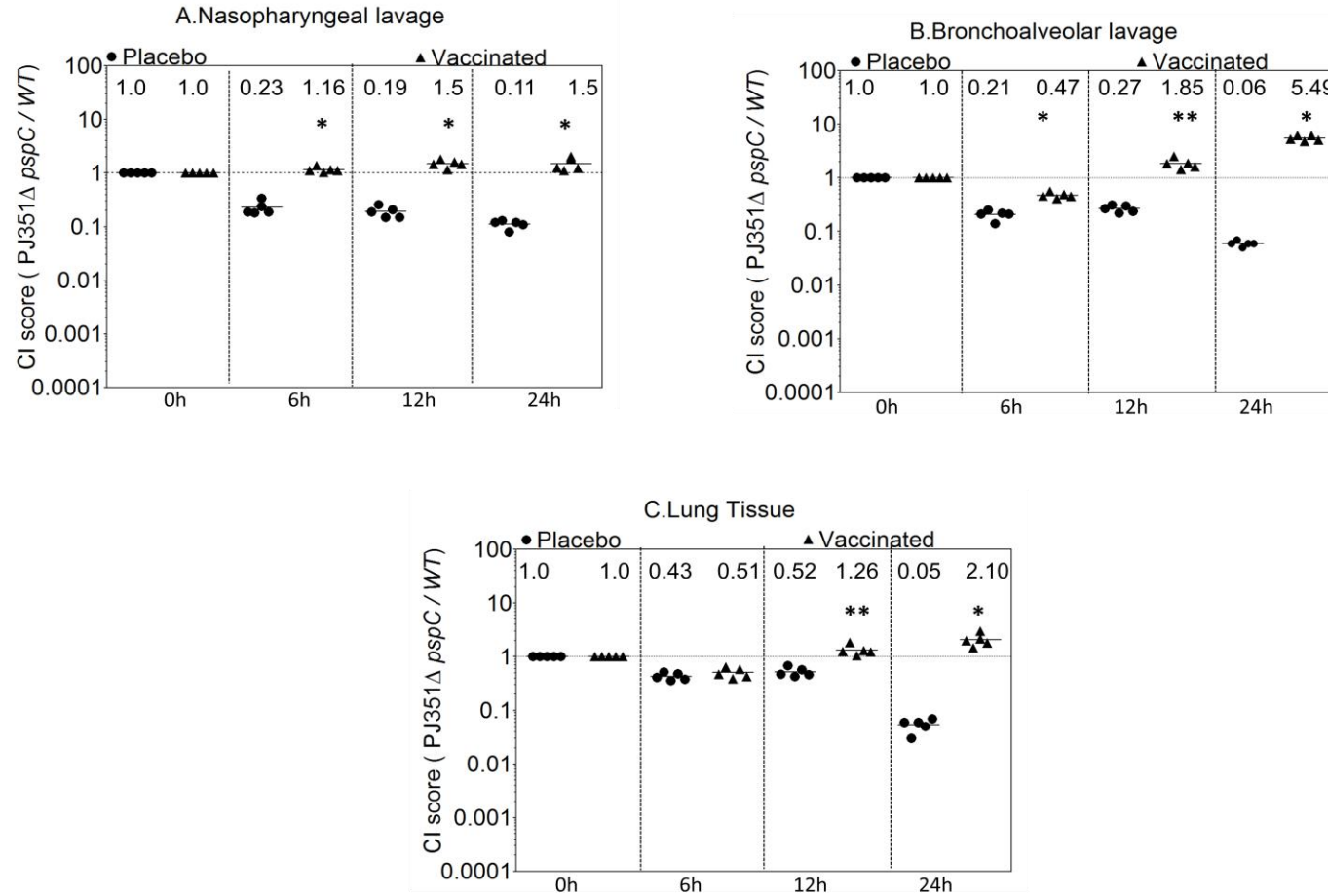


Figure 3.18. Protection against intranasal challenge with *S. pneumoniae* PJ351. Placebo and vaccinated mice subcutaneously injected with saponin+PBS and rPspC+saponin+PBS, respectively, were intranasally inoculated with PJ351 wild-type and PJ351 Δ pspC strains. Pneumococci were recovered from 0, 6, 12 and 24h post infection from A) Nasopharynx B) BAL fluid and C) Lungs of mice. The *in vivo* competitive index (CI) was calculated as described in the text. Values less than 1 indicate attenuation of the knockout and those greater than 1 indicate out-competition of the wild-type strain. Each symbol represents the CI obtained from an individual animal. A Mann-Whitney test was used for comparison of mean CI in placebo and vaccinated mice. Data represent average CIs from a single experiment. * $p \leq 0.05$, ** $p \leq 0.01$.

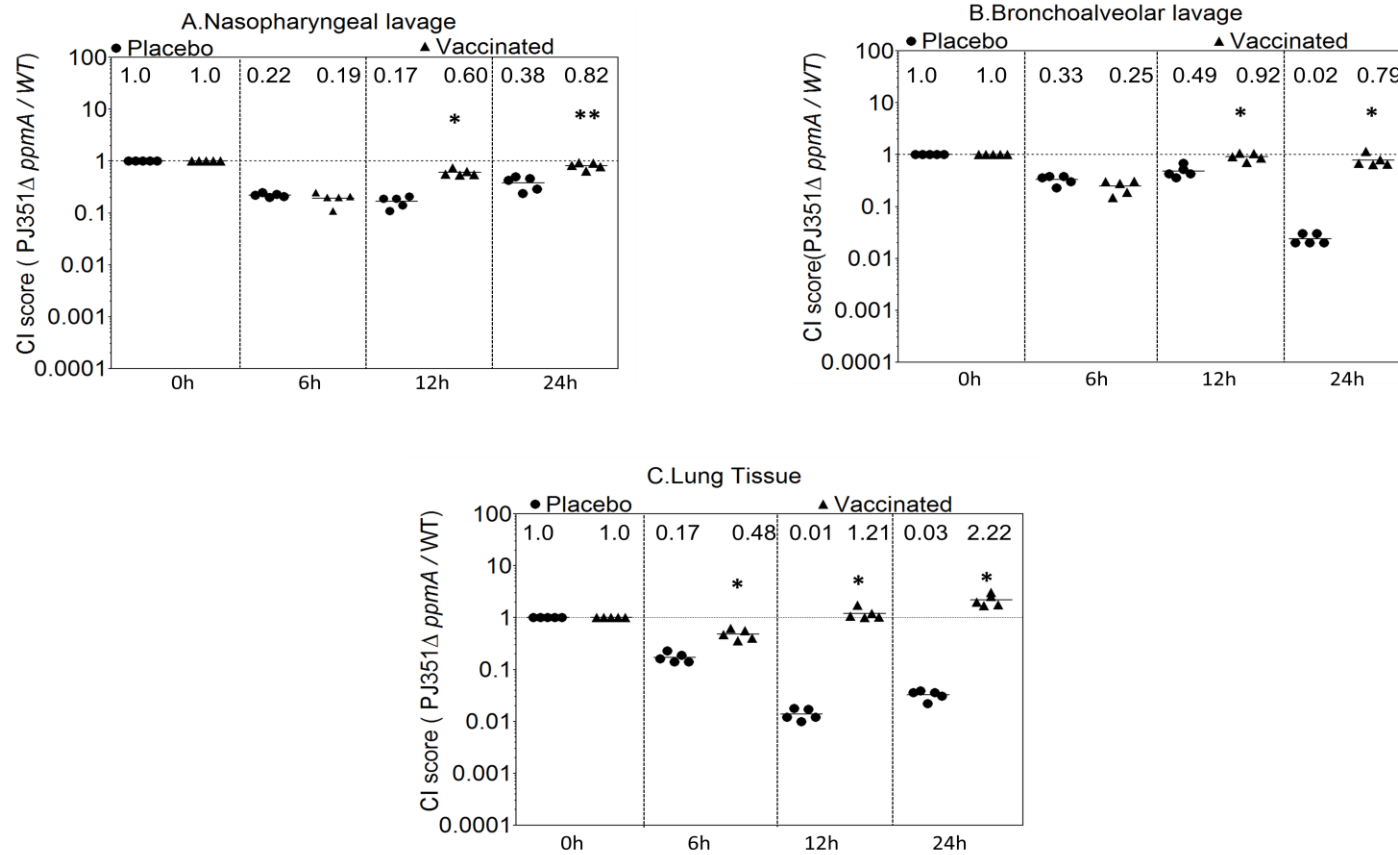


Figure 3.19 Protection against intranasal challenge with *S. pneumoniae* PJ351. Placebo and vaccinated mice subcutaneously injected with saponin+PBS and rPpmA+saponin+PBS, respectively, were intranasally inoculated with PJ351 wild-type and PJ351 Δ ppmA strains. Pneumococci were recovered at 0, 6, 12 and 24h post infection from A) Nasopharynx, B) BAL fluid and C) Lungs of mice. The *in vivo* competitive index (CI) was calculated as described in the text. Values less than 1 indicate attenuation of the knockout and those greater than 1 indicate out-competition of the wild-type strain. Each symbol represents the CI obtained from an individual animal. A Mann-Whitney test was used for comparison of mean CI in placebo and vaccinated mice. Data represent average CIs from a single experiment. * $p \leq 0.05$, ** $p \leq 0.01$.

To demonstrate the reliability of our model, we compared CV's based on colony counts with CV's based on competitive indices for PJ351 Δ *pspC*/PJ351::*rpsL* or PJ351 Δ *ppmA*/PJ351::*rpsL* strains from the nasopharynx, BAL fluid and lungs of placebo and PspC or PpmA vaccinated mice. Table 3.4 A-C indicates that CV's for raw bacterial counts in all sites sampled were within the range of 0-200%, compared to a range of 0-30% for CV's of CI scores. These results suggest that bacterial dispersion based on raw bacterial counts of PJ351 Δ *pspC*/PJ351::*rpsL* between individual mice are high in comparison to that based on competitive indices. Similar results were also observed when CV's derived from colony counts of PJ351 Δ *ppmA*/PJ351::*rpsL*, were compared to those derived from CI scores (Table 3.5 A-C).

Table 3.4 Standard deviations and coefficients of variations based on the pneumococcal CFUs and competitive index scores of PJ351 Δ pspC/PJ351::rpsL strains in the nasopharynx, BAL fluid and lungs.

	A.NASOPHARYNX															
	PLACEBO (PJ351ΔpspC/PJ351::rpsL)								VACCINATED (PJ351ΔpspC/PJ351::rpsL)							
	Pneumococcal CFUs				Competitive index scores				Pneumococcal CFUs				Competitive index scores			
	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours
Mean	121002	12599	15387	7551	1.0	0.23	0.19	0.11	118647	4936	4640	1105	1.0	1.16	1.50	1.50
Std. Deviation	70523	15359	15143	2205	0.0	0.07	0.05	0.02	72230	5717	3150	1160	0.0	0.13	0.24	0.43
Lower 95% Ci of mean	33435	-6472	-3415	4813	1.0	0.15	0.14	0.09	28962	-2162	729	-335	1.0	1.00	1.20	0.97
Upper 95% Ci of mean	208569	31670	34190	10288	1.0	0.31	0.25	0.14	208332	12034	8551	2545	1.0	1.32	1.79	2.04
Coefficient of variation (CV)	58%	122%	98%	29%	0%	29%	24%	17%	61%	116%	68%	105%	0%	11%	16%	29%

	B.BAL															
	PLACEBO (PJ351ΔpspC/PJ351::rpsL)								VACCINATED (PJ351ΔpspC/PJ351::rpsL)							
	Pneumococcal CFUs				Competitive index scores				Pneumococcal CFUs				Competitive index scores			
	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours
Mean	107000	121040	42177	186625	1.0	0.21	0.27	0.06	101541	72746	14672	5332	1.0	0.47	1.85	5.49
Std. Deviation	54405	122515	23016	282753	0.0	0.04	0.04	0.01	32638	124926	11289	4033	0.0	0.06	0.41	0.62
Lower 95% Ci of mean	39446	-31083	13599	-164461	1.0	0.16	0.22	0.05	61015	-82371	654.6	324	1.0	0.41	1.34	4.72
Upper 95% Ci of mean	174553	273163	70755	537711	1.0	0.26	0.32	0.07	142067	227863	28689	10340	1.0	0.54	2.36	6.27
Coefficient of variation (CV)	51%	101%	55%	152%	0%	20%	14%	12%	32%	171%	77%	76%	0%	12%	22%	11%

	B.LUNGS															
	PLACEBO (PJ351 Δ pspC/PJ351:: <i>rpsL</i>)								VACCINATED (PJ351 Δ pspC/PJ351:: <i>rpsL</i>)							
	Pneumococcal CFUs				Competitive index scores				Pneumococcal CFUs				Competitive index scores			
	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours
Mean	88140	78500	82751	54260	1.0	0.43	0.52	0.05	86020	79316	44083	4608	1.0	0.51	1.26	2.10
Std. Deviation	44531	116061	125235	32498	0.0	0.07	0.10	0.02	64944	59000	19466	4091	0.0	0.11	0.39	0.58
Lower 95% Ci of mean	32847	-65609	-72749	13908	1.0	0.35	0.39	0.04	5381	6058	19912	-472	1.0	0.37	0.77	1.38
Upper 95% Ci of mean	143433	222610	238252	94612	1.0	0.51	0.65	0.07	166659	152575	68254	9688	1.0	0.64	1.74	2.82
Coefficient of variation (CV)	51%	148%	151%	60%	0%	16%	20%	28%	76%	74%	44%	89%	0%	22%	31%	28%

Table 3.5 Standard deviations and coefficients of variations based on the pneumococcal CFUs and competitive index scores of PJ351 Δ ppmA/PJ351::*rpsL* strains in the nasopharynx, BAL fluid and lungs.

