Drivers of Nasopharyngeal Pneumococcal Colonisation: Investigation Using an Experimental Human Challenge Model

Thesis submitted in accordance with the requirements of the Liverpool School of Tropical Medicine for the degree of Doctor of Medicine (MD)

By

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Declaration

This thesis is the result of my own work and effort. The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

Research in this thesis was carried out at the Liverpool School of Tropical Medicine. For the Hand to Nose study presented in this thesis I developed this project from the initial stages; I wrote the protocol and all study documents for ethical submission. I presented the project to the Research Ethics Committee and secured approval. I arranged sponsorship of the study from Liverpool School of Tropical Medicine. I co-ordinated and managed both studies in this project working with clinicians, research nurses, laboratory staff and the Clinical Research Facility (CRU) at the Royal Liverpool and Broadgreen University Hospital Trust (RLBUHT). Working within the clinical research team I lead recruitment of volunteers, clinical sample taking and symptoms data collection.

In some instances, work was carried out in collaboration with other colleagues. The table below details in full the attribution of work and responsibility related to the project.

Activity	Responsibility- Hand to Nose Study	Responsibility- New Serotypes Study
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Clinician cover for safety monitoring of participants	**Victoria Connor** Hugh Adler Seher Zaidi	**Victoria Connor** Hugh Adler Seher Zaidi
Study oversight and management	**Victoria Connor**	**Victoria Connor** Helen Hill
Recruitment, sample taking, symptoms data collection	**Victoria Connor** Caz Hales Helen Hill Angie Hyder-Wright Hugh Adler Seher Zaidi Rachel Robinson Cath Lowe	**Victoria Connor** Caz Hales Helen Hill Angie Hyder-Wright Hugh Adler Seher Zaidi Rachel Robinson Cath Lowe
Inoculum preparation and nasal wash sample processing and pneumococcal detection (culture)	Sherin Pojar Esther German Elena Mitsi Elissavet Nikolaou	Sherin Pojar Esther German Elena Mitsi Elissavet Nikolaou
Viral throat swab processing	Liverpool Clinical Laboratories	NA
Research blood sample processing	NA	Esther German Carla Solórzano Gonzalez
Pneumococcal DNA extraction	**Victoria Connor**	**Victoria Connor** Sherin Pojar Esther German Carla Solórzano Gonzalez Elena Mitsi
qPCR process	**Victoria Connor**	Elissavet Nikolaou
ELISA	NA	**Victoria Connor**
Statistical analysis	**Victoria Connor**	**Victoria Connor**

Conflicts of Interest

Both studies described in this thesis were either partly or fully funded by the Medical Research Council (MRC) (MR/M011569/1). Unilever (consumer goods company) partly funded the Hand to Nose study outlined in Chapter 3, representatives from this company participated in the design of this study. This participation included participation in preliminary talks about the feasibility of using the pneumococcal challenge model to investigate hand transmission and sharing of protocols for standard handwashing studies used by Unilever. There was also review and some comments given about the first draft of the study protocol which the study team reviewed and only considered if it would improve the scientific strength of the study.

The conduct of both studies, analysis and presentation of results and the decision to disseminate results were solely determined by the authors, without influence from any funding source.

Presentations, Awards and Publications

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- **V. Connor**, E. German, S. Pojar, E. Mitsi, C. Hales, E. Nikolaou, A. Hyder-Wright, H. Adler, S. Zaidi, H. Hill, S.P. Jochems, H. Burhan, T. Tobery, J. Rylance and D.M. Ferreira. Hands are Vehicles for Transmission of *Streptococcus pneumoniae* in Novel Controlled Human Infection Study. **European Respiratory Journal - in press; accepted 01/08/2018**

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Abbreviations

The following list may be useful for abbreviations used throughout the thesis, all are explained at their first use.

AE	Adverse event
AOM	Acute otitis media
ARI	Acute respiratory infection
AUC	Area under the curve
CAP	Community acquired pneumonia
CFU	Colony forming units
CHIM	Controlled human infection model
CI	Confidence intervals
COPD	Chronic Obstructive Pulmonary Disease
COSHH	Control of Substances Hazardous to Health
CPS	Capsular polysaccharide
CRF	Case report form
CRM	Cross-reactive material
CRU	Clinical Research Unit
CTIMP	Clinical trials of investigation medicinal products
DMSC	Data monitoring and safety committee
EHPC	Experimental human pneumococcal colonisation/ carriage
GP	General Practitioner
Hand to Nose study	Hand to Nose transmission of Streptococcus pneumoniae in healthy participants – pilot study
hMPV	Human Metapneumovirus
hRV	Human Rhinovirus
IgG	Immunoglobulin
IL	Interleukin
IPD	Invasive pneumococcal disease
IRAS	Integrated Research Application System
ISRCTN	International Standard Randomised Controlled Trials number
ITT	Intention-to-treat
LRTI	Lower respiratory tract infection
LSTM	Liverpool School of Tropical Medicine
M&M	Miles and Misra method
MHRA	Medicines and Healthcare Products Regulatory Authority

New Serotypes study	The effect of different serotypes of pneumococcus on colonisation in healthy participants
NHS	National Health Service
NIHR	National Institute of Health Research
NPS	Nasopharyngeal swab
NVT	Non-vaccine serotype - not included in PCV
NW	Nasal wash
OM	Otitis media
PATH	Program for Appropriate Technology in Health
PcpA	Protein choline-binding protein
PCV	Pneumococcal conjugate vaccine
PhtD	Pneumococcal histidine triad protein D
PI	Primary investigator
PIL	Patient information leaflet
Ply	Pneumolysin
PPI	Patient and public engagement
PPV	Polysaccharide pneumococcal vaccine
PS	Polysaccharide
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
qPCR	Quantitative polymerase chain reaction
RCT	Randomised control trial
RD&I	Research, development and innovation department
REC	Research and Ethics Committee
RLBUHT	Royal Liverpool and Broadgreen Hospital Trust
RSV	Respiratory Syncytial Virus
SAE	Serious adverse event
SD	Standard deviation
SOP	Standard operating protocols
STGG	Skim milk, tryptone, glucose, glycerol medium
TNF	Tumour necrosis factor
TOPS	The over-volunteering protection system
TSC	Trial steering committee
UK	United Kingdom
URT	Upper respiratory tract
URTI	Upper respiratory tract infection
USA	United States of America
VT	Vaccine serotype included in PCV
VTM	Viral transport medium
WHO	World Health Organisation

Abstract

Introduction *Streptococcus pneumoniae* (or pneumococcus) is a common commensal (coloniser) of the human upper respiratory tract. Colonisation is likely a prerequisite for respiratory tract infections and invasive pneumococcal disease. Colonisation also has a significant role in the horizontal spread of this pathogen within communities, but paradoxically could also lead to boosting of the host's immune system. We use the unique experimental human pneumococcal challenge (EHPC) model to study pneumococcal transmission and colonisation in healthy adults. This novel study design allows us to investigate bacteriological and immune factors associated with colonisation and to examine the density and duration of colonisation episodes.

Project Aims 1) Investigation of the transmission dynamics of *Streptococcus pneumoniae*. Can the hands can be a vector for transmission of *S. pneumoniae* into the nasopharynx, leading to colonisation? Does concurrent asymptomatic viral infection affect transmission? 2) Investigation of the propensity of two pneumococcal serotypes to cause experimental pneumococcal colonisation, to improve the generalisability of the model and to investigate if immunological responses to serotype 6B are similar to other serotypes. We also wanted to investigate if colonisation is an asymptomatic process in healthy adults? How do the host's polysaccharide specific antibody responses affect colonisation?

Main findings Using our unique controlled human pneumococcal challenge model, we have demonstrated the viability of transmission of pneumococcus from the hand into the nasopharynx, leading to colonisation. We were unable to investigate the relationship between colonisation acquisition and concurrent viral infection due to the

absence of viral infection in our participants. The data presented in this thesis showed that the experimental human pneumococcal carriage model can successfully investigate transmission dynamics of pneumococcus. We also demonstrated the varying propensity of two pneumococcal serotypes, 23F and 15B to experimentally colonise the nasopharynx of healthy adults. Nasopharyngeal colonisation was shown not to cause nasal symptoms; however, the data suggested that colonisation may cause a cough in healthy adults. No relationship was found between the level of serum IgG to 15B capsular polysaccharide at screening and colonisation outcome after intranasal inoculation. Nasopharyngeal colonisation with 15B was however, found to boost polysaccharide specific immunity; colonisation positive participants had a significant increase in serum IgG levels to 15B capsular PS.

Implications Data presented in this thesis suggest that good hand hygiene practices, already known to reduce enteric bacterial and viral disease, may also prevent the spread of pneumococcus which is thought to be spread primarily through aerosolisation. Results support epidemiological studies which have shown the varying propensity of different pneumococcal serotypes to cause colonisation. We can build upon this work by investigating serotypes *in vitro* and *in vivo* to understand bacterial factors that impact the pneumococcus' ability to colonise the nasopharynx in humans. The EHPC model will be useful in further studies to better understand the dynamics and drivers of pneumococcal transmission, bacterial factors which support successful colonisation and host responses to pneumococcal exposure and colonisation.

1 Introduction

This thesis focuses on investigating the drivers of nasopharyngeal pneumococcal colonisation. Two studies were conducted, and the clinical aspects of these studies will predominantly be discussed in this thesis. Both studies used the experimental human pneumococcal challenge model to answer research questions which are outlined at the end of the introduction. To form a rationale for these studies, a literature review was conducted on pneumococcal colonisation, transmission of *Streptococcus pneumoniae* and pneumococcal disease and is presented below along with description of gaps in the literature. Following this introduction, the methods section outlines the broad methods relevant to both studies described in this thesis. Chapter 3 and 4 describe the results of the two studies conducted as part of this MD project. The final chapter (Chapter 5) is a general discussion of the main findings of both studies, a methodological critique of the work conducted and the implications of the findings with an outline of possible future work in this field.

1.1 Overview

Streptococcus pneumoniae (or pneumococcus) is Gram-positive bacterium which is a common cause of respiratory tract infections and invasive disease worldwide. Pneumococcus is also a common commensal (coloniser) of the human upper respiratory tract. The majority of pneumococcal serotypes have a polysaccharide capsule (CPS) that surrounds the cell wall [1]. There are over 90 different serotypes described, each with a biochemically unique polysaccharide capsule. In most

serotypes this capsule is attached to the cell wall of the pneumococcus by covalent bonds [2]. Epidemiological studies have shown that most serotypes can cause disease, however, the majority of pneumococcal infections are secondary to a minority of serotypes [3]. This thick layer of CPS improves the organism's ability to evade the host's defences and is required for invasive infection [4]. The mechanisms by which the capsule aids in evading the host's defences include:

- Repelling anionic mucus with it's negatively charged polysaccharide capsule which allows the bacteria to escape the nasal mucus [5 6]
- Inhibition of phagocytosis by innate immune cells again by electrostatic repulsion [6]
- Helping to escape neutrophil net traps [6]
- Inhibition of complement and helps reduce recognition by immunoglobulins[6]

The World Health Organisation (WHO) named *Streptococcus pneumoniae* as a priority pathogen in 2017 because it poses a high level of threat to human health [7]. Invasive disease occurs when pneumococcus proliferates in areas of the body such as the middle ear, sinuses, blood stream and lungs (Figure 1) [8]. Meningitis, sepsis and pneumonia are the predominant invasive diseases caused by pneumococcus and are more common in high risk groups including elderly people, patients with immunodeficiencies and young children [9].

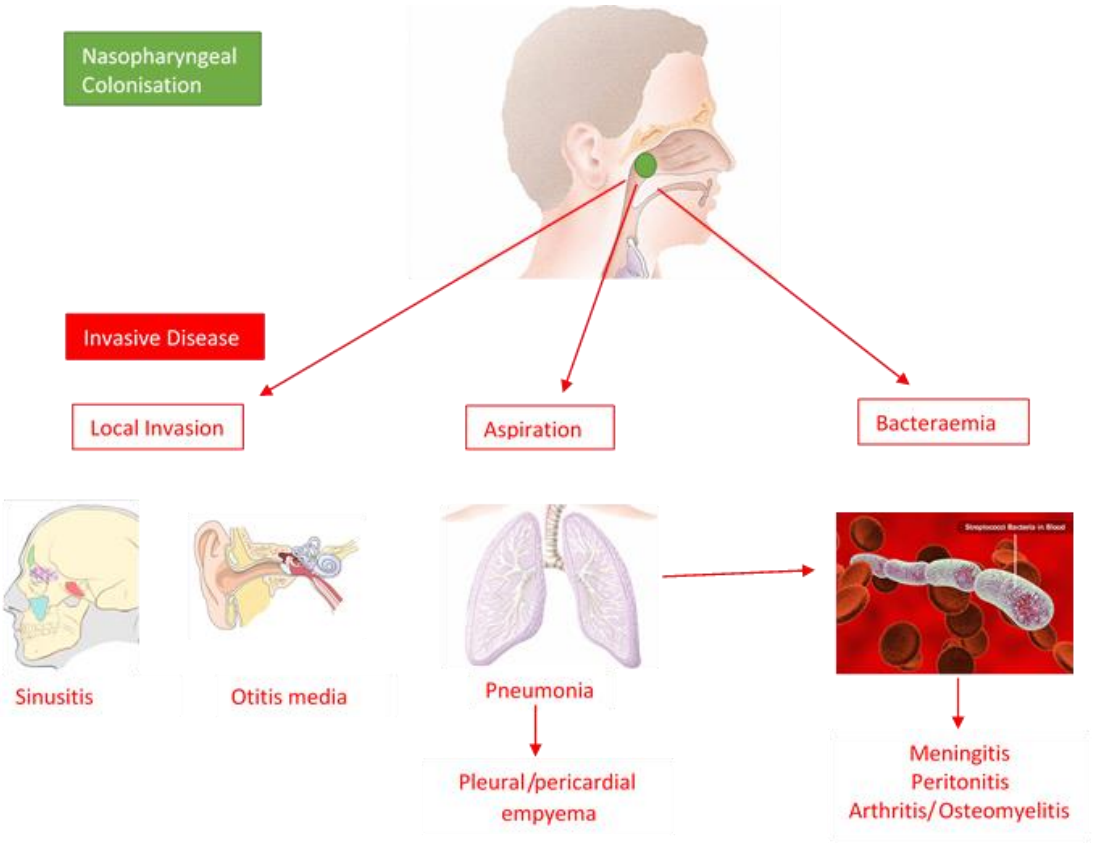

Figure 1*:* Pathogenesis of pneumococcal disease
Streptococcus pneumoniae colonises the nasopharynx, this often leads to clearance following a local immune response. Local spread and progression to otitis media is common in children. Children have the highest rates of nasopharyngeal pneumococcal colonisation. Aspiration into normally sterile alveoli can lead to pneumonia or into the blood stream leading to bacteraemia. Complications such as meningitis and empyema can also occur.[19]

Transmission and acquisition of this pathogen and its colonisation in the nasopharynx is likely a pre-requisite for the development of infectious disease. Colonisation also has a significant role in horizontal spread of this pathogen within communities and could also lead to immune protection. Controlled human infection studies have shown that colonisation can be an immunising event; an increase in both anti-pneumococcal antibody and T cell specific responses have been shown following colonisation [10].

Pneumococcus is a leading cause of lower respiratory tract infection and pneumonia worldwide. Definitive microbiological diagnosis is often difficult and antibiotic resistance is increasing. Strategies to prevent pneumococcal disease are becoming increasingly important. Therefore, this thesis focusses on investigating drivers of pneumococcal colonisation acquisition which may be blocked to reduce pneumococcal burden.

1.2 Colonisation

1.2.1 Pneumococcal colonisation

Stable colonisation of the human nasopharynx with *S. pneumoniae* is a common human phenomenon with 40-95% of infants and 10-25% of adults colonised at any time[11] [12-15]. The upper respiratory tract is also an ecological niche for many other bacterial species which colonise it, including the pneumococcus [16]. Rates of pneumococcal acquisition and colonisation vary greatly by age, geographical location and socioeconomic background [9].

Colonisation with pneumococcus is a dynamic process. Multiple pneumococcal serotypes can colonise the nasopharynx both simultaneously and sequentially but there is usually a predominant current colonising serotype [17]. In addition, interspecies competition between resident flora such as alpha-haemolytic *Streptococci* inhibit potential colonisers including *S. pneumoniae*, *H. influenzae* and *S. aureus.* This leads to a constantly changing composition of the nasopharyngeal flora. I t is poorly understood why this leads to dynamism in the nasopharyngeal microbiome rather than a static state dominated by α haemolytic streptococci [9].

Colonisation requires that the pathogen penetrates the mucous barrier which overlies the epithelium and avoids mechanical clearance mechanisms [5]. Robust binding to host cellular carbohydrates and proteins is mediated by cell-wall associated proteins such as pneumococcal surface adhesins [5] . The bacterium must

also survive and replicate despite host cellular and humoral defences. Selective pressures have led to niche adaptation and may increase virulence (see Figure 2) [5]. These local host responses play an important role in regulating all pathogens including pneumococcus in the upper airway. People who mount a poor mucosal immune response may subsequently develop persistent or recurrent colonisation episodes [18] [19]. Conversely a quick and efficient local immune response can result in elimination of colonisation and prevention of re-colonisation [18] [19].

