Experimental human pneumococcal carriage

Thesis submitted in accordance with the requirements of the

University of Liverpool for the degree of Doctor in Philosophy

by

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Declaration

This thesis is the result of my own work and effort. In some instances, work was done in collaboration with other colleagues and institutions. Table I details in full the attribution of work and responsibility related to the project.

The research in this thesis was carried out at the Liverpool School of Tropical Medicine.

Chapter 6: Density and duration of experimental human pneumococcal carriage is also presented in the thesis of Amelieke JH Cremers at the Radboud University Medical Centre, Nijmegen, The Netherlands. The remaining chapters contained in this thesis have not been presented, nor are currently being presented, either wholly or in part, for any other degree or qualification.

Table I: People that have contributed to the work presented in this thesis

Activity	Responsibility
Recruitment, sample taking, inoculation, symptoms data collection	Angela D. Wright, Andrea M. Collins, Carole Hancock, David Shaw (Liverpool School of Tropical Medicine)
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Genetic comparison of two 23F strains and detection of *amiC* frameshift mutation	Ankur B. Dalia (Tufts University School of Medicine, Boston)
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Anti-PspC IgG ELISA	Michael Garner-Jones (Liverpool School of Tropical Medicine)
Depletion and purification of antibodies from nasal wash and sera samples	Angela D. Wright and Michael Garner-Jones
Factor H binding and antibody binding assays and flow cytometry analysis	Nicholas Coombes, Michael Garner-Jones; Adriana T. Moreno (Instituto Butantan)
Epithelial pIgR and PAFr expression by flow cytometry	Sarah Glennie (University of Bristol)
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Abstract

Pneumococcal disease is preceded by nasopharyngeal colonization, which is also the source of transmission. Current pneumococcal conjugate vaccines protect against invasive disease and reduce carriage in children, but are less effective against mucosal disease and have limited serotype coverage. There is an urgent need for new vaccines and colonization has been suggested as an alternative endpoint in vaccine licensure. Experimental human pneumococcal carriage, although potentially risky, offers a way to examine colonization in the context of vaccination. Experimental carriage also allows the investigation of the impact of a pathogen on the immunological complexity and normal microbiota of humans, both of which cannot be done using animal models. We developed a safe and reproducible method of experimental human pneumococcal carriage, described bacteriological and immune factors associated with carriage, and examined the density and duration of experimental carriage.

The data presented in this thesis show that experimental human pneumococcal carriage was safe and reproducible. There were important bacteriological differences between pneumococcal strains that affected carriage. Asymptomatic upper respiratory tract viral infection increased both the risk of pneumococcal colonization and the levels of mucosal Factor H, leading to increased colonization density. This model will be useful in further studies of pneumococcal pathogenicity and host protection against carriage and disease. The model may also be used to select vaccine candidates by protective efficacy in blocking experimental carriage.

Papers arising from work presented in this thesis

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Gritzfeld JF*, Cremers AJH*, Ferwerda G, Ferreira DM, Kadioglu A, et al. (2014). Density and duration of experimental human pneumococcal carriage. Clin Microbiol Infec 20: O1145-O1151

Gritzfeld JF, Cremers AK, Gordon SB (2013). Detection limits in pneumococcal carriage. Pediatr Infect Dis J 32: 425-426

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Papers arising during the course of this PhD

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Cremers JH, Zomer AL, **Gritzfeld JF**, Ferwerda G, van Hijum SAFT, et al. (2014). The adult nasopharyngeal microbiome as a determinant of pneumococcal acquisition. Microbiome 2: 44

Wall EC, **Gritzfeld JF**, Scarborough M, Ajdukiewicz KMB, Mukaka M, et al. (2014). Genomic pneumococcal load and CSF cytokines are not related to outcome in Malawian adults with meningitis. J Infection 69: 440-446

Shak JR, Cremers AJH, **Gritzfeld JF**, de Jonge MI, Hermans PWM, et al. (2014). Impact of experimental human pneumococcal carriage on nasopharyngeal bacterial densities in healthy adults. PLoS One 9: e98829

Neill DR, Coward WR, **Gritzfeld JF**, Richards L, Garcia-Garcia FJ, et al. (2014). Density and duration of pneumococcal carriage is maintained by transforming growth factor β1and T regulatory cells. Am J Respir Crit Care Med 189: 1250-1259

Ferreira DM, Neill DR, Bangert M, **Gritzfeld JF**, Green N, et al. (2013). Controlled human infection and re-challenge with *Streptococcus pneumoniae* reveals the protective efficacy of carriage in healthy adults. Am J Respir Crit Care Med 187: 855-864

Wright AK, Bangert M, **Gritzfeld JF**, Ferreira DM, Jambo KC, Wright AD, Collins AM, Gordon SB (2013). Experimental human pneumococcal carriage augments IL-17A-dependent T-cell defence of the lung. *PLoS Pathog* 9(3), e1003274

*****Authors contributed equally to this work

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Abbreviations

Abbreviations are explained at their first use. The following list may also be useful.

BAL	bronchoalveolar lavage
CAP	community-acquired bacterial pneumonia
CbpA	choline-binding protein
CFU	colony forming units
ChoP	phosphorylcholine
EHPC	experimental human pneumococcal carriage
ELISA	enzyme-linked immunosorbent assay
EMEM	eagles minimal essential media
FH	factor H
IPD	invasive pneumococcal disease
IVIG	intravenous immunoglobulin
lytA	autolysin
M&M	miles and misra quantification
MFI	mean fluorescence intensity
OD	optical density
OPA	opsonophagocytic killing assay
PBS	phosphate buffered saline
PcpA	pneumococcal choline-binding protein A
PCV	pneumococcal conjugate vaccine
PNPP	p-nitrophenyl phosphate
PspA	pneumococcal surface protein A
PspC	pneumococcal surface protein C
qPCR	quantitative real-time polymerase chain reaction
rPAF	receptor for platelet-activating factor
RSV	respiratory syncytial virus
SLPI	secretory leukocyte protease inhibitor
STGG	skim milk, tryptone, glucose, glycerol media
THY	todd-hewitt broth with 0.5% yeast extract
URT	upper respiratory tract

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Chapter 1

Introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae (pneumococcus) is an encapsulated Gram positive diplococcus with more than 90 structurally and serologically distinct pneumococcal serotypes (Figure 1.1). The serotype is determined by the polysaccharide capsule which is the outermost layer of the cell and is made of repeating units of simple sugars. Of the more than 90 described polysaccharide capsular types, all but four are negatively charged due to acidic sugars, phosphate, or pyruvate, with the remainder being neutral [1].

Approximately 20 serotypes are associated with 70% of invasive pneumococcal disease (IPD) worldwide and around 10 of these serotypes account for most paediatric infections [2]. However, disease only occurs following colonization of the nasopharynx. The asymptomatic carriage state, therefore, is the most important interaction between bacterium and host as it is both the reservoir for spreading the bacteria within the community and the source of disease.

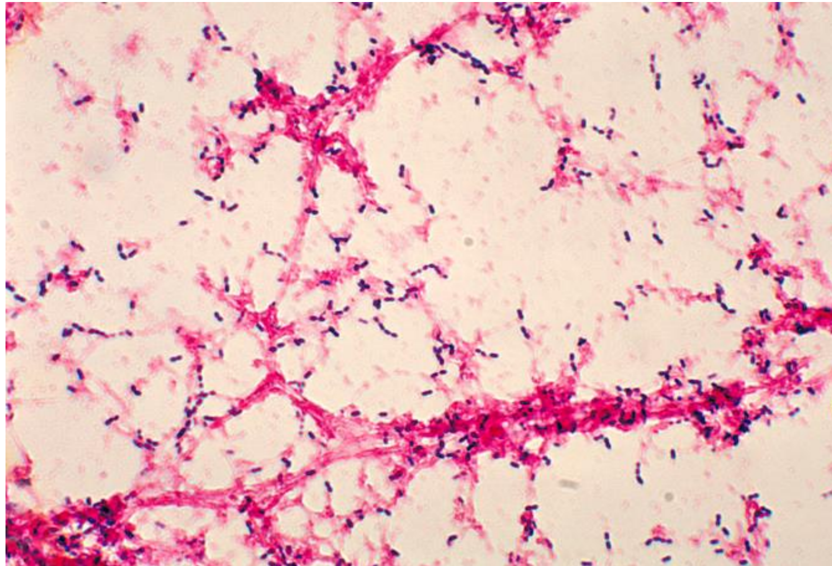

Figure 1.1: Photomicrograph of *S. pneumoniae* grown from blood culture (taken from CDC Public Health Image Library, image ID# 2896).

1.1.1 Epidemiology of pneumococcal carriage

Pneumococcal carriage rates are dependent on an individual's age and vary by geographic area. In children, carriage rates range from 27% in developed countries to 85% in developing countries [3]. In developing countries, children can become colonized within days of birth. In The Gambia >80% of children have acquired pneumococcus within 33 days of birth [4]. In contrast, less than 50% of children from developed nations are likely to become colonized within the first year of life [5]. After the first year, carriage incidence rises steadily until around 4 years of age and then decreases to around 10% in adults [6].

Not only does carriage incidence decrease with age, but so also does the duration of carriage. Large variations in the duration of carriage have been observed in different cohorts. In a study done in neonates from Alabama in the 1970s, duration of carriage ranged from one to 17 months and, on average, the younger the infant was at the time of acquisition, the longer a strain was likely to be carried [7]. This trend was also observed in Sweden where the mean carriage duration of penicillin-resistant pneumococci in children under five was 43 days as compared to 25 days in those over the age of five [8].

Pneumococcal serogroup is also important to carriage duration. Both the Alabama study and the Swedish study, as well as others [9,10], have found that duration of carriage is longer for common serotypes such as 6 and 23. In the Swedish study there was no longer a difference in duration for either age or serogroup beyond the age of five.

1.1.1.1 Risk factors associated with carriage

Risk factors for pneumococcal carriage and disease are multi-factorial and can include crowding, the environment, and socio-economic factors [11-13].

1.1.1.1.2 Crowding

Crowding is very important in the spread of pneumococcus and particularly so in young children who are both in close contact with other young children and have a poorly developed immune system. In The Netherlands, children attending day care centres have been found to be at a 1.6- to 3.4-fold increased risk of nasopharyngeal colonization than those not attending [14].

1.1.1.1.2 Environment

Exposure to smoke increases the risk of pneumococcal carriage. Smoking, both passive and active, increases carriage in the smoking parent and the child exposed to the smoke [15-17]. In Australian Aboriginal adults, carriage is also associated with the frequency of sitting at an outside fire [18].

1.1.1.1.3 Socioeconomics

Socioeconomic factors can influence transmission. Overcrowded housing combined with inferior hygiene facilitates transmission, allowing pneumococcus to sustain in the community [18,19]. Aerosol transmission has been demonstrated in a ferret model where ferrets carrying *S. pneumoniae* were able to transmit the bacteria to other ferrets in the same cage or up to 1m away [20]. This could be comparable to the conditions people face in overcrowded, unsanitary housing.

In rural Alaskan communities, a lack of in-home running water has been associated with increased carriage in children under 10 [21]. It is suggested that conserving water for cooking and dish washing decreases handwashing, especially in children who need assistance. Hand contamination in Australian Indigenous children also correlates with carriage; Aboriginal children living in remote areas were 2 times more likely to be carrying pneumococcus and 8 times more likely to have hand contamination than children attending urban child-care centers [22].

Sharing beverages could also increase carriage rates. In a study of Israeli army recruits, frequent sharing of a drinking bottle was shown to be a strong risk factor for pneumococcal carriage [23].

1.1.2 Epidemiology of pneumococcal disease

Similar to the pattern observed with carriage, IPD rates also correlate with age (Figure 1.2) and disease rates and mortality vary geographically, with the majority of deaths occurring in Asia and Africa.

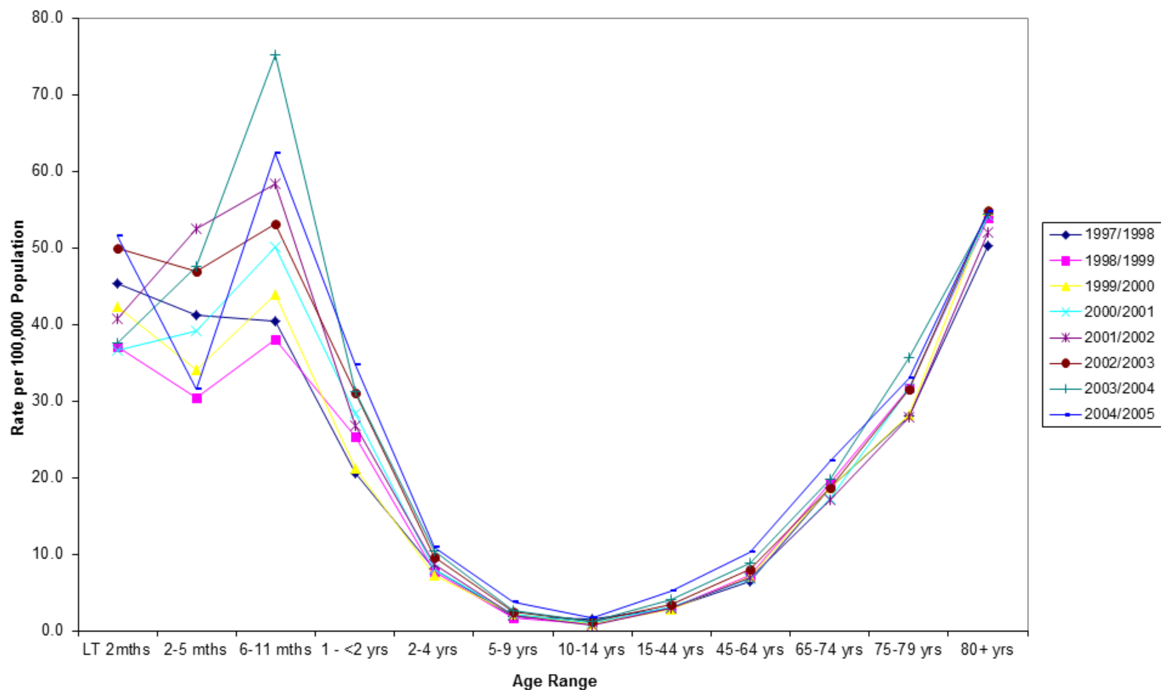

Figure 1.2: Invasive pneumococcal disease (IPD) incidence rate per 100,000 in England and Wales. Reproduced with permission from Public Health England, July 2012.

Prior to the introduction of the conjugate vaccine, the incidence of IPD in children less than 2 years of age was 44.4/100,000 (range 11.3-104.4) per year in Europe and 167/100,000 in the United States [3,24]. In comparison, the incidence in the same population in Mozambique was 797/100,000. However, following the introduction of the conjugate vaccine IPD incidence dropped drastically in some countries. In the United States the overall incidence of IPD in children under 5 dropped to 23.6/100,000 by 2007 [25]. And in South Africa, the overall incidence of IPD in children under 2 declined from 54.8 cases per 100,000 to 17.0 cases in 2012 [26].

In adults, pneumonia is also one of the leading causes of infectious death [27]. In American and European adults, *S. pneumoniae* causes approximately 30-50% of community-acquired bacterial pneumonia (CAP) [3]. In the United States alone, CAP accounts for more than

600,000 hospitalizations and 59,000 deaths [28]. As more and more of the general population reaches the age of 65, the burden of disease in this age group will only increase.

1.1.3 The association of pneumococcal carriage with disease

The spectrum of disease caused by *S. pneumoniae* is wide and varied. Following colonization, the pneumococcus can spread and cause non-invasive illness, such as otitis media or sinusitis, or it can spread to the lungs, blood or meninges, causing pneumonia, bacteraemia, or meningitis. Disease does not occur without an initial colonization step (Figure 1.3).

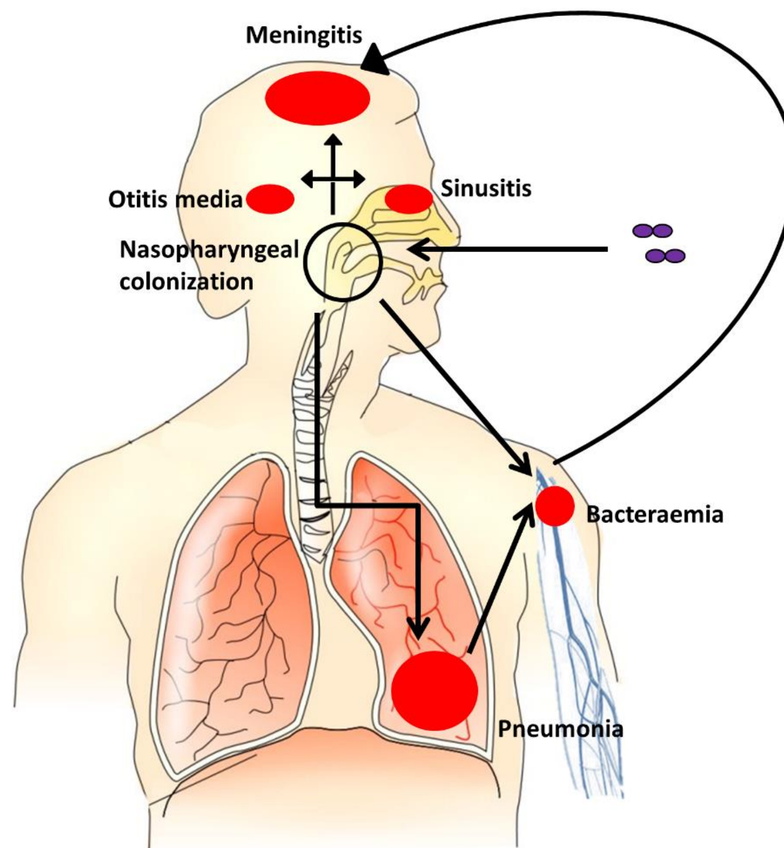

Figure 1.3: Pathogenesis of pneumococcal disease.

1.1.3.1 Association between carriage and disease in animal models

The first proof of the association between carriage and disease comes from animal models. Using a chinchilla model, Linder et al. demonstrated that intranasal inoculation of pneumococci leads to otitis media [29]. Similar results were seen in rats and then in ferrets that were first infected with influenza virus and then challenged with pneumococci [30,31]. Colonization was also shown to lead to invasive disease in mice [32,33].

1.1.3.2 Association between carriage and disease in humans

The first study in humans to show a link between recent acquisition of pneumococci and pneumococcal disease was done by Gray et al. who followed 82 infants from birth up to 2 years of age [7]. In these infants, serotypes causing disease were similar to commonly carried serotypes but disease was mainly associated with a newly acquired serotype; 74% of infections were caused by serotypes carried less than a month before disease. In a larger study of 329 children followed from 2 months to 2 years of age, pneumococcal acute otitis media was also associated with the newly acquired disease-causing serotype [34].

1.1.3.3 Association of serotype with the incidence of carriage and disease

The difference in disease virulence between serotypes is best illustrated by serotype 1 as it is a major cause of IPD and has been associated with outbreaks yet it is rarely found in healthy carriers [35]. This is in comparison to serotypes 6B and 23F which are frequently carried and have low invasive potential [36]. Sleeman et al. demonstrated that attack rate varies by capsule and an inverse relationship exists between attack rate and carriage duration [9].

One explanation for the differences in attack rate between serotypes is the capsular structure. Serotypes that are more heavily encapsulated are more resistant to neutrophil-mediated killing and persist for longer in the nasopharynx [37]. Pneumococcal surface charge has been shown to be one of the mechanisms behind this, with a more negative surface charge correlated with increased resistance to nonopsonic killing and increased carriage prevalence [38].

1.2 Mechanisms of *S. pneumoniae* carriage

For *S. pneumoniae* to access epithelial surfaces and establish carriage it must first reduce entrapment in the nasal cavity mucus secretions. This is achieved through expression of the polysaccharide capsule [39,40].

1.2.1 Expression of capsule during colonization

The polysaccharide capsule is differentially controlled based on the host niche. Pneumococcal isolates lacking capsule rarely cause disease and, in a mouse model, nonencapsulated derivatives are unable to colonize when compared to an encapsulated parent strain [41,42].

While sufficient capsule is needed to evade clearance mechanisms, excessive capsule has been shown to inhibit adherence to the epithelium [43,44]. To achieve a balance, the pneumococcus spontaneously switches its expression between two phenotypes - opaque and transparent - which are distinguished by differences in colony opacity [45].

Differences in colony opacity are due to the production of capsular polysaccharide; in the opaque form there is a 1.2- to 5.6-fold greater amount of capsular polysaccharide as compared to the transparent form [46]. The thinner capsule of the transparent phenotype provides better access to the epithelium and, compared to the opaque variant, enhances adherence to buccal epithelial cells [47]. This difference in colonization capability was demonstrated in infant rats where rats that received the transparent phenotype had higher rates of carriage than those that received the opaque phenotype [45]. In the rats that received the opaque inoculum, all colonies were transparent by 7 days post-inoculation.

Hammerschmidt et al. used electron microscopy to visualize pneumococci during contact with epithelial cells and found that pneumococci in contact with, and starting to enter, cells lacked capsule [48]. Once pneumococcus has accessed the epithelial surface it uses surface proteins to bind to host cell-surface carbohydrates and proteins and initiate colonization.

1.2.2 Pneumococcal surface proteins involved in carriage

Underneath the capsule lies the cell wall which acts as an anchor for cell-wall-associated surface proteins responsible for assisting with pneumococcal adherence to cell-surface carbohydrates [49-51]. Adherence to the nasal epithelium is initiated when the pneumococci bind to cell-surface carbohydrates such as N-acetylglucosamine-β-(1,4)-galactose [52,53]. This initial binding is mediated by a number of bacterial structures, including phosphorylcholine (ChoP), choline-binding protein A (CbpA), and adhesins.

1.2.2.1 Choline-binding proteins mediate adherence

ChoP, a component of both cell-wall associated and lipotechoic acids, mediates adherence to the receptor for platelet-activating factor (rPAF) and is the anchor for a number of choline-binding proteins [49]. ChoP and these choline-binding proteins, including CbpA, are found in larger amounts on transparent pneumococci due to the increased amount of techoic acid in this phenotype [46,54]. This may account for the increased adherence and better colonization of the transparent phenotype.

CbpA, also known as pneumococcal surface protein C (PspC), binds to the polymeric Ig receptor which mediates adherence and aids in the migration of pneumococci through the

mucosal barrier [55]. A PspC knockout mutant has shown reduced epithelial cell binding and decreased nasopharyngeal colonization when compared to wild-type [54]. In addition, PspC binds factor H (FH), a regulator of the alternative complement pathway, enabling the pneumococci to evade the host immune response and aiding adherence to epithelial cells [56-58].

Another choline-binding protein, pneumococcal choline-binding protein A (PcpA), is also able to facilitate pneumococcal adherence to nasopharyngeal and lung epithelial cells [59].

1.2.2.2 Lipoproteins mediate adherence to cell-surface carbohydrates

Pneumococcal lipoproteins are essential for both substrate transport and pneumococcal adherence. Pneumococcal surface adhesin A (PsaA) binds to E-cadherin, a transmembrane glycoprotein, and antibodies to PsaA have been shown to reduce pneumococcal adherence to nasopharyngeal epithelial cells [50,60]. Another group of lipoproteins, the Ami-AliA/AliB oligopeptide permease, was necessary for nasopharyngeal colonization in a mouse model [61]. Mutations in the *ami* locus have been suggested to decrease pneumococcal adherence to pulmonary epithelial cells as a result of reduced binding to a glycoconjugate receptor on lung epithelial or vascular endothelial cells [62].

1.2.2.3 Protease production increases attachment

Pneumococcal IgA1 protease has been shown to increase bacterial attachment to epithelial cells *in vivo* by cleaving human IgA1, causing a change in surface charge and resulting in ChoP interacting with rPAF [63].

1.2.2.4 Adhesins assist in binding to the extracellular matrix

Two adhesins important for the binding of pneumococci to the extracellular matrix are pneumococcal adhesion and virulence factor (PavA) and enolase. PavA, found on the outer cell surface, binds fibronectin and has been shown to be necessary for long-term carriage of *S. pneumoniae* D39 in a murine carriage model [64,65]. Enolase, an anchorless surface protein, binds plasminogen [66]. A third component of the extracellular matrix, vitronectin, was recently shown to interact with PspC, allowing pneumococci to use vitronectin as a molecular bridge and facilitating adherence [67,68].

1.2.2.5 Neuraminidase facilitates persistence in the nasopharynx

Colonization can also be enhanced by the pneumococcal enzyme neuraminidase (NanA). NanA cleaves N-acetylneuraminic acid from mucin thereby decreasing the viscosity of the mucus and increasing colonization, and it can expose host epithelial cell surface receptors

for pneumococcus [69,70]. The synergism that exists between pneumococci and respiratory viruses, such as influenza, in causing illness may be related to the action of neuraminidase and the increased adherence of pneumococci [71,72].

1.3 Immunity to *S. pneumoniae*

Innate immunity is the body's first line of defence against a foreign pathogen. It is immediate, non-specific and evolutionarily conserved [73]. However, if the pathogen is able to evade or overcome the innate defences, the adaptive immune system is activated. Natural immunity against the pneumococcus is a complex interaction between the innate and adaptive immune systems.

1.3.1 Innate immunity to the pneumococcus

When the body first encounters a pathogen the aim is simply to prevent its entry. The epithelial surfaces of the body provide a physical barrier to stop the pathogen and the mucosal lining of the epithelium traps pathogens in mucus. However, the mucosal epithelium also secretes a number of chemicals that can kill pathogens or inhibit their growth.

1.3.1.1 Antimicrobial peptides against pneumococcus

Three peptides are the most abundant antimicrobial factors in airway secretions [74]. Lysozyme is abundant in both upper and lower airway secretions and has been shown *in vitro* to inhibit the growth of clinical isolates of *S. pneumoniae* [75]. However, the pneumococcus has two enzymes, PgdA, a *N*-acetylglucosamine deacetylase, and Adr, an *O*-acetyl transferase, that counter this mechanism by modifying the peptidoglycan structure [76]. This modification has a significant fitness cost for the pneumococcus but this is outweighed by the resulting elimination of the lysozyme target. Lactoferrin is also present in nasal secretions and acts by depleting iron and restricting bacterial growth however pneumococcal surface protein A (PspA) can bind it, preventing it from killing pneumococci [77,78]. Secretory leukocyte protease inhibitor (SLPI) is secreted from submucosal glands and is thought to destabilize bacterial membranes as a result of its cationic charge [79].

Two other important antimicrobial peptide families in mucosal host defence are the defensins and the cathelicidins. There are two main defensin subfamilies, α- and β-defensins, and both work by lysing the bacteria [80]. Both the α-defensins (human neutrophil proteins 1 to 3(HNP)) and the β- defensins (human β- defensin-2 and 3 (hBD)) have been shown to have an antimicrobial effect on *S. pneumoniae in vitro* [81,82]. Human

cathelicidin LL-37 also acts by disrupting the bacterial membrane [83]. Pneumococci have been shown to induce hBDs in human pulmonary epithelial cells [84] and the pneumococcal toxin pneumolysin (PLY) has been shown to induce the release of LL-37 from human lung mast cells, which reduced pneumococcal viability [85].

In the lung, the pulmonary surfactant proteins A (SP-A) and D (SP-D), members of the collectin family, are found in the fluid that bathes the epithelial surfaces of the lung. SP-A and SP-D can bind and agglutinate a number of pathogens as well as act as opsonins [86]. In mice that were SP-D deficient, pneumococcal colonization and infection in the upper and lower respiratory tract increased [87]. The collectins, including SP-A and SP-D, are also known as pattern recognition receptors (PRRs) and they, along with numerous other PRRs, play a crucial role in host defence.

1.3.1.2 The role of pattern recognition receptors in defence against pneumococcus

When pathogens manage to breach the epithelium, the immune system must be able to distinguish self from non-self. To do this, it uses a variety of PRRs which can be expressed on the host cell surface, intracellularly, or secreted and which recognize highly conserved microbial structures known as pathogen-associated molecular patterns (PAMPs) [88]. PRRs include soluble receptors, like a number of components of the complement system, and membrane-bound receptors, including members of the Toll-like receptor (TLR) family, as well as the NOD-like receptors and cytosolic DNA sensors.

1.3.1.3 Complement system action against pneumococcus

The complement system consists of more than 30 circulating and membrane-bound proteins that, upon activation, initiate an enzyme cascade that eventually results in pathogen death. There are three overlapping pathways through which complement is activated: the classical pathway, the alternative pathway, and the lectin pathway. All three pathways converge on the activation of C3 to lead to the same effector molecules [89]. Complement activation results in cleavage of C3 to C3b which is deposited on the surface of the pathogen and processed to iC3b, both of which are important opsonins [90]. Pathogens are then eliminated either through opsonophagocytosis, membrane attack complex-mediated lysis, and/or the induction of inflammation.

1.3.1.3.1 Complement pathways involved in protection against pneumococcus

The classical pathway is activated by direct binding of C1q to the pathogen surface or to antibody-antigen complexes. Brown et al. demonstrated that the classical pathway is the

most important complement pathway for innate immunity to pneumococcus in mice and it is partially activated by the binding of natural IgM to bacteria [91]. However, there are other mechanisms that can activate the classical pathway during pneumococcal infection. These include the acute phase PRRs C-reactive protein (CRP) and serum amyloid protein (SAP), which bind ChoP [92-94], and SIGN-R1, a cell surface lectin, which binds capsule [95].

The alternative pathway is spontaneously activated at low levels but will only amplify when attached to a foreign surface. During pneumococcal infection, the alternative pathway is responsible for amplification of complement activation, leading to increased C3 deposition [91]. The pneumococcal cell wall can activate the alternative pathway, inducing the inflammatory reaction and cytokine production [96-98].

The lectin pathway is initiated by binding of the mannose-binding lectin (MBL), a soluble receptor, to PAMPs, mainly carbohydrates, on the microbial surface. There are two other recognition molecules, ficolin (1, 2 and 3) and collectin kidney 1 that, along with MBL, can form a complex with three MBL-associated serine proteases (MASPs) [99,100]. Until recently, the role of the lectin pathway in defence against pneumococcus appeared negligible. However, it has now been shown in mice that MASP-2, ficolin, and collectin are key PRRs that can activate complement via the lectin pathway and protect against pneumococcal disease [101,102].

1.3.1.3.2 The role of complement in the progression from carriage to disease

Humans with complement deficiencies are at an increased risk of IPD [103] but until recently it was not known if complement could prevent colonization from leading to sepsis. Using a murine model, Bogaert et al. showed that complement-depletion did not impact colonization density but mice were more likely to progress from carriage to disease than control or neutrophil-depleted mice [104]. This demonstrates a critical role for complement in stopping colonization from progressing to disease.

1.3.1.3.3 Pneumococcal mechanisms used to resist complement

Given the importance of complement in innate immunity, it is not surprising that the pneumococcus has developed a number of mechanisms to resist it (Figure 1.4).

1.3.1.3.3.1 Polysaccharide capsule

The pneumococcal capsule has been shown to have numerous effects on complement activity against the pneumococcus. Hyams et al. [105] demonstrated that capsule inhibits the binding of IgG, IgM, and CRP to *S. pneumoniae* which, in turn, inhibits classical pathway

activity. C3b/iC3b opsonization by the alternative pathway and the conversion of C3b to iC3b on the bacterial surface were also impaired, as was phagocytosis mediated by complement, IgG, and nonopsonic phagocytic receptors [105].

1.3.1.3.3.2 Serotype

Different serotypes vary in their susceptibility to complement deposition and there exists an association between serotype prevalence in carriage and resistance to nonopsonic neutrophil-mediated killing [37,106,107]. Opsonophagocytosis can also vary between serotypes and has been linked with serotype-specific mortality [43,106,108]. However, serotype alone does not determine the effect of complement-mediated immunity, rather it is a combination of serotype and genetic background [106,109].

1.3.1.3.3.3 Proteins

A number of pneumococcal proteins also inhibit complement-mediated immunity. It has long been known that PspA inhibits complement activation but only recently was it discovered that it does so by blocking CRP binding to ChoP, thereby decreasing complement activation [110-112].

PspC mediates immune evasion by binding human FH and C3 [113,114]. The interaction between FH and PspC protects the pneumococcus from complement-mediated clearance [115]. However, FH binding varies by serotype and is negatively correlated with C3b/iC3b deposition, with serotype invasiveness associated with high levels of FH binding and resistance to complement [116].

PLY, a key pneumococcal virulence factor that activates the classical pathway, directs complement activation away from the bacterial surface and prevents C3 deposition on the cell [117,118]. Recently, Ali et al. found that PLY can also trigger complement activation via the lectin pathway [119].

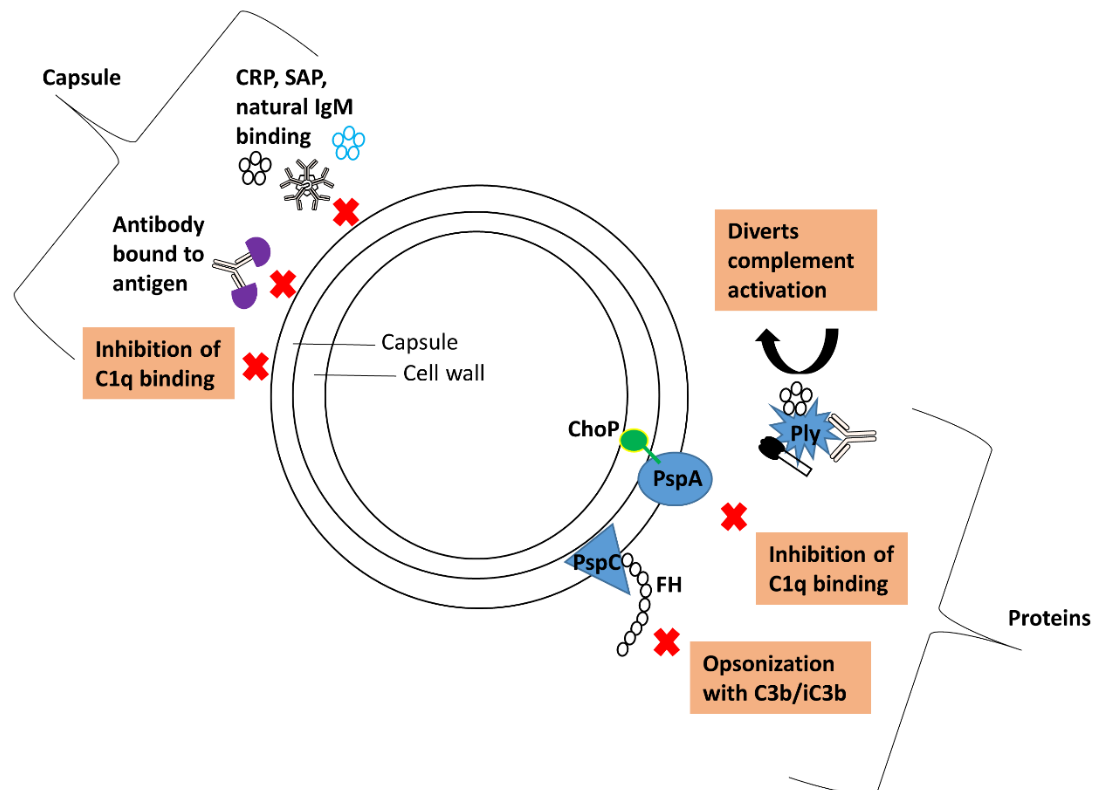

Figure 1.4: Pneumococcal mechanisms used to resist complement.

1.3.1.3.3.4 Biofilm

Biofilms are increasingly being studied as they are known to be important in resistance to host-defence and antimicrobials. For *S. pneumoniae*, *in vitro* growth as a biofilm has been shown to impair C3b deposition and target both the classical and alternative pathways through impaired activation of CRP and C1q and increased recruitment of FH [120].

1.3.1.4 Toll-like receptors involved in protection against pneumococcus

As PRRs, the role of TLRs is to detect pathogens and initiate the immune response by leading to the production of mediators such as cytokines and chemokines. There are 10 known TLRs in humans, of which three have been described in protection against the pneumococcus.

1.3.1.4.1 TLR2

TLR2 is located on the cell surface and was thought to recognize pneumococcal peptidoglycan and lipotechoic acid until recently when it was suggested that both peptidoglycan and lipotechoic acid may stimulate inflammatory responses by TLR2-independent mechanisms [121-123]. Instead, lipoproteins may be the major pneumococcal TLR2 ligand [124]. Regardless of the ligand, TLR2 is important in protection against both

carriage and disease [125,126]. In TLR2-deficient mice, clearance of colonization is impaired and susceptibility to pneumococcal meningitis is increased, with intensified disease severity and bacterial numbers [127-129]. However, the importance of TLR2 does not extend to protection against pneumococcal pneumonia [130].

1.3.1.4.2 TLR4

TLR4 is also found on the cell surface and has been shown to interact with PLY [131]. However, its role in carriage clearance is unclear; Malley et al. [132] found TLR4-deficient mice had increased colonization density and risk of disease however, van Rossum et al. [127] saw no difference between wild-type and TLR4-deficient mice. *In vivo* disease studies have found TLR4-deficient mice have less upper respiratory tract (URT) cell apoptosis and higher bacterial loads in their lungs [131,133]. Recently, McNeela et al. [134] found that PLY directly activates innate immune cells, independently of TLR4, and there is no interaction of TLR4 with PLY.

1.3.1.4.3 TLR9

TLR9 is expressed within endosomal compartments and plays a protective role in the lung; TLR9-deficient mice are more susceptible to pneumococcal pneumonia and have an increased bacterial load in the lung [135].

TLRs are a crucial link between innate and adaptive immunity. TLR recognition of a PAMP induces production of inflammatory molecules through the activation of an intracellular pathway including nuclear factor-κB, mitogen-activated protein kinases and interferon-regulatory factors which initiate the activation of the adaptive immune response [136].

1.3.2 Adaptive immunity to the pneumococcus

Although slow to start, the adaptive immune system is highly specific and has memory, and is comprised of a humoral response and a cell-mediated response.

1.3.2.1 Humoral immunity against pneumococcus

Humoral immunity consists of the production of antibodies by B cells. In children, antibodies to pneumococcal capsular polysaccharide arise through natural exposure and following vaccination with a conjugate vaccine but are serotype-specific [137,138]. In adults, anticapsular IgG can be detected in the serum following carriage [139]. However, in animal models protection against colonization and clearance of carriage were both independent of antibody [140,141].

In the mucosa, the dominant antibody is IgA and it is thought to mediate protection by blocking adherence to the host tissues. There are two forms of IgA, IgA1 and IgA2, with IgA1 making up approximately 90% of the total IgA in the URT [142]. A number of common respiratory pathogens have an IgA1 protease which, in the case of *S. pneumoniae*, cleaves human IgA1, increasing adherence to respiratory epithelial cells *in vitro* and preventing phagocytic killing [63,143] .

1.3.2.1.1 Agglutination of pneumococcus

Another important role for antibodies in the mucosa is agglutination of bacteria into a cluster that can then be cleared by the host. This was recently demonstrated in mice where, following passive immunization with IgG and intra-nasal challenge with pneumococcus, colonization was prevented, as was transmission between littermates [144]. When mice were immunized with human IgA1, colonization was only prevented following challenge with an IgA1-protease-deficient mutant; challenge with an IgA1-protease-producing strain did not result in agglutination, again highlighting the role of pneumococcal virulence factors in evasion of the host immune response.

1.3.2.2 Cellular immunity against pneumococcus

If a pathogen crosses the epithelial barrier, it will often be immediately recognized by a phagocyte. Macrophages are a phagocytic cell found throughout the body, including the lung, and are derived from monocytes. Upon activation they recruit another phagocyte, the neutrophil, which is a short-lived cell found in the blood. Macrophages and neutrophils display a number of cell-surface receptors which recognize the surface molecules of pathogens, leading to phagocytosis.

1.3.2.2.1 Macrophages

In mice, recruitment of monocytes/macrophages to the upper airway cleared pneumococcal colonization by phagocytosis [145]. The retention of macrophages in the nasopharynx was the result of an innate cytokine, macrophage migration inhibitory factor (MIF). In MIF-deficient mice, carriage was prolonged and was correlated with reduced numbers of macrophages [146]. MIF activity has also been shown to inhibit alveolar macrophage migration in a rabbit model of pneumococcal pneumonia [147].

Alveolar macrophages are very important in protection against pneumonia. In a mouse pneumonia model, depletion of alveolar macrophages resulted in increased disease susceptibility and higher mortality [148]. But the ability of macrophages to clear bacteria is finite and when they fail to control infection the recruitment of neutrophils becomes

critical for bacterial clearance [148]. At this point the role of the macrophage changes and it begins to regulate the immune response through the production of cytokines.

1.3.2.2.2 Neutrophils

Neutrophils are crucial for phagocytic clearance of pneumococci. During pneumococcal colonization in mice, there is an influx of neutrophils into the nasal space [127]. When these neutrophils interact with pneumococcal PLY, the resulting neutrophil lysis enhances the delivery of pneumococcal antigen to the nasal-associated lymphoid tissue [149]. This neutrophil lysis combined with pneumococcal degradation by neutrophils alone results in rapid clearance of the pneumococcus from the nasopharynx.

The importance of neutrophils in carriage clearance also extends to disease. In a mouse model of colonization, neutrophil depletion resulted in a 50% death rate with a normally asymptomatic carriage strain [149]. In models of disease, depletion of neutrophils in mice increased susceptibility to pneumococcal infection [150,151].

1.3.2.2.3 CD4+ T cells

Trzciński et al. [141] and McCool et al. [140] have found that protection against colonization and clearance of carriage using murine models, respectively, were independent of antibody. Other studies have shown that in mice lacking immunoglobulin, intranasal immunization with a killed, nonencapsulated whole cell vaccine, a cell wall polysaccharide, or a selection of pneumococcal proteins, protected against colonization [152-154]. Further examination demonstrated that both protection from, and clearance of, carriage required CD4+ T cells in these models [141,153,155]. The CD4+ T cell subset responsible for protection against colonization in mice was shown to be Th17 [156].

1.3.2.2.4 Th17 cells

Interleukin-17A (IL-17A), a cytokine that induces neutrophil recruitment and activation, is secreted by the Th17 subset of CD4+ T helper cells. In mice, expression of IL-17A was correlated with increased clearance of a secondary carriage episode and levels in the blood of mice immunized with killed pneumococcal whole cell antigen prior to challenge were inversely correlated with carriage density [156]. Protection against colonization was neutrophil-dependent and recombinant human IL-17A enhanced antibody-dependent and -independent pneumococcal killing *in vitro* [156].

Zhang et al. further confirmed the importance of Th17 cells in clearance of colonization [157]. The recruitment of monocytes/macrophages to the upper airway during colonization

was dependent on the production of IL-17A-expressing CD4+ T cells via TLR2 signalling. In mice that had previously been colonized, clearance was enhanced by an influx of neutrophils.

1.3.3 The effect of carriage on defence against future carriage and disease

Although colonization can generate an immune response that, in the majority of the population, prevents disease and leads to clearance of carriage, it is still not clear how this impacts on subsequent carriage and disease. In a mouse model of carriage, mice that had cleared carriage and were re-challenged with the same strain had similar colonization density to mice that were not initially colonized, even though they had generated a whole immunoglobulin response to the strain [140]. In contrast, a separate study found that when mice were challenged following carriage, mucosal protection and protection from sepsis required both CD4+ T cells and antibody [158].

Immunological protection against subsequent disease following carriage has also been demonstrated in mice; carriage of a PLY negative mutant strain significantly increased survival following invasive pneumococcal challenge and also resulted in cross-serotype protection [159]. In a pneumonia challenge model, prior pneumococcal colonization prevented bacteraemia by antibody-mediated phagocytosis which led to protection from lethal pneumonia [160].

In humans, carriage is known to be an immunizing event [161]. In the absence of infection, carriage has been shown to induce immunoglobulins against pneumococcal capsular polysaccharide and proteins in the serum [137,162]. In Gambian infants that were followed longitudinally, the rate of reacquisition and the duration of carriage, even with the same serotype, did not differ [4]. However, in this natural setting where sampling is intermittent, it is possible that carriage episodes can be misclassified or missed completely.

1.4 Vaccination against *S. pneumoniae*

Current pneumococcal vaccines have had great success in decreasing IPD but do not confer optimal protection against non-bacteraemic pneumonia and otitis media which account for the largest burden of disease (Figure 1.5) [163,164]. Current pneumococcal vaccines target the polysaccharide capsule because it is the target of the mature human immune response and it influences pathogen transmission [165], the epidemiology of infection [166] and disease virulence [167].

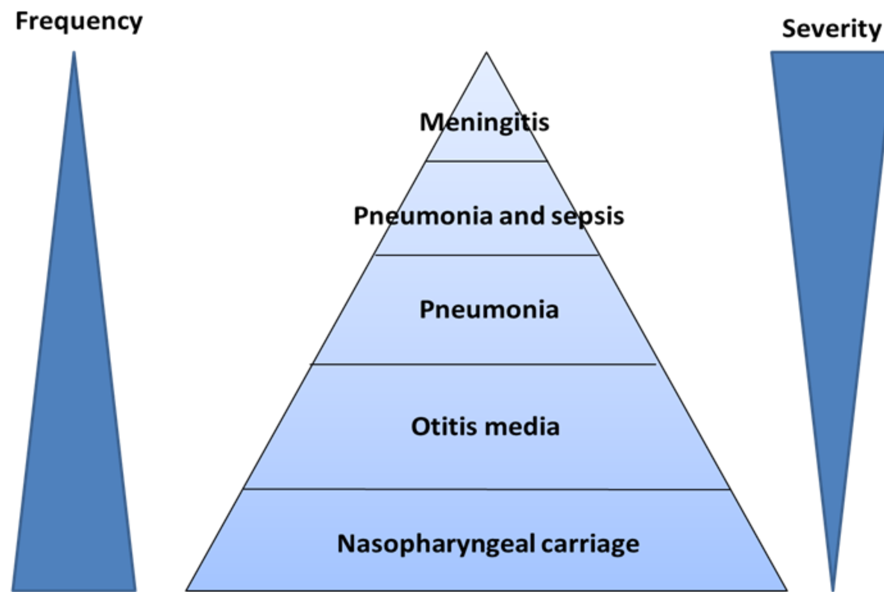

Figure 1.5: Burden of pneumococcal disease demonstrating the inverse relationship between frequency and severity.

1.4.1 Early attempts at pneumococcal vaccination

The first vaccine against the pneumococcus consisted of heat killed, locally isolated, uncharacterized pneumococcal strains that had been suspended in saline and were given subcutaneously. The first clinical trials were conducted in 1911 in South Africa among mine workers where pneumococcal pneumonia had a case fatality rate of 20 to 40% [168]. These first whole cell vaccine trials had vaccine efficacies ranging from 25% to 82% and were generally considered to have been positive [169,170]. This was an important period in pneumococcal vaccine development as vaccine preparation, administration and dosage methods were developed, as were methods for detecting and measuring protective immunity.

1.4.2 Pneumococcal polysaccharide vaccines

Polysaccharide vaccines were first tested in 1945 on students at an army-air force school [171]. The results were promising and the vaccine efficacy, at 84%, clearly prevented pneumococcal pneumonia due to the vaccine serotypes. However, penicillin became readily available at the same time as the vaccine was developed and the vaccine was quickly forgotten and withdrawn.

When the widespread availability and overuse of penicillin led to a dramatic increase in pneumococcal antibiotic resistance, interest in developing new pneumococcal vaccines was renewed. Based on the study in 1945 that found links between immunization with pneumococcal polysaccharide capsule and protection from pneumonia [171], work was done to develop a new vaccine against the pneumococcus.

In 1977 a 14-valent polysaccharide vaccine was licensed for use in adults in the United States. This was expanded to a 23-valent pneumococcal polysaccharide vaccine (PPV23) in 1983 which is currently licensed for use in adults and in children over the age of 2 with underlying medical conditions. A 0.5 ml dose of the vaccine contains 25 µg of purified capsular polysaccharide from each of 23 serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F) and includes the most common drug-resistant serotypes [172].

1.4.2.1 Pneumococcal polysaccharide vaccine efficacy

Despite numerous trials over many years, the effectiveness of PPV23 in protection against pneumonia and IPD is controversial and inconclusive. It is difficult to measure the efficacy and effectiveness because of the low frequency of invasive infection, the inaccuracy of the diagnostic criteria for pneumococcal pneumonia and the variation with age and underlying illness.

A recent meta-analysis that looked at the efficacy of PPV23 in adults found the relative risk for presumptive pneumococcal pneumonia to be 0.64 [95% Confidence Interval 0.43-0.96] but with a significant heterogeneity between trials ($P<0.001$) due to variable trial quality [173]. In trials of higher quality there was little evidence of vaccine protection among the elderly and at-risk groups (1.04 [0.78-1.38]). These findings suggest that pneumococcal vaccination is not effective in preventing pneumonia, even among the groups for whom the vaccine is recommended.

The limited success of the polysaccharide vaccine in its target group has been overshadowed by its failure to induce immune memory in children. Pneumococcal polysaccharides are unable to bind with class II major histocompatibility complex and are therefore T cell independent antigens. A long-term protective immune response in infants and children requires T cell help and the generation of immunological memory which is achieved by conjugating a polysaccharide to a protein.

1.4.5 Pneumococcal conjugate vaccines

Conjugation of purified pneumococcal polysaccharide to a nontoxic carrier protein was based on the success of the *Haemophilus influenzae* type B vaccine where use of a conjugate vaccine lead to a rapid reduction in disease [174]. The first pneumococcal conjugate vaccine on the market was the 7-valent pneumococcal conjugate vaccine (PCV7) (Prevnar) which offered protection against serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F [175].

In 2009, conjugate vaccines containing 10 (Synflorix, PCV10) or 13 (Prevnar, PCV13) capsular polysaccharides were introduced and have led to the gradual replacement of PCV7. Serotypes 1, 5, and 7F have been added to both PCV10 and PCV13; PCV13 also includes 3, 6A, and 19A.

PCV10 and PCV13 cover >70% of serotypes that cause IPD in children from all geographic regions [3]. Both have been licensed to protect against IPD, pneumonia and acute otitis media in infants and children from 6 weeks of age to 5 years. PCV13 has also been licensed for the prevention of pneumococcal disease in adults over 50 years of age [176].

1.4.5.1 Pneumococcal conjugate vaccine efficacy

Pneumococcal conjugate vaccines are protective against invasive pneumococcal diseases caused by vaccine serotypes [25,177]. Mucosal and systemic anti-capsular antibody responses are induced by the vaccine [178] leading to protection from invasive disease, however, the vaccine is less effective against mucosal diseases such as otitis media and pneumonia.

1.4.5.1.1 Immune correlates of protection

Immune correlates of protection for the conjugate vaccine are based on detecting anti-capsular antibody to polysaccharide using enzyme-linked immunosorbent assay (ELISA) and opsonophagocytosis, since it is essential in host defence against the pneumococcus. The World Health Organization (WHO) has proposed a level of 0.35 µg of IgG anticapsular antibody per ml for protection against IPD [179,180]. The opsonophagocytic killing assay (OPA) was developed to evaluate pneumococcal vaccines as immunogenicity and protection were not closely associated. Antibodies opsonize pneumococci (and protect the host) and an OPA can be used to measure the ability of the serum to opsonize the bacteria.

1.4.5.1.2 Protection against carriage

The impact of conjugate vaccines on carriage has been shown in a number of populations. In American Indian infants, a colonization study nested within a phase 3 efficacy trial showed a decrease in vaccine-type carriage after the primary vaccination at 7 and 12 months [181]. In another nested carriage study, Gambian children were also protected against vaccine-type carriage following vaccination [182].

In both of these populations there was also a decrease in carriage density following PCV7 vaccination, with the decrease in rural Gambia seen across different age groups [181,183].

1.4.5.1.3 Protection against invasive disease

The real benefit of conjugate vaccines has been in reduction of invasive disease. In the first PCV7 trial in Northern California, invasive disease caused by vaccine serotypes was reduced by 87.3% in children under 1, by 58.1% in children under 2 and by 62.4% in children less than 5 years of age [177]. Similar decreases have been seen all over the world including South Africa where the vaccine efficacy in infants was 83% [184].

1.4.5.1.4 Protection against mucosal disease

Conjugate vaccines are less effective against mucosal disease. This may be a result of difficulty in diagnosing pneumonia or it could be due to differences between mucosal and systemic defence.

In a study of Finnish infants, those vaccinated with PCV7 had a 6% reduction in the number of acute otitis media episodes as compared to those not vaccinated [185]. The actual incidence of acute otitis media due to vaccine serotypes was reduced by 57%. This is similar to the 64% reduction in vaccine serotype otitis media in American Indian infants following vaccination with PCV7 [186].

All-cause pneumonia admissions were reduced in children under the age of 2 in both the United States and parts of Europe following vaccination with PCV7 [187,188]. In Gambian children aged 6-51 weeks, the efficacy of PCV9 was 37% against radiologically confirmed pneumonia and 16% against mortality [189]. In Filipino children under 2, PCV11 reduced community-acquired radiologically confirmed pneumonia by 23% [190].