	A.NASOPHARYNX															
	PLACEBO (PJ351 Δ ppmA/PJ351:: <i>rpsL</i>)								VACCINATED (PJ351 Δ ppmA/PJ351:: <i>rpsL</i>)							
	Pneumococcal CFUs				Competitive index scores				Pneumococcal CFUs				Competitive index scores			
	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours
Mean	185380	7909	15087	7384	1.0	0.22	0.17	0.38	179840	8580	6181	3272	1.0	0.19	0.60	0.82
Std. Deviation	234631	3813	11385	9818	0.0	0.02	0.04	0.11	127064	5200	5219	2578	0.0	0.05	0.08	0.12
Lower 95% Ci of mean	-105955	3175	950	-4807	1.0	0.20	0.12	0.24	22068	2123	-299	70	1.0	0.13	0.50	0.67
Upper 95% Ci of mean	476715	12643	29223	19575	1.0	0.25	0.22	0.52	337612	15036	12661	6473	1.0	0.26	0.71	0.97
Coefficient of variation (CV)	127%	48%	75%	133%	0%	9%	25%	29%	71%	61%	84%	79%	0%	26%	14%	15%

	B.BAL															
	PLACEBO (PJ351ΔppmA/PJ351::rpsL)								VACCINATED (PJ351ΔppmA/PJ351::rpsL)							
	Pneumococcal CFUs				Competitive index scores				Pneumococcal CFUs				Competitive index scores			
	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours
Mean	71708	67581	12178	68560	1.0	0.33	0.49	0.02	71483	45396	4693	2224	1.0	0.25	0.92	0.79
Std. Deviation	49251	78083	9171	83131	0.0	0.06	0.13	0.01	39643	44409	3659	1757	0.0	0.07	0.15	0.21
Lower 95% Ci of mean	10554	-29372	791	-34661	1.0	0.25	0.33	0.01	22260	-9745	150	41	1.0	0.16	0.07	0.53
Upper 95% Ci of mean	132862	164534	23565	171781	1.0	0.41	0.64	0.03	120707	100538	9237	4406	1.0	0.34	0.74	1.05
Coefficient of variation (CV %)	69%	116%	75%	121%	0%	20%	26%	23%	55%	98%	78%	79%	0%	29%	16%	27%

	C.LUNGS															
	PLACEBO (PJ351ΔppmA/PJ351::rpsL)								VACCINATED (PJ351ΔppmA/PJ351::rpsL)							
	Pneumococcal CFUs				Competitive index scores				Pneumococcal CFUs				Competitive index scores			
	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours	0 hours	6 hours	12 hours	24 hours
Mean	71483	45396	4693	2224	1.0	0.17	0.01	0.033	97300	14083	2855	19304	1.0	0.48	1.21	2.22
Std. Deviation	39643	44409	3659	1757	0.0	0.05	0.004	0.007	40814	9383	2404	18333	0.0	0.11	0.32	0.59
Lower 95% Ci of mean	22260	-9745	150	41	1.0	0.12	0.002	0.02	46622	2432	-129.7	-3460	1.0	0.35	0.82	1.49
Upper 95% Ci of mean	120707	100538	9237	4406	1.0	0.22	0.008	0.04	147978	25734	5840	42068	1.0	0.67	1.61	2.96
Coefficient of variation (CV %)	55%	98%	78%	79%	0%	22%	27%	20%	42%	67%	84%	95%	0%	22%	26%	26%

3.3.2 Antibody responses against pneumococcal proteins

In order to analyse antibody responses after subcutaneous immunisations, serum was collected from mice 2 weeks after the final immunisation by way of cardiac puncture and analysed by ELISA. Analysis showed the presence of high antibody specific IgG titres in immunised mice as compared to the saponin placebo group: PspC, 0.02 ± 0.02 U/mL vs 832 ± 375 U/mL, $p < 0.0001$ and PpmA, 0.02 ± 0.02 U/mL vs 1162 ± 190 U/mL, $p < 0.0001$. There was, however, no detectable relationship between antibody concentration in the individual vaccinated animals and the competitive index ratios between the wild-type strains and isogenic knockouts.

3.3.3 *PspC* and *ppmA* knockouts in relation to the wild-type D39 strain

During the first 12 hours, placebo and rPspC vaccinated mice carried an equal ratio of knockout and wild-type bacteria in the nasopharynx. After 24 hours, the CI in the placebo mice dropped significantly compared to the vaccinated mice ($p = 0.0012$) (Figure 3.20 A). No changes in competitive indices were observed in the bronchoalveolar tract of placebo and vaccinated mice at 0 and 6 hours as there was an equal ratio of the knockout to wild-type pneumococci. A significant reduction in the CI of placebo mice was observed at 12 and 24 hours (12 hours, $p = 0.0079$ and 24 hours, $p = 0.0114$) (Figure 3.20 B). No difference in CIs was observed at 0 and 6 hours in the lungs of placebo and vaccinated mice as there was an equal ratio of the knockout to wild-type pneumococci. Comparison of CI scores from the lungs of mice at 12 hours and 24 hours revealed a significant reduction in placebo mice compared to an increase in vaccinated mice (12 hours, $p = 0.0079$ and 24 hours, $p = 0.0079$) (Figure 3.20 C).

In the nasopharynx of mice vaccinated with rPpmA, we observed a reduction in the CI at all-time points in placebo mice compared to the vaccinated mice. We observed a reduction in CI scores at all time points in the nasopharynx of placebo mice compared to the vaccinated mice (6 hours, $p = 0.0117$; 12 hours, $p = 0.0079$ and 24 hours, $p = 0.0119$) (Figure 3.21 A). There were no significant differences in CIs between the placebo and vaccinated mice in the BAL fluid and lungs at all-time points observed (Figures 3.21 B and C).

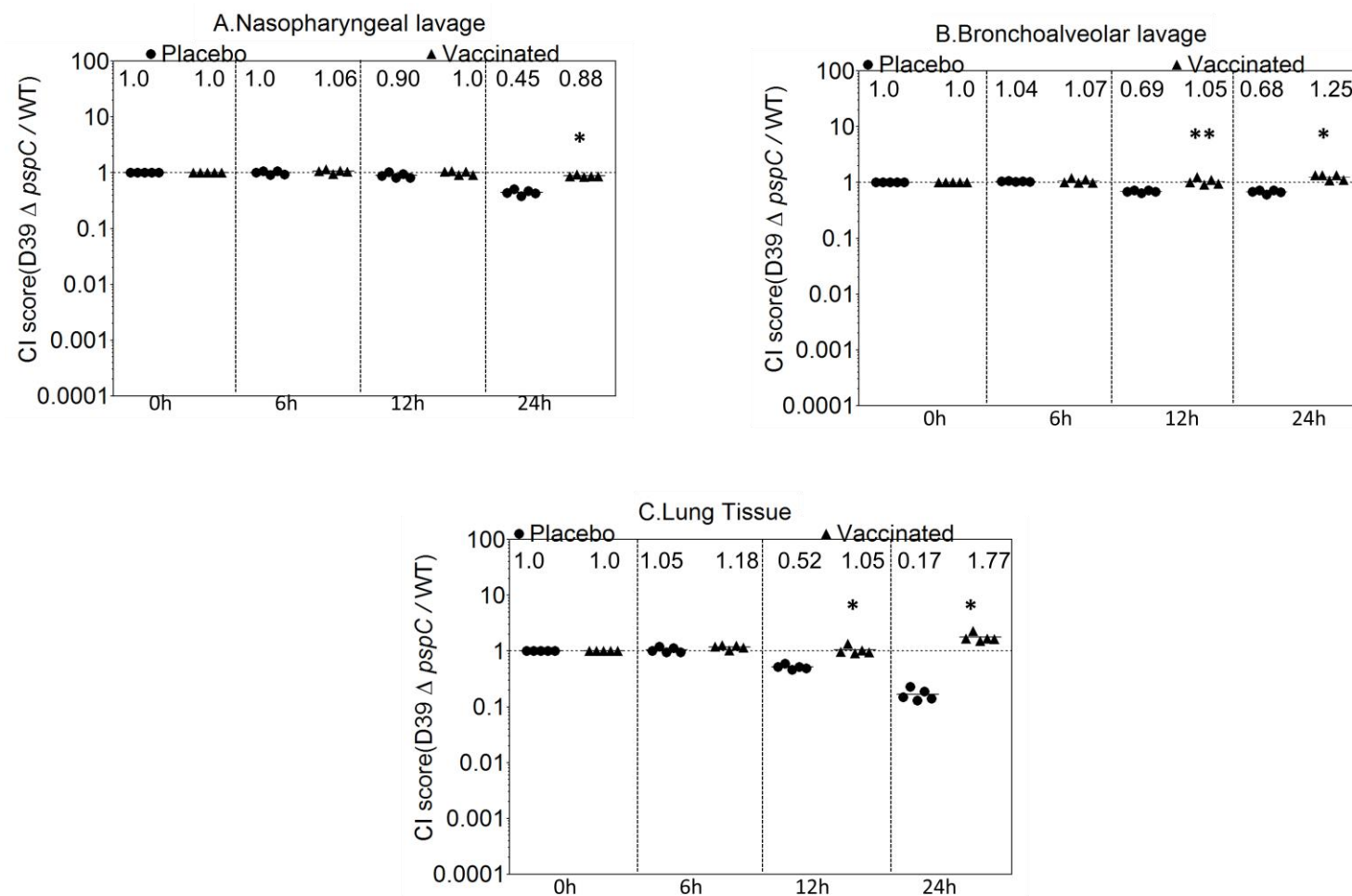


Figure 3.20. Protection against intranasal challenge with *S. pneumoniae* D39 Placebo and vaccinated mice subcutaneously injected with saponin+PBS and rPspC+saponin+PBS, respectively were intranasally inoculated with an equal amount of 10^6 CFU of D39 streptomycin-resistant wild-type and D39 Δ pspC strains per 50 μ L dose. Pneumococci were recovered at 0, 6, 12 and 24h post infection from A) Nasopharynx, B) BAL fluid and C) Lungs of mice. The *in vivo* competitive index (CI) was calculated as described in the text. Each symbol represents the CI obtained from an individual animal. Values less than 1 indicate attenuation of the knockout and those greater than 1 indicate out-competition of the wild-type strain. Each symbol represents the CI obtained from an individual animal. A Mann-Whitney test was used for comparison of mean CI in placebo and vaccinated mice. Data represent average CIs from a single experiment. * $p \leq 0.05$, ** $p \leq 0.01$.

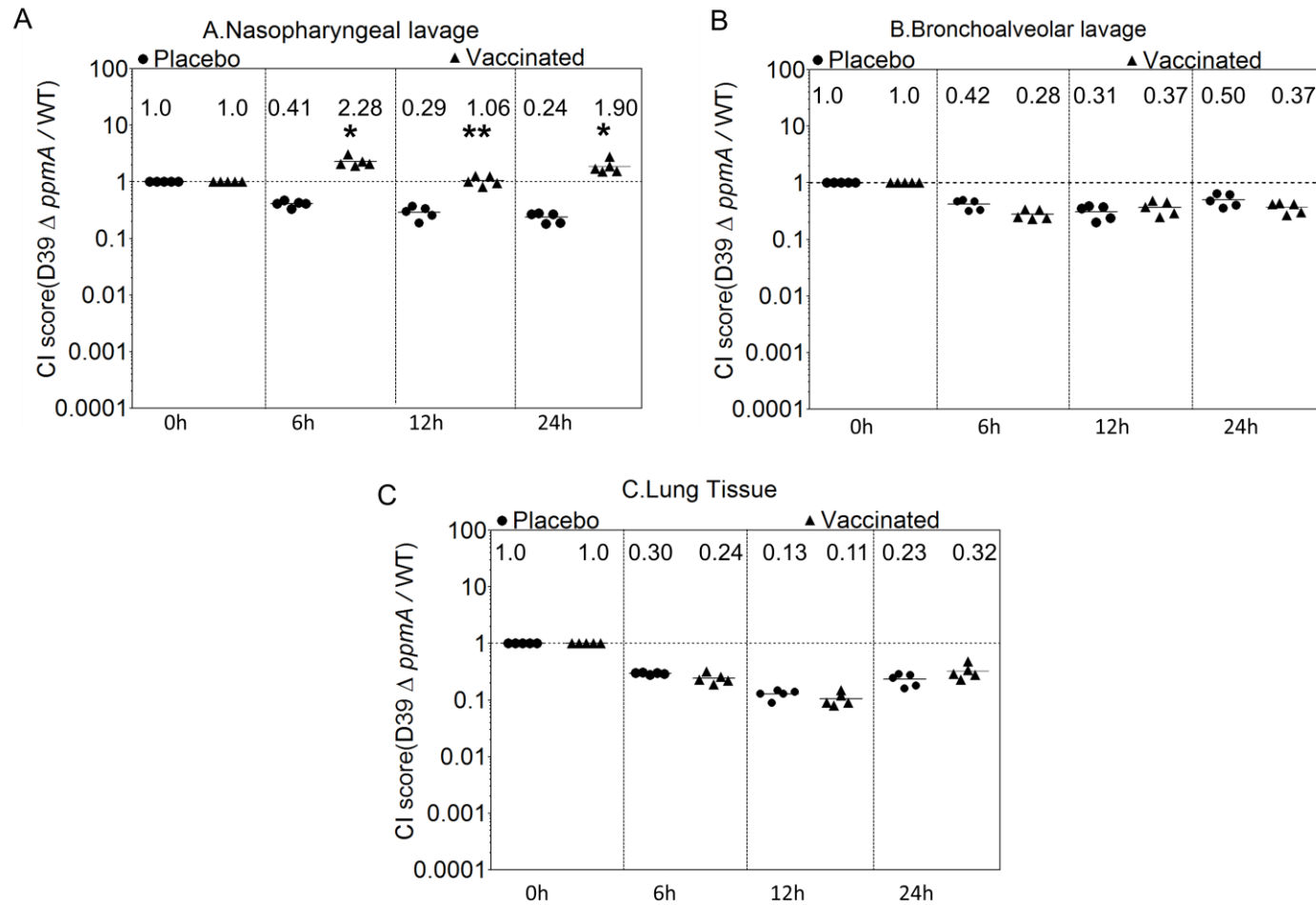


Figure 3.21. Protection against intranasal challenge with *S. pneumoniae* D39. Placebo and vaccinated mice subcutaneously injected with saponin+PBS and rPpmA+saponin+PBS, respectively were intranasally inoculated with D39 wild-type and D39 Δ ppmA strains. Pneumococci were recovered at 0, 6, 12 and 24h post infection from A) Nasopharynx, B) BAL fluid and C) Lungs of mice. The *in vivo* competitive index (CI) was calculated as described in the text. Each symbol represents the CI obtained from an individual animal. Values less than 1 indicate attenuation of the knockout and those greater than 1 indicate out-competition of the wild-type strain. Each symbol represents the CI obtained from an individual animal. A Mann-Whitney test was used for comparison of mean CI in placebo and vaccinated mice. Data represent average CIs from a single experiment. * $p \leq 0.05$, ** $p \leq 0.01$.