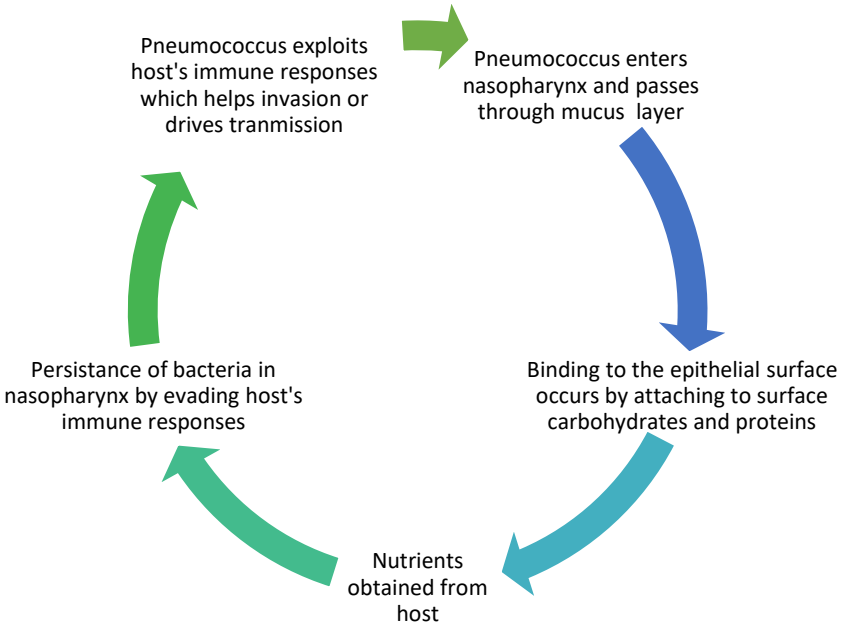

Figure 2: The stages of pneumococcal colonisation of upper respiratory tract adapted from Siegel et al [5]

Streptococcus pneumoniae colonised the nasopharynx by initially entering at the nose and passes through a layer of mucus. When the bacteria reach the epithelial surface, they bind to surface carbohydrates and proteins. Following this, to allow for replication, pneumococcus obtains nutrients which can involve exploiting inflammation produced by the host. Persistence of pneumococcus also includes circumvention of both cellular and humoral immune responses. Following this the pneumococcus can use these responses to persist and lead to colonisation invade the host potentially leading to disease Evasion of host immune responses also allows for exit from the host which can drive transmission. In addition, growth during colonisation leads to increased bacterial densities which can increase the likelihood of transmission. Shifts in colonisation density, co-infection with viruses and interactions with other commensal and pathogenic bacteria in the nasopharynx can affect stages of this cycle. Viral co-infection increases bacterial load and mucus production and therefore leads to increased shedding [20]. The success of the pneumococcus also requires interaction with nasopharyngeal microbiota, these interactions can either be co-operative or competitive [20].

1.2.2 Pneumococcal colonisation and impact on immunity

The highest rates of pneumococcal colonisation are observed in infants. Following this, colonisation rates decrease with increasing age. Interestingly, in low and middle-income countries, the highest rates of colonisation are observed at 2 months of age (80%) with a gradual reduction observed until 3 years of age, followed by a more dramatic reduction in rates following this [13]. Conversely, in high-income countries, less than 50% of children under 1 year of age have been found to be colonised, a peak is observed at 3 years of age where colonisation rates of 60% have been reported [11]. Differences in colonisation rates between high and middle-lower income countries continue into adulthood. High income countries report adult colonisation rates of approximately 10% compared to low-and middle-income countries reporting colonisation rates up to 40% [21].

It is hypothesised that the reduction of colonisation rates with increasing age is due to the development of specific immunity which partially protects older children and adults against colonisation. General reduction in rates of pneumococcal colonisation and disease around the second and third years of life coincides with the development of humoral and cellular responses to pneumococcal capsular polysaccharides and protein antigens. This has been generally thought to be the immune response mounted in unvaccinated children in response to pneumococcal exposure [22]. The fact that immunisation with a polysaccharide conjugate vaccine (PCV) has been shown to reduce pneumococcal colonisation and produces a serotype-specific antibody response supports this view [22]. However, there is some evidence from longitudinal

follow up of children, during their first year of life, that colonisation protects against subsequent colonisation episodes [23]. This protection is serotype-independent and is observed prior to maturation of capsule-specific antibodies. It is likely that a combination of serotype-dependent and serotype-independent immune mechanisms explain how colonisation is controlled with increasing age.

Repeated exposure to pneumococcus and episodes of pneumococcal colonisation are likely to boost immune defences and contribute to lower rates of colonisation and disease [24]. Murine models have shown that antigen specific T-cell and specific antibody responses develop during colonisation and protect against subsequent re-colonisation [25-28]. Mice who had been previously colonised with pneumococcus showed earlier clearance of the pathogen when re-colonised [25]. This correlated with higher levels of luminal neutrophils compared to those observed in mice being colonised for the first time [25]. A further study showed a high level of protection against fatal invasive disease in mice which had previously cleared a colonisation episode [26]. This study reported that higher numbers of CD4+ cells and increased levels of interleukin 17A (IL17A) in the lungs were associated with a reduction the number of pneumococcal found in the lungs of pre-colonised mice [26]. However, a conflicting murine study reported that a previous colonisation episode protected against death from subsequent severe pneumonia, mainly by reducing rates of bacteraemia [28]. This protection remained when mice were depleted of CD4 cells prior to colonisation but was lost in antibody deficient mice. This suggests that the protection against bacteraemia following pneumonia may not be dependent on

CD4+ T-cells but could be related to antibody-mediated phagocytosis of the bacteria from the blood [28].

Pneumococcal colonisation in children without any invasive infection is associated with higher serum levels of immunoglobulins against pneumococcal proteins and capsular polysaccharide [12 24 29]. Controlled human infection studies, which have been developed over the last 10-20 years, have been able to improve the understanding of the immune responses resulting from pneumococcal colonisation [29 30]. One of these models is the Experimental Human Pneumococcal Challenge model (EHPC) which has been established over the last 9 years at the Liverpool School of Tropical Medicine (LSTM). This model uses serotype 6B *S. pneumoniae* to establish colonisation in approximately 50% of healthy participants following nasopharyngeal challenge with 80,000 colony forming units (CFU) per nostril [10].

One EHPC study showed that specific immune mucosal responses are elicited following exposure to pneumococcus even in the absence of colonisation. These results support the possibility that exposure to low doses of pneumococcus is potentially immunising at the mucosal surface [24]. It also reported an increased anti-pneumococcal polysaccharide immunoglobulin response (IgG and IgA) nasally in participants following inoculation and an increase in IgG levels found in fluid obtained from the lungs of these participants [24].

A similar human controlled infection study showed that a previous colonisation episode was significantly protective against reacquisition of colonisation by the same

pneumococcal serotype (6B) [10]. Following the initial pneumococcus exposure (inoculation) an increase in IgG to several pneumococcal proteins was observed in all participants, the largest of which were observed in colonisation positive participants [24]. Increased levels of IgG to the 6B anti-capsular polysaccharide were also found but only in colonisation positive participants [24]. Ten colonisation positive participants were inoculated for a second time with the same serotype up to 11 months following clearance of the first colonisation episode (re-challenged). Eighty percent of participants (8/10) were found to have significantly increased level of IgG to both proteins and polysaccharides which protected against reacquisition of colonisation [10]. This is an important finding as it suggests that the immunising effect of a single episode of pneumococcal colonisation is functionally significant. These results can may have significant implications of future vaccine strategies; they support the development of pneumococcal mucosal vaccine strategies. However, the relative importance of protection against pneumococcal colonisation and its association with the reduction of mucosal infections and invasive pneumococcal disease is still unclear.

1.2.1 Pneumococcal colonisation and disease

Colonisation is important as it is believed to be a pre-requisite of infection and is the primary reservoir for transmission but can also be a source of immunising exposure and immunological boosting against pneumococcal infection in both children and adults [10 29 31]. Most colonisation episodes will not lead to a subsequent disease episode. The progression from stable nasopharyngeal colonisation to invasive

disease is enhanced by local inflammation caused by cytokines such as interleukin 1 and tumour necrosis factor (TNF). The inflammatory cascade that follows the production of these factors lead to a change in the number of receptors on epithelial and endothelial cells [20 32]. Invasion of the pneumococci follows due to pneumococcal cell-wall choline binding to one of these upregulated receptors namely platelet-activating-factor receptor, which in turn induces internalisation of the pneumococci [20 32]. In addition, choline-binding protein A (PspC) on the pneumococcus interacts with Ig receptors, on cytokine-activated human cells [9 33]. This leads to increased migration though the mucosal barrier [33] .

Colonisation by pneumococcus is often asymptomatic but it can progress to respiratory or even systemic disease [9]**.** Observational studies show a direct link between pneumococcal disease and colonisation at the individual level [31]. Most commonly this link is seen with mild mucosal infections (predominantly AOM) but some reports suggest a link between colonisation and pneumonia or invasive pneumococcal disease (IPD) [31]. One study shows a disproportionally high prevalence of colonisation in children affected with pneumococcal disease [31]. Another study set in Pakistan found that 94% (101/108) of children diagnosed with IPD were carrying the bacteria in their nasopharynx compared to 52% (69/133) colonisation rate in healthy controls [34]. the study found in 99% (69/70) of cases there was concordance between the serotype cultured from the nasopharynx and that causing invasive disease[34]. A similar study in The Gambia found comparable results; 90% (73/81) of children with IPD were found to have pneumococcal colonisation compared to 76% (86/113) colonisation rates in healthy controls (chi squared, 6.99; $P<0.01$)[35].

However, it is difficult to prove a temporal relationship between pneumococcal colonisation and subsequent IPD.

It is believed that pneumococcus is more likely to cause disease soon after colonisation of the upper airway before there is time for the body to mount a cell-mediated response and for antibodies to develop. Longitudinal colonisation patterns were studied in a cohort from birth to 24 months of age; serial throat and nasopharyngeal swabs were taken to determine colonisation status [36]. They found that infection usually occurred within 30 days of the acquisition of pneumococcal colonisation with a new serotype (74% of infections [23/31]) and found that disease following prolonged colonisation was rare [36].

Evidence suggests that pneumococcal serotypes differ in their duration of colonisation and invasiveness. Some serotypes are rarely found in colonisation but have high invasiveness. For these serotypes it is hypothesised that they may only colonise the nasopharynx for a short duration which is difficult to see prior to disease and therefore difficult for temporal relationship to be proven [31]. A further hypothesis is that these serotypes may colonise at lower densities and are therefore not detected in epidemiological studies [37]. Using a meta-analysis, researchers showed that for some serotypes there is an inverse correlation between invasive disease and colonisation prevalence. In this study, the most invasive serotypes were the least likely to be found to colonise the nasopharynx and the most frequent colonisers were the least likely to cause invasive disease [37]. Research suggests that there may be a specific density needed for the transition from colonisation to disease or a common

factor which allows for both, one study found that patients with pneumococcal pneumonia had higher densities of pneumococcal nasopharyngeal colonisation compared to asymptomatic colonised controls [38].

Recent technical advancements have also allowed more in-depth research into the dynamics of colonisation episodes with multiple pneumococcal serotypes. Evidence e suggests that children that are colonised with multiple pneumococcal serotypes have higher overall density of colonisation than those with a single serotype colonisation episode [39]. A further study used lytA qPCR and molecular serotyping to investigate the prevalence of pneumococcal serotypes in colonisation episodes. They found that 30% of colonised children (89/299) were colonised with 2 or more pneumococcal serotypes [40]. The authors concluded that multiple pneumococcal serotypes may be transmitted between children as a complex mixed community and colonise the nasopharynx in the same way rather than as a single serotype [40]. High density colonisation has also been hypothesised as a risk for invasive disease. A surveillance study carried out in South Africa found that higher colonisation density was associated with viral co-infection (adjusted odds ratio [OR], 1.7; 95%CI, 1.1-2.6) and invasive pneumococcal pneumonia (adjusted OR, 2.3; 95% CI, 1.3-4.0) [41].

1.3 Transmission of *Streptococcus pneumoniae*

Historically transmission of *S. pneumoniae* was thought to occur primarily due to inhalation of infected respiratory droplets from person-to person. However, spread of pneumococcus by various transmission methods are biologically plausible including aerosol, droplet or indirect contact. The relative frequency of these different modes of bacterial transmission and their links to pneumococcal colonisation or disease in humans is poorly understood [42]. Epidemiological data suggest that transmission is enhanced when there is close contact with a carrier and is more likely to occur with concurrent viral respiratory tract infections [8] [43]. *S. pneumoniae* outbreaks have been well documented in day care centres, military camps, prisons and nursing homes [8]. To allow for successful implementation of methods to reduce transmission we first need to understand better the mechanisms underlying pneumococcal transmission into the nasopharynx.

It has been suggested that in young adults pneumococcal transmission may occur through saliva by sharing drinking glasses and bottles [44]. This study investigated pneumococcal colonisation prevalence in an Israeli Army training base and possible risk factors for colonisation [44]. They reported that sharing of a drinking glass/bottle was common practice with 48% of participants reporting frequent sharing. They reported that frequent sharing of a drinking glass/bottle was a strong and independent risk factor for pneumococcal colonisation. The study also concluded that there was no evidence of a correlation between hand wash frequency and

colonisation. The authors suggest that pneumococci may be transmitted in saliva in adults.

More recently our understanding of the process of pneumococcal transmission has improved following the development of murine models which have successfully studied transmission dynamics. However, current understanding of the dynamics of human-to-human transmission is still poor and needs further investigation. A recent randomised controlled trial examined the effects of nasopharyngeal bacterial colonisation during a viral URT co-infection in 151 children. The study used the live attenuated influenza vaccine (LAIV) as a surrogate for mild URT viral infection. The results suggested that the use of this vaccine may increase bacterial densities in the nasopharynx [45]. The authors suggest that due to the absence of safety concerns, following the widespread use of the LAIV, that LAIV could be used as a tool to investigate the dynamics of pneumococcal transmission in the future [45].

1.3.1 Rodent models investigating transmission

Initial rodent models investigating the dynamics of pneumococcal transmission depended on influenza co-infection to increase pneumococcal transmission. One study in 2010 used a model of transmission in ferrets. The benefit of using ferrets for pneumococcal transmission studies lies in the fact that they sneeze which allows for airborne transmission [46]. This study showed that ferrets which had previously been infected with influenza virus had higher rates of pneumococcal disease and transmission [46]. In addition, in a further experiment they intranasally inoculated contact ferrets (no pneumococcal colonisation) with Influenza A virus prior to contact

with colonised ferrets. This pre-existing viral infection promoted pneumococcal acquisition and allowed acquisition of colonisation over longer distances [46].

Other groups have studied transmission dynamics using murine models. One group analysed transmission from index pups colonised with pneumococcus at 4 days old to contact pups from the same litter who had previously been infected with influenza [47]. They found that younger age, close contact and viral co-infection all increased transmission [47]. This group also found that influenza increased bacterial titres in both the inoculated donor mice and the index mice [47]. Furthermore, using neutrophil depletion they showed that higher bacterial numbers during colonisation promoted transmission, as did nasopharyngeal inflammation in the contact pups (demonstrated by cytokine production) [48].

More recently another group also using a murine model, suggest that increased transmission during concurrent influenza infection is likely secondary to increased bacterial shedding rather than solely due to higher bacterial titres in donor mice during viral co-infection [49]. Shedding was found to increase with levels of inflammation observed in the upper respiratory tract in response to influenza infection [49]. A further study supported this finding by reporting that reduction of inflammation using intra-nasal dexamethasone reduced shedding and transmission [50].

More recently a murine model has been developed which can investigate the transmission dynamics during pneumococcal mono-infection. This model allows for

examination solely of pneumococcal factors and host responses that can impact on transmission. In 2016 this model was published and showed that bacterial shedding peaked over the first 4 days post inoculation of index pups. This correlates with a peak of inflammation in the upper respiratory tracts due to colonisation [51]. This study also reported that transmission within a litter was enhanced when there was a high ratio of colonised pups to un-colonised contact pups [51]. Colonisation density significantly affected level of shedding and rates of transmission were proportional to the level of shedding observed [51].

1.3.2 Healthy carrier transmission

There is evidence of *S. pneumoniae* spread within families. One study which looked at 64 families for a period of 8 weeks to 52 weeks found 25 episodes of transmission of a single serotype of *S. pneumoniae* from one family member to another [52]. They also saw rapid spread of pneumococcus between family members if a new serotype was introduced to the family; 7/25 transmission episodes took place within 2 weeks of a new serotype entering the household [52]. They also described 2 different and distinctive patterns of spread of *S. pneumoniae* in these families; (1) apparent concurrent acquisition of colonisation of a specific serotype of pneumococcus by two or more members of the family and (2) the prolonged colonisation by one member of the family with sudden spread to several others in the household [52]. They suggested that a specific event could facilitate dissemination of the bacteria; they hypothesised that simultaneous viral illness could be this event, but this was difficult to investigate as there were three times more viral episodes as episodes of *S.*

pneumoniae colonisation [52]. However, they did find that when evaluating the 25 episodes of transfer of *S. pneumoniae* a presumed donor and recipient could be identified [52]. Investigating these presumed donors, they found in 14 of the 25 episodes the donor had symptoms of upper respiratory tract infections (URTI) during the 2-week period where transmission could have occurred [52]. They hypothesised that increased production of respiratory secretions or another mechanism associated with presumed viral illness may play a role in increased transmission [52].