1.4.5.1.5 Indirect effects of pneumococcal conjugate vaccination

Aside from the direct immunological protection as a result of vaccination, PCV success is also a result of herd protection. The reduction in carriage of vaccine serotypes in children resulted in a reduction in the number of circulating vaccine serotypes (and hence

population exposure), so much so that, one year after the introduction of the original PCV7 vaccine, the reduction in disease in the United States was greater than the percentage of children who had been vaccinated [177]. This disease reduction was seen in young infants that were too young to receive the vaccine [191], in children that were not vaccinated, and in older children and adults too old to have received the vaccine [192].

1.4.5.2 Drawbacks of pneumococcal conjugate vaccines

Despite the significant impact of pneumococcal conjugate vaccines on invasive disease there are a number of clinical drawbacks.

The first of these is the lack of universal serotype coverage. Protection from current vaccines is limited to the serotypes they contain and the prevalence of these serotypes varies geographically.

Secondly, as vaccination reduces carriage of vaccine-type pneumococci, the void is being filled by non-vaccine-type pneumococci, a phenomenon known as serotype replacement. As with serotype prevalence, the rate of replacement also varies geographically. In most American children and older adults, the increase in carriage of non-vaccine-types has had little effect on increasing IPD by these serotypes [193]. However, an increase in IPD caused by non-vaccine serotypes has been seen in Alaskan children where the IPD rate caused by non-vaccine-types has increased 140% as compared to the pre-vaccine period [194].

In adults with HIV, the switch to IPD caused by non-vaccine-types has been quite dramatic, a 44% increase between 1999 and 2003, and demonstrates the ability of non-vaccine-types to cause disease in immunosuppressed hosts [195]. There has also been a change in the clinical presentation of disease following conjugate vaccination as complicated pneumonias, such as parapneumonic empyema, seem to be on the rise [196,197]. Continuing surveillance is the key to determining if PCV vaccines are having the desired effect on disease reduction over the long term.

One other major drawback is cost. Conjugate vaccines are expensive and difficult to produce which limits their implementation in most developing countries [198]. To combat this, the GAVI Vaccine Alliance has helped introduce the pneumococcal conjugate vaccine into the national immunization programs of more than 30 countries and has launched an Advance Market Commitment to speed up the development and availability of pneumococcal vaccines to the developing world [199]. As part of this commitment, conjugate vaccines will be supplied to developing countries for 10 years at $3.50 per dose.

1.4.6 Novel pneumococcal vaccine development

Due to the high costs and difficult manufacturing complexity of PCVs, an affordable vaccine that confers broad protection against pneumococcal disease is a major priority.

1.4.6.1 Live attenuated vaccines

A live immunization may offer both mucosal and systemic immunity. A phase I dose escalation trial of three recombinant attenuated *Salmonella enterica* serovar Typhi vaccine vectors expressing PspA was recently completed in the USA [200]. Unfortunately while all three vaccines were safe and well-tolerated, the immune response was limited which the authors suggest may be due to high pre-existing antibody titers to *S.* Typhi.

Another live attenuated pneumococcal vaccine was recently shown to protect against otitis media, pneumonia and invasive disease in a serotype-independent manner [201]. Crucially, this vaccine, which was generated by deleting genes necessary for microbial adaptation to the host environment, was protective against mucosal disease in mice and chinchillas.

A whole cell vaccine, similar to what was first used in 1911, would be both affordable to produce and would offer serotype-independent protection. In mice, subcutaneous injection of an inactivated whole cell vaccine was protective against nasopharyngeal colonization and aspiration-sepsis [202]. It was also shown to induce IgG and Th17 immune responses in mice against 23 clinical isolates of diverse multilocus sequence type and serotype [203]. This vaccine has recently been produced to current Good Manufacturing Practice standards [204] and, in a phase I trial with 14 healthy adults, 8 developed serum antibody responses post-vaccination that passively protected mice following intravenous infection with serotype 3 [205].

1.4.6.2 Protein vaccines

A number of pneumococcal proteins have been shown to be protective against carriage and invasive disease in animal models but their efficacy in humans has not yet been established.

An ideal protein antigen should be found on the cell surface, expressed at all pathogenesis stages and be highly conserved between serotypes. In order to fit these criteria it is likely that a protein vaccine would be made up of a number of different proteins. A few studies have examined the protective effect of protein combinations and found that in many cases combinations of multiple proteins demonstrate a synergistic protective effect whereas the proteins on their own have little effect on protection from disease [206,207].

An effective protein vaccine could provide coverage worldwide and give serotype-independent protection, helping to eliminate the threat of serotype replacement. Since proteins can be engineered efficiently and cheaply, vaccines could be produced at a much lower cost in comparison to PCVs making them much more affordable for developing countries.

If these vaccines do provide serotype-independent protection it is possible that carriage will be completely eliminated. Whether it is eliminated or simply reduced, it is crucial to have a better understanding of the pneumococcus and the mechanisms behind carriage in order to aid vaccine development.

1.5. The role of co-colonizers in *S. pneumoniae* carriage

The nasopharynx, aside from being the residence of the pneumococcus, is home to millions of other bacteria and viruses. This complex and ever-changing community of microbes influences the ability of the pneumococcus to colonize and become established in the nasopharynx.

1.5.5.1 Intraspecies competition

Intraspecies competition and co-colonization can impact pneumococcal carriage. Horizontal gene transfer amongst pneumococci is very common and can be facilitated by co-colonization [208]. In a longitudinal study of carriage in Danish children, acquisition of a new serotype was weak when the child was already colonized [209]. This dataset was examined further, along with longitudinal carriage data from American Indian and Gambian infants, and, when combined, the data showed strong between-strain competition which occurred at acquisition [210]. However, using a mouse model of co-colonization, Marks et al. recently demonstrated that strains with a lower ability to colonize are better able to persist when a different strain with a high colonization ability is already established, thus potentially enhancing the ability of unencapsulated and rare serotypes to colonize [211].

The mechanisms behind pneumococcal competition are not completely understood but bacteriocins, which are small, antimicrobial peptides produced by numerous bacterial species, are thought to be important. The *blp* locus of pneumococci encodes both a bacteriocin peptide and an immunity peptide and, when the pneumococci is missing these peptides, it is unable to compete against a parent strain or another serotype in a mouse model of co-colonization [212].

1.5.5.2 Co-colonization of pneumococcus and respiratory viruses

It has long been reported that there is an association between influenza infection and bacterial pneumonia [213-215]. The synergism between virus and pneumococcus can also be extended to other respiratory viruses including human metapneumovirus and respiratory syncytial virus (RSV) [216,217]. Co-infection of influenza and pneumococcus has been shown to increase pneumococcal adherence to the epithelium and synergistically stimulate type I interferons, inhibiting clearance and resulting in a higher bacterial load [72,218]. This was observed in Vietnamese children with radiologically confirmed pneumonia, who had a marked increase in density of colonizing pneumococci if co-infected with influenza A, RSV, or rhinovirus, and, more recently, in South Africans being treated for acute lower respiratory tract infection where there was a relationship between high pneumococcal colonization density and respiratory virus co-infection [219,220]. This increase in density can not only increase the risk of transmission to normally sterile parts of the body, it can also increase the risk of transmission within the population [20,221,222].

1.5.5.3 Co-colonization of pneumococcus and *Staphylococcus aureus*

Although *Staphylococcus aureus* resides in the anterior nares, there exists an inverse correlation between carriage of *S. pneumoniae* and *S. aureus* [16,223,224]. *In vitro* experiments suggested hydrogen peroxide (H_2O_2) produced by the pneumococcus was the cause however, this was not replicated in a neonatal rat model of co-colonization, nor was it predictive of co-colonization in children [225-227]. This inverse relationship has also been found between carriage of *S. aureus* and vaccine-type pneumococci [16,224]. However, there are conflicting results following introduction of the pneumococcal conjugate vaccine; some studies found *S. aureus* carriage remained steady, while others found it increased [228,229]. Interestingly, this inverse correlation is missing in people with HIV, suggesting an immunological mechanism is involved [230,231].

1.5.5.4 Co-colonization of pneumococcus and Gram negatives

I*n vitro* H_2O_2 killing by pneumococcus has also been demonstrated for *H. influenzae*, *Moraxella catarrhalis*, and *Neisseria meningitidis* [232] and, just as the killing of *S. aureus* is not mirrored *in vivo*, neither is it indicative of the interaction between *S. pneumoniae* and *H. influenzae*. Lysenko et al. showed co-colonization in a mouse model resulted in rapid clearance of the pneumococcus, which was associated with increased neutrophil recruitment [233]. Recently, Margolis et al. demonstrated a more synergistic relationship; in a neonatal rat model, density of *H. influenzae* was increased if *S. pneumoniae* was

present [234]. The impact of density has been extended in a study of Peruvian children where a positive correlation was found between carriage densities [223].

1.5.5.5 The nasopharyngeal microbiome

Classical studies of co-colonization have relied on *in vitro* experimentation and have mainly focused on potential pathogens. But the microbiota of the human nasopharynx is a complex community of harmless commensals intermingled with potential pathogens, and is generally assumed to be beneficial to the host [235]. Microbiome analysis can reveal the intricate balance of the polymicrobial state and might help us understand the progression from asymptomatic colonization to disease. A recent study of healthy Dutch children found the nasopharyngeal microbiota varied strongly with season and was highly diverse [236]. Further investigation of the paediatric nasopharyngeal niche by a 16S rDNA sequencing approach revealed associations between pneumococcal carriage and specific bacterial genera and found that microbial communities with pneumococcus were less diverse and less even [237].

1.6 Detection of *S. pneumoniae* carriage

Detection of carriage is of great importance as it could be used as an endpoint in vaccine trials. Limitations of detection in conventional microbiology have led to the development of molecular based detection systems. Microarray and immunoblot methods have been used to enhance detection of multiple serotypes from nasopharyngeal samples [238,239]. The development of PCR-based serotyping systems could aid surveillance of vaccine-targeted serotypes and help overcome difficulties associated with serological testing.

1.6.1 Nasopharyngeal sample collection

As pneumococci reside in the posterior nasopharynx, the ideal sampling method must reach there; anterior nasal swabs will not work.

1.6.1.1 Sampling methods in children and infants

In children, a nasopharyngeal swab (NPS) has been shown to be better at detecting nasopharyngeal pathogens than an oropharyngeal swab (OPS) but the transoral route is better tolerated [240]. Nasal wash has been shown to have higher sensitivity in pneumococcal detection than NPS but is less well tolerated [241]. The current WHO recommendation for detecting nasopharyngeal carriage of *S. pneumoniae* in infants and children is the NPS [242].

1.6.1.2 Sampling methods in adults

Lieberman et al. [243] showed that samples taken from the nasopharynx of adults, either a NPS or nasal wash, detected 59% more pneumococcal colonization than OPS. Similarly, nasopharyngeal aspirate cultures were shown to be positive more often for pneumococci than OPS cultures but results were best when both specimens were taken [244]. Recently, nasal wash was shown to be more comfortable and better able to detect pneumococci and other potential respiratory pathogens than NPS [245]. However, this method of sampling is dependent on a fresh sample, rather than a frozen one [246]. The current WHO recommendation is to collect both NPS and OPS samples if possible and if not, then the NPS sample should be the one taken [242].

Since the updated WHO recommendations in early 2014, a few studies have focused on the usefulness of the OPS. In a study of healthy Italian adolescents, *S. pneumoniae* was found in 35.8% of OPS samples as compared to 3.5% of NPS [247]. This study did not use culture to detect pneumococci but relied on detection of the autolysin (*lytA*) and capsular (*cpsA*) genes using real-time polymerase chain reaction (PCR).

Similarly, Trzciński et al. [248] compared paired trans-oral and nasopharyngeal swabs using conventional and molecular methods in healthy adults. After primary culture, significantly more NPS samples were culture-positive as compared to OPS (P<0.001) but following culture enrichment significantly more OPS samples were positive for pneumococci (P<0.001) by quantitative real-time PCR (qPCR).

1.6.2 Methods used to detect pneumococcal carriage

1.6.2.1 Microbiological culture

Microbiological culture is the current gold standard for detection of pneumococci. A sample should be plated on defibrinated blood agar (sheep, horse, or goat) supplemented with 2.5-5 µg/ml gentamicin, which is used to prevent the growth of other organisms [242]. Following overnight incubation at 37°C in 5% CO_2, alpha-haemolytic, draughtsman-like colonies should be sub-cultured for optochin sensitivity and bile solubility (Figure 1.6). A Gram stain should be performed to confirm the presence of Gram positive diplococci.

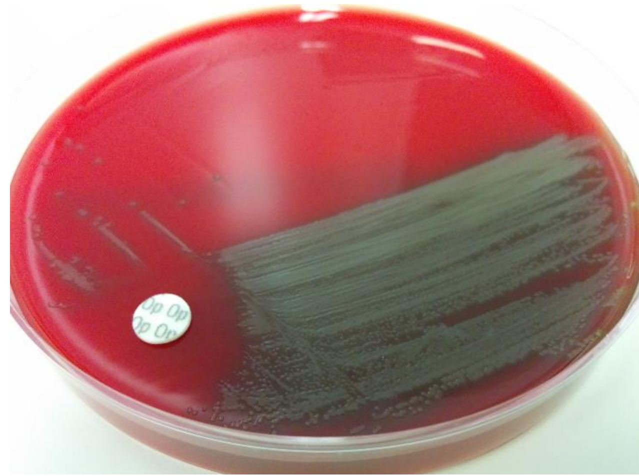

Figure 1.6: Overnight growth of *S. pneumoniae* on a blood agar plate. A zone of inhibition can be seen around the optochin disk.

1.6.2.2 Culture enrichment

Culture enrichment can be used to enhance the detection of pneumococcus [249,250] but it is not currently recommended by the WHO and it cannot be used to determine density of colonization [242]. In the study by Trzciński et al. culture enrichment did not impact on the NPS results but enhanced pneumococcal detection in OPS samples. It is possible that the enrichment step was detecting the transient presence of pneumococci rather than actual colonization, especially since the volunteers were parents of 24-month old children.

1.6.2.3 Non-culture based detection methods

Non-culture based methods of detection can overcome some of the issues associated with detecting pneumococcus in the nasopharynx including the requirement of viable organisms and the difficulty of distinguishing between pneumococci and other streptococci, including members of the viridans group [251].

A number of PCR-based assays targeting numerous pneumococcal genes have been developed but many have had issues with specificity [252-255]. The most widely used real-time PCR assay at present targets the autolysin gene *lytA*. However, a *lytA*-like gene has been found in other streptococcal species [256]. The ability of pneumococci to share genetic information with other oral streptococci warrants further development of non-culture detection techniques.

One possible technique is multilocus sequence typing (MLST). MLST can provide information on the genetic relatedness of isolates by identifying pneumococcal clones and

clonal complexes [257]. It has been shown to be very useful in identifying non-serotypeable and atypical pneumococci [258,259].

1.6.2.4 Serotyping

Detection of the serotypes involved in carriage has traditionally been done by the Quellung method but latex agglutination, which is easier to interpret and cheaper than Quellung, is equally as effective [4,239]. However, as of 2014, the WHO continues to recommend the Quellung reaction as the core method for serotyping pneumococcal isolates.

New serotyping methods that are based on genotypic detection, rather than phenotypic detection, are currently being developed. Microarray is an example of a genotypic method that can not only determine pneumococcal serotype but can also be used to detect multiple serotype carriage [239].

1.6.3 Determining pneumococcal carriage density

Detection of carriage has long been important, especially in the context of vaccine trials. Recently, the importance of carriage density following vaccination, as well as the relationship between carriage density and disease, has been gaining attention.

1.6.3.1 Methods to determine density

Historically, density was determined using semi-quantitative colony counts, where bacterial load was categorized according to the number of colonies in each quadrant of an agar plate [260]. However, a quantitative value can easily be obtained by serially diluting a sample on agar, although this may not be sensitive enough to detect changes at low density [261].

The development of real-time PCR has provided a molecular method able to detect low-level density. But a drawback of PCR is that it cannot distinguish between live and dead pneumococci. It may be that culture and qPCR are complementary in detection of pneumococcal carriage density.

1.6.3.2 Role of density in disease and transmission

Determination of density is also important because of the link between carriage and disease. It has been suggested that there may be a point at which the density of pneumococci in the nasopharynx greatly increases the chance of disease within the host and, potentially, transmission.

An inverse correlation between nasopharyngeal density and age exists which may explain why children are such efficient transmitters of the pneumococcus [183]. In Vietnamese

children, a high pneumococcal density in the nasopharynx was associated with radiologically confirmed pneumonia and viral co-infection [219]. In adults with CAP, qPCR on sputum samples suggested that clinical infection correlated with increased pneumococcal load, and in adults with lower respiratory tract infection, density greater than 10^5 colony forming units (CFU)/ml was suggested to indicate clinically significant infection [262,263]. However, both these studies used the *ply* gene which is not an ideal target as it has been identified in oral streptococci [264]. Using the gene fragment Spn9802 as a target, another study of adults with CAP also determined 10^4 DNA copies/mL was a clinically significant cutoff in nasopharyngeal aspirate samples [265]. More recently, a study of HIV-infected South African adults suggested a nasopharyngeal pneumococcal load ≥8 x 10^3 copies/mL could be used to diagnose pneumococcal pneumonia [266].

This link between density and disease suggests that simply detecting carriage as a binary variable is no longer enough. Detecting changes in density will be vital when testing novel vaccines as an impact on carriage density could alter both disease and transmission.

1.7 Models of *S. pneumoniae* carriage

Pneumococcal carriage and/or disease does not occur naturally in animals, with the exception of serotype 3 which has been found in the respiratory tract of horses [267]. Even though animals are not natural hosts for pneumococcus, animal models have been essential in the study of pneumococcal carriage and disease. Not only have they been used to examine the mechanisms of disease pathogenesis and the role of host and bacterial factors, but they have also been important in testing novel vaccine candidates, providing safety and efficacy data to support progression to human clinical trials. To be successful, an *in vivo* animal model must be well characterized, predictive, and use clear, measurable readouts.

1.7.1 Mouse model of carriage

The most commonly used animal model for pneumococcal carriage is the mouse model. Generally, mice are inoculated intranasally by dropping the bacteria onto the nares. Carriage can be established using multiple serotypes and both density and duration can be measured [268].

The murine model has been shown to be similar to experimental human carriage; using a clinical isolate, colonization was achieved with a similar minimum dose ($<10^4$ CFU), the average duration was several weeks, and the episode was a self-limited event [140].

1.7.2 Infant rat and chinchilla models of carriage

Infant rat models have been used to investigate differences in the abilities of transparent and opaque strains to colonize [45]. They have also been used to examine transmission by inoculating infant rats and putting them in a cage with uninoculated infant rats [269].

Chinchillas have been used to investigate the interaction between respiratory viruses and the pneumococcus in both colonization and otitis media models [270,271].

1.7.3 Nonhuman primate model of carriage

In the past, nonhuman primate models focused on pneumonia or sepsis however a rhesus macaque model of carriage was recently developed. In this model, carriage was self-limiting and lasted up to 7 weeks in more than 60% of animals at a dose of $1x10^6$ CFU/naris [272]. Nonhuman primate models are the animal model closest to resembling human disease but are both cost-prohibitive and difficult to get approval for.

Unfortunately, no animal model can mimic the natural interaction between a pathogen and its human host and therefore cannot guarantee an authentic human response. This has resulted in the development of human challenge models.

1.8 Experimental human challenge studies

Controlled human challenge experiments allow us to examine the impact of a pathogen on the immunological complexity and normal microbiota of humans, both of which cannot be done using animal models. They also offer the ability to follow infection from the initial interaction between host and pathogen - something not possible during natural infection.

Human challenge experiments have been used to study disease pathogenesis, progress development of antimicrobials, and to evaluate vaccine efficacy as a pre-cursor to large-scale trials. Bacteria, viruses, and parasites have all been used in experimental infection models, causing illness that is either self-limiting or can be treated within a short time frame.

The most famous human challenge experiment to date was performed in 1796 [273]. Edward Jenner took a scraping from a cowpox lesion on the hand of dairymaid and inoculated it into an 8-year old boy. Two months later he inoculated the boy again, this time with smallpox, and the boy did not get ill, demonstrating protection against disease.

1.8.1 Ethical framework behind human challenge models

There were no written guidelines for performing human challenge experiments in 1796. Today, challenge models involving humans require strong ethical and legal frameworks.

In 2001, Miller and Grady [274] published an ethical framework that could be used to evaluate human infection models. In the framework they explore the rationale behind challenge experiments, suggesting that a challenge model used to investigate novel vaccine candidates could, not only, limit the number of people exposed to a candidate vaccine in field trials, but could also increase the efficiency and reduce the time and cost of vaccine development. However they should not be performed if the work could be done using an animal model.

They also discuss the importance of evaluating the level of risk to both the volunteer and their contacts and of creating a plan for how the risks might be minimized. Symptoms, both physical and psychological, need to be monitored.

When it comes to recruitment, it is important to only include those that have a solid understanding of what is involved and can give informed consent. This means excluding vulnerable populations, such as children and incompetent adults, and ensuring that financial compensation is appropriate for the participation required but will not entice someone to overlook the risks and join the study.

One final ethical consideration suggested by Miller and Grady is how to treat isolation. In some cases it is necessary to isolate volunteers in order to prevent transmission but this interferes with the ethical practice of allowing a volunteer to withdraw from a research study at any time. This must be dealt with during the informed consent process and must not impact on a volunteer’s right to withdraw following the period of isolation.

1.8.1.1 Regulation of human challenge models

At present, human challenge models are handled differently in the UK and the USA. In the UK, human infection studies are the responsibility of the study sponsor and ethics committee and are not regulated by the Medicines and Healthcare Related Products Agency (MHRA). In the USA, a live organism used in humans falls under federal regulations and requires an Investigational New Drug application [275].

1.8.2 Human challenge models with potential respiratory pathogens

A number of potential bacterial respiratory pathogens have been used in human infection experiments to further what was discovered in animal models. For example, a human

carriage model of *S. aureus* was used to confirm the importance of a protein found to be a major determinant of nasal colonization in the mouse [276].

Experimental carriage of *Neisseria lactamica*, a commensal related to *N. meningitidis*, has been shown to restrict acquisition of meningococcal carriage and induce cross-reactive opsonophagocytic antibodies to the meningococcus [277]. Experimental carriage of nontypeable *H. influenzae* resulted in two genes, *licA* and *igaB*, being switched from phase-off to phase-on, suggesting a role for these genes in early colonization [278].

The common thread between these pathogens is they all exclusively infect humans, limiting the conclusions that can be drawn from animal models of colonization and necessitating the use of human challenge.

1.8.3 Experimental human pneumococcal carriage

1.8.3.1 Experimental human pneumococcal carriage in the United States

The first experimental human pneumococcal carriage (EHPC) study was performed in the United States [161]. Healthy adults were recruited if they satisfied the following inclusion criteria: aged between 18 and 40 years old, HIV-seronegative, non-pregnant, having an intact spleen, no history of pneumococcal vaccination, no penicillin allergy, no history of recurrent respiratory tract infections or chronic illnesses, non-smoker, consume <3 alcoholic beverages per day, no close contact with individuals at increased risk for pneumococcal disease, and lack of pneumococcal colonization for 2-4 weeks prior to challenge as determined by nasal and throat cultures.

Serotypes 23F and 6B were used in dose-escalation studies to determine an optimal colonizing dose and to examine the antibody response to carriage. Nasal wash and serum were taken before challenge and every 2 weeks after, until carriage was not detected for two consecutive visits. The serum antibody response to pneumococcal polysaccharides, which are in the PCV, was minimal and the specific antibody levels in serum did not predict if an individual would become colonized. Pre-existing high levels of PspA, a potential vaccine candidate, correlated with protection against carriage of the experimental strain, suggesting a role for the protein in preventing pneumococcal carriage in humans.

Candidate vaccine antigens were also assessed for their immunogenicity in the EHPC model [279]. Serum samples were taken before, and 1 month following, challenge, and then every 2 weeks until carriage had cleared. The serum antibodies to seven different antigens were measured but only two, CbpA and PspA, showed an increase in serum IgG during carriage.

The immune response to both proteins induced measureable strain-to-strain cross-reactivity. These studies focused on serum IgG responses and so mucosal antibody responses still need to be investigated.

1.8.3.2 Experimental human pneumococcal carriage in England

The first EHPC trials in England used the same serotype 23F strain as that used by McCool et al. in the USA but a different serotype 6B strain [161,280]. Healthy adult volunteers challenged with pneumococcus which did not result in carriage had increased immunoglobulin responses in nasal wash and bronchoalveolar lavage (BAL), but not blood, 6 weeks after challenge [281]. In nasal wash, both IgG and IgA were increased compared to baseline and IgG anti-PspA was increased in nasal wash after 6B challenge and in BAL after 23F challenge. The frequency of pneumococcal specific, cytokine producing, CD4+ T cells in BAL or blood did not change after challenge. This suggests that pneumococcal exposure may immunize mucosal surfaces by enhancing anti-protein immunoglobulin responses, but not cellular or capsular responses.

1.8.3.3 Applications of the experimental human pneumococcal carriage model

The value of the EHPC model extends beyond the exploration of host immunity to include microbial interactions in the nasopharynx as well as vaccine discovery and testing (Table 1.1).

Table 1.1 Summary of EHPC model applications

Applications of the EHPC Model	Investigations undertaken
Host Pressure on *S. pneumoniae*	Exploration of cellular, innate and adaptive humoral immune responses in relation to pneumococcal carriage
Microbial Interactions	Examine changes in the host milieu following inoculation with pneumococcus
Vaccine Development and Testing of Novel Vaccines	Detection of a reduction or elimination in pneumococcal nasopharyngeal carriage
Live Whole Bacteria Responses to Inoculation	Determination of specific immune responses to pneumococcal antigens

1.8.3.4 Usefulness of an experimental human pneumococcal carriage model in novel vaccine testing

There is a number of design complexities involved in testing new pneumococcal vaccines. One problem is the lack of licensing criteria. Another is that opsonophagocytic assays used to detect anti-capsular antibody to polysaccharide may not be the best way to determine immune correlates of protection. It is also difficult to trial a new vaccine using clinical disease endpoints as it is not ethical to administer a placebo vaccine when the existing conjugate vaccine is very effective. Non-inferiority trials comparing a new vaccine against a conjugate vaccine and using clinical end-points would require very large sample sizes and be prohibitively expensive.

It is still uncertain how the next generation of vaccines will protect against the pneumococcus. A reduction in carriage density or duration, a complete elimination of nasopharyngeal colonization, or a prevention of bacterial invasion, are all possibilities. Using a reduction in or elimination of carriage as an endpoint could be a useful mechanism to test a vaccine with cross serotype protection and mucosal immunogenicity. As the EHPC model is based on the detection of carriage, it could be very useful in comparing new vaccines.

1.8.3.4.1 Carriage as an endpoint in vaccine trials

An international consultation recently examined the potential for using colonization as an alternative endpoint in vaccine licensure [282]. They suggest five reasons a colonization endpoint is important in a phase 3 or 4 vaccine trial: carriage is the necessary precursor to disease and an effect on colonization could serve as an indication of protection against pneumococcal disease [283]; vaccine impact in the community depends on the level of direct protection against colonization; colonization incidence and prevalence is higher than disease meaning smaller sample sizes; a shorter sample collection period means confounding factors are less likely to interfere with the measurement of vaccine efficacy; endpoints can be more directly estimated for individual serotypes [284].

An EHPC model could be used to determine the protective effect of a novel vaccine against carriage in a small and controlled population in comparison to placebo groups and/or the current PCV. Carriage can be followed weekly and assessed by acquisition, density and/or duration. Mucosal samples, such as nasal wash and BAL, could be collected alongside systemic samples to assess mucosal immune responses and help establish new immune correlates of protection.

Using EHPC to detect a reduction in carriage and/or carriage density following vaccination could inform on the potential ability of the vaccine to decrease transmission and achieve herd protection. It could also provide proof of concept and be a stepping stone to pursuing large and expensive clinical trials.

1.8.3.5 Usefulness of an experimental human pneumococcal carriage model as a vaccine model

EHPC offers, not only an opportunity to test vaccine efficacy but also, the chance to measure the immunogenicity of the live whole bacteria used for pneumococcal immunization via the intranasal route (Table 1.1). The nature of the model allows the investigation of cellular responses, innate and adaptive humoral immune responses, and microbiological interactions in the upper and lower respiratory tracts and systemic circulation [285].

Mucosally active vaccines should enhance both mucosal and systemic immunity for protection against pneumococcal infection [286]. A mucosal vaccine is an attractive option as it mimics the natural route of exposure to pneumococcus. By using the EHPC model to deliver whole bacteria, specific immune responses to pneumococcal antigens can be examined and these results can be applied to the development of a potential mucosal vaccine.

The EHPC model can be used to determine changes within the nasopharynx that occur during initial acquisition and the establishment of pneumococcal carriage. This includes changes within the inoculated pneumococcus in response to the host milieu as well as challenges the pneumococcus faces when confronted with other commensals that are already established in the nasopharynx.

1.9 Project aims

There were three main aims in this project, each with a number of specific objectives.

Aim 1: To further develop an experimental human pneumococcal carriage model

Objectives:

- Determine an appropriate inoculation dose to achieve carriage by dose-response tests
- Test the reproducibility of the model at the chosen dose
- Examine the presence of symptoms following experimental challenge
- Determine the protective effect of a carriage episode on subsequent carriage

Aim 2: To discover factors associated with experimental carriage

Objectives:

- Explore differences between two pneumococcal serotypes used in the model
- Examine the host innate immune response following experimental challenge
- Determine the role of respiratory viruses in experimental carriage establishment

Aim 3: To explore the density and duration of experimental carriage

Objectives:

- Compare detection of carriage by culture and qPCR
- Compare detection of carriage density by culture and qPCR
- Assess carriage density during a carriage episode

Chapter 2

Materials and methods

2.1 Initial model development

The first step in developing an experimental carriage model was to determine the nasopharyngeal sampling method that would be used for detection. As part of my MSc (Medical Microbiology) thesis, I compared the sensitivities of the nasopharyngeal swab and nasal wash in detecting potential respiratory pathogens and showed that the nasal wash was significantly more likely to detect pathogens than the swab using microbiological culture and was also the more comfortable sampling method [245]. The other part of my MSc project was to begin developing the experimental carriage model. Using a serotype 23F strain, we challenged one volunteer with 9,300 CFU; carriage was not detected by culture or PCR [287].

Following this first attempt at experimental carriage, we performed a series of pilot studies. These pilot studies were used to refine the experimental carriage method in preparation for the studies described in Chapter 3 and were completed prior to the start of this PhD (Table 2.1, page 39). During these studies I was responsible for preparing the inoculum and detecting pneumococcal carriage.

In study 1, volunteers were intranasally challenged with two doses of a serotype 23F strain, approximately two weeks apart [281]. Nasal wash, serum and BAL samples were taken 2

weeks before challenge and again 6 weeks after the second challenge dose. Nasal wash was also collected 1 week before inoculation and on days 2, 4, and 7 after each challenge. The two doses were similar (10,448±5289 and 13,226±4215 CFU/100 µl) and the carriage rate was 11% (1/9).

For study 2, bronchoscopy prior to challenge was removed and volunteers were again double-challenged with serotype 23F using a similar sampling schedule to study 1. There was no carriage in this group (0/6) at doses of 9,933±2828 and 18,000±3471 (unpublished data).

Because of the low carriage rates with 23F in studies 1 and 2, a serotype 6B was tested. In study 3, volunteers had nasal wash samples taken one week prior to challenge and then on days 2, 7, 21, 28, and 35 after challenge. The carriage rate was 71% (5/7) at a dose of 33,286±12,829 (unpublished data).

Based on the high carriage rate with serotype 6B in study 3, volunteers in study 4 were again challenged with serotype 6B but bronchoscopy was re-added two weeks prior to, and 6 weeks after, challenge [281]. There were no carriers in this group (0/9) at a dose of 44,889±12,085. Bronchoscopy with bronchoalveolar lavage has been shown to cause inflammation in the lower respiratory tract so bronchoscopy prior to challenge was stopped following this study in case the inflammation was affecting the ability of the pneumococci to colonize [288].

Study 5 was similar to study 3. Volunteers were again challenged with serotype 6B but there was no bronchoscopy. The carriage rate was 22% (2/9) at a dose of 28,789±6877 (unpublished data).

Study 6 was used to examine to effect of experimental carriage on reacquisition of the same strain. Three carriage positive volunteers from study 3 returned 6-11 months after the initial inoculation to be re-challenged with the homologous 6B strain; all three were protected from reacquisition of carriage [289]. This is discussed further in Chapter 4.

Finally, studies 7 and 8 were used to test the inoculum stocks that had been prepared for the Dose-Ranging study described in Chapter 3. In study 7 the carriage rate following challenge with 6B was 25% (1/4); in study 8 the carriage rate after challenge with 23F was 50% (2/4) (unpublished data).

Table 2.1 Summary of EHPC pilot studies

STUDY	SEROTYPE	DESCRIPTION[1]	DATES	N	CARRIERS	DOSE (CFU/ 100 µl) (±SD)[2]	CONCLUSION
1	23F	Bronchoscopy	Nov 2009 – Feb 2010	9	11% (1/9)	10,448 ± 5289 13,226 ± 4215	Repeat without bronchoscopy
2	23F	No Bronchoscopy	Mar 2010 – Apr 2010	6	0	9,933 ± 2828 18,000 ± 3471	Try different serotype
3	6B	No Bronchoscopy	Oct 2010	7	71% (5/7)	33,286 ± 12829	Add bronchoscopy prior to challenge
4	6B	Bronchoscopy	Jan 2011 – Mar 2011	9	0	44,889 ± 12085	Repeat without bronchoscopy
5	6B	No Bronchoscopy	Mar 2011 – Aug 2011	9	22% (2/9)	28,789 ± 6877	Method successful, use in future studies
6	6B	6B Re-challenge	Jun 2011 – Sep 2011	3	0	29,356 ± 5885	Carriage protective against reacquisition of same strain
7	6B	Inoculum Test	Sep 2011	4	25% (1/4)	23,541 ± 8084	Inoculum ready for use in dose-escalating study
8	23F	Inoculum Test	Sep 2011	4	50% (2/4)	38,083 ± 7025	Inoculum ready for use in dose-escalating study

[1]Bronchoscopy as a descriptor relates to bronchoscopy done prior to challenge
[2]Volunteers in studies 1 and 2 were challenged twice, approximately 2 weeks apart

2.2 Clinical procedures

2.2.1 Recruitment and ethics

Healthy adult volunteers were enrolled with informed consent to an Experimental Human Pneumococcal Carriage trial using one of two pneumococcal serotypes, 6B or 23F. Participants were recruited to the study if they were:

- between the ages of 18-60
- spoke fluent English
- were able to communicate easily by both mobile telephone and text message

They were excluded from the study if they:

- had close contact with at-risk individuals (young children, immunosuppressed adults, elderly, chronic ill health)
- were a current smoker or had a significant smoking history (>10 pack years)
- had asthma or other respiratory disease
- were pregnant
- were allergic to penicillin
- were involved in another clinical trial (unless observational or in a non-interventional phase)
- were unable to give fully informed consent

Ethical approval was obtained from the National Health Service Research Ethics Committee (11/NW/0592) and the studies were sponsored by the Royal Liverpool and Broadgreen University Hospitals Trust. Recruitment occurred through poster advertisements and on the University of Liverpool website, according to the NHS Research Ethics Committee guidance. All volunteer appointments took place at the Clinical Research Unit located in the Royal Liverpool University Hospital.

2.2.2 Study schedules

An initial screening visit was scheduled for one week prior to pneumococcal challenge. This visit included a focused clinical history and targeted clinical examination. If a previously unrecognized abnormality was found, the volunteer was excluded from the study and an appropriate investigation was arranged through primary care.

During initial screening a full blood count was obtained to ensure the white cell count was within normal range before inoculation. Prior to inoculation, volunteers were educated on

the risks involved with participation and were provided with an emergency patient leaflet, digital thermometer, emergency telephone numbers and a 3 day course of amoxicillin.

2.2.2.1 Dose-Ranging study schedule

At the screening appointment for the Dose-Ranging study, nasal wash, serum, saliva and urine samples were taken (Figure 2.1). A viral throat swab was taken at the screening and day 2 appointments for the 6B $4x10^4$ CFU/naris dose and all the 23F doses. For the first five doses, volunteers naturally carrying pneumococcus at the pre-screen appointment were excluded from the study. Natural carriers screened as part of the $3.2x10^5$ dose for either 6B or 23F were followed in parallel to challenged volunteers.

Inoculation occurred one week after screening and then samples were taken on days 2, 7 and 14 (Figure 2.1). In a subset of volunteers additional blood samples were taken (Appendix D). Carriage positive volunteers were invited to have a bronchoscopy following antibiotics and carriage clearance.

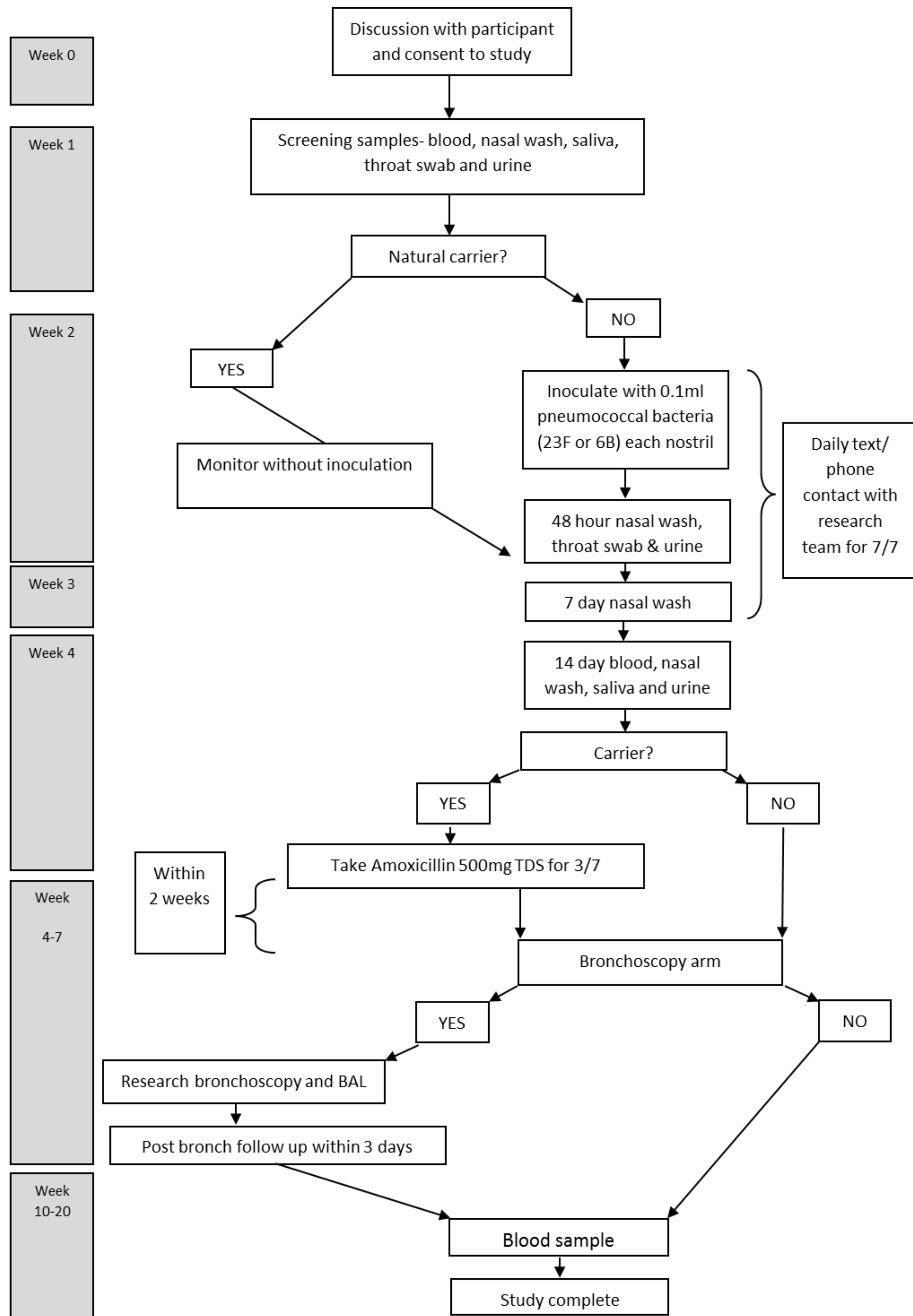

Figure 2.1: Flow chart of Dose-Ranging study appointments. The throat swab was not taken for the 6B $1x10^4$ and $2x10^4$ dose groups. Only natural carriers screened as part of the $3.2x10^5$ dose for either 6B or 23F were followed in parallel to those that were challenged.

2.2.2.2 Reproducibility study schedule

Volunteers recruited to the Reproducibility study followed a similar schedule to the Dose-Ranging study, including additional blood samples in a subset of volunteers (Appendix E). However, there were three changes: the day 2 throat swab was removed based on results from the Dose-Ranging study, the study was extended by 3 weeks to better examine carriage duration, and bronchoscopy was offered to all participants, regardless of carriage status (Figure 2.2). All natural carriers were followed in parallel to challenged volunteers.

2.2.2.3 Re-challenge study schedules

Volunteers followed the same schedule of appointments as the Dose-Ranging study with one exception: those re-challenged with the homologous 6B serotype completed the study after day 7. This was done because carriage status was determined by this point.

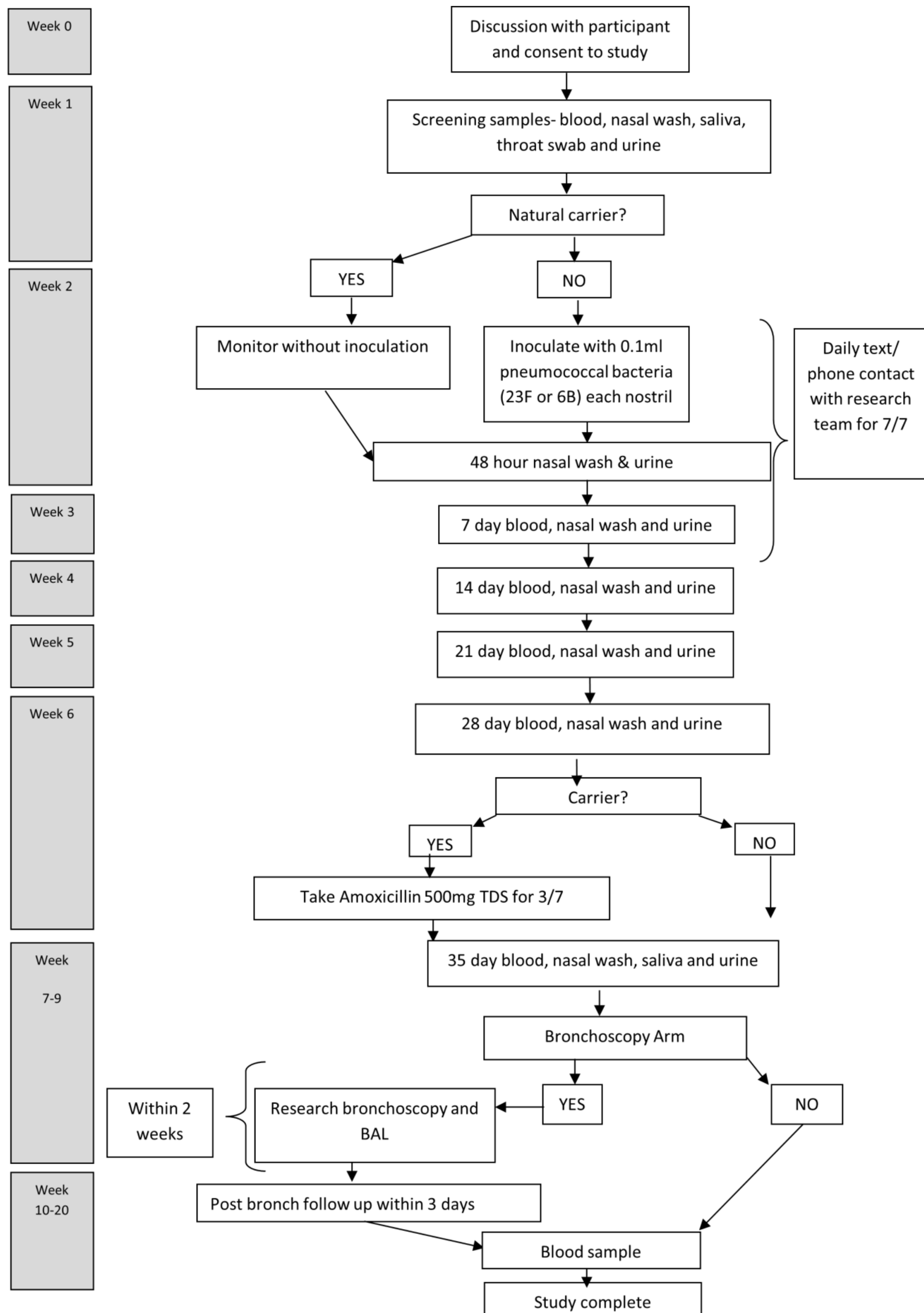

Figure 2.2: Flow chart of Reproducibility study appointments. All volunteers, regardless of carriage status, were offered a bronchoscopy but participation was optional.

2.2.3 Safety monitoring

While the risk to healthy volunteers of developing pneumococcal infection (i.e. sinusitis, otitis media, pneumonia, bacteraemia or meningitis) was very low, the study was designed to minimise risk by appropriate study design, volunteer education and rigorous safety procedures.

Following experimental challenge, volunteers were required to send a text message to the clinical team by 2pm every day for a week. If the text was not received by 2pm the volunteer was contacted to ensure his/her well-being. If he/she did not respond, the allocated next of kin was contacted.

Each volunteer received an emergency pack at the inoculation appointment. This pack contained a post-inoculation information sheet with all the necessary emergency contact information, a thermometer, and a course of amoxicillin. The amoxicillin was only to be taken under three circumstances: if he/she was carrying pneumococcus at the end of the study; in the event he/she was unwell and was instructed to do so by the research team; or if he/she was unwell and unable to contact the research team. The volunteer was encouraged to keep the emergency pack with them at all times during the study.

The clinical team was available 24/7 with nurse contact during working hours and two doctors available out of hours. All queries were dealt with by telephone call and/or immediate review. Provisions were available for immediate, direct admission to the Infectious Disease ward at the Royal Liverpool University Hospital, if needed.

2.2.3.1 Data monitoring and safety committee

As an additional measure of protection a Data Monitoring and Safety Committee, consisting of clinicians and scientists from outside the research group, was set up. The committee received updates on recruitment and a weekly safety report that contained the bacterial dose each volunteer received and whether any symptoms or illness were reported. This report allowed the safety of the study to be externally critiqued without bias. The committee was also available for consultation in the event of any adverse events.

2.2.4 Symptom reporting

2.2.4.1 Passive reporting of symptoms

To examine symptoms related to experimental challenge, we asked volunteers to report any URT symptoms to the clinical team at every appointment. This passive data collection was done for nine of the twelve groups in the Dose-Ranging study: the first five serotype 6B doses and the first four serotype 23F doses. Any complaints were recorded in the case notes.

2.2.4.2 Active reporting of symptoms

To better understand if symptoms were related to carriage or to the inoculation itself, we planned to recruit 10 volunteers to a saline control arm. These volunteers did not know they were being challenged with saline and were required to actively complete a daily symptom log (Figure 2.3) on the day of challenge and for 7 days post-challenge.

To compare symptoms following saline challenge with symptoms following bacterial challenge, volunteers that received the 3.2×10^5 6B dose and the 1.6×10^5 and 3.2×10^5 23F doses in the Dose-Ranging study, as well as the volunteers in the Reproducibility study, also filled out a daily symptom log.

Version 1: January 2012 Daily Symptom Log

Please mark a cross on the point of the scale that represents your symptoms

Day 0: Inoculation

Key to symptoms

1: None- to an occasional limited episode
2:
3: Mild- steady symptoms but easily tolerated
4:
5: Moderately bothersome- symptoms hard to tolerate/ may interfere with daily activities and/ or sleep
6:
7: Unbearably severe- symptoms are so bad/ cannot function all of the time

Assessment of nasal symptoms severity

1 2 3 4 5 6 7

Sneezing

Runny nose

Congestion/stuffiness

Itchy nose

Postnasal drip

(nasal secretions running down the back of throat)

Total nasal symptoms

Assessment of non-nasal symptom severity

1 2 3 4 5 6 7

Eye symptoms

Throat symptoms

Cough

Ear symptoms

Headache

Overall assessment of both nasal and non-nasal symptom severity

EHPC Study REC Ref 11/NW/0592

Figure 2.3: Daily symptom log. Volunteers in the 6B 3.2x10^5 dose group, the 23F 1.6x10^5 and 3.2x10^5 dose groups, the saline control arm and the Reproducibility study filled out a daily symptom log on the day of challenge (Day 0) and for 7 days post-challenge. Adapted from Spector et al. [290].

2.3 Laboratory procedures

2.3.1 Bacterial strains and growth conditions

Clinical isolates of serotype 6B strain BHN418 (GenBank accession number ASHP00000000.1) (a gift of Prof. P Hermans, Radboud University Nijmegen) and serotype 23F strain P833 (a gift of Prof. JN Weiser, University of Pennsylvania) [161] were cultured on Columbia Blood Agar with horse blood (Oxoid) and incubated at 37°C overnight in 5% CO_2. The following day a "parent" bead stock was made by scraping all the overnight growth from the blood agar plate and adding it to a cryovial containing ceramic beads and a cryopreservative fluid (Technical Service Consultants Ltd.). Vials were stored at -80°C and were tested for contamination and colony uniformity before being used to prepare inoculation stocks. This "parent" stock was used to prepare all subsequent inoculation stocks.

Pneumococcal 23F strain P1123 was isolated from the nasopharynx of a volunteer during experimental carriage in the study performed by McCool et al. [161]. It is a derivative of the 23F inoculum (P833) that was used to challenge volunteers in that study.

S. pneumoniae serotype 2 strain D39 was used in the pneumococcal adherence and internalization assay in Chapter 7. D39 pneumococci were grown in Todd-Hewitt broth (Oxoid) supplemented with 0.5% yeast extract (Becton Dickinson) (THY) at 37°C in 5% CO_2 until reaching an optical density (OD) of 0.4-0.5 nm at OD_{600}. Bacteria were either used immediately for flow binding assays, or re-suspended in THY 10% glycerol and stored at -80°C for adherence assays.

2.3.2 Experimental pneumococcal challenge

2.3.2.1 Inoculum stock preparation

Preparation of inoculum stocks was done in a clean environment, using a dedicated fumehood, incubator, and pipettes. It was crucial that no animal products were inoculated into volunteers so Vegitone Infusion broth (Fluka), a vegetable-based growth medium, was used to grow the inoculum.

From the "parent" bead stock, the desired serotype was streaked for heavy growth onto a blood plate, ensuring that the entire plate was covered (Figure 2.4). The plate was then incubated overnight at 37°C, 5% CO_2. The following day half of the plate was swabbed and mixed in 12 ml of warmed Vegitone. This was repeated for the other half of the plate. The

cultures were incubated at 37°C for at least 2 hours or until a visual change in turbidity was detected.

The 12 ml of culture was then added to 40 ml of warmed Vegitone medium and the OD was measured and adjusted to 0.15 at OD_{600}nm. The stock was quantified at this point using the Miles and Misra method (M&M, see section 2.3.2.2) and incubated at 37°C, 5% CO_2. OD readings and M&M quantification were done every hour until an OD_{600} of 0.25, early-mid-log phase, was reached. To one of the tubes 10% sterile glycerol was added and 1 ml aliquots were prepared and stored at -80°C. The other tube was centrifuged at 3345 *x* g for 15 minutes, the supernatant was removed, and the pellet was resuspended in 22.5 ml of Vegitone to concentrate the bacteria. 10% sterile glycerol was added and 1ml aliquots were prepared and stored at -80°C.

After a minimum of 48 hours in the freezer, three stock aliquots were quantified using the M&M method to ensure reproducibility. This value was then used to dilute the stock to the desired inoculum dose.