The variability of each measurement of bacterial numbers was tested by calculating CV's from the nasopharynx, BAL fluid and lungs of mice. Data analysis for raw bacterial counts for mice inoculated with D39 Δ *pspC*/D39::*rpsL* strains indicated a large spread in bacterial counts as determined by CV's of higher than 100%. In comparison, data analysis for competitive index scores indicated little variation in data as a result of lower CV's (Table 3.6 A-C). Likewise for data analysis for mice inoculated with D39 Δ *ppmA*/D39::*rpsL* strains indicated a similar pattern of results (Table 3.7 A-C).

Table 3.6 Standard deviations and coefficients of variations based on pneumococcal CFUs and competitive index scores of D39 Δ pspC/D39::rpsL strains in the nasopharynx, BAL fluid and lungs.

	A.NASOPHARYNX															
	PLACEBO (D39ΔpspC/D39::rpsL)								VACCINATED (D39ΔpspC/D39::rpsL)							
	Pneumococcal CFUs				Competitive index scores				Pneumococcal CFUs				Competitive index scores			
	0 hrs	6 hrs	12 hrs	24hrs	0hrs	6 hrs	12 hrs	24 hrs	0hrs	6 hrs	12hrs	24 hrs	0hrs	6 hrs	12 hrs	24 hrs
Mean	134824	41880	17130	26516	1.0	1.0	0.90	0.45	129187	40746	18794	15248	1.0	1.06	1.00	0.88
Std. Deviation	175425	57847	17211	42776	0.0	0.08	0.09	0.05	77358	73187	14530	7452	0.0	0.07	0.08	0.04
Lower 95% Ci of mean	-82997	-29947	-4240	-26597	1.0	0.91	0.79	0.39	33134	-50128	752	5995	1.0	0.97	0.90	0.83
Upper 95% Ci of mean	352644	113707	38500	79629	1.0	1.10	1.01	0.51	225240	131620	36836	24501	1.0	1.15	1.10	0.92
Coefficient of variation (CV)	130%	138%	100%	161%	0%	8%	10%	11%	60%	180%	77%	49%	0%	7%	8%	4%

	B.BAL															
	PLACEBO (D39ΔpspC/D39::rpsL)								VACCINATED (D39ΔpspC/D39::rpsL)							
	Pneumococcal CFUs				Competitive index scores				Pneumococcal CFUs				Competitive index scores			
	0 hrs	6 hrs	12 hrs	24 hrs	0 hrs	6 hrs	12 hrs	24 hrs	0 hrs	6 hrs	12 hrs	24 hrs	0 hrs	6 hrs	12hrs	24hrs
Mean	493619	288000	105543	105152	1.0	1.04	0.69	0.68	459487	320980	85500	68220	1.0	1.07	1.05	1.25
Std. Deviation	484074	138116	118126	150055	0.0	0.02	0.03	0.05	323907	313159	56901	51934	0.0	0.10	0.13	0.13
Lower 95% Ci of mean	-107441	116506	-41131	-81167	1.0	1.01	0.65	0.62	57301	-67861	14848	3735	1.0	0.94	0.88	1.09
Upper 95% Ci of mean	1e+006	459494	252216	291471	1.0	1.07	0.73	0.74	861672	709821	156152	132705	1.0	1.19	1.21	1.41
Coefficient of variation (CV)	98%	48%	112%	143%	0%	2%	5%	7%	70%	98%	67%	76%	0%	9%	12%	11%

	C.LUNGS															
	PLACEBO (D39ΔpspC/D39::rpsL)								VACCINATED (D39ΔpspC/D39::rpsL)							
	Pneumococcal CFUs				Competitive index scores				Pneumococcal CFUs				Competitive index scores			
	0hrs	6 hrs	12 hrs	24 hrs	0hrs	6hrs	12hrs	24 hrs	0 hrs	6 hrs	12hrs	24 hrs	0 hrs	6 hrs	12 hrs	24 hrs
Mean	152215	113802	23195	143528	1.0	1.05	0.52	0.17	165799	118190	13237	28850	1.0	1.18	1.05	1.77
Std. Deviation	114912	109734	20518	273549	0.0	0.11	0.05	0.04	159520	113245	3378	39392	0.0	0.10	0.17	0.31
Lower 95% Ci of mean	9533	-22452	-2281	-196129	1.0	0.92	0.45	0.12	-32272	-22422	9042	-20062	1.0	1.06	0.83	1.39
Upper 95% Ci of mean	294898	250056	48671	483185	1.0	1.20	0.58	0.22	363870	258802	17431	77762	1.0	1.30	1.26	2.16
Coefficient of variation (CV)	75%	96%	88%	191%	0%	11%	10%	25%	96%	96%	26%	137%	0%	8%	17%	17%

Table 3.7 Standard deviations and coefficients of variations based on pneumococcal CFUs and competitive index scores of D39 Δ ppmA/D39::rpsL strains in the nasopharynx, BAL fluid and lungs.

	A.NASOPHARYNX															
	PLACEBO (D39ΔppmA/D39::rpsL)								VACCINATED (D39ΔppmA/D39::rpsL)							
	Pneumococcal CFUs				Competitive index scores				Pneumococcal CFUs				Competitive index scores			
	0 hrs	6 hrs	12 hrs	24 hrs	0hrs	6 hrs	12 hrs	24 hrs	0 hrs	6 hrs	12 hrs	24 hrs	0hrs	6 hrs	12 hrs	24 hrs
Mean	159460	66185	13782	30526	1.0	0.41	0.29	0.24	146240	31441	5063	11492	1.0	2.28	1.06	1.90
Std. Deviation	139445	41221	14661	31248	0.0	0.05	0.07	0.05	98951	28226	4576	9852	0.0	0.45	0.20	0.52
Lower 95% Ci of mean	-13685	15002	-4423	-8274	1.0	0.35	0.21	0.18	23376	-3607	-619.2	-741.4	1.0	1.73	0.09	1.25
Upper 95% Ci of mean	332605	117367	31986	69326	1.0	0.47	0.38	0.30	269104	66489	10745	23725	1.0	2.84	0.81	2.55
Coefficient of variation (CV)	87%	62%	106%	102%	0%	12%	24%	20%	68%	90%	90%	86%	0%	20%	19%	28%

	B.BAL															
	PLACEBO (D39ΔppmA/D39::rpsL)								VACCINATED (D39ΔppmA/D39::rpsL)							
	Pneumococcal CFUs				Competitive index scores				Pneumococcal CFUs				Competitive index scores			
	0 hrs	6 hrs	12hrs	24 hrs	0hrs	6 hrs	12 hrs	24 hrs	0hrs	6 hrs	12 hrs	24hrs	0hrs	6hrs	12hrs	24hrs
Mean	354666	247773	326622	264353	1.0	0.42	0.31	0.50	310583	216640	318728	335160	1.0	0.28	0.34	0.37
Std. Deviation	545277	177659	260548	558930	0.0	0.09	0.08	0.13	192482	204972	426809	450831	0.0	0.05	0.10	0.08
Lower 95% Ci of mean	-322389	27179	3107	-429654	1.0	0.50	0.21	0.34	71584	-37867	-211228	-224623	1.0	0.21	0.24	0.27
Upper 95% Ci of mean	1e+006	468367	650136	958360	1.0	0.13	0.42	0.66	549583	471147	848683	894943	1.0	0.34	0.49	0.47
Coefficient of variation (CV)	154%	72%	80%	211%	0%	21%	27%	26%	62%	95%	134%	135%	0%	19%	27%	21%

	C.LUNGS															
	PLACEBO (D39 Δ ppmA/D39:: <i>rpsL</i>)								VACCINATED (D39 Δ ppmA/D39:: <i>rpsL</i>)							
	Pneumococcal CFUs				Competitive index scores				Pneumococcal CFUs				Competitive index scores			
	0 hrs	6 hrs	12 hrs	24 hrs	0 hrs	6 hrs	12 hrs	24 hrs	0 hrs	6 hrs	12 hrs	24 hrs	0 hrs	6 hrs	12 hrs	24 hrs
Mean	90242	99985	346000	342401	1.0	0.30	0.13	0.23	87716	165197	368380	515061	1.0	0.24	0.11	0.32
Std. Deviation	69639	29651	141458	449646	0.0	0.01	0.02	0.06	25064	125864	563566	693319	0.0	0.05	0.03	0.10
Lower 95% Ci of mean	3773	63168	170355	-215911	1.0	0.28	0.10	0.16	56595	8915	-331383	-345813	1.0	0.18	0.07	0.21
Upper 95% Ci of mean	176710	136802	521645	900713	1.0	0.31	0.16	0.31	118838	321478	1e+006	1e+006	1.0	0.31	0.14	0.44
Coefficient of variation (CV)	77%	30%	41%	131%	0%	4%	18%	25%	29%	76%	153%	134%	0%	20%	27%	30%

3.3.4 Antibody responses against pneumococcal proteins

Antibody analysis indicated significant differences in antibody concentrations of sera between placebo and immunised mice: PspC, 0.020 ± 0.020 U/mL vs 1908 ± 1936 U/mL, $p < 0.0001$ and PpmA: 0.020 ± 0.020 U/mL vs 1265 ± 227 U/mL, $p < 0.0001$. There was, however, no detectable relationship between antibody concentration in the vaccinated animals and the CI scores between the wild-type strains and isogenic knockouts.

3.4 GROWTH CHARACTERISTICS OF PNEUMOCOCCAL KNOCKOUTS RELATIVE TO WILD-TYPE STRAINS IN A CO INOCULATION MODEL IN UNVACCINATED MICE AT A SINGLE TIME POINT

To further elucidate the relevance and potential use of this model, a single time point (T = 24h) in which the vaccine effect was most pronounced was chosen for the remainder of the experiments. Additional proteins of the cell wall and membrane bound class (SlrA, PspA and IgA1 bacterial protease) were assessed for their potential to protect mice from infection with the pneumococcus using our mouse model. A group of 30 unvaccinated mice (10 mice per protein) were intranasally inoculated with a 1:1 ratio of 10^6 CFU of *S. pneumoniae* wild-type and knockout strains. Animals were sacrificed at 24 hours post inoculation, the number of CFU in the nasopharynx, BAL fluid and lungs were enumerated, and the differences in the bacterial load in the placebo group were determined.

3.4.1 Virulence characteristics of *pspA*, *igA1* and *slrA* knockouts in relation to the PJ351 wild-type strain in unvaccinated mice

The recovery of all knockout bacteria in the PJ351 strain was significantly lower compared to the wild-type strain in the nasopharynx. After 24 hours, the *slrA* knockout in the nasopharynx was significantly reduced by 10-fold, in relation to the wild-type strain ($p = 0.0005$). Similarly in the BAL fluid, the knockout was significantly reduced by 15-fold in relation to the wild-type ($p = 0.0005$). The effect of the *slrA* knockout was most noticeable in the lung tissue, which was reduced by 17-fold in relation to the wild-type ($p = 0.0005$) (Figure 3.22 A). The *pspA* knockout was less able to colonise the nasopharynx of mice compared to the parent strain following 24 hours of inoculation (6-fold, $p = 0.0089$). A more pronounced effect of the *pspA* knockout was observed in the BAL fluid (33-fold, $p = 0.0146$) and lungs (31-fold, $p = 0.0118$) (Figure 3.22 B). Similarly, the *igA1* knockout was significantly attenuated in its ability to colonise the nasopharynx of mice (2-fold, $p < 0.0001$). There was an observation of a significant difference between the wild-type and the *igA1* knockout in the BAL fluid (8-fold, $p < 0.0001$) and lungs (11-fold, $p < 0.0001$) (Figure 3.22 C).

3.4.2 Virulence characteristics of *pspA*, *igA1* and *slrA* knockouts in relation to the D39 wild-type strain in unvaccinated mice

Deletion of D39 Δ *slrA* resulted in a reduction of the bacterial load in the nasopharynx of mice compared to the wild-type (4 fold, $p < 0.0001$). We found no marked reduction in bacterial load of D39 Δ *slrA* compared to the wild-type strain in the BAL fluid and lungs (Figure 3.23 A). Likewise, the *pspA* knockout was attenuated in its ability to colonise the nasopharynx of mice (9-fold, $p < 0.0001$). There was also no marked reduction in bacterial load of D39 Δ *pspA* compared to the parent strain in the BAL fluid and lungs (Figure 3.23 B). The *igA1* knockout was reduced by half in relation to the wild-type strain in the nasopharynx of mice ($p < 0.0001$). Deletion of *igA1* did not attenuate the ability of the D39 strain to grow in the BAL fluid and lungs of mice (Figure 3.23 C).