Another study assessed pneumococcal transmission in Muslim pilgrims completing the Hajj [53]. They took nasopharyngeal swabs and administered a questionnaire to 3203 subjects (1590 at beginning-Hajj and 1613 at end-Hajj) they found that there was a statistically significant increase in nasal colonisation between the beginning and end of the Hajj (4.4 % vs 7.5%; prevalence ratio 1.7, 95% CI 1.3-2.3) but did not investigate the possible routes of transmission [53]. This likely indicates there was increased transmission of pneumococcus during the Hajj from person-to person. An overall increase of colonisation was observed rather than the increase of a specific serotype which reduces the possibility that a single invasive clone could have expanded during the Hajj [53]. They also found that there was a lack of association between duration of time at the Hajj and likelihood of colonisation [53]. This is in keeping with results of the study above which suggest that transmission leading to colonisation happens relatively quickly following contact and that there may be an all-or-nothing protective response, however, further study is needed to understand this further [52].

1.3.3 Disease transmission

Patients with pneumococcal pneumonia are usually considered a relatively low contagion risk; hospitalised patients with pneumococcal infections are not treated under isolation and health care workers do not take increased infection control measures [8]. However, there have been reports of epidemics previously in Africa and Canada [54-56]. In Ghana there was a steady increase of incidence of pneumococcal meningitis from 2000-2003 [55]. The researchers concluded that the *S. pneumoniae* ST217 clonal complex showed a high propensity to cause meningitis and that evidence of increasing incidences suggested that the lineage had high epidemic potential [55]. Following a review of cases with suspected bacterial meningitis between 2002-2005 in Burkina Faso it was reported that pneumococcal meningitis was occurring in an epidemic pattern. An average of 38 cases of *S. pneumoniae* infection was identified each month during the meningitis epidemic season compared to average of 8.7 cases/month at other times of the year [56]. Of the 48 pneumococci that were tested, 41% (21/48) were identified as serotype 1, with the remaining identified as 15 different serotypes [56]. In Canada during 2000/2001 there was a report of pneumonia epidemic caused by a virulent strain of streptococcus pneumonia serotype 1 [54]. A total of 84 cases of pneumococcal pneumonia were identified, of these 34/84 (40%) occurred in adults aged 20-64 years and majority were severe infections with 75/84 needing hospitalisation [54].

Another study investigated thirteen clusters of acute otitis media (AOM) in siblings and analysed the bacterial pathogens causing disease in these siblings [57]. Following

comparison of the pneumococcal isolates from each sibling pair they found 100% homology between the siblings and antibiotic susceptibility testing were able to show homology between pairs of organisms from siblings [57]. This provides evidence that there is person-to-person transmission among siblings with AOM, but what the current study could not show is what impact the disease process of AOM has on the transmission in these cases.

Despite these reports, outbreaks of pneumococcal infection are generally uncommon and usually are observed in high risk populations such as nursing home residents [58], day care centres [59], prisons [60] and residents in homeless shelters [61]. One interesting cohort study investigated an outbreak of multi-drug resistant pneumococcal pneumonia in an American nursing home in 1996 [58]. The study reported that 23% of residents and 3% of employees were colonised with a multidrug-resistant *S. pneumoniae,* serotype 23F. Evidence suggested that the transmission route was likely person-to-person transmission from staff to residents. This was due to two main reasons; firstly, residents that were colonised or had developed pneumonia from this bacterial serotype were randomly distributed throughout the facility and secondly two colonised residents were bedbound with no exposure to any other resident or visitors [58]. However, they did not investigate how this transmission from staff to residents took place. They noted that one colonised staff member, who had widespread contact with residents, had a febrile respiratory illness during the period which was treated with antibiotics and hypothesised that working during this illness may have been the cause of the spread.

An outbreak of pneumococcal pneumonia in children was observed in the United Kingdom (UK) in 2006 [57]. Initially three cases of pneumococcal pneumonia were reported in an English primary school. Children were aged 4-5. Following identification of this outbreak, school contacts and those living in the same household of these children were given rifampicin chemoprophylaxis but unfortunately despite this intervention two further cases were reported from classmates in the same school [57]. All five cases were caused by pneumococcal serotype 1 which supports the hypothesis that there was transmission between these subjects which lead to disease [57]. Following the second outbreak throat swabs were obtained from cases and contacts, only one further carrier of *S. pneumoniae* serotype 1 was identified [57]. However, the authors conclude that this colonisation rate may be under-reported due to sampling technique. Colonisation was only determined from oropharyngeal swabs rather than nasopharyngeal swabs due to NP swabs being an unpleasant procedure for children [57]. In many of these reported outbreaks of pneumococcal disease, close contact in crowded conditions was often hypothesised as a major risk factor for the transmission.

1.3.4 *Streptococcus pneumoniae* survival in the environment

To better understand how pathogens are transmitted, it is important for us to understand how long pathogens can persist on inanimate objects. This is specifically important in the health care setting for deciding on the appropriate treatment of surfaces. The longer pathogens can persist on a surface, the longer it is a possible

source of transmission which can endanger patients or be further spread by health care workers [62].

There is evidence to suggest that in the hospital environment many inanimate objects such as computer keyboards, bed rails and tap handles can be reservoirs for pathogen transmission [63] [64]. A study evaluated 144 samples from computer keyboards and tap handles in a medical intensive care unit to investigate if they were reservoirs of nosocomial pathogens [63]. The colonisation rate for keyboards was 24%, and for taps was 11% [63]. Pathogens recovered included *Staphylococcus aureus* (49%), *Enterococcus* 18% and *Enterobacter* 12%. A further study investigated survival and transfer of bacteria in laboratory conditions, they found a variable degree of pathogen transfer from contaminated objects to the hands, with the highest rates of transmission observed with *E.coli, Salmonella spp., and Staph aureus* [65].

Kramer et al [62] found that most Gram-positive bacteria can survive on dry surfaces for months, and that Gram-negative bacteria have been reported to persist longer on average than Gram-positive bacteria. When evaluating environmental factors that prolonged persistence on objects they found that lower temperatures (4-6 degrees centigrade) and high humidity (>70%) were both associated with longer persistence for most bacteria [62].

Data specific to *S. pneumoniae* are few. It has been reported that pneumococcus can persist for up to 28 days outside the human host [62] [66](see Table 1). Prolonged survival was reported when the bacteria are stored in dry conditions and at lower

temperatures [66]. More recently a study investigated the survival and infectivity of *S. pneumoniae* following desiccation [67]. They found that direct contact with respiratory droplets containing pneumococci may not be needed for transmission of this pathogen [67]. Desiccated pneumococci were recovered and following this were able to colonise the nasopharynx of mice following intranasal inoculation[67]. This suggests that inanimate objects could be a source of pneumococcal transmission [67]. Only one study was found which specifically investigated pneumococcal survival time on hands and fomites [68]. Pneumococcus was suspended in either serum broth (10% horse serum in Brain heart infusion broth) or Mueller-Hinton broth. Three volunteer's hands were exposed to pneumococcus. They reported a significant reduction in pneumococcal counts after only 3 minutes [68]. However, pneumococcus could still be recovered 3 hours after exposure when suspended in serum broth. Interestingly when testing pneumococcal survival on fomites, despite substantial loss of viable pneumococcus following exposure, pneumococcus could still be recovered from a glass plate up to 15 hours after exposure and from a plastic toy at the final eight-hour sampling point [68].

Table 1: Survival time of Streptococcus pneumoniae [62 66]

Origin of Streptococcus pneumonia bacteria	Temperature stored at	Stored in Dry conditions (Survival time in days)	Stored in moist conditions (Survival time in days)
Infectious human pus mixed with room dust [66]	15-20°C	12 but not 16	5 but not 8
Infectious blood of a rabbit mixed with room dust [66]	15-20°C	1 but not 3	1 but not 3
Infectious sputum from patient mixed with room dust [66]	15-20°C	20 but not 25	12 but not 16
Infectious sputum from 42 patients and divided into 2 portions [66]	4°C	28	N/A
Infectious sputum from 42 patients and divided into 2 portions [66]	Room temperature	7	N/A

Recently emerging evidence suggests that capsular type can also effect shedding and transmission of pneumococcus. A recent murine model examined whether pneumococcal capsule contributes to viability of pneumococcus outside the host [7]. This study showed that the capsule supports pneumococcal viability during starvation conditions (24 hours in phosphate-buffered saline at 25°C) [7]. Pneumococcal serotype affected viability during these nutrient-poor conditions and a decrease in capsule thickness and amount of CPS was observed following starvation [7]. It was confirmed that serotype differences in survival were due to capsular type, rather than a result of genetic background by testing capsular-switch mutants [7]. This

study suggests a possible new role of the pneumococcal capsule; pneumococcus may be able to utilise their capsule as a nutrient source to maintain viability when the organism is being transmitted between hosts. A further murine study found that capsule type and amount affected shedding of pneumococcus during a colonisation episode and this was associated with the rate of host-to-host transmission [69]. The authors highlighted that those specific serotypes which are strongly bound by mucin are shed less by the host and therefore are less likely to be transmitted [69].

The results of these recent studies suggest that capsular type may lead to varying rates of pneumococcal transmission and could explain why different serotypes have varying propensity to cause outbreaks of disease. Serotypes which can survive for longer in the environment could increase the likelihood of acquisition, colonisation and potentially disease in a new human host.

1.3.5 Hand to nose transmission of *S. pneumoniae*

Hand washing interventions can reduce the transmission of disease spread by the faecal-oral route, and is an effective and feasible means of reducing rates of gastroenteric infections in developing countries [70]. Hand washing interventions may also be a promising intervention against acute respiratory infections. The hands can be vectors for respiratory microorganisms which are shed from the nose and mouth to a new host's mucous membranes [70]. A meta-analysis of 8 studies conducted in 2006 showed that hand washing interventions could reduce the risk of acute respiratory infections (ARI's) by around 16% (95% CI 11-21%) [70]. However, the quality of the studies examined was poor and had geographical limitations; none of the

studies were conducted in developing countries and 6/8 studies were conducted in the USA. Therefore, the generalisability r of the findings are uncertain [70].

When evaluating the possible impact of transmission of infection by self-inoculation, it is important to consider the prevalence of face-touching behaviour. Self-inoculation is a type of contact transmission where contaminated hands make contact with other parts of the body such as the mouth, eyes and nostrils ,which subsequently introduces bacteria into those sites [71]. An observational study from 1973 monitored a total of 124 adults in a lecture theatre or Sunday school environment for 30-50 minute periods [72]. They observed 29 episodes of nose picking (0.33 hr^{-1}) and 33 episodes of eye rubbing (0.37 hr^{-1}) [72].

Two more recent studies reported much higher rates of hand to face contact [71 73]. Nicas et al in 2008 conducted an observational study looking at the hand touching rate of 10 volunteers. Participants sat alone at a desk for a 3-hour period performing office-type work and the frequency of hand contact with the eyes, nostrils and lips was examined. The average total contact rate per hour was 15.7 [73]. They noted that there was significant inter-individual variability in total hand contact with facial membranes [73]. This was shown by a 35-fold difference in the range limits (lowest total hand to face contacts 3 vs. highest 104) [73].

Kwok et al [71] observed 26 students' face touching behaviour during two 2-hour lectures. They found that on average each of the students touched their face 23 times per hour; of these 44% (1024/2346) involved contact with a mucous membrane [71].

Of the 1024 touches that involved mucous membranes, there was found to be a fairly even split between the mouth (36% 327/1024), nose (31% 318/10247 and eyes (27% 273/1024) with 6% (61/1024) of touches being a combination of mucous membrane contact [71] (Figure 3)**.** The students involved in this study were medical students who had previously had one 4-hour infection control lecture which involved teaching about hand hygiene, aseptic technique and transmission-based precautions. Kwok et al concluded that due to the high frequency of mouth and nose touching observed in this study, hand hygiene is an essential preventative method to break colonisation and transmission cycles [71].

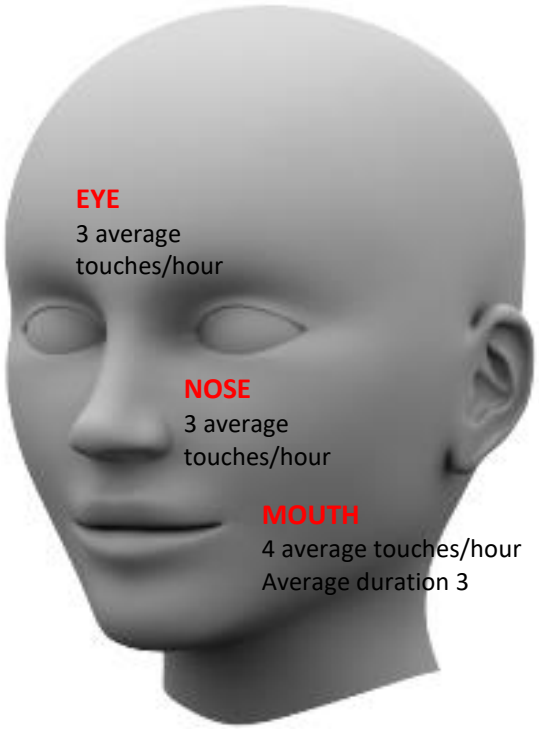

Figure 3: Average number touches of mucosal surfaces observed over 1 hour period adapted from Kwok et al [71]
Results from a behavioural study involving medical students in Australia. Face-touching behaviour was observed via videotape recording; frequency of hand to face contact was analysed.

The above studies support the hypothesis that hands could be an important vector for bacterial transmission to facial mucous membranes. *S. pneumoniae* colonises the upper airway; through touching of contaminated surfaces, sneezing, coughing and rubbing/picking of the nose pneumococcus could contaminate the hands. Due to this it is biologically plausible that hand to face contact could be a significant transmission pathway. There is very little research investigating the link between pneumococcus hand contamination and rates of nasal colonisation currently.

Only two relevant studies in humans were found; the first found pneumococcal nasal colonisation in 83% (67/81) of children studied in Papua New Guinea with a corresponding hand contamination rate of 22% (18/81) [74]. They also found that 17/20 children who were found to have hand contamination with pneumococcus had the same serotype colonising their nasopharynx [74]. In conjunction with testing children, the study also evaluated pneumococcal hand contamination of mothers; two mothers had pneumococcus recovered from their hands but there was no concordance with the serotype found colonising their children's nasopharynges [74].

The second study compared the rates of pneumococcal hand contamination and nasal colonisation in two groups of children in Australia; a remote aboriginal community group (n=89) and an urban child care centre group (n=294) [42]. They found a two-fold increase of nasal colonisation rates in the remote group compared to the urban group (90%, 80/89 positive for colonisation remote group vs. 43%. 125/294 urban group) using nasal swabs [42]. This correlated with higher hand contamination rates in the remote group; the remote group were >9 times more likely than the

urban group to have pneumococcal hand contamination (37%, 33/89 positive hand swab remote group vs. 4%, 13/294 urban group) [42]. This suggests that pneumococcal shedding during a colonisation episode is possible and that the hands could be a possible vehicle for pneumococcal transmission in the community.

Rodent models investigating pneumococcal transmission are adding to the current understanding of the extent of airborne transmission versus contact-dependent transmission. Transmission secondary to infected secretions from a person colonised with pneumococcus may involve direct person-to-person spread or may involve contaminated surfaces or fomites [20]. A recent study demonstrated that an environmental reservoir of pneumococcus can facilitate transmission [50]. In this study colonised mice were kept in separate cages from un-colonised mice. Both sets of mice had an uncolonised mother; three times per day mice were switched between cages but were never in contact with each other [50]. Over 50% transmission rate was observed; the investigators concluded that contamination of the mother's teats with pneumococcus was the source of transmission between the colonised and uncolonised mice. They confirmed that the bedding was not an environmental source of pneumococcal transmission by doing a further experiment. When they moved the mothers between cages of colonised and uncolonised mice transmission was still seen suggesting that pneumococcus was likely acquired from the surface of the mothers [50].

1.3.6 Reduction of transmission

The benefits of hand washing for reducing the spread of respiratory bacterial pathogens is uncertain with the evidence currently available [42]. Previous intervention studies using hand hygiene interventions to reduce viral transmission pathways support the hypothesis that both direct and indirect contact are important in transmission of viruses from person to person. A randomised control trial looking at the effect of infection control measures in child care centres showed only a 5% overall reduction of incidence of "colds" [75].

There is limited research assessing whether hand hygiene interventions and infection control teaching can reduce upper respiratory bacterial pathogen transmission. A cluster randomised control trial (RCT) conducted in 20 child care centres in Australia investigated this question[76]. Ten centres (219 children) had one infection control training session for staff and had regular follow up visits by researchers to implement 20 second hand washing, barrier nose-wiping, removal of contaminated toys and non-touch sunscreen application [76]. The other 10 centres (235 children) acted as controls with no intervention given. The researchers found no reduction in transmission of bacterial pathogens (adjusted incidence rate ratio, IIR, 0.97 95% CI 0.88, 1.08) or respiratory illness in the intervention group (adjusted IIR 1.00 95% CI 0.93, 1.01) [76]. Transmission of bacterial pathogens (*S. pneumoniae* and *H. influenza*) was assessed with nasal swabs undertaken every 2 weeks for 6 months and childhood illness was reported by parents. However, interpretation of these findings was limited by poor uptake of the hand hygiene practices in the intervention centres

(proportion of hygiene practices consistent with guidelines; weighted mean difference 14%, 95% CI 9, 19).