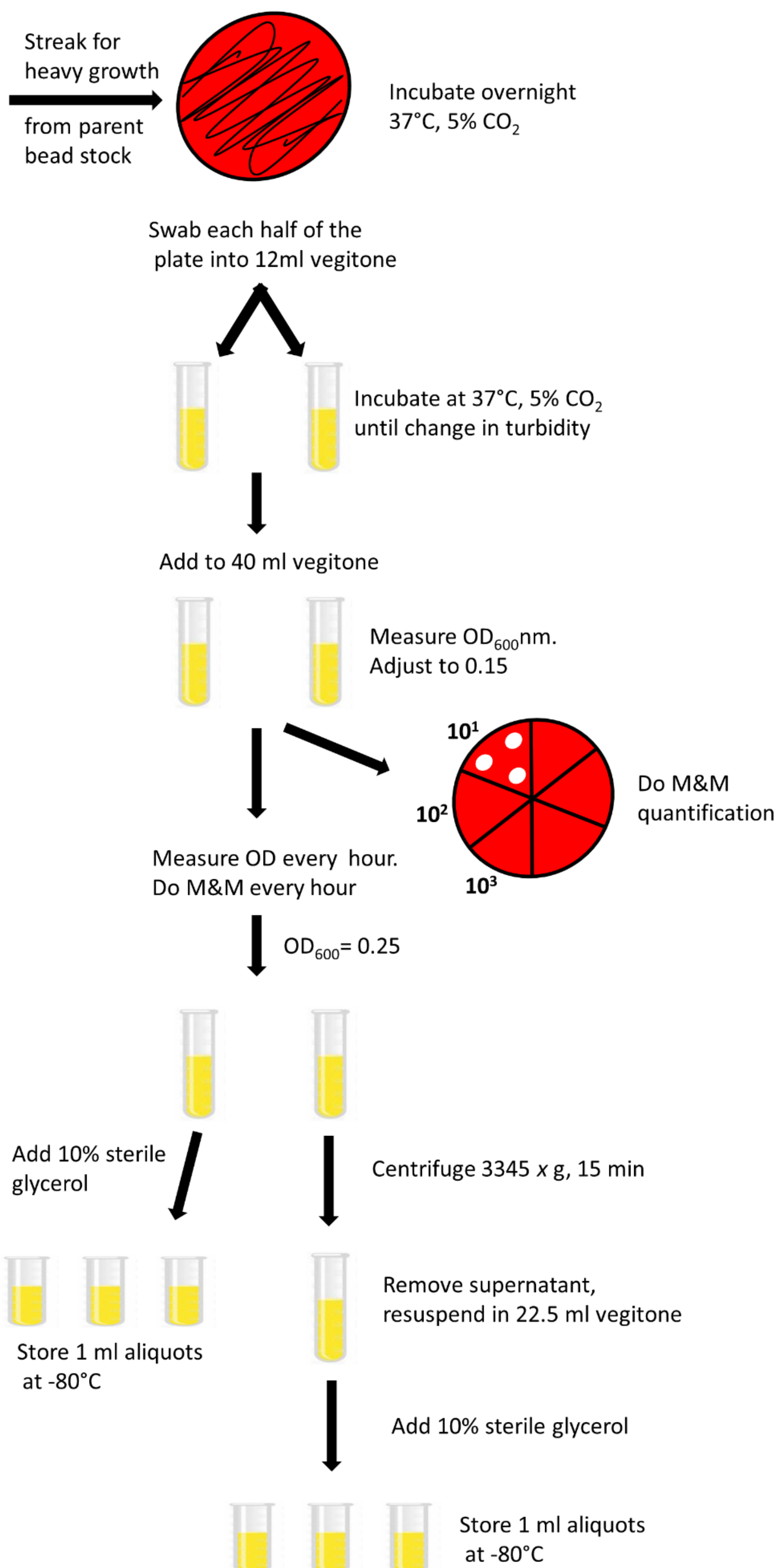

Figure 2.4: Flow chart diagram of inoculum stock preparation

2.3.2.1.1 Confirmation of inoculum serotype and sensitivity

Prior to use in a human carriage study, bacterial stock purity, penicillin sensitivity and serotype were confirmed by an independent reference laboratory (Public Health England, UK).

2.3.2.2 Quantification of *S. pneumoniae* – Miles and Misra method

A modification of the M&M method was used to perform viability counts [291]. A blood agar plate was divided into six sections and labelled 1 to 6. In a 96 well U-bottom plate (Corning Inc.), 20 µl of bacteria was added to 180 µl of sterile phosphate buffered saline (PBS) and serially diluted (ten-fold) until a dilution of 10^6 was reached. Three drops of 10 µl from each dilution were placed on the corresponding section of the plate and allowed to dry. The plate was then inverted and incubated overnight at 37°C, 5% CO_2.

The next day, visible colonies in each dilution section were counted and recorded (Figure 2.5).

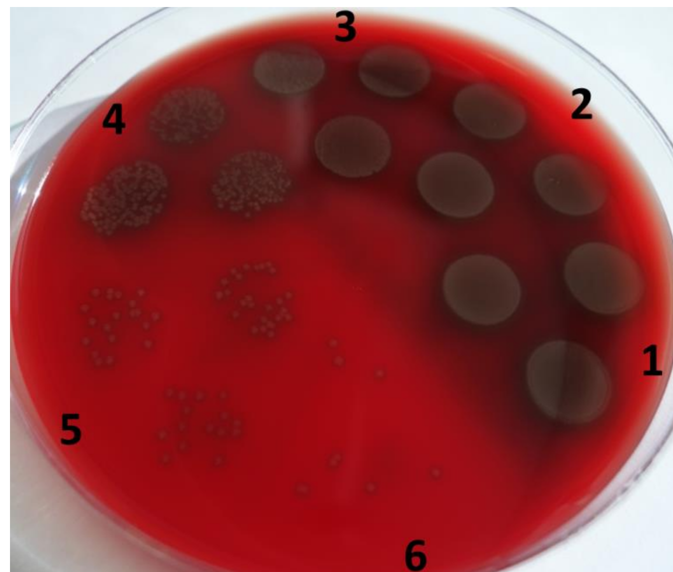

Figure 2.5: Miles and Misra bacterial quantification. Three 10 µl drops from a serial dilution were placed on the corresponding dilution section of the plate. The plate was then inverted and incubated overnight at 37°C, 5% CO_2. Individual colonies can be counted in the 5th dilution section.

2.3.2.2.1 How to calculate CFU per ml

The dilution section with a count between 30 and 300 was used to determine the CFU/ml with the formula:

$$\frac{\text{CFU}}{\text{ml}} = \left(\frac{(\text{average number of colonies in section})\ \text{x}\ (\text{dilution factor})}{\text{the volume of the drop plated}}\right) \text{x}\ 1000$$

2.3.2.4 Determination of inoculum dose

The dilution of the inoculum stock to the desired dose had to be determined prior to the start of an experimental carriage study. This was done using the calculated density of the inoculum stock after 48 hours at -80°C and plugging it into:

$$C1V1 = C2V2$$

where C1 = the density of the inoculum stock
V1= the unknown volume the stock must be diluted in
C2 = the desired quantity for inoculation
V2 = the desired total volume of the final inoculation

2.3.2.4.1 Quantification of inoculum dose

Once the dilution volume for the inoculum stock was determined, quantification by M&M was performed; this was considered the “pre-inoculation” count. It takes approximately 30 minutes to complete the entire inoculation procedure and pneumococci suspended in saline will start to die in this interval. To account for this, the diluted inoculum sat at room temperature for 30 minutes and then a “post-inoculation" quantification was performed. The average of the before and after counts was used to determine the final inoculated concentration.

An inoculum dose was considered out of protocol if it was less than half or more than double the desired concentration.

2.3.2.5 Preparation of inoculum on day of challenge

A half an hour before a scheduled inoculation appointment an aliquot of the desired serotype was thawed, centrifuged at 17,000 *x* g for 3 minutes, and the bacterial pellet was washed before being re-suspended and diluted in sterile, hospital grade 0.9% saline to reach the desired concentration of bacteria.

Once prepared, the inoculum was quantified using a modified version of M&M. Instead of plating the dilution as drops within a sector, each dilution had its own plate and the three 10 µl drops were placed in a line on a slightly inclined plate and allowed to run down the length of the plate (Figure 2.6).

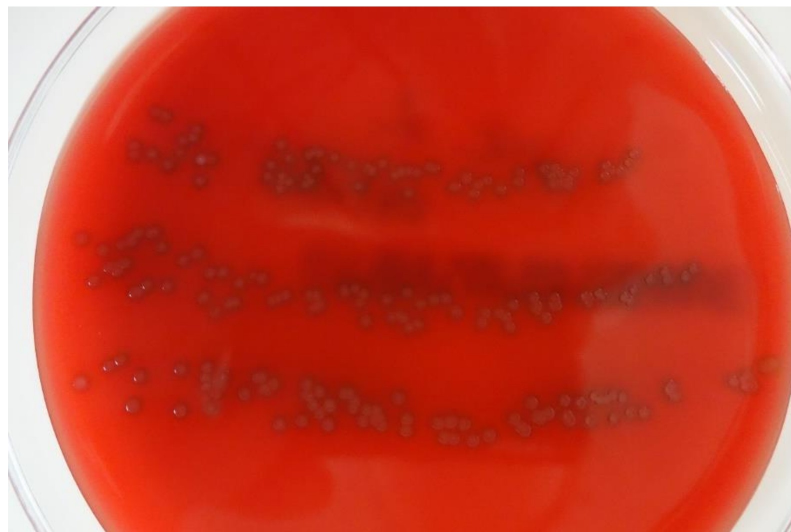

Figure 2.6: Quantification of inoculum on blood agar plate. Three 10 µl drops from the inoculum were dropped in a line on a plate and the plate was tilted slightly to allow the drops to run down it. This was done before and after the inoculation appointment. All colonies were counted and used to calculate inoculation dose.

The prepared inoculum was then taken to the Clinical Research Unit at the Royal Liverpool University Hospital. Following completion of all volunteer inoculations, the inoculum was taken back to the laboratory for the post-inoculation quantification.

2.3.2.6 Nasopharyngeal inoculation

Intra-nasal inoculation with *S. pneumoniae* was carried out by a Research Nurse. The inoculation was performed while the volunteer was seated comfortably in a semi-recumbent position. With the head tilted back slightly, the tip of a P200 pipette was inserted just inside the nasal cavity and 100 µl of the bacterial inoculum was slowly dispersed across the nasal mucosa in a circular motion (Figure 2.7).

Figure 2.7: Inoculation of the nasal mucosa with 100 µl of *S. pneumoniae*. Volunteers were seated in a semi-recumbent position, with the head tilted slightly back. The tip of a P200 pipette was inserted just inside the nasal cavity and 100 µl of the bacterial inoculum was slowly dispersed across the nasal mucosa in a circular motion.

During the inoculation, it was important that the pipette tip did not come in contact with the nasal mucosa as a disruption in the integrity of the epithelium could result in bacteria entering the bloodstream. It was also important that the inoculum was not expelled too far back in the nasal cavity as it would run down the throat and be swallowed instead of remaining in the nasopharynx.

The inoculation was repeated for the other nostril and then the volunteer remained in the semi-recumbent position for 10 minutes without sniffing or blowing the nose.

2.3.2.7 Nasal wash sampling method

Nasal wash was used to sample the nasopharynx of volunteers. Samples were collected one week before inoculation and at all visits post-inoculation [292,293]. A volunteer was seated comfortably and the head was tilted back 30° from the vertical. The volunteer was then asked to take a deep breath in and hold their breath whilst pushing their tongue up and backwards against the roof of the mouth. A syringe filled with 20 ml of sterile saline was inserted into the anterior nasal space and 5 ml of saline was expelled. The volunteer then leaned forward immediately and expelled the fluid by exhaling rapidly through the nose into a foil bowl (Figure 2.8).

The procedure was repeated three more times so that each naris had been washed twice and a total of 20 ml was used. Following collection the samples were pooled in a centrifuge tube and transported to the laboratory at room temperature.

Figure 2.8: Nasal wash. A syringe filled with 20 ml of sterile saline was inserted into the anterior nasal space and 5 ml of saline was expelled. The volunteer then leaned forward and expelled the saline by exhaling rapidly through the nose into a foil bowl. Picture reproduced with permission of Dr. J. Rylance.

2.3.2.7.1 Adaptations for the nasal wash procedure

In some cases the nasal wash method needed to be adapted to obtain an adequate sample. If a volunteer had a blocked/congested nose and the saline ran out anteriorly after insertion, the volunteer blew the nose and then the syringe was inserted a little further back and the head was tilted back further. If a volunteer tasted saline during the nasal wash then the connection between the posterior nasopharynx and oropharynx had not been

adequately closed. The procedure was then explained again, with an emphasis on pushing the tongue against the roof of the mouth. In either case if the yield was less than 5 ml the procedure was repeated using up to an extra 20 ml.

2.3.2.8 Nasal wash sample processing

Prior to centrifugation, nasal wash samples were examined for debris or mucus. If a nasal wash sample contained a substantial amount of mucus, the sample was vortexed to break it up. Any large pieces of mucus were then removed with a pipette, ensuring as little saline as possible was removed.

Nasal wash samples were centrifuged at 3345 *x* g for 10 minutes. Following centrifugation the volume of supernatant was recorded and then stored in 1 ml aliquots at -80°C.

The nasal wash bacterial pellet was re-suspended in 100 µl of skim milk, tryptone, glucose, glycerol (STGG) medium (Appendix A) and the total volume in the centrifuge tube was determined. A 20 µl drop of the sample was streaked onto Columbia Horse Blood Agar containing 4 µg/ml gentamicin (Sigma). If the nasal wash was post-inoculation 10 µl was serially diluted in a 96 well U-bottom plate and M&M quantification to determine the density of pneumococcal carriage (section 2.3.3.2) was performed using blood agar containing 4 µg/ml gentamicin.

The nasal wash pellet was then diluted by adding 800 µl of STGG and 25 µl was streaked onto both blood and chocolate agar (Columbia Agar with Chocolated Horse Blood, Oxoid) to determine co-colonising flora. The remainder of the sample was split into two cryovials and stored at -80°C. All plates were incubated overnight at 37°C, 5% CO_2.

2.3.2.8.1 Removal of microbial flora from nasal wash plates

The nasopharynx is not a sterile environment and microbial flora can obscure the presence of *S. pneumoniae* (Figure 2.9A). To remove any microbial flora it was crucial to add gentamicin (4 µg/ml) to the blood agar plate for both the M&M quantification and the concentrated nasal wash pellet (Figure 2.9B).

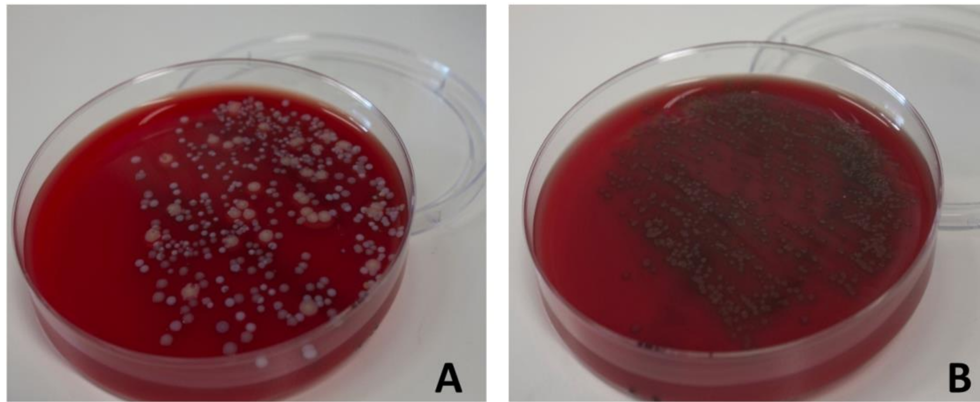

Figure 2.9: Blood agar plate inoculated with nasal wash, without and with gentamicin added to the media. Blood agar plates inoculated with nasal wash can be difficult to read due to the presence of other nasopharyngeal flora. (A) A blood agar plate with a nasal wash sample. Growth of microbial flora hides the pneumococci. (B) Gentamicin was added to a blood agar plate before the same nasal wash sample was streaked out. Pneumococci are clearly visible and all other microbial flora has been removed.

2.3.3 Detection of pneumococcal carriage

2.3.3.1 Detection of pneumococcal carriage by culture

Following overnight incubation plates were examined for the presence of *S. pneumoniae*. Any alpha haemolytic, draughtsman-like colonies were sub-cultured for purity. A Gram stain was performed and optochin sensitivity and bile solubility were tested.

Serotype confirmation was performed using latex agglutination (Statens Serum Institute). If serotype 6 or 23 was detected at any time point, the volunteer was considered carriage positive.

2.3.3.2 Measurement of pneumococcal carriage density

M&M was used to determine carriage density by culture. To calculate the CFU/ml of nasal wash returned, the CFU/µl was determined in the same way as described in section 2.3.2.2.1. The CFU/µl was then multiplied by the volume of the bacterial pellet suspension after the addition of 100 µl of STGG. This value was then divided by the amount of nasal wash returned by the volunteer to obtain CFU/ml of nasal wash [292].

$$\frac{\text{CFU}}{\text{ml nasal wash}} = \frac{\frac{\text{CFU}}{\mu\text{l}} \text{ x STGG total pellet volume}}{\text{nasal wash volume returned}}$$

We chose to divide the CFU by the amount of nasal wash returned, rather than the amount of saline instilled, because the return volume contained the pneumococci and varied between volunteers. There is a possibility that a nasal wash misses pneumococci that are tightly adhered to the epithelium however, Wu et al. [268] showed in adult mice that the CFU recovered from a nasal wash strongly correlated with the CFU from a paired homogenized nasal tissue sample taken after the wash.

2.3.3.3 Bacterial DNA extraction

Directly after the nasal wash collection, 2 ml was added to 4 ml of RNAprotect Bacteria Reagent (Qiagen). After 5 minutes incubation at room temperature the sample was transferred to the laboratory on ice and stored at -80°C until processing.

Thawed samples were pelleted in a 2 ml tube by three centrifugation steps at 19,090 *x* g for 20 minutes at 4°C. The pellet was resuspended in 0.3 ml lysis buffer with protease (Agowa Mag mini DNA extraction kit, LGC Genomics), 50 mg sterilized zirconia/silica beads (diameter 0.1 mm, Biospec Products) and 0.3 ml phenol (Phenol BioUltra, Sigma-Aldrich). The sample was mechanically disrupted by bead beating in a TissueLyser LT (Qiagen) twice at 50 Hz for 2 minutes. After centrifugation the aqueous phase was transferred to a sterile 1.5 ml tube. Binding buffer was added in twice the volume of the aqueous phase plus 10 µl of magnetic beads, after which the sample was incubated in a mixing machine for 30 minutes at room temperature. The magnetic beads were washed with 200 µl of both wash buffer 1 and 2 and eluted with 63 µl elution buffer according to the manufacturer's instructions.

2.3.3.4 Quantification of pneumococcal DNA by qPCR

Determination of carriage density by qPCR was performed through partial amplification of the *lytA* gene. The primer and probe sequences were: forward primer 5'-ACGCAATCTAGCAGATGAAGCA-3'; reverse primer 5'-TCGTGCGTTTTAATTCCAGCT-3'; probe 5'-(FAM)-GCCGAAAACGCTTGATACAGGGAG-(BHQ1)-3' as previously published [253]. The 20 µl PCR mix consisted of 1x TaqMan® Universal PCR Master Mix (Life Technologies), 0.1 µM of each primer, 0.1 µM probe and 1 µl of the extracted DNA. Thermal cycling was performed in an ABI 7500 Fast Real-Time PCR System (Life Technologies) under cycling conditions: 2 minutes 50°C, 10 minutes 95°C and 40 cycles of 15 sec 95°C and 1 minute 60°C.

A standard curve of a 10-fold dilution series of genomic DNA extracted from *S. pneumoniae* (TIGR4, ATCC BAA-334) was used. The genomic DNA was extracted using the Qiagen

Genomic-tip 20/G Kit (Qiagen), and quantified by a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific). The conversion from weight pneumococcal DNA to number of DNA copies *S. pneumoniae* was based on the weight of one genome copy TIGR4 calculated by the genome length in base pairs times the weight of a DNA base pair (650 Dalton). The lower limit of detection (LLOD) of the method was set at 40 cycles. A nasal wash was considered positive if both duplicates yielded a qPCR signal below 40 cycles.

2.3.4 *In vitro* assays

2.3.4.1 Microarray

Microarray was used to investigate the potential of multiple serotype carriage in one volunteer. The bacterial pellet from the pre-screen and days 2, 7, and 14 visits, was diluted 1:10 and 1:100 and 50 µl of each dilution, as well as a neat suspension, were spread onto selective agar plates (Colistin sulphate, Oxolinic acid, blood agar; Oxoid) and incubated overnight at 37°C in 5% CO_2. All colonies from the plate with the highest density of distinct non-confluent colony growth were scraped into 1 ml of sterile PBS. DNA extractions were performed using the QIAamp DNA Mini Kit (Qiagen), including lysis buffer (20 mM Tris/HCl, 2 mM EDTA, 1% v/v Triton, 20 mg/ml lysozyme) and RNase treatment as previously described [239].

Molecular serotyping was performed using the BµG@S SP-CPSv1.4.0 microarray. Briefly, DNA samples were fluorescently labelled and hybridized to the Agilent 8×15K format microarray according to manufacturer's instructions for the Agilent genomic DNA ULS labelling and oligo aCGH hybridisation reagent kits.

Microarray data was statistically analysed using a Bayesian hierarchical model which calculates the probabilities of serotype combinations based on the data from the microarray and determines the serotype, or combination of serotypes, present in the sample [294].

2.3.4.2 Determination of phase morphology

Pneumococcal isolates were streaked on tryptic soy agar plates containing catalase and incubated at 37°C in the presence of CO_2. Determination of phase morphology was done under magnification and transmitted, oblique illumination as previously described [45]. Briefly, transparent colonies were bluish in colour and appeared smaller and more transparent in the centre, due to a central depression, giving the impression of a

"bullseye". Opaque colonies were whitish in colour and slightly bigger, appearing more uniform and with a domed shape.

2.3.4.3 Complement deposition assay

Pneumococci were cultured in THY broth at 37°C, 5% CO_2 to an OD_{600} of 0.4. Bacteria were then centrifuged at 3,350 *x* g for 10 minutes, the supernatant removed, and the pellet resuspended in 1 ml of PBS. 100 µl of the pneumococcal suspension was added to two Eppendorf tubes and centrifuged at 17,000 *x* g for 3 minutes. The supernatant was removed and one pellet was resuspended in 1% gelatine veronal buffer (GVB) (Sigma). This was the control tube which was resuspended in 1% GVB at every step. The experimental condition pellet was resuspended in 20% normal human sera (plasma from buffy coats) and then both tubes were incubated at 37°C, 5% CO_2 for 30 minutes.

Bacteria were centrifuged at 17,000 *x* g for 3 minutes, the supernatant removed and the experimental tube re-suspended in a 1:300 dilution of IgG2a mouse-anti-human-C3 (Abcam) for 30 minutes at 37°C, 5% CO_2. Following this incubation, bacteria were centrifuged at 17,000 *x* g for 3 minutes, the supernatant removed, and the experimental tube resuspended in a 1:500 dilution of anti-mouse IgG, IgA, IgM-FITC (Sigma) for 30 minutes at 4°C in the absence of light. Bacteria were then centrifuged at 17,000 *x* g for 3 minutes, washed with PBS, re-suspended in PBS, and stored at 4°C in the absence of light until acquisition.

Samples were acquired using a nine-colour LSR II flow cytometer (BD). The Mean Fluorescence Intensity (MFI) (±SD) was calculated as the percentage of the bacterial population positive for C3 deposition multiplied by the geometric mean fluorescence of the total cell population.

2.3.4.4 Measurement of anti-pneumococcal polysaccharide antibodies in intravenous immunoglobulin by ELISA

To measure the level of polysaccharide antibodies to serotypes 6B and 23F in intravenous immunoglobulin (IVIG), 96-well plates were coated with 5 µg/ml of purified 6B or 23F polysaccharide (Statens Serum Institute) for 5 hours at 37°C. All samples were diluted in 10% foetal bovine serum in PBS (PBS-F) and plates were washed 3 times with PBS-Tween 0.05% between each step using a microplate washer (Wellwash 4 MK2, Thermo Scientific).

In a separate 96-well plate, IVIG was diluted in PBS-F containing 10 µg/ml of cell wall polysaccharide (CWPS Multi, Statens Serum Institute) and incubated at room temperature

for 30 minutes. When CWPS Multi is used, separate adsorption with the 22F capsule is not required. Pneumococcal reference serum lot 89-SF5 (U.S. Food and Drug Administration) was used as a standard. Diluted/adsorbed samples were then transferred to the pre-coated plates and incubated overnight at 4°C. Bound antibodies were detected using goat anti-human IgG conjugated with alkaline phosphatase (Sigma) for 2 hours at room temperature. 0.5 mg/ml of p-nitrophenyl phosphate (PNPP) was added as a substrate. Absorbance was measured at 405 nm using a FLUOstar OMEGA plate reader (BMG Labtech). Samples were run in triplicate, twice.

2.3.4.5 Opsonophagocytic killing assay

2.3.4.5.1 Isolation of neutrophils from peripheral blood

Ethical approval to take peripheral blood from healthy adults was acquired through the Liverpool School of Tropical Medicine Research Tissue Bank (REC ref. 11/H1002/9).

15 ml of blood was obtained from healthy volunteers in heparin containing tubes (BD). Neutrophils were isolated from the blood using a standard density gradient separation method. First, 15 ml of Histopaque 1119 (Sigma) was added to a 50 ml falcon tube followed by carefully layering an equal volume of Histopaque 1077 (Sigma) on top. 15 ml of whole blood was then carefully layered on top of the Histopaque gradient and the tube was centrifuged at room temperature for 30 minutes at 677 *x* g with the brake off. Following centrifugation, the top two layers were removed with a Pasteur pipette and discarded and the neutrophil layer was transferred to a fresh 50 ml tube, taking care not to carry over red blood cells. The tube with the neutrophils was topped up to 50 ml with Hanks' Balanced Salt Solution (HBSS) without calcium or magnesium (-/-) (Gibco) and washed by centrifugation at 200 *x* g for 10 minutes.

If the cell pellet showed signs of residual red blood cell contamination, 2 ml of RBC lysis buffer 1x (Biolegend) solution was added and the pellet was incubated at room temperature, protected from light, for 10 minutes. The tube was then topped up to 50 ml with HBSS-/- and centrifuged at 200 *x* g for 10 minutes. This step was repeated and then the cell pellet was resuspended in 0.5 ml of HBSS-/- and the cell number and viability was assessed using Trypan Blue and a haemocytometer.

2.3.4.5.2 Neutrophil opsonophagocytic killing assay

Aliquots of 6B and 23F pneumococci were thawed, centrifuged at 17,000 *x* g for 3 minutes and diluted to $5x10^4$ CFU/ml in HBSS with Ca^{2+} and Mg^{2+} (+/+) (Gibco). IVIG (Grifols Lot 26NLVH1) was used as a source of pathogen-specific antibody and was diluted 1:8 in HBSS+/+. 500 µl of diluted IVIG was mixed with 500 µl of diluted 6B pneumococci in a bijoux tube. This was repeated for serotype 23F. 500 µl of both pneumococcal suspensions were also added to 500 µl of HBSS+/+ as non-opsonized controls. All four tubes were then placed in a shaking incubator (100 RPM; Sarstedt TPM-2 Shaker) at 37°C for 20 minutes. As two serotypes were being tested, quantification of the diluted pneumococcal suspensions was performed as previously described (Chapter 2, section 2.3.2.2) to ensure the same number of pneumococci had been added for both serotypes.

Following opsonization, 20 µl of the bacterial suspension was added to a 96 well plate with 10 µl of baby rabbit complement (Pel-Freez Biologicals). Neutrophils were diluted to $1.7x10^6$ cells in Opsonization Buffer B (OBB) containing HBSS+/+, 1% GVB (Sigma) and 5% heat inactivated foetal bovine serum (Invitrogen) and 30 µl was then added to each well, giving a multiplicity of infection of 1:100 (1 pneumococcus to 100 phagocytes). 30 µl of OBB was added to each well for a final volume of 80 µl/well. Control wells contained non-opsonized bacteria

Plates were incubated at 37°C for 45 minutes on a shaking platform (200 RPM). Phagocytosis was stopped by resting plates on ice for 10 minutes. 10 µl of the reaction mixture was tilt-plated onto a blood agar plate in triplicate. Plates were incubated overnight at 37°C, 5% CO_2 before counting bacterial colonies. Results were expressed as percent killing which was determined by the average CFU recovered from the control wells minus the average CFU recovered from the experimental wells, divided by the average CFU from the control wells.

2.3.4.6 Sequencing

2.3.4.6.1 Genetic comparison of the 6B and 23F strains

The 23F inoculum strain (P833) was sequenced using the Roche 454 platform at the Centre for Genomic Research, University of Liverpool. Annotations were transferred from *S. pneumoniae* 23F, ST81 to the assembled 23F genome using Rapid Annotation Transfer Tool (RATT) [295]. The genome of 23F was compared to the publicly available 6B genome [280] in the Artemis Comparison Tool (ACT) [296] to identify areas of genomic difference, focusing on known adherence factors.

The accession number for *S. pneumoniae* 23F, ST81 is FM211187.

2.3.4.6.2 Genetic comparison of 23F strains P833 and P1123

The two 23F strains (P833 and P1123) were also sequenced on an Illumina Hi-Seq as previously described [297].

All analysis of Illumina data was performed using CLC Main Workbench 6 (CLC bio, Boston, MA). First, *de novo* assembly was performed on the reads from P833 and then the reads from P833 and P1123 were mapped to the resulting contig sequences. Single nucleotide polymorphism (SNP) and deletion-insertion polymorphism (DIP) analysis was then performed and true genetic variations were scored as being present in the P1123 mapping while being absent from the P833 mapping.

2.3.4.7 Determination of a mutation in *amiC*

2.3.4.7.1 Confirmation of amiC mutation by sequencing

The presence/absence of a frameshift mutation in the *amiC* gene was determined by PCR amplification and sequencing using ABD233 (5'-GAATCCAAATGGCTGTAACAGGAGC-3') and ABD234 (5'-AACCTATGCTAATACACCAGTTCTTCAGG-3').

2.3.4.7.2 Phenotypic confirmation of a mutation in the ami locus

Mutations in the *ami* locus have been shown to confer resistance to aminopterin [298]. To phenotypically assess the presence/absence of the *amiC* mutation in the 23F strains (P833 and P1123), the two strains were serially diluted on blood agar containing $2x10^{-6}$ M aminopterin and examined for growth, indicative of aminopterin resistance, following incubation.

2.3.4.8 Pneumococcal adherence and internalization assays

Nasopharyngeal human carcinoma epithelial cells (Detroit 562 ATCC-CCL-138) were grown to confluence in Eagle's Minimal Essential Media (EMEM) supplemented with 10% foetal bovine serum, 2 mM L-glutamine (Sigma), 40 U/ml penicillin and 40 µg/ml streptomycin (Sigma). Cells were grown to confluence and then monolayers were released by incubation with EDTA/trypsin (Invitrogen). Cells were re-suspended in 20 ml of complete medium and counted on a haemocytometer. Cell cultures were seeded at $1x10^{5}$ cells/ml in a 24 well plate.

Confluent monolayers of Detroit 562 epithelial cells were washed three times with warmed PBS and then 500 µl of antibiotic free EMEM was added to each well. Bacterial suspensions

were added to each well at a density of $1x10^6$ CFU for serotypes 6B and 23F and $2x10^6$ CFU for strain D39. Plates were then gently shaken and 20 µl was obtained from each well for dilution and initial CFU quantification via M&M on blood agar plates. After allowing for adherence at 37°C, 5% CO_2 for 3 hours, the wells were washed five times with PBS to remove non-adherent bacteria and cells were either lysed with 300 µl of 1% saponin (Sigma) for 10 minutes (for adherence) or treated with 100 µg/ml of ampicillin in EMEM (1 ml per well) for 3 hours at 37°C, 5% CO_2 before lysing (for internalization). Recovered bacteria were quantified by serial dilution on blood agar plates. Plates were incubated at 37°C 5% CO_2 overnight and initial and recovered CFU were counted.

2.3.4.8.1 Epithelial cell inflammation

For inflamed epithelium, cells were stimulated with interleukin-1β (IL-1β), tumor necrosis factor (TNF) and interferon gamma (IFN-γ) (50 ng/ml each) in 500 µl of complete EMEM for 16-18 hours at 37°C 5% CO_2. Cells were then washed 3 times with warmed PBS and 500 µl of EMEM without antibiotics was added to each well before addition of pneumococci.

2.3.4.8.2 Epithelial pIgR and rPAF expression by flow cytometry

Stimulated (inflamed) and non-stimulated (non-inflamed) cells were scraped from plate wells after washing with warmed PBS. Tubes containing cells were centrifuged for 5 minutes at 836 *x* g and cells were washed again to ensure epithelial cells were in a single cell suspension. Cell suspensions were incubated with platelet-activating factor receptor (rPAF) human monoclonal antibody (1:62.5) and polymeric immunoglobulin receptor (pIgR) rabbit polyclonal antibody (1:25) (Cambridge Bioscience) at 4˚C for 15 minutes. Anti-human PE conjugate and anti-rabbit FITC-conjugate antibodies were added for 15 minutes at 4˚C. Cells were washed and stored at 4˚C prior to acquisition using a BD LSR II flow cytometer. 10,000 gated events were recorded. PE goat anti-mouse IgG2a (1:250) and FITC donkey anti-rabbit IgG (1:125) (Biolegend) were used as isotype controls.

2.3.4.9 Detection and identification of URT viruses

Oropharyngeal swabs were collected 5 days prior to intranasal inoculation and immediately put in viral transport medium (VTM) (MWE). Viral multiplex PCR was performed as described elsewhere [299,300]. Briefly, viral RNA was extracted from 200 µl of VTM and eluted into 85 µl buffer using the standard Pathogen Complex 200 protocol on the QIAsymphony (QIAGEN). The eluates were then analysed using four multiplex PCR assays on the LC480 real-time PCR machine (Roche Diagnostics). The assay panel covered the

qualitative detection of influenza A and B, RSV, human metapneumovirus, human rhinovirus, parainfluenza viruses 1-4, and coronaviruses OC43, NL63, 229E, HKU1.

2.3.4.10 Levels of FH, lactoferrin, SLPI, and beta defensin 2 in nasal wash and anti-PspC in serum

To measure levels of FH, 96-well plates were coated with nasal wash samples serially diluted in carbonate-bicarbonate buffer. Purified human FH (125 ng/ml) (Calbiochem) was used as a standard and plates were incubated overnight at 4°C. All plates were washed 3 times with PBS-Tween 0.05% between each step using a microplate washer (Wellwash 4 MK2, Thermo Scientific). Plates were blocked with PBS-1% BSA for 30 minutes before incubating with goat anti-FH antibodies (1:15000 dilution) (Calbiochem) for 1 hour. This was followed by HRP-conjugated anti-goat IgG (1:5000 dilution) (R&D Systems, Abingdon, UK) for 1 hour. Plates were developed with TMB Substrate Reagent Set (BD).

Levels of lactoferrin (AssayPro), SLPI (R&D) and beta defensin 2 (Antigenix America) were measured by sandwich ELISAs following manufacturer's recommendations. All plates were washed 3 times with PBS-Tween 0.05% between each step using the microplate washer. 96-well plates were coated with anti-human lactoferrin (2 µg/ml) (AssayPro) or SLPI (0.5 µg/ml) (R&D) and incubated at 4°C overnight. Plates were blocked with PBS-1% BSA before nasal wash samples serially diluted in PBS-0.1% BSA were added. Human lactoferrin (80 ng/ml) (Assaypro) and recombinant human SLPI (30 ng/ml) (R&D) were used as standards. Plates were incubated at room temperature for 2 hours. Biotinylated rabbit anti-lactoferrin (2 µg/ml) or goat anti-SLPI (0.5 µg/ml) were used for detection and plates were incubated for 2 hours at room temperature. Streptavidin-alkaline phosphatase (1:1000) (AbD Serotec Ltd) was added and plates were incubated at room temperature for 1 hour prior to development with 0.5 mg/ml of PNPP.

Beta defensin 2 levels were measured using the Human BD-2 Super X ELISA kit as per manufacturer's instructions (Antigenix America). Briefly, neat nasal wash samples were added to a 96-well plate pre-coated with BD-2 capture antibody. Recombinant human BD-2 (2 ng/ml) was used as a standard. Biotin detection antibody (0.1 µg/ml) was added to each well and the plate was incubated at room temperature for 1 hour. Plates were washed 4 times with the provided wash buffer and streptavidin-HRP conjugate was added (1:1500), followed by incubation at room temperature for 30 minutes. Plates were developed with TMB Substrate solution.

For the FH, lactoferrin, SLPI, and BD-2 assays, absorbance was measured at 450 nm using a FLUOstar OMEGA plate reader (BMG Labtech).

Levels of anti-PspC IgG were measured by ELISA using plates coated with recombinant purified PspC (1 µg/ml) [289]. Eight-fold serial dilutions of serum samples in 0.1% BSA were added and incubated for 2 hours at room temperature. PspC-specific IgG antibodies were detected using alkaline-phosphatase conjugated goat anti-human IgG (Sigma). 0.5 mg/ml of PNPP was added as a substrate and absorbance was measured at 405 nm. Anti-PspC IgG concentrations were calculated using a reference sera sample with a known anti-PspC concentration assigned in the laboratory of Prof. Helena Käyhty (National Institute for Health and Welfare, Helsinki, Finland). The sample concentration was calculated using the last dilution of the sample with OD>0.1.

All samples were run in triplicate, and samples with a CV of greater than 15% were repeated. A 4-parameter fit was used to generate the standard curve.

2.3.4.11 Depletion and purification of antibodies from nasal wash and sera samples

Nasal wash samples from 10 volunteers were pooled and IgG and IgA were depleted by anti-human IgG and anti-human IgA agarose (Sigma-Aldrich), respectively. Samples were slowly added to an agarose column and then washed with 0.01 M sodium phosphate buffer. Flow-through fractions were used in pneumococcal adherence assays. A dot blot was used to confirm antibody depletion. Briefly, 4 µl of purified sample was spotted onto a nitrocellulose membrane (Sigma) and left to dry. The membrane was blocked by soaking in 5% milk in tris-buffered saline with Tween20 (TBS-T) for 30 minutes at room temperature. Following blocking, the membrane was incubated with anti-human IgG-alkaline phosphatase (1:2000) or anti-human IgA-alkaline phosphatase (1:2000) and incubated for 1.5 hours at room temperature. The membrane was washed with TBS-T for 10 minutes and developed with TMB substrate (BioRad).

Serum samples from 7 volunteers were used for anti-PspC IgG purification. First, purified total IgG was obtained from individual samples by HiTrap protein G affinity column (GE Healthcare). Purified samples were then loaded in CNBr-activated Sepharose coupled with recombinant purified PspC. Coupling of the PspC ligand (1 mg of ligand PspC to 200 mg of sepharose) and anti-PspC purification was performed as per manufacturer’s instructions. To confirm anti-PspC IgG purification, ELISAs were performed using plates coated with PspC as described in section 2.3.4.10 [289].

2.3.4.12 FH binding and antibody binding assays

Bacterial stocks were washed and re-suspended in PBS. For inhibition of FH binding, 10^7 CFU were re-suspended in 100 µl of individual nasal wash samples, purified IgA and IgG samples (undiluted) or purified anti-PspC IgG (1:2 diluted) and incubated for 45 minutes at 37°C. To evaluate the saturating concentration of FH binding and for positive control of inhibition of FH binding experiments, bacterial pellets were re-suspended in 100 µl of purified human FH (Calbiochem) (3 to 40 µg/ml) and incubated for 45 minutes at 37°C.

Samples were washed with PBS and incubated in 100 µl of goat anti-FH (1:200) (Calbiochem) for 45 minutes at 37°C before wash and incubation in 100 µl of FITC-conjugated anti-goat (1:500) (Sigma) at 4°C in the dark for 30 minutes. Samples were washed twice and re-suspended in 500 µl of PBS- 4% paraformaldehyde and stored at 4°C prior to acquisition using a BD LSR II flow cytometer. 20,000 bacterial events were acquired and samples were gated relative to a negative control containing no anti-FH. Anti-PspC IgG binding was evaluated by incubating bacterial pellets with individually purified human anti-PspC IgG samples for 45 minutes at 37°C before detection using anti-human IgG FITC antibody (1:10000, Sigma). As FH binding to *S. pneumoniae* is often biphasic with strongly positive and weakly negative populations of bacteria, results are presented as fluorescence index (FI: percentage of positive bacteria multiplied by the geometric mean fluorescence index (MFI) in arbitrary units).

2.3.4.13 Anti-PspC antibody epitope mapping

Overlapping peptide arrays containing 15-mer peptides with a frameshift of four residues corresponding to the amino acid sequence of PspC3 (GenBank accession no EF424119) were synthesized in a slide support (CelluSpots, Intavis). Peptide arrays were incubated individually with 29 sera samples collected from 18 volunteers inoculated with pneumococcus or from mice immunized with 3 doses of 5 µg of PspC3 containing 50 µg of Alum as previously described [301]. Concentration of pneumococcal specific antibodies was standardized so that each sample used for incubation contained 10 µg/mL of PspC IgG. Detection was performed using alkaline phosphatase conjugated anti-mouse IgG and anti-human IgG (Sigma). MTT 0.12M (Methylthiazolyldiphenyl-tetrazolium bromide), BCIP 0.16M (5- Bromo-4-chloro-3-indolyl phosphate) and $MgCl_2$ 1M in citrate-buffered saline pH 7.0 was used for development.

2.3.5 Mouse model of colonization

All animal experiments were completed by Ankur B. Dalia at Tufts University in Boston, Massachusetts and were done in accordance with NIH guidelines, the Animal Welfare Act and US federal law. Tufts University School of Medicine's Institutional Animal Care and Use Committee approved the experimental protocol "B2011-57" that was used for this study. All animals were housed in a centralized and AAALAC-accredited research animal facility that is fully staffed with trained husbandry, technical and veterinary personnel.

Bacterial cultures were grown in THY broth to an OD_{600} of ~0.6 and then washed and concentrated in PBS. 10^7 bacteria in 10 µl were intranasally inoculated into both nares of anesthetized (isoflurane) 4-6 week old Swiss Webster mice. Five days post-inoculation, mice were euthanized, the trachea cannulated, and 500 µl of PBS instilled. Nasal wash was collected and plated for quantitative culture. The lower limit for detection in these assays was 10 CFUs.

Chapter 3

Dose-dependency and reproducibility of an experimental human pneumococcal carriage model

3.1 Introduction

Numerous studies have examined natural pneumococcal carriage and it is well known that rates vary by age and geographic area [5]. Limited data exist on the immune response to carriage because it is difficult to capture an entire carriage episode in nature. Previous EHPC studies have found an association between humoral responses and carriage [161,279] and shown that pneumococcal exposure without carriage immunizes mucosal surfaces [281].

In order to further examine both mucosal and systemic immunity in the upper and lower airway, we designed a human model of experimental pneumococcal carriage. Such a model could be used as an immunological probe of carriage, as a live mucosal vaccine model, and as a surrogate of protection for testing novel pneumococcal vaccines.

To develop a standardized, reproducible model of carriage, we aimed to construct a dose-response curve using pneumococcal serotypes 6B and 23F. We predicted that carriage rates of 50% or greater would allow the model to have high sensitivity for vaccine efficacy with small study numbers. To ensure the dose and the model were reproducible, a target carriage rate and dose were chosen based on the dose-response curve and tested in an independent group of volunteers. To investigate whether experimental carriage was

symptomatic, a control group of volunteers were challenged with 0.9% saline; volunteers from all groups were asked to report symptoms.

3.2 Materials and methods

3.2.1 Recruitment

Inclusion and exclusion recruitment criteria can be found in Chapter 2, section 2.2.1. The Patient Information Sheet is in Appendix B and the consent form is in Appendix C.

3.2.1.1 Dose-Ranging study

We planned to recruit 120 healthy adult volunteers; 60 for challenge with the 6B strain and 60 with the 23F. For each serotype, the volunteers were subdivided into six groups of 10 and each group was challenged with 0.1 ml of pneumococcus per nostril at an increasing dose.

The targeted challenge dose for the initial 10 volunteers was $1x10^4$ CFU/naris and this doubled for the following groups: $2x10^4$, $4x10^4$, $8x10^4$, $1.6x10^5$, and $3.2x10^5$.

3.2.1.2 Reproducibility study

We recruited 24 volunteers to determine the reproducibility of the model (Reproducibility study). The inoculation dose was chosen based on the results of the Dose-Ranging study with the target set as the dose at which 50% carriage was achieved.

3.2.2 Study schedules

Study schedules are described in Chapter 2, section 2.2.2.

3.2.3 Nasopharyngeal inoculation

Preparation of the inoculum stock and determination of dose can be found in Chapter 2, sections 2.3.2.1 and 2.3.2.4. The inoculation method is described in Chapter 2, section 2.3.2.6.

3.2.4 Detection of carriage rate and density of carriage

Nasal wash samples were collected and processed as described in Chapter 2, sections 2.3.2.7 and 2.3.2.8. Detection of pneumococcal carriage by culture and measurement of carriage density were performed as described in Chapter 2, sections 2.3.3.1 and 2.3.3.2, respectively.

3.2.5 Safety and symptoms

Details on volunteer safety are given in Chapter 2, section 2.2.3. Details on symptom reporting are given in Chapter 2, section 2.2.4.

3.2.6 Microarray

The microarray method and analysis was performed as described in Chapter 2, section 2.3.4.1.

3.2.7 Statistical analysis

The Fisher's exact test was used to analyse colonization rates, and both passive and active symptom complaints. Differences in 6B colonization density were analysed using a one-way ANOVA with Bonferroni's post-test. A Kaplan-Meier curve was generated for time to carriage clearance. Graph and statistical analysis was performed using GraphPad prism version 5.0 (California, USA). All *P* values were two-tailed and considered significant if $P \leq 0.05$.

3.3 Results

3.3.1 Dose-Ranging study

We recruited and challenged 120 volunteers. Sixty were challenged with a serotype 6B strain and 60 with a serotype 23F strain, according to protocol, with minimal adverse effects. The average age of the volunteers challenged with 6B (23.1 ±5.9 years) was similar to those challenged with 23F (21.4 ±3.9 years) as was the male:female ratio in each group (6B 28:32; 23F 27:33).

During the initial screening process, 11% (18/157) of volunteers were natural carriers; 10 during screening for 6B challenge, 7 during screening for 23F challenge, and 1 during screening for mock challenge.

3.3.1.1 Inoculation doses were within the targeted range

Inoculation doses complied with protocol if they fell within a halving or a doubling of the target amount; all inoculation doses were within the targeted range (Table 3.1). The average inoculation dose per group can be seen in Table 3.1. When the doses were compared across the two serotypes, only the $4x10^4$ (*P*<0.0001) dose and the $1.6x10^5$ (*P*=0.0002) dose were significantly different.

Table 3.1 Average inoculation dose per group for both serotype 6B and 23F

Dose Group (CFU/naris)	Serotype 6B Average Dose (± SD)		Serotype 23F Average Dose (± SD)	
$1x10^4$	10,650	± 704	10,127	± 196
$2x10^4$	23,433	± 3,637	21,184	± 1,671
$4x10^4$	53,042	± 3,266	37,167	± 8,886
$8x10^4$	89,833	± 10,603	86,567	± 3,298
$1.6x10^5$	133,266	± 3,492	150,000	± 6,286
$3.2x10^5$	312,500	± 13,106	309,166	± 31,193

3.3.1.2 Serotype 6B was more successful at establishing carriage

Serotypes 6B and 23F had significantly different rates of colonization, regardless of dose (P<0.0001, Fisher's exact test) (Figure 3.1). From the $4x10^4$ dose onwards, percent colonization for those challenged with 6B was greater than 50%. Colonization with 23F never rose above 10% and was not detected at the $1x10^4$, $1.6x10^5$, or $3.2x10^5$ doses.

For serotype 6B at least one volunteer was carriage positive at every dose and the carriage rate was dose-dependent (Figure 3.1). Of the 6B carriage positive volunteers, 70% (19/27) remained carriage positive for more than two weeks.

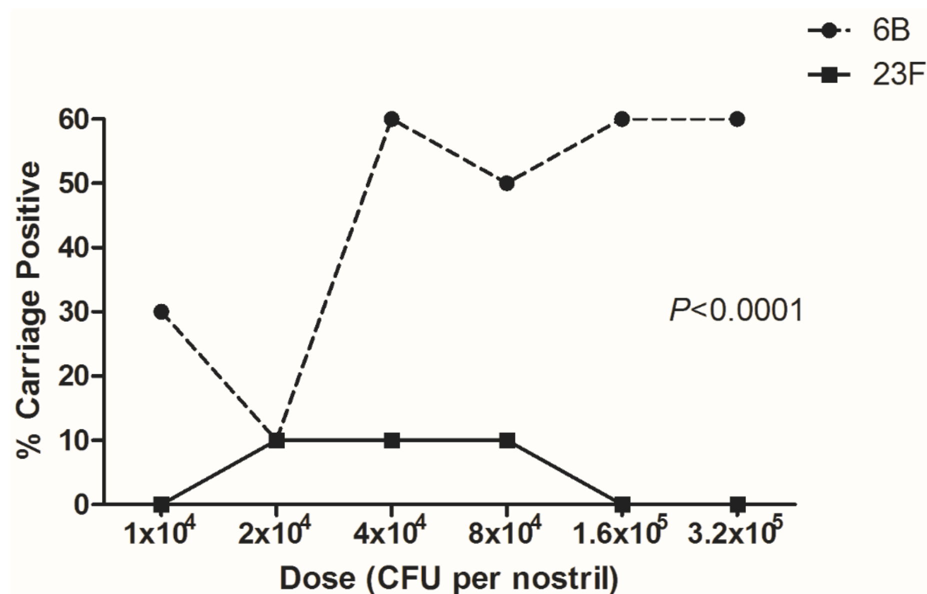

Figure 3.1: **Experimental Human Pneumococcal Carriage Dose-Ranging curve.** Groups of 10 volunteers were intranasally inoculated with either serotype 6B (circle) or 23F (square) over a range of six doses, starting with $1x10^4$ CFU per nostril and doubling up to $3.2x10^5$. Percent colonization represents the number of volunteers in the group positive for pneumococcal carriage at any time point following inoculation. A P value ≤0.05 was considered significant using a Fisher's exact test on overall carriage rates.

3.3.1.3 Carriage density was not a function of inoculation dose

Since an inoculation dose above $4x10^4$ did not significantly alter the carriage rate, we measured carriage density to determine if it would increase in parallel with the increasing inoculation dose. There was no significant difference in density of 6B colonization at any time point for doses over $4x10^4$ ($P>0.05$ 1-way ANOVA) (Figure 3.2). Carriage density remained stable over the two week sampling period. Density of 23F colonization could not be analysed due to the low rates of carriage. Similarly, the 6B $1x10^4$ and $2x10^4$ doses were removed from analysis because of the low colonization numbers.

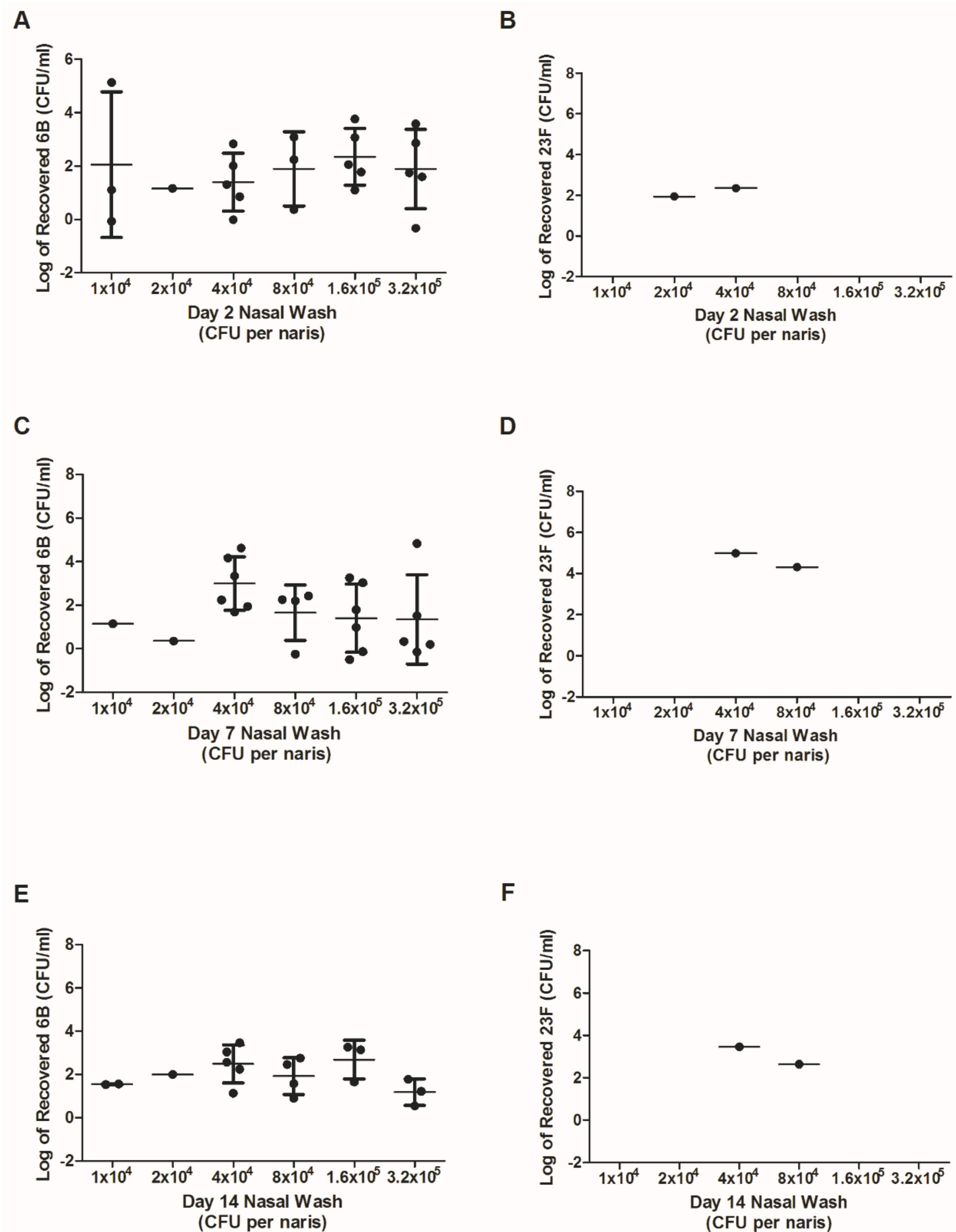

Figure 3.2: **Density of carriage is not a function of inoculated dose and shows stability over time.** Nasal washes were performed on day 2 (A, B), day 7 (C, D) and day 14 (E, F) post-inoculation to determine carriage status and density. Nasal wash was serially diluted and pneumococcal colonies were quantified on blood agar. Density is reported as CFU/ml of nasal wash returned. Data bars represent the mean ± SD. A *P* value ≤0.05 was considered significant using a one-way ANOVA test with Bonferroni's post-test to compare the 6B doses above $4x10^4$.