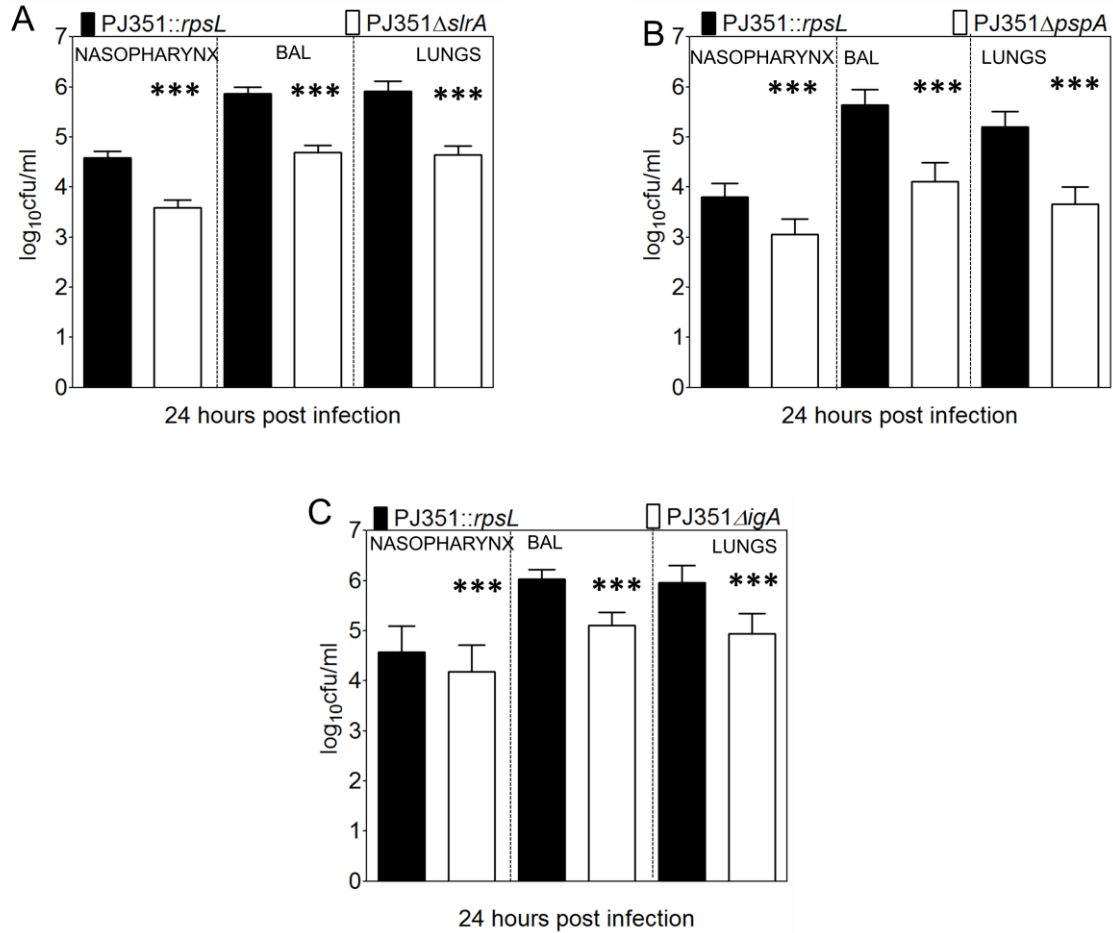


Figure 3.22. Intranasal challenge of unvaccinated mice with streptomycin-resistant pneumococcal strain PJ351 (PJ351::rpsL) and knockout type strains PJ351ΔslrA, PJ351ΔpspA and PJ351ΔigaA1. Female NMRI mice were infected intranasally with an equal amount of 10⁶ CFU of PJ351 streptomycin-resistant wild-type and knockout strains per 50μL dose. Groups of 10 mice were co-infected with a 1:1 mixture of wild-type and knockout strain. Pneumococci were recovered at 24h post infection from the (A) Nasopharynx, (B) BAL fluid and (C) Lung tissue. Statistical significance of changes in CFU between the wild-type and knockout strain were assessed using the Mann-Whitney test. ***p ≤ 0.0001.

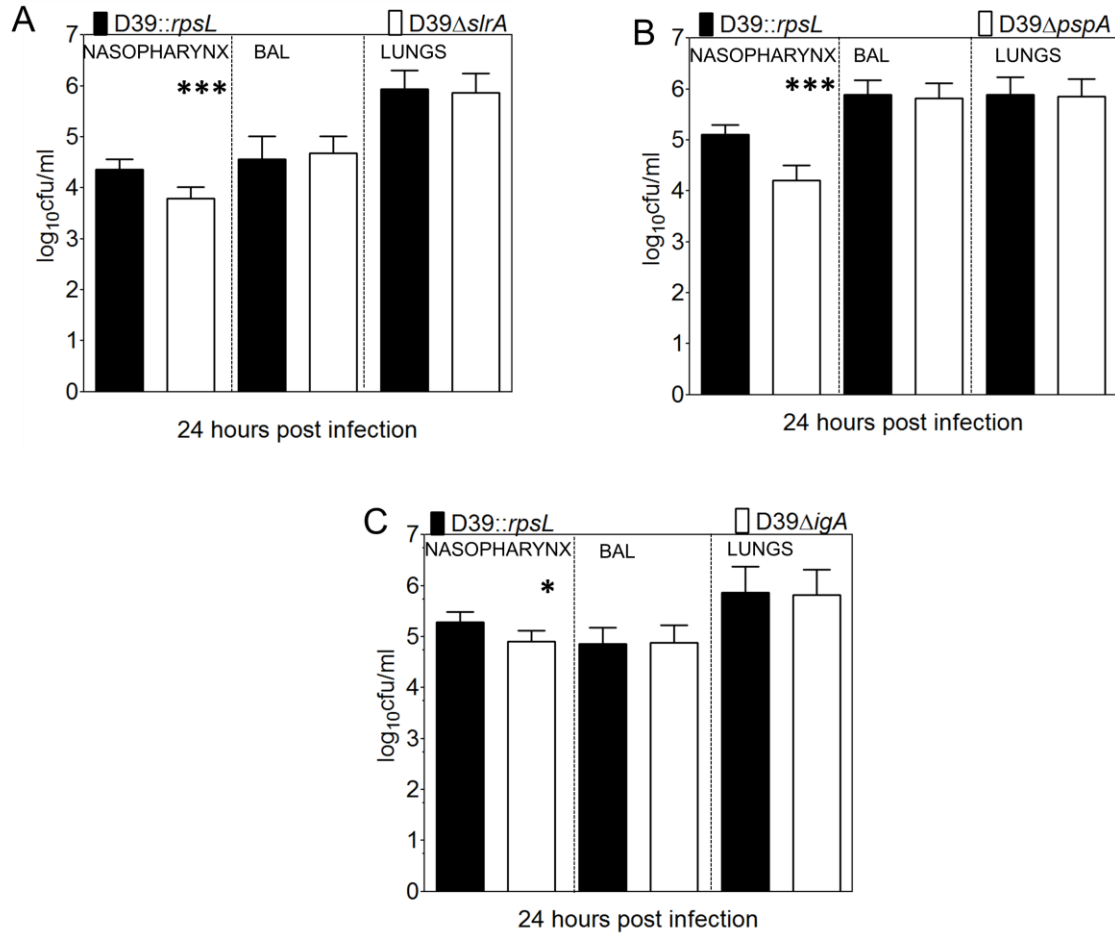


Figure 3.23. Intranasal challenge of unvaccinated mice with streptomycin-resistant pneumococcal strain D39 (D39::rpsL) and knockout type strains D39ΔslrA, D39ΔpspA and D39ΔigaA1. Female NMRI mice were infected intranasally with an equal amount of 10^6 CFU of D39 streptomycin-resistant wild-type and knockout strains per 50μL dose. Groups of 10 mice were co-infected with a 1:1 mixture of wild-type and knockout strain. Pneumococci were recovered at 24h post infection from the (A) Nasopharynx, (B) BAL fluid and (C) Lung tissue. Statistical significance of changes in CFU between the wild-type and knockout strain were assessed using the Mann-Whitney test. * $p \leq 0.05$, *** $p \leq 0.0001$.

3.5 IMPACT OF VACCINATION ON THE OUTGROWTH OF WILD-TYPE PNEUMOCOCCI IN RELATION TO KNOCKOUTS IN CO-INOCULATED VACCINATED MICE

In order to determine whether the recombinant proteins would elicit a protective immune response, a competitive index analysis of the knockout strains and their isogenic PJ351 and D39 wild-type strains was performed. NMRI mice were immunised separately with 50 µg of each protein (PspA, IgA1 bacterial protease or SlrA) and four weeks later, challenged with 1:1 ratio of knockout strains and wild-type strains at 10^6 of total bacteria (knockout and wild-type strains combined at a 1:1 ratio). Animals were sacrificed at 24 hours post inoculation, CI scores were performed as described in chapter 2.

3.5.1 Impact of vaccination on *slrA*, *pspA* or *iga1* knockouts in relation to the wild-type PJ351 strain in vaccinated and unvaccinated mice

No significant changes in competitive indices were observed in the nasopharynx of placebo and SlrA vaccinated mice ($p = 0.5966$). The CI score from the BAL fluid of placebo mice and SlrA vaccinated mice revealed that there was no significant difference between the groups ($p = 1.000$). In the lung tissues of placebo and SlrA vaccinated mice, we observed no significant differences in the CI scores ($p = 0.1198$) (Figure 3.24 A).

There were also no observed changes in competitive indices in the nasopharynx of placebo and PspA vaccinated mice ($p = 0.1523$). Likewise, there was no significant difference in the comparison of CI scores in the BAL fluid of placebo mice and PspA vaccinated mice ($p = 0.7618$). There were also no significant differences in the CI scores from the lung tissues between the placebo and vaccinated mice ($p = 0.1301$) (Figure 3.24.B).

Similarly, in the nasopharynx of placebo mice and IgA vaccinated mice, there were no observed changes in the CI scores in both placebo and vaccinated mice ($p = 0.3840$). No significant differences were observed between the CI scores of placebo and vaccinated mice ($p = 0.3254$). Statistical comparison of the CI scores from the lung tissues of placebo and vaccinated mice showed a non-significant difference between the groups ($p = 0.3285$) (Figure 3.24.C).

Further, CV's calculated on mean bacterial counts for $\Delta slrA$, $\Delta pspA$, ΔigA and their parent strain from the nasopharynx, BAL fluid and lungs of placebo and vaccinated mice indicated high variation in data. On the other hand, CV's calculated on CI scores indicated low variation in data for all strains in the placebo and vaccinated mice as evidenced by CV's less than 30% (Table 3.8 A-I).

3.5.2 *SlrA*, *pspA* or *igA1* knockouts in relation to the wild-type D39 strain

There was no significant difference in CI scores in the nasopharynx of mice between placebo and *SlrA* vaccinated mice ($p = 0.5463$). Comparison of the CI scores from the BAL fluid of placebo and *SlrA* vaccinated mice indicated no significant changes between the two groups ($p = 0.2558$). In the lung tissue of placebo and *SlrA* vaccinated mice we observed no significant differences in the CI scores ($p = 0.1110$) (Figure 3.25.A).

No significant changes in competitive indices were also observed in the nasopharynx of placebo and *PspA* vaccinated mice ($p = 0.2676$). Similarly, no significant differences were observed in the BAL fluid of placebo and vaccinated mice ($p = 0.0887$). The same trend was also observed in the lung tissue of placebo and vaccinated mice in which there were no significant differences in the CI scores between the groups ($p = 0.7748$) (Figure 3.25.B).

In contrast, a significant reduction in the CI of placebo mice was observed compared to an increase in CI of *IgA* vaccinated mice ($p = 0.0002$). Statistical comparison of the CI scores from the BAL fluid of placebo and vaccinated mice showed that there were no significant differences between the two groups ($p = 0.7618$). Same results were seen in the lung tissues of placebo and *IgA* vaccinated mice. Statistical comparison of vaccinated and placebo mice showed a non-significant difference in CI scores ($p = 0.2404$) (Figure 3.25.C).

CV's calculated for mean bacterial counts of all strains from the nasopharynx, BAL fluid and lungs of placebo and vaccinated mice revealed high dispersion in data, compared to CV's calculated for CI scores (Table 3.9 A-I).

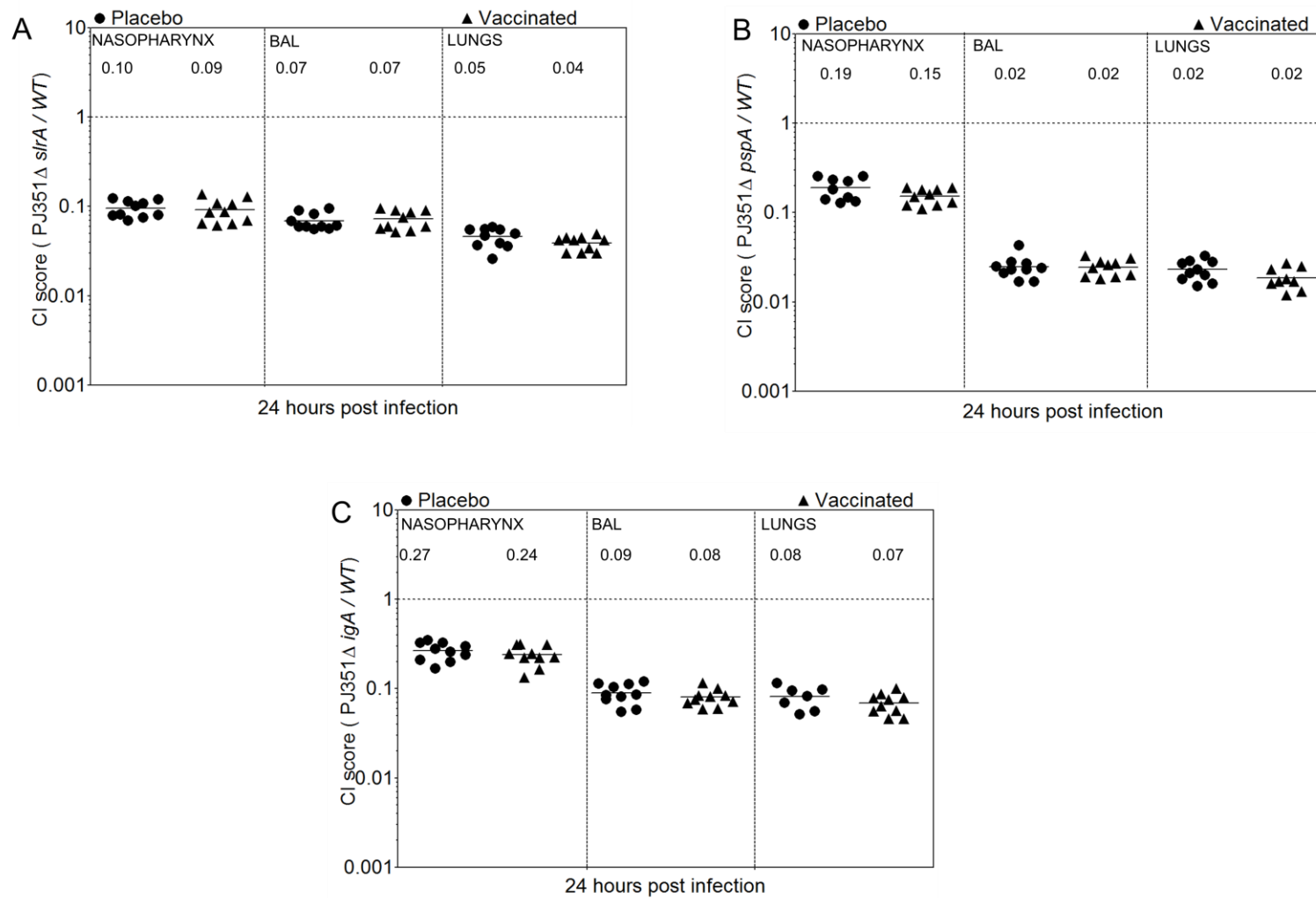


Figure 3.24. Protection against intranasal challenge with *S. pneumoniae* PJ351. Groups of 20 mice equally distributed into the vaccinated and placebo groups were subcutaneously injected with saponin+PBS+ (A) rSlrA, (B) rPspA or (C) rIgA1 bacterial protease and saponin+PBS, respectively. Intranasal co-inoculation was with a 1:1 mixture of PJ351 wild-type and PJ351 knockout strains. Pneumococci were recovered at 24h post infection from the nasopharynx, BAL fluid and lung tissue. A value of 1 indicates that both strains are proliferating equally. Values less than 1 indicate attenuation of the knockout and those greater than 1 indicate out-competition of the wild-type strain. A Mann-Whitney test was used for comparison of mean CI in placebo and vaccinated mice. Data represent average CIs from a single experiment.