1.4 Pneumococcal disease treatment and prevention

1.4.1 Pathogenicity

Streptococcus pneumoniae was first described by George M Sternberg who isolated it in 1880 from a colonised individual [77]. He also described recovery of the organism from the saliva of healthy students which established that the pneumococcus is part of the normal human microflora [77]. *S. pneumoniae* was first described as a cause of lobar pneumonia in 1883 by Friedlander and Talamon. Diagnosis of pneumococcal pneumonia improved following the development of the Gram stain as a method to differentiate bacterial species in 1884. It is a Gram-positive bacterium which has the potential to cause invasive infections and is considered to be an extracellular bacterial pathogen [78]. Extracellular pathogens can replicate or persist on mucosal surfaces in the human body or in tissues outside host cells. They can also spread quickly or establish an infection if they successfully contend with host humoral defences and cellular immune mechanisms (e.g. T cells and phagocytes) [78]. Due to this, the ability of extracellular pathogens to evade clearance by the body's humoral and cellular immune defence mechanisms is an important determinant of pathogenicity.

The pneumococcus can release a pore-forming toxin called pneumolysin; which at low levels can induce apoptosis of cells and high levels cause lysis of all cells with cholesterol in their membrane [79]. This pathogen also has pro-inflammatory cell wall components such as C-polysaccharide and F-antigen. These release tissue damaging enzymes such as neuraminidase and have adhesins, such as pneumococcal surface protein A (PspA), that can bind to cell surface carbohydrates which play important roles in combating the host's immune responses [80].

The polysaccharide capsule surrounds the pneumococcal cell wall which is comprised of peptidoglycan and teichoic acid. This capsule protects the bacteria from phagocytosis by obstructing leukocyte fixation onto the cell wall [1]. Pneumococcal serotypes each have chemically distinct capsules which can affect:

- Tendency to cause outbreaks,
- Antibiotic resistance profiles,
- Likelihood of causing invasive disease (serotypes 1 and 7E are prominent serotypes for invasive disease),
- Prevalence of mucosal disease or nasopharyngeal colonisation; different serotypes have varying propensity to cause colonisation and invasive disease [1 2].

Geographical variation in serotypes is often dependent on the period studied and differences in age distribution between host's; serotypes most commonly observed in young children include 6B, 9V, 14, 19F and 23F [1 2 80]. These differences in serotypes

may be due to the quantity and degree of encapsulation of the polysaccharide capsule. Thicker capsules protect against neutrophil-mediated killing *in vitro*, and may prevent clearance of colonisation by the host [2]. Other genetic factors relating to the pneumococcus are also likely to impact on virulence such as bacterial adhesins.

1.4.2 Pneumococcal disease

Streptococcus pneumoniae is the most common cause of acute otitis media, sinusitis and pneumonia worldwide and also causes a significant number of cases of meningitis [81]. Epidemiological studies have shown that there is an inverse relationship between the frequency and severity of types of pneumococcal disease (see Figure 4).

The archetypal presentation of a patient with pneumococcal pneumonia has been historically described as symptoms including sudden onset of chills and pleuritic chest pain, closely followed by fever and rusty sputum production. However, many present with more non-specific symptoms especially in the elderly and in young children[81]. Pneumococcal pneumonia can progress to pneumococcal bacteraemia in some patients, rates of bacteraemia have been estimated at 20-25% [82] and mortality from bacteraemia has stayed high at 20-30% despite antibiotic treatment [83].

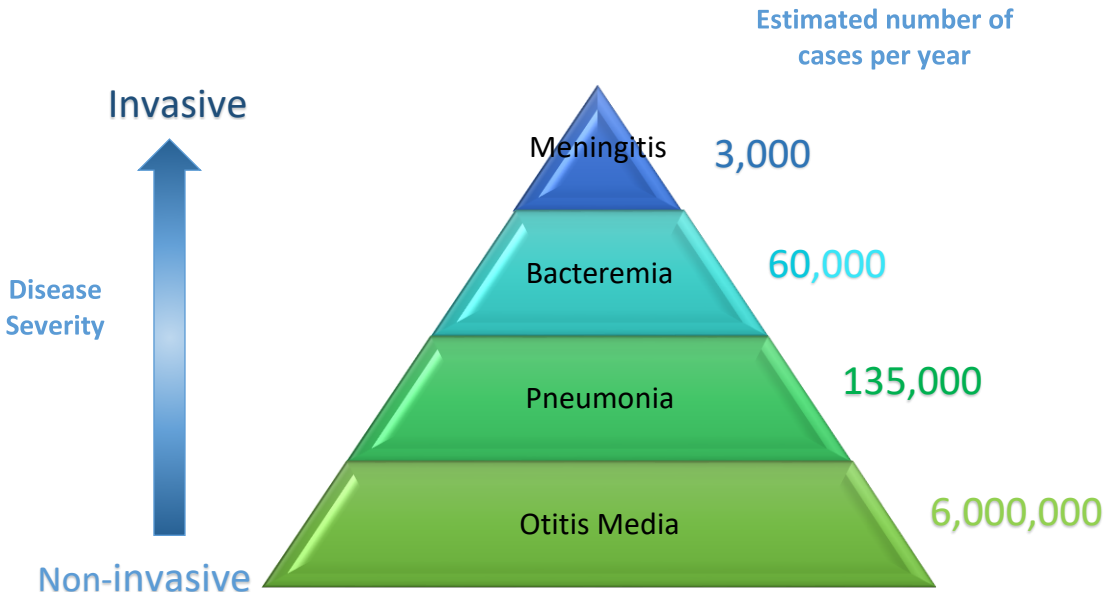

Figure 4*:* Burden of pneumococcal disease adapted from Edwards and Griffin [84].
Estimated pneumococcal disease cases per year in adults and children in USA taken from CDC report 2002 (www.cdc.gov/nip/publications/surv-manual/chapt09_pneumo.pdf)

Pneumococcus is considered to be the most common cause of CAP worldwide and in the UK is the most common cause of CAP in children ≤2 years of age [85]. Timely and effective management of pneumococcal pneumonia can be difficult. It is hindered by low rates of microbiological pathogen confirmation in patients with suspected pneumonia and lower respiratory tract infections (LRTI) [86 87]. Following spread of the pneumococcus to a sterile site it can also cause IPD which encompasses a range of diseases including bacteraemia, meningitis, empyema and septic arthritis.

Penicillin has been the mainstay of antibiotic treatment for any pneumococcal infection for over half a century [81]. However, increasing antibiotic resistance is a growing problem worldwide. *S. pneumoniae* is no exception to this and we have

observed increasing antibiotic resistance develop specifically in serotypes that have high prevalence in children for example serotypes 6, 14, 19 and 23.

Due to these diagnostic difficulties and increasing problems with antibiotic resistance, strategies to prevent pneumococcal disease are becoming increasingly important.

1.4.2.1 Burden in UK and worldwide and risk factors for disease

The WHO estimates that 1.6 million deaths are caused by pneumococci annually, with over a million of these deaths attributed to pneumococcal pneumonia in children under the age of 5 in developing countries [81 88]. The global pneumococcal disease burden in children, especially under 5 years of age is well documented. In 2000 it was estimated that there were 13.9 million cases of pneumococcal pneumonia per year in this age group. The burden of pneumococcal disease in adults is less well known.

Over the last 100 years, the incidence of pneumococcal pneumonia has stayed stable but a significant improvement in mortality rates has been observed following widespread antibiotic use [89]. In the United Kingdom (UK) and high-income countries, the yearly incidence of community acquired pneumonia (CAP) is approximately 1% of which half is attributable to *S. pneumoniae*.

Overall incidence of pneumococcal pneumonia is 5 in 1000, with the incidence being significantly higher in high risk populations such as infants and the elderly [81]. Pneumococcal pneumonia can progress to invasive pneumococcal disease (IPD) (e.g. bacteraemia). The annual incidence of this is 10-20 cases per 100,000 individuals annually in North America and Europe [81]. The risk of IPD increases by more than 20 times this rate in young children attending day care centres [90]. In Western countries, the incidence of pneumococcal disease rises in the winter months and the increased rates of viral respiratory infections observed during this period could be the pre-disposing factor for this [81].

Both age and gender are found to be important risk factors for pneumococcal pneumonia [81]. The incidence of pneumococcal pneumonia is up to 50 times higher in the elderly (over age of 65) and in the very young (under age of 2), with a male: female ratio of approximately 1.5-2:1 [81]. Many underlying co-morbidities have been found to pre-dispose patients to pneumococcal disease. A case- control study investigated this retrospectively. They compared 63 men with culture proven pneumococcal infections to 130 uninfected control patients and calculated relative risks using logistic regression analysis [91]. They found that the following conditions were statistically significant independent risk factors (relative risks shown in brackets); dementia (5.82), seizure disorders (4.38), heart failure (3.83), cerebrovascular disease (3.82, institutionalisation (3.13) and chronic obstructive pulmonary disease (COPD; 2.38) [91]. Concurrent respiratory tract infection, especially influenza viral infection, is also a risk factor for pneumococcal pneumonia [81].

1.4.3 Pneumococcal disease prevention: current pneumococcal vaccines

In the UK, there are currently three vaccine formulations licenced for the prevention of pneumococcal infections; the pneumococcal polysaccharide vaccine (PPV23) and two pneumococcal conjugate vaccines (PCV10 and PCV13) [92]. See Table 2 for comparisons of these vaccines.

Table 2: Comparison of currently licenced pneumococcal vaccine formulations in the UK
Table reports specific aspects of the pneumococcal vaccines which have been, or currently are licenced for use in the UK. Further discussion about vaccine immunological profile and efficacy discussed in sections 1.4.3 and 1.4.4.

	PCV 7	PCV13	PPV23
Serotypes covered	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F.	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F.	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.
UK Vaccination recommendation	*From 2006-2010* [93]*:* recommended for all children at 8 weeks old, 16 weeks old and 1 year old.	*Since 2010* [93]*:* recommended for all children at 8 weeks old, 16 weeks old and 1 year old.	*Since 2003* [93]*:* recommended for all over 65's and younger adults who have chronic illnesses that put them at higher risk of invasive pneumococcal disease.
Effective against *S. pneumoniae*	Invasive pneumococcal disease. Reduction in nasopharyngeal colonisation, leading to reduction in transmission of vaccine serotypes (in turn reduced incidence of mucosal disease). Moderately effective against mucosal disease such as pneumonia and AOM [94].		Invasive pneumococcal disease. Non-conclusive evidence on protection against pneumococcal pneumonia or other mucosal infections[92].

The currently used PPV contains polysaccharide from 23 different serotypes (PPV23). PPV23 elicits a T-cell independent, humoral immune response. Evidence suggests this protects against IPD but there is debate about this vaccine's effectiveness against pneumonia [92]. The immune response to PPV is not long lasting and does not show a booster response upon challenge with native polysaccharide. Due to this, protection induced by this vaccine is limited. A major limitation is lack of efficacy in infants under 2 years of age whose immune systems are immature [95]. PPV immunisation does not protect against nasopharyngeal colonisation by pneumococcus, therefore no herd immunity was observed after vaccine implementation. Herd immunity is the indirect protection against an infectious disease whereby individuals who are not immune can benefit and which occurs when many of the population are immune following vaccination.

A meta-analysis which evaluated 25 studies (18RCTs and 7 non-RCTs) found strong evidence that PPV is effective against IPD with overall efficacy of 74% (95% CI 56% to 85%) [96]. No statistical heterogeneity was found when all RCTs were included in analysis However, there was a statistical difference between heterogeneity of studies conduction among different populations such as healthy adults in low-income countries ($P < 0.01$) [96]. Results of effectiveness against pneumonia were inconclusive [96]. Efficacy against all-cause pneumonia in low-income countries was reported (OR 0.54, 95% CI 0.43 to 0.67) [96]. However, this was not the case in high-income countries in the general population (OR 0.71, 95% CI 0.45 to 1.12) or those with chronic disease (OR 0.93, 95% CI 0.73 to 1.1) [96]. A further meta-analysis supported this finding and concluded that PPV did not appear to be effective against pneumonia even among

elderly patients or adults with chronic disease for whom the vaccination is recommended [97].

Some randomised control trials have found protective effect of the PPV against pneumonia; this prospective randomised study included 1006 nursing home residents. It reported a statistically significant reduction in pneumococcal pneumonia cases in the vaccine arm compared to control (14/502 ,2.8% vs 37/504, 7.3%; P<0.001) [98]. Due to this specific study population (mean age of 84, nursing home residents many with co-morbidities), it is difficult to generalise the findings to larger population. In addition, a non-randomised observational study of 27,204 individuals aged ≥60 years in Spain reported that recent vaccination with PPV23 (<5 years ago) reduced risks of bacteraemic pneumococcal CAP (hazard ratio [HR], 0.38; 95% confidence interval [CI], .09–1.68) and non-bacteraemic pneumococcal CAP (HR, 0.52; 95% CI, .29–.92)[99]. This was only observed following sub-analysis of patients who had received the vaccine within the last 5 years, when evaluating the full cohort, no protective effect was observed [99].

PVC13 is the most commonly used polysaccharide conjugate vaccine worldwide; this vaccine covers pneumococcal 13 serotypes [100]. PCV13 contains purified polysaccharides of the capsular antigens of each of the 13 pneumococcal serotypes covered. These are individually conjugated (coupled) with a nontoxic diphtheria toxin (CRM197, CRM cross-reactive material). The conjugation of the capsular polysaccharide to a carrier protein in PCV13, unlike PPV, activates a T-cell dependent antibody response which leads to mucosal immunity and immunological memory [92]

[101]. Previous studies suggest that this leads to immunity for the individual user but also leads to herd immunity [92]. Global vaccine strategies have been very successful, and 132 countries have introduced PCV into their national immunisation programs (see Figure 5.

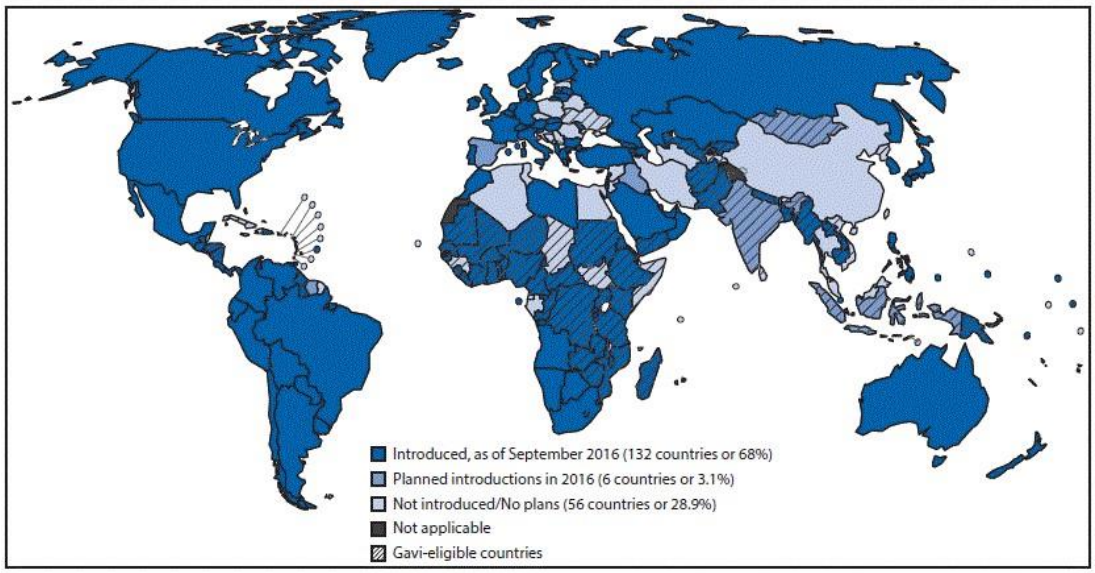

Figure 5: Worldwide current or planned implementation of PCV into national immunization schedule as of September 2016.
Taken from World Health Organization, Immunization Vaccines and Biologicals Database, September 2016. http://www.who.int/immunization/monitoring_surveillance/data/en [102]

Since the introduction of childhood PCV there has been a decrease in IPD and pneumonia in children and similar reduction in rates of IPD have been observed in older adults [92]. A large randomised control trial, which included 85,000 adults aged 65 years of age or older was conducted by a PCV vaccine manufacturer [103]. This study showed 45.6% (95% confidence interval [CI]=21.8-62.5%) efficacy of PCV13 against all vaccine-type pneumococcal pneumonia and 75% (CI=41.4-90.8%) efficacy against vaccine-type IPD in adults ≥65 years of age [103].