3.3.2 Reproducibility study

Due to the lack of carriage with serotype 23F in the Dose-Ranging study, we did not move forward with it in the model.

Since the carriage rates for serotype 6B at the $4x10^4$, $8x10^4$, $1.6x10^5$ and $3.2x10^5$ doses were not significantly different, we could have chosen any of these four to test the reproducibility of the model. The $8x10^4$ dose was chosen because it resulted in 50% carriage and meant that if the dose was halved or doubled, a similar carriage rate would still be achieved (Figure 3.1).

Twenty-four volunteers were challenged with serotype 6B. The average age of the volunteers was 22.3 ±2.9 years and the male:female ratio was 9:15. During the initial screening process, one natural carrier was detected and was followed in parallel with the challenged cohort.

3.3.2.1 The model was reproducible above a dose of $4x10^4$ CFU/naris

The average inoculation dose with serotype 6B in the Reproducibility study was 61,944 ±4603 CFU/naris. Following challenge, 42% (10/24) of volunteers were carriage positive by microbiological culture.

To determine if the model was reproducible, a confidence interval for the mean carriage rate of the model was determined. By allowing the assumption of no difference in carriage rate above the $4x10^4$ dose in the Dose-Ranging study, we combined the carriage rates from those four doses with the carriage rate from the Reproducibility study. The mean carriage rate of the model was 52% with a 95% confidence interval of 40-64%.

3.3.2.2 Carriage density was stable up to one month post-challenge

Carriage density remained stable over time (*P*=0.8, one-way ANOVA) at an average of 100 CFU/ml (Figure 3.3), similar to the Dose-Ranging study.

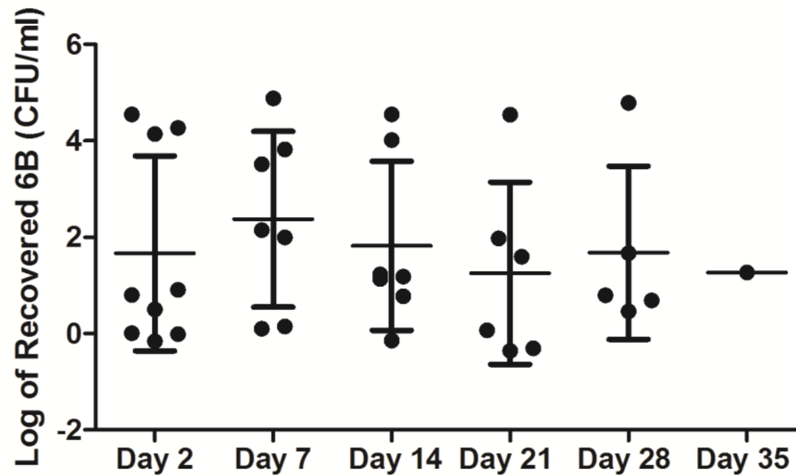

Figure 3.3: Density of carriage, when present, is stable up to one month post-challenge. Nasal washes were taken on day 2 post-inoculation and then weekly up to day 35 to determine carriage status and density. Nasal wash was serially diluted and pneumococcal colonies were quantified on blood agar. Density is reported as of CFU/ml of nasal wash returned. Data bars represent the mean ± SD. A *P* value ≤0.05 was considered significant using a one-way ANOVA test to compare density at days 2 to 28. All volunteers took antibiotics after day 28 except one.

3.3.2.3 Half of all carriers had cleared carriage one month after challenge

Carriage clearance was considered the first negative nasal wash after which all successive washes were negative. Median carriage duration by Kaplan-Meier analysis was 31.5 days (Figure 3.4). By day 14, 20% (2/10) of carriage positive volunteers had cleared carriage; by day 28, 50% had cleared carriage (5/10). All volunteers still positive at day 28 were required to take 500 mg of amoxicillin for 3 days to clear carriage however, one volunteer did not take antibiotics until after the day 35 nasal wash; this volunteer was the only carrier at day 35 (Figure 3.4).

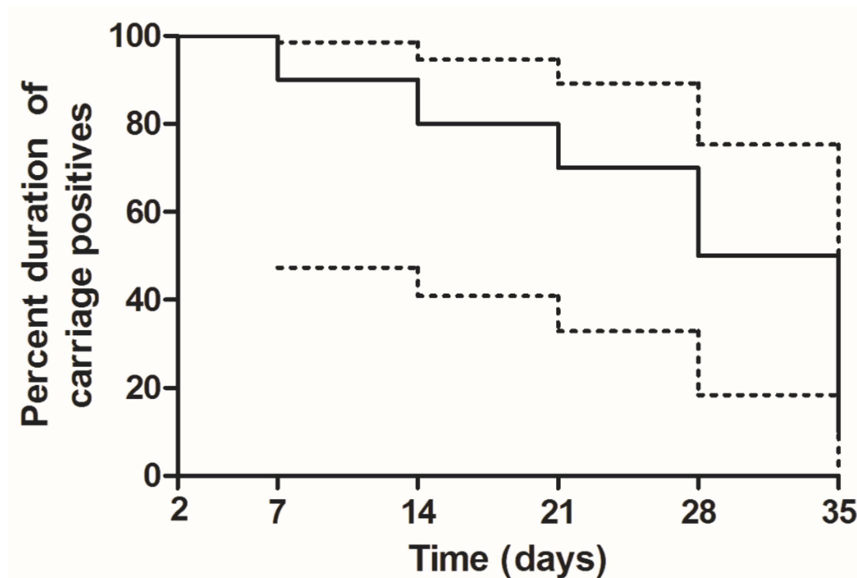

Figure 3.4: Kaplan-Meier survival curve for time to carriage clearance. Carriage clearance was considered the first negative nasal wash after which all successive washes were negative. One volunteer was still a carrier at the final appointment (day 35). All other volunteers took antibiotics to clear carriage after day 28. The 95% confidence interval is represented by the dotted lines.

3.3.2.4 Detection of potential co-colonization

One volunteer was carriage negative by culture prior to inoculation and was subsequently challenged with serotype 6B. At days 2, 7, 14, and 28, serotype 6 was not detected by latex agglutination but the volunteer was carriage positive for serogroup 9.

To determine if co-colonization was being missed by culture and latex agglutination, microarray was performed using the pre-screen and days 2, 7, and 14 nasal wash bacterial pellets. Serotype 6B was not detected at any time point; serotype 9N was detected at days 2, 7 and 14 but not at the pre-inoculation screen (Table 3.2).

Table 3.2 Determination of serotype in a natural carrier challenged with serotype 6B

Visit	Serotype detected by culture (latex agglutination)	Pneumococcal serotypes detected by microarray
Pre-inoculation	none	none
Day 2	9	9N
Day 7	9	9N
Day 14	9	9N

3.3.3 Experimental carriage was not symptomatic

3.3.3.1 Passive symptom detection

90 volunteers were asked by the clinical team at each appointment if they had experienced any URT symptoms in the preceding days. 22.2% (20/90) of volunteers reported symptoms. Of these 20 volunteers, 25% were carriers (5/20) (Table 3.3). All five symptomatic carriers had been challenged with 6B and symptoms were not related to a specific dose (one at $1x10^4$, two at $8x10^4$, two at $1.6x10^5$). None of the 23F carriers complained of symptoms. Because the number of carriers complaining of symptoms was too small for individual serotype analysis, we compared the presence of symptoms between carriers (5/24) and non-carriers (15/66), regardless of serotype, and found no significant difference (P=0.99).

Investigations were undertaken for any reported systemic symptoms, including fever. Of the three 6B carriage positive volunteers that reported systemic symptoms, none showed evidence of pneumococcal disease. One volunteer had symptoms consistent with concurrent viral infection. Another volunteer complained of gastric pain on deep

inspiration and, after meeting with the study doctor and undergoing multiple investigations, was advised to take antibiotics. The third volunteer was diagnosed clinically with tonsillitis. All volunteers had rapid resolution of symptoms.

Table 3.3 Passive symptom detection following challenge with the first five 6B doses and the first four 23F doses in the Dose-Ranging study

				All symptoms*		Systemic symptoms**		Local symptoms***	
Challenge serotype	Challenge dose (CFU/ 100 µl)	Age (±SD)	Gender (M:F)	Carriage positive	Carriage Negative	Carriage positive	Carriage Negative	Carriage positive	Carriage Negative
6B	$1x10^4$	26.2 (±5.8)	5:5	33% (1/3)	0% (0/7)	33% (1/3)	0% (0/7)	0% (0/3)	0% (0/7)
6B	$2x10^4$	22.6 (±3.3)	6:4	0% (0/1)	0% (0/9)	0% (0/1)	0% (0/9)	0% (0/1)	0% (0/9)
6B	$4x10^4$	21.8 (±1.5)	5:5	0% (0/6)	0% (0/4)	0% (0/6)	0% (0/4)	0% (0/6)	0% (0/4)
6B	$8x10^4$	25.4 (±11.8)	2:8	40% (2/5)	40% (2/5)	20% (1/5)	0% (0/5)	40% (2/5)	40% (2/5)
6B	$1.6x10^5$	20.1 (±1.9)	4:6	33% (2/6)	50% (2/4)	17% (1/6)	25% (1/4)	17% (1/6)	25% (1/4)
23F	$1x10^4$	19.9 (±1.7)	4:6	-	30% (3/10)	-	0% (0/10)	-	30% (3/10)
23F	$2x10^4$	20.0 (±1.7)	3:7	0% (0/1)	33% (3/9)	0% (0/1)	0% (0/9)	0% (0/1)	33% (3/9)
23F	$4x10^4$	20.5 (±1.6)	5:5	0% (0/1)	33% (3/9)	0% (0/1)	11% (1/9)	0% (0/1)	22% (2/9)
23F	$8x10^4$	21.6 (±1.8)	7:3	0% (0/1)	22% (2/9)	0% (0/1)	11% (1/9)	0% (0/1)	11% (1/9)

*All symptoms were statistically compared using Fisher's exact test
**Systemic symptoms included: fever, tonsillitis, headache, gastric pain, neck pain
***Local symptoms included symptoms related to the ears, nose, and/or throat

3.3.3.2 Active symptom detection

To further analyse if symptoms related to challenge or carriage, 10 volunteers were challenged with sterile saline and completed a symptom log on the day of challenge and for 7 days post-challenge. For comparison, volunteers from the Dose-Ranging 6B $3.2x10^5$ group and the Dose-Ranging 23F $1.6x10^5$ and $3.2x10^5$ groups, as well as volunteers from the Reproducibility study ($8x10^4$), filled out the daily symptom log.

The average age of the saline control group was 23.1 ± 9.1 years and the male:female ratio was 5:5. Fifty-four volunteers completed the daily symptom log; 8 challenged with saline, 17 with serotype 23F, and 29 with serotype 6B. Of the 17 volunteers challenged with serotype 23F, 9 were from the $1.6x10^5$ dose group and 8 from the $3.2x10^5$ dose group. Of the 29 volunteers challenged with serotype 6B, 10 were from the $3.2x10^5$ dose group and 19 were challenged with $8x10^4$ as part of the Reproducibility study. None of the volunteers challenged with 23F were carriage positive; 14 challenged with 6B were carriage positive.

3.3.3.2.1 Non-nasal symptoms

There were five categories related to non-nasal symptoms: eyes, throat, cough, ears, and headache. The score awarded at inoculation (day 0) was considered the volunteer's baseline score. At any point in the following seven days, if the score went above the baseline, the volunteer was considered symptomatic. Due to the small number of volunteers in each group, scores for the five categories were combined for an overall non-nasal symptom score.

Overall, 55.5% (30/54) of volunteers reported non-nasal symptoms. Of these, 11.1% (6/54) were challenged with saline, 16.7% (9/54) with 23F, and 27.8% (15/54) with 6B. To examine if challenge without carriage was symptomatic, we compared the saline challenge group with the two 23F challenge groups. 75% (6/8) of saline challenged volunteers were symptomatic, compared to 66.7% (6/9) of 23F $1.6x10^5$ (P=0.99) and 37.5% (3/8) of 23F $3.2x10^5$ (P=0.31) (Figure 3.5A). There was no significant difference between the saline challenge group and the pneumococcal challenge groups.

To determine if carriage was symptomatic, we compared the 6B carriers and non-carriers. 57% (8/14) of carriers were symptomatic; 83.3% (5/6) were challenged with $3.2x10^5$ and 37.5% (3/8) with $8x10^4$ (Figure 3.5B). 46.7% (7/15) of non-carriers were symptomatic. There was a significant difference in the report of non-nasal symptoms between carriers and non-carriers in the $3.2x10^5$ group (P=0.048) but not the $8x10^4$ group (P=0.37).

There were only four complaints that scored higher than 5 or “moderately bothersome”: one from a volunteer in the saline challenge group that complained of a cough; one from a carriage positive volunteer in the 6B 3.2x10^5 dose group that had a sore throat; and two from carriage negative volunteers: one in the 6B 8x10^4 dose group complained of ear symptoms and one in the 23F 1.6x10^5 dose group complained of a headache.

3.3.3.2.1 Nasal symptoms

There were five categories related to local (nasal) symptoms: sneezing, runny nose, congestion/stuffiness, itchy nose and post nasal drip. As with the non-nasal symptoms, the score awarded at inoculation (day 0) was considered the volunteer’s baseline score. At any point in the following seven days, if the score went above the baseline, the volunteer was considered symptomatic. Due to the small number of volunteers in each group, scores for the five categories were combined for an overall nasal symptom score.

In total, 61.1% (33/54) of volunteers complained of nasal symptoms. Of these, 11.1% (6/54) were challenged with saline, 16.7% (9/54) with 23F, and 33.3% (18/54) with 6B. To examine if challenge without carriage was symptomatic, we again compared the saline challenge group with the two 23F challenge groups. 75% (6/8) of saline challenged volunteers were symptomatic, compared to 44.4% (4/9) of 23F 1.6x10^5 (P=0.33) and 62.5% (5/8) of 23F 3.2x10^5 (P=0.99) (Figure 3.5A). There was no significant difference between the saline challenge group and the pneumococcal challenge groups in the presence of nasal symptoms following inoculation.

To determine if carriage resulted in nasal symptoms, we compared the 6B carriers and non-carriers. 78.6% (11/14) of 6B carriers were symptomatic with 83.3% (5/6) challenged with 3.2x10^5 and 75% (6/8) with 8x10^4 (Figure 3.5B). Just as in the non-nasal symptoms analysis, 46.7% (7/15) of non-carriers were symptomatic. There was no significant difference in the report of nasal symptoms between carriers and non-carriers in the 3.2x10^5 group (P=0.19) or the 8x10^4 group (P=0.63).

There were also no 6B carriage positive or saline challenged volunteers that scored any nasal symptoms higher than 4 or “mild” but for carriage negatives, there were a number of symptoms scored higher than 5. From the 6B 8x10^4 dose group, there was one complaint of congestion and one of an itchy nose. From the 23F 1.6x10^5 dose group there were eight nasal symptom complaints scored higher than 5: 1 sneezing, 2 runny nose, 2 congestion, 1 itchy nose, and 2 postnasal drip.

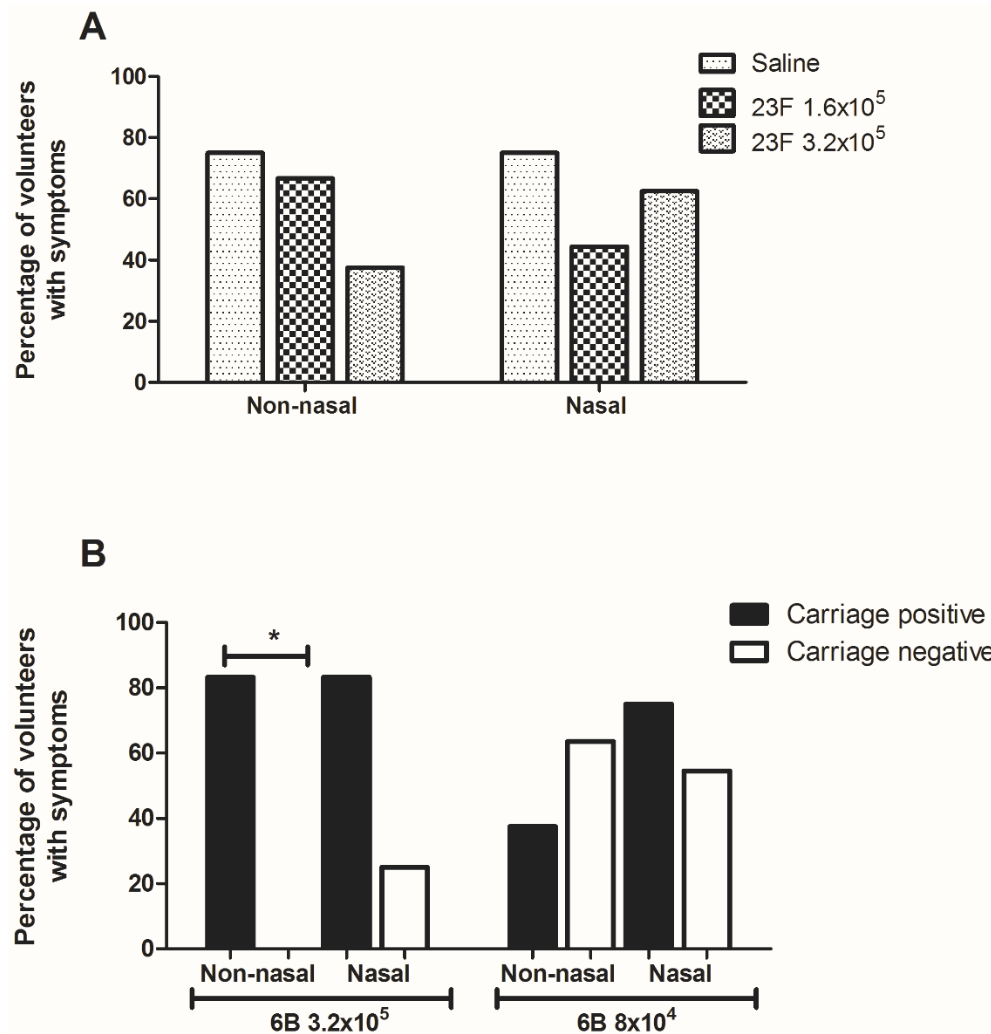

Figure 3.5: Percentage of volunteers with actively reported symptoms. Volunteers filled out a daily symptom log on the day of challenge and for 7 days post-challenge. The score at inoculation was considered the baseline score. At any point in the seven days, if the score went above baseline, the volunteer was considered symptomatic. (A) Volunteers challenged with saline were compared to carriage negative volunteers challenged with 23F. There was no significant difference between the saline challenge group and the pneumococcal challenge groups in the presence of non-nasal or nasal symptoms. (B) Symptom complaints were compared between the 6B carriage positives and carriage negatives. There was a significant difference in non-nasal symptoms between carriers and non-carriers in the $3.2x10^5$ group but not the $8x10^4$ group. There was no significant difference in nasal symptoms. A *P* value ≤0.05 was considered significant using a Fisher's exact test. *denotes significance.

3.4 Discussion

We determined an appropriate inoculation dose to achieve 50% carriage, tested the reproducibility of the model at the chosen dose, and compared the presence of symptoms following experimental challenge in carriers, non-carriers, and saline challenged volunteers.

3.4.1 Dose-dependent establishment of carriage

We have developed a model of experimental human pneumococcal carriage. For serotype 6B, carriage establishment was dose-dependent until $4x10^4$ CFU/naris, after which carriage rates stabilized around 50%. Carriage rates for serotype 23F remained at or below 10%, regardless of dose.

The first human carriage studies in the USA used a range of doses for challenge with 6B – $4.4x10^4$ to $7.8x10^4$ CFU – with a carriage rate of 75% (6/8) [161,279]. These doses and the carriage rate were similar to what we found: 60% (6/10) carriage at $4x10^4$ CFU and 50% carriage (5/10) at $8x10^4$ CFU; however the 6B strains were not the same. Three doses were used for challenge with 23F in the USA studies: $5x10^3$, $7x10^3$, or $1.7x10^4$ with carriage rates of 0% (0/4), 50% (3/6) and 75% (3/4), respectively. In comparison, the carriage rate in this study at a similar dose ($2x10^4$) was only 10% (1/10). This was surprising as the 23F strain used here was given to us by the group that performed the USA study and was therefore thought to be the same. The different carriage rates of 6B and 23F were unexpected and are investigated further in Chapter 5.

3.4.2 Experimental carriage was reproducible

We hypothesized that carriage rates would increase in a linear fashion along with dose. However, carriage rates actually plateaued after $4x10^4$ CFU/naris. Based on these results, we chose $8x10^4$ CFU/naris as the challenge dose for the Reproducibility study because it resulted in a 50% carriage rate and if the desired dose was halved or doubled, the carriage rate would remain around 50%.

At an average dose of 61,944 (±4603) CFU/naris, 42% (10/24) of volunteers became colonized. We demonstrated that experimental carriage was reproducible with a mean carriage rate of 52% and a 95% confidence interval of 40-64%. To calculate the mean carriage rate, we combined the overall carriage rate from the four doses above $4x10^4$ in the Dose-Ranging study (23/40) with the carriage rate from the Reproducibility study (10/24). We pooled the carriage rates of the four doses from the Dose-Ranging study because they

were not significantly different above $4x10^4$ and combined this with the Reproducibility study carriage rate because the dose was chosen based on the Dose-Ranging results.

3.4.2.1 Detecting potential pneumococcal co-colonization

A volunteer that was carriage negative at the pre-screen appointment was challenged with serotype 6B. At 2 days post-challenge serogroup 9 was detected but not serogroup 6. Microarray was used to determine if co-colonization with both serogroups was being missed by culture. Serotype 9N was not detected in the pre-screen sample but was detected at days 2, 7 and 14. Serotype 6B was not detected at any time point. It is not possible to determine if serotype 9N was acquired before challenge or between challenge and the day 2 nasal wash however, it is important to know that the culture results matched the microarray results.

If serotype 9N was present when the volunteer was challenged with serotype 6B, it is possible that the 9N outcompeted 6B. Competition between pneumococcal strains has been suggested to be behind the increase in non-vaccine serotypes following vaccination and competition experiments in a mouse model of carriage have shown that carriage of one strain can reduce colonization by a second strain [303]. Dawid et al. [212] have demonstrated that bacteriocins, specifically the *blpMN* operon, may be the mechanism behind intraspecies competition.

3.4.3 Experimental carriage was not symptomatic

Symptom reporting was split into two groups, passive and active. For passive symptom detection, volunteers reported any symptoms directly to the clinical team at each appointment. To address whether carriage was symptomatic, we combined all carriers and non-carriers, regardless of serotype, because the numbers were too low for individual serotype analysis. In this group, symptoms did not correlate with carriage.

For all symptoms combined, 6B $1x10^4$ was the only dosing group in which more carriage positive than carriage negative volunteers reported symptoms. Across all dosing groups and both serotypes, an equal number of carriage positive and carriage negative volunteers complained of systemic symptoms. For local symptoms, there was also an equal number of complaints from carriage positive and negative volunteers challenged with 6B. However, this was not the case following challenge with 23F; eight carriage negative volunteers complained of local symptoms as compared to zero carriage positive volunteers.

For active symptom detection, volunteers completed a daily symptom log. Using these scores, we first evaluated whether challenge without carriage was symptomatic. For both non-nasal and nasal symptoms, there was no difference in symptoms between volunteers challenged with saline and volunteers challenged with serotype 23F. Next, we examined if carriage was symptomatic. Carriage was not associated with increased nasal symptoms but was associated with increased non-nasal symptoms in the 6B $3.2x10^5$ group. No 23F carriers completed a daily symptom log so we could not compare nasal symptoms between carriers of the two serotypes however, the percentage of 23F non-carrier volunteers complaining of nasal symptoms (53%) was higher than the 6B challenged non-carriers (46%).

The percentage of volunteers that complained of symptoms following challenge with saline was similar to those challenged with pneumococci. The volume of saline inoculated is very small and unlikely to cause symptoms, therefore it is possible that challenge itself has a psychological effect whereby the idea of being colonized leads to symptoms.

The observation that 23F carriage negative volunteers complained more often of local or nasal symptoms in both passive and active detection, respectively, is not surprising given that Wright et al. [281] demonstrated that experimental challenge with serotype 23F was immunizing in the absence of carriage. Although the number of volunteers analysed was small, this suggests that nasal symptoms following challenge with serotype 23F may be related to carriage clearance, not carriage establishment. Challenge with a different serotype 23F strain as well as other serotypes, such as serotype 3 which has a very thick capsule, could inform on whether serotype plays a role in symptoms related to carriage clearance, rather than carriage establishment.

Chapter 4

The protective effect of a carriage episode on subsequent experimental carriage

4.1 Introduction

In the majority of the population, pneumococcal carriage generates an immune response that prevents disease and leads to carriage clearance. In infants and adults, carriage leads to an increase in both anticapsular and antiprotein antibody levels in serum [137,161,162], which are associated with a reduction in natural carriage.

Direct re-challenge to understand how a primary carriage episode affects reacquisition of the same or a different serotype is difficult to study in nature because sampling is intermittent, meaning carriage episodes can be misclassified or missed completely. In mice, prior colonization has been shown to result in cross-serotype protection against subsequent carriage and invasive disease [159].

We attempted direct re-challenge using the experimental human pneumococcal carriage model. To determine within-serotype protection from carriage, a subset of volunteers that had been successfully colonized with serotype 6B were re-challenged with the same serotype 6B strain. To examine cross-serotype protection from carriage, volunteers in whom we had detected natural pneumococcal carriage at an initial visit were challenged with serotype 6B once natural carriage had cleared.

4.2 Materials and methods

4.2.1 Recruitment

Inclusion and exclusion recruitment criteria can be found in Chapter 2, section 2.2.1. The Patient Information sheet is in Appendix B and the consent form is in Appendix C.

4.2.1.1 Homologous re-challenge

Up to 11 months following initial nasopharyngeal challenge, a subset of successfully colonized volunteers from pilot studies 6 or 7 (described in Chapter 2, section 2.1) and the Dose-Ranging study (Chapter 3) were recruited for re-challenge with the homologous 6B serotype. The target dose was $4x10^4$ CFU/naris.

4.2.1.2 Heterologous challenge

Up to 15 months following a natural carriage episode with a strain different from the 6B challenge strain, volunteers were re-recruited for heterologous challenge with serotype 6B at a dose of $4x10^4$ CFU/naris.

4.2.2 Study schedules

Study schedules can be found in Chapter 2, section 2.2.2 and section 2.2.2.3.

4.2.3 Nasopharyngeal inoculation

Preparation of the inoculum stock and determination of dose can be found in Chapter 2, sections 2.3.2.1 and 2.3.2.4. The inoculation method is described in Chapter 2, section 2.3.2.6.

4.2.4 Detection of carriage

Nasal wash samples were collected and processed as described in Chapter 2, sections 2.3.2.7 and 2.3.2.8, respectively. Detection of pneumococcal carriage by culture and measurement of carriage density were performed as described in Chapter 2, sections 2.3.3.1 and 2.3.3.2, respectively.

4.2.5 Statistical analysis

Protection against reacquisition of carriage was analysed using the Fisher’s exact test. A one-way ANOVA with Bonferroni's post-test was used to examine carriage density over time. The Mann Whitney test was used to compare the carriage density between two carriage episodes and compare two inoculation doses. Graph and statistical analysis was

performed using GraphPad prism version 5.0 (California, USA). All *P* values were two-tailed and considered significant if $P \leq 0.05$.

4.3 Results

4.3.1 Re-challenge with the homologous serotype 6B was protective against reacquisition of carriage

Ten volunteers that had previously been carriage positive for serotype 6B were re-challenged, four from previous pilot studies and six from the Dose-Ranging study. The average age of the volunteers was 23.1 ± 3.1 years and the male:female ratio was 4:6 (Table 4.1).

Table 4.1 Details of volunteers re-challenged with serotype 6B

Volunteer	Age (± SD)	Gender	Study	Dose (CFU/100 µl)	Interval* (weeks)
1	21	Female	Pilot 6	35,167	32
2	21	Male	Pilot 6	23,400	43
3	19	Male	Pilot 6	29,500	48
4	23	Female	Pilot 7	37,166	31
5	21	Female	Dose-Ranging	37,000	15
6	26	Female	Dose-Ranging	37,000	17
7	21	Female	Dose-Ranging	37,000	18
8	25	Male	Dose-Ranging	39,500	18
9	25	Female	Dose-Ranging	39,500	16
10	29	Male	Dose-Ranging	39,500	18
	23.1 ± 3.1	M:F 4:6		35,473 ± 5,164	

* Interval time (weeks) between initial carriage episode and re-challenge
Values are mean ± SD

Volunteers were re-challenged with the same 6B strain that was used for the initial challenge at an average inoculation dose of 35,473 (±5,164) CFU/naris. None of the challenged volunteers became colonized (0% carriage rate). A previous carriage episode significantly protected against reacquisition of carriage of the same strain (*P*=0.01, Fisher's exact test).

4.3.2 Challenge with a heterologous serotype was not protective against reacquisition of carriage

Eight volunteers in whom we had detected natural pneumococcal carriage returned between 38 and 67 weeks after the natural carriage episode to be challenged with serotype 6B. The average age of the volunteers was 26.8 ± 13.6 years and the male:female ratio was 4:4 and the average inoculation dose was 35,375 (±2,651) CFU/naris (Table 4.2).

50% (4/8) of the volunteers became colonized with 6B. Serotypes carried at the initial screening appointment included: 3 (x3), 6, 15, 19, and 33 (x2) (Table 4.2). Of the carriage negative volunteers, one had previously carried a serogroup 6 strain, while the other three had carried a serotype 3 (x2) or 33. In this cohort, a previous carriage episode was not protective against reacquisition of carriage of a heterologous strain (*P*=0.08, Fisher's exact test).

Table 4.2 Reacquisition of carriage following heterologous experimental challenge

Volunteer	Age (± SD)	Gender	Dose (± SD)	Interval* (weeks)	Serotype at initial screen	Experimental Carriage
1	21	Male	41,667	44	3	Yes
2	19	Female	34,000	61	15	Yes
3	22	Female	34,000	46	19	Yes
4	22	Male	34,000	67	33	Yes
5	20	Female	34,000	53	3	No
6	20	Male	35,667	53	3	No
7	59	Male	34,000	60	6	No
8	31	Female	35,667	38	33	No
	26.8 ± 13.6	M:F 4:4	35,375 ± 2,651			

* Interval time (weeks) between initial carriage episode and re-challenge.

4.3.3 Carriage density following experimental challenge was similar to density following a recent natural carriage episode

Although we do not know if we captured natural carriage at the beginning, middle, or end of the episode, we wanted to determine if natural carriage density was similar to experimental carriage density. We compared the natural carriage density from two volunteers at three time points with the experimental carriage density from the same volunteers at three time points post-challenge. There was no significant difference in density between the two carriage episodes (*P*=0.9, Mann Whitney test) (Figure 4.1).

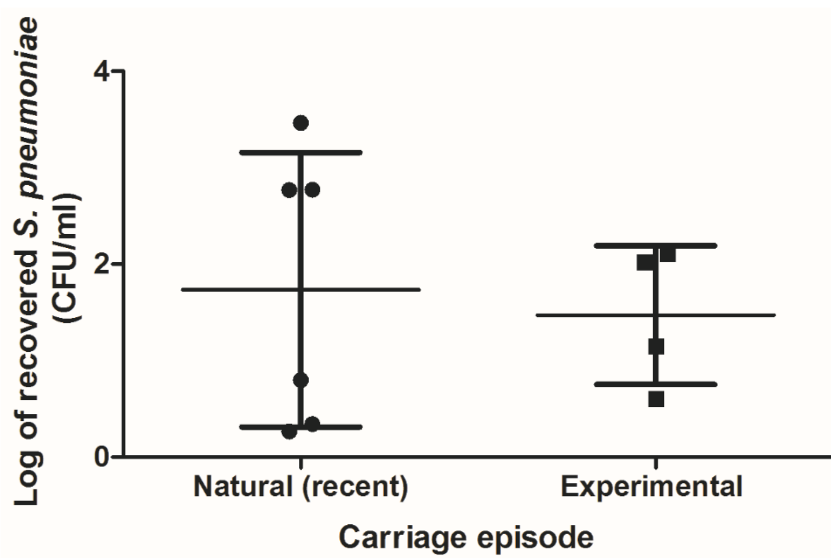

Figure 4.1: Carriage density is similar between natural carriage and experimental carriage. Carriage density from two volunteers was determined at three visits during natural carriage and compared to the density from the same volunteers at three visits after experimental challenge. Nasal washes were taken to determine carriage status and density. Nasal wash was serially diluted and pneumococcal colonies were quantified on blood agar. Density is reported as CFU/ml of nasal wash returned. Closed circles represent natural carriage density; closed squares represent experimental carriage density.

As there was no difference in overall density between two carriage episodes, we wanted to determine if a recent natural carriage episode would decrease carriage density during an experimental carriage episode. There was not enough density data from the recent natural carriage episodes to do this directly, so we compared carriage density over time from the Reproducibility study volunteers (Chapter 3, section 3.3.2.2) - considered the primary carriage episode - with the experimental carriage density from the recent natural carriers that were carriage positive following pneumococcal challenge- considered a secondary carriage episode (Figure 4.2). Although the inoculation dose between the two groups was different (61,944±4603 vs. 35,375±2651, $P<0.0001$, Mann Whitney test) there was no difference in density at any time point ($P=0.7$, one-way ANOVA).

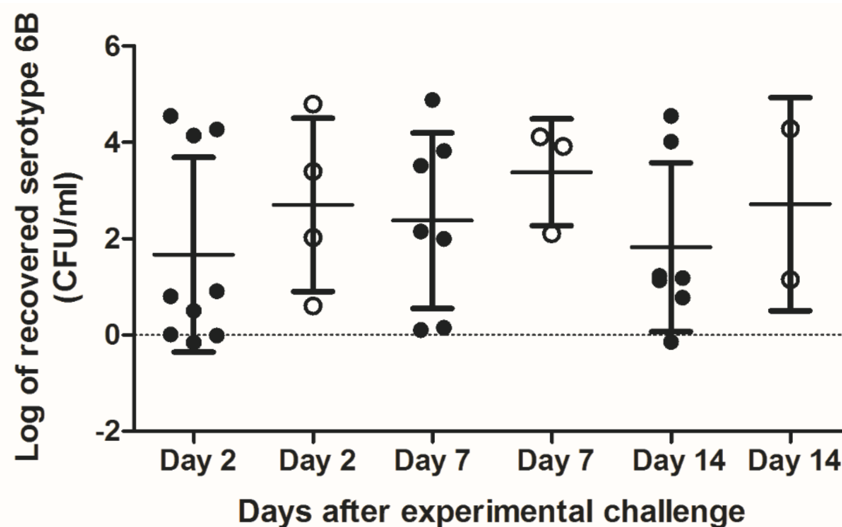

Figure 4.2: Carriage density is similar between an initial experimental carriage episode and an experimental carriage episode following recent natural carriage. Nasal washes were taken at days 2, 7 and 14 post-challenge to determine carriage status and density. Nasal wash was serially diluted and pneumococcal colonies were quantified on blood agar. Density is reported as CFU/ml of nasal wash returned. Closed circles represent carriage density from an initial experimental carriage episode; open circles represent carriage density during experimental carriage following a recent natural carriage episode. Data bars represent the mean ± SD. A P value ≤0.05 was considered significant using a one-way ANOVA with Bonferroni's post-test.

4.4 Discussion

We determined the protective effect of a carriage episode on subsequent carriage.

4.4.1 Carriage was protective against reacquisition of the same serotype

We showed that a single carriage episode is protective against subsequent carriage of the same serotype. None of the volunteers that were re-challenged with serotype 6B up to 48 weeks after an initial carriage episode with 6B were re-colonized (0% carriage rate). In comparison, the carriage rate at the same dose in the Dose-Ranging study was 60%.

Protection against carriage reacquisition has been shown in Bangladeshi infants that were followed for 1 year after birth. In these infants, prior carriage was associated with serotype-independent protection against reacquisition of serotypes 6A, 6B, 15B and C, and 19F [307]. In a cohort of Israeli children aged 1-5 years, prior colonization with serotypes 6A, 14, and 23F was associated with a lower risk of reacquisition of the homologous type [308]. In these children, protection against serotypes 14 and 23F was correlated with increased serotype-specific antibodies. The difference in protective mechanisms between these two populations is likely related to the age of the children; the immune system of the Bangladeshi infants has not matured enough to generate serotype-specific antibodies.

4.4.2 Carriage was not protective against acquisition of a different serotype

Only 50% of volunteers that had cleared natural carriage and were subsequently challenged with 6B became colonized. Two of the volunteers that carried serotypes 3 and 33, respectively, in the recent natural carriage episode were protected against carriage following challenge. However, another two volunteers also initially carrying these 2 serotypes became colonized with 6B following challenge.

Cross-protective immunity has been shown within serogroup but not across serotype. Vaccination with PCV13, which contains serotypes 6A and 6B, induced functional antibody responses to 6C and showed cross-protection between serotypes 7A and 7F [310]. Intranasal immunization of mice with a live attenuated pneumococcal strain containing a capsular polysaccharide mutation protected against subsequent challenge with a different strain [158]. Although capsular serotypes do not elicit cross-protection, pneumococcal

proteins such as PspA do. Serum from humans immunized with recombinant PspA has been shown to protect mice against infection with three different serotypes [311].

4.4.3 Previous carriage was not associated with reduced density of subsequent carriage

Not only has previous carriage been shown to impact reacquisition, it has also been shown to alter carriage duration. A longitudinal carriage study of infants in rural Northwestern Thailand found that colonization with serotype 14 or 19F was associated with reduced duration of subsequent carriage episodes with the same serotype [312]. We have not yet evaluated the impact of a primary carriage episode on the duration of subsequent carriage in healthy adults; however we have looked at its impact on carriage density. There was no difference in carriage density between a recent natural carriage episode and an experimental carriage episode.

We showed in Chapter 3 (section 3.3.1.3) that carriage density was not a function of inoculation dose so to examine density over time we compared carriage density from the Reproducibility study volunteers with carriage density from the volunteers that had cleared natural carriage and were experimentally colonized. Again, there was no difference in carriage density. One of the issues in comparing natural carriage density to experimental carriage density is that we don’t know where in the carriage episode the natural carrier was when the sample was taken: carriage could have been recently acquired or it could already have been established for a few weeks.

Chapter 5

Bacteriological and genetic factors influence experimental human pneumococcal carriage

5.1 Introduction

Rates of *S. pneumoniae* carriage and disease determined in epidemiological studies depend on age, geographic area, socio-economic factors, and the host's innate and adaptive immune response [19,139,156,313,314]. But carriage is not solely dependent on the host and few human studies have examined the bacteriological determinants of carriage acquisition.

It has previously been demonstrated that pneumococcal serotype plays a large role in determining carriage as some serotypes are frequently carried and have low invasive potential whilst others are more commonly found in disease [35,36,315]. It is also known that the capsular phenotype switches between transparent and opaque according to location. The transparent phenotype expresses more cell wall teichoic acid, aiding adherence and stable colonization in the nasopharynx; the opaque phenotype, more common in invasive disease, produces higher quantities of capsular polysaccharide, resulting in a decrease in opsonophagocytic killing [45,47,316,317]. Attachment to the epithelium and acquisition of nutrients are also crucial to colonization and survival in the nasopharynx [61,318].

The differing propensity of pneumococcal strains to establish carriage is of importance in transmission and disease. As discussed in Chapter 3, the different carriage rates following experimental challenge with 6B or 23F were unexpected. To investigate why the 23F strain

was less able to colonize than the 6B strain in this model, we assessed bacteriological factors thought to associate with carriage acquisition and compared them across the two strains.

5.2 Materials and methods

5.2.1 Recruitment and ethics

Inclusion and exclusion recruitment criteria can be found in Chapter 2, section 2.2.1. The Patient Information Sheet is in Appendix B and the consent form is in Appendix C.

Ethical information for the animal experiments can be found in Chapter 2, section 2.3.5.

5.2.2 Bacterial strains and growth conditions

Details on the 6B (BHN418) and 23F (P833) strains used for inoculation and the 23F P1123 strain can be found in Chapter 2, section 2.3.1.

5.2.3 Inoculation and sampling

Preparation of the inoculum stock and determination of dose can be found in Chapter 2, sections 2.3.2.1 and 2.3.2.4. The inoculation method is described in Chapter 2, section 2.3.2.6.

Nasal wash samples were collected and processed as described in Chapter 2, sections 2.3.2.7 and 2.3.2.8.

5.2.4 Determination of phase morphology

The method used to determine phase morphology was performed as described in Chapter 2, section 2.3.4.2.

5.2.5 Complement deposition assay

The complement deposition assay was performed as described in Chapter 2, section 2.3.4.3.

5.2.6 Measurement of anti-pneumococcal polysaccharide antibodies in intravenous immunoglobulin by ELISA

The ELISA used to measure the level of polysaccharide antibodies to serotypes 6B and 23F in IVIG was performed as described in Chapter 2, section 2.3.4.4.

5.2.7 Opsonophagocytic killing assay

Isolation of neutrophils from peripheral blood was performed as described in Chapter 2, section 2.3.4.5.1.

The neutrophil opsonophagocytic killing assay was performed as described in Chapter 2, section 2.3.4.5.2.

5.2.8 Sequencing

5.2.8.1 Genetic comparison of the 6B and 23F strains

The genetic comparison of the 6B and 23F strains was performed as described in Chapter 2, section 2.3.4.6.1.

5.2.8.2 Genetic comparison of 23F strains P833 and P1123

The genetic comparison of the two 23F strains (P833 and P1123) was performed as described in Chapter 2, section 2.3.4.6.2.

5.2.9 Determination of a mutation in *amiC*

5.2.9.1 Confirmation of *amiC* mutation by sequencing

The sequencing method used to confirm the presence/absence of a frameshift mutation in the *amiC* gene was performed as described in Chapter 2, section 2.3.4.7.1.

5.2.9.2 Phenotypic confirmation of a mutation in the *ami* locus

Phenotypic confirmation of the *amiC* mutation was performed as described in Chapter 2, section 2.3.4.7.2.

5.2.10 Pneumococcal adherence assay

The pneumococcal adherence assay was performed as described in Chapter 2, section 2.3.4.8.

5.2.11 Mouse model of colonization

The mouse model of colonization was performed as described in Chapter 2, section 2.3.5.

5.2.12 Statistical analysis

Data was compared using the Fisher's exact test, unpaired Student's *t* test, Mann Whitney test, and the one-way ANOVA. Flow cytometric data was analysed using FlowJo software version 7.6.5 (Treestar Inc). Graph and statistical analysis was performed using GraphPad prism version 5.0 (California, USA). All *P* values are two-tailed and considered significant if $P \leq 0.05$.

5.3 Results

5.3.1 Transparent colonies were the dominant phenotype in the 6B inoculum stock

To determine if phase variation could partially explain why the 6B strain was better at colonization, both inoculum stocks were examined under magnification. Transparent colonies were the dominant phenotype in the 6B inoculum stock but the population was a mix of transparent and opaque (Figure 5.1). The opacity of the 23F inoculum could not be determined even though the appearance of the colonies was more homogenous than the 6B.

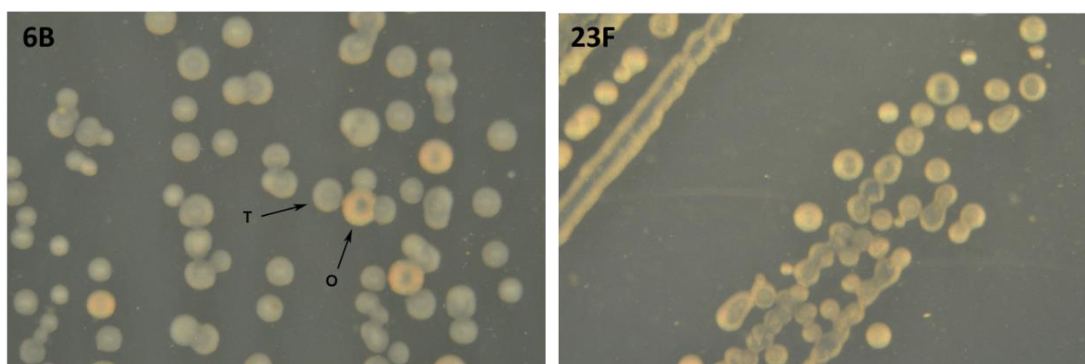

Figure 5.1: Transparent colonies are the dominant phenotype in the 6B inoculum stock. 6B and 23F inoculum stock colony morphologies were determined under oblique, transmitted illumination. Arrows denote transparent (T) and opaque (O) colonies. Magnification, 10x.

Eight 6B and three 23F isolates recovered at 48 hours post-challenge from eleven individual volunteers were examined to see if carriage altered phenotype. Four out of eight 6B isolates were uniformly transparent by 48 hours. Two isolates were a mixed population with a higher number of transparent colonies as compared to opaque – similar to the inoculum - and two isolates were a mixed 50/50 population of transparent and opaque. Similarly to the inoculum, the 23F isolates could not be characterized due to a high degree of autolysis. However, all three isolates appeared uniform and were comparable to the inoculum.

5.3.2 The 23F inoculum strain was more susceptible to complement deposition as compared to the 6B strain

In a flow based assay of C3b binding, we found that C3 deposition varied significantly between the two strains with markedly less C3 deposited on 6B (MFI: 74.35±0.5 SD) as compared to 23F (152.2±3.1) (*P*<0.001) (Figure 5.2).

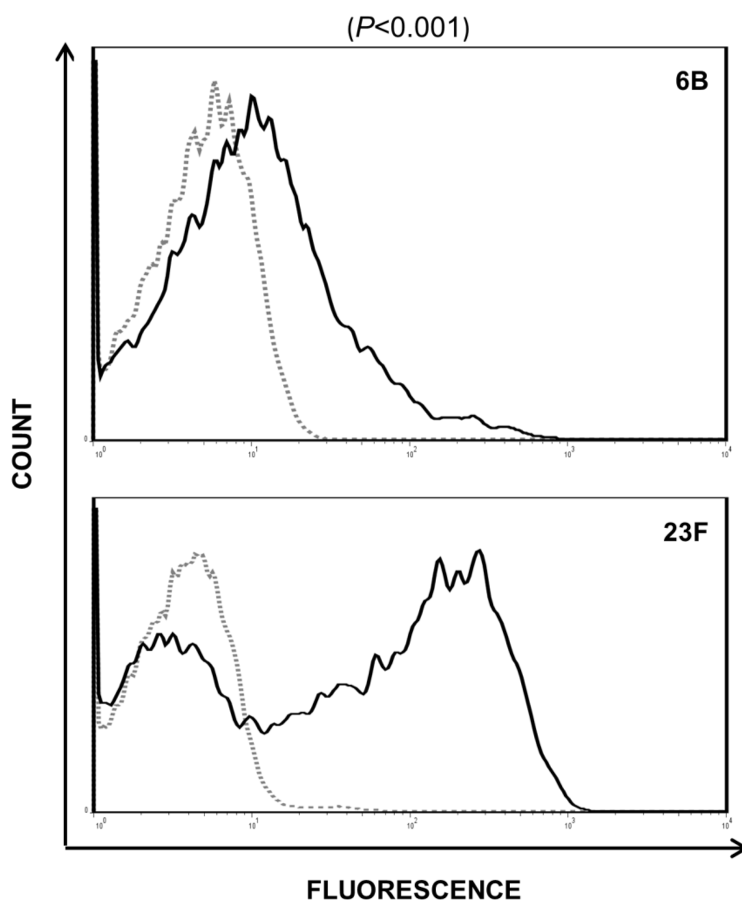

Figure 5.2: **The 23F inoculum strain of pneumococci is more susceptible to complement deposition than the 6B strain using a flow based assay of C3b binding.** Pneumococci were grown to an OD_{600}nm of 0.4. Human serum (20%), mouse-anti-human C3, and anti-mouse FITC were added at separate steps, with a control included for each. C3 deposition and Mean Fluorescence Intensity (MFI) were measured by flow cytometry. Grey broken lines represent control samples incubated with PBS. Solid black lines represent samples incubated with 20% human serum. MFI (±SD) was calculated as the percentage of the bacterial population positive for C3 deposition multiplied by the geometric mean fluorescence of the total cell population. A *P* value ≤0.05 was considered significant using an unpaired Student’s *t* test.

5.3.3 Opsonophagocytic killing did not differ between the 6B and 23F strains

Prior to comparing the opsonophagocytosis of the 6B and 23F strains, we determined the antibody concentration in IVIG to the 6B and 23F polysaccharides. There was no significant difference in the amount of anti-polysaccharide IgG to either serotype in the IVIG (*P*=0.06) (Figure 5.3).

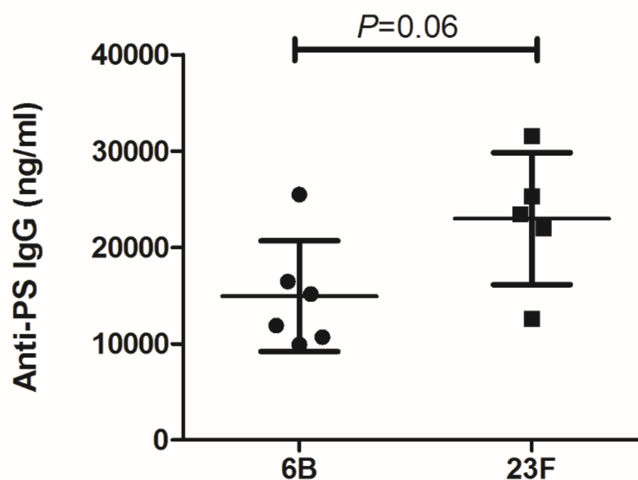

Figure 5.3: The amount of anti-polysaccharide IgG in IVIG is similar for both the 6B and 23F strains. An ELISA was performed using 6B or 23F pneumococci to measure specific IgG in IVIG. Values are triplicates from two different experiments. A *P* value ≤0.05 was considered significant using an unpaired Student's *t* test.

Using IVIG as the source of antibody in the opsonophagocytosis assay, there was no difference in the killing of 23F and 6B (21.5% vs. 37.9%) (*P*=0.7) (Figure 5.4).

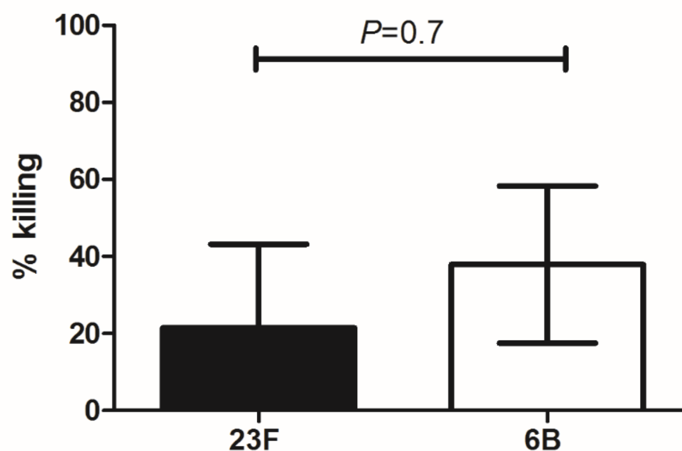

Figure 5.4: The percentage of opsonophagocytic killing does not differ between the 6B and 23F strains. Pneumococci were opsonized with intravenous immunoglobulin (IVIG) and added to freshly derived peripheral blood neutrophils and baby rabbit complement. Following incubation, pneumococci were quantified on blood agar plates. Percent killing was determined by taking the average CFU recovered from the control wells minus the average CFU recovered from the experimental wells, divided by the average CFU from the control wells. Killing is the average from three experiments using the blood of three different volunteers. A *P* value ≤0.05 was considered significant using a Mann Whitney test.

5.3.4 Two genetic mutations in the 23F strain are relevant for adherence

We compared the whole genome sequences of 6B and 23F to look for differences in known adherence factors. The *pcpA* gene was unique to the 6B strain when the genomes of the two serotypes were compared.