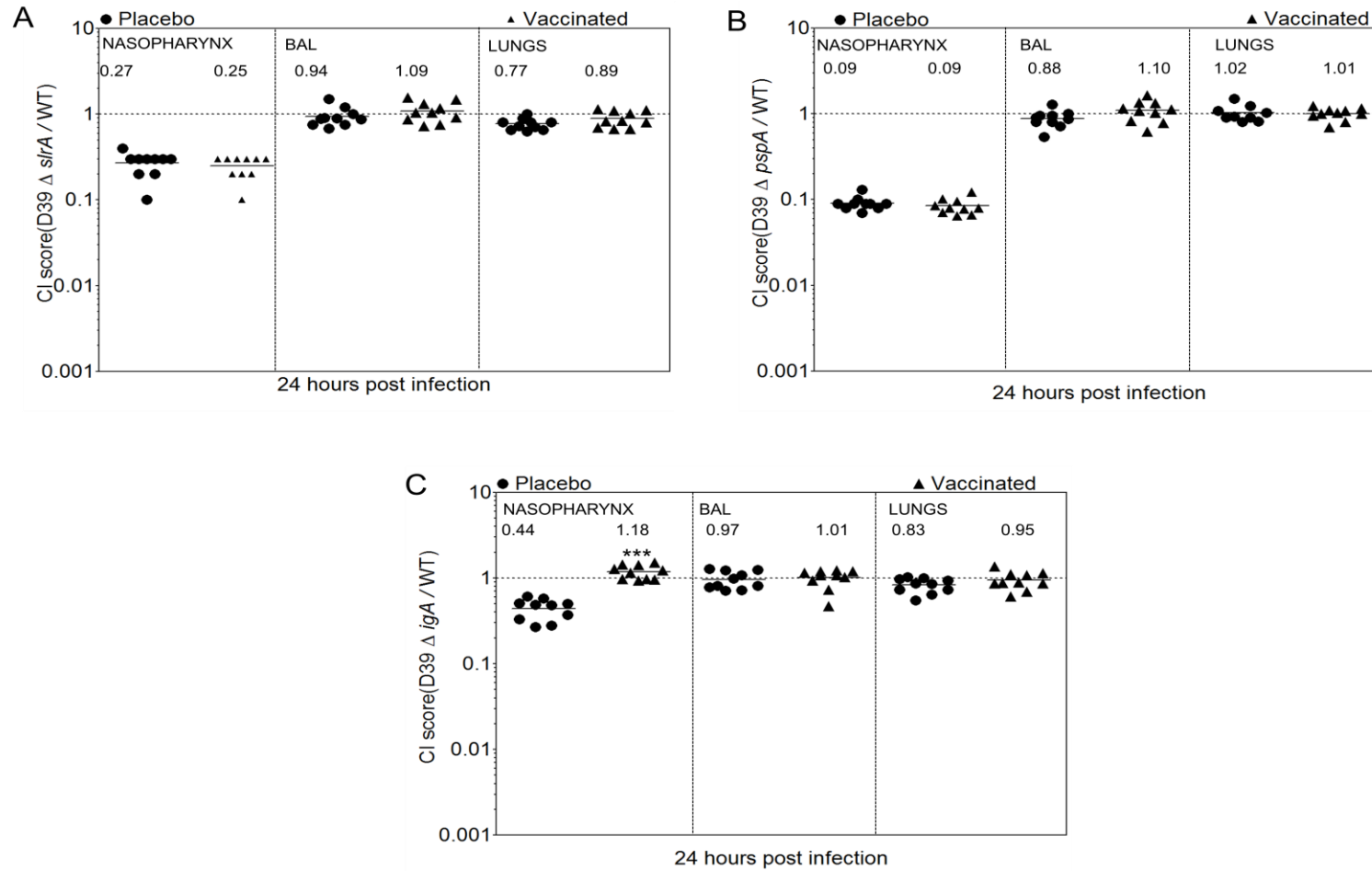


Figure 3.25. Protection against intranasal challenge with *S. pneumoniae* D39. Groups of 20 mice equally distributed into the vaccinated and placebo groups were subcutaneously injected with saponin+PBS+ (A) rSlrA, (B) rPspA or (C) rIgA1 bacterial protease and saponin+PBS, respectively. Mice were subsequently inoculated with a 1:1 mixture of D39 wild-type and D39 knockout strains. Pneumococci were recovered at 24h post infection from the nasopharynx, BAL fluid and lung tissue. A value of 1 indicates that both strains are proliferating equally. Values less than 1 indicate attenuation of the knockout and those greater than 1 indicate out-competition of the wild-type strain. A Mann-Whitney test was used for comparison of mean CI in placebo and vaccinated mice. Data represent average CIs from a single experiment. *** $p \leq 0.0001$.

Table 3.8 Standard deviations and coefficients of variations based on pneumococcal CFUs and competitive index scores of PJ351Δ*slrA*/PJ351::*rpsL*, PJ351Δ*pspA*/PJ351::*rpsL* and PJ351Δ*igA1*/PJ351::*rpsL* strains in the nasopharynx, BAL fluid and lungs.

	A.NASOPHARYNX				B.BAL				C.LUNGS			
	PLACEBO (PJ351Δ <i>slrA</i> / PJ351:: <i>rpsL</i>)		VACCINATED (PJ351Δ <i>slrA</i> / PJ351:: <i>rpsL</i>)		PLACEBO (PJ351Δ <i>slrA</i> / PJ351:: <i>rpsL</i>)		VACCINATED (PJ351Δ <i>slrA</i> / PJ351:: <i>rpsL</i>)		PLACEBO (PJ351Δ <i>slrA</i> / PJ351:: <i>rpsL</i>)		VACCINATED (PJ351Δ <i>slrA</i> / PJ351:: <i>rpsL</i>)	
	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score
Mean	38111	0.01	3909	0.09	726330	0.07	49443	0.07	820810	0.05	43707	0.04
Std. Deviation	45546	0.02	5298	0.03	831319	0.02	61579	0.02	1.52e+006	0.01	70248	0.01
Lower 95% Ci of mean	5529	0.08	118.8	0.07	131635	0.06	5392	0.06	-262628	0.04	-6546	0.03
Upper 95% Ci of mean	70693	0.11	7699	0.11	1.32e+006	0.08	93494	0.09	1.90e+006	0.05	93960	0.04
Coefficient of variation (CV)	120%	21%	136%	30%	114%	21%	125%	24%	185%	24%	161%	19%

	D.NASOPHARYNX				E.BAL				F.LUNGS			
	PLACEBO (PJ351Δ <i>pspA</i> / PJ351:: <i>rpsL</i>)		VACCINATED (PJ351Δ <i>pspA</i> / PJ351:: <i>rpsL</i>)		PLACEBO (PJ351Δ <i>pspA</i> / PJ351:: <i>rpsL</i>)		VACCINATED (PJ351Δ <i>pspA</i> / PJ351:: <i>rpsL</i>)		PLACEBO (PJ351Δ <i>pspA</i> / PJ351:: <i>rpsL</i>)		VACCINATED (PJ351Δ <i>pspA</i> / PJ351:: <i>rpsL</i>)	
	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score
Mean	6262	0.19	1132	0.15	434900	0.02	12938	0.02	158650	0.02	4520	0.02
Std. Deviation	5487	0.05	1147	0.03	459556	0.01	17681	0.01	159962	0.01	5472	0.01
Lower 95% Ci of mean	2044	0.15	249.6	0.13	106150	0.02	290.2	0.02	44219	0.02	604.9	0.02
Upper 95% Ci of mean	10480	0.23	2013	0.18	763650	0.03	25586	0.03	273080	0.03	8434	0.02
Coefficient of variation (CV)	88%	28%	101%	20%	106%	30%	137%	22%	101%	26%	121%	28%

	G.NASOPHARYNX				H.BAL				I.LUNGS			
	PLACEBO (PJ351ΔigA1/ PJ351::rpsL)		VACCINATED (PJ351ΔigA1/ PJ351::rpsL)		PLACEBO (PJ351ΔigA1/ PJ351::rpsL)		VACCINATED (PJ351ΔigA1/ PJ351::rpsL)		PLACEBO (PJ351ΔigA1/ PJ351::rpsL)		VACCINATED (PJ351ΔigA1/ PJ351::rpsL)	
	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score
Mean	36908	0.27	15289	0.24	1.086e+006	0.09	127200	0.08	909063	0.08	88118	0.07
Std. Deviation	86495	0.06	36714	0.06	582835	0.02	103651	0.02	1.07e+006	0.02	131820	0.02
Lower 95% Ci of mean	-29578	0.22	-12932	0.20	598482	0.07	40544	0.07	12728	0.06	-22088	0.06
Upper 95% Ci of mean	103394	0.31	43510	0.28	1.57e+006	0.11	213856	0.09	1.81e+006	0.10	198323	0.08
Coefficient of variation (CV)	234%	23%	240%	26%	54%	26%	81%	22%	118%	29%	149%	26%

Table 3.9 Standard deviations and coefficients of variations based on pneumococcal CFUs and competitive index scores of D39ΔslrA/D39::rpsL, D39ΔpspA/D39::rpsL and D39ΔigA1/D39::rpsL strains in the nasopharynx, BAL fluid and lungs.

	A.NASOPHARYNX				B.BAL				C.LUNGS			
	PLACEBO (D39ΔslrA/D39::rpsL)		VACCINATED (D39ΔslrA/ D39::rpsL)		PLACEBO (D39ΔslrA/ D39::rpsL)		VACCINATED (D39ΔslrA/ D39::rpsL)		PLACEBO (D39ΔslrA/ D39::rpsL)		VACCINATED (D39ΔslrA/ D39::rpsL)	
	pneumococcal CFUs	CI score	pneumococcal CFUs	CI score	pneumococcal CFUs	CI score	pneumococcal CFUs	CI score	pneumococcal CFUs	CI score	pneumococcal CFUs	CI score
Mean	22650	0.27	6124	0.25	36104	0.94	48247	1.09	853205	0.77	730492	0.89
Std. Deviation	13701	0.08	4304	0.07	66206	0.24	55536	0.29	1.166e+006	0.12	1.038e+006	0.19
Lower 95% Ci of mean	12849	0.21	3045	0.20	-11258	0.77	8519	0.88	18888	0.68	-11912	0.75
Upper 95% Ci of mean	32451	0.33	9203	0.30	83466	1.12	87975	1.30	1.688e+006	0.86	1.473e+006	1.03
Coefficient of variation (CV)	60%	30%	70%	28%	183%	26%	115%	27%	137%	16%	142%	21%

	D.NASOPHARYNX				E.BAL				F.LUNGS			
	PLACEBO (D39 Δ pspA/ D39:: <i>rpsL</i>)		VACCINATED (D39 Δ pspA/ D39:: <i>rpsL</i>)		PLACEBO (D39 Δ pspA/ D39:: <i>rpsL</i>)		VACCINATED (D39 Δ pspA/ D39:: <i>rpsL</i>)		PLACEBO (D39 Δ pspA/ D39:: <i>rpsL</i>)		VACCINATED (D39 Δ pspA/ D39:: <i>rpsL</i>)	
	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score
Mean	127620	0.09	15867	0.09	777350	0.88	663130	1.10	770278	1.02	718089	1.01
Std. Deviation	71882	0.02	15380	0.02	703577	0.20	616596	0.30	922811	0.23	833373	0.16
Lower 95% Ci of mean	76198	0.08	4865	0.07	274037	0.74	222040	0.88	60944	0.85	77503	0.89
Upper 95% Ci of mean	179042	0.10	26869	0.10	1.281e+006	1.02	1.104e+006	1.32	1.480e+006	1.20	1.359e+006	1.12
Coefficient of variation (CV)	56%	18%	97%	21%	91%	22%	93%	28%	120%	22%	116%	16%

	G.NASOPHARYNX				H.BAL				I.LUNGS			
	PLACEBO (D39 Δ igA1/ D39:: <i>rpsL</i>)		VACCINATED (D39 Δ igA1/ D39:: <i>rpsL</i>)		PLACEBO (D39 Δ igA1/ D39:: <i>rpsL</i>)		VACCINATED (D39 Δ igA1/ D39:: <i>rpsL</i>)		PLACEBO (D39 Δ igA1/ D39:: <i>rpsL</i>)		VACCINATED (D39 Δ igA1/ D39:: <i>rpsL</i>)	
	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score	Pneumococcal CFUs	CI score
Mean	193860	0.44	81140	1.18	70987	0.97	74929	1.01	740628	0.83	652867	0.95
Std. Deviation	109436	0.12	49300	0.22	77949	0.23	92344	0.24	1.625e+006	0.16	1.427e+006	0.22
Lower 95% Ci of mean	115574	0.36	45873	1.03	15225	0.80	8869	0.84	-422152	0.72	-367670	0.79
Upper 95% Ci of mean	272146	0.53	116407	1.34	126749	1.13	140989	1.18	1.903e+006	0.95	1.673e+006	1.11
Coefficient of variation (CV)	56%	27%	61%	19%	110%	24%	123%	24%	219%	20%	219%	24%

3.5.3 Antibody responses against pneumococcal proteins

In order to analyse antibody responses after subcutaneous immunisations, serum was collected from mice by way of cardiac puncture and analysed by ELISA. We observed a significant difference in antibody concentrations between sera from the placebo mice and immunised mice 24 hours after challenge with PJ351 (IgA1 bacterial protease: 0.020 ± 0.020 U/mL vs 595 ± 315 U/mL; $p < 0.0001$; PspA: 0.020 ± 0.020 U/mL vs 1457 ± 581 U/mL; $p < 0.0001$ and SlrA: 0.020 ± 0.020 U/mL vs 882 ± 165 U/mL) and D39 (IgA1 bacterial protease: 0.020 ± 0.020 U/mL vs 706 ± 329 U/mL; $p < 0.0001$; PspA: 0.020 ± 0.020 U/mL vs 1539 ± 822 and SlrA: 0.020 ± 0.020 U/mL vs $794 \pm$ U/mL). However, there was no detectable relationship between antibody concentration in the vaccinated animals and the CI ratios between the wild-type strains and isogenic knockouts.