1.4.4 Rationale behind new vaccine development

Despite the success of the PCV and PPV immunisations in reducing pneumococcal disease and mortality worldwide, the widespread use of these vaccines and addition into public immunisation programmes has highlighted several limitations [92 101 104]. These include:

- **Protective immunity is limited to the specific serotypes** contained in each of the PPV23 and PCV13 – there are currently over 90 known serotypes of *S. pneumoniae.*
- **'Serotype replacement'** is a major limitation of the PCVs. Among asymptomatic carriers, the prevalence of non-vaccine serotypes ([NVT] non PCV 13 serotypes) has increased significantly (serotype replacement). However, there does not seem to be any impact of overall pneumococcal colonisation rates [105]. A review, also reported an increase in NVT pneumococcal disease [105]. The true amount of replacement may be underestimated due to biases in the pre-vaccine colonisation data. Surveillance systems may underestimate the prevalence of serotypes with lower invasive potential. Because these systems monitor invasive pneumococcal disease, serotypes which do not cause disease are likely to be underreported. Epidemiology studies can also underestimate prevalence of pneumococcal serotypes which have short colonisation durations. This is because surveillance tests for colonisation often are undertaken weekly or less frequently in these studies[105]. In addition, simultaneous colonisation with

multiple pneumococcal serotypes has been increasingly reported with improvements in molecular techniques for pneumococcal detection [40]. Due to this, NVT may have been more prevalent colonisers but at lower density in a multiple serotype colonisation episode and therefore under detected.

- **Limited efficacy against certain serotypes** covered in PCV13 (serotype 1 and 5) [92]. Clinical trials in South Africa [106 107] and The Gambia [108 109] failed to show evidence of efficacy of PCV against serotype 1. A further study, reported PCV13's vaccine effectiveness at 62% and 66% against serotype 1 and 3 respectively although confidence intervals spanned zero [110]. A further study found that the vaccine effectiveness for serotype 3 was not significant (26%, 95% CI, -69%-68%) [111]. In developing countries serotype 1 and 3 continue to be prevalent serotypes causing IPD therefore the lack of efficacy is problematic.
- **Geographic variation in protection** from both PCV13 and PPV23 due to varying serotype distribution worldwide. The current vaccines favour covering serotypes that are most prevalent in western countries such as USA or Europe rather than the serotypes that cause most of invasive disease in developing countries [9]. This was a larger issue with the first PCV vaccine; PCV 7 than for PCV13.
- **Hypo-responsiveness to the capsular polysaccharide** is observed with PCV immunisation (serotype-specific) if children had an episode of pneumococcal nasal colonisation shortly before the first dose of PCV [112 113]. This is only partially overcome after the 2nd or 3rd dose of the vaccine. There has also been reports that repeated doses of bacterial polysaccharides may induce a state

of immune tolerance or hypo-responsiveness. All studies, except one [114], reported that serotype-specific pneumococcal antibody concentrations were lower after the second dose of PPV compared the first dose [115-119] .

- **Complexity and high costs of protein-conjugate vaccines** incurred by pharmaceutical companies and purchasers may impact on the future development of these vaccines to expand coverage to more serotypes [92].
- **Transient efficacy in healthy elderly.** Clinical effectiveness of PPV in older adults is likely to diminish over time. The reduction in pneumococcal capsular antibodies over time following vaccination has been shown in PPV [120]. Reduction in clinical effectiveness over time is also seen in other unconjugated polysaccharide vaccines [121]
- **Lack of proven efficacy in very frail elderly or immunocompromised.** Evidence suggests that PPV23 is effective in preventing invasive pneumococcal disease in healthy young adults and in the healthy older population [122]. However, the vaccines effectiveness in the immunocompromised and very elderly has not been demonstrated [123] **[122] ,**

1.4.5 Novel vaccine development

Limitations of the current vaccines available for protection of pneumococcal disease have driven the development of novel vaccines some of which would be serotype-independent. New vaccine approaches include new polysaccharide conjugate technologies, pneumococcal whole cell vaccines and vaccines which are based on highly-conserved noncapsular protein antigens [92] [124] [125]. Due to the limitations

discussed above, there are specific criteria any new vaccine must satisfy to make a significant impact on disease (target product profile);

- New vaccines should be highly conserved and develop immunity to all pneumococcal serotypes
- There should be evidence to suggest mucosal immunity and immunity against nasopharyngeal colonisation
- The vaccines should have evidence of immunogenicity with evidence of non-inferiority to a licensed pneumococcal vaccine
- The vaccines should induce antibody and cell mediated immune responses
- The immunity should be long-lasting
- The vaccine should be designed to prevent disease in the majority of the population including children under 2 years of age
- The vaccine should be low cost to produce

One possible new vaccine approach is modification of the conjugate vaccine; an international non-profit organisation (PATH) is investigating two alternatives; firstly to develop a conserved pneumococcal protein which would be used as a carrier for a specific number or polysaccharides which could be chosen and changed to meet specific geographical needs [124]. Secondly, they are looking at ways to accelerate the development of vaccines that target serotypes which are more prevalent in developing countries and are also developing new strategies to reduce the cost of manufacturing [124].

An alternative strategy is the use of broadly conserved protein combinations for vaccines. The pneumococcus has multiple proteins exposed on its surface which could be used as possible vaccine antigens. Well-studied examples of these are pneumococcal surface protein A (PspA) and choline-binding protein A (PspC). While other virulence factors that are also at the forefront of protein-based vaccine development include choline-binding protein (PcpA), pneumolysin (Ply) and pneumococcal histidine triad protein D (PhtD) [92] [124]. Research into these protein-based serotype independent subunit vaccines have reached varying stages of advancement. Recombinant PspA has been studied in a phase 1 clinical trial where it was found to be safe in humans and was also immunogenic [126]. This study used a passive transfer murine challenge model as a surrogate for protection of humans [126]. Human participants were administered recombinant PspA, pre- and post-immune serum samples were examined [126]. The authors reported that human antibody to PspA could protect mice from pneumococcal infection [126] .

Another promising area of research is whole cell pneumococcal vaccines, either in live attenuated form or killed form, in which many pneumococcal antigens would be present at once [92] [124]. Potential benefits of a killed bacteria whole-cell vaccine development would be the very low cost of manufacture and that it could protect against all serotypes and could lead to comprehensive mucosal and systemic immunity [124] [127].

1.4.5.1 Controlled human infection studies

Controlled human infections models (CHIM) involve the experimental infection of study participants with a pathogen. They provide the opportunity for researchers to study organisms, their incubation periods and clinical disease. More recently these studies have been used to accelerate the development of new drugs and vaccines for infectious diseases by adding significant data prior to large scale efficacy trials. The first controlled infection studies were described in the 17th century. The ethics of these preliminary CHIM studies were questionable with the most infamous example of Edward Jenner inoculating his gardeners son with cowpox in 1796, and following this repeatedly with smallpox lesion material [128].

There have been significant improvements in ethical considerations of all research and specifically CHIM studies since these preliminary studies; CHIM studies must conform to a strict ethical framework and go through rigorous independent review prior to starting. However, some still argue that CHIM studies are inherently unethical due to concerns about non-maleficence [129 130]. There are significant global health benefits from these studies but to the individuals involved there is an inherent risk of illness [128]. In addition, similar to phase one studies, there are no direct benefits to participants. Due to this, CHIM studies can only be carried out to investigate treatable or self-limiting diseases [128].

The experimental human pneumococcal challenge (EHPC) model is different to the majority of CHIM by having colonisation rather than infection as its primary endpoint.

Due to this, from an ethical and clinical standpoint, the inherent risks and potential for harm is much lower. The Liverpool EHPC team have inoculated over 1000 participants with pneumococcus over the last 9 years with no related significant adverse events.

1.4.6 Experimental Human Pneumococcal Colonisation studies and their use for vaccine research

A major roadblock in the process of developing new protein vaccines has been a means of prioritising between proposed vaccine candidates [131]. Nasopharyngeal colonisation is likely the source of pneumococcal transmission into sterile sites and therefore is likely a prerequisite for invasive disease [31 131]. It has therefore been proposed as a marker for vaccine efficacy [31 131]. For new vaccines due to come to the market, protection against colonisation may predict the overall protective effect against mucosal or invasive disease [101]. Experimental human pneumococcal colonisation (EHPC) can be induced in humans. This was first shown by McCool et al [29] in 2002; this group successfully induced nasopharyngeal colonisation following pneumococcal inoculation with serotypes 6B and 23F and showed that controlled human infection with pneumococcus was safe [29 30].

Following these initial studies in the United States of America (USA), a safe and reproducible EHPC model was establish at LSTM, which can be used to test the protection induced by vaccination against nasopharyngeal colonisation [101]. Preliminary studies investigated what the optimal sampling method would be to identify nasopharyngeal pneumococcal colonisation. The team compared

nasopharyngeal swab (NPS) to nasal wash for the detection of potential respiratory pathogens. This study showed that nasal wash was more comfortable for volunteers and was significantly more likely to detect pathogens than NPS [132].

The model allows assessment of the immune responses at the mucosal surface and systemically following inoculation with live whole bacteria. It can also be useful to help evaluate and compare new vaccines that are possibly coming to market; it could evaluate pneumococcal colonisation protection and any change in nasopharyngeal flora following vaccination [101]. To increase the generalisability of the model and its use for vaccine development, it is important to test multiple serotypes of *S. pneumoniae*.

It has been previously reported that pneumococcal serotype has a significant role in determining colonisation. Some serotypes are more likely to be carried but have low potential to cause infectious or invasive disease. Conversely others are more commonly observed in pneumococcal disease [133]. A previous human pneumococcal challenge study performed by the Weiser group in the USA used serotype 23F P833. They inoculated participants with either 5,000 CFU/naris, 7,000 CFU/naris or 17,000 CFU/naris to assess if there was a dose dependant relationship to colonisation acquisition and achieved colonisation rates of 43% (6/14) with a duration of colonisation ranging from 27-122 days [29]. Following this, our team (Liverpool EHPC team), retested the 23F serotype in addition to serotype 6B in a dose-ranging human challenge study. Colonisation rates for 23F in this study were poor for all inoculum doses (Figure 6) [134].

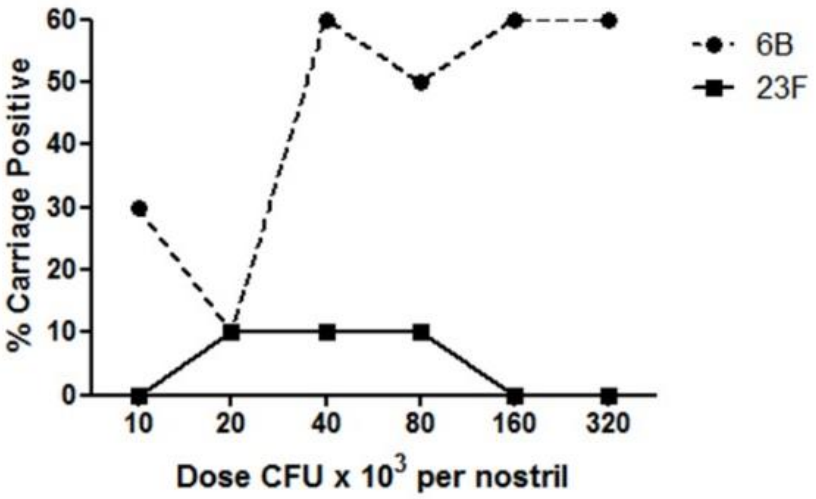

Figure 6: Dose ranging curve for serotypes 6B and 23F (unpublished work from J. Gritzfeld thesis)
10 participants were inoculated with either serotype 6B (circle) or 23F (square) for each of the six doses from $1x10^4$ CFU/naris to $3.2x10^5$ CFU/naris. Y axis shows percentage of colonisation positive participants in each group defined as number of participates found to be an experimental coloniser of pneumococcus in nasal washes at any time point following inoculation.

Due to the poor colonisation rates observed above, the 23F serotype used was sequenced to look for a cause of these results. It was discovered that this serotype differed from the original P833 serotype tested in the USA [29]. Murine models had previously demonstrated that mutations in *amiC* gene could significantly affect the ability of the pneumococcus to colonise. It was found that there was a frameshift mutation in the *amiC* gene in the 23F serotype used in this initial study, which was hypothesised to be the cause of these poor colonisation rates. However, for the 'new serotypes' study (see below) the serotype used was a naturally-derived isolate of P833; P1121. This contained a stable form of the wild-type *amiC* gene; so we expected to be able to reproduce findings from the US-based group of 50% colonisation rates at 7,000 CFU/naris and 75% at 17,000 CFU/naris [29].

1.5 Project aims

This project focuses on improving knowledge about the acquisition of *S. pneumoniae* colonisation using controlled human infection trials.

Aim 1: To investigate whether the hands can be a vector for transmission of *S. pneumoniae* from hands into nasopharynx, leading to colonisation.

a) We will also investigate whether asymptomatic viral infection at the time of exposure to pneumococcus affects acquisition of pneumococcal colonisation following transmission from hands to nose

Transmission of *S. pneumoniae* from person-to-person is thought to occur due to airborne respiratory droplets. Epidemiological data provide evidence that transmission is influenced by overcrowding and concurrent viral respiratory tract infections [8] [43]. There are no published data investigating specifically how *Streptococcus pneumoniae* is spread and testing different routes of transmission. **(Chapter 4: HAND TO NOSE STUDY)**

Aim 2: To investigate the propensity for two different pneumococcal serotypes (23F and 15B) to cause experimental pneumococcal colonisation in healthy adults

a) We will investigate whether exposure to pneumococcus or pneumococcal colonisation is symptomatic in healthy adults and

b) Investigate the host's polysaccharide specific antibody (IgG) response following experimental challenge

To improve our knowledge about nasal pneumococcal colonisation we planned to extend the EHPC model by colonising participants' nasopharynx with different serotypes of *S. pneumoniae* (23F and a non-vaccine type [15B] were tested). Success in this would ensure that the model is more generalisable and useful for further development of vaccine testing studies. Specifically, the development of a colonisation model with a non-vaccine type serotype would be an important step for vaccine testing. New pneumococcal vaccines will need to demonstrate an impact on reducing colonisation, and protein-based vaccines will need to show efficacy against serotypes not covered by PCV13. **(Chapter 5: NEW SEROTYPES STUDY)**

2 Methods

2.1 Overview

This chapter describes the methods relevant to both studies described in this thesis. It covers methodology used to investigate the main aims of the thesis outlined at the end of Chapter 1. Specific methods relevant to one study are considered separately in each chapter for ease of reading. The chapter is divided into subsections which cover study set up, clinical procedures, methodology and laboratory processes.

2.2 Study set up: Research in the National Health Service (NHS)

2.2.1 Sponsorship

All clinical research undertaken in the NHS requires a sponsor. The definitions of a sponsor and roles and responsibilities are detailed in the Research Governance Framework, Edition 2 2005 [135] (guidelines in place when New Strains study was set up) and later by UK Policy Framework for Health and Social Care Research 2017 [136] (see Table 3).The sponsor needs to be an individual, organisation or group which is a legal entity involved in either the funding, running, hosting of the research or employing the research staff.

Sponsorship can be sole (in the case of Hand to Nose study- single sponsorship by LSTM) or co-sponsored by more than one organisation (in the case of New Serotypes study- joint sponsorship between LSTM and Royal Liverpool and Broadgreen University Hospital Trust [RLBUHT]). Co-sponsorship involves a pre-agreed division of

the sponsor responsibilities prior to initiation of the study. Agreement for LSTM or RLBUHT to sponsor a research study involves the research team completing an application to the organisation's research governance department. Initially sponsorship in principle is obtained. Full sponsorship is only agreed once the organisation receives documentary evidence of ethical and relevant national regulatory approvals. No study activities should be completed prior to full approval and sponsorship.

Table 3: Definitions and responsibilities of a sponsor taken from Research Governance Framework [135] and UK policy for Health and Social Care Research [136]

	Research Governance Framework for Health and Social Care (Second edition, 2005) [135]	UK Policy Framework for Health and Social Care Research (2017) [136]
Definition	'Individual, organisation or group taking on responsibility for securing the arrangements to initiate, manage and finance a study. A group of individuals and/or organisations may take on sponsorship responsibilities and distribute them by agreement among the members of the group, provided that, collectively, they make arrangements to allocate all the responsibilities in this research governance framework that are relevant to the study.' [135]	'The sponsor is the individual, organisation or partnership that takes on overall responsibility for proportionate, effective arrangements being in place to set up, run and report a research project. All health and social care research has a sponsor.' [136]
Responsibilities	Main responsibility of the sponsor is to assure the quality of the research, including ensuring participant's wellbeing, all legal requirements are met, the research team are adequate to carry out the work and have resources and arrangements in place for the study to be completed. More detailed set of responsibilities are set out in the Research Governance Framework for Health and Social Care, Second edition 2005 [135] and the UK Policy Framework for Health and Social Care Research 2017 [136].	

2.2.2 Health Research Authority (HRA)

HRA approval process for research trials was fully implemented in the UK in March 2016. This new system was designed to combine the independent ethical opinion/approval by the Research Ethics Committee (REC) and a review of research studies against NHS standards [137]. This is an assessment of governance and legal requirements needed for each study which is undertaken by specific HRA staff, previously undertaken by individual NHS Research Development and Innovation (RD&I) offices.

HRA approval was only required for the Hand to Nose study because the New Serotypes study received ethical approval before this new system was fully implemented. HRA application is encompassed into the Integrated Research Application System (IRAS) form, the research team also provides the HRA with Statement of Activities and Statement of Events forms which together aim to capture all information about study activities and study related information at a local level.

There are many steps involved in obtaining HRA approval for a clinical trial. Figure 7 below shows the main steps in obtaining approval. The Hand to Nose study gained HRA approval in February 2017. For the New Serotypes study which was approved pre-implementation of HRA approval, the sole responsibility for ensuring the study complied with all legal and governance requirements fell to RLBUHT RD&I department, see below section 2.2.4.