5.3.4.1 The 23F inoculum also contained a frameshift mutation in *amiC*

The 23F strain used for inoculation was chosen because it successfully established carriage in a human experimental model in the USA [161]. However, it was recently discovered that a proportion of the inoculum in the USA study contained a frameshift mutation in the *amiC* gene (personal communication, Ankur B. Dalia and Prof. JN Weiser). We analysed the 23F inoculum used in this study, as well as the 6B inoculum, for the presence of a frameshift mutation in *amiC*. The 23F inoculum contained the *amiC* mutation while the 6B inoculum contained a wild-type *amiC* (Figure 5.5A).

Functional proof of the *amiC* frameshift mutation in the 23F inoculum strain was obtained by testing for resistance to aminopterin. Mutations in the *ami* locus confer resistance to aminopterin [298]. Serotype 23F strain P1123, isolated from the nasopharynx of a volunteer in the USA study following challenge with a 23F P833 strain [161], was used as a comparator because it is the same serotype and it contains a wild-type (wt) *amiC*. In the presence of aminopterin, the *amiC* mutant P833 strain showed no inhibition of growth, confirming the presence of the *amiC* mutation (Figure 5.5B).

A

6B CAA TAT CCA TCT ATG ATT GTC AGC TCT GCT ATT ACT GGT TTG ATT GGT TTG GTT

Q Y P S M I V S S A I T G L I G L V

23F CAA TAT CCA TCT ATG ATT GTC AGC TCT GC[ ]TAT TAC TGG TTT GAT TGG TTT GGT T

Q Y P S M I V S S C Y Y W F D W F G

B

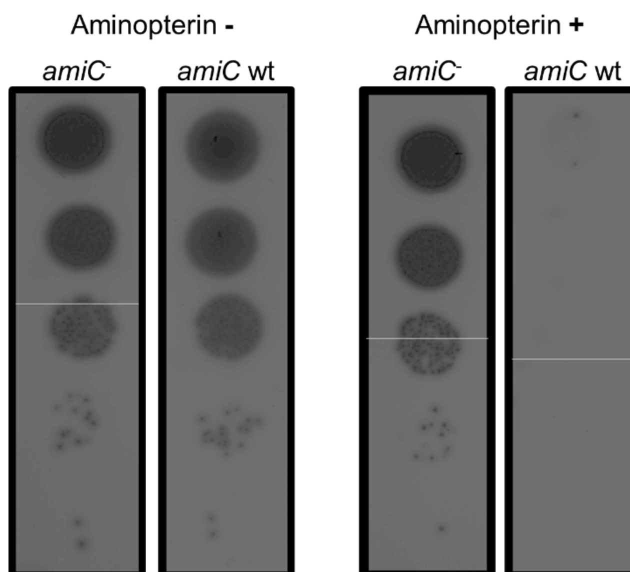

Figure 5.5: Genes unique to the 6B strain include those important for colonization. (A) Sequences of *amiC* wild-type 6B and *amiC* - 23F P833. A single base pair insertion resulting in a frameshift mutation in the 23F P833 strain is highlighted by a square box. The corresponding change in amino acids is shown below. (B) Aminopterin sensitivity of the 23F inoculum strain P833 (*amiC* -) and the derivative wild-type (wt) strain P1123 (*amiC* wt). Ten-fold dilutions of the two 23F strains were plated on blood agar containing $2x10^{-6}$M aminopterin.

5.3.4.2 There were six genetic differences between the serotype 23F inoculum and a derivative strain

We compared the sequences of the *amiC* mutant 23F inoculum (P833) and the *amiC* wild-type derivative 23F strain (P1123) to determine if there were any other genetic differences that may impact adherence. There were five other genetic differences between the *amiC* mutant inoculum P833 strain and the *amiC* wild-type derivative P1123 strain (Table 5.1). None of the genes are known to play a role in pneumococcal adherence.

Table 5.1 Five genetic lesions between P833 and P1123 resulting in an amino acid change

TIGR4 annotation	P833 > P1123 codon change	AA change*	AA in TIGR4	Annotated Gene Function	Description
SP_1514	TTG > TTT	L5F	F	F0F1 ATP Synthase subunit C	Energy metabolism [319]
SP_0416	AAG > GAG	K21E	E	MarR Family transcriptional regulator	Response to antibiotic and oxidative stress
SP_1550	CAT > CGT	H248R	R	Glutathione S transferase	Detoxification
SP_1179	AAT > AAG	N267K	K	Ribonucleotide diphosphate reductase, alpha subunit	Nucleic acid metabolism [320]
SP_1243	ATC > AGC	I157S	S	Glucose-6-phosphate-1 dehydrogenase	Pentose metabolism [321]

* Displayed as (AA in P833) (AA position within protein) (AA in P1123).

5.3.5 The 23F strain had decreased adherence to Detroit 562 epithelial cells as compared to the 6B strain

We used an *in vitro* epithelial cell model to compare the adherence of serotypes 6B and 23F and found that the 6B strain was more likely to adhere to nasopharyngeal cells than the 23F strain (P=0.0013) (Figure 5.6A).

As both *amiC* and *pcpA* have been shown to play roles in pneumococcal adherence, we also compared the 23F inoculum strain containing the *amiC* mutation (P833) with a derivative 23F strain containing wild-type *amiC* (P1123). The 23F strain with the wild-type *amiC* was more likely to adhere to nasopharyngeal cells than the 23F inoculum with the *amiC* mutation (P=0.0024) (Figure 5.6B).

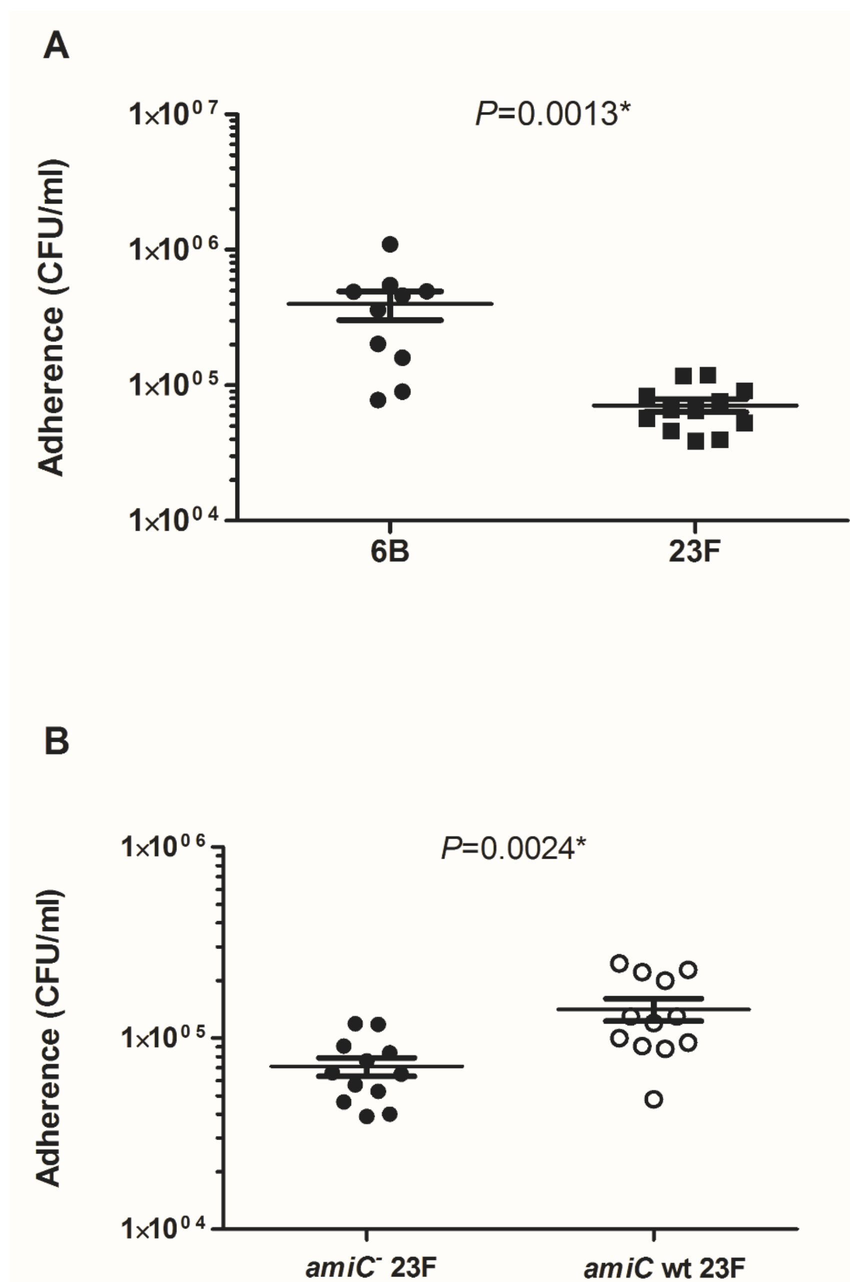

Figure 5.6: **A 23F strain of pneumococci is less adherent to Detroit 562 epithelial cells *in vitro* than a 6B strain and a derivative 23F strain**. 1x10^6 CFU of 6B or 23F pneumococci were added to confluent Detroit 562 nasopharyngeal epithelial cells and incubated. Following incubation, cells were lysed with saponin and adherent bacteria were quantified by serial dilution on blood agar. (A) Comparison of serotype 6B (closed circles) and serotype 23F (closed squares). (B) Comparison of serotype 23F (P833) containing the *amiC* mutation (closed circles) and serotype 23F (P1123) containing wild-type *amiC* (open circles). Data bars represent the mean ± SD. (*) denotes statistical significance using an unpaired Student's *t* test where $P \leq 0.05$ was considered significant.

5.3.6 An *amiC* mutant did not establish sustained carriage in a mouse model of colonization

A mouse model of colonization was used to determine if the frameshift mutation in *amiC* affected nasopharyngeal colonization. We compared the ability of the *amiC* wild-type strain (P1123) and the *amiC* mutant strain (P833) to colonize mice, keeping in mind that, although P1123 is a derivative of P833, they are not isogenic (Table 5.1).

At 5 days post-inoculation, the *amiC* wild-type strain (P1123) had successfully established carriage, while the *amiC* mutant strain (P833) had not, suggesting that *amiC* is critical for colonization of the URT in this mouse model (Figure 5.7).

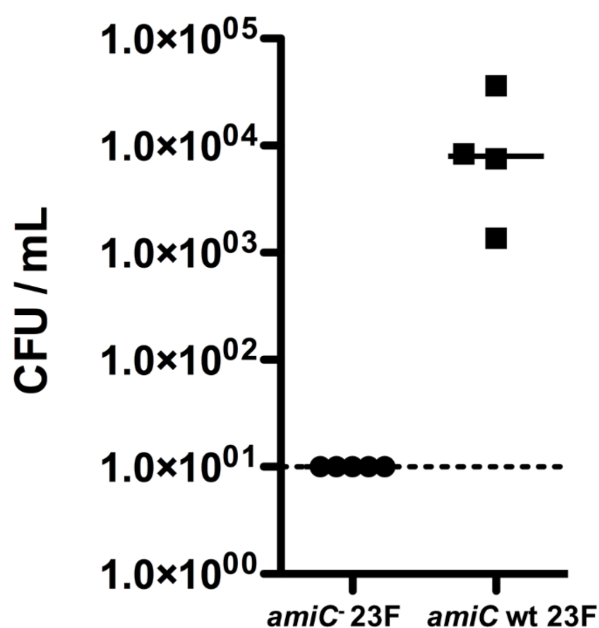

Figure 5.7: *AmiC* is critical for colonization in a mouse model of colonization. Mice were intranasally inoculated with 10^7 CFUs of the 23F *amiC* mutant strain (P833) or the *amiC* wild-type strain (P1123). Colonization burden was determined 5 days post-inoculation. The horizontal line represents the median. The dotted line represents 10 CFUs, the limit of detection.

5.4 Discussion

We determined bacteriological differences between the 6B and 23F inoculation strains that may have contributed to the different experimental carriage rates seen in Chapter 3.

5.4.1 Serotype 23F was more susceptible to complement deposition but this did not translate to increased killing

The 23F inoculum strain examined in this thesis was more susceptible to complement deposition than the 6B strain. The difference in susceptibility to complement deposition between serotypes has previously been shown, as has an association between serotype prevalence in carriage and resistance to nonopsonic neutrophil-mediated killing [37,106]. Melin et al. [106] have shown a significant correlation between antibody concentrations to PspA, PspC, and pneumococcal histidine triad D (PhtD) in normal human sera and C3 deposition on pneumococci. As we did not measure antibody levels in the human serum it is possible that these could be the reasons for the difference in deposition.

Opsonophagocytosis can also vary between serotypes and has been linked with serotype-specific mortality [43,106,108]. However, we did not see a difference in killing between the 6B strain and the 23F strain, even though there was more complement deposited on the surface of 23F. Similar levels of anti-polysaccharide specific antibodies in IVIG suggest the difference in killing is not related to IgG. Hyams et al. [105] showed that capsule can prevent phagocytosis, mediated, not only by complement receptors and IgG receptors, but also by nonopsonic receptors. Together with the suggestion by Melin et al. [106] that the capsule type might be more important than the genetic background in determining resistance to opsonophagocytosis, it is likely that the differences in opsonophagocytosis between 6B and 23F are the result of a number of factors.

5.4.2 Two genes shown to have roles in adherence were attenuated in serotype 23F

When we compared the whole genome sequences of the 6B and 23F strains we found a mutation in the *pcpA* gene of 23F. PcpA is a choline-binding protein that has been shown to facilitate pneumococcal adherence to nasopharyngeal and lung epithelial cells [59]. A recent study found 11% of carriage isolates and 3% of invasive disease isolates were missing the *pcpA* gene [280]. Upon examination of a pneumococcal genome database (Malawi-Liverpool-Wellcome Trust Clinical Research Programme), only 39% (55/140) of

isolates contained the *pcpA* gene (personal communication, Jen Cornick). Taken together, it is likely that *pcpA* is not crucial for survival in the nasopharynx.

Serotype 23F also had a frameshift mutation in *amiC*. AmiC is a pneumococcal transmembrane protein that has been shown to play a role in both adherence and oligopeptide transport [62,323]. Recently, a high throughput transposon sequencing approach used to characterize the genes important for colonization in the mouse found that the *amiC* mutant was highly attenuated in the TIGR4 pneumococcal strain [324]. We also searched the aforementioned pneumococcal genome database for *amiC* and found all 140 isolates contained a full length *amiC* gene.

5.4.2.1 Serotype 23F had decreased adherence *in vitro* and decreased carriage in a mouse model

The 23F inoculum strain with the *amiC* mutation was less likely to adhere to nasopharyngeal cells than both the 6B strain and the derivative 23F strain. In a mouse model of colonization, the 23F inoculum with the *amiC* mutation did not carry whereas the derivative isolate with a full length *amiC* did.

Differences in adhesion have been shown in relation to the site of isolation, with strains derived from otitis media and healthy nasal carriage having better adhesive capacity to human pharyngeal cells as compared to strains from septicaemia or meningitis patients [318]. Similarly, strains of the same capsule type adhered better when isolated from the nasopharynx of otitis media patients as compared to those isolated from the blood or cerebrospinal fluid. These differences in adherence may largely be due to phase variation and the greater ability of the transparent phenotype to adhere to epithelial cells, rather than the genetic background of each capsule [47]. However, in this case, it is possible that the decreased adherence of 23F was a result of mutation, in either *pcpA* or *amiC*, rather than phenotype, especially since the phenotype of 23F was indeterminable.

The much higher 23F carriage rate in the USA study [161], as compared to the rate reported here, was likely due to a mixed inoculum containing both *amiC* wild-type and mutant cells. All strains recovered from colonized volunteers in the USA study were wild-type for *amiC*, suggesting that the wild-type *amiC* strongly outcompeted the *amiC* mutant (Dalia and Weiser, personal communication). Conversely, in this study the inoculum and isolates from all colonized volunteers had no detectable level of *amiC* wild-type. Despite this, we found colonization with the *amiC* mutant strain in 5% of volunteers.

Chapter 6

Density and duration of experimental human pneumococcal carriage

6.1 Introduction

Understanding carriage is important as it is implicated in both person-to-person transmission and spread within the body, potentially leading to meningitis, bacteremia, or pneumonia. To what extent density and duration of pneumococcal carriage affect the risk of transmission and invasive disease is under increasing scrutiny [266,328].

Detection of live pneumococci by culture is the current gold standard [242]. Culture and molecular methods can be combined to increase carriage detection, including detection of multiple serotypes [239]. This can be achieved by including an enrichment step prior to culture and molecular analysis or by combining data from both culture and molecular methods, which would allow density to be determined for each method separately [249].

Understanding carriage is also important in the context of vaccination. Pneumococcal conjugate vaccines have resulted in a decrease in invasive disease and carriage of vaccine-serotype pneumococci [181,329]. While carriage of vaccine-type pneumococci has declined, carriage of non-vaccine-type pneumococci has increased [330,331]. The long-term impact of vaccination can be measured using carriage data, which can estimate post-vaccine changes in invasive disease incidence [332]. Colonization has been suggested as an endpoint because it is relatively easy to measure and is more common than disease,

therefore requiring a much smaller sample size in a vaccine trial [282,284,333]. To identify small changes in density or duration of colonization, sensitive methods to accurately detect these changes will be important in estimating vaccine effects.

We longitudinally followed pneumococcal carriage episodes and compared detection of carriage by culture and qPCR. We focused on density and duration in experimental carriers but also compared both parameters in natural carriers that were followed in parallel.

6.2 Materials and methods

6.2.1 Recruitment and ethics

Inclusion and exclusion recruitment criteria can be found in Chapter 2, section 2.2.1. The Patient Information Sheet is in Appendix B and the consent form is in Appendix C.

6.2.2 Inoculation

Preparation of the inoculum stock and determination of dose can be found in Chapter 2, sections 2.3.2.1 and 2.3.2.4. The inoculation method is described in Chapter 2, section 2.3.2.6.

6.2.3 Quantification of pneumococci by culture

Nasal wash samples analysed in this chapter were from volunteers in both Dose-Ranging and Reproducibility studies. Nasal wash samples were collected and processed as described in Chapter 2, sections 2.3.2.7 and 2.3.2.8. If pneumococci were detected at the pre-inoculation screen, the volunteer was not challenged but returned for a nasal wash at the same time points as those who were.

Carriage density by culture was determined as described in Chapter 2, section 2.3.3.2.

6.2.4 Bacterial DNA extraction

The bacterial DNA extraction method was performed as described in Chapter 2, section 2.3.3.3.

6.2.5 Quantification of pneumococcal DNA by qPCR

Determination of carriage density by qPCR was performed described in Chapter 2, section 2.3.3.4.

6.2.6 Statistical analysis

Differences in proportion of samples positive for carriage were statistically tested by a Chi-square test (Fisher's exact if <10 cases in a cell). Quantitative data was compared using Spearman's rank correlation coefficient. Graph and statistical analyses were performed using GraphPad prism version 5.0 (California, USA). All *P* values are two-tailed and considered significant if $P \leq 0.05$.

6.3 Results

6.3.1 Comparison of culture and qPCR in the detection of pneumococci

331 nasal wash samples collected from 79 volunteers were tested by microbiological culture and qPCR for the presence of *S. pneumoniae*. The proportion of samples positive for carriage by qPCR was significantly more than the proportion of samples positive for carriage by culture (42.6% vs. 27.5%, $P<0.0001$) (Table 6.1). The sensitivity of detection by qPCR was 92.3% (84/91) compared with culture, and the specificity was 75.9% (183/241). In 2.1% of samples, pneumococci were detected by culture but not qPCR and in 17.2% of samples pneumococci were only detected by qPCR.

Table 6.1 Comparison of bacterial culture and qPCR in detection of *S. pneumoniae*

	Culture positive (%)	**Culture negative (%)**	**Total**
qPCR positive (%)	84 (25.4%)	57 (17.2%)	141 (42.6%)
qPCR negative (%)	7 (2.1%)	183 (55.3%)	190 (57.4%)
Total	91 (27.5%)	241 (72.8%)	331 (100%)

6.3.1.1 The number of volunteers positive for carriage by qPCR was more than those positive for carriage by culture

The proportion of carriage positive volunteers was also determined over time (Figure 6.1). Across all time points, the number of volunteers positive for carriage by qPCR was more than those positive for carriage by culture, but this was only significant at 2 days post-challenge (*P*=0.009).

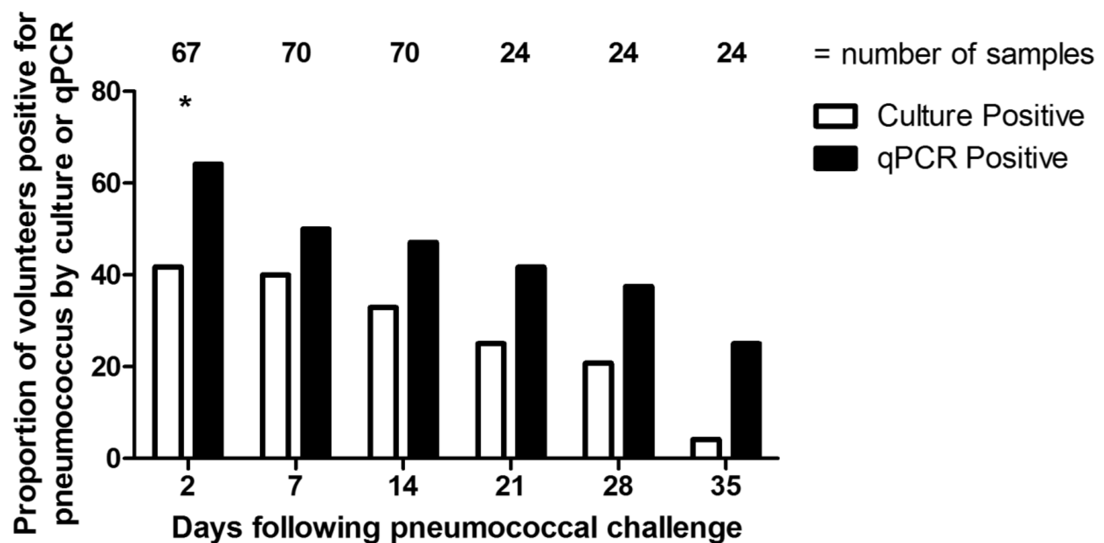

Figure 6.1: Proportion of carriage positive volunteers detected by bacterial culture or qPCR over time. For detection by culture, nasal wash samples were plated on blood agar with gentamicin. For qPCR detection, nasal wash was added to RNA protect and frozen until DNA extraction. A volunteer was considered positive by culture (white bar) if Gram positive, alpha-hemolytic, optochin-sensitive colonies were detected after 48 hours. Serotype was confirmed by latex agglutination. The target gene for qPCR was *lytA*. Samples with a Ct value <40 were considered qPCR positive (black bar). The number of samples analysed are indicated above each time point.*Statistical significance (*P* ≤0.05 using a Chi-square test or Fisher's exact if <10 cases in a cell).

6.3.2 Correlation of density detected by culture and qPCR

The correlation of pneumococcal density between culture and qPCR was determined for 147 samples. One sample was excluded from analysis because the number of colonies on the culture plate was too high for accurate determination of CFU/ml. Quantification of pneumococci by culture and qPCR were positively correlated (r_s=0.73, P<0.0001) (Figure 6.2). The detection limit for qPCR was the number of copies still detectable after 40 cycles, or 10^1 DNA copies/ml. The limit of detection for culture was set at the lowest density detected, or 0.2 CFU/ml.

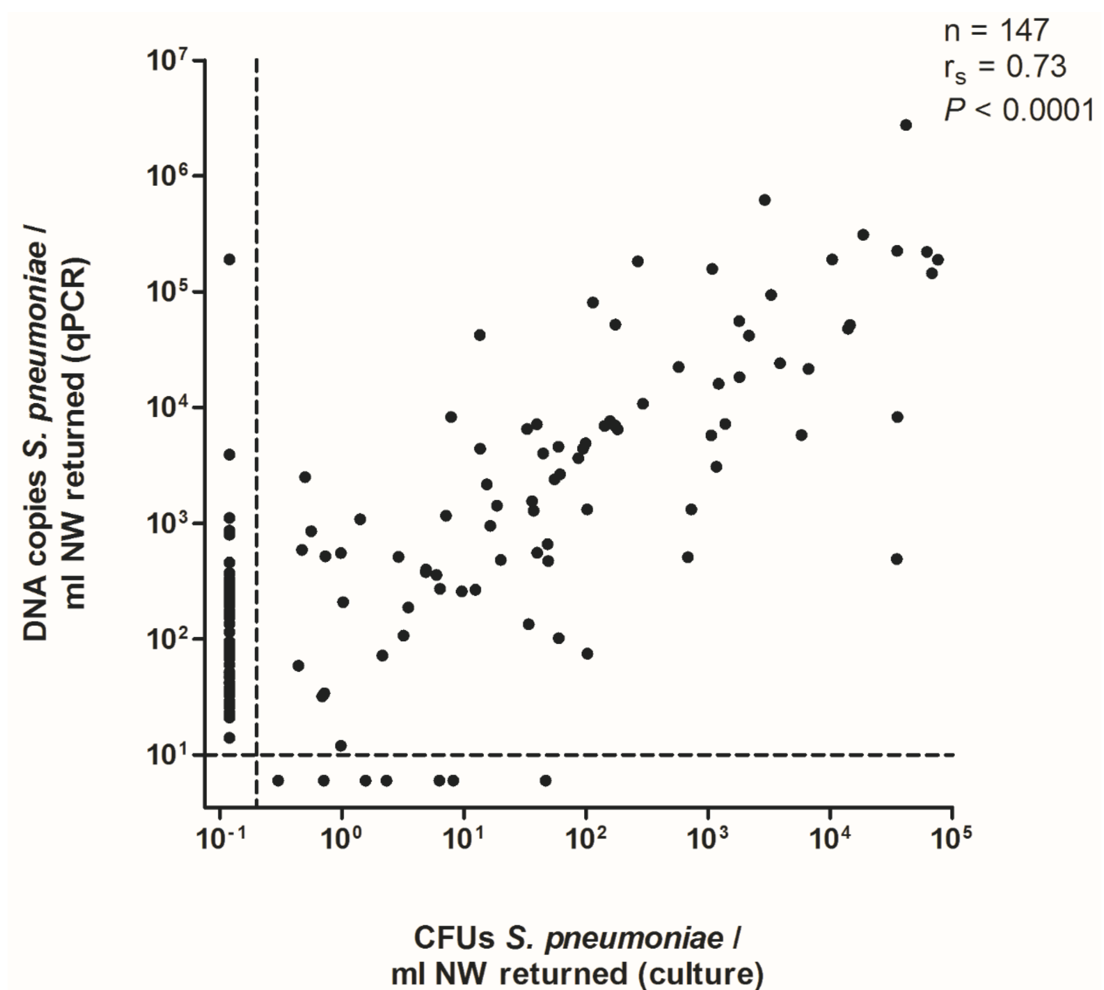

Figure 6.2: Correlation between bacterial culture and qPCR in quantifying *S. pneumoniae* in nasal wash samples. Quantification of pneumococci by culture and qPCR are positively correlated. The Spearman rank correlation coefficient for samples positive by both culture and qPCR is 0.73. Dotted lines represent lower limits of detection. $P \leq 0.05$ was considered significant.

6.3.2.1 Pneumococcal detection by culture, stratified by qPCR density

When pneumococcal detection by culture was stratified by qPCR density, the difference between the two methods became apparent. If the qPCR density was greater than 10^3 copies/ml, 94.8% (55/58) of samples were both culture and qPCR positive (Table 6.2). However, at densities ranging between 10^1 and 10^2, only 34.55% (29/84) of qPCR positive samples were also positive by culture (P<0.0001). Below the qPCR limit of detection, 7.7% (7/91) of samples were still positive by culture. In all but one of these samples, the culture density was lower than 10 CFU/ml.

Table 6.2 Detection of pneumococci in nasal wash by bacterial culture and qPCR, categorized according to qPCR density

Density by qPCR (copies/ml)	No. culture positive/No. qPCR positive (%)
<10	7/0
10^1	6/37 (16.2)
10^2	23/47 (48.9)
10^3	30/32 (93.8)
10^4	14/14 (100)
10^5	11/12 (91.7)
Total	91/142 (64.1)

6.3.3 Fluctuations in carriage density are accurately detected when both culture and qPCR are used for detection

Culture and qPCR were used to examine pneumococcal detection over time in four experimentally colonized and three naturally colonized volunteers. The fluctuations in density during a carriage episode were similar for both experimentally and naturally colonized volunteers (Figure 6.3). In Figure 6.3A, volunteer 1 had two consecutive time points (days 7 and 14) that were culture negative but qPCR positive. Contrastingly, volunteer 2 was culture positive but qPCR negative on day 28. In both cases, the carriage status varied depending on the method. This variation was also seen in natural carriers (Figure 6.3C). Both methods were beneficial when determining the length of a carriage episode. Volunteers 3 and 4 (Figure 6.3B) had at least two samples positive by both culture and qPCR but then at least three consecutive samples negative by culture but positive by qPCR. This pattern was also seen in natural carrier serotype 33 (Figure 6.3C).

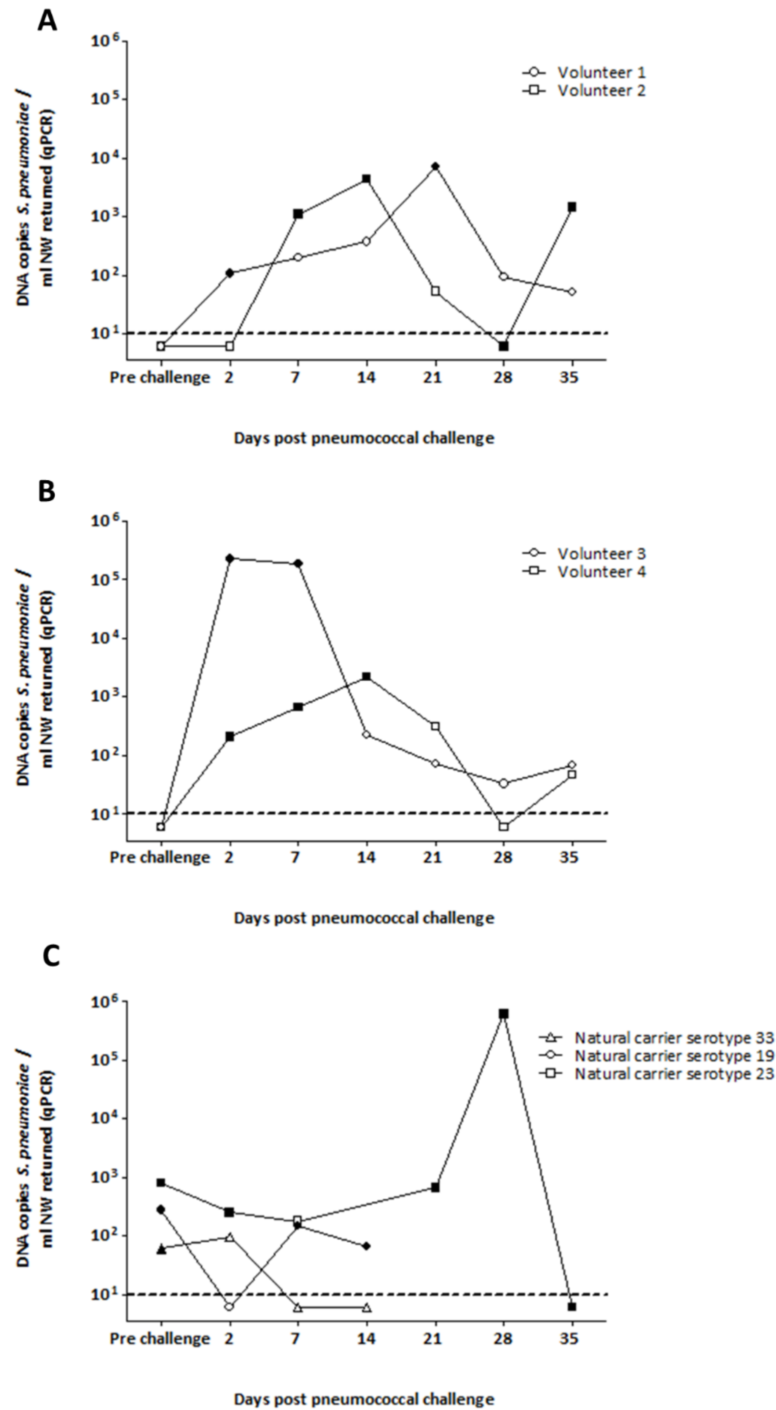

Figure 6.3: Culture and qPCR are complementary for following a carriage episode. At low density, carriage results can vary by method, which can impact on (A) the number of carriage episodes and (B) the duration of carriage episodes. (C) Natural carriage episodes mimic the variation seen in experimental carriage. Filled shapes: carriage positive by culture. Open shapes: carriage negative by culture. Dotted lines represent lower limits of detection. NW, nasal wash.

6.4 Discussion

Longitudinal nasal wash samples from adults who were intranasally challenged with pneumococci were assessed for carriage density by culture and qPCR.

6.4.1 A higher pneumococcal carriage rate was detected using qPCR, as compared with culture

The proportion of samples positive for carriage by qPCR was significantly more than the proportion of samples positive for carriage by culture. Detection of pneumococcal carriage by culture is the gold standard [242]. By isolating the pneumococcus, further tests can be performed to determine serotype and antibiotic sensitivity. Culture is also more feasible in resource-limited settings. Non-culture methods are increasingly being used to detect pneumococci. Several studies have focused on gene targets using qPCR, with *lytA* the most widely used, and have shown that a qPCR-based method is more sensitive than culture [223,253]. There have been concerns over the specificity of *lytA* and the possibility that it may not discriminate between *S. pneumoniae* and viridans streptococci however, our specificity (75.9%) was similar to that published by Carvalho et al. [253] in CSF samples (70%). To increase sensitivity, some studies include an enrichment step but this cannot be used when quantification is an endpoint [248,249].

A lower detection rate in culture compared with qPCR could be explained by the potential for subclinical -or low density- carriage. Turner et al. [239] showed detection of multiple serotypes in nasopharyngeal samples is significantly underestimated when using the standard WHO protocol which is able to detect a predominant serotype but is not efficient at detecting low-abundance serotypes. This links with serotype replacement where it is unclear if the increase in non-vaccine-type pneumococci is due to the elimination of vaccine-type pneumococci or if the replacement is because non-vaccine-types are carried at low density and are simply “unmasked” when vaccine types are inhibited or removed [181,330]. Low density carriage could explain the differences between culture and qPCR seen here; at a density of less than 10^2, the low abundance is missed by culture but picked up by qPCR.

6.4.2 Culture and qPCR were complementary in determining pneumococcal carriage density, as well as the number and duration of carriage episodes

The number of pneumococcal *lytA* DNA copies were generally 1 to 2 log^{10} higher than the corresponding CFU count. It has been shown in children that at a density of less than 10^5 CFU/ml, significantly less carriers were detected by culture, not only for pneumococcus but also for *S. aureus* and *H. influenzae* [223]. However, as we have shown here and discussed previously [246], we are able to detect carriage by culture at <10 copies/ml and suggest that, when using a fresh nasal wash sample, culture and qPCR are equally sensitive until 10^2 CFU/ml.

The parallel decrease of culture positivity rates and pneumococcal densities measured by qPCR, suggests that qPCR is more suitable for detecting low levels of carriage. However, qPCR is not informative on viability of pneumococci in the nasopharynx. As seen in Figure 6.3B, continuous qPCR detection when culture results are negative may represent prolonged low density carriage, living cells that are in a culture-unfavourable metabolic state, or remaining pneumococcal debris. And intermittent culture positive results could be misconstrued as separate carriage episodes when they are actually a single carriage episode as supported by qPCR detection (Figure 6.3A). By applying both methods, the chance of missing pneumococcal carriage may be intercepted. In our controlled environment, the most advantageous approach to longitudinally study a carriage episode is to use both methods.

Inconsistencies between culture and qPCR detection could also be explained by decreasing success rates at the threshold of detection. Culture densities were low (< 10 CFU/ml) in 6/7 qPCR negative samples, and in 54 out of 57 culture negative samples qPCR densities were below 10^3 copies/ml. The higher density inconsistencies could be attributed to experimental error; culture positive/qPCR negative samples could be the result of faulty DNA extraction or the exclusion of qPCR signals below 10 copies/ml (> 40 cycles). Four of the seven exclusively culture positive samples were analysed again using a different *lytA* primer set but remained negative for pneumococcal DNA [334]. We chose a threshold of 40 cycles which was used in the development of the *lytA* qPCR assay. We observed that the success rate of detection of the standard curve was still 100% at 40 cycles, and that the standard curve still followed a straight line at 40 cycles. In addition, almost all samples

detected between 35 and 40 cycles were positive in both duplicates. These observations imply that the lower limit of detection of our method had not yet been reached at 40 cycles. This cut-off is two cycles higher than that used in the study with children, possibly explaining the differences in detection limits between the two studies [223,253].

Our study surveyed adult pneumococcal carriage in consecutive nasal wash samples. However, this sampling method is less suited for those who are unable to actively participate in sample collection (e.g. young children). Further studies would need to determine whether our observations hold true for different sampling methods and populations.

The strength of this data is the determination of both culture and qPCR density during a controlled carriage episode. We observed a high correlation between the two methods in calculating density. Absolute numbers were 1 to 2 log^{10} lower by culture, which is in line with observations previously reported by Albrich et al. [266]. The difference may be due to the inability of qPCR to distinguish between live and dead bacteria - an important distinction in low density samples. Lower culture density may also be a result of the pneumococcal tendency to grow in pairs and chains. More than one pneumococcus can form a single colony but qPCR will detect each individual bacterium.

Carriage is a dynamic event and we have shown that an experimental human carriage model mimics a natural carriage episode. The data from our study indicate that culture and qPCR methods are highly complementary when studying pneumococcal carriage episodes, especially when the carriage density is low. This is important information for future vaccine studies where carriage density or duration may be an endpoint.

Chapter 7

Modulation of nasopharyngeal innate defences by viral co-infection predisposes individuals to experimental pneumococcal carriage

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7.1 Introduction

Secondary bacterial infections with *S. pneumoniae* are a major cause of morbidity and mortality during pandemic influenza [335]. Pneumococcus commonly colonizes the URT in healthy individuals but viral infections transform this normally harmless commensal organism into a potentially fatal pathogen by increasing pneumococcal transmission [20], carriage density [219,336] and host disease susceptibility [218]. The current threat of influenza pandemics, increasing antibiotic resistance, and the burden of co-infection in the young and aged populations make it critical to understand how viral infection increases susceptibility to pneumococcal disease.

Pneumococcal colonization at the mucosal surface is a prerequisite of invasive disease [7,337]. Children with radiologically confirmed pneumonia have a marked increase in the density of colonizing pneumococci if co-infected with influenza A, RSV or rhinovirus [219]. Several possible mechanisms may account for increased nasopharyngeal pneumococcal density, including influenza-induced damage to the epithelium and/or alterations in host cellular responses to bacterial pathogens [338,339]. Viral infections reduce mucociliary velocity, denude epithelial surfaces and expose basement membranes, and modulate chemokine and innate defences [340-342].

We used an EHPC model [292] to investigate the relationship between asymptomatic URT viral infections and pneumococcal colonization in humans. We also investigated a possible mechanism by which virus could modulate mucosal defences and increase colonization by measuring levels of several soluble innate factors at the nasal mucosa, including human FH. FH is a soluble protein found in human plasma that suppresses the alternative complement pathway [115]. The pneumococcus binds FH via PspC [113], facilitating adherence to epithelial cells [58]. PspC also interacts with the human polymeric immunoglobulin receptor (pIgR) to promote bacterial adherence to, and invasion of, epithelial cells, as well as binding to the secretory component of immunoglobulin A (sIgA) [56]. As a result of these important interactions with the host immune system PspC has been proposed as a vaccine candidate to block pneumococcal carriage [18].

7.2 Materials and methods

7.2.1 Recruitment and ethics

Inclusion and exclusion recruitment criteria can be found in Chapter 2, section 2.2.1. The Patient Information Sheet is in Appendix B and the consent form is in Appendix C.

Volunteers from both the Dose-Ranging and Reproducibility studies were included in this chapter as were 40 volunteers from a separate study. These 40 volunteers from the separate study followed a similar schedule as those in the above two studies with one major exception: they were vaccinated with the Hepatitis A (Avaxim) vaccine four weeks prior to pneumococcal challenge.

7.2.2 Inoculation

Preparation of the inoculum stock and determination of dose can be found in Chapter 2, sections 2.3.2.1 and 2.3.2.4. The inoculation method is described in Chapter 2, section 2.3.2.6.

7.2.3 Quantification of pneumococci by culture

Nasal wash samples were collected and processed as described in Chapter 2, sections 2.3.2.7 and 2.3.2.8. Detection of pneumococcal carriage by culture and measurement of carriage density were performed as described in Chapter 2, sections 2.3.3.1 and 2.3.3.2, respectively.

7.2.4 Detection and identification of URT viruses

URT viruses were detected as described in Chapter 2, section 2.3.4.9.

7.2.5 Levels of FH, lactoferrin, SLPI, and beta defensin 2 in nasal wash and anti-PspC in serum

The methods used to determine the levels of FH, lactoferrin, SLPI, beta defensin 2, and anti-PspC were performed as described in Chapter 2, section 2.3.4.10.

7.2.6 Depletion and purification of antibodies from nasal wash and sera samples

The methods used to deplete and purify antibodies from nasal wash and sera samples were performed as described in Chapter 2, section 2.3.4.11.

7.2.7 Bacterial strains and growth conditions

Details on the 6B (BHN418) strain used for inoculation and the *S. pneumoniae* serotype 2 strain D39 used in the pneumococcal adherence and internalization assay can be found in Chapter 2, section 2.3.1.

7.2.8 FH binding and antibody binding assays

The FH binding assay and the antibody binding assay were performed as described in Chapter 2, section 2.3.4.12.

7.2.9 Epithelial cell assays

7.2.9.1 Epithelial cell growth and inflammation of epithelium

Detroit 562 epithelial cells were grown and maintained as described in Chapter 2, section 2.3.4.8. Inflammation of the epithelium was performed as described in Chapter 2, section 2.3.4.8.1.

7.2.9.2 Epithelial pIgR and rPAF expression by flow cytometry

Determination of epithelial pIgR and rPAF expression was performed as described in Chapter 2, section 2.3.4.8.2.

7.2.9.3 Pneumococcal adherence and internalization assays

The pneumococcal adherence and internalization assays were performed as described in Chapter 2, section 2.3.4.8.

7.2.10 Anti-PspC antibody epitope mapping

The antibody epitope mapping method was performed as described in Chapter 2, section 2.3.4.13.

7.2.11 Statistical analysis

Fisher's exact test was used to compare the proportion of carriers that were virus positive and virus negative. Pearson's correlation coefficient was used to compare log transformed FH levels and pneumococcal carriage density. Flow Cytometry data were analysed using Flow Jo v7.6.1, except for the epithelial pIgR and rPAF expression which was analysed using v10. To account for possible correlation among the repeated measurements in carriage status and density, a generalized estimating equation (GEE) model was employed for the data analysis. For the GEE analysis of carriage presence (Yes or No), binomial distribution and logit link function were used. For the GEE analysis of carriage density, log transformed

density was used as the dependent variable and normal distribution and identity link function were used. Three GEE models were estimated to assess the effects of FH levels and virus status on carriage status and density, separately and jointly. Model 1 has virus status (positive and negative) and day (Day 2, 7 and 14) as predictors; Model 2 has FH levels in log scale and day as predictors; Model 3 has FH levels and virus status, day, and interaction between FH levels and virus as predictors. Differences were considered significant if $P \leq 0.05$. Statistical analyses and graphical presentations were performed using Graphpad Prism5 and SAS9.3.

7.3 Results

7.3.1 Asymptomatic URT viral infections increased susceptibility to pneumococcal colonization

One hundred and one healthy volunteers (age 18-50) were challenged with pneumococcus between November 2011 and April 2014. The average age (mean±SD) of volunteers was 23±6 years and 38% were male (38/101). Oropharyngeal swabs taken 5 days prior to challenge were analysed by PCR for the presence of URT viruses. Nasal wash samples were taken 2, 7 and 14 days post-inoculation and analysed for the presence of pneumococcal carriage by classical microbiology. Experimental carriage of 6B pneumococcus was detected in 52 out of 101 volunteers (51%), as defined by the presence of pneumococci in nasal wash at any time point post challenge.

We investigated whether the presence of virus predisposed certain individuals to colonization. There were no significant differences between virus positive and virus negative volunteers with regards to age (22±3 vs. 23±7 years, P=0.5), gender (M:F 6:14 vs. 32:49, P=0.6), or inoculation dose (P=0.9). Amongst virus positive volunteers, 75% became colonized (15/20) as compared to 46% virus negative volunteers (37/81) (P=0.02) (Figure 7.1A). We could not find an association between the presence of virus and increased density (Figure 7.1B). There was also no association between virus presence and season (Figure 7.1C). Only infections caused by a single virus were detected. The detected viruses amongst colonized volunteers were rhinovirus (8/15), coronavirus (4/15), RSV (2/15) and parainfluenza virus (1/15); similarly non-colonized individuals were positive for rhinovirus (3/5), coronavirus (1/5) and adenovirus (1/5).

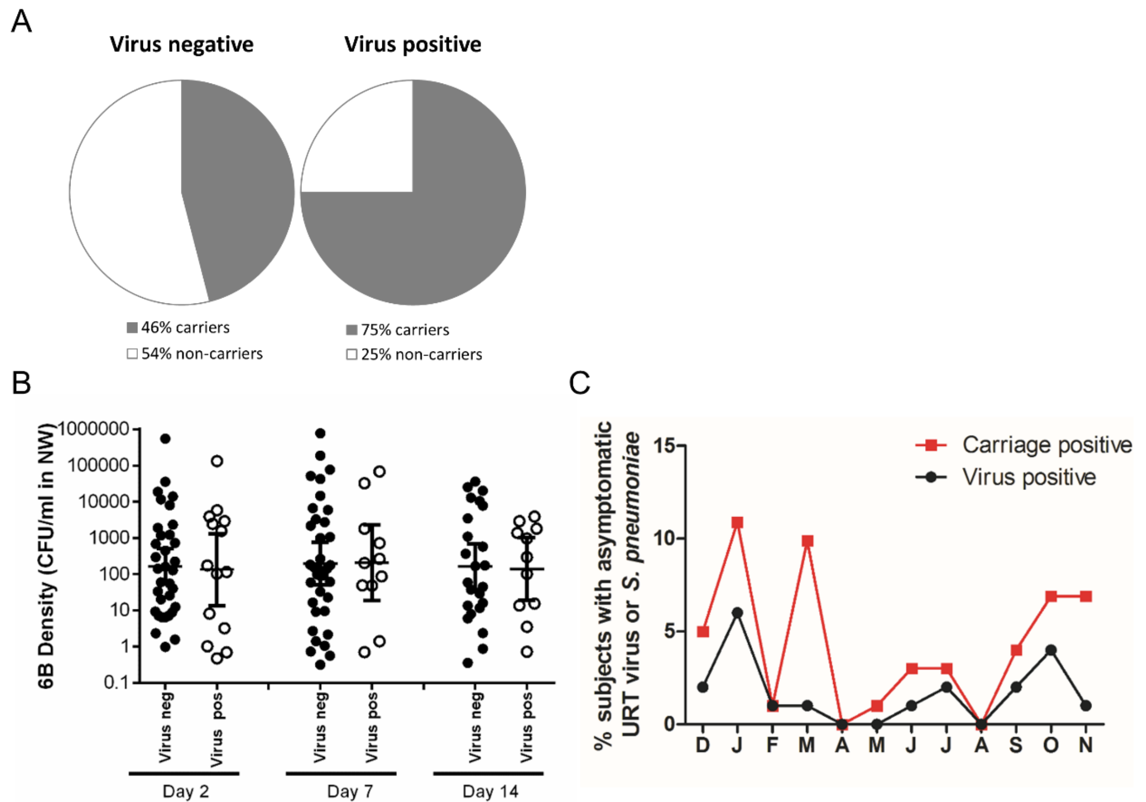


Figure 7.1: Asymptomatic upper respiratory tract (URT) viral infections are associated with susceptibility to pneumococcal colonization but not increased density. (A) One hundred and one healthy volunteers were screened for URT viruses by multiplex PCR 5 days before intranasal inoculation of 6B pneumococcus. The percentage of carriage positive (carriers) and carriage negative (non-carriers) volunteers are shown for virus negative or virus positive. Amongst virus positive volunteers 75% became colonized as compared to 46% virus negative volunteers (*P*=0.02, using Fisher's exact test). (B) Colonization density recovered from the nasopharynx was measured in nasal wash samples collected 2, 7 and 14 days following pneumococcal inoculation and is expressed as CFU/ml recovered from nasal wash of virus negative and virus positive groups. There was no difference in colonization density between virus positive and virus negative volunteers. (C) The percentage of volunteers positive for asymptomatic URT virus or *S. pneumoniae* were analysed per month. There was no association between virus presence and season.

7.3.2 Levels of mucosal FH were increased in individuals co-infected with virus and pneumococci and associated with increased colonization density

We investigated levels of mucosal FH, SLPI, β-defensin 2 and lactoferrin in nasal wash to determine whether modulation of these innate factors by a virus could predispose individuals to pneumococcal colonization. Levels of FH (Figure 7.2A), were slightly higher in the nasal wash samples of volunteers co-infected with virus and pneumococci compared to virus only individuals (P=0.04). In contrast, levels of the antimicrobial factor SLPI (Figure 7.2B), beta defensin 2 (Figure 7.2C) and lactoferrin (Figure 7.2D) were not significantly different.

As virally infected individuals colonized with pneumococci had slightly higher levels of FH we investigated the relationship between FH and bacterial colonization density. We found that individuals with high mucosal FH levels had increased 6B colonization density (Figure 7.2E, P=0.005).

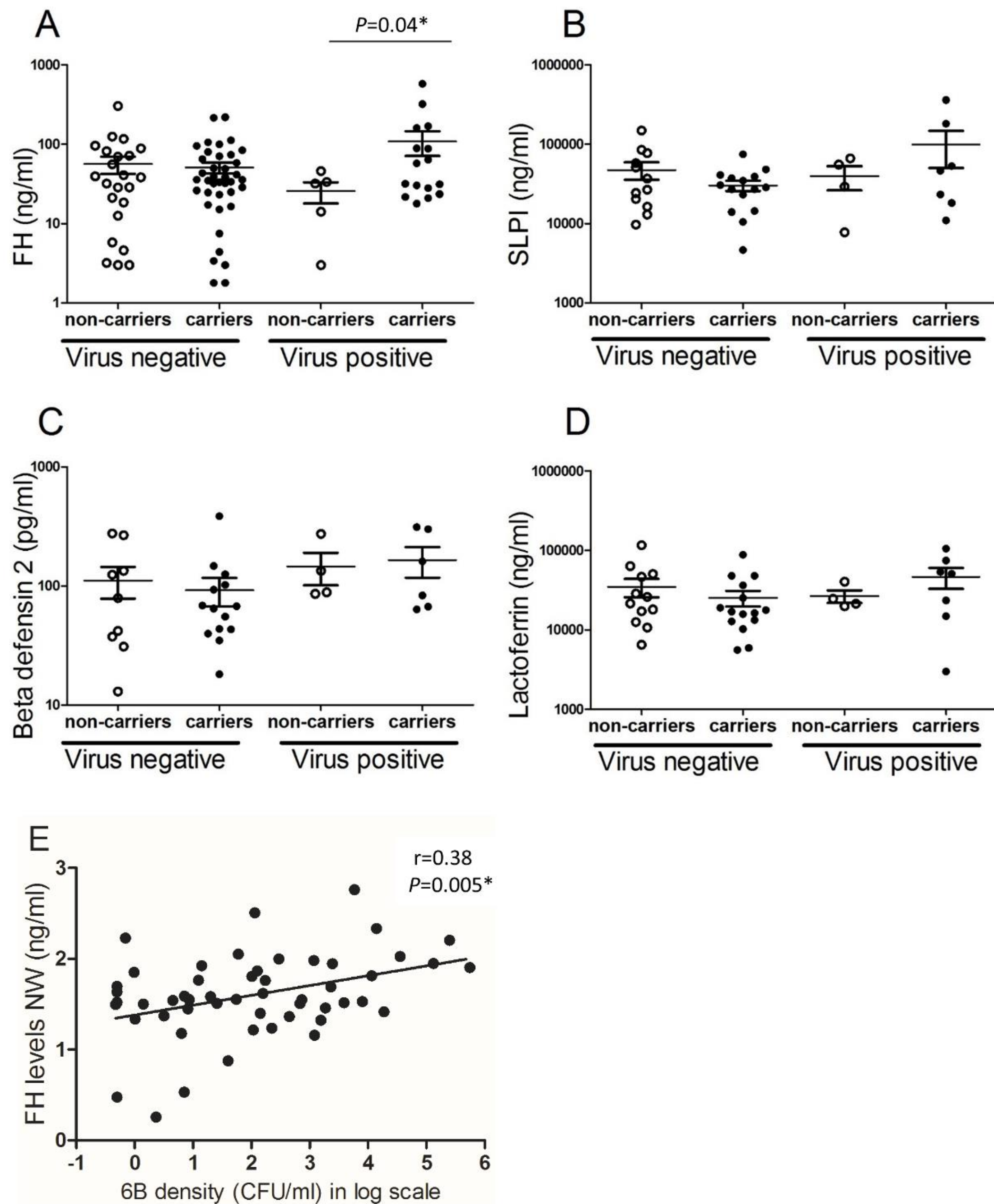


Figure 7.2: Levels of mucosal FH are increased in individuals co-infected with virus and pneumococci and correlate with colonization density. Mucosal levels of (A) FH (B) SLPI (C) beta defensin 2 and (D) lactoferrin were measured in nasal wash samples obtained 2 days after pneumococcal challenge with 6B. Levels are expressed in ng/ml and results are stratified by pneumococcal carriage and absence or presence of virus. Statistical differences were analysed using an unpaired Student's *t* test (**P* ≤0.05). (E) We observed a positive correlation between levels of FH and pneumococcal colonization density (CFU/ml) recovered from nasal wash 2 days after inoculation using Pearson's correlation test. *P* value and the correlation coefficient (r) is presented.