CHAPTER 4 DISCUSSION

4.1 Development of an intranasal pneumonia co-inoculation model

The first part of this work was to establish an intranasal pneumonia co-inoculation model in NMRI mice for at least two *S. pneumoniae* strains, PJ351 (serotype 1) and D39 (serotype 2). We aimed to 1) Use clinically relevant strains that were relatively virulent in mice 2) Mimic the natural course of disease in humans 3) Reduce effects of variability between mice and 4) Reduce the number of mice required for an experiment.

Mouse and pneumococcal strain selection were imperative to the success of our study. The outbred mouse strain was chosen in this study based on a number of reasons. Firstly, outbred mouse strains are representative of a human population which is heterogeneous in nature, making them the desirable strain to work with. Secondly, inbred strains are known to produce very variable results in response to pneumococcal infection whereas the survival times of different strains of outbred mice were more consistent (Gingles *et al.* 2001). Finally, we used NMRI mice due to the availability of a strong colony of NMRI mice in our institution.

An ideal pneumococcal strain for our study was one that was clinically responsible for pneumococcal infection in children less than five years old and relatively virulent in mice. Data in this thesis indicated that PJ351 (serotype 1) and D39 (serotype 2) were more virulent than TIGR4 (serotype 4) as indicated by higher density values of 4.44×10^5 CFU in the lungs compared to 1.42×10^4 CFU at the end of 24 hours, for the TIGR4 strain. These observations indicated pneumococcal strain-specific behaviour *in vivo* as described previously by Stol *et al.* In their development of a non-invasive mouse model of otitis media, they found that TIGR4 (serotype 4) was more virulent to BALB/c mice, followed by SME215 (serotype 19F), PJ1324 (serotype 6B) and D39 (serotype 2) (Stol *et al.* 2009).

Serotype 4 is one of the serotypes responsible for 5% of pneumococcal infections in children <5 years old and in the elderly (Silberbauer *et al.* 2011) and it is one of the 13 serotypes included in the pneumococcal conjugate vaccine. The challenge of BALB/c mice with serotype 4 resulted in low-level bacteraemia that slowly lead to meningitis; making this strain important in studies involving the pathology of meningitis (Orihuela *et al.* 2003). In our

studies, pathogenesis of TIGR4 from the nasopharynx to lungs following intranasal inoculation was variable. There was successful colonisation of the nasopharynx, but the growth of TIGR4 in the bronchoalveolar tract and lungs was defined by a reduction in recovery of bacterial numbers at different time points. This was not surprising because TIGR4 is considered a low capsule-producing strain, which can be easily cleared from lungs and subsequently in the bronchoalveolar tract with the aid of phagocytes (Orihuela *et al.* 2003). When choosing a pneumococcal strain to work with, a virulent strain is an appropriate choice because it leads to successful colonisation and a higher dose of inoculum is not required to initiate infection. The coefficient of variation indicated high variability in the ability of TIGR4 to infect the bronchoalveolar tract and lung tissue, compared to PJ351 and D39.

Serotype 1 is responsible for about 21% of pneumococcal infections causing invasive disease in children > 5 years of age in Sub-Saharan Africa (Ba *et al.* 2014). It is perhaps more clinically relevant than D39, as it includes many more strains found to cause invasive pneumococcal disease in humans (Brueggemann & Spratt 2003). Serotype 1 is exceptionally virulent in NMRI mice and is not readily cleared by host's immune responses. Serotype 2 is also a highly virulent serotype and is an important cause of invasive disease in Sub-Saharan Africa, accounting for about 6% of pneumococcal infections in children less than five years of age (Donkor *et al.* 2013). The challenge of MF1 and CD1 mice with serotype 2 results in septicaemia with the end-point often being death. Our data indicated that PJ351 and D39 were more able to invade the lung mucosae following I.N inoculation, with densities of 10^5 CFU being recovered at the end of 24 hours. PJ351 and D39 offer the opportunity to study protection by antibodies at the nasopharynx and lung mucosae, as a model of non-bacteraemic pneumonia.

The intranasal route was the ideal method of inducing infection in NMRI mice since it follows the natural course of disease in the human species, in that disease stems from the nasopharynx. In this thesis, the success of the intranasal route was evident as nasopharyngeal colonisation was maintained throughout the course of the experiment, following intranasal inoculation with 10^6 CFU of pneumococci. Thus, in the placebo group, initial counts for PJ351 and D39 were 10^5 CFU and dropped by only a log to 10^4 CFU at each time point. Numbers of bacteria (between 10^5 and 10^6 CFU) in the BAL fluid and lungs were recovered

for the duration of the experiment, thus demonstrating efficient infection following intranasal inoculation.

The cornerstone of animal testing is a three pronged strategy of replacement, reduction and refinement. Whilst it is extremely difficult to replace animal models in vaccine testing, since the complete functioning of an intact immune system cannot be emulated *ex vivo*, there is still considerable room for the improvements through reduction of the sample size required and refinement of the read out systems to measure the vaccine efficacy. In this thesis, we refined the intranasal pneumonia mouse model by bringing forward the time point for terminating the experiment and by extending the co-inoculation setup to include vaccinated mice. In contrast to previously described mouse models of disease, the 24 hour time point was selected since our main endpoint was bacterial outgrowth and survival. Previous data indicated that mice challenged I.N. with 50µL of 4.3×10^6 CFU of serotype 1 resulted in heavy lung infection and bacteraemia, with 100% deaths occurring between 24 and 30 hours after challenge (Jakobsen *et al.* 1999). In another study, outbred MF1 mice intranasally infected with 50 µL of 10^6 CFU of D39 displayed initial signs of clinical disease at 24 hours and mice survived up until 34 hours (Kerr *et al.* 2004). In a separate study, outbred CD1 mice intranasally challenged with 50 µL of 10^7 CFU of D39 had a median survival time of 48 hours (Cohen *et al.* 2012). Beyond the 24 hour time point was inhumane as mice had the potential of becoming septic or moribund. In this study, the 24 hour time point was used based on data from other studies indicating that beyond the 24 hour time point, most outbred strains infected with an inoculum of 10^6 CFU had the potential to develop septicaemia which results in death. Refinement in this model replaced survival time in the following way: 1) Numbers of colonising bacteria are derived entirely from bacterial counts rather than survival of the host animals and it can therefore distinguish between knockout strains whose attenuation is too subtle to be detected by survival time. Furthermore, survival time relies on the number of animals surviving the infection whereas the competitive index is obtained from the numbers of bacteria before and after the infection, providing a figure that is based on a much larger sample size, 2) The quantitative counts are performed on multiple sample types (nasopharyngeal lavage, bronchoalveolar tract lavage fluid and lung tissue), which yields more data per mouse to scrutinise and 3) By co-inoculating and using the ratio of the two strains in a particular mouse, one eliminates the sources of variability in the procedures, such as inoculum uptake and sample collection. Considering the reduced number of mice used in

this model (5 animals per time point), the variability and precision in this model is acceptable, in that the CV's of the CI values in a co-inoculation situation ranged from 0-30% depending on the strain and protein used. Whereas the CV's of colony counts within a group for a particular bacterial strain ranged from 0-200%. These refinements contribute to the reduction in sample size. Thus for experiments reported in Figures 3.14-3.21, 5 mice per time totalling point 20 mice per experimental group were used compared to the approximately 89 mice per time point (356 mice per experimental group) that would have been required if the wild-type and knockout strains were inoculated into different mice as determined by a post-hoc sample size calculation ($\mu_1=52764$; $\mu_2=72165$; $SD=92095$, power= 0.80, $\alpha=0.05$).

One of the concerns with this model is the possibility of competition between the wild-type and knockout strains. However, several factors support its use: 1) Both strains were inoculated at a low (non-competitive) dose and given ample space to grow at a same rate, further, the two strains are essentially the same strain with respect to most factors e.g. capsule type, bacteriocin production (Son *et al.* 2011) and hence the immune system does not see them as different, and the strains are unlikely to react to each other in a different way, 2) In the co-inoculation setup, the knockout remains constant to indicate that the calibrator (which is the knockout) is not influenced by a decrease in the wild-type strain and 3) The knockout strain is invisible to the vaccine induced host immune response, thus it is not targeted by antibodies.

4.1.2 Construction of pneumococcal deletion knockouts in PJ351 and D39 strains

PpmA, *slrA*, *pspA*, *pspC*, *stkP* and *iga1* are found in all pneumococcal strains and their flanking sequences are highly similar in strains chosen for this study (PJ351 and D39). As such, it was possible to use the same primer design for constructing PJ351 and D39 knockouts strains. The successful creation of knockouts, in general, is due to a deletion strategy used by (Hermans *et al.* 2006) that allows for in-frame deletion. The success of this cloning strategy is hinged on the ability to remove fragments of equal length to the inserted cassettes to eliminate the possibility of any polar effects. Although it would have been beneficial to this study to conduct genotypic verification and genetic complementation, there were some budget and time constraints that prohibited that possibility. The recovery of all knockout strains was significantly lower compared to the wild-type strains in the

nasopharynx 24 h after co-inoculation of unvaccinated mice with equal proportions of a PJ351 or D39 wild-type and the isogenic knockout strains. Thus, no genetic rearrangements were expected in resulting knockouts: The observed behaviour of the knockout strains were supported by publications related to similar knockouts as discussed in more detail in section 4.2.

The limitation of our model is that we can only target non-essential genes which show no attenuation of growth *in vitro* when knocked out using the model. It is also important to note that most essential genes are housekeeping genes (involved in protein synthesis, glycolysis etc.) (Riddle *et al.* 1997) which are unlikely to make good vaccine components. Since they are not surface exposed (cytoplasmic), they are not specific and likely to cross-react with similar antigens on the surface of other organisms, including those that make up the host commensal flora (Peng & Gao 2014; Klein & Jones 1994). However, there are a few exceptions such as StkP and PcsB that are surface exposed proteins (Giefing *et al.* 2008) and have been demonstrated to contribute to pneumococcal virulence and identified as potential vaccine candidates. Despite the limitation of our model, it is important to note that 66% of all genes in the pneumococcal genome are non-essential (Bijlsma *et al.* 2007) and therefore despite the limitation of only testing non-essential genes with similar *in vitro* growth characteristic, the model can still be applied to a significant portion of the genome. In support of this statement is work that was done by others such as Hermans *et al.* (2006), Kerr *et al.* (2004) and Cron *et al.* (2011). In their studies, using the co-inoculation model, they uncovered novel non-essential genes (*slrA*, *ppmA*, *ami-aliA/aliB* Permease and two DHH Subfamily 1 Proteins) that were demonstrated to contribute to pneumococcal virulence.

4.2 Usefulness of the intranasal pneumococcal pneumonia co-inoculation model to study pneumococcal virulence

An added benefit of this model, is that we were able to analyse differences in the behaviour of the wild-type and knockout strains in the unvaccinated mice which enables us to gain insight into the function of these proteins with respect to their roles in virulence, such as the ability of the knockouts to establish colonisation of the NP, infection of the BAL fluid and lungs.

Pneumococcal surface protein C (PspC) is one of the well-studied proteins which is preferentially expressed in the transparent phase favoured in the nasopharynx (Rosenow *et al.* 1997). In our mouse model, PJ351- and D39 *pspC* knockout strains were attenuated in their ability to colonise the nasopharynx. Our data are comparable to previously published data that demonstrated the role of PspC in nasopharyngeal colonisation in a rat and mouse models (Rosenow *et al.* 1997; Balachandran *et al.* 2002).

The bronchoalveolar tract is a transitional pathway of bacteria from the nasopharynx to the lungs. It holds valuable information about the status of the lungs in infected animals. In our co-inoculation pneumococcal pneumonia mouse model, PJ351 *pspC* and D39 *pspC* knockouts displayed an inability to colonise the bronchoalveolar tract and grow out in the lungs of mice. The contribution of PspC on the growth of the pneumococcus in the bronchoalveolar tract has been previously studied. In a study concentrating on the role that proteins have in the progression of disease, *pspC* was instrumental in the transition of the bacteria from the nasopharynx to the lungs (Orihuela *et al.* 2004). On the other hand, there are conflicting results regarding the role of PspC on lung infection. Using an MF-1 fatal pneumonia mouse model, strain specificity in virulence of *pspC* was demonstrated. Serotypes 2, 3 and 19 deficient in *pspC* behaved similarly to their isogenic wild-type strains; whereas *pspC* serotype 4 deficient strain was easily cleared from the system (Kerr *et al.* 2006). A *pspC* knockout in a D39 background was not attenuated in a CBA/N fatal pneumonia mouse model (Ogunniyi *et al.* 2007). The discrepancy in our results with another data is probably due to the use of challenge models that rely on survival as an endpoint. In this regard, our intranasal pneumonia co-inoculation model is more relevant to determine the role of PspC on pneumonia because our main endpoint was bacterial growth.