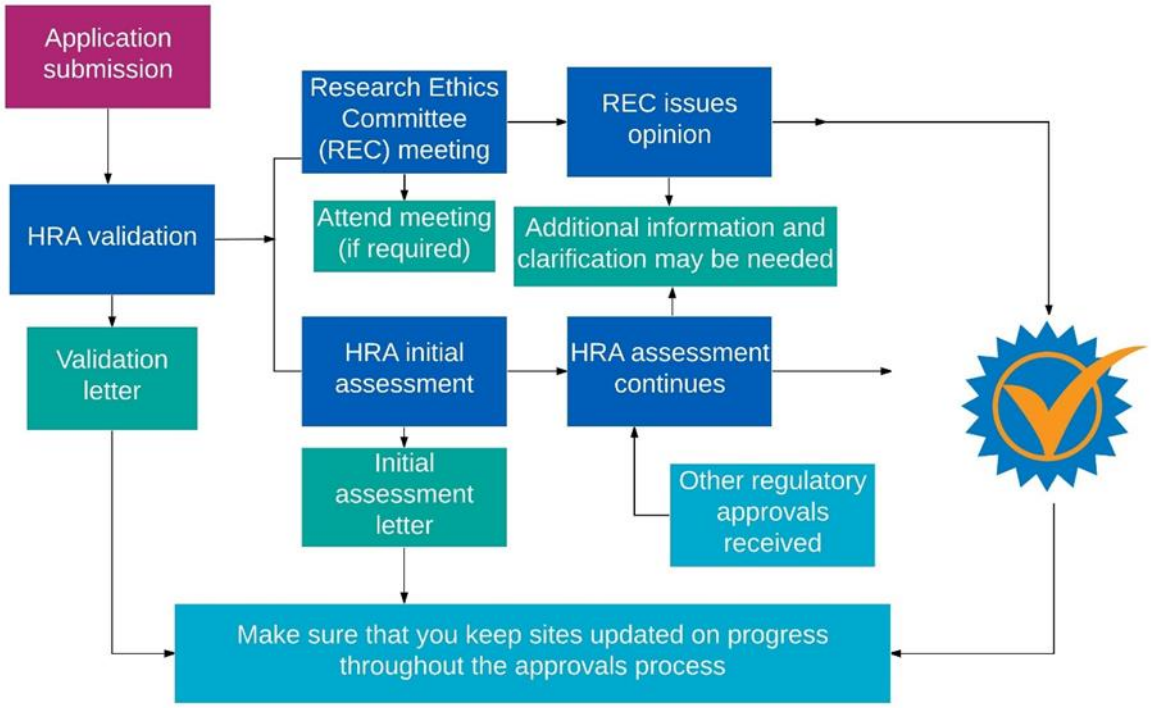

Figure 7: HRA approval process applies to all research projects taking place in the NHS in England [112]
HRA approval encompasses assessment of governance and legal compliance and independent ethical opinion by the Research Ethics Committee.

2.2.3 Ethics: Integrated Research Application System (IRAS) and Research and Ethics Committee (REC)

One of the HRA's main functions is ensuring that research undertaken in the NHS is ethically acceptable. To do this they have multiple RECs countrywide. They aim to protect research participants by ensuring that studies are carried out in an ethical way and ensure that participants rights, safety, dignity and wellbeing is protected. In addition, they aim to ensure that research being undertaking is of potential benefit to science, society and the participants. Both studies in this thesis (Hand to Nose [Chapter 3] and New Serotypes [Chapter 4]) required full REC approval because these studies involved human volunteers and were carried out in NHS sites.

A separate IRAS form was created for each study and was the main basis of the REC application. Following this, the research team attended a REC meeting date, this gave the REC an opportunity to ask questions about the study and give feedback on changes that they would like the study team to make to documents provided or IRAS form. Ethical approvals for the studies were granted as shown in Table 4 below.

Table 4: REC study approvals and other approvals/registrations for research studies within this thesis

Study	REC	IRAS number	Sponsor	Other approvals/registrations
Hand to Nose study **Chapter 3**	Liverpool (East) 17/NW/0054 Approved: 02.02.2017	221034	LSTM	ISRTN: 12909224 registered 27.06.2017 NIHR portfolio: 33503 HRA approval: 22.02.2017 RD&I: 5376 approved 17.03.2017
New Serotypes study **Chapter 4**	Liverpool (East) 15/NW/0931 Approved: 25.01.2016	193680	LSTM and RLBUHT	ISRTN: 68323432 registered 15.08.2017 NIHR portfolio: 20815 RD&I: 5124 approved 08.08.2016

2.2.4 Research Development and Innovation Department (RD&I)

Both studies discussed in this thesis were carried out in the Clinical Research Unit (CRU) based in RLBUHT. As the site of these research studies RLBUHT has a legal obligation to ensure that they are aware of all research undertaken on the site and

have carried out certain assessments prior to study initiation. The CRU is a Medicines and Healthcare Products Regulatory Agency (MHRA) accredited phase one research unit at RLBUHT. All participant visits for both studies were conducted in this unit.

Final RD&I approval was provided for both studies once all other approvals were in place including:

- REC approval
- Finance team/ RD&I business team approval (contracts finalised)
- HRA approval (only Hand to Nose study as this step only implemented March 2016)
- Directorate managers approval
- CRU approval
- Evidence of sponsorship in place
- Evidence of liability insurance in place
- Evidence of Principle Investigator/Chief Investigator contract with RLBUHT

2.2.5 Patient and Public Involvement (PPI)

PPI is becoming increasingly important in research, and there are many benefits to the research team and public with this involvement. Some of the many benefits include:

- Supporting recruitment and consent

- Demonstrating to funders that the research topic is important and relevant to the public and patients
- Demonstrating to funders that the study design and documents are acceptable to the public
- Identifying possible ethical issues with the design of the study

The EHPC team have been studying pneumococcal colonisation in Liverpool over the last 9 years. The clinical and lab EHPC team understand the importance of PPI in research and we strived to ensure we had continual PPI events planned throughout both study periods.

We attended many public engagement events; these events gave us the opportunity to provide information about the research being undertaken and improve local knowledge about research. This included us attending public open days, science fairs which are open to the public, having regular display stands and face to face engagement with the public at local gymnasiums and libraries. We also created a newsletter which was disseminated to previous research participants, colleagues and members of the public to which provided updates on findings of finished studies and future studies.

We have also developed a role within the team for research ambassadors; individuals who previously participated in one of the EHPC studies. For the studies described in this thesis, research ambassadors were used to sense-check promotional and

information materials, to provide lay-input into the study protocols and to assist with recruitment events.

A relatively new addition to our PPI work was utilising social media platforms as a way of involving the public in the research undertaken. We used multiple platforms including Facebook and Twitter to regularly upload information about our face-to-face research events the public could attend and update followers about current trials which were open to recruitment. This has impacted on the demographics of our participants; the majority tended to be under 30 years of age who were more likely to have an active social media connection [138].

2.3 Clinical procedures

2.3.1 Trial designs

The two studies in this thesis used a human challenge model study design. Participants were challenged with live pneumococcus either intranasally (inoculated) or on their hands (exposure). All participants were seen prior to this challenge for screening and then were followed up for a period of up to 14 days post challenge. Nasal wash and blood samples were obtained to assess for nasopharyngeal pneumococcal colonisation and to investigate the immune response to challenge with or without colonisation (sample obtained discussed further below).

Study specific trial design with study design flow charts are outlined in Chapters 3 and 4 (sections 3.2.2 and 4.2.2).

2.3.2 Ethical considerations

Autonomy

The study team ensured that participants were given sufficient information, written and spoken, without the use of medical jargon or other language requiring specialist knowledge. This ensured that participants could understand the research objectives and the risks and benefits of any procedures. Participants were always given enough time to consider the information before consenting to any involvement. We ensured that all participants did not feel pressured or persuaded into participating in the research study. Participants were financially compensated for their participation in the studies. Offering this payment was not intended to unduly influence participation but to compensate participants for their time and travel expenses incurred due to taking part in the trial.

Participants that were recruited via the Consent4Consent database (see below section 2.3.3) were contacted regarding the research as they had previously expressed an interest in research. These participants had already given their consent to be contacted about future research that they may be eligible for. Participants were informed that they had the right to withdraw their consent and therefore withdraw from the study at any time without giving reason.

Non-maleficence

The research team had a responsibility to minimise the risk of harm to the participants. We ensured that all researchers in the team had sufficient knowledge about the proposed interventions and potential risks. In addition, strict safety procedures were in place for both studies described in this thesis. See sections 2.3.6 and 2.3.7 for further details on participant safety during the trial.

Beneficence

There are no direct benefits to the participants when taking part in the study. However, participants may have benefited from a better understanding of clinical research and from a sense of contributing to valuable medical research.

Justice

Justice is balanced with non-maleficence. We ensure that the research is open to all individuals, but important exclusion criteria are in place, primarily to protect individuals from undue risk.

2.3.3 Recruitment and advertising

Prior to the start of the study the research team ensured that there were strategies in place to meet recruitment targets. Recruitment strategies used for both studies described in this thesis are detailed in Table 5. Figure 8 shows a flow chart of the

participant recruitment process. Advertising for the studies was not aimed at any vulnerable adults or those that may have lacked capacity to consent to take part in research. Participants were asked to gift their samples for use in future studies and to share with collaborators internationally. A continuous consent approach was used throughout the study; participants were asked at each visit if they were willing to continue.

Table 5: Recruitment strategies for both Hand to Nose study and New Serotypes study

Recruitment activity	Description
Flyers and recruitment posters	Flyers and recruitment posters were put up on notice boards, table displays and around local public areas and local universities
Social media adverts	Facebook and Twitter announcements
Electronic notice boards	Announcements on local university's electronic notice/announcement boards.
Face-to-face recruitment drives	Included: local university fresher's fairs, local university open days and events at local halls of residences, libraries and gymnasiums. Interested people were given study recruitment flyers and were briefly spoken to about the purpose of the research and what taking part involved. **Face to face recruitment drives were the most fruitful recruitment strategy for our team. During the 2016 fresher's events at local universities we obtained contact details of over 1400 potential participants.**
Consent4Consent Database	Consent4Consent Database is a secure database of volunteers, created by RLBUHT, who had given their permission to be contacted about future research projects.

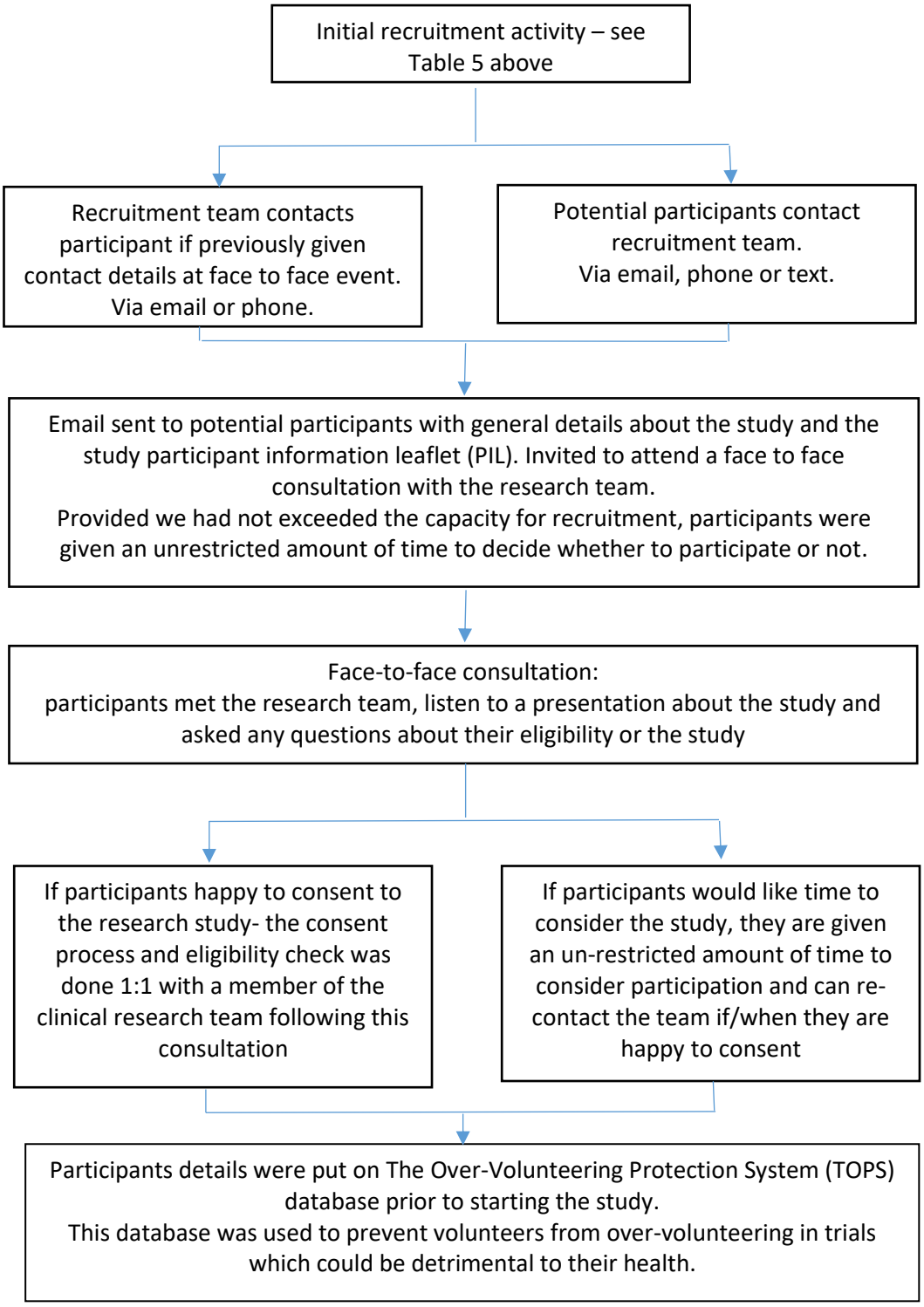

Figure 8: Flow chart of participant recruitment
Following initial expression of interest in taking part in research, participants were given written information about the study and all were seen for a face-to-face discussion about the study prior to consent.

2.3.4 Inclusion and exclusion criteria

Any specific inclusion and exclusion criteria for the Hand to Nose study and New Serotypes study are described in Chapter 3 (Hand to Nose) and Chapter 4 (New Serotypes).

Inclusion Criteria- common to both studies described in this thesis
• Adults aged 18-50 years **To minimise the risk of pneumococcal infection, and to allow comparison with previously publishe experimental work done by the EHPC group**
• Fluent spoken English **To ensure all participants have a comprehensive understanding of the research project and their proposed involvement**

Exclusion Criteria- common to both studies described in this thesis

- History of major pneumococcal illness (other severe bacterial infections included if participant fully recovered)
- History of drug or alcohol abuse
- History of smoking, defined as:
  - Current regular smoker (smokes daily/ smokes > 5 cigarettes per week)
  - Recent smoker i.e. within the last 6 months
  - Ex-smoker with a significant smoking history (>10 pack years)
- Asthma (current and on treatment or not on treatment but symptomatic) and chronic respiratory diseases– both due to propensity to infection and due to possible confounding effect of medications such as corticosteroids.
- Any other acute or chronic medical diagnosis that is known to increase the risk of bacterial infections or could lead to hospitalisation during the study period- at the discretion of study team
- Taking daily medications that may affect the immune system such as steroids, steroid nasal spray, or retinoids
- On any courses of medications which may affect the immune system such as chemotherapy, biologics, radiotherapy
- Current illness, acute illness within 3 days prior to inoculation or antibiotic treatment within 2 weeks of inoculation- both due to propensity to infection and to minimise any effect on inoculation or
  transmission
- Currently pregnant
- Diabetes- type 1 and type 2 (current diagnosis)
- Involved in another clinical trial unless observational or in follow-up (non-interventional) phase- both due to propensity to infection and to minimise effect on inoculation or transmission
- Significant anaemia, thrombocytopenia, neutropenia, neutrophilia, leucocytosis- as per results of full blood count test at screening
- Previously undiagnosed abnormality found on screening clinical examination such as murmur

To minimise risk of possible pneumococcal infection following inoculation or exposure

- Allergy to penicillin/amoxicillin

For safety of the participants- pneumococcal pathogen used in the studies fully sensitive to penicillins and are therefore given to participants during the study as emergency supply if unwell or to attempt to clear carriage at the end of the study

- Close physical contact with at risk individuals (children under 5 years of age, immunosuppressed adults, elderly, chronic ill health)

To minimise pneumococcal transmission and possible subsequent infection risk to others in the Community

- Have been involved in an EHPC clinical trial involving pneumococcal inoculation in the last 3 years
- Previous pneumococcal vaccination
- Taking daily medications that may affect the inoculation such as long-term antibiotics

To ensure homogeneity and interpretability of immunological endpoints of the participant population with respect to previous pneumococcal immunisation or disease

2.3.5 Study schedule

Common parts of the study schedule for the Hand to Nose study and New Serotypes study are described below, specific details for each study are detailed in Chapter 3 for Hand to Nose Study (section3.2.5) and Chapter 4 for New Serotypes study (section 4.2.2).