Using a GEE model we examined the association of virus presence and mucosal FH levels, individually as well as jointly, with carriage presence and density (Table 7.1). When we examined the effects of virus presence and mucosal FH levels individually (GEE model 1 and 2), virus presence was associated with a 2.83 times increase in the odds of becoming colonized (*P*=0.023) and a doubling in FH levels was associated with a 2.54 times increase in the geometric mean of carriage density (*P*=0.002). When we examined their effects jointly (GEE model 3), we found an interaction. Specifically, amongst virus positives a doubling in FH levels was associated with a 9.33 times increase in the odds of carriage (*P*=0.034) and a 4.26 times increase in the geometric mean of carriage density (*P*=0.009).

Table 7.1 Association of virus presence and mucosal FH levels with carriage presence and carriage density

Model$	Variable	Carriage presence (Odds ratio [95% CI])	*P* value	Carriage density (Ratio [95% CI])	*P* value
Model 1	Virus positive	2.83 [1.15-6.96]	0.023	0.96 [0.43-2.17]	0.930
Model 2	FH levels	1.95 [0.82-4.64]	0.133	2.54 [1.43-4.51]	0.002
Model 3	FH levels (Virus negative)	1.20 [0.43-3.34]	0.727	2.36 [1.06-5.25]	0.035
	FH levels (Virus positive)	9.33 [1.19-73.31]	0.034	4.26 [1.43-12.68]	0.009

$ GEE model was used for the data analysis. Model 1 has virus status (positive and negative) and day (Day 2, 7 and 14) as fixed effects; Model 2 has FH levels in log scale and day as fixed effects; Model 3 has FH levels and virus status, day, and interaction between FH levels and virus as fixed effects.

7.3.3 Nasal wash fluid and epithelium inflammation increased pneumococcal adherence and internalization

We used a model of non-inflamed and inflamed nasopharyngeal epithelium to investigate the role of mucosal FH in pneumococcal adherence during health and co-infection, respectively. Inflammation of epithelium was confirmed by increased expression of pIgR (Figure 7.3A) and rPAF (Figure 7.3B).

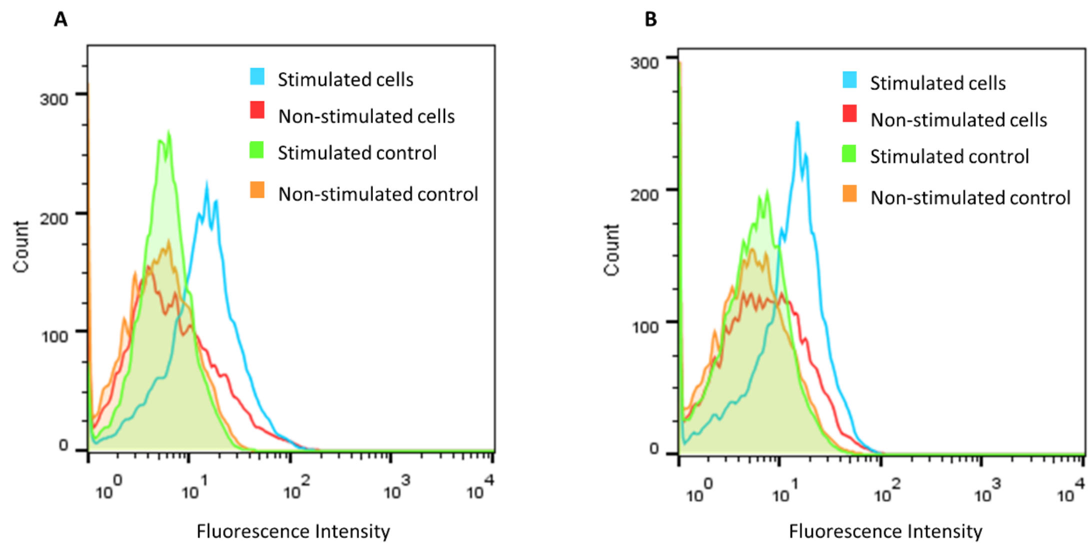


Figure 7.3: Confirmation of epithelium inflammation. Stimulated (blue) and non-stimulated epithelial cells (red) were incubated with (A) pIgR and (B) rPAF to confirm inflammation of epithelium through increased receptor expression. FITC donkey anti-rabbit IgG and PE goat anti-mouse IgG2a were used as isotype controls, respectively.

We observed more than a two-fold increase in pneumococcal adherence of D39 to inflamed epithelium compared to non-inflamed epithelium (Figure 7.4A, black squares). We tested whether pure FH or nasal wash containing FH would influence bacterial adherence. Pneumococcal IgA1 protease cleaves IgA1 antibodies found in nasal wash [281] and promotes pneumococcal adherence [143]. Because of this important mechanism we used nasal wash samples depleted of IgG or IgA.

Pre-treatment of bacteria with pure FH increased D39 pneumococcal adherence to non-inflamed and inflamed epithelium (Figure 7.4A, open dots) and adherence was dependent on PspC expression (Figure 7.4B). Pre-treatment of bacteria with nasal wash samples depleted of IgA (Figure 7.4A, grey dots) or IgG (Figure 7.4A, black dots) equally increased pneumococcal adherence to nasopharyngeal cells, and this effect was independent of PspC expression (Figure 7.4B). We observed increased internalization of bacteria when pre-treated with FH (Figure 7.4C, open dots), nasal wash IgA-depleted (Figure 7.4C, grey dots) or nasal wash IgG-depleted (Figure 7.4C, black dots). This effect was more marked when the epithelium was inflamed.

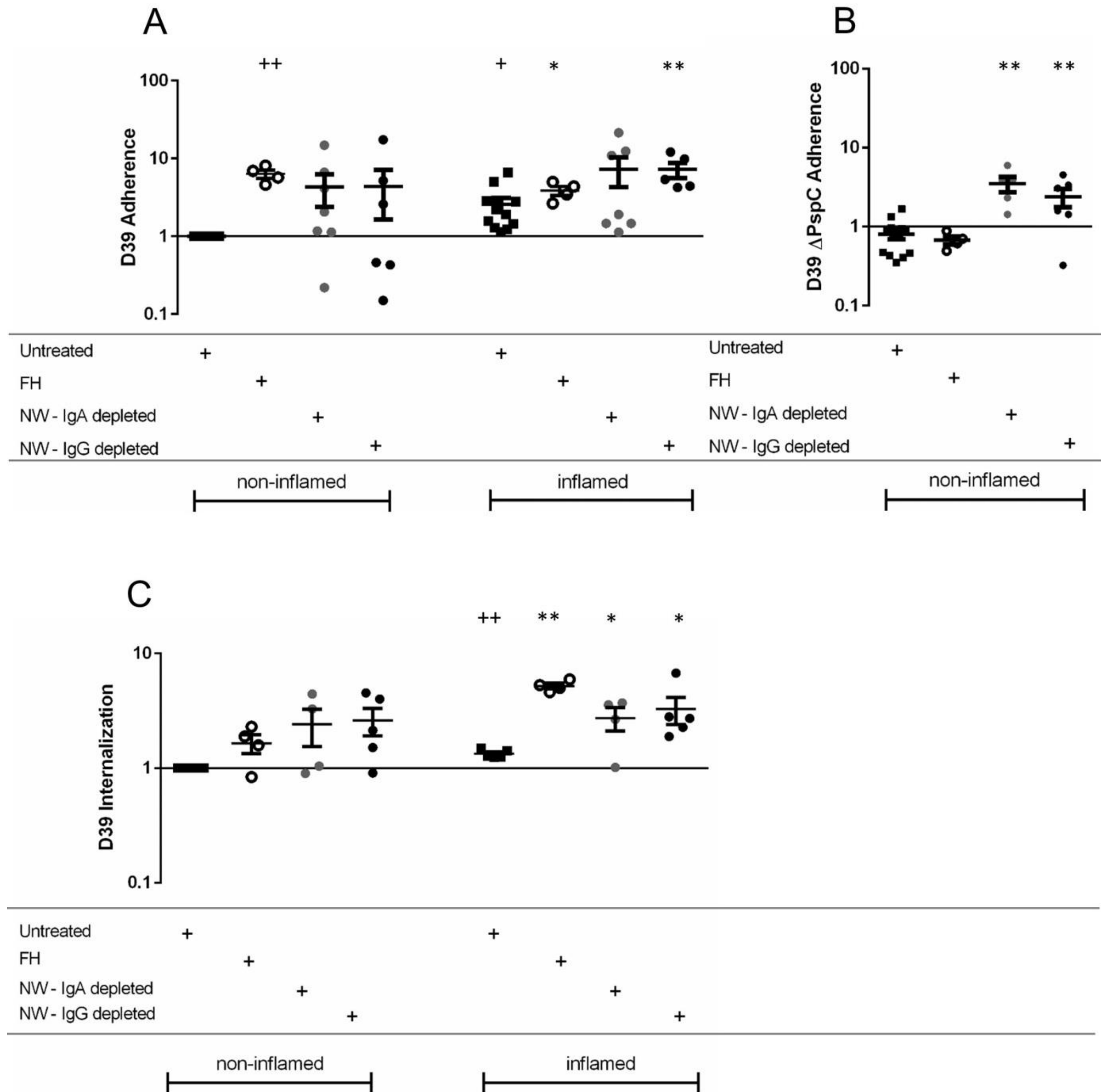


Figure 7.4: Pneumococcal epithelial adherence and internalization are increased in the presence of human nasal wash or Factor H. Adherence of (A) D39 and (B) D39ΔPspC to human pharyngeal epithelial cells (D562) was evaluated in non-inflamed and inflamed epithelium. Epithelial adherence (A) and internalization (C) of pneumococci was increased following treatment of bacteria with human purified FH (open dots), nasal wash depleted of IgA (grey dots), nasal wash depleted of IgG (black dots) when compared to untreated bacteria (black squares). Epithelial adherence (B) was not increased when D39ΔPspC was pre-treated with FH (open dots). All conditions were performed in duplicate and experiments were repeated at least twice. For each experiment, the average recovered CFU from the control condition (adherence of D39 untreated bacteria to non-inflamed cells) was used to calculate fold change of all remaining conditions. **+** represents statistical

significance compared to control condition using an unpaired *t* test (**+**$P \le 0.05$ and **++** $P \le 0.005$). *represents statistical significance compared to untreated bacteria of same epithelial condition using an unpaired Student's *t* test (*$P \le 0.05$ and **$P \le 0.005$). All conditions were performed in duplicate and experiments were performed at least twice. For each experiment, the average recovered CFU from the control condition (adherence of D39 untreated bacteria to non-inflamed cells) was used to calculate fold change for all remaining conditions.

7.3.4 Anti-PspC IgG partially blocked FH binding to pneumococcus and pneumococcal adherence and internalization to pharyngeal cells

We investigated whether anti-PspC antibodies purified from individuals participating in the EHPC model could block FH binding to pneumococcus *in vitro* and circumvent the increased adherence of pneumococcus during inflammation. The specific binding of FH via PspC to our laboratory strain was confirmed (Figure 7.5A and B).

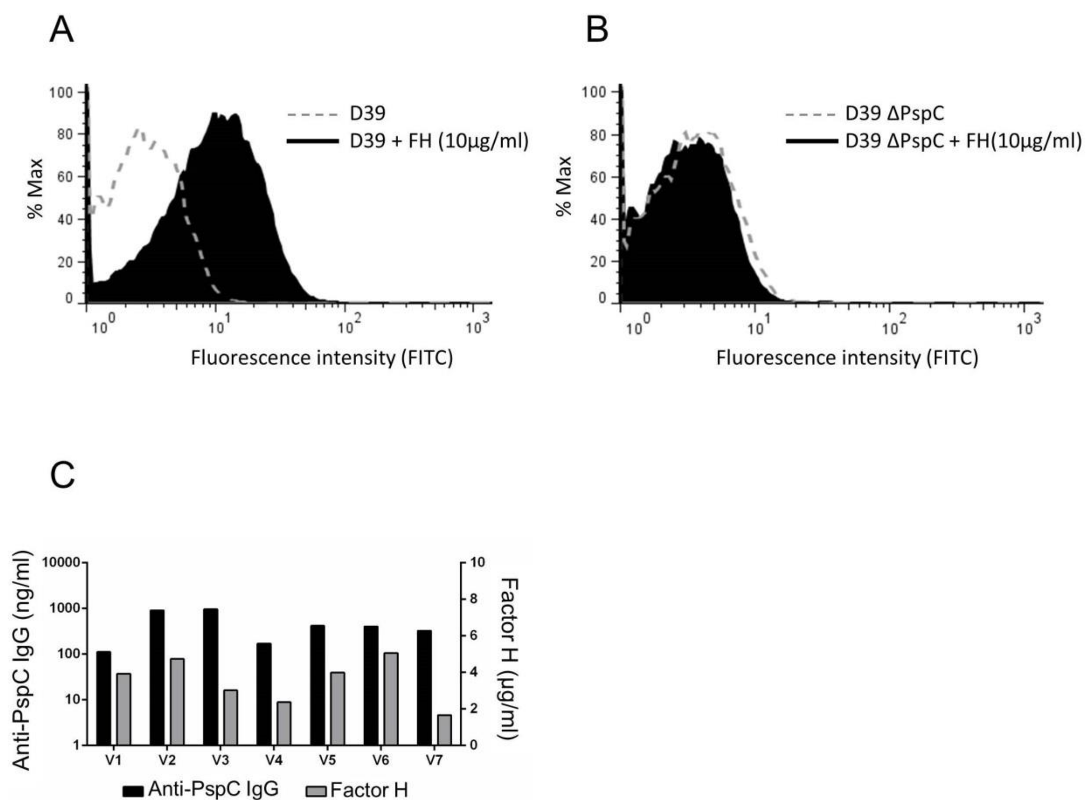


Figure 7.5: Binding of human FH to pneumococcus is mediated by PspC. (A) D39 and (B) D39 ΔPspC were employed in FH binding assays. Fluorescence intensity of bacteria (dotted line) and bacteria incubated with 10 μg/ml of FH (filled black) are shown on the Y axis. (C) Levels of anti-PspC IgG and FH were measured in samples purified from serum of 7 volunteers. The two-step purification process was able to purify anti-PspC specific IgG but FH from sera could not be entirely removed.

Anti-PspC antibodies were isolated from serum where they are more abundant than in nasal wash. Flow cytometric analysis indicated that samples from each subject had anti-PspC IgG that bound effectively to pneumococcus (Figure 7.6A, *P*=0.01 using unpaired *t* test). Levels of IgG binding were variable among samples and related to anti-PspC IgG levels measured by ELISA (Figure 7.5C). Purified samples also had measurable levels of FH remaining after the anti-PspC IgG purification process (Figure 7.5C). Purified samples were incubated with bacteria prior to addition of FH in order to block PspC-FH interaction. We observed a partial inhibition of FH binding to pneumococcus by purified human anti-PspC IgG samples (Figure 7.6B).

We then investigated whether purified anti-PspC IgG could block pneumococcal adherence and internalization to host epithelial cells. Epithelial adherence was reduced by purified anti-PspC IgG (Figure 7.6C, grey dots) containing 3.5 µg/ml of residual FH (residual from anti-PspC purification process, Figure 7.6C) but not in the presence of higher levels of FH (20 µg/ml) (Figure 7.6C, open dots). This interaction was specific to PspC as treatment with anti-PspC IgG did not alter adherence of D39 ΔPspC (Figure 7.6D, grey dots). Internalization of D39 was inhibited by anti-PspC IgG (Figure 7.6E, grey dots) but not by purified total IgG (open squares).

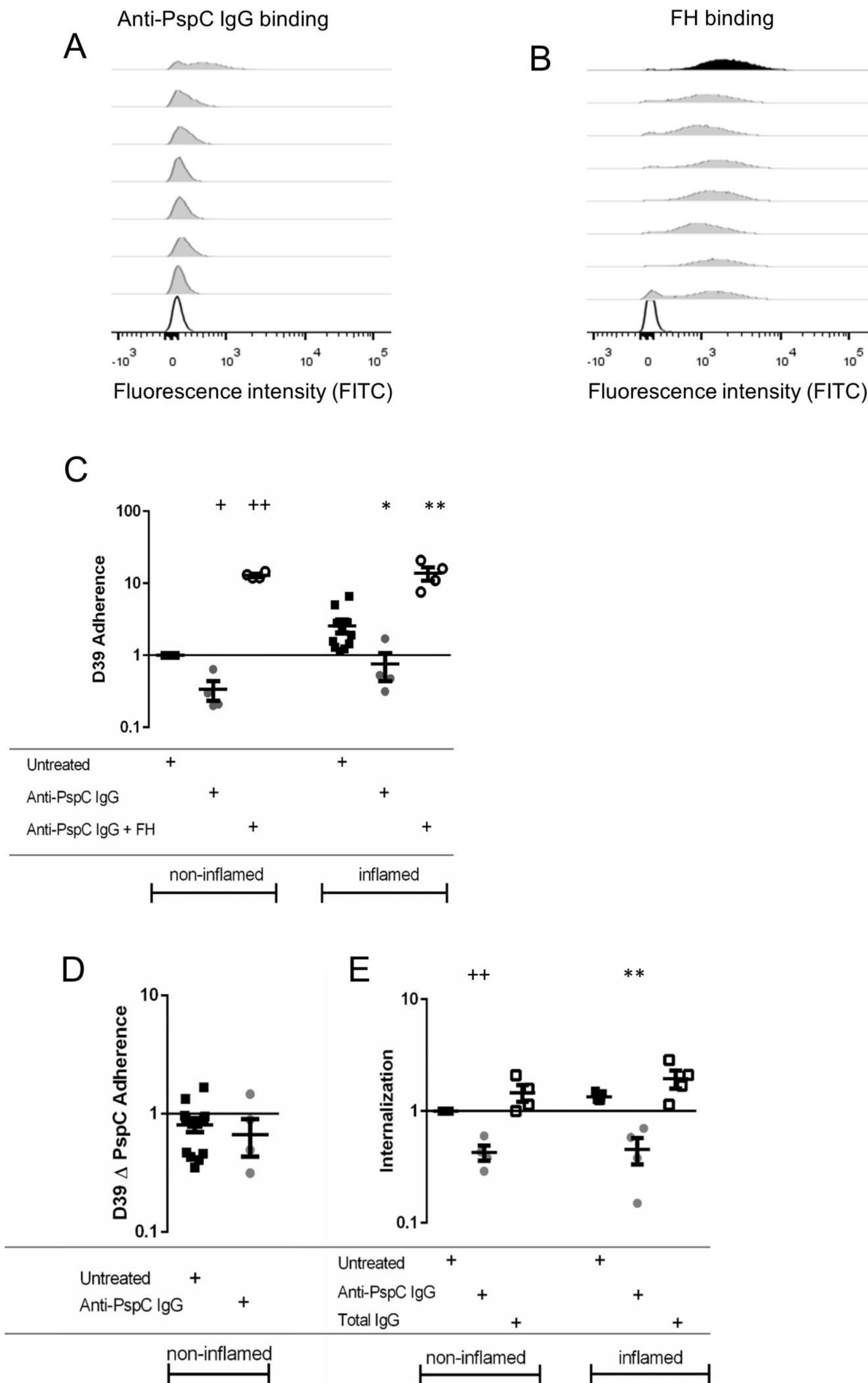


Figure 7.6: Purified human anti-PspC IgG binds to pneumococcus and partially inhibits Factor H binding and adherence to human pharyngeal epithelial cells. (A) We measured anti-PspC IgG binding to D39 and (B) inhibition of FH binding by purified anti-PspC. Each row represents one subject. Negative controls (without anti-PspC or FH) are represented by unfilled histograms (A and B). For the inhibition of FH assay the positive control (without

anti-PspC and with FH) is represented by black filled histogram. (C) Epithelial adherence of D39 was evaluated following treatment of bacteria with purified anti-PspC IgG containing low levels of FH (3.5 µg/ml residual from purification of serum samples, grey dots) or with anti-PspC IgG before addition of high levels of FH (10 µg/ml, open dots) and compared to untreated bacteria. (D) Adherence of D39ΔPspC untreated (black squares) or treated with purified anti-PspC IgG (grey dots) was evaluated. (E) D39 epithelial internalization was measured in untreated bacteria (black squares) and bacteria treated with the purified anti-PspC IgG (grey dots) or the total IgG purified from the same serum samples as control for antibody specificity (open squares). **+** represents statistical significance compared to control condition using an unpaired *t* test (**+***P*≤0.05 and **++***P*≤0.01). **represents statistical significance compared to untreated bacteria on inflamed epithelial condition (black squares) using an unpaired *t* test (**P*≤0.05 and ***P*≤0.005).

7.3.5 Anti-PspC epitope mapping revealed lack of human antibodies recognizing the binding site for FH after intranasal exposure to pneumococcus

To explore reasons for the inefficient blocking of the FH-pneumococcus interaction by anti-PspC IgG we used peptide arrays to define the epitopes of PspC recognized by human antibodies. We used serum from 18 healthy adults challenged with pneumococcus to probe peptide arrays containing the entire sequence of PspC group 3 (PspC3 - 491/00 strain). We observed that despite high levels of anti-PspC antibodies only one out of the 29 samples had antibodies recognizing the binding sites for FH which supports the partial inhibition of FH binding to pneumococcus observed *in vitro* (Figure 7.6B). Secretory IgA (sIgA) binding sites were recognized by all samples (Figure 7.7).

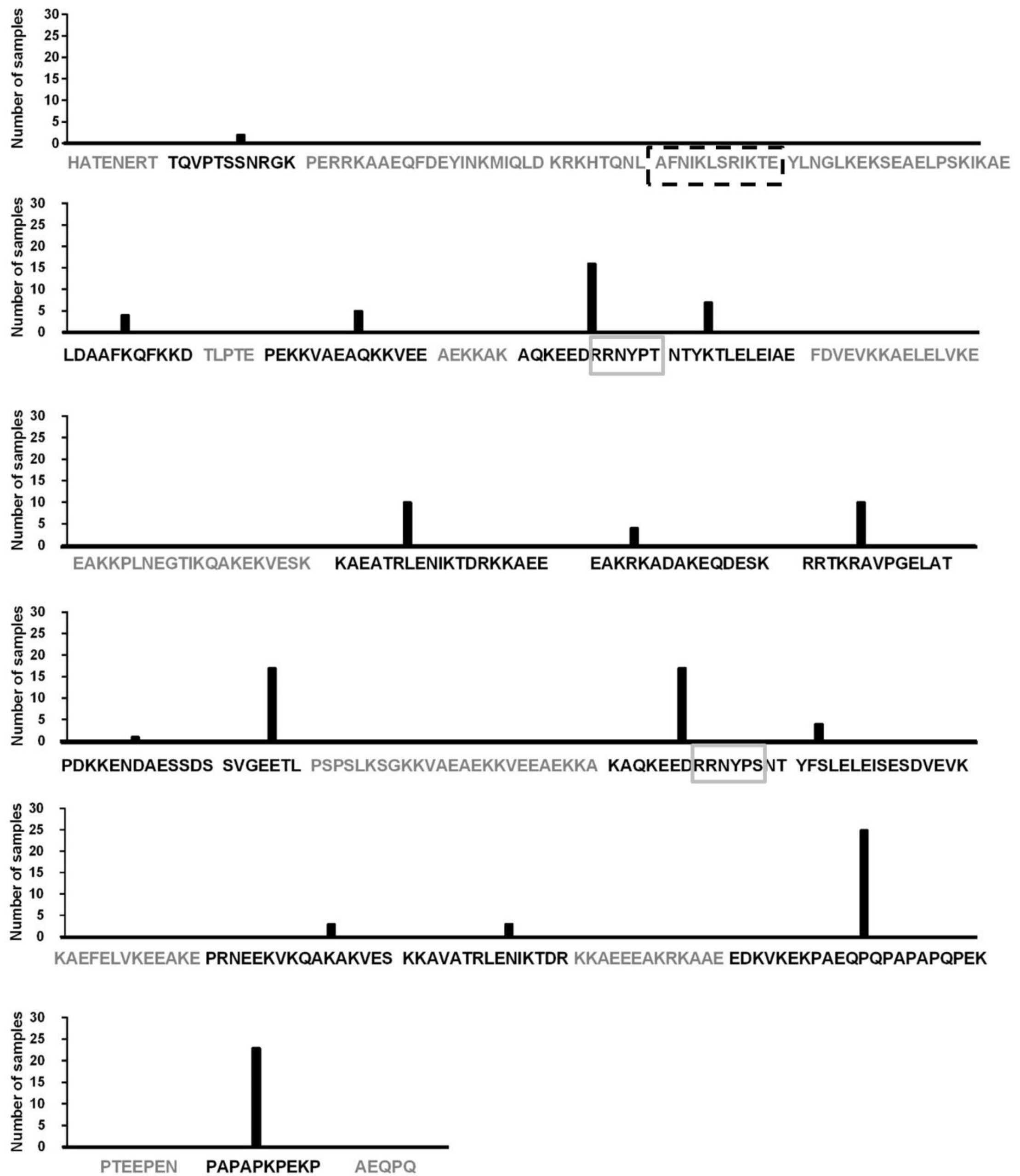


Figure 7.7: PspC epitope mapping using sera of healthy adults exposed to pneumococcus reveals the lack of antibodies to the FH binding site. The amino acid sequence of PspC group 3 variant was spotted on peptide arrays and is represented on the X axis. Peptide arrays covering the N-terminal region until the proline-rich region were probed with 29 samples from 18 volunteers. The number of samples that recognized each peptide is represented on the Y axis. Sequences of the peptides recognized by human sera are in black and the ones not recognized are in grey. Binding sites for FH and sIgA are represented in dashed black and continuous grey boxes, respectively.

PspC-FH binding is specific to humans and does not occur in mice [343,344]. We repeated the peptide arrays using sera from mice immunized with PspC3 to test the hypothesis that PspC would not be masked by binding of FH and therefore mice would produce antibodies against this region. We found that sera from immunized mice recognized the binding site for FH as well as all the other peptides already identified by human sera (Figure 7.8). These findings support our previous results and suggest that PspC-FH is as an important interaction that occurs in the nasopharynx during pneumococcal colonization.

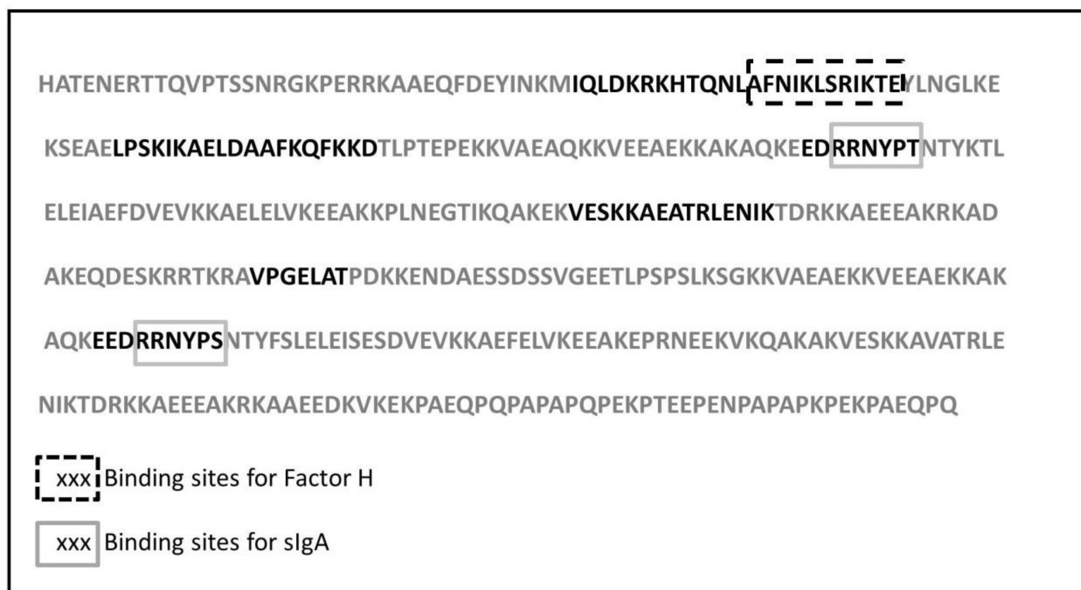


Figure 7.8: PspC epitope mapping using sera from mice immunized with recombinant PspC3 reveals the presence of antibodies to the FH binding site. Sera from mice immunized with the recombinant protein PspC3 was used to probe peptide arrays covering the N-terminal region until the proline-rich region of PspC group 3. Sequences of the peptides recognized by murine sera are shown in black and the ones not recognized are shown in grey. Binding sites for FH and sIgA are represented in dashed black and continuous grey boxes, respectively.

7.4 Discussion

We aimed to determine the role of respiratory viruses in experimental carriage establishment and examine how their presence might impact several soluble innate factors.

7.4.1 Virus was associated with increased likelihood of colonization and levels of FH

S. pneumoniae has evolved several strategies to adhere to host cells and evade host complement and immune attack. We have shown that virus co-infection was associated with three times increased odds of becoming colonized. In the presence of virus, mucosal levels of FH were increased and this may partly explain the predisposition of virally infected individuals to pneumococcal carriage in our EHPC model. Increased FH levels were also associated with increased carriage density.

Our results corroborate previous reports showing that carriage isolates presented greater binding to FH than systemic isolates [345] which supports the relevance of the pneumococcus-FH interaction at the upper airway. However, we could not observe a correlation between viral co-infection and increased colonization density. This is in contrast to findings recently published by Wolter et al. who found a relationship between increased pneumococcal colonization density and respiratory virus co-infection in South Africans being treated for acute lower respiratory tract infection [220]. This could be the result of differences in recruitment; our study included healthy, asymptomatic adults, whereas the South African study recruited hospitalized individuals of any age, including 41% less than 2 years and 51% who were HIV positive.

Using a GEE model we found an interaction between virus presence, susceptibility to colonization, increased colonization density and increased FH levels. This is the first time that asymptomatic URT viral infection has been directly associated with increased pneumococcal colonization and increased mucosal FH levels in healthy adults.

We were unable to detect FH expression in epithelial cells after *in vitro* stimulation with nasal wash samples containing virus (all values were zero). Previous transcriptome of epithelial cells have not found expression of the FH gene to be up regulated after virus infection [346]. It is therefore likely that increased FH levels at the mucosa were not

sourced by increased local expression but by leakage from blood stream into the nasal mucosa due to inflammation caused by pneumococcus virus co-infection [342].

7.4.2 Epithelial inflammation and FH binding resulted in increased pneumococcal adherence to the epithelium

We investigated the effect of nasal wash containing FH on pneumococcal adherence to non-inflamed and inflamed epithelium. We observed that treatment of pneumococcus with nasal wash containing several innate factors, including FH, promoted increased epithelial attachment and internalization. This effect was more pronounced when bacteria were treated with purified human FH or the epithelium was inflamed.

We used a pneumococcal strain lacking expression of PspC (D39 ΔPspC) to show that PspC mediates the effect of FH on epithelial adherence. Although D39 expresses PspC3 and the 6B strain used for human challenge expresses PspC9, there is a 58% similarity in the N terminal (first 110 amino acids) between the PspC amino acid sequences of both strains. With D39, increased adherence promoted by nasal wash was not only caused by existing FH in these samples. We observed no difference in adherence when nasal wash samples depleted of IgG or IgA were used to treat D39 ΔPspC and therefore the PspC-sIgA interaction was not the main mechanism for this increased adherence either. There are several pneumococcal specific host-pathogen interactions that could play a role in epithelial attachment [347]. The interaction between PspC and vitronectin has recently been highlighted as an important mechanism for pneumococcal adhesion [348].

We hypothesize that in the presence of anti-PspC IgG and low levels of FH, there is decreased pneumococcal adherence as anti-PspC antibodies block adherence mediated by other factors such as vitronectin (Figure 7.9, middle). FH continues to facilitate low levels of adherence because human anti-PspC antibodies do not recognize the FH binding site. When FH levels are high, pneumococcal adherence increases due to more interactions between PspC and FH (Figure 7.9, right). Decreased adherence caused by the binding of anti-PspC antibodies to other factors such as vitronectin is masked by this increase in Pspc-FH binding and adherence.

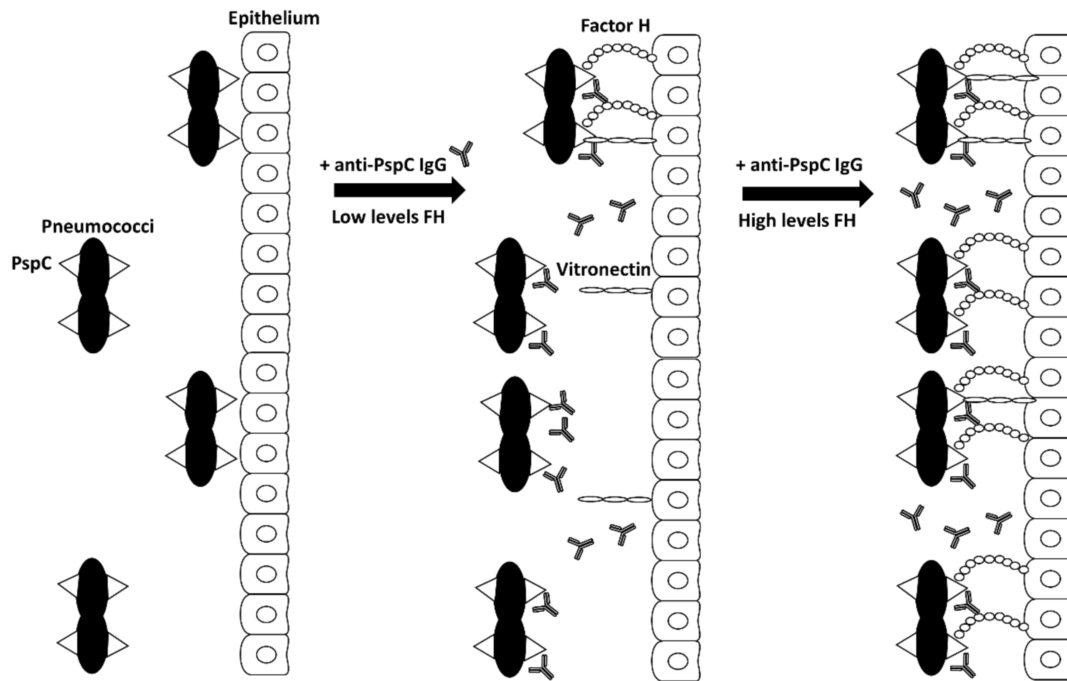


Figure 7.9: Schematic model of the proposed relationship between PspC, FH, anti-PspC IgG, and pneumococcal adherence. Left column: untreated condition – no FH or other factors such as vitronectin are present. Adherence is moderate. Middle column: in the presence of anti-PspC and low levels of FH there is decreased adherence because anti-PspC antibodies block pneumococcal adherence to the epithelium mediated by other factors, such as vitronectin. Human anti-PspC antibodies do not recognize the FH binding site of PspC and therefore the FH-PspC interaction will continue to facilitate low levels of pneumococcal adherence. Right column: in the presence of anti-PspC and high levels of FH there is increased adherence due to increased interactions between PspC and FH.

7.4.3 PspC epitope mapping revealed individuals lacked antibodies against the FH binding region

Since we observed that the PspC-FH interaction was associated with high rates and densities of carriage in humans co-infected with virus, we tested whether antibodies to the vaccine candidate PspC could block this interaction. PspC-based vaccines are protective against both invasive pulmonary disease and colonization in murine models of infection [349,350]. We observed a partial blocking of FH binding to pneumococcus by flow cytometry analysis and epithelial adherence assay. Anti-PspC IgG purified from serum blocked bacterial attachment when low levels of FH were present in the assay. These

results could be explained by the fact that healthy adults do not have antibodies specific to the PspC region that binds FH. By using sera from mice immunized with PspC in these assays we found that human carriage induces anti-PspC antibodies to the same epitopes as the ones induced by parenteral immunization with purified protein in mice. Two major differences were that i) mice did not have antibodies against the proline-rich region of PspC whilst this was one of the most recognized regions by human antibodies and ii) human antibodies did not recognize the binding site for FH on PspC whilst immunized mice did. Cross-reactive antibodies against the proline-rich region of the PspA could explain the high levels of recognition of the PspC proline-rich region in humans [351,352]. We have previously observed that colonization increased mucosal IgG antibodies to the N-terminal region of PspA but not PspC [289]. Most importantly, the fact that humans have an inefficient presentation of the PspC region that binds to FH, strongly suggests that FH binding to pneumococcus during colonization covers this site of the PspC antigen.

Our findings confirm previous reports that PspC is the major, if not the only, protein that binds to FH. PspC-FH interaction allows pneumococci to protect themselves from the complement system [353] as well as facilitate pneumococcal adherence and uptake by human epithelial cells [354]. Increased adherence to lung epithelial cells was also reported when pneumococci were pre-incubated with FH [355] and could explain the associated high burden of pneumonia following influenza infections [356].

Polymorphisms in the FH gene have been associated with increased susceptibility to *S. aureus* colonization in humans [357]. Binding to human FH has also been described as an important host-pathogen interaction for *N. meningitidis* [358] and a FH binding protein is a component in the first licensed vaccine against *N. meningitidis* serogroup B [359-361]. Point mutations that eliminate FH binding have been shown to enhance protective antibody responses to vaccination using this meningococcal FH binding protein [362]. Our results support the use of PspC as a mucosal vaccine candidate and highlight that mutations in the FH binding site which allow antibody generation against this region should be considered for any vaccine based on PspC. Blocking PspC-FH interaction with specific PspC antibodies at the mucosa has the potential to reduce viral associated pneumococcal colonization and burden of pneumonia associated with viral infections.

Chapter 8

General discussion

8.1 Introduction

Experimental human pneumococcal carriage offers a plausible model of natural carriage and a potential model for use in vaccine development. The technique is valuable but complex and involves clinical risk by introducing a pathogen into a human. A model of carriage will allow accurate determination of the immunological correlates of protection, the immunizing effect of carriage and the effect of host pressure on the pathogen in the nasopharyngeal niche. Further, methods of carriage detection used in epidemiologic studies, and including vaccine studies, can be compared.

This thesis had three aims:

1. To further develop an experimental human pneumococcal carriage model
2. To discover factors associated with experimental carriage
3. To explore the density and duration of experimental carriage

8.2 Summary and discussion of findings

8.2.1 Development of an experimental human pneumococcal carriage model

We have developed a model of experimental human pneumococcal carriage using *S. pneumoniae* serotype 6B. Carriage establishment was dose-dependent until 4×10^4

CFU/naris, after which carriage rates stabilized around 50% up to a dose of 3.2×10^5 CFU/naris. Carriage rates for serotype 23F remained at or below 10%, regardless of dose. Carriage density was independent of dose for serotype 6B and could not be determined for serotype 23F because there was minimal carriage. The lack of a relationship between challenge dose and carriage density reassured us that higher doses did not result in increased density and thus, increased risk of disease and transmission. An independent relationship between carriage density and challenge dose was previously shown in an adult mouse model of colonization and we have now demonstrated it in humans [268].

The natural carriage rate during the initial screening process of the Dose-Ranging study was 11% (18/157). This is similar to what was found in the UK from 2001-2002 [6]. However, a recent study of children and their household contacts attending the same general practices in Hertfordshire, UK found that adult carriage rates have dropped from 7.6% to 3.4% following the introduction of PCV13 into the UK childhood vaccination schedule [302]. This decrease may not be evident in our study because our population was largely made up of university students who have less contact with vaccinated children than parents, especially as contact with children was a study exclusion criterion.

In this model, the dose (10^4) required for 50% of volunteers to become colonized was lower than what is used in animal models of carriage (10^{5-7}) and resulted in safe, sustained carriage [159,268]. The next step is to examine the dose-dependency of a number of serotypes, both ones common in carriage and ones more often found in disease.

8.2.1.1 Experimental carriage was reproducible

We demonstrated that a carriage rate of 50% was reproducible using a target dose of 8×10^4 CFU/naris. By combining carriage rates from the Dose-Ranging study above 4×10^4 CFU with the carriage rate from the Reproducibility study, we achieved a mean carriage rate of 52% with a 95% confidence interval of 40-64%. Reproducibility was very important for the model to be successful as a mechanism for novel vaccine testing.

One month after challenge in the Reproducibility study, 50% of carriage positive volunteers had cleared carriage. In those still carrying at day 28, carriage density was stable. This duration is slightly longer than the 27 day median carriage duration seen by Högberg et al. [8] in Swedish adults. However, because we gave antibiotics at day 28 to eliminate carriage, we don't know the true length of the experimental carriage episodes. It is possible the

duration is more comparable to the USA study which reported duration of experimental carriage was between 27 and 122 days [161].

8.2.1.2 Experimental carriage was not symptomatic

Experimental carriage was not symptomatic. Regardless of whether volunteers passively reported symptoms to the clinical team or actively filled out a daily symptom log, there was no difference in the presence of nasal symptoms between carriers and non-carriers. There was also no difference in the percentage of volunteers that complained of symptoms following challenge with saline as compared to those challenged with pneumococci.

Although we did not see a correlation between symptoms and experimental carriage in adults, a number of studies have shown that carriage is symptomatic in children. In Kenyan children, URT symptoms, cough or coryza have been shown to be significant risk factors for carriage of pneumococcus, with coryza also a risk factor for *H. influenzae* carriage [304,305]. However, as these were both cross-sectional studies it is not known whether colonization resulted in symptoms or the presence of virus resulted in colonization.

Recently, the relationship between rhinorrhea, colonization, and viral infections were examined in children attending daycare [306]. Rhinitis symptoms were associated with *H. influenzae* presence and density but not with *S. pneumoniae*. Nonetheless there was a significant association between both viral presence and load and pneumococcal presence and density. The alterations in the nasopharynx during viral infection may increase the detection of pneumococci, as well as the density, leading to an increase in transmission and may explain why symptomatic children who are heavily colonized with pneumococci are such spreaders.

8.2.1.3 Experimental carriage was protective against reacquisition of the same serotype

We have shown that a single carriage episode was protective against subsequent carriage of the same serotype. Volunteers that were re-challenged with serotype 6B up to 48 weeks after an initial carriage episode with 6B were not colonized (0% carriage rate). In comparison, only 50% of volunteers were protected against challenge with serotype 6B following clearance of a natural carriage episode, the same rate as primary study volunteers.

We have since examined the immune response behind protection from reacquisition in healthy adults and found that experimental carriage increased both mucosal and systemic IgG levels to pneumococcal capsule and proteins [289]. These responses protected mice against invasive disease in a serotype-independent manner following passive transfer of sera from colonized individuals. However, two of the ten volunteers challenged with the homologous serotype 6B did not have increased levels of IgG, suggesting protection requires more than capsular and protein antibodies and lends support to a role for mucosal CD4+ T cells in protection against carriage, as has been shown in mouse models [156]. This is also consistent with a mathematical model proposed by Cobey and Lipsitch [309] in which anticapsular immunity acquired during colonization is weak and only confers 30 to 60% reduction in susceptibility to future carriage of that type.

The difference in carriage reacquisition after heterologous challenge, as compared to after homologous challenge, is likely a result of individual differences in immunity although we can't exclude the effect of cross-protection between pneumococcal proteins. To further examine the differences related to reacquisition, the levels of IgG to polysaccharides and proteins prior to re-challenge in the four volunteers could be measured. It would also be informative to compare the levels between those that carried and those that didn't, irrespective of serotype. As the number of natural carriers that were re-challenged was quite small, increasing the numbers would enhance these findings.

8.2.1.3.1 Previous carriage was not associated with reduced density of subsequent carriage

There was no difference in carriage density between a recent natural carriage episode and an experimental carriage episode. As not all serotypes alter future carriage acquisition or duration, it is likely that there are also differences between serotypes when it comes to carriage density. Therefore it is not entirely surprising that recent carriage did not impact secondary carriage density, especially since challenge was with a different serotype. We couldn't examine if challenge with the homologous serotype would have the same result because it did not result in carriage however, this has been performed in a mouse model of colonization. Using the same strain for both primary challenge and subsequent re-challenge, there was no difference in colonization density between two consecutive carriage episodes [140]. Combining our human results with the mouse data, we suggest that what determines carriage density is the result of a delicate balance within the microenvironment of the nasopharynx and may be impacted by the microbial flora. While

colonization establishment may be determined by the immune system, it is possible that carriage density is a function of nutrient availability and is not related to previous carriage.

To further explore how density is altered by reacquisition, successive carriage episodes would need to be evaluated. This should include challenge with different serotypes and, if possible, the homologous serotype. As we were not able to establish carriage with the homologous type, this may be difficult but could be attempted using a different strain or by re-challenging more than a year after the first carriage episode. If density is influenced by the microbial flora, a better understanding of the nasopharyngeal microbiota, both before, during, and after carriage, may further inform on carriage density.

8.2.2 Factors involved in establishment of experimental carriage

8.2.2.1 Bacteriological differences between the 6B and 23F inoculum strains

We investigated differences between the 6B and 23F serotypes used in the model in an effort to determine why 6B successfully established carriage and 23F did not (Table 8.1). Transparent colonies were the dominant phenotype in the 6B inoculum stock however, the opacity of the 23F inoculum could not be determined. The 23F strain was more susceptible to complement deposition than the 6B strain. When we compared the whole genome sequences of the 6B and 23F strains we found a mutation in the *pcpA* gene of 23F as well as a frameshift mutation in *amiC*. The 23F inoculum strain with the mutations was less likely to adhere to nasopharyngeal cells than both the 6B strain and a derivative 23F strain that had a full length *amiC*. In a mouse model of colonization, the 23F inoculum with the *amiC* mutation did not carry whereas the derivative isolate with a full length *amiC* did.

Table 8.1 Summary of investigated differences between the 6B and 23F inoculum strains

		6B (BHN418)	23F (P833)
Phase phenotype	Transparent	++	indeterminate
	Opaque	+	indeterminate
Complement deposition		+	++
Genes involved in adherence and nutrient uptake	*PcpA*	+	-
	AmiC	+	-
Adherence (D562 cells)		++	+
Colonization in a mouse model		+	-
Experimental colonization in humans		+++	+

+/- represents presence/absence, >1 + indicates a larger difference

The first difference between the two inoculum serotypes listed in Table 8.1 is the phenotype. The 6B phase phenotype was largely transparent however, the phenotype of the 23F strain could not be characterized due to a high degree of autolysis. Transparent colonies have been shown to have higher rates of autolysis than opaque variants [45], which suggests that the 23F strain was also transparent. Since transparent variants are better at colonizing the mouse nasopharynx than opaque variants, it is unlikely that phenotype is the reason for the different carriage rates of the 6B and 23F strains. However, excluding phenotype does not exclude the capsule itself. It is possible that the amount of capsule may be a contributing factor to the different colonization rates. Capsule size should be determined for both serotypes.

The second major difference between the two strains was complement deposition, with more complement deposited on 23F than on 6B. An experimental chinchilla model of acute otitis media was used to compare high- and low-level C3-binding of 6A isolates. Isolates with low-level C3-binding were significantly more likely to cause experimental otitis media than high-level C3-binding isolates [322]. However, when the animals were challenged intranasally with either isolate, there was no difference in the density of colonization between the groups suggesting that nasopharyngeal colonization is independent of C3 surface binding in chinchillas. If colonization in humans is also independent of complement deposition, this would suggest that the increased complement deposition on 23F was not the reason for the different colonization rates.

The third major difference between the 6B and 23F inoculum strains were the mutations in the *amiC* and *pcpA* genes of 23F. It is likely that these mutations led to the differences in adherence and in colonization, both in the mouse and human models. In order to better understand the role of *pcpA* and *amiC* in human carriage and to determine which gene was responsible for the reduced carriage of serotype 23F, both mutations need to be repaired to wild-type in the 23F inoculum strain. In parallel, knockout mutants of both genes should be created in the 6B inoculum strain. These strains should then be tested in both a mouse and human model of colonization.

These experiments will address if either gene was the cause of the reduced 23F carriage rate however, they will not determine if the role of *amiC* in colonization is related to its involvement in nutrient transport or adherence. Pneumococci have complex growth requirements and are auxotrophic for a number of amino acids [325]. Acquisition of the necessary amino acids commonly occurs through oligopeptide uptake and can be mediated through the permease Ami [326]. The mutation in *amiC* could be limiting the 23F strain's access to nutrients or causing an inability to compete with the commensal flora for nutrients, resulting in a low colonization rate. We hypothesize that the ability of the mutant to establish colonization may relate to the diversity of the nasopharyngeal microbiome. If the nasopharyngeal flora was able to alter the metabolic composition in the nasopharynx, the necessity for oligopeptide transport via the Ami-AliA/AliB permease may have been diminished and the strain's ability to colonize restored. Early analysis of the pre-inoculation microbiome of two 23F carriage positive volunteers found an abundance of *Prevotella* species was positively associated with carriage establishment (Amelieke Cremers, unpublished data).

In line with this finding, Hathaway et al. [327] recently showed that the AliB-like ORF 2 of non-encapsulated *S. pneumoniae* binds a peptide found in *Prevotella* species. It is suggested that this interaction then mediates early colonization. If this role in colonization could be extended to encapsulated pneumococci, it could explain the association between 23F carriage establishment and increased presence of *Prevotella* species however, no definitive conclusions can be drawn due to the small number of carriage positive samples in this study. To determine if an *amiC* mutant could survive in the nasopharynx as a result of specific species in the microbiome, namely *Prevotella*, nasal wash fluid could be used as a medium to grow *amiC* wild-type and mutant strains. If growth of the mutant strains is inhibited as compared to the wild-type strains, different concentrations of *Prevotella*

species could be added to the nasal wash media to determine if there is a concentration at which growth of the *amiC* mutant is restored.

8.2.2.2 Asymptomatic URT virus was associated with increased likelihood of colonization

We have shown that the presence of virus in the nasopharynx was associated with a three times increase in the odds of becoming colonized and an increase in mucosal levels of FH. However, levels of the antimicrobial factor SLPI, antimicrobial beta defensin 2, and lactoferrin were not altered following challenge. Using a GEE model, we found an interaction between virus presence, susceptibility to colonization, increased colonization density and increased FH levels. This is the first time that asymptomatic URT viral infection has been directly associated with increased pneumococcal colonization and increased mucosal FH levels in healthy adults. Using an *in vitro* epithelial model, epithelial inflammation and FH binding resulted in increased pneumococcal adherence to the epithelium. Binding was partially blocked by antibodies targeting the FH-binding protein PspC. PspC epitope mapping revealed individuals lacked antibodies against the FH binding region.

The lack of antibodies generated against the FH binding region suggests that a mutation in the FH binding site would result in antibody generation which may block the PspC-FH interaction. Pneumococcal strains with mutations in the FH binding site could be used to challenge healthy adult volunteers and the serum from colonized volunteers could be analysed for the presence of antibodies against the FH binding region.

The immunological mechanisms behind carriage are wide and varied. Although we did not see a role for the innate factors SLPI, β-defensin 2, and lactoferrin in carriage establishment or clearance, Neill et al. [363] have shown that levels of transforming growth factor (TGF)-β1 and interleukin (IL)-10 are higher in volunteers that establish pneumococcal carriage following challenge, than in those who don’t. The role of cytokines in carriage establishment/clearance requires further investigation.

8.2.3 Density and duration of experimental carriage

We compared culture and qPCR in the detection of pneumococci and found that the proportion of samples positive for carriage by qPCR was significantly more than the proportion of samples positive for carriage by culture. Across all visits, the number of

volunteers positive for carriage by qPCR was more than those positive for carriage by culture, but this was only significant at 2 days post-challenge.

We have shown that quantification of pneumococci by culture and qPCR was positively correlated. At a qPCR density greater than 10^3 copies/ml, 94.8% of samples were both culture and qPCR positive. Below the qPCR limit of detection, 7.7% of samples were still positive by culture.

We also used culture and qPCR to compare experimental and natural carriage and found that fluctuations in density during a carriage episode were similar for both experimentally and naturally colonized volunteers. The two methods were complementary when determining the length of a carriage episode. However, because we only followed volunteers for one month, we don't know the true length of an experimental carriage episode using this model. Duration could easily be determined by following carriage positive volunteers until they naturally cleared carriage, instead of having them take antibiotics after 28 days.