Putative proteinase maturation protein (PpmA) is a surface associated lipoprotein that is conserved in most pneumococcus strains. In this study, we confirmed the contribution of PpmA on colonisation in both the PJ351 and D39 strains. We also indicated that pneumococcal knockouts of PJ351 and D39 lacking the gene encoding PpmA were easily cleared from the upper airways and lungs. This is in agreement with Cron *et al* who demonstrated the contribution of PpmA on colonisation in the genetic background strains of D39 and TIGR4 (Cron *et al.* 2009). In another study, mice infected with pneumococcal knockouts deficient in PpmA survived longer than mice infected with the wild-type strain (Overweg *et al.* 2000), thus indicating their contribution in virulence of mice.

PspA is an extensively studied protein in the family of choline binding proteins. Our results clearly demonstrated that deletion of *pspA* in the PJ351 and D39 strains resulted in the inability of the strain to colonise the nasopharynx of mice, thus indicating its contribution in the early stages of infection, colonisation. Our results concur with a previous study that showed that PspA-deficient pneumococci had a reduced ability to infect the nasopharynx of mice (Ogunniyi *et al.* 2007). We further went on to demonstrate the involvement of PspA in PJ351 on growth in the bronchoalveolar tract and lungs of mice. This was evidenced by the reduced ability of the PJ351 strain lacking PspA to grow in the bronchoalveolar tract and lungs of mice. In support of our data, is a study by Hava and Camilli that showed the inability of the TIGR4 strain lacking the *pspA* gene to grow in the lungs of mice; thus indicating its role in the establishment of pneumonia (Hava & Camilli 2002). Another study that is in support of our data is by Ren *et al*, who showed that a WU2 strain (serotype 3) deficient in PspA had significant reductions in virulence compared to its isogenic wild-type strain (Ren *et al.* 2003). We, however, failed to demonstrate the contribution of PspA in the D39 background on growth in the bronchoalveolar tract and lungs of mice. A study by Orihuela *et al* supports our findings (Orihuela *et al.* 2004). They showed that a D39 PspA deficient strain infected the lungs of mice in a similar manner as the wild-type strain. Berry and Paton further revealed no significant differences in growth following infection with 10^5 CFU of a D39 strain deficient in PspA and its isogenic wild-type strain (Berry & Paton 2000), thus indicating that PspA did not attenuate the D39 strain. This contrasting information suggests that the virulence of PspA is dependent on the strain-specific background. An explanation for strain-specific behaviour is due to the presence or absence of other virulence genes directly or indirectly contributing to observed phenotypes (Cron *et al.* 2011).

Streptococcal lipoprotein rotamase A (SlrA) is a surface-exposed lipoprotein that promotes colonisation by the pneumococcus. Using our intranasal pneumococcal pneumonia co-inoculation model, we showed that SlrA contributed on colonisation of the PJ351 and D39 pneumococci. This was indicated by the reduced ability of the PJ351 and D39 strains deficient in *slrA* to colonise the nasopharynx of mice. This finding is corroborated by results of a previous study showing that SlrA is required for colonisation by the pneumococcus (Hermans *et al.* 2006). We further demonstrated the involvement of *slrA* on the growth of the pneumococcus in the bronchoalveolar tract and lungs of mice. We showed that the PJ351 strain lacking *slrA* was attenuated in its ability to grow in the bronchoalveolar tract and lungs of mice. On the other hand, we failed to demonstrate the contribution of *slrA* on the growth of D39 in the bronchoalveolar tract and lungs of mice. This was evidenced by the equal recovery of the Δ *slrA* and wild-type strains from the bronchoalveolar lavage fluid and lungs of mice. Our data are comparable to a study by Hermans *et al.*; by using an invasive mouse pneumonia model, they revealed non-significant differences in the survival times between mice challenged with D39 and the *slrA* knockout (Hermans *et al.* 2006). We thus conclude that SlrA is involved in the establishment of colonisation, assists in the transition of the PJ351 pneumococcus from the nasopharynx to the lungs and in the establishment of pneumococcal pneumonia.

Bacterial IgA1 protease is a surfaced exposed protein that is present in all pneumococcal strains. Using our intranasal pneumonia co-inoculation model, we were able to demonstrate that bacterial IgA1 protease plays a role in bacterial colonisation since the PJ351- and D39 *igA1* deficient strains were attenuated in their ability to colonise the nasopharynx of mice. Our data is in accordance with other studies that have demonstrated the role of IgA1 protease in colonisation by the pneumococcus (Weiser *et al.* 2003). In their study, by using human anti-capsular polysaccharide IgA1, they showed that IgA1 protease modified IgA1 antibody so that it promoted rather than inhibited pneumococcal adherence to epithelial cells. In our model, the ability of IgA1 protease in the PJ351 strain to infect the bronchoalveolar tract and lungs was demonstrated by a reduced inability of the PJ351 Δ *igA* strain to grow in the bronchoalveolar tract and lungs of mice. A study by Chiavolini *et al.*, also demonstrated the prolonged survival of mice infected with a null *igA1* strain compared to a TIGR4 wild-type strain thus indicating its role towards certain stages of virulence (Chiavolini *et al.* 2003). On the other hand, the role of IgA1 protease in the growth of the

D39 strain in the BAL fluid and lungs were not successfully demonstrated. D39 strains deficient in *igA1* were equal in their ability to replicate in the bronchoalveolar tract and lungs of mice. Our data is in accordance with the signature tagged mutagenesis data of Lau *et al*, who did not find that zinc metalloproteinases genes contributed to the virulence of serotype 3 WU2 strain (Lau *et al*. 2001). This difference in data suggests that the virulence of IgA1 might be dependent on the strain-specific background, as already discussed in text.

4.3 Usefulness of the intranasal pneumococcal pneumonia co-inoculation model to study protection of pneumococcal proteins

In order to demonstrate the sensitivity of our model in evaluating the efficacy of vaccine candidate molecules, we co-inoculated vaccinated mice with either wild-type PJ351 or D39 and their isogenic knockouts. We suggest that a lot of assays might not be sensitive enough to show protection of vaccine candidate pneumococcal proteins in a lethal challenge model with survival as an endpoint. As a result, the potential of some protein antigens might have been overlooked in previous studies by testing their efficacy in mouse models that rely on survival as an endpoint.

PspC is a promising candidate for the composition of a protein vaccine against *S. pneumoniae* (Lima *et al.* 2013). In our model, PspC has been shown to elicit a significant level of protection against infection by the pneumococcus. Using a setup that allows the infection of the knockout and wild-type strains into one mouse, we demonstrated protection of PspC against pneumococcal infection. Other studies, however, were not sensitive enough to show protection of a single recombinant PspC in a lethal challenge model with survival as an endpoint. In a study done by Ferreira *et al.*, no significant changes in pneumococcal CFU in the lungs and no significant survival were observed among all mice vaccinated with any PspC formulation, using an invasive intranasal mouse model (Ferreira *et al.* 2009), despite this protein being implicated in the process of pneumonia. In another study in which mice were vaccinated with recombinant PspC and challenged intraperitoneally with a lethal dose of 10^7 CFU of D39, recombinant PspC did not elicit significant protection against the pneumococcus over that of the placebo (Ogunniyi *et al.* 2001). In a separate study, in lieu of demonstrating the protective efficacy of PspC, mice were intraperitoneally vaccinated with the protein and subsequently challenged with different doses of 10^2 , 10^5 and 10^6 CFU of D39 and 6A. Mice were, however, not significantly protected against pneumococcal infection (Ogunniyi *et al.* 2007). This data clearly demonstrates limitations of invasive mouse models in demonstrating the efficacy of single recombinant PspC against pneumococcal infections compared to our intranasal pneumonia co-inoculation mouse model.

PpmA is a promising vaccine candidate, which has been included in studies evaluating natural responses in children towards pneumococcal proteins (Lebon *et al.* 2011). PpmA is a lipoprotein that is embedded in the cell membrane and therefore not detectable on the surface

of the pneumococcus. Further, surface accessibility to exogenous antibody has been demonstrated to be hindered by the presence of the cell wall and capsule (Gor *et al.* 2005), thus making it a poor vaccine candidate. However, our intranasal pneumonia co-inoculation model shows that the PpmA protein has the potential of protecting mice against colonisation and pneumonia. The PJ351 bacterial counts recovered from the nasopharynx, BAL fluid and lungs of mice were significantly reduced, whereas the D39 counts were only significantly lowered in the nasopharynx of mice. In support of our results is an *in vitro* study that showed that PpmA antibodies facilitated phagocytosis of serotypes 4, 6A, 9V, 14, 18C, 19F, and 23F (Overweg *et al.* 2000), thus indicating the potential of PpmA in protecting mice against pneumococcal infection. On the other hand, current mouse models of disease have failed to demonstrate the protective potential of PpmA. PpmA did not afford any protection in mice against lethal systemic infection with 500 CFU of a serotype 3 strain (Gor *et al.* 2005). Another group failed to demonstrate its protective efficacy in a co-inoculation pneumonia mouse model (Audouy *et al.* 2007). In their study, there were no significant reductions in the D39 bacterial numbers in mice vaccinated with PpmA compared to placebo mice. Comparing our data to these studies, it seems the difference in results was dependent on the type of mouse model used. PpmA did not afford any protection in a mouse model of lethal infection as revealed in the Gor *et al.* study whereas in our mouse model, which relied on relative bacterial outgrowth as an endpoint, protection was successfully demonstrated. Audouy *et al.* used counts which have a high CV between mice from each group. We used CI's which have low CV's between mice in each group. Therefore, our model was statistically more sensitive.

SlrA is an immunogenic protein that elicits antibody responses early in life (Lebon *et al.* 2011). SlrA was, however, not protective against colonisation and pneumonia with the PJ351 and D39 strains in our mouse model. Given the results obtained for PpmA, it was expected that SlrA could elicit some protective potential against pneumococcal infection. However, a dissimilarity in results might indicate a difference in relative expression levels of SlrA and PpmA and capsular structure (Jomaa *et al.* 2005).

Bacterial IgA1 has a high affinity to secretory IgA which is predominant in the nasopharynx of human, thus it follows that bacterial IgA should contribute to the stages of colonisation rather than invasive disease. In our intranasal pneumonia co-inoculation model, we clearly showed the protective efficacy of bacterial IgA against pneumococcal

colonisation. This was indicated by a reduction in the D39 bacterial counts in the nasopharynx of mice following immunisation with bacterial IgA1. Our data does not match the results obtained by Audouy *et al*, who demonstrated that bacterial counts in the nasopharynx were not lowered in the presence of monovalent bacterial IgA1, in a pneumonia mouse model (Audouy *et al*. 2007). Audouy *et al* used single inoculation of bacteria which has a lower statistical power due to variability in the inoculation preparation and sampling as opposed to our model which used co-inoculation of bacteria and thus increasing the precision with which measurements are made.

PspA is an important candidate for an alternative vaccine against pneumococcal infections, as demonstrated in pre-clinical and phase I studies (Vadesilho *et al*. 2014). Surprisingly, in our model the protective efficacy of PspA was not demonstrated. Our data are in accordance to work done by McDaniels *et al*, explaining that PspA was not protective against the virulent strain D39-serotype 2 (Mcdaniel *et al*. 1991). In her paper, Shilpa Basavanna also explained that recombinant PspA did not elicit protective antibodies against the D39 strain (Basavanna *et al*. 2009). Further work by the Intercell team also showed the inability of PspA to protect mice against serotype 1 and 4 (Intercell AG fact sheet 2005). Different from our data is work by Wu *et al*, who showed that intranasal immunisation of mice with PspA protected them against lethal infection with an A66 strain-serotype 3 (Wu *et al*. 1996). Recent work by Kong *et al* also revealed the protective capabilities of a nanogel based PspA vaccine against infection with an A66 strain (Kong *et al*. 2013). The likely explanation for our data, based on this information, is that PspA is not protective against virulent pneumococcal strains such as serotype 1 and 2. For instance, pathogenesis of pneumococcal strains in NMRI mice revealed that serotype 3 was detected late in the blood compared to serotype 1 and 2 (Saeland *et al*. 2000), thus indicating that it was less virulent. In BALB/c mice, serotype 3 caused pneumonia, whereas serotype 2 resulted in high-grade septicaemia (Orihuela *et al*. 2003). These studies explain serotype 3 as being less virulent than serotype 1 and 2, thus supporting our claims. In this thesis, we successfully demonstrated the efficacy of most of our proteins in protecting mice against colonisation and pneumococcal pneumonia.

In this thesis, antibody concentrations at the time of challenge in vaccinated mice were within the range of 595-1908 U/mL compared to a range of 0.020-0.020 U/L in placebo

mice. This demonstrated the efficacy of most of our proteins in protecting mice against colonisation and pneumococcal pneumonia. However, there was no detectable relationship between antibody concentration in vaccinated mice and the competitive index ratios between the wild-type and knockout strains. The observation was that at certain time point's bacterial CFU numbers recovered from the nasopharynx, BAL fluid and lungs were lower in vaccinated mice compared to placebo mice. The importance of antibodies in protecting against IPD and colonisation can be demonstrated by the success of pneumococcal conjugate vaccines (Feldman & Anderson 2014). However, there is overwhelming evidence that indicates that aside from antibodies, there is another mechanism that depends on CD4⁺ T cell (Malley 2010; Trzcinski *et al.* 2005) and TH₁₇ responses (Moffitt *et al.* 2011). Case in point is a study that revealed that most unimmunised children at 36 months in the USA have antibody concentrations that are not at the protective level, yet disease from serotypes 10 and 14 is almost 10 fold lower at that age compared to immunised children at 12 months (Lipsitch *et al.* 2005). In mice, the ability to clear colonisation between a mouse strain deficient in antibody production and the wild-type mouse strain was similar. There was also no correlation between the density of colonisation and amounts of serum or of mucosal antibodies. This finding precluded the involvement of antibodies in clearance of pneumococcal colonisation, thus attributing it to other effectors of protection (McCool & Weiser 2004).

One feature of this model is that by using the knockout strain as a calibrator, one can achieve an idea as to the level of protection by the vaccine, and gain some hint at the mechanism behind the protection. For example, in the presence of a host immune response, the colony counts of wild-type strain can be reduced relative to the knockout by two related mechanism. The target antigen can be neutralised with respect to its normal functioning by bound antibody, or antigen target recognition can elicit clearance of the wild-type strain. Whilst both can occur concurrently and are interrelated, it is noteworthy, that the maximum effect on the CI by target neutralisation alone is a CI of 1, where the wild-type has been reduced to the same levels as the knockout strain. In cases where the CI is >1 in the vaccinated mice, any reduction of the wild-type over and above the levels of the knockout strain must be achieved by opsonophagocytic killing. Thus, in this model, CIs >1 denote the most promising antigen candidates.

What is clear from the ELISA and mouse model results is that protection against colonisation and pneumonia might be antibody mediated and there could also be a strong cell mediated immune component. In support of our findings are studies by (Lu *et al.* 2011; Lu *et al.* 2009) that indicate that the subcutaneous route of immunisation can protect mice from mucosal colonisation by triggering both the antibody and cell mediated immune response. Thus subcutaneous tissues can effectively serve as effective antigen presenting sites for mucosal immune responses to antigens. Another study, conducted in humans, that aimed to determine whether PcsB, StkP, PsaA were able to induce adaptive immune responses in the elderly group, indicated that the elderly have a naturally acquired humoral and cellular immune response to pneumococcal proteins (Schmid *et al.* 2011). This study indicates that both mechanisms of protection observed in humans could also be observed in mice models, as might be the case in our study.

4.4 CONCLUSION

We have successfully established a mouse model to evaluate pneumococcal candidate vaccines that offer several benefits over existing models. These includes refined endpoints that are able to detect vaccine efficacy with greater sensitivity than conventional endpoint models and the use of internal controls/calibrators that are able to reduce the variance in sampling that can reduce the numbers of mice required per time point by a factor of 18. The model offers the benefit of being able to establish information on the contribution of the target antigens towards virulence. A concern with the model is the variability in the response of the studied antigens in different pneumococcal host backgrounds. This coupled with the labour involved in the construction of the knockouts and the intensive sampling and colony counting, makes this a difficult model to implement in an industrial high throughput screening scenario.

4.4.1 Potential future directions

- The difference in attenuation levels observed between the PJ351 and D39 strains led to the speculation of whether differential expression of other pneumococcal genes, in the absence of *pspC*, *ppmA*, *slrA*, *iga* and *pspA*, could attribute to the observed results. It might be beneficial in future to analyse the expression of other pneumococcal genes in the PJ351 and D39 strains during establishment of colonisation, transition from the nasopharynx to lungs and establishment of pneumococcal pneumonia.
- Based on the suggested mechanism of action for PspC, PpmA and bacterial IgA1 protease reported in this study, we speculated that protection afforded by immunisation of mice with the various protein antigens is antibody mediated. We suggest that passive immunisation challenge experiments should be conducted in future to support the speculations.

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APPENDIX 1. Validation of Enzyme Linked Immunosorbent assay (ELISA)

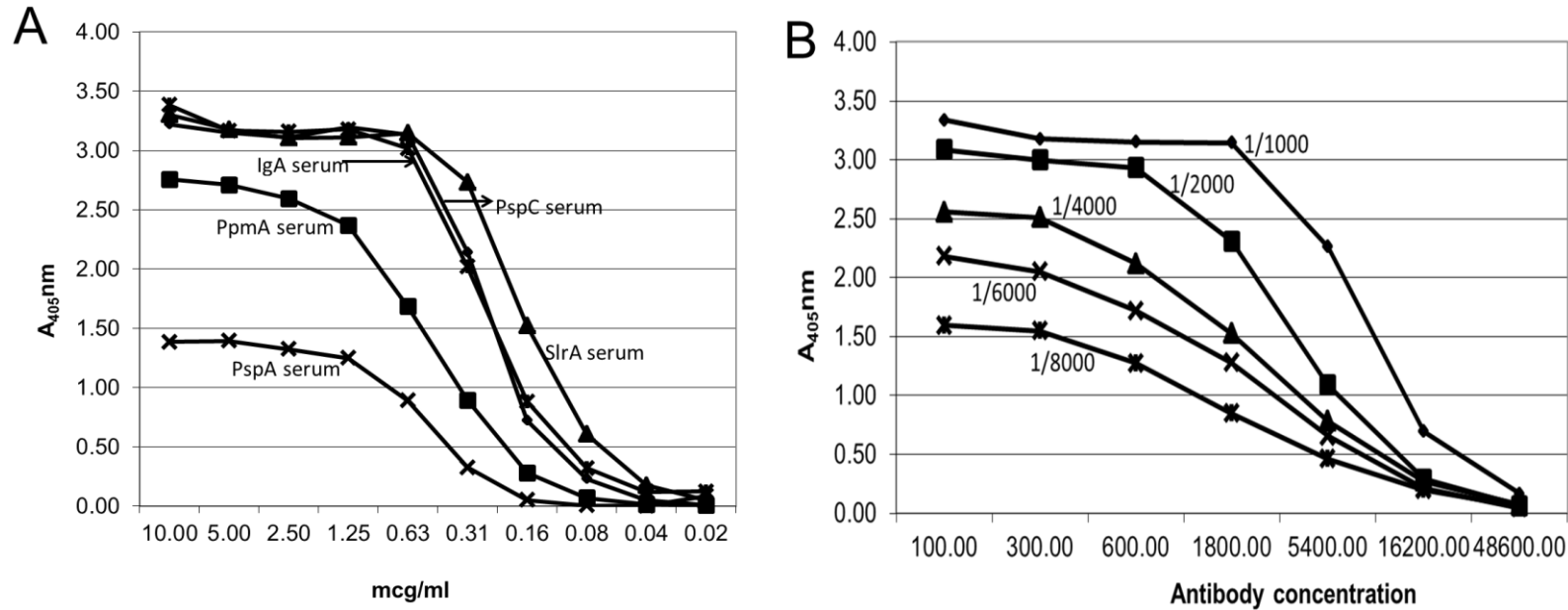


Figure 1.1. Selection of the optimal concentrations of antigens and anti-mouse alkaline phosphatase conjugate. (A). PspC, PspA, PpmA, SlrA and IgA were adsorbed onto the surface of a microtiter plate in increasing concentrations (0.01-10 $\mu\text{g/mL}$) and dropped into microtiter plates 100 μL per well (4 wells for each concentration) breadthways. Following overnight incubation at 4 $^{\circ}\text{C}$, the normal steps for ELISA were followed as mentioned in section 3.10 in materials and methods. (B). Microtitre plates were coated overnight at 4 $^{\circ}\text{C}$ with 0.625 $\mu\text{g/mL}$ of PspC and SlrA. PspA, IgA and PpmA were coated overnight with coating concentrations of 5 $\mu\text{g/mL}$ dissolved in PBS. Following 2 hours incubation at 37 $^{\circ}\text{C}$, plates were washed three times with PBS-T and alkaline phosphatase conjugated anti-mouse IgG was diluted at 1:1000-1:8000, dropped into coated wells lengthways and incubated at 37 $^{\circ}\text{C}$ for 2 hours. Following incubation at 37 $^{\circ}\text{C}$, ELISA was completed using the protocol in section 3.10 in materials and methods.

Table 1.1 Results of optimization of ELISA assays

Performance characteristic	Expected acceptance criteria	Optimization results	Comments
Background	All blanks should be <0.1	Range: 0.080 – 0.085	
Stability of assay (inter-assay precision)	Quality control sera should be within 2 SD of established value or %CV <20	High QC: CV=8-10% Low QC: CV=0-4%	Samples were tested on different plates and time points.
Intra-assay precision	Intra-plate well-to-well CV must be <10%	Sample 1: 92 wells, mean OD = 1.77, SD = 0.077, CV = 4% Sample 2: 92 wells, mean OD = 1.85, SD = 0.075, CV = 4%	2 different samples on multiple wells within the plate
Linearity	Goodness of fit of the transformed reference curve to a straight line. An R^2 value between 0.99 and 1 is expected	$R^2 = 0.99$ for all standard curves	

APPENDIX 2: CULTURE MEDIA, BUFFERS AND REAGENTS

YT agar composition (per litre)

Add the following to 800 mL H₂O

yeast extract 5 g

tryptone 8 g

NaCl 5 g

Agar, 15 g

Adjust pH to 7.0 with NaOH

Adjust volume to 1 L with dH₂O

Sterilise by autoclaving

LB broth composition (per litre)

Add the following to 800 mL H₂O

yeast extract 5 g

tryptone 10 g

NaCl 10 g

Adjust pH to 7.5 with NaOH

Adjust volume to 1 L with dH₂O

Sterilise by autoclaving

Todd Hewitt broth

Add the following to mL H₂O

Todd Hewitt broth

Yeast extract

Transformation buffer I (200 mL)

30 mM Potassium acetate 0.588 g

100 mM Rubidium chloride 2.42 g

10 mM Calcium Chloride 0.294 g

50 mM Manganese Chloride 2.0 g

15% Glycerol 30m L

pH 5.8 with dilute acetic acid and filter sterilise

Transformation buffer II (100 mL)

10mM MOPS 0.21 g

75mM Calcium chloride 1.1 g

10mM Rubidium chloride 0.121 g

15% Glycerol 15 mL

pH 6.5 with dilute NaOH and filter sterilise

50 X TAE buffer (1 litre)

Dissolve 242 g Tris in 500 mL H₂O.

Add 100 mL 0.5 M Na₂EDTA (pH 8.0) and 57.1 mL glacial acetic acid.

Adjust volume to 1 litre with H₂O.

Store at room temperature

1M Tris (1 Litre)

Dissolve 121.14 g Tris (hydroxymethyl) aminomethane (Tris, MW=121.14) in 800 mL H₂O.

Adjust pH to 8.0 by adding concentrated HCl.

Adjust volume to 1 litre with H₂O

Sterilise by autoclaving.

Store at room temperature

0.5 M Na₂EDTA (1 Litre)

Dissolve 186.12 g disodium ethylenediaminetetraacetate-2H₂O in 800 mL H₂O; stir vigorously on a magnetic stirrer.

Adjust to pH 8.0 with NaOH (~ 20g NaOH pellets) and adjust the volume to 1 litre with H₂O.

Sterilise by autoclaving

Store at room temperature

5M NaCl (1 Litre)

Dissolve 292.2 g sodium chloride in 800 mL H₂O.

Adjust volume to 1 litre with H₂O

Sterilise by autoclaving.

Store at room temperature

3M NaOAc (1 Litre)

Dissolve 408.24 g sodium acetate-3H₂O in 800 mL H₂O.

Adjust to pH 5.2 with glacial acetic acid.

Adjust volume to 1 Litre with H₂O

Sterilise by autoclaving

Store at room temperature

0.1 M IPTG (50 mL)

Dissolve 1.19 g isopropyl β-D-thiogalactopyranoside in 40 mL H₂O.

Adjust volume to 50mL with H₂O

Sterilise by filtration.

Store at -20°C.

X-gal (20 mg/mL)

Dissolve 400 mg 5-Bromo-4-Chloro-3-Indolyl-β-D-galactoside (X-gal) in 20 mL N', N'-dimethylformamide.

Store protected from light at -20°C.

10% SDS- (1 Litre)

Dissolve 100g sodium dodecyl sulphate crystals (SDS) in 900 mL H₂O.

Heat to 68°C to solute the crystals

Adjust pH to 7.2 with HCl

Adjust volume to 1 litre with H₂O

Lysozyme (10 mg/mL)

Dissolve 0.1 g of lysozyme in 10 mL of dH₂O

Proteinase K (10mg/mL)

Dissolve 0.1 g of proteinase K in 10 mL of dH₂O

Store at -20°C

Ampicillin (50 mg/mL)

Dissolve 0.5 g of ampicillin in 10 mL of dH₂O

Store at -20°C

Trimethoprim (50 mg/mL)

Dissolve 0.5 g of trimethoprim in 10 mL of dH₂O

Store at -20°C

APPENDIX 3: FORMULAS, REAGENTS AND EQUIPMENT

Formula used for calculations

Needed grams= Concentration x Molecular weight x final volume

Conversions

mg -g= divide by 100

mg - μ g=multiply by 1000

μ g-ng= divide by 1000

mL-l= multiply by 1000

mM-m=multiply by 1000

Reagents

Absolute ethanol

Acrodisc syringe filters (0.2 μ m supor membrane)

Acetic acid

Agarose

Ampicillin

Autoclavable micropipettes

Bacto tryptone

Bacto agar

Bacto Todd Hewitt broth

Bromophenol blue

Calcium chloride

Chloroform

CTAB

Disposable plastic pipettes

Disposable inoculating loops and needles

Eppendorf tubes –RNase and DNase free (0.2, 0.6, 1.5 mL)

EDTA

Expand long template PCR system

Glycerol
IPTG
Isopropanol
Lysozyme
Manganese (II) chloride
MOPS
Molecular weight markers
Petri dishes
Potassium acetate
PCR nucleotide mix
Proteinase K
PCR nucleotide mix
Primer pairs
Qiaquick gel extraction kit (Qiagen)
Rubidium chloride
Rapid DNA ligation kit
Restriction enzymes
SDS
Sodium Chloride
StrataPrep plasmid miniprep kit (Stratagene/Agilent technologies)
Tris base ultrapure
Trimethoprim
X-gal

Equipment

CO₂ incubator
Centrifuge machine
Dry bath
ELPHO power supply
Eppendorf master cycler gradient
Incubator

Orbital shaker

pH meter

Spectrophotometer

UV transilluminator

Weighing machine

Water bath

APPENDIX 4 ANIMAL ETHICS CERTIFICATE

AESC3



STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2012/05/2B

APPLICANT: Ms K W Lebogo

DEPARTMENT: Mineral Metabolism Research Unit

PROJECT TITLE: Pre-clinical evaluation of pneumococcal vaccine candidate molecules in an mouse colonization model

Number and Species

286 NMRI female mice

Approval was given for the use of animals for the project described above at an AESC meeting held on **28 February 2012**. This approval remains valid until **28 February 2014**.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form.

Signed:  Date: 13/3/2012
(Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed:  Date: 13/03/2012
(Registered Veterinarian)

cc: Supervisor:
Director: CAS