Following consent, participants were given a study schedule by the research team. This included an initial screening visit which was scheduled for the week prior to pneumococcal inoculation or exposure (see Table 6 for screening safety assessments). During the initial screening, additional study samples were also obtained to allow for baseline assessment. These included nasal wash to assess for natural pneumococcal colonisation and viral throat swab for both studies. A week after the screening visit, all participants who had passed screening came back for day 0 which included pneumococcal challenge (inoculation or exposure).

Table 6: Screening safety assessments

Assessment	Rationale	Action taken if abnormal
Focused clinical history with medication review and clinical examination	Ensure no abnormality that could increase risk of pneumococcal infection or would put participant at increased risk in the case of infection.	**Significant abnormality:** volunteer was excluded from the study and the study doctor arranged appropriate investigations or treatment through primary care. **Minor abnormality:** if no potential to increase infection risk, volunteer included in study and study team informed primary care for follow up.
Urinary pregnancy test	Rule out pregnancy which could increase risks associated with pneumococcal infection	Excluded from study if positive.
Full blood count blood test	Ensure no abnormality that could increase risk of pneumococcal infection or would put participant at increased risk in the case of infection. No specific cut off values were used when evaluating full blood count results. Results were taken into context of clinical history and examination.	**Significant abnormality:** volunteer was excluded from the study and the study doctor arranged appropriate investigations or treatment through primary care. **Minor abnormality:** if no potential to increase infection risk, volunteer included in study and study team informed primary care for follow up.

2.3.6 Safety

Pneumococcus is responsible for infections including otitis media, sinusitis, pneumonia, bacteraemia and meningitis. Exposing patients to pneumococcus gives a theoretical risk to participants of these infections. However, experience from previous pneumococcal challenge studies that have been run in Liverpool, suggests that the risk to healthy volunteers of developing an infection is very low. 10% adults experience natural colonisation at any time; while the incidence of invasive disease

is 20/100,000 patient years). Nevertheless, both studies reported in this thesis were designed to ensure any risk was minimised as follows:

- **Experienced study team** (in pneumococcal human challenge studies) undertaking the work.
- **Careful serotype selection and dosing.** Serotypes are all fully sequenced and have had antibiotic resistance testing by Public Health England. Dosing for these studies was based on a previous dose ranging study undertaken by the EHPC team [134 139]. The team has experience of inoculating volunteers with doses of serotype 6B pneumococcus between 10,000-320,000 CFU/naris [134 139]. We have shown that inoculating at these doses is safe and colonisation rates are reproducible [134 139]. Further information on serotype selection and dosing can be found in Chapter 3 and 4.
- **Study design.** The New Serotypes study had a dose escalation period built into the study design. This ensured safety monitoring of at least 7 days post exposure of the first group receiving a new dose prior to any further inoculations (see Figure 13, Chapter 4). For both the Hand to Nose study and the New Serotypes studies, participants were put into groups of up to 24 participants. Groups were inoculated at least a week apart to ensure that we could minimise exposure if any safety concerns were raised.
- **Participant selection and exclusion criteria.** For both studies, strict exclusion criteria must be met, and safety screen visit was carried out to ensure participants do not have any un-diagnosed conditions which could put them

at increased risk (previously explained under the inclusion/exclusion heading).

- **Participant education**. Participants were educated about potential risks involved in the study and signs and symptoms to look out for during the study at numerous times. See Appendix A for participant information leaflets.
- **Rigorous safety procedures.** Each volunteer received a safety information leaflet, business card with emergency contact information, thermometer and 3-day course of amoxicillin. Participants were advised to keep this pack with them at all times during the study. They were advised to take amoxicillin1) in the event they were unwell and were instructed to by the research team 2) if they were unwell and unable to contact the research team 3) if they had carried pneumococcus during the study and did not have 2 clear nasal washes prior to the last visit. Daily monitoring was also undertaken; participants were required to contact the study team with their temperature reading and any symptoms before midday for 7 days post exposure/inoculation. If the study team had not heard from a participant by midday, the volunteer was contacted to ensure their wellbeing. If they did not respond an allocated daily contact person/next of kin was contacted.
- **24-hour emergency telephone contact available.** A member of the clinical team was available for participants 24 hours a day, 7 days per week. This emergency number was covered by a research nurse during working hours and a medical consultant out of hours. Safety queries were either dealt with by phone advice or face to face review could be arranged.

- **Weekly safety report.** This report included study number of each participant, bacterial dose and serotype, date of inoculation, if they were a carrier of the bacteria post inoculation and details of any symptoms or illness that had been reported.

Risks to researchers during the study are also very low. The main potential risks include needle stick injury to the clinical staff during venepuncture procedures and biological/chemical hazards within the laboratory for the lab staff. Bacterial stock preparation is undertaken in a hood to reduce risk of staff pneumococcal colonisation. During inoculation/exposure procedure staff also ensure that the bacterial stock is only open to the environment for minimal amount of time to ensure bacteria are not aerosolised and keep risk of pneumococcal colonisation of staff to a minimum. To ensure these risks are at a minimum, only experienced staff carried out procedures, within their competencies, in accordance with standard operating procedures regulated by good clinical practice and national guidelines. Appropriate risk and Control of Substances Hazardous to Health (COSHH) assessments are in place for all laboratory procedures. All laboratory work was conducted in an appropriately rated laboratory in line with health and safety regulations for research with human tissues/infectious agents.

2.3.7 Safety monitoring

The following definitions were used in both Hand to Nose study and New Stains study (taken from HRA):

- **Adverse Event (AE):** Any untoward medical occurrence in a participant to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product.
- **Serious Adverse Event (SAE)** A serious adverse event is any untoward medical occurrence that:
    - Results in death
    - Is life-threatening
    - Required inpatient hospitalisation or prolongation of existing hospitalisation
    - Results in persistent or significant disability/incapacity
    - Other 'important medical events' may also be considered serious if they jeopardise the participant or require an intervention to prevent one of the above consequences

There are strict guidelines for monitoring safety of participants taking part in research studies and reporting any safety concerns; Table 7 details the procedure for informing REC of any safety events. The Hand to Nose study and New Serotypes study were not clinical trials of an investigational medicinal product (CTIMP). Accordingly, the REC was only informed about SAEs that were related to the study and unexpected. In addition to this all SAEs were recorded and reported to the DMSC and sponsors (within 24hrs). SAE were monitored and reported until the end of the participant's follow-up.

Non-serious adverse events were collected systematically during the research and recorded in the case report form and in the weekly safety report. Participants in the New Serotypes study were also asked to keep a log of symptoms since new serotypes of pneumococcus were being investigated. The results were summarised and reported to the DMSC. Safety reporting continued until the participant's last visit.

Table 7: Safety reporting to REC for non-CTIMP research studies; guidelines taken from the HRA [140]

	Who	When
SAE	CI or sponsor to the REC which issued favourable opinion	Within 15 days of the CI becoming aware of the event
Urgent safety measures	CI or Sponsor to the REC which issued favourable opinion	Immediately by telephone and then in writing within 3 days
Progress reports	To be submitted by sponsor, sponsor's legal representative or Chief Investigator. Must always be signed by the CI. To the REC which issued favourable opinion	Annually (starting 12 months after the date of the favourable opinion)
Declaration of the conclusion or early termination of the research	CI or Sponsor to the REC which issued favourable opinion	Within 90 days (conclusion). Within 15 days (early termination).
Summary of final report	CI or Sponsor to the REC which issued favourable opinion	Within one year of the conclusion of the research.

2.3.8 Data Management and Safety Committee (DSMC)

The EHPC team has an established Data Management and Safety Committee (DMSC) who are available for advice and support for the research team. DSMC should be an independent group of experts which can advise the study team using their expertise and can give recommendations. This committee was used as an additional safety measure for our studies. It consisted of 3 members (including at least 1 statistician) who are independent of the study team. The DMSC were supplied with a safety report at the end of the study, in the event of an SAE, or if requested at any time by the PI or DMSC members. Review of this safety report allows for critique of the safety of the studies without any possible reporting bias by study staff deciding to only escalate certain events to the committee. In the event of a SAE the DMSC and sponsor were also informed within 24 hours of PI becoming aware.

2.3.9 Nasopharyngeal pneumococcal challenge

Participants in both the studies reported in this thesis were exposed to pneumococcal bacteria (pneumococcal challenge). On the day of pneumococcal challenge participants were specifically asked to report any upper respiratory tract (URT) symptoms to the clinical team and an oral temperature was taken as an extra safety check. If the participant reported any URT symptoms or had an abnormal temperature (>38 °C) they would be reviewed by one of the study doctors who would undertake a full clinical history and examination and decide about the safety of the participant to undergo the pneumococcal challenge. The pneumococcal challenge methods are described in Chapter 3 and 4, sections 3.2.7 and 4.2.1.

2.3.10 Nasal wash sampling method

Nasal wash was used for detection and quantification of pneumococcal colonisation. For this procedure, participants were seated, with their head tilted back to 30°, and were asked to hold their tongue at the roof of their mouths. Participants were asked to take a deep breath in and hold this breath (to avoid lung aspiration). Following this, 5mls of sterile 0.9% sodium chloride (saline) was instilled into one nostril, then collected into a sterile foil bowl following leaning forward and blowing the saline out of the nose while releasing the held breath (see Figure 9).

This same procedure was then repeated three more times, in total twice in each nostril, with a total of 20ml of saline used. The sample was then transferred into a 50ml centrifuge tube (Falcon, Thermo Fisher scientific, USA) for transportation to the laboratory. If less than 10ml of sample was obtained, the procedure was repeated to obtain an adequate specimen. Up to 10mls of extra saline was used if needed.

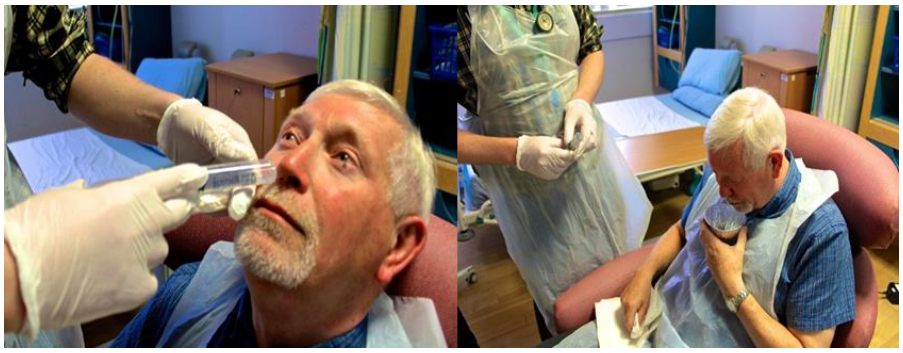

Figure 9: Nasal wash procedure
A syringe is filled with 20mls of saline, this is inserted into the nasal cavity and 5mls of saline is instilled into the nasopharynx. The participant has their head tilted back to approximately 30° while the saline is expelled and then leans forward to collect the expelled saline into a foil bowl. Picture reproduced with permission from EHPC team volunteer. Consent gained prior to photographs taken.

2.3.11 Viral swab sampling method

Swabs (flock swab, FLOQSwabs™) were taken from the palatopharyngeal arch using a tongue depressor, then placed immediately in viral transport medium (universal transport medium) and put on ice for transfer to the laboratory. Throat swabs preceded nasal washes to minimise oropharyngeal contamination with nasal pathogens (Figure 10).

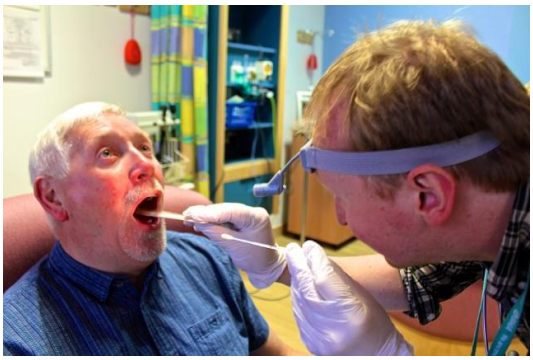

Figure 10: Viral throat swab procedure.
A sterile medical swab is used to sample the oropharynx. A wooden tongue depressor was used if needed. Picture was reproduced with permission from EHPC team. Volunteer consent was gained prior to photography.

2.3.12 Blood sampling method

Blood samples were obtained by venepuncture from the arm. The skin was cleaned prior to the procedure (CloraPrep® chlorhexidine gluconate 20mg/ml and isopropyl alcohol 0.7ml/ml), and samples were collected using a vacutainer system (Becton Dickinson, Plymouth, UK) (Figure 11).

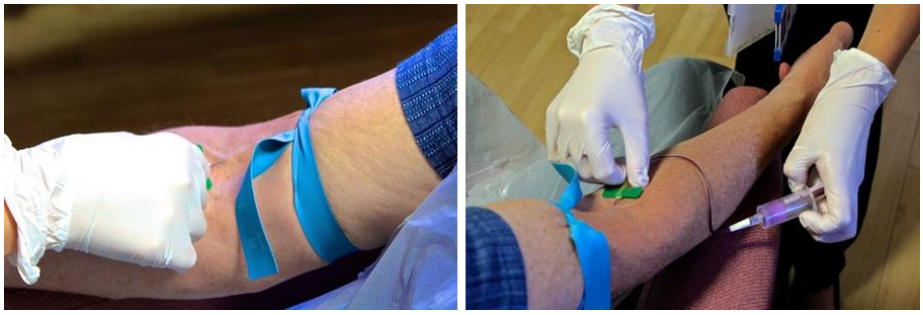

Figure 11: Venepuncture procedure
Participant's blood was usually taken from the antecubital fossa. The skin was cleaned prior to the procedure and samples collected using a vacutainer system. Picture reproduced with permission from EHPC team. Volunteer consent was gained prior to photography.

2.4 Laboratory procedures

2.4.1 Pneumococcal stock preparation (batch)

For each serotype (15B, 23F and 6B) a 'parent' bead stock was prepared which was then used to prepare all subsequent inoculation stocks. For preparation of the 'parent' stock, clinical isolates of each serotype were cultured on Columbia Blood Agar with horse blood (Oxoid, UK). This was then incubated overnight at 37°C in 5% carbon dioxide CO_2. Following this the bacteria growth was added to Microbank™ vials (Pro-lab Diagnostics, USA). These cryovials contain ceramic beads and a unique cryo-preservative fluid which has been proven to allow for longer survival of fastidious cultures and higher quantitative recoveries and stored at -80°C [141]. Prior to preparing any inoculum stocks from this 'parent' stock, it was checked for contamination by plating of the stock and checking for any non-pneumococcal bacterial growth and colony uniformity.

The preparation of the batch pneumococcal stock was undertaken in a fume hood with dedicated incubator and pipettes to ensure no contamination. Using the 'parent' stock, each serotype was plated on blood agar and incubated overnight at 37°C in 5% CO_2. Bacterial colonies which grew overnight were then mixed with Vegitone broth (a vegetable-based growth medium, Sigma-Aldrich, UK) and grown to mid-log phase. These were then stored in aliquots of 1ml following the addition of 10% sterile glycerol at -80°C.

The determination of colony forming units (CFU) per ml is carried out using a slight modification of the Miles and Misra method [142]. A blood agar plate was initially divided into 6 sections and labelled as shown in Figure 12. Using a 96 well U-bottom plate (Corning Inc, Germany), 180ul of sterile saline was mixed with 20ul of bacteria. Serial dilutions were performed to 10^6. Three 10ul drops from each dilution were placed on the corresponding section of the blood agar plate (e.g. 10^4 in section 4). Following time for drying, the plate was incubated for 9-16 hours at 37°C, 5% CO_2. The following day the number of visible colonies in each section was counted.

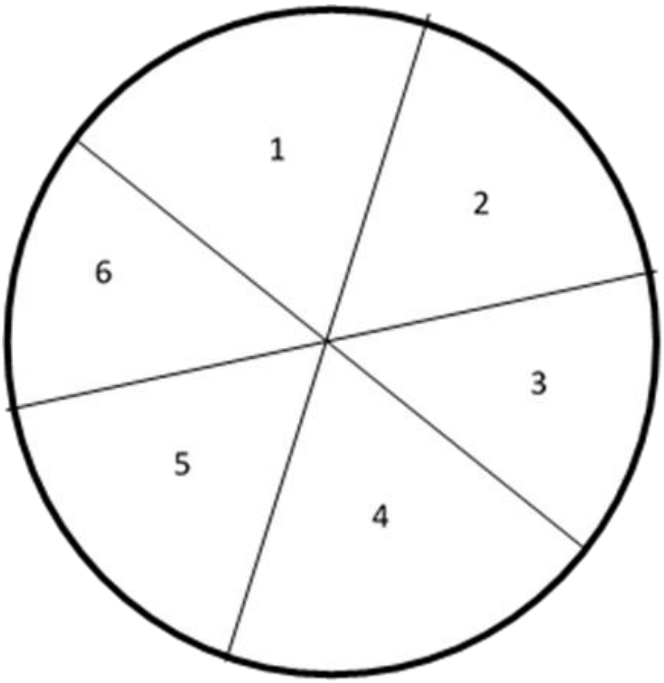

Figure 12: Miles and Misra plates for determination of CFU/ml
Three 10µl drops from serial dilution of inoculum were placed into the corresponding section of the blood agar plate. The plate was then inverted and incubated overnight. The following day the number of visible individual colonies in each section was counted.

Calculation of the final dose (CFU/ml) is determined using the least dilute section where the number of individual colonies can be counted using the following formula;

$$\text{CFU/ml} \left[\frac{(\text{number of visible colonies}/3) \times (\text{dilution factor of section})}{\text{volume of the drop plated}} \right] \times \text{volume of bacteria stock used for challenge}$$

Prior to human challenge, a sample of the bacterial stock was sent to an independent reference laboratory (Public Health England, UK) to confirm bacterial stock purity, serotype and antibiotic sensitivity.

2.4.2 Preparation of pneumococcal stock on day of challenge

Participants in the Hand to Nose study and New Serotypes studies were put into groups. Each group was challenged on the same day in groups of up to 7 participants per challenge. This helped to minimise variability between different bacterial challenge stocks being used throughout the study. In addition, for all participants the bacterial challenge stock came from the same 'parent' stock and same pneumococcal batch stock made.

30 minutes prior to the scheduled pneumococcal challenge appointments the bacterial aliquot was thawed. This was spun down at 17000g for 3 minutes, followed by a wash step in normal saline. The bacterial pellet was re-suspended in 0.9% saline to the desired concentration based on prior CFU quantification. Bacterial suspension densities were prospectively quantified by Miles and Misra (M&M) serial dilution, both from defrosted stock, and from a control sample which was transported to and from the clinical site. This established the loss of bacterial viability related to transport. Two independent counts were performed per sample.

2.4.3 Nasal wash sample processing

Nasal wash samples were transported to the laboratory and were processed within one hour of the sample being taken to minimise loss of viability. Any large pieces of mucus were removed (after vortexing to break up the debris or mucus) prior to processing with as little loss of saline as possible. The nasal wash sample was then

centrifuged for 10 minutes at 3345g, the supernatant volume was recorded and then stored as 1ml aliquots at -80°C.

The nasal wash pellets were mixed with 100µl of skim milk, tryptone, glucose, glycerol medium (STGG), and the STGG plus nasal wash pellet volume was recorded. 20µl of this was then streaked onto a blood agar plate with gentamicin (4µg/ml). 10µl was then used for M&M to assess possible colonisation density. The remaining nasal wash pellet was diluted with 8.2ml STGG; a further 25µl was streaked onto blood agar plates to assess for co-colonising flora. All plates were incubated overnight at 37°C in 5% CO_2. The remainder of the sample was divided into 3 cryovials and stored at -80°C.

2.4.4 Detection of pneumococcal colonisation by culture

Pneumococcal growth was confirmed by visual appearance of the colonies (draughtsman-like colony morphology), presence of α-haemolysis, Gram-positive staining, optochin sensitivity and bile solubility.

Serotype was assessed using latex agglutination (commercial kit from Statens Serum Institute, Denmark). A participant was labelled as colonisation positive if the serotype was proven to be the type experimentally challenged with (6B for Hand to Nose and 23F or 15B for New Serotypes). If there was growth of any other serotypes the participant was labelled as a natural carrier of pneumococcus.

2.4.5 Detection of pneumococcal colonisation by quantitative polymerase chain reaction (qPCR)

2.4.5.1 Bacterial DNA extraction

Stored samples were thawed and 300µl nasal wash pellet suspension was centrifuged at 20,238g for 7 minutes. The pellet was re-suspended with lysis buffer and protease mix (300µl; 1-part protease to 6-parts of lysis buffer [LGC Genomics GmbH, Germany]), 100µl of zirconium beads and 300µl of phenol, and disrupted using a TissueLyser (Qiagen, Germany), twice at maximum speed for 3 minutes (with cooling on ice in between). Following a further centrifuge for 10 minutes at 9391g to ensure separation of phases. The aqueous phase was mixed with binding buffer (600µl) and magnetic beads (10µl), then vortexed and incubated for 30-90 minutes in a gyratory rocker (Stuart™ SSM3 Gyratory Rocker) at room temperature. The sample and magnetic beads were washed twice with 2 washer buffers (200µl each) and bacterial DNA was eluted with 63µl of elution buffer. This buffer was the final step which detaches the extracted DNA from the magnetic beads and makes it soluble for recovery.

2.4.5.2 Quantification of pneumococcal DNA by qPCR

Determination of colonisation status and the density of colonisation episode by qPCR was performed through partial amplification of the lytA gene. The master mix included 12µl of DEEPC-treated water, 0.225µl of forward primer (5'-

ACGCAATCTAGCAGATGAAGCA-3'), 0.225µl of reverse primer (5'-TCGTGCGTTTTAATTCCAGCT-3'), 0.125µl probe (5'-(FAM)-GCCGAAAACGCTTGATACAGGGAG-(BHQ)-3') and 12.5µl of TaqMan® Universal PCR Master Mix (Life Technologies). In each well, 22.5µl of the master mix was mixed with 2.5µl of extracted DNA. On each plate, two negative controls used 25µl master mix only, and each plate contained a standard curve of 10-fold dilutions of genomic DNA extracted from *S. pneumoniae* (10^6-10^1*)*. Samples were assayed as duplicates, using thermal cycling conditions: 10 minutes at 95°C for DNA denaturation followed by 40 cycles of 15 seconds at 95°C and then finally 1 minute at 60°C. All qPCR thermal cycling was performed in the same ABI 7500 Fast Real-Time qPCR system (Life Technologies, UK).

The lower limit of detection was set at 40 cycles (CT). The qPCR plate was repeated if: 1) there was DNA detected in either of the negative control wells or; 2) any of the standards between 10^6-10^2 were not detected or; 3) both 10^1 standard wells were not detected or 4) the scatter of the standard results around the fitted regression line was poor ($R^2 \leq 0.98$, slope was not between -3.1 and -3.6) or 5) the efficiency was not between 90% and 110%.

2.4.6 Detection and identification of upper respiratory tract viruses

Viral RNA was extracted from 200µl of viral transport medium (VTM), in which the oropharyngeal swab was stored and eluted into buffer using standard protocols for the QIAcube or QIAsymphony instruments. The samples were analysed for detection of viral RNA using four multiplex real-time PCR assays which amplify the viral

genome. All PCR assays were run using the Roche LC480 Instrument (Roche Diagnostics, UK). The four assay panels cover the detection of:

1. Influenza A and Influenza B
2. Parainfluenza 1-4, Adenovirus
3. Human Metapneumovirus (hMPV), Respiratory Syncytial Virus (RSV), Human Rhinovirus (hRV)
4. Coronaviruses OC43, NL63, 229E, HKU1

3 Hand to Nose Transmission of *Streptococcus pneumoniae* in Healthy Participants – Pilot Study (Hand to Nose)

This chapter was accepted for publication in a peer review journal (European Respiratory Journal) on 1st August 2018 and is currently in press.

3.1 Introduction

In pneumococcal epidemiology, transmission routes of *S. pneumoniae* between individuals remains poorly understood. It is hypothesised that transmission occurs due to inhalation of contaminated respiratory droplets [8]. It is unclear if the hands or fomites (inanimate object capable of carrying infectious organisms) have a role in pneumococcal transmission. If direct transmission is implicated in the spread of pneumococcus the relative contributions of direct and indirect transmission modes to pneumococcal colonisation and disease are unknown. Current understanding of the transmission of this important clinical pathogen is described in Chapter 1 (section 1.3).

The hands have been identified as vehicles for the transmission of respiratory pathogens previously. Multiple studies have identified respiratory viral pathogens on hands and fomites [62 70 72 143 144],with persistence on dry inanimate surfaces ranging from 3 hours for coronavirus and up to 3 months for adenovirus [62]. When evaluating bacterial respiratory pathogens, Gram-negative species such as klebsiella species and *Pseudomonas aeruginosa* have been shown to survive on inanimate surfaces for months [62]. However, others only last for days such as *Haemophilus influenzae* (12 days) [66].

Evidence is limited on the survival of pneumococcus on hands and fomites. One study exposed the hands of 3 adult volunteers, a glass plate and a plastic ball to pneumococcus suspended in two different media (Serum broth [10% horse serum in Brain Heart Infusion Broth, Oxoid] and Mueller-Hinton broth [Oxoid]) [68]. Persistence of pneumococcus on the skin was reported for up to 3 hours post exposure when suspended in Serum Broth, but with substantially lower pneumococcal counts than the initial bacterial stock [68]. Viable pneumococcus was also recovered from a glass plate at 15 hours post contamination. For the plastic ball there was an 85% average loss of pneumococci (266 CFU) at 4 hours but reported ongoing recoverable bacteria 8 hours post contamination.

Many epidemiological studies have examined natural pneumococcal carriage in the nasopharynx and transmission of infection has been described in case studies of outbreaks or epidemics of specific pneumococcal serotypes in communities [54-56]. Healthy carrier transmission is less well understood. Observational studies suggest that person-to-person transmission occurs when in close contact with carriers such as within family groups [52] and could be increased with overcrowding for example during the Hajj [53].

There is emerging evidence from murine models which investigate pneumococcal transmission from host to host. Most of these models have investigated pneumococcal transmission during influenza A co-infection, as this enhances pneumococcal shedding and therefore leads to higher acquisition rates by new hosts [49]. More recently evidence from an infant murine model describing shedding and

transmission during pneumococcal mono-infection has been published [51]. Using this model Zafar et al [51]reported that bacterial shedding was highest in pups intranasally infected with pneumococcus at age four days and that it peaks over the first four days post inoculation. They also reported that transmission was more likely to occur when there was a high ratio of colonisation positive pups to colonisation negative pups [51].

We wanted to explore further possible mechanisms of human-to-human transmission and acquisition of nasopharyngeal pneumococcal colonisation. To do this we have developed our human pneumococcal challenge model to assess the feasibility of self-inoculation as a possible mechanism for pneumococcal transmission.

This chapter addresses the following question: Can the hands be vehicles for direct transmission of pneumococcus from the hands to nostrils leading to experimental pneumococcal colonisation?

3.2 Methods

3.2.1 Study set up

Hand to nose study set up procedures and approvals gained can be found in Chapter 2, section 2.2.

3.2.2 Trial design

Non-specific EHPC trial design information is described in Chapter 2, section2.3.1, section and ethical principles which were considered prior to the study initiation are outlined in section 2.3.2.

The Hand to Nose study involved human participants being experimentally exposed to *Streptococcus pneumoniae* on their hands and asked to facilitate transmission of bacteria to the nasopharynx. Participants were followed up to evaluate if they had acquired nasopharyngeal pneumococcal colonisation using nasal washes at day 2, 6 and 9. There was an assessment of two different transmission methods and the impact of wet bacterial transmission versus transmission following drying.

The dose of pneumococcus used for the exposure procedures was 3.2×10^6 CFU. In a previous EHPC study [139] participants were intranasally inoculated with up to 3.2×10^5 CFU/naris (6.4×10^5 CFU total) with no significant adverse events. Pre-study experiments showed that there was a 1-2 log drop in pneumococcal counts recovered from the hand after exposure when the bacteria were wet and

immediately after drying (data not shown). Therefore, we estimated that we could expose participants' hands to 6.4 x 10^6 CFU and still be confident that it would be safe for participants. However, as this is the first study of this type, we decided to use half this dose (3.2x10^6 CFU) to ensure safety further.

All participants were exposed to serotype 6B *Streptococcus pneumoniae.* The first forty volunteers were randomly allocated, on the day of exposure, to one of four different transmission groups;

1. Sniffing bacterial residue after air-drying of the hands- Group A/ 'dry sniff'
2. Pick/poke nose with finger exposed to wet bacteria residue- Group B/ 'wet poke'
3. Sniffing wet bacterial residue- Group C/ 'wet sniff'
4. Pick/poke nose with finger exposed to dried bacterial residue 'dry poke'

Randomisation was computer-generated and occurred in blocks of 6. An independent co-ordinator from LSTM's Tropical Clinical Trial Unit produced the randomisation schedule. Two members of staff that were not involved in the conduct of the study produced sealed envelopes containing the group allocations. On the day of exposure, a clinical team member opened the envelope in from of the participant and informed them which transmission group they would be in.

Following the results from these first 4 groups, the trial steering committee (TSC), discussed the results and decided which group would be taken on to complete a larger number of participants (see Figure 13).

Figure 13: Study Design Flow Chart

The first 40 participants were randomly allocated, on the day of exposure, to one of the four transmission groups. Rates of colonisation for each of these transmission groups were discussed by the trial steering committee following the completion of the first 40 participants. One group was chosen to take forward to an extended cohort for better precision of estimated rates of colonisation.

3.2.3 Recruitment

Recruitment strategies and where the study was advertised is outlined in Chapter 2, section 2.3.3.

3.2.4 Inclusion/Exclusion criteria

Inclusion and exclusion recruitment criteria can be found in Chapter 2, section 2.3.4. For the Hand to Nose study some additional specific inclusion criteria and exclusion criteria are outlined below.

Table 8: Inclusion and exclusion criteria specific to Hand to Nose study

Inclusion Criteria
• Access to their own mobile telephone **To ensure safety and timely communication.** Also, to ensure the participants can follow the protocol by contacting us daily with temperature and any symptoms during the first 7 days post exposure to pneumococcus
• Capacity to give informed consent **To ensure it is clear to the research ethics committee that we will only be undertaking this research in adults who have capacity to make their own decision about entering the study**
Exclusion Criteria
• Any acute dermatological illness or skin injury affecting the hands or face **To ensure no confounding effects of topical medications or increased propensity to skin infections**
• Natural carriers of pneumococcus as determined at screening visit **To minimise the potentially positive or negative effects on pneumococcal challenge**

3.2.5 Study schedule

Non-specific study schedule details are outlined in Chapter 2, section 2.3.5. In the Hand to Nose study following pneumococcal exposure, participants were followed up for 3 further visits on day 2, 6 and 9 post exposure visits. These follow up visits entailed only a nasal wash sample. See Figure 14 for full appointment schedule for Hand to Nose study.

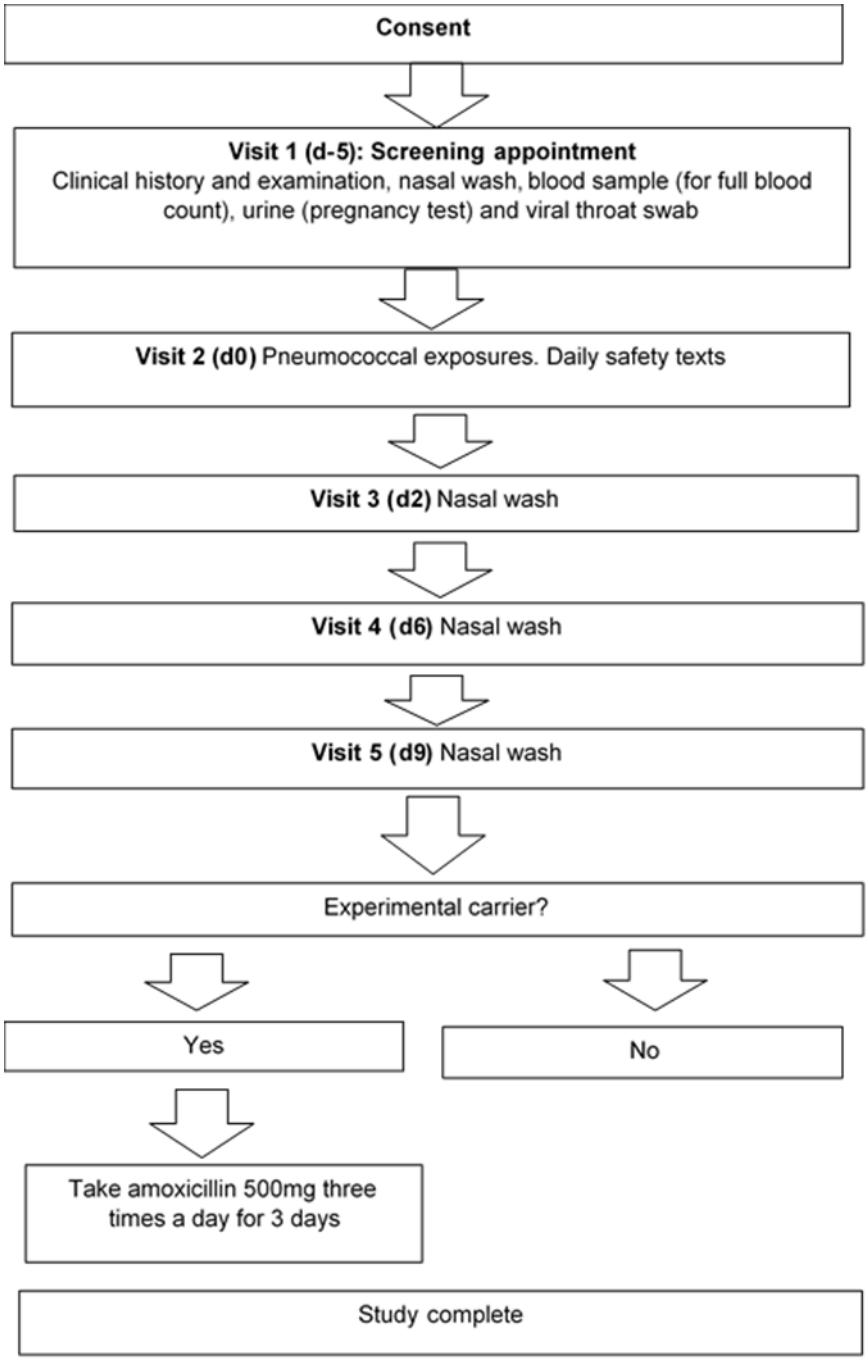

Figure 14: Hand to Nose