As discussed in Chapter 1 section 1.6.1.2, a recent study by Trzciński et al. [248] suggested that the trans-oral swab may be better than the nasopharyngeal swab in detecting carriage in adults. We have taken trans-oral swabs alongside nasal washes as part of a recent study and will compare the detection of pneumococci using both conventional and molecular methods. Although pneumococcal quantification by culture and qPCR was positively correlated, there was a difference between the two methods at detecting low density carriage (Table 6.2). Including an enrichment step might decrease the number of culture negative, qPCR positive samples however, this would have to be performed after the initial quantification by culture and qPCR.

8.3 Implications

Although this experimental carriage model has been very successful in adults, it would be unsuitable for use in children because of the higher incidence of disease. It is also unknown if showing a decrease in carriage in adults after vaccination would be predictive of a similar decrease in children, the elderly, or the immunocompromised. Because the immune system and nasopharyngeal flora of every person differs, a level of variability is introduced into the model which requires careful consideration when screening volunteers and determining sample sizes.

8.3.1 Implications for testing novel vaccines and vaccine development

Current pneumococcal conjugate vaccines have greatly decreased the incidence of IPD, more through herd protection than through a direct effect on immunized individuals. But this decrease has led to an increase in non-vaccine serotypes. Further serotype replacement, as well as high production cost and a lack of serotype diversity, makes global implementation of conjugate vaccines difficult. New vaccines are urgently needed to overcome the problems with conjugate vaccines. The safe and reproducible experimental carriage model presented here could be used to down-select candidate pneumococcal vaccines with carriage prevention as a surrogate of vaccine-induced immunity.

For the first time, we have shown that asymptomatic URT viral infection is directly associated with increased pneumococcal colonization and increased mucosal FH levels in healthy adults. We suggest that FH binding to PspC *in vivo* masks the FH binding region, enabling FH to facilitate pneumococcal attachment to the epithelium during viral infection despite the presence of anti-PspC antibodies. A PspC vaccine that blocks the PspC-FH interaction with specific PspC antibodies at the mucosa could reduce pneumococcal colonization and may have enhanced protection in those with underlying viral infection.

8.3.2 Implications for detection of pneumococcal carriage

The suggestion that colonization is a good endpoint in vaccine trials is based on the idea that it is relatively easy to measure and is more common than disease, therefore requiring a much smaller sample size [282,284,333]. However, if the endpoint is a reduction in carriage density or duration, sensitive methods to accurately detect these changes will be important in estimating vaccine effects. We demonstrated that culture and qPCR were complementary in the detection of carriage density and duration. This will be important when novel vaccines are tested using experimental carriage and detection of carriage density or duration is crucial to the success of the trial.

8.4 Future work

8.4.1 Novel vaccine testing

We have developed a safe and reproducible model of experimental human pneumococcal carriage that could be used as a vaccine development tool. The first planned test of the model is a double-blind, randomized, controlled trial testing the protective effect of the current conjugate vaccine PCV13 (Prevenar) against colonization in healthy adult volunteers. Following that trial, we plan to test novel vaccine candidates for protection against acquisition of pneumococcus in healthy adults.

8.4.2 Pneumococcal biology

We have shown the experimental carriage model works well with a serotype 6B strain. To increase the scope of the model, a dose-response curve for a number of serotypes should be completed. Serotypes that cause carriage and are clinically relevant, as well as serotypes that have variable capsular expression should be included. We have shown that carriage differences might not be due to serotype alone so sequence type and genetic background should also be considered when choosing which serotypes to test.

The role of specific genes and virulence factors in human carriage can be examined by challenging with auxotrophic mutants. There is a precedent for using genetically modified bacteria in human challenge studies; the role of the staphylococcal cell-wall protein clumping factor B in colonization was investigated by intra-nasally challenging human volunteers with a wild-type *S. aureus* strain and a single-locus knockout mutant [276].

The idea of a microbiological bottleneck - in which disease is a monoclonal event [319] - is critical in pneumococcal transmission and, hence, vaccine escape. To try and answer this, healthy adults could be challenged with a mixed strain inoculum, containing similar and diverse strains, using sequencing to determine strain recovery. Mixed inoculation could also be used to investigate intraspecies competition, comparing different capsular and protein expressing strains.

8.4.3 Host susceptibility to experimental carriage

We have demonstrated successful experimental carriage in healthy adult volunteers however, healthy adults do not have high rates of carriage or disease and are not the target population of current vaccination strategies. The elderly, in comparison, have low carriage rates but high disease incidence. To better understand changes in susceptibility to carriage

associated with increasing age, an elderly cohort should be challenged with pneumococcus and the immunological response induced by carriage should be analysed.

Because carriage rates and disease incidence also vary by geographic area, it is important to test the model in different locations, such as Africa. In these studies, the environment (such as exposure to smoke during cooking) and co-morbidities (such as HIV status) may alter the outcome of the model and need to be taken into consideration.

Developing a successful experimental carriage model in these groups will allow novel vaccine candidates to be tested in some of the most-at-risk populations.

8.5 Conclusion

We have presented here a model of experimental human pneumococcal carriage that is safe and reproducible. In trying to determine what predicts experimental carriage, we have shown that mucosal innate factors are likely not involved, however the presence of virus increases the risk of becoming colonized. The model presented here could be used to test novel vaccines with experimental carriage as an endpoint, and to continue investigating the host and bacterial factors involved in establishing experimental human pneumococcal carriage.

References

1. Kamerling JP. Pneumococcal polysaccharides: a chemical view. In: Tomasz A (2000) *Streptococcus pneumoniae*: molecular biology & mechanisms of disease: Mary Ann Liebert.
2. Reingold A, Cutts, F., Kamau, T., Levine, O., O'Brien, K., Ignacio Santos Preciado, J., Schrag, S. (2006) Detailed review paper on pneumococcal conjugate vaccine: presented to the WHO Strategic Group of Experts on Immunization.
3. WHO (2012) Pneumococcal vaccines WHO position paper - 2012 - Recommendations. Vaccine 30: 4717-4718.
4. Hill PC, Cheung YB, Akisanya A, Sankareh K, Lahai G, et al. (2008) Nasopharyngeal carriage of *Streptococcus pneumoniae* in Gambian infants: a longitudinal study. Clin Infect Dis 46: 807-814.
5. Regev-Yochay G, Raz M, Dagan R, Porat N, Shainberg B, et al. (2004) Nasopharyngeal carriage of *Streptococcus pneumoniae* by adults and children in community and family settings. Clin Infect Dis 38: 632-639.
6. Hussain M, Melegaro A, Pebody RG, George R, Edmunds WJ, et al. (2005) A longitudinal household study of *Streptococcus pneumoniae* nasopharyngeal carriage in a UK setting. Epidemiol Infect 133: 891-898.
7. Gray BM, Converse GM, 3rd, Dillon HC, Jr. (1980) Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. J Infect Dis 142: 923-933.
8. Hogberg L, Geli P, Ringberg H, Melander E, Lipsitch M, et al. (2007) Age- and serogroup-related differences in observed durations of nasopharyngeal carriage of penicillin-resistant pneumococci. J Clin Microbiol 45: 948-952.
9. Sleeman KL, Griffiths D, Shackley F, Diggle L, Gupta S, et al. (2006) Capsular serotype-specific attack rates and duration of carriage of *Streptococcus pneumoniae* in a population of children. J Infect Dis 194: 682-688.
10. Smith T, Lehmann D, Montgomery J, Gratten M, Riley ID, et al. (1993) Acquisition and invasiveness of different serotypes of *Streptococcus pneumoniae* in young children. Epidemiol Infect 111: 27-39.
11. Bogaert D, De Groot R, Hermans PW (2004) *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet Infect Dis 4: 144-154.
12. Ghaffar F, Friedland IR, McCracken GH, Jr. (1999) Dynamics of nasopharyngeal colonization by *Streptococcus pneumoniae*. Pediatr Infect Dis J 18: 638-646.
13. O'Brien KL, Santosham M (2004) Potential impact of conjugate pneumococcal vaccines on pediatric pneumococcal diseases. Am J Epidemiol 159: 634-644.
14. Bogaert D, Engelen MN, Timmers-Reker AJ, Elzenaar KP, Peerbooms PG, et al. (2001) Pneumococcal carriage in children in The Netherlands: a molecular epidemiological study. J Clin Microbiol 39: 3316-3320.
15. Greenberg D, Givon-Lavi N, Broides A, Blancovich I, Peled N, et al. (2006) The contribution of smoking and exposure to tobacco smoke to *Streptococcus pneumoniae* and *Haemophilus influenzae* carriage in children and their mothers. Clin Infect Dis 42: 897-903.
16. Bogaert D, van Belkum A, Sluijter M, Luijendijk A, de Groot R, et al. (2004) Colonisation by *Streptococcus pneumoniae* and *Staphylococcus aureus* in healthy children. Lancet 363: 1871-1872.

17. Coles CL, Kanungo R, Rahmathullah L, Thulasiraj RD, Katz J, et al. (2001) Pneumococcal nasopharyngeal colonization in young South Indian infants. Pediatr Infect Dis J 20: 289-295.
18. Mackenzie GA, Leach AJ, Carapetis JR, Fisher J, Morris PS (2010) Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults: cross-sectional surveys in a population with high rates of pneumococcal disease. BMC Infect Dis 10: 304.
19. Principi N, Marchisio P, Schito GC, Mannelli S (1999) Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Ascanius Project Collaborative Group. Pediatr Infect Dis J 18: 517-523.
20. McCullers JA, McAuley JL, Browall S, Iverson AR, Boyd KL, et al. (2010) Influenza enhances susceptibility to natural acquisition of and disease due to *Streptococcus pneumoniae* in ferrets. J Infect Dis 202: 1287-1295.
21. Reisman J, Rudolph K, Bruden D, Hurlburt D, Bruce MG, et al. (2014) Risk factors for pneumococcal colonization of the nasopharynx in Alaska native adults and children J Ped Infect Dis 3: 104-111.
22. Stubbs E, Hare K, Wilson C, Morris P, Leach AJ (2005) *Streptococcus pneumoniae* and noncapsular *Haemophilus influenzae* nasal carriage and hand contamination in children: a comparison of two populations at risk of otitis media. Pediatr Infect Dis J 24: 423-428.
23. Levine H, Zarka S, Dagan R, Sela T, Rozhavski V, et al. (2012) Transmission of *Streptococcus pneumoniae* in adults may occur through saliva. Epidemiol Infect 140: 561-565.
24. Isaacman DJ, McIntosh ED, Reinert RR (2010) Burden of invasive pneumococcal disease and serotype distribution among *Streptococcus pneumoniae* isolates in young children in Europe: impact of the 7-valent pneumococcal conjugate vaccine and considerations for future conjugate vaccines. Int J Infect Dis 14: e197-209.
25. Pilishvili T, Lexau C, Farley MM, Hadler J, Harrison LH, et al. (2010) Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. J Infect Dis 201: 32-41.
26. von Gottberg A, de Gouveia L, Tempia S, Quan V, Meiring S, et al. (2014) Effects of vaccination on invasive pneumococcal disease in South Africa. N Engl J Med 371: 1889-1899.
27. WHO (2008) The Global Burden of Disease: 2004 Update. http://www.who.int/healthinfo/global_burden_disease/2004_report_update/en/index.html.
28. Jackson ML, Neuzil KM, Thompson WW, Shay DK, Yu O, et al. (2004) The burden of community-acquired pneumonia in seniors: results of a population-based study. Clin Infect Dis 39: 1642-1650.
29. Linder TE, Daniels RL, Lim DJ, DeMaria TF (1994) Effect of intranasal inoculation of *Streptococcus pneumoniae* on the structure of the surface carbohydrates of the chinchilla eustachian tube and middle ear mucosa. Microb Pathog 16: 435-441.
30. van der Ven LT, van den Dobbelsteen GP, Nagarajah B, van Dijken H, Dortant PM, et al. (1999) A new rat model of otitis media caused by *Streptococcus pneumoniae*: conditions and application in immunization protocols. Infect Immun 67: 6098-6103.
31. Peltola VT, Boyd KL, McAuley JL, Rehg JE, McCullers JA (2006) Bacterial sinusitis and otitis media following influenza virus infection in ferrets. Infect Immun 74: 2562-2567.
32. Kadioglu A, Gingles NA, Grattan K, Kerr A, Mitchell TJ, et al. (2000) Host cellular immune response to pneumococcal lung infection in mice. Infect Immun 68: 492-501.

33. Ogunniyi AD, LeMessurier KS, Graham RM, Watt JM, Briles DE, et al. (2007) Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of *Streptococcus pneumoniae* D39 in a mouse model. Infect Immun 75: 1843-1851.
34. Syrjanen RK, Auranen KJ, Leino TM, Kilpi TM, Makela PH (2005) Pneumococcal acute otitis media in relation to pneumococcal nasopharyngeal carriage. Pediatr Infect Dis J 24: 801-806.
35. Brueggemann AB, Spratt BG (2003) Geographic distribution and clonal diversity of *Streptococcus pneumoniae* serotype 1 isolates. J Clin Microbiol 41: 4966-4970.
36. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, et al. (2003) Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. J Infect Dis 187: 1424-1432.
37. Weinberger DM, Trzcinski K, Lu YJ, Bogaert D, Brandes A, et al. (2009) Pneumococcal capsular polysaccharide structure predicts serotype prevalence. PLoS Pathog 5: e1000476.
38. Li Y, Weinberger DM, Thompson CM, Trzcinski K, Lipsitch M (2013) Surface charge of *Streptococcus pneumoniae* predicts serotype distribution. Infect Immun 81: 4519-4524.
39. Nelson AL, Roche AM, Gould JM, Chim K, Ratner AJ, et al. (2007) Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. Infect Immun 75: 83-90.
40. Wood WB, Jr., Smith MR (1949) The inhibition of surface phagocytosis by the capsular slime layer of pneumococcus type III. J Exp Med 90: 85-96.
41. Magee AD, Yother J (2001) Requirement for capsule in colonization by *Streptococcus pneumoniae*. Infect Immun 69: 3755-3761.
42. Malley R, Lipsitch M, Stack A, Saladino R, Fleisher G, et al. (2001) Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. Infect Immun 69: 4870-4873.
43. Winkelstein JA, Abramovitz AS, Tomasz A (1980) Activation of C3 via the alternative complement pathway results in fixation of C3b to the pneumococcal cell wall. J Immunol 124: 2502-2506.
44. Talbot UM, Paton AW, Paton JC (1996) Uptake of *Streptococcus pneumoniae* by respiratory epithelial cells. Infect Immun 64: 3772-3777.
45. Weiser JN, Austrian R, Sreenivasan PK, Masure HR (1994) Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. Infect Immun 62: 2582-2589.
46. Kim JO, Weiser JN (1998) Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. J Infect Dis 177: 368-377.
47. Cundell DR, Weiser JN, Shen J, Young A, Tuomanen EI (1995) Relationship between colonial morphology and adherence of *Streptococcus pneumoniae*. Infect Immun 63: 757-761.
48. Hammerschmidt S, Wolff S, Hocke A, Rosseau S, Muller E, et al. (2005) Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. Infect Immun 73: 4653-4667.
49. Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila I, Tuomanen EI (1995) *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. Nature 377: 435-438.

50. Romero-Steiner S, Pilishvili T, Sampson JS, Johnson SE, Stinson A, et al. (2003) Inhibition of pneumococcal adherence to human nasopharyngeal epithelial cells by anti-PsaA antibodies. Clin Diagn Lab Immunol 10: 246-251.
51. Hammerschmidt S (2006) Adherence molecules of pathogenic pneumococci. Curr Opin Microbiol 9: 12-20.
52. Andersson B, Dahmen J, Frejd T, Leffler H, Magnusson G, et al. (1983) Identification of an active disaccharide unit of a glycoconjugate receptor for pneumococci attaching to human pharyngeal epithelial cells. J Exp Med 158: 559-570.
53. Krivan HC, Roberts DD, Ginsburg V (1988) Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc beta 1-4Gal found in some glycolipids. Proc Natl Acad Sci U S A 85: 6157-6161.
54. Rosenow C, Ryan P, Weiser JN, Johnson S, Fontan P, et al. (1997) Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. Mol Microbiol 25: 819-829.
55. Zhang JR, Mostov KE, Lamm ME, Nanno M, Shimida S, et al. (2000) The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. Cell 102: 827-837.
56. Dave S, Carmicle S, Hammerschmidt S, Pangburn MK, McDaniel LS (2004) Dual roles of PspC, a surface protein of *Streptococcus pneumoniae*, in binding human secretory IgA and factor H. J Immunol 173: 471-477.
57. Quin LR, Carmicle S, Dave S, Pangburn MK, Evenhuis JP, et al. (2005) In vivo binding of complement regulator factor H by *Streptococcus pneumoniae*. J Infect Dis 192: 1996-2003.
58. Hammerschmidt S, Agarwal V, Kunert A, Haelbich S, Skerka C, et al. (2007) The host immune regulator factor H interacts via two contact sites with the PspC protein of *Streptococcus pneumoniae* and mediates adhesion to host epithelial cells. J Immunol 178: 5848-5858.
59. Khan MN, Sharma SK, Filkins LM, Pichichero ME (2012) PcpA of *Streptococcus pneumoniae* mediates adherence to nasopharyngeal and lung epithelial cells and elicits functional antibodies in humans. Microbes Infect 14: 1102-1110.
60. Anderton JM, Rajam G, Romero-Steiner S, Summer S, Kowalczyk AP, et al. (2007) E-cadherin is a receptor for the common protein pneumococcal surface adhesin A (PsaA) of *Streptococcus pneumoniae*. Microb Pathog 42: 225-236.
61. Kerr AR, Adrian PV, Estevao S, de Groot R, Alloing G, et al. (2004) The Ami-AliA/AliB permease of *Streptococcus pneumoniae* is involved in nasopharyngeal colonization but not in invasive disease. Infect Immun 72: 3902-3906.
62. Cundell DR, Pearce BJ, Sandros J, Naughton AM, Masure HR (1995) Peptide permeases from *Streptococcus pneumoniae* affect adherence to eucaryotic cells. Infect Immun 63: 2493-2498.
63. Weiser JN, Bae D, Fasching C, Scamurra RW, Ratner AJ, et al. (2003) Antibody-enhanced pneumococcal adherence requires IgA1 protease. Proc Natl Acad Sci U S A 100: 4215-4220.
64. Pracht D, Elm C, Gerber J, Bergmann S, Rohde M, et al. (2005) PavA of *Streptococcus pneumoniae* modulates adherence, invasion, and meningeal inflammation. Infect Immun 73: 2680-2689.
65. Kadioglu A, Brewin H, Hartel T, Brittan JL, Klein M, et al. (2010) Pneumococcal protein PavA is important for nasopharyngeal carriage and development of sepsis. Mol Oral Microbiol 25: 50-60.
66. Bergmann S, Rohde M, Chhatwal GS, Hammerschmidt S (2001) alpha-Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. Mol Microbiol 40: 1273-1287.

67. Bergmann S, Lang A, Rohde M, Agarwal V, Rennemeier C, et al. (2009) Integrin-linked kinase is required for vitronectin-mediated internalization of *Streptococcus pneumoniae* by host cells. J Cell Sci 122: 256-267.
68. Voss S, Hallstrom T, Saleh M, Burchhardt G, Pribyl T, et al. (2013) The choline-binding protein PspC of *Streptococcus pneumoniae* interacts with the C-terminal heparin-binding domain of vitronectin. J Biol Chem 288: 15614-15627.
69. Tong HH, Blue LE, James MA, DeMaria TF (2000) Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. Infect Immun 68: 921-924.
70. Manco S, Hernon F, Yesilkaya H, Paton JC, Andrew PW, et al. (2006) Pneumococcal neuraminidases A and B both have essential roles during infection of the respiratory tract and sepsis. Infect Immun 74: 4014-4020.
71. McCullers JA, Bartmess KC (2003) Role of neuraminidase in lethal synergism between influenza virus and *Streptococcus pneumoniae*. J Infect Dis 187: 1000-1009.
72. Peltola VT, Murti KG, McCullers JA (2005) Influenza virus neuraminidase contributes to secondary bacterial pneumonia. J Infect Dis 192: 249-257.
73. Ausubel FM (2005) Are innate immune signaling pathways in plants and animals conserved? Nat Immunol 6: 973-979.
74. Travis SM, Singh PK, Welsh MJ (2001) Antimicrobial peptides and proteins in the innate defense of the airway surface. Curr Opin Immunol 13: 89-95.
75. Lee HY, Andalibi A, Webster P, Moon SK, Teufert K, et al. (2004) Antimicrobial activity of innate immune molecules against *Streptococcus pneumoniae*, Moraxella catarrhalis and nontypeable *Haemophilus influenzae*. BMC Infect Dis 4: 12.
76. Davis KM, Akinbi HT, Standish AJ, Weiser JN (2008) Resistance to mucosal lysozyme compensates for the fitness deficit of peptidoglycan modifications by *Streptococcus pneumoniae*. PLoS Pathog 4: e1000241.
77. Ward PP, Conneely OM (2004) Lactoferrin: role in iron homeostasis and host defense against microbial infection. Biometals 17: 203-208.
78. Shaper M, Hollingshead SK, Benjamin WH, Jr., Briles DE (2004) PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin [corrected]. Infect Immun 72: 5031-5040.
79. Scott A, Weldon S, Taggart CC (2011) SLPI and elafin: multifunctional antiproteases of the WFDC family. Biochem Soc Trans 39: 1437-1440.
80. Ganz T (2003) Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol 3: 710-720.
81. Beiter K, Wartha F, Hurwitz R, Normark S, Zychlinsky A, et al. (2008) The capsule sensitizes *Streptococcus pneumoniae* to alpha-defensins human neutrophil proteins 1 to 3. Infect Immun 76: 3710-3716.
82. Scharf S, Zahlten J, Szymanski K, Hippenstiel S, Suttorp N, et al. (2012) *Streptococcus pneumoniae* induces human beta-defensin-2 and -3 in human lung epithelium. Exp Lung Res 38: 100-110.
83. Steinstraesser L, Kraneburg U, Jacobsen F, Al-Benna S (2011) Host defense peptides and their antimicrobial-immunomodulatory duality. Immunobiology 216: 322-333.
84. Doss M, White MR, Tecle T, Hartshorn KL (2010) Human defensins and LL-37 in mucosal immunity. J Leukoc Biol 87: 79-92.
85. Cruse G, Fernandes VE, de Salort J, Pankhania D, Marinas MS, et al. (2010) Human lung mast cells mediate pneumococcal cell death in response to activation by pneumolysin. J Immunol 184: 7108-7115.

86. Lawson PR, Reid KB (2000) The roles of surfactant proteins A and D in innate immunity. Immunol Rev 173: 66-78.
87. Jounblat R, Clark H, Eggleton P, Hawgood S, Andrew PW, et al. (2005) The role of surfactant protein D in the colonisation of the respiratory tract and onset of bacteraemia during pneumococcal pneumonia. Respir Res 6: 126.
88. Janeway CA, Jr., Medzhitov R (2002) Innate immune recognition. Annu Rev Immunol 20: 197-216.
89. Murphy KP, Janeway C, Travers P, Walport M, Mowat A, et al. (2012) Janeway's Immunobiology. New York: Garland Science. xix, 868 p. p.
90. Walport MJ (2001) Complement. First of two parts. N Engl J Med 344: 1058-1066.
91. Brown JS, Hussell T, Gilliland SM, Holden DW, Paton JC, et al. (2002) The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. Proc Natl Acad Sci U S A 99: 16969-16974.
92. Volanakis JE, Kaplan MH (1971) Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. Proc Soc Exp Biol Med 136: 612-614.
93. Yuste J, Botto M, Bottoms SE, Brown JS (2007) Serum amyloid P aids complement-mediated immunity to *Streptococcus pneumoniae*. PLoS Pathog 3: 1208-1219.
94. Szalai AJ, Briles DE, Volanakis JE (1995) Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in transgenic mice. J Immunol 155: 2557-2563.
95. Kang YS, Do Y, Lee HK, Park SH, Cheong C, et al. (2006) A dominant complement fixation pathway for pneumococcal polysaccharides initiated by SIGN-R1 interacting with C1q. Cell 125: 47-58.
96. Winkelstein JA, Tomasz A (1978) Activation of the alternative complement pathway by pneumococcal cell wall teichoic acid. J Immunol 120: 174-178.
97. Hummell DS, Swift AJ, Tomasz A, Winkelstein JA (1985) Activation of the alternative complement pathway by pneumococcal lipoteichoic acid. Infect Immun 47: 384-387.
98. Bruyn GA, Zegers BJ, van Furth R (1992) Mechanisms of host defense against infection with *Streptococcus pneumoniae*. Clin Infect Dis 14: 251-262.
99. Fujita T (2002) Evolution of the lectin-complement pathway and its role in innate immunity. Nat Rev Immunol 2: 346-353.
100. Hansen S, Selman L, Palaniyar N, Ziegler K, Brandt J, et al. (2010) Collectin 11 (CL-11, CL-K1) is a MASP-1/3-associated plasma collectin with microbial-binding activity. J Immunol 185: 6096-6104.
101. Ali YM, Lynch NJ, Haleem KS, Fujita T, Endo Y, et al. (2012) The lectin pathway of complement activation is a critical component of the innate immune response to pneumococcal infection. PLoS Pathog 8: e1002793.
102. Endo Y, Takahashi M, Iwaki D, Ishida Y, Nakazawa N, et al. (2012) Mice deficient in ficolin, a lectin complement pathway recognition molecule, are susceptible to *Streptococcus pneumoniae* infection. J Immunol 189: 5860-5866.
103. Picard C, Puel A, Bustamante J, Ku CL, Casanova JL (2003) Primary immunodeficiencies associated with pneumococcal disease. Curr Opin Allergy Clin Immunol 3: 451-459.
104. Bogaert D, Thompson CM, Trzcinski K, Malley R, Lipsitch M (2010) The role of complement in innate and adaptive immunity to pneumococcal colonization and sepsis in a murine model. Vaccine 28: 681-685.
105. Hyams C, Camberlein E, Cohen JM, Bax K, Brown JS (2010) The *Streptococcus pneumoniae* capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. Infect Immun 78: 704-715.

106. Melin M, Trzcinski K, Antonio M, Meri S, Adegbola R, et al. (2010) Serotype-related variation in susceptibility to complement deposition and opsonophagocytosis among clinical isolates of *Streptococcus pneumoniae*. Infect Immun 78: 5252-5261.
107. Hyams C, Yuste J, Bax K, Camberlein E, Weiser JN, et al. (2010) *Streptococcus pneumoniae* resistance to complement-mediated immunity is dependent on the capsular serotype. Infect Immun 78: 716-725.
108. Hostetter MK (1986) Serotypic variations among virulent pneumococci in deposition and degradation of covalently bound C3b: implications for phagocytosis and antibody production. J Infect Dis 153: 682-693.
109. Hyams C, Opel S, Hanage W, Yuste J, Bax K, et al. (2011) Effects of *Streptococcus pneumoniae* strain background on complement resistance. PLoS One 6: e24581.
110. Tu AH, Fulgham RL, McCrory MA, Briles DE, Szalai AJ (1999) Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. Infect Immun 67: 4720-4724.
111. Ren B, Szalai AJ, Hollingshead SK, Briles DE (2004) Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect Immun 72: 114-122.
112. Mukerji R, Mirza S, Roche AM, Widener RW, Croney CM, et al. (2012) Pneumococcal surface protein A inhibits complement deposition on the pneumococcal surface by competing with the binding of C-reactive protein to cell-surface phosphocholine. J Immunol 189: 5327-5335.
113. Dave S, Brooks-Walter A, Pangburn MK, McDaniel LS (2001) PspC, a pneumococcal surface protein, binds human factor H. Infect Immun 69: 3435-3437.
114. Smith BL, Hostetter MK (2000) C3 as substrate for adhesion of *Streptococcus pneumoniae*. J Infect Dis 182: 497-508.
115. Jarva H, Janulczyk R, Hellwage J, Zipfel PF, Bjorck L, et al. (2002) *Streptococcus pneumoniae* evades complement attack and opsonophagocytosis by expressing the pspC locus-encoded Hic protein that binds to short consensus repeats 8-11 of factor H. J Immunol 168: 1886-1894.
116. Hyams C, Trzcinski K, Camberlein E, Weinberger DM, Chimalapati S, et al. (2013) *Streptococcus pneumoniae* capsular serotype invasiveness correlates with the degree of factor H binding and opsonization with C3b/iC3b. Infect Immun 81: 354-363.
117. Mitchell TJ, Andrew PW, Saunders FK, Smith AN, Boulnois GJ (1991) Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein. Mol Microbiol 5: 1883-1888.
118. Yuste J, Botto M, Paton JC, Holden DW, Brown JS (2005) Additive inhibition of complement deposition by pneumolysin and PspA facilitates *Streptococcus pneumoniae* septicemia. J Immunol 175: 1813-1819.
119. Ali YM, Kenawy HI, Muhammad A, Sim RB, Andrew PW, et al. (2013) Human L-ficolin, a recognition molecule of the lectin activation pathway of complement, activates complement by binding to pneumolysin, the major toxin of *Streptococcus pneumoniae*. PLoS One 8: e82583.
120. Domenech M, Ramos-Sevillano E, Garcia E, Moscoso M, Yuste J (2013) Biofilm formation avoids complement immunity and phagocytosis of *Streptococcus pneumoniae*. Infect Immun 81: 2606-2615.
121. Davis KM, Nakamura S, Weiser JN (2011) Nod2 sensing of lysozyme-digested peptidoglycan promotes macrophage recruitment and clearance of *S. pneumoniae* colonization in mice. Journal of Clinical Investigation 121: 3666-3676.

122. Opitz B, Puschel A, Schmeck B, Hocke AC, Rosseau S, et al. (2004) Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized *Streptococcus pneumoniae*. J Biol Chem 279: 36426-36432.
123. Gisch N, Kohler T, Ulmer AJ, Muthing J, Pribyl T, et al. (2013) Structural reevaluation of *Streptococcus pneumoniae* Lipoteichoic acid and new insights into its immunostimulatory potency. J Biol Chem 288: 15654-15667.
124. Tomlinson G, Chimalapati S, Pollard T, Lapp T, Cohen J, et al. (2014) TLR-mediated inflammatory responses to *Streptococcus pneumoniae* are highly dependent on surface expression of bacterial lipoproteins. J Immunol 193: 3736-3745.
125. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, et al. (1999) Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 163: 1-5.
126. Schroder NW, Morath S, Alexander C, Hamann L, Hartung T, et al. (2003) Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. J Biol Chem 278: 15587-15594.
127. van Rossum AM, Lysenko ES, Weiser JN (2005) Host and bacterial factors contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine model. Infect Immun 73: 7718-7726.
128. Echchannaoui H, Frei K, Schnell C, Leib SL, Zimmerli W, et al. (2002) Toll-like receptor 2-deficient mice are highly susceptible to *Streptococcus pneumoniae* meningitis because of reduced bacterial clearing and enhanced inflammation. J Infect Dis 186: 798-806.
129. Koedel U, Angele B, Rupprecht T, Wagner H, Roggenkamp A, et al. (2003) Toll-like receptor 2 participates in mediation of immune response in experimental pneumococcal meningitis. J Immunol 170: 438-444.
130. Knapp S, Wieland CW, van 't Veer C, Takeuchi O, Akira S, et al. (2004) Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense. J Immunol 172: 3132-3138.
131. Srivastava A, Henneke P, Visintin A, Morse SC, Martin V, et al. (2005) The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease. Infect Immun 73: 6479-6487.
132. Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, et al. (2003) Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proc Natl Acad Sci U S A 100: 1966-1971.
133. Branger J, Knapp S, Weijer S, Leemans JC, Pater JM, et al. (2004) Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. Infect Immun 72: 788-794.
134. McNeela EA, Burke A, Neill DR, Baxter C, Fernandes VE, et al. (2010) Pneumolysin activates the NLRP3 inflammasome and promotes proinflammatory cytokines independently of TLR4. PLoS Pathog 6: e1001191.
135. Albiger B, Dahlberg S, Sandgren A, Wartha F, Beiter K, et al. (2007) Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. Cell Microbiol 9: 633-644.
136. Kumar H, Kawai T, Akira S (2011) Pathogen recognition by the innate immune system. Int Rev Immunol 30: 16-34.
137. Soininen A, Pursiainen H, Kilpi T, Kayhty H (2001) Natural development of antibodies to pneumococcal capsular polysaccharides depends on the serotype: association

with pneumococcal carriage and acute otitis media in young children. J Infect Dis 184: 569-576.

138. Malley R (2010) Antibody and cell-mediated immunity to *Streptococcus pneumoniae*: implications for vaccine development. J Mol Med (Berl) 88: 135-142.
139. Goldblatt D, Hussain M, Andrews N, Ashton L, Virta C, et al. (2005) Antibody responses to nasopharyngeal carriage of *Streptococcus pneumoniae* in adults: a longitudinal household study. J Infect Dis 192: 387-393.
140. McCool TL, Weiser JN (2004) Limited role of antibody in clearance of *Streptococcus pneumoniae* in a murine model of colonization. Infect Immun 72: 5807-5813.
141. Trzcinski K, Thompson C, Malley R, Lipsitch M (2005) Antibodies to conserved pneumococcal antigens correlate with, but are not required for, protection against pneumococcal colonization induced by prior exposure in a mouse model. Infect Immun 73: 7043-7046.
142. Kirkeby L, Rasmussen TT, Reinholdt J, Kilian M (2000) Immunoglobulins in nasal secretions of healthy humans: structural integrity of secretory immunoglobulin A1 (IgA1) and occurrence of neutralizing antibodies to IgA1 proteases of nasal bacteria. Clin Diagn Lab Immunol 7: 31-39.
143. Janoff EN, Rubins JB, Fasching C, Charboneau D, Rahkola JT, et al. (2014) Pneumococcal IgA1 protease subverts specific protection by human IgA1. Mucosal Immunol 7: 249-256.
144. Roche AM, Richard AL, Rahkola JT, Janoff EN, Weiser JN (2015) Antibody blocks acquisition of bacterial colonization through agglutination. Mucosal Immunol 8: 176-185.
145. Zhang Z, Clarke TB, Weiser JN (2009) Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. J Clin Invest 119: 1899-1909.
146. Das R, LaRose MI, Hergott CB, Leng L, Bucala R, et al. (2014) Macrophage migration inhibitory factor promotes clearance of pneumococcal colonization. J Immunol 193: 764-772.
147. Cantey JR, Hand WL (1974) Cell-mediated immunity after bacterial infection of the lower respiratory tract. J Clin Invest 54: 1125-1134.
148. Knapp S, Leemans JC, Florquin S, Branger J, Maris NA, et al. (2003) Alveolar macrophages have a protective antiinflammatory role during murine pneumococcal pneumonia. Am J Respir Crit Care Med 167: 171-179.
149. Matthias KA, Roche AM, Standish AJ, Shchepetov M, Weiser JN (2008) Neutrophil-toxin interactions promote antigen delivery and mucosal clearance of *Streptococcus pneumoniae*. J Immunol 180: 6246-6254.
150. Sun K, Salmon SL, Lotz SA, Metzger DW (2007) Interleukin-12 promotes gamma interferon-dependent neutrophil recruitment in the lung and improves protection against respiratory *Streptococcus pneumoniae* infection. Infect Immun 75: 1196-1202.
151. Garvy BA, Harmsen AG (1996) The importance of neutrophils in resistance to pneumococcal pneumonia in adult and neonatal mice. Inflammation 20: 499-512.
152. Malley R, Srivastava A, Lipsitch M, Thompson CM, Watkins C, et al. (2006) Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. Infect Immun 74: 2187-2195.
153. Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, et al. (2005) CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. Proc Natl Acad Sci U S A 102: 4848-4853.
154. Basset A, Thompson CM, Hollingshead SK, Briles DE, Ades EW, et al. (2007) Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization

elicited by intranasal immunization with purified pneumococcal proteins. Infect Immun 75: 5460-5464.
155. Trzcinski K, Thompson CM, Srivastava A, Basset A, Malley R, et al. (2008) Protection against nasopharyngeal colonization by *Streptococcus pneumoniae* is mediated by antigen-specific CD4+ T cells. Infect Immun 76: 2678-2684.
156. Lu YJ, Gross J, Bogaert D, Finn A, Bagrade L, et al. (2008) Interleukin-17A mediates acquired immunity to pneumococcal colonization. PLoS Pathog 4: e1000159.
157. Zhang Z, Clarke TB, Weiser JN (2009) Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. Journal of Clinical Investigation 119: 1899-1909.
158. Roche AM, King SJ, Weiser JN (2007) Live attenuated *Streptococcus pneumoniae* strains induce serotype-independent mucosal and systemic protection in mice. Infect Immun 75: 2469-2475.
159. Richards L, Ferreira DM, Miyaji EN, Andrew PW, Kadioglu A (2010) The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. Immunobiology 215: 251-263.
160. Cohen JM, Khandavilli S, Camberlein E, Hyams C, Baxendale HE, et al. (2011) Protective contributions against invasive *Streptococcus pneumoniae* pneumonia of antibody and Th17-cell responses to nasopharyngeal colonisation. PLoS One 6: e25558.
161. McCool TL, Cate TR, Moy G, Weiser JN (2002) The immune response to pneumococcal proteins during experimental human carriage. J Exp Med 195: 359-365.
162. Holmlund E, Quiambao B, Ollgren J, Jaakkola T, Neyt C, et al. (2009) Antibodies to pneumococcal proteins PhtD, CbpA, and LytC in Filipino pregnant women and their infants in relation to pneumococcal carriage. Clin Vaccine Immunol 16: 916-923.
163. Melegaro A, Edmunds WJ, Pebody R, Miller E, George R (2006) The current burden of pneumococcal disease in England and Wales. J Infect 52: 37-48.
164. Morrow A, De Wals P, Petit G, Guay M, Erickson LJ (2007) The burden of pneumococcal disease in the Canadian population before routine use of the seven-valent pneumococcal conjugate vaccine. Can J Infect Dis Med Microbiol 18: 121-127.
165. Melegaro A, Choi Y, Pebody R, Gay N (2007) Pneumococcal carriage in United Kingdom families: estimating serotype-specific transmission parameters from longitudinal data. Am J Epidemiol 166: 228-235.
166. Scott JA, Hall AJ, Dagan R, Dixon JM, Eykyn SJ, et al. (1996) Serogroup-specific epidemiology of *Streptococcus pneumoniae*: associations with age, sex, and geography in 7,000 episodes of invasive disease. Clin Infect Dis 22: 973-981.
167. Austrian R (1981) Some Observations on the Pneumococcus and on the Current Status of Pneumococcal Disease and Its Prevention. Reviews of Infectious Diseases 3: S1-S17.
168. Wright AE, Morgan WP, Colebrook L, Dodgson RW (1914) Observations on prophylatic inocculation against pneumococcus infections, and on the results which have been achieved by it. Lancet 1: 1-10.
169. Cecil RL, Austin JH (1918) Results of Prophylactic Inoculation against Pneumococcus in 12,519 Men. J Exp Med 28: 19-41.
170. Maynard G (1915) Pneumonia inoculation experiment no. III. Med J S Afr 11: 36-38.
171. Macleod CM, Hodges RG, Heidelberger M, Bernhard WG (1945) Prevention of Pneumococcal Pneumonia by Immunization with Specific Capsular Polysaccharides. J Exp Med 82: 445-465.
172. WHO (2008) 23-valent pneumococcal polysaccharide vaccine. WHO position paper. Wkly Epidemiol Rec 83: 373-384.

173. Huss A, Scott P, Stuck AE, Trotter C, Egger M (2009) Efficacy of pneumococcal vaccination in adults: a meta-analysis. CMAJ 180: 48-58.
174. Peltola H, Kilpi T, Anttila M (1992) Rapid disappearance of *Haemophilus influenzae* type b meningitis after routine childhood immunisation with conjugate vaccines. Lancet 340: 592-594.
175. Black S, Shinefield H, Fireman B, Lewis E, Ray P, et al. (2000) Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. Pediatr Infect Dis J 19: 187-195.
176. FDA (2011) Summary basis for regulatory action- Prevnar 13. http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm201667.htm.
177. Black SB, Shinefield HR, Hansen J, Elvin L, Laufer D, et al. (2001) Postlicensure evaluation of the effectiveness of seven valent pneumococcal conjugate vaccine. Pediatr Infect Dis J 20: 1105-1107.
178. Gordon SB, Kayhty H, Molyneux ME, Haikala R, Nurkka A, et al. (2007) Pneumococcal conjugate vaccine is immunogenic in lung fluid of HIV-infected and immunocompetent adults. Journal of Allergy and Clinical Immunology 120: 208-210.
179. Jodar L, Butler J, Carlone G, Dagan R, Goldblatt D, et al. (2003) Serological criteria for evaluation and licensure of new pneumococcal conjugate vaccine formulations for use in infants. Vaccine 21: 3265-3272.
180. Siber GR, Chang I, Baker S, Fernsten P, O'Brien KL, et al. (2007) Estimating the protective concentration of anti-pneumococcal capsular polysaccharide antibodies. Vaccine 25: 3816-3826.
181. O'Brien KL, Millar EV, Zell ER, Bronsdon M, Weatherholtz R, et al. (2007) Effect of pneumococcal conjugate vaccine on nasopharyngeal colonization among immunized and unimmunized children in a community-randomized trial. J Infect Dis 196: 1211-1220.
182. Cheung YB, Zaman SM, Nsekpong ED, Van Beneden CA, Adegbola RA, et al. (2009) Nasopharyngeal carriage of *Streptococcus pneumoniae* in Gambian children who participated in a 9-valent pneumococcal conjugate vaccine trial and in their younger siblings. Pediatr Infect Dis J 28: 990-995.
183. Roca A, Bottomley C, Hill PC, Bojang A, Egere U, et al. (2012) Effect of age and vaccination with a pneumococcal conjugate vaccine on the density of pneumococcal nasopharyngeal carriage. Clin Infect Dis 55: 816-824.
184. Klugman KP, Madhi SA, Huebner RE, Kohberger R, Mbelle N, et al. (2003) A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. N Engl J Med 349: 1341-1348.
185. Eskola J, Kilpi T, Palmu A, Jokinen J, Haapakoski J, et al. (2001) Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med 344: 403-409.
186. O'Brien KL, David AB, Chandran A, Moulton LH, Reid R, et al. (2008) Randomized, controlled trial efficacy of pneumococcal conjugate vaccine against otitis media among Navajo and White Mountain Apache infants. Pediatr Infect Dis J 27: 71-73.
187. Grijalva CG, Nuorti JP, Arbogast PG, Martin SW, Edwards KM, et al. (2007) Decline in pneumonia admissions after routine childhood immunisation with pneumococcal conjugate vaccine in the USA: a time-series analysis. Lancet 369: 1179-1186.
188. Patrzalek M, Albrecht P, Sobczynski M (2010) Significant decline in pneumonia admission rate after the introduction of routine 2+1 dose schedule heptavalent

pneumococcal conjugate vaccine (PCV7) in children under 5 years of age in Kielce, Poland. Eur J Clin Microbiol Infect Dis 29: 787-792.
189. Cutts FT, Zaman SM, Enwere G, Jaffar S, Levine OS, et al. (2005) Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial. Lancet 365: 1139-1146.
190. Lucero MG, Nohynek H, Williams G, Tallo V, Simoes EA, et al. (2009) Efficacy of an 11-valent pneumococcal conjugate vaccine against radiologically confirmed pneumonia among children less than 2 years of age in the Philippines: a randomized, double-blind, placebo-controlled trial. Pediatr Infect Dis J 28: 455-462.
191. Poehling KA, Talbot TR, Griffin MR, Craig AS, Whitney CG, et al. (2006) Invasive pneumococcal disease among infants before and after introduction of pneumococcal conjugate vaccine. JAMA 295: 1668-1674.
192. Black S, Shinefield H, Baxter R, Austrian R, Bracken L, et al. (2004) Postlicensure surveillance for pneumococcal invasive disease after use of heptavalent pneumococcal conjugate vaccine in Northern California Kaiser Permanente. Pediatr Infect Dis J 23: 485-489.
193. Hicks LA, Harrison LH, Flannery B, Hadler JL, Schaffner W, et al. (2007) Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998-2004. J Infect Dis 196: 1346-1354.
194. Singleton RJ, Hennessy TW, Bulkow LR, Hammitt LL, Zulz T, et al. (2007) Invasive pneumococcal disease caused by nonvaccine serotypes among alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA 297: 1784-1792.
195. Flannery B, Heffernan RT, Harrison LH, Ray SM, Reingold AL, et al. (2006) Changes in invasive Pneumococcal disease among HIV-infected adults living in the era of childhood pneumococcal immunization. Ann Intern Med 144: 1-9.
196. Tan TQ, Mason EO, Jr., Wald ER, Barson WJ, Schutze GE, et al. (2002) Clinical characteristics of children with complicated pneumonia caused by *Streptococcus pneumoniae*. Pediatrics 110: 1-6.
197. Byington CL, Korgenski K, Daly J, Ampofo K, Pavia A, et al. (2006) Impact of the pneumococcal conjugate vaccine on pneumococcal parapneumonic empyema. Pediatr Infect Dis J 25: 250-254.
198. Ginsburg AS, Alderson MR (2011) New conjugate vaccines for the prevention of pneumococcal disease in developing countries. Drugs Today (Barc) 47: 207-214.
199. Gavi (2014) About the pneumococcal advance market commitment. http://www.gavi.org/funding/pneumococcal-amc/about/: Accessed November 14, 2014.
200. Frey SE, Lottenbach KR, Hill H, Blevins TP, Yu Y, et al. (2013) A Phase I, dose-escalation trial in adults of three recombinant attenuated Salmonella Typhi vaccine vectors producing *Streptococcus pneumoniae* surface protein antigen PspA. Vaccine 31: 4874-4880.
201. Rosch JW, Iverson AR, Humann J, Mann B, Gao G, et al. (2014) A live-attenuated pneumococcal vaccine elicits CD4+ T-cell dependent class switching and provides serotype independent protection against acute otitis media. EMBO Mol Med 6: 141-154.
202. Lu YJ, Leite L, Goncalves VM, Dias Wde O, Liberman C, et al. (2010) GMP-grade pneumococcal whole-cell vaccine injected subcutaneously protects mice from nasopharyngeal colonization and fatal aspiration-sepsis. Vaccine 28: 7468-7475.

203. Moffitt KL, Yadav P, Weinberger DM, Anderson PW, Malley R (2012) Broad antibody and T cell reactivity induced by a pneumococcal whole-cell vaccine. Vaccine 30: 4316-4322.
204. Goncalves VM, Dias WO, Campos IB, Liberman C, Sbrogio-Almeida ME, et al. (2014) Development of a whole cell pneumococcal vaccine: BPL inactivation, cGMP production, and stability. Vaccine 32: 1113-1120.
205. Briles D, King J, Hale Y, Keech C, Malley R, et al. Immune sera from adults immunized with killed whole-cell non-encapsulated vaccine protects mice from fatal infection with type 3 pneumococci. Poster (OP-165) presented at International Symposium on Pneumococci and Pneumococcal Diseases Mar 9-13, 2014. Hyderabad, India.
206. Ogunniyi AD, Grabowicz M, Briles DE, Cook J, Paton JC (2007) Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. Infect Immun 75: 350-357.
207. Briles DE, Hollingshead SK, Paton JC, Ades EW, Novak L, et al. (2003) Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae*. J Infect Dis 188: 339-348.
208. Donkor ES, Bishop CJ, Gould K, Hinds J, Antonio M, et al. (2011) High levels of recombination among *Streptococcus pneumoniae* isolates from the Gambia. MBio 2: e00040-00011.
209. Auranen K, Mehtala J, Tanskanen A, M SK (2010) Between-strain competition in acquisition and clearance of pneumococcal carriage--epidemiologic evidence from a longitudinal study of day-care children. Am J Epidemiol 171: 169-176.
210. Mehtala J, Antonio M, Kaltoft MS, O'Brien KL, Auranen K (2013) Competition Between *Streptococcus pneumoniae* Strains: Implications for Vaccine-Induced Replacement in Colonization and Disease. Epidemiology 24: 522-529.
211. Marks LR, Reddinger RM, Hakansson AP (2012) High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in *Streptococcus pneumoniae*. MBio 3.
212. Dawid S, Roche AM, Weiser JN (2007) The blp bacteriocins of *Streptococcus pneumoniae* mediate intraspecies competition both in vitro and in vivo. Infect Immun 75: 443-451.
213. Hament JM, Kimpen JL, Fleer A, Wolfs TF (1999) Respiratory viral infection predisposing for bacterial disease: a concise review. FEMS Immunol Med Microbiol 26: 189-195.
214. McCullers JA (2006) Insights into the interaction between influenza virus and pneumococcus. Clin Microbiol Rev 19: 571-582.
215. Madhi SA, Klugman KP, Vaccine Trialist G (2004) A role for *Streptococcus pneumoniae* in virus-associated pneumonia. Nat Med 10: 811-813.
216. Kukavica-Ibrulj I, Hamelin ME, Prince GA, Gagnon C, Bergeron Y, et al. (2009) Infection with human metapneumovirus predisposes mice to severe pneumococcal pneumonia. J Virol 83: 1341-1349.
217. Watson M, Gilmour R, Menzies R, Ferson M, McIntyre P, et al. (2006) The association of respiratory viruses, temperature, and other climatic parameters with the incidence of invasive pneumococcal disease in Sydney, Australia. Clin Infect Dis 42: 211-215.
218. Nakamura S, Davis KM, Weiser JN (2011) Synergistic stimulation of type I interferons during influenza virus coinfection promotes *Streptococcus pneumoniae* colonization in mice. J Clin Invest 121: 3657-3665.
219. Vu HT, Yoshida LM, Suzuki M, Nguyen HA, Nguyen CD, et al. (2011) Association between nasopharyngeal load of *Streptococcus pneumoniae*, viral coinfection, and

radiologically confirmed pneumonia in Vietnamese children. Pediatr Infect Dis J 30: 11-18.
220. Wolter N, Tempia S, Cohen C, Madhi SA, Venter M, et al. (2014) High nasopharyngeal pneumococcal density, increased by viral coinfection, is associated with invasive pneumococcal pneumonia. J Infect Dis 210: 1649-1657.
221. Diavatopoulos DA, Short KR, Price JT, Wilksch JJ, Brown LE, et al. (2010) Influenza A virus facilitates *Streptococcus pneumoniae* transmission and disease. FASEB J 24: 1789-1798.
222. Short KR, Reading PC, Wang N, Diavatopoulos DA, Wijburg OL (2012) Increased nasopharyngeal bacterial titers and local inflammation facilitate transmission of *Streptococcus pneumoniae*. MBio 3.
223. Chien YW, Vidal JE, Grijalva CG, Bozio C, Edwards KM, et al. (2013) Density interactions among *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* in the nasopharynx of young Peruvian children. Pediatr Infect Dis J 32: 72-77.
224. Regev-Yochay G, Dagan R, Raz M, Carmeli Y, Shainberg B, et al. (2004) Association between carriage of *Streptococcus pneumoniae* and *Staphylococcus aureus* in Children. JAMA 292: 716-720.
225. Regev-Yochay G, Trzcinski K, Thompson CM, Malley R, Lipsitch M (2006) Interference between *Streptococcus pneumoniae* and *Staphylococcus aureus*: In vitro hydrogen peroxide-mediated killing by *Streptococcus pneumoniae*. J Bacteriol 188: 4996-5001.
226. Margolis E (2009) Hydrogen peroxide-mediated interference competition by *Streptococcus pneumoniae* has no significant effect on *Staphylococcus aureus* nasal colonization of neonatal rats. J Bacteriol 191: 571-575.
227. Regev-Yochay G, Malley R, Rubinstein E, Raz M, Dagan R, et al. (2008) In vitro bactericidal activity of *Streptococcus pneumoniae* and bactericidal susceptibility of *Staphylococcus aureus* strains isolated from cocolonized versus noncocolonized children. J Clin Microbiol 46: 747-749.
228. Dunne EM, Manning J, Russell FM, Robins-Browne RM, Mulholland EK, et al. (2012) Effect of pneumococcal vaccination on nasopharyngeal carriage of *Streptococcus pneumoniae*, *Haemophilus influenzae*, Moraxella catarrhalis, and *Staphylococcus aureus* in Fijian children. J Clin Microbiol 50: 1034-1038.
229. Spijkerman J, Prevaes SM, van Gils EJ, Veenhoven RH, Bruin JP, et al. (2012) Long-term effects of pneumococcal conjugate vaccine on nasopharyngeal carriage of *S. pneumoniae*, S. aureus, *H. influenzae* and M. catarrhalis. PLoS One 7: e39730.
230. Madhi SA, Adrian P, Kuwanda L, Cutland C, Albrich WC, et al. (2007) Long-term effect of pneumococcal conjugate vaccine on nasopharyngeal colonization by *Streptococcus pneumoniae*--and associated interactions with *Staphylococcus aureus* and *Haemophilus influenzae* colonization--in HIV-Infected and HIV-uninfected children. J Infect Dis 196: 1662-1666.
231. McNally LM, Jeena PM, Gajee K, Sturm AW, Tomkins AM, et al. (2006) Lack of association between the nasopharyngeal carriage of *Streptococcus pneumoniae* and *Staphylococcus aureus* in HIV-1-infected South African children. J Infect Dis 194: 385-390.
232. Pericone CD, Overweg K, Hermans PW, Weiser JN (2000) Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. Infect Immun 68: 3990-3997.
233. Lysenko ES, Ratner AJ, Nelson AL, Weiser JN (2005) The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. PLoS Pathog 1: e1.

234. Margolis E, Yates A, Levin BR (2010) The ecology of nasal colonization of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*: the role of competition and interactions with host's immune response. BMC Microbiol 10: 59.
235. Blaser MJ, Falkow S (2009) What are the consequences of the disappearing human microbiota? Nat Rev Microbiol 7: 887-894.
236. Bogaert D, Keijser B, Huse S, Rossen J, Veenhoven R, et al. (2011) Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. PLoS One 6: e17035.
237. Laufer AS, Metlay JP, Gent JF, Fennie KP, Kong Y, et al. (2011) Microbial communities of the upper respiratory tract and otitis media in children. MBio 2: e00245-00210.
238. Bronsdon MA, O'Brien KL, Facklam RR, Whitney CG, Schwartz B, et al. (2004) Immunoblot method to detect *Streptococcus pneumoniae* and identify multiple serotypes from nasopharyngeal secretions. J Clin Microbiol 42: 1596-1600.
239. Turner P, Hinds J, Turner C, Jankhot A, Gould K, et al. (2011) Improved detection of nasopharyngeal cocolonization by multiple pneumococcal serotypes by use of latex agglutination or molecular serotyping by microarray. J Clin Microbiol 49: 1784-1789.
240. van der Veen EL, Rovers MM, Leverstein-van Hall MA, Sanders EA, Schilder AG (2006) Influence of sampling technique on detection of potential pathogens in the nasopharynx. Arch Otolaryngol Head Neck Surg 132: 752-755.
241. Abdullahi O, Wanjiru E, Musyimi R, Glass N, Scott JA (2007) Validation of nasopharyngeal sampling and culture techniques for detection of *Streptococcus pneumoniae* in children in Kenya. J Clin Microbiol 45: 3408-3410.
242. Satzke C, Turner P, Virolainen-Julkunen A, Adrian PV, Antonio M, et al. (2013) Standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae*: Updated recommendations from the World Health Organization Pneumococcal Carriage Working Group. Vaccine 32: 165-179.
243. Lieberman D, Shleyfer E, Castel H, Terry A, Harman-Boehm I, et al. (2006) Nasopharyngeal versus oropharyngeal sampling for isolation of potential respiratory pathogens in adults. J Clin Microbiol 44: 525-528.
244. Watt JP, O'Brien KL, Katz S, Bronsdon MA, Elliott J, et al. (2004) Nasopharyngeal versus oropharyngeal sampling for detection of pneumococcal carriage in adults. J Clin Microbiol 42: 4974-4976.
245. Gritzfeld JF, Roberts P, Roche L, El Batrawy S, Gordon SB (2011) Comparison between nasopharyngeal swab and nasal wash, using culture and PCR, in the detection of potential respiratory pathogens. BMC Res Notes 4: 122.
246. Gritzfeld JF, Gordon SB, Cremers A (2013) Detection limits in pneumococcal carriage. Pediatr Infect Dis J 32: 425-426.
247. Principi N, Terranova L, Zampiero A, Manzoni F, Senatore L, et al. (2014) Oropharyngeal and nasopharyngeal sampling for the detection of adolescent *Streptococcus pneumoniae* carriers. J Med Microbiol 63: 393-398.
248. Trzcinski K, Bogaert D, Wyllie A, Chu ML, van der Ende A, et al. (2013) Superiority of trans-oral over trans-nasal sampling in detecting *Streptococcus pneumoniae* colonization in adults. PLoS One 8: e60520.
249. da Gloria Carvalho M, Pimenta FC, Jackson D, Roundtree A, Ahmad Y, et al. (2010) Revisiting pneumococcal carriage by use of broth enrichment and PCR techniques for enhanced detection of carriage and serotypes. J Clin Microbiol 48: 1611-1618.
250. Lankinen KS, Salo P, Rapola S, Salo E, Takala AK, et al. (1997) Pneumococcal capsular antigen detection after enrichment culture: an alternative to culture methods in epidemiologic research. Am J Trop Med Hyg 56: 211-215.

251. Ikryannikova LN, Lapin KN, Malakhova MV, Filimonova AV, Ilina EN, et al. (2011) Misidentification of alpha-hemolytic streptococci by routine tests in clinical practice. Infect Genet Evol 11: 1709-1715.
252. Whatmore AM, Efstratiou A, Pickerill AP, Broughton K, Woodard G, et al. (2000) Genetic relationships between clinical isolates of *Streptococcus pneumoniae*, Streptococcus oralis, and Streptococcus mitis: characterization of "Atypical" pneumococci and organisms allied to S. mitis harboring *S. pneumoniae* virulence factor-encoding genes. Infect Immun 68: 1374-1382.
253. Carvalho Mda G, Tondella ML, McCaustland K, Weidlich L, McGee L, et al. (2007) Evaluation and improvement of real-time PCR assays targeting lytA, ply, and psaA genes for detection of pneumococcal DNA. J Clin Microbiol 45: 2460-2466.
254. Arbique JC, Poyart C, Trieu-Cuot P, Quesne G, Carvalho Mda G, et al. (2004) Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of Streptococcus pseudopneumoniae sp. nov. J Clin Microbiol 42: 4686-4696.
255. Zhang Q, Ma Q, Su D, Li Q, Yao W, et al. (2010) Identification of horizontal gene transfer and recombination of PsaA gene in streptococcus mitis group. Microbiol Immunol 54: 313-319.
256. Romero P, Lopez R, Garcia E (2004) Characterization of LytA-like N-acetylmuramoyl-L-alanine amidases from two new Streptococcus mitis bacteriophages provides insights into the properties of the major pneumococcal autolysin. J Bacteriol 186: 8229-8239.
257. Enright MC, Spratt BG (1998) A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology 144 (Pt 11): 3049-3060.
258. Hanage WP, Kaijalainen T, Herva E, Saukkoriipi A, Syrjanen R, et al. (2005) Using multilocus sequence data to define the pneumococcus. Journal of Bacteriology 187: 6223-6230.
259. Simoes AS, Sa-Leao R, Eleveld MJ, Tavares DA, Carrico JA, et al. (2010) Highly penicillin-resistant multidrug-resistant pneumococcus-like strains colonizing children in Oeiras, Portugal: genomic characteristics and implications for surveillance. J Clin Microbiol 48: 238-246.
260. Turner P, Turner C, Kaewcharernnet N, Mon NY, Goldblatt D, et al. (2011) A prospective study of urinary pneumococcal antigen detection in healthy Karen mothers with high rates of pneumococcal nasopharyngeal carriage. BMC Infect Dis 11: 108.
261. Smith-Vaughan H, Byun R, Nadkarni M, Jacques NA, Hunter N, et al. (2006) Measuring nasal bacterial load and its association with otitis media. BMC Ear Nose Throat Disord 6: 10.
262. Yang S, Lin S, Khalil A, Gaydos C, Nuemberger E, et al. (2005) Quantitative PCR assay using sputum samples for rapid diagnosis of pneumococcal pneumonia in adult emergency department patients. J Clin Microbiol 43: 3221-3226.
263. Kais M, Spindler C, Kalin M, Ortqvist A, Giske CG (2006) Quantitative detection of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and Moraxella catarrhalis in lower respiratory tract samples by real-time PCR. Diagn Microbiol Infect Dis 55: 169-178.
264. Neeleman C, Klaassen CH, Klomberg DM, de Valk HA, Mouton JW (2004) Pneumolysin is a key factor in misidentification of macrolide-resistant *Streptococcus pneumoniae* and is a putative virulence factor of S. mitis and other streptococci. J Clin Microbiol 42: 4355-4357.

265. Abdeldaim GM, Stralin K, Olcen P, Blomberg J, Herrmann B (2008) Toward a quantitative DNA-based definition of pneumococcal pneumonia: a comparison of *Streptococcus pneumoniae* target genes, with special reference to the Spn9802 fragment. Diagn Microbiol Infect Dis 60: 143-150.
266. Albrich WC, Madhi SA, Adrian PV, van Niekerk N, Mareletsi T, et al. (2012) Use of a rapid test of pneumococcal colonization density to diagnose pneumococcal pneumonia. Clin Infect Dis 54: 601-609.
267. Benson CE, Sweeney CR (1984) Isolation of *Streptococcus pneumoniae* type 3 from equine species. J Clin Microbiol 20: 1028-1030.
268. Wu HY, Virolainen A, Mathews B, King J, Russell MW, et al. (1997) Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice. Microb Pathog 23: 127-137.
269. Malley R, Stack AM, Ferretti ML, Thompson CM, Saladino RA (1998) Anticapsular polysaccharide antibodies and nasopharyngeal colonization with *Streptococcus pneumoniae* in infant rats. J Infect Dis 178: 878-882.
270. Tong HH, Weiser JN, James MA, DeMaria TF (2001) Effect of influenza A virus infection on nasopharyngeal colonization and otitis media induced by transparent or opaque phenotype variants of *Streptococcus pneumoniae* in the chinchilla model. Infect Immun 69: 602-606.
271. Giebink GS, Berzins IK, Marker SC, Schiffman G (1980) Experimental otitis media after nasal inoculation of *Streptococcus pneumoniae* and influenza A virus in chinchillas. Infect Immun 30: 445-450.
272. Philipp MT, Doyle LA, Martin DS, Plauche GB, Phillippi-Falkenstein KM, et al. (2012) A rhesus macaque model of *Streptococcus pneumoniae* carriage. J Med Primatol 41: 60-66.
273. Riedel S (2005) Edward Jenner and the history of smallpox and vaccination. Proc (Bayl Univ Med Cent) 18: 21-25.
274. Miller FG, Grady C (2001) The ethical challenge of infection-inducing challenge experiments. Clin Infect Dis 33: 1028-1033.
275. Pollard AJ, Savulescu J, Oxford J, Hill AV, Levine MM, et al. (2012) Human microbial challenge: the ultimate animal model. Lancet Infect Dis 12: 903-905.
276. Wertheim HF, Walsh E, Choudhurry R, Melles DC, Boelens HA, et al. (2008) Key role for clumping factor B in *Staphylococcus aureus* nasal colonization of humans. PLoS Med 5: e17.
277. Evans CM, Pratt CB, Matheson M, Vaughan TE, Findlow J, et al. (2011) Nasopharyngeal colonization by Neisseria lactamica and induction of protective immunity against *Neisseria meningitidis*. Clin Infect Dis 52: 70-77.
278. Poole J, Foster E, Chaloner K, Hunt J, Jennings MP, et al. (2013) Analysis of nontypeable *Haemophilus influenzae* phase-variable genes during experimental human nasopharyngeal colonization. J Infect Dis 208: 720-727.
279. McCool TL, Cate TR, Tuomanen EI, Adrian P, Mitchell TJ, et al. (2003) Serum immunoglobulin G response to candidate vaccine antigens during experimental human pneumococcal colonization. Infect Immun 71: 5724-5732.
280. Browall S, Norman M, Tangrot J, Galanis I, Sjostrom K, et al. (2014) Intraclonal variations among *Streptococcus pneumoniae* isolates influence the likelihood of invasive disease in children. J Infect Dis 209: 377-388.
281. Wright AK, Ferreira DM, Gritzfeld JF, Wright AD, Armitage K, et al. (2012) Human nasal challenge with *Streptococcus pneumoniae* is immunising in the absence of carriage. PLoS Pathog 8: e1002622.
282. Goldblatt D, Ramakrishnan M, O'Brien K, Grp P-WM (2013) Using the impact of pneumococcal vaccines on nasopharyngeal carriage to aid licensing and vaccine

implementation; A Pneumocarr meeting report March 27-28, 2012, Geneva. Vaccine 32: 146-152.
283. Simell B, Auranen K, Kayhty H, Goldblatt D, Dagan R, et al. (2012) The fundamental link between pneumococcal carriage and disease. Expert Rev Vaccines 11: 841-855.
284. Auranen K, Rinta-Kokko H, Goldblatt D, Nohynek H, O'Brien KL, et al. (2013) Colonisation endpoints in *Streptococcus pneumoniae* vaccine trials. Vaccine 32: 153-158.
285. Ferreira DM, Jambo KC, Gordon SB (2011) Experimental human pneumococcal carriage models for vaccine research. Trends Microbiol 19: 464-470.
286. Jambo KC, Sepako E, Heyderman RS, Gordon SB (2010) Potential role for mucosally active vaccines against pneumococcal pneumonia. Trends Microbiol 18: 81-89.
287. Gritzfeld JF (2009) Sampling and detection methods used in nasopharyngeal carriage. MSc Medical Microbiology: University of Liverpool.
288. Von Essen SG, Robbins RA, Spurzem JR, Thompson AB, McGranaghan SS, et al. (1991) Bronchoscopy with bronchoalveolar lavage causes neutrophil recruitment to the lower respiratory tract. Am Rev Respir Dis 144: 848-854.
289. Ferreira DM, Neill DR, Bangert M, Gritzfeld JF, Green N, et al. (2013) Controlled human infection and rechallenge with *Streptococcus pneumoniae* reveals the protective efficacy of carriage in healthy adults. Am J Respir Crit Care Med 187: 855-864.
290. Spector SL, Nicklas RA, Chapman JA, Bernstein IL, Berger WE, et al. (2003) Symptom severity assessment of allergic rhinitis: part 1. Ann Allergy Asthma Immunol 91: 105-114.
291. Miles AA, Misra SS, Irwin JO (1938) The estimation of the bactericidal power of the blood. J Hyg (Lond) 38: 732-749.
292. Gritzfeld JF, Wright AD, Collins AM, Pennington SH, Wright AK, et al. (2013) Experimental human pneumococcal carriage. J Vis Exp.
293. Naclerio RM, Meier HL, Kagey-Sobotka A, Adkinson NF, Jr., Meyers DA, et al. (1983) Mediator release after nasal airway challenge with allergen. Am Rev Respir Dis 128: 597-602.
294. Newton R, Hinds J, Wernisch L (2011) Empirical Bayesian models for analysing molecular serotyping microarrays. BMC Bioinformatics 12: 88.
295. Otto TD, Dillon GP, Degrave WS, Berriman M (2011) RATT: Rapid Annotation Transfer Tool. Nucleic Acids Res 39: e57.
296. Carver TJ, Rutherford KM, Berriman M, Rajandream MA, Barrell BG, et al. (2005) ACT: the Artemis Comparison Tool. Bioinformatics 21: 3422-3423.
297. Lazinski DW, Camilli A (2013) Homopolymer tail-mediated ligation PCR: a streamlined and highly efficient method for DNA cloning and library construction. Biotechniques 54: 25-34.
298. Tiraby G, Sicard MA (1973) Integration efficiency in DNA-induced transformation of Pneumococcus. II. Genetic studies of mutant integrating all the markers with a high efficiency. Genetics 75: 35-48.
299. Bezerra PG, Britto MC, Correia JB, Duarte Mdo C, Fonceca AM, et al. (2011) Viral and atypical bacterial detection in acute respiratory infection in children under five years. PLoS One 6: e18928.
300. Hopkins MJ, Moorcroft JF, Correia JB, Hart IJ (2011) Using the full spectral capacity (six channels) of a real-time PCR instrument can simplify diagnostic laboratory screening and typing protocols for pandemic H1N1 influenza. Influenza and Other Respiratory Viruses 5: 110-114.
301. Vadesilho CF, Ferreira DM, Gordon SB, Briles DE, Moreno AT, et al. (2014) Mapping of Epitopes Recognized by Antibodies Induced by Immunization of Mice with PspA and PspC. Clin Vaccine Immunol 21: 940-948.

302. van Hoek AJ, Sheppard CL, Andrews NJ, Waight PA, Slack MP, et al. (2014) Pneumococcal carriage in children and adults two years after introduction of the thirteen valent pneumococcal conjugate vaccine in England. Vaccine 32: 4349-4355.
303. Lipsitch M, Dykes JK, Johnson SE, Ades EW, King J, et al. (2000) Competition among *Streptococcus pneumoniae* for intranasal colonization in a mouse model. Vaccine 18: 2895-2901.
304. Abdullahi O, Nyiro J, Lewa P, Slack M, Scott JA (2008) The descriptive epidemiology of *Streptococcus pneumoniae* and *Haemophilus influenzae* nasopharyngeal carriage in children and adults in Kilifi district, Kenya. Pediatr Infect Dis J 27: 59-64.
305. Abdullahi O, Karani A, Tigoi CC, Mugo D, Kungu S, et al. (2012) The prevalence and risk factors for pneumococcal colonization of the nasopharynx among children in Kilifi District, Kenya. PLoS One 7: e30787.
306. Rodrigues F, Foster D, Nicoli E, Trotter C, Vipond B, et al. (2013) Relationships between rhinitis symptoms, respiratory viral infections and nasopharyngeal colonization with *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* in children attending daycare. Pediatr Infect Dis J 32: 227-232.
307. Granat SM, Ollgren J, Herva E, Mia Z, Auranen K, et al. (2009) Epidemiological evidence for serotype-independent acquired immunity to pneumococcal carriage. J Infect Dis 200: 99-106.
308. Weinberger DM, Dagan R, Givon-Lavi N, Regev-Yochay G, Malley R, et al. (2008) Epidemiologic evidence for serotype-specific acquired immunity to pneumococcal carriage. J Infect Dis 197: 1511-1518.
309. Cobey S, Lipsitch M (2012) Niche and neutral effects of acquired immunity permit coexistence of pneumococcal serotypes. Science 335: 1376-1380.
310. Cooper D, Yu X, Sidhu M, Nahm MH, Fernsten P, et al. (2011) The 13-valent pneumococcal conjugate vaccine (PCV13) elicits cross-functional opsonophagocytic killing responses in humans to *Streptococcus pneumoniae* serotypes 6C and 7A. Vaccine 29: 7207-7211.
311. Briles DE, Hollingshead SK, King J, Swift A, Braun PA, et al. (2000) Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J Infect Dis 182: 1694-1701.
312. Turner P, Turner C, Jankhot A, Helen N, Lee SJ, et al. (2012) A longitudinal study of *Streptococcus pneumoniae* carriage in a cohort of infants and their mothers on the Thailand-Myanmar border. PLoS One 7: e38271.
313. Bogaert D, Weinberger D, Thompson C, Lipsitch M, Malley R (2009) Impaired innate and adaptive immunity to *Streptococcus pneumoniae* and its effect on colonization in an infant mouse model. Infect Immun 77: 1613-1622.
314. Garcia-Rodriguez JA, Fresnadillo Martinez MJ (2002) Dynamics of nasopharyngeal colonization by potential respiratory pathogens. J Antimicrob Chemother 50 Suppl S2: 59-73.
315. Balicer RD, Zarka S, Levine H, Klement E, Sela T, et al. (2010) Control of *Streptococcus pneumoniae* serotype 5 epidemic of severe pneumonia among young army recruits by mass antibiotic treatment and vaccination. Vaccine 28: 5591-5596.
316. Weiser JN, Bae D, Epino H, Gordon SB, Kapoor M, et al. (2001) Changes in availability of oxygen accentuate differences in capsular polysaccharide expression by phenotypic variants and clinical isolates of *Streptococcus pneumoniae*. Infect Immun 69: 5430-5439.

317. Kim JO, Romero-Steiner S, Sorensen UB, Blom J, Carvalho M, et al. (1999) Relationship between cell surface carbohydrates and intrastrain variation on opsonophagocytosis of *Streptococcus pneumoniae*. Infect Immun 67: 2327-2333.
318. Andersson B, Eriksson B, Falsen E, Fogh A, Hanson LA, et al. (1981) Adhesion of *Streptococcus pneumoniae* to human pharyngeal epithelial cells in vitro: differences in adhesive capacity among strains isolated from subjects with otitis media, septicemia, or meningitis or from healthy carriers. Infect Immun 32: 311-317.
319. Gerlini A, Colomba L, Furi L, Braccini T, Manso AS, et al. (2014) The role of host and microbial factors in the pathogenesis of pneumococcal bacteraemia arising from a single bacterial cell bottleneck. PLoS Pathog 10: e1004026.
320. Sun X, Ge F, Xiao CL, Yin XF, Ge R, et al. (2010) Phosphoproteomic analysis reveals the multiple roles of phosphorylation in pathogenic bacterium *Streptococcus pneumoniae*. J Proteome Res 9: 275-282.
321. Kloosterman TG, Hendriksen WT, Bijlsma JJ, Bootsma HJ, van Hijum SA, et al. (2006) Regulation of glutamine and glutamate metabolism by GlnR and GlnA in *Streptococcus pneumoniae*. J Biol Chem 281: 25097-25109.
322. Sabharwal V, Ram S, Figueira M, Park IH, Pelton SI (2009) Role of complement in host defense against pneumococcal otitis media. Infect Immun 77: 1121-1127.
323. Alloing G, de Philip P, Claverys JP (1994) Three highly homologous membrane-bound lipoproteins participate in oligopeptide transport by the Ami system of the gram-positive *Streptococcus pneumoniae*. J Mol Biol 241: 44-58.
324. van Opijnen T, Camilli A (2012) A fine scale phenotype-genotype virulence map of a bacterial pathogen. Genome Res 22: 2541-2551.
325. Tettelin H, Nelson KE, Paulsen IT, Eisen JA, Read TD, et al. (2001) Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science 293: 498-506.
326. Claverys JP, Grossiord B, Alloing G (2000) Is the Ami-AliA/B oligopeptide permease of *Streptococcus pneumoniae* involved in sensing environmental conditions? Res Microbiol 151: 457-463.
327. Hathaway LJ, Battig P, Reber S, Rotzetter JU, Aebi S, et al. (2014) *Streptococcus pneumoniae* detects and responds to foreign bacterial peptide fragments in its environment. Open Biol 4: 130224.
328. Stralin K, Herrmann B, Abdeldaim G, Olcen P, Holmberg H, et al. (2014) Comparison of sputum and nasopharyngeal aspirate samples and of the PCR gene targets lytA and Spn9802 for quantitative PCR for rapid detection of pneumococcal pneumonia. J Clin Microbiol 52: 83-89.
329. Mbelle N, Huebner RE, Wasas AD, Kimura A, Chang I, et al. (1999) Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. J Infect Dis 180: 1171-1176.
330. Weinberger DM, Malley R, Lipsitch M (2011) Serotype replacement in disease after pneumococcal vaccination. Lancet 378: 1962-1973.
331. Bottomley C, Roca A, Hill PC, Greenwood B, Isham V (2013) A mathematical model of serotype replacement in pneumococcal carriage following vaccination. J R Soc Interface 10: 20130786.
332. Weinberger DM, Bruden DT, Grant LR, Lipsitch M, O'Brien KL, et al. (2013) Using pneumococcal carriage data to monitor postvaccination changes in invasive disease. Am J Epidemiol 178: 1488-1495.
333. Auranen K, Rinta-Kokko H, Goldblatt D, Nohynek H, O'Brien KL, et al. (2013) Design questions for *Streptococcus pneumoniae* vaccine trials with a colonisation endpoint. Vaccine 32: 159-164.

334. McAvin JC, Reilly PA, Roudabush RM, Barnes WJ, Salmen A, et al. (2001) Sensitive and specific method for rapid identification of *Streptococcus pneumoniae* using real-time fluorescence PCR. J Clin Microbiol 39: 3446-3451.
335. Morens DM, Taubenberger JK, Fauci AS (2008) Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. J Infect Dis 198: 962-970.
336. Short KR, Diavatopoulos DA, Thornton R, Pedersen J, Strugnell RA, et al. (2011) Influenza virus induces bacterial and nonbacterial otitis media. J Infect Dis 204: 1857-1865.
337. Bogaert D, De GR, Hermans PW (2004) *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet InfectDis 4: 144.
338. Didierlaurent A, Goulding J, Patel S, Snelgrove R, Low L, et al. (2008) Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. J Exp Med 205: 323-329.
339. Hussell T, Goulding J (2010) Structured regulation of inflammation during respiratory viral infection. Lancet Infect Dis 10: 360-366.
340. Pittet LA, Hall-Stoodley L, Rutkowski MR, Harmsen AG (2010) Influenza virus infection decreases tracheal mucociliary velocity and clearance of *Streptococcus pneumoniae*. Am J Respir Cell Mol Biol 42: 450-460.
341. Faure M, Rabourdin-Combe C (2011) Innate immunity modulation in virus entry. Current Opinion in Virology 1: 6-12.
342. Vareille M, Kieninger E, Edwards MR, Regamey N (2011) The airway epithelium: soldier in the fight against respiratory viruses. Clin Microbiol Rev 24: 210-229.
343. Lu L, Ma Z, Jokiranta TS, Whitney AR, DeLeo FR, et al. (2008) Species-specific interaction of *Streptococcus pneumoniae* with human complement factor H. J Immunol 181: 7138-7146.
344. Hammerschmidt S, Tillig MP, Wolff S, Vaerman JP, Chhatwal GS (2000) Species-specific binding of human secretory component to SpsA protein of *Streptococcus pneumoniae* via a hexapeptide motif. Mol Microbiol 36: 726-736.
345. Quin LR, Onwubiko C, Carmicle S, McDaniel LS (2006) Interaction of clinical isolates of *Streptococcus pneumoniae* with human complement factor H. FEMS Microbiol Lett 264: 98-103.
346. Kimaro Mlacha SZ, Peret TC, Kumar N, Romero-Steiner S, Dunning Hotopp JC, et al. (2013) Transcriptional adaptation of pneumococci and human pharyngeal cells in the presence of a virus infection. BMC Genomics 14: 378.
347. Kadioglu A, Weiser JN, Paton JC, Andrew PW (2008) The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. Nat Rev Microbiol 6: 288-301.
348. Voss S, Hallstrom T, Saleh M, Burchhardt G, Pribyl T, et al. (2013) The Choline-binding Protein PspC of *Streptococcus pneumoniae* Interacts with the C-terminal Heparin-binding Domain of Vitronectin. Journal of Biological Chemistry 288: 15614-15627.
349. Ferreira DM, Darrieux M, Silva DA, Leite LC, Ferreira JM, Jr., et al. (2009) Characterization of protective mucosal and systemic immune responses elicited by pneumococcal surface protein PspA and PspC nasal vaccines against a respiratory pneumococcal challenge in mice. Clin Vaccine Immunol 16: 636-645.
350. Hernani Mde L, Ferreira PC, Ferreira DM, Miyaji EN, Ho PL, et al. (2011) Nasal immunization of mice with Lactobacillus casei expressing the pneumococcal surface protein C primes the immune system and decreases pneumococcal nasopharyngeal colonization in mice. FEMS Immunol Med Microbiol 62: 263-272.
351. Brooks-Walter A, Briles DE, Hollingshead SK (1999) The pspC gene of *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-reactive

antibodies to PspA and provides immunity to pneumococcal bacteremia. Infect Immun 67: 6533-6542.

352. Melin M, Coan P, Hollingshead S (2012) Development of cross-reactive antibodies to the proline-rich region of pneumococcal surface protein A in children. Vaccine 30: 7157-7160.
353. Yuste J, Khandavilli S, Ansari N, Muttardi K, Ismail L, et al. (2010) The effects of PspC on complement-mediated immunity to *Streptococcus pneumoniae* vary with strain background and capsular serotype. Infect Immun 78: 283-292.
354. Agarwal V, Asmat TM, Luo S, Jensch I, Zipfel PF, et al. (2010) Complement regulator Factor H mediates a two-step uptake of *Streptococcus pneumoniae* by human cells. Journal of Biological Chemistry 285: 23486-23495.
355. Quin LR, Onwubiko C, Moore QC, Mills MF, McDaniel LS, et al. (2007) Factor H binding to PspC of *Streptococcus pneumoniae* increases adherence to human cell lines in vitro and enhances invasion of mouse lungs in vivo. Infect Immun 75: 4082-4087.
356. Fleming-Dutra KE, Taylor T, Link-Gelles R, Garg S, Jhung MA, et al. (2013) Effect of the 2009 influenza A(H1N1) pandemic on invasive pneumococcal pneumonia. J Infect Dis 207: 1135-1143.
357. Emonts M, Uitterlinden AG, Nouwen JL, Kardys I, Maat MP, et al. (2008) Host polymorphisms in interleukin 4, complement factor H, and C-reactive protein associated with nasal carriage of *Staphylococcus aureus* and occurrence of boils. J Infect Dis 197: 1244-1253.
358. Madico G, Welsch JA, Lewis LA, McNaughton A, Perlman DH, et al. (2006) The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance. J Immunol 177: 501-510.
359. Snape MD, Dawson T, Oster P, Evans A, John TM, et al. (2010) Immunogenicity of two investigational serogroup B meningococcal vaccines in the first year of life: a randomized comparative trial. Pediatr Infect Dis J 29: e71-79.
360. Santolaya ME, O'Ryan ML, Valenzuela MT, Prado V, Vergara R, et al. (2012) Immunogenicity and tolerability of a multicomponent meningococcal serogroup B (4CMenB) vaccine in healthy adolescents in Chile: a phase 2b/3 randomised, observer-blind, placebo-controlled study. Lancet 379: 617-624.
361. Pollard AJ, Riordan A, Ramsay M (2014) Group B meningococcal vaccine: recommendations for UK use. Lancet 383: 1103-1104.
362. Beernink PT, Shaughnessy J, Braga EM, Liu Q, Rice PA, et al. (2011) A meningococcal factor H binding protein mutant that eliminates factor H binding enhances protective antibody responses to vaccination. J Immunol 186: 3606-3614.
363. Neill DR, Coward WR, Gritzfeld JF, Richards L, Garcia-Garcia FJ, et al. (2014) Density and duration of pneumococcal carriage is maintained by transforming growth factor beta1 and T regulatory cells. Am J Respir Crit Care Med 189: 1250-1259.

Appendices

Appendix A: Recipe for STGG medium

Tryptone soya broth (Oxoid CM0129)	6 g
Skim milk powder (Oxoid L31)	4 g
Glucose	1 g
Glycerol (Sigma G5516)	20 ml
Distilled water	200 ml

Mix together the tryptone soya broth, skim milk powder, glucose, and distilled water. Slowly add the glycerol. Autoclave at 15 lb/in^2 and 121°C for 15 minutes. Store bottle at 4°C. Before removing any media, vortex the bottle to resuspend the pellet that develops during storage.

Appendix B: Patient information sheet

Experimental Human Pneumococcal Carriage

Investigator	Designation	Contact telephone
Professor Stephen Gordon	Principle Investigator	0151 705 3169
Dr Andrea Collins	Research Registrar	0151 705 3712
Angela Wright	Research Nurse	0151 706 4856
David Shaw	Research Nurse	0151 706 4856
Carole Hancock	Research Nurse	0151 706 3381

Patient information sheet

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

PART 1 WHAT IS THE PURPOSE OF THE STUDY?

We are interested in developing new and better ways to protect the body against a bug, or bacterium, called *Streptococcus pneumoniae* (also known as pneumococcus). In most people pneumococcus can occasionally be found harmlessly inhabiting the nose where it does not cause any problem (pneumococcal carriage). About 10% of adults carry pneumococcus at any one time, and almost all adults experience an episode of carriage at least once per year. Carriage acts as a natural vaccine, boosting immunity against pneumococcal infection in adults and children. Disease in young adults is rare - less than 10 cases per 100 000 people per year. When pneumococcus does cause problems, usually in young children or elderly people, it can be very serious as it is the bacterium responsible for diseases such as pneumonia, sepsis and meningitis, which kill millions of children around the world each year. There is already a vaccine against pneumococcus which helps prevent sepsis and meningitis but it is less effective against pneumonia than we would like.

In order to develop an effective vaccine against pneumonia, we need to understand how pneumococcal carriage acts to boost immunity. We need to demonstrate that we can reproduce carriage in healthy volunteers and then prevent it with new vaccines. Our aim in this study is to inoculate people with varied doses of pneumococcal bacteria to determine the optimum dose for carriage (dose-ranging study) and then reproduce these findings in

sufficient participants to be sure that we have a reproducible method (reproducibility study).

What is involved?

We will inoculate small doses of bacteria into the nostrils of volunteers known to have no current natural pneumococcal carriage. We will then collect samples from these people, some of whom will establish carriage for a period (days to weeks), in order to answer questions about immune defence and then use the information in our work to develop a better vaccine to protect against pneumonia. We will offer antibiotics to clear carriage to all volunteers. The samples we collect will include upper airway (nasal wash, throat swab and saliva), lower airway (bronchoscopy and lavage) and systemic (blood, urine) samples relevant to defence against infection. The number of samples that you will have depends on which part of the study you are in and is explained below –

Study	Blood samples	Urine samples	Nasal wash samples	Saliva samples	Throat swabs	Bronchoscopy
Dose ranging	3	3	4	2	2	optional
Reproducibility	7	7	7	2	2	optional
Re-challenge	2	0	4	0	0	optional
Enriched sampling (in addition to those above)	5	0	0	0	0	optional

Who may take part?

You will be able to join the study if you are fit and healthy and not a current regular smoker.

Who may NOT take part?

People who have/are:

- Current regular smokers (smoke on a daily basis)
- Significant smoking history defined as someone who has previously smoked more than 20 cigarettes per day for 10 years (or the equivalent)
- Asthma/respiratory disease
- Pregnant
- Already involved in another clinical trial
- Allergic to penicillin/amoxicillin
- On medication that may affect the immune system in any way e.g steroids, steroid
- nasal spray Regular contact with 'at-risk' individuals – this means children, immunosuppressed adults, elderly or those with chronic ill health

Do I have to take part?

No, you do not have to take part. It is entirely up to you to decide whether or not to take part in this study. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive at this hospital. You will be able to choose if samples and information collected up to that point may be used or should be destroyed.

PART 2 WHAT DO I HAVE TO DO?

Clinic

After reading through the information if you decide you would like to take part we will arrange to see you in a clinic to discuss the trial further. During that appointment we will ask some routine questions about your medical health and we will want to listen to your heart and lungs. If you decide that you wish to take part we will ask you to sign a consent form. We will then ask your permission to take a wash from your nose, a sample of your saliva, throat swab, urine and a blood test. This will all be done to make sure you are fit to take part in the study, and to see if you are one of the 10% of people who already carry pneumococcus in their nose.

Measurements of immune response in the nose

The nasal wash is similar to a process used in yoga for nasal hygiene. Salty water is gently instilled in to the nose (sucked by breathing in yoga) and retained for a few seconds before being allowed to run out. As with a mouth wash, this process washes out some secretions and cells from the nose that can be used to measure immune responses. Inevitably, this process feels like having water up your nose, but warm saline is less likely to cause sneeze or irritation than cold water, and the process is safe.

Nasal carriage of pneumococcus

What we are now interested in is whether there is a change in your body's immune system when you have pneumococcus in your nose. We will invite you to the clinic where we will put a small amount of liquid in your nose that contains live pneumococcus in order to mimic natural pneumococcal carriage. We do not expect that this will cause you any symptoms as previous similar studies have not had any problems. As a precaution, however, we will provide you with an information sheet about warning symptoms or signs and there will be a doctor or nurse available by telephone day 24 hours a day in case you have any concerns. We will also provide you with a course of antibiotics to keep with you, in case you feel unwell. We will see you after the inoculation to repeat the nasal wash and see if pneumococcus is still present in your nose. Each day, we will ask you to contact the research team by phone or text to ensure that all is well.

We will then follow you up once a week for a period (up to 4 weeks in the rechallenge and dose-ranging study; up to 8 weeks in the reproducibility study) for sample collection (nasal wash/saliva/throat swab/urine/blood) to monitor the presence or absence of pneumococci in your nose. If you are a carrier, we will ask you to take a 3 day course of antibiotics to be sure that the pneumococcus has been cleared at the end. One final visit will be arranged in the dose ranging and reproducibility study between 10 and 20 weeks after the start of the study.

In volunteers that successfully carry the bacteria, we will offer the chance to repeat this inoculation with the same strain or a different strain 2-18 months following carriage (this is called re-challenge). If you wish to participate in the re-challenge, you will be asked to complete a further consent form, your participation is optional.

Volunteers that naturally carry the bacteria at their initial sampling, will be invited to return 2-18 months after for inoculation with 6B or 23F strain of pneumococcus, assuming they are no longer naturally carrying the bacteria. If you wish to participate, you will be asked to complete a further consent form, your participation is optional. The sampling is the same as for the re-challenge (see above table), lasting 14 days in total post inoculation.

As part of the study, you will have the option of participating in extra sampling. This includes extra blood samples throughout the study and/or bronchoscopy at the end of the study.

What does enriched sampling involve?

Extra blood samples will be taken on five extra occasions during the study; this will involve extra visits to the hospital. This extra time and inconvenience is accounted for in the remuneration.

What is a bronchoscopy and lavage?

A bronchoscopy means a camera test to look inside the lungs and a lavage is washing of a small segment of the right lung. The test is carried out as a day procedure in the hospital; to prepare for the test **you must not eat or drink for 4 hours** before arrival. In order to make the camera test comfortable and controlled, we first put local anaesthetic (both jelly and spray) inside your nose and mouth to numb the lining. We will also offer you some sedation to make you more relaxed if you would like. We do not do the test under a general anaesthetic. During the test we provide supplementary oxygen to all people, along with oxygen, pulse and blood pressure monitoring to ensure that you are safe.

We pass the bronchoscope (a flexible tube of the same diameter as a ball-point pen) either through the nose or mouth to the back of the throat. Using a channel in the bronchoscope, we then put local anaesthetic on the voice box (this will make you splutter) and in the main airways. As a result of this anaesthetic, you will not feel the bronchoscope inside your lungs at all. When the bronchoscope is in the correct position, we wash a sub-segment of the right lung. We use 200mls of saline which is about the same as a cup of tea. The warm saline is introduced and withdrawn using gentle hand suction and as with the nasal wash, we collect some lung secretions and cells (bronchoalveolar lavage or BAL) in order to test immune function. It generally takes about 15-20 minutes to prepare a volunteer for bronchoscopy, including giving the anaesthetic and sedation, and 7 minutes to complete the bronchoscopy and lavage.

After bronchoscopy, because you will have had medication to numb your throat and maybe even sedation, we will observe you for at least 2 hours. After this time, the medication will have worn off and you will be allowed to eat and drink again as normal before going home. Later in the evening after bronchoscopy, some people get a pain (mild

pleurisy) under their right arm which can last for the first 12-24hours. This is caused by inflammation in the area that lies above the part of the right lung where the lavage was done and is best alleviated using paracetamol.

What laboratory investigations will be done on samples collected?

All of your samples (blood, nasal wash, urine, saliva, throat swab and BAL) will be used to determine the organisms (bacteria and viruses) present and the immune responses that defend the body, particularly the lung, against infection. The tests on the samples will be done in the Liverpool School of Tropical Medicine and in collaborating laboratories including those at the Royal Liverpool University Hospital. All of your identifiable information will be removed from the samples; they will be given a unique number that does not reveal your identity.

How much will I get paid?

We do not want anybody to be persuaded to take part because of the money we offer. The money is to remunerate volunteers for the time, inconvenience, loss of income, risks and possible discomfort that taking part may cause.

Activity	Approximate Time	Payment
Screening appointments (clinical exam, blood tests, nasal wash, throat swab and saliva sample)	30min	£30 per visit
Inoculation with pneumococcus (includes email/telephone contact and completion of daily symptom log)	30 minutes	£50
Weekly follow up visit (Review by researcher, blood, urine and nasal wash)	15 minutes	£20 per visit
Enriched sampling limb: blood sample	10 minutes	£10 per visit
Bronchoscopy and BAL (including follow up visit)	4 hours	£100

What if I change my mind?

You will be asked to sign a consent form at the beginning of the study, and a further form before bronchoscopy. You have the right to change your mind, or withdraw your consent, at any time during the study. This will have no effect on your future care within the NHS. Please note that should you decide to withdraw from the study, you will be paid for the amount completed up to that point.

What are the benefits of taking part?

It is unlikely that there will be any direct benefit to you as a result of taking part except that the nasal swabs may identify unexpected bacteria which could be treated. If this occurs, you will be referred for the appropriate treatment in either the Infectious Disease or Respiratory service in the Royal Liverpool University Hospital. In all cases, you will be a

valuable part of a research study that we hope will eventually lead to the development of a new vaccine against pneumonia.

What are the risks?

The risks that you should consider *before* participation in this study are:

(i) Risks associated with nasal washing or bronchoscopy (ii) Risks associated with inoculation with live bacteria.

- **Risks associated with nasal washing**

Nasal washing carries minimal risk and is a part of common yoga *as* described above. The British Lung Foundation (BLF) describes bronchoscopy as a safe procedure which carries little risk (BLF patient information sheet available on request). Most people do not suffer any ill effects but of those that do, the common side effects are a sore throat and hoarseness for a few hours, or perhaps nasal discomfort and a minor nose bleed after the test. This is the reason we keep an eye on you in the hospital for a few hours after the test to make sure you are not experiencing any of these problems. It is possible that you will experience a drop in blood oxygen or breathlessness during the procedure and if these occur, we will stop immediately. As mentioned above, some people experience mild pleurisy (pain with fever) for some hours during the night following the procedure. This is best controlled by paracetamol.

- **Risks associated with inoculation with live bacteria**

Experimental studies of this type have never yet resulted in infection but the bacteria are live and so infection either of you or your close contacts is possible. We do however all commonly naturally carry this bacterium in our nose from time-to-time, especially as children. We do not expect that carrying the bacteria in your nose will cause any illness in you or your contacts but this is one of the reasons we want to make sure you are healthy (with a good immune system) before taking part and we will not recruit people in close contact with young children or vulnerable adults. The pneumococcus is a bacterium that can cause serious diseases such as pneumonia, sepsis and meningitis. It is therefore important that you are aware of this risk and clearly understand the safety information that you are given. In the event, should you feel unwell, you will have an emergency information leaflet to advise you on what to do and the precise symptoms to be concerned about including fever, drowsiness and ear-ache. You can speak to a doctor involved in the project at any time by telephone. You will also be given a course of antibiotics (that you will have at home and carry with you at all times) to take in the event that you develop any symptoms (as per emergency information leaflet) that may be related to the pneumococcal bacteria. If you carry the bacteria in your nose then at the end of 4 weeks, we will give you antibiotics to be absolutely sure that there are no bacteria left.

What if there is a problem?

You will have the contact numbers and email addresses for the Research Registrar and Nurse who will be available for contact to answer any questions. They will also be available 24 hours a day in the unlikely event of an emergency. Any medical care needed will be provided in the Royal Liverpool University Hospital and this could include any test or

treatment needed up to and including hospital admission with CT scan, chest X-ray, lumbar puncture and intravenous antibiotics. Further, the study is sponsored (insured) by the Royal Liverpool University Hospital and the Liverpool School of Tropical Medicine. Compensation for any injury caused by taking part in this study will be in accordance with the guidelines of the Association of the British Pharmaceutical Industry (ABPI). Broadly speaking the ABPI guidelines recommend that 'the sponsor', without legal commitment, should compensate you without you having to prove that it is at fault. This applies in cases where it is likely that such injury results from giving any new drug or any other procedure carried out in accordance with the protocol for the study. 'The sponsor' will not compensate you where such injury results from any procedure carried out which is not in accordance with the protocol for the study. Your right at law to claim compensation for injury where you can prove negligence is not affected. Copies of these guidelines are available on request.

Will my details be kept confidential?

Yes. We need to collect information about your medical history and any relevant family history to make sure you are fit to take part in the study. We will also collect clinical data relating to the results of blood tests, swabs and bronchoscopies. It is important that the research doctors in charge of the study continue to have access to your personal information so you can be followed up properly, or contacted during/after the trial. One reason that we might need to contact you is if we find an important immune problem whilst testing your samples; this would mean that the Doctors would decode your specific volunteer identification number in order to contact you to inform you of the issue/defect. This ability to access personal information will be limited to Dr Stephen Gordon, Dr Andrea Collins and Sr Angela Wright. We will ask your permission to inform your GP that you are taking part in the trial as this may be relevant to your medical care outside the trial. Any information of value to your care will be conveyed to you and to your GP. All information we collect will be kept secure and confidential. Your research notes will be kept separate from your NHS notes. All data will be collected and stored within the Royal Liverpool University Hospital and the Liverpool School of Tropical Medicine. It will be stored for a period of 4 years after publication or longer if required by a publishing journal. No publication or presentation regarding this study will contain information that can be identified as from any particular volunteer.

In the laboratory, all information that identifies you will be removed and replaced with a code so that laboratory staff will not know the individual identity of samples collected.

What if I wish to complain about the way in which this study has been conducted?

If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you and are not compromised in any way because you have taken part in a research study. If you have any complaints or concerns please contact your study doctor (see below details).

Further questions?

If you have any further questions about this trial please contact Prof Stephen Gordon (Principal Investigator) on 0151 705 3169, Dr Andrea Collins (Study Dr) on 0151 705 3712 or Sr Angela Wright on 0151 706 4856 adwright@liverpool.ac.uk (Research Nurse) during normal working hours.

In the event of an emergency please contact Professor Gordon via the Royal Liverpool University Hospital Switchboard (0151 706 2000).

You will be given a copy of this information sheet and of your signed consent form to keep.

Thank you.

Appendix C: Consent form

Experimental Human Pneumococcal Carriage
Protocol A

Study Number:	
Study Subject	

Consent Form

If you agree with each sentence below, please INITIAL **the box:** **INITIALS**

1. I confirm that I have read and understand the information sheet dated 14th December 2012, version 4, for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily ☐

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

3. I understand that the relevant section of my medical notes and data collected during the study, may be looked at by individuals from regulatory authorities or from the NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to access my records ☐

4. I agree to my GP being informed of my participation in the study. ☐

5. I agree to provide details of a contact who, in the event of an emergency, could be contacted on my behalf ☐

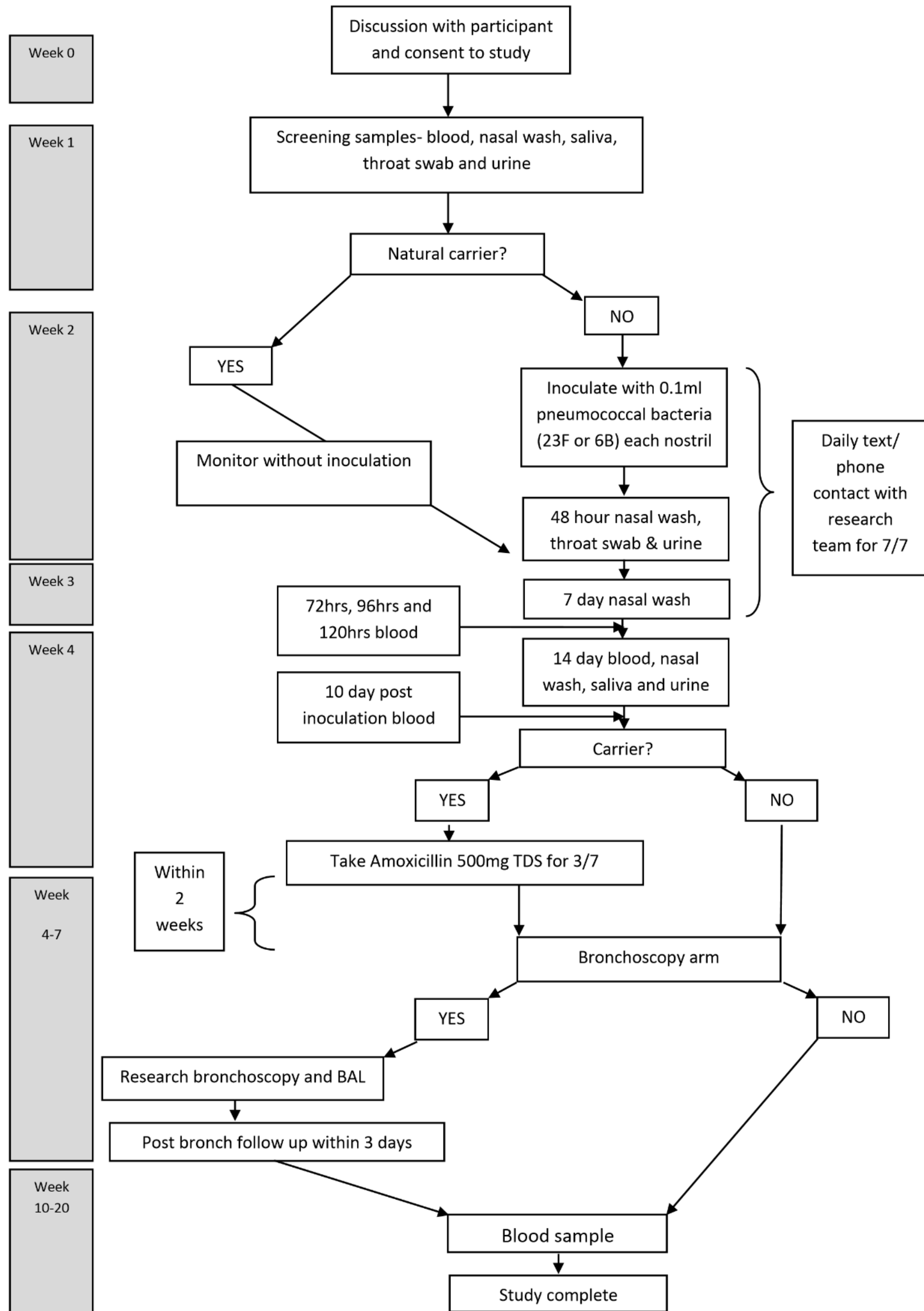
6. I agree to take part in this study . ☐

Please print and sign your name below and add today's date:

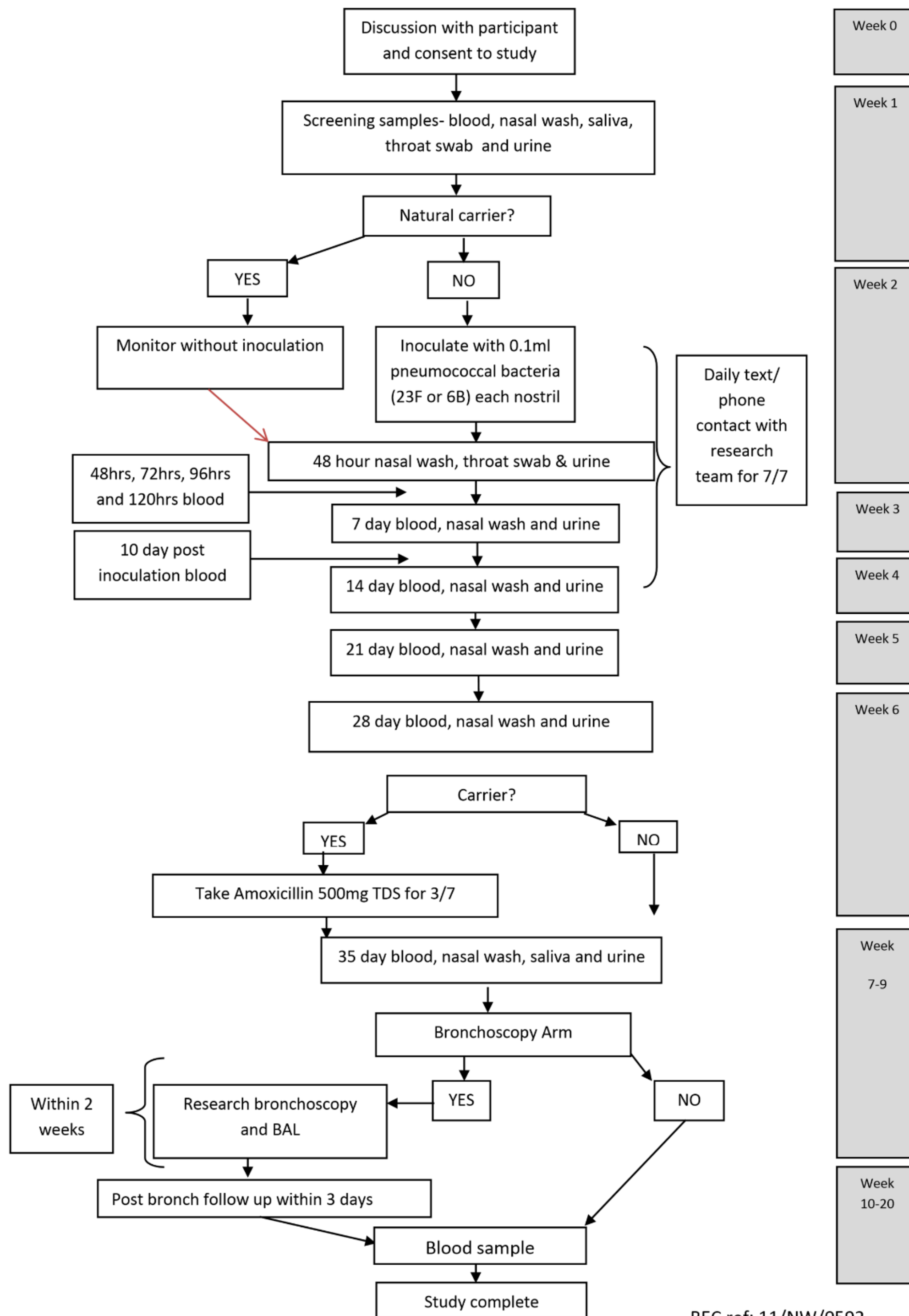
…………………………………	…………………………………	……………………
Name of patient	Signature	Date
…………………………………	…………………………………	……………………
Name of person taking consent	Signature	Date

1 copy for patient: 1 for CRF: 1 to be filed in the hospital notes

Appendix D: Dose-Ranging study schedule with additional bloods



Appendix E: Reproducibility study schedule with additional bloods



REC ref: 11/NW/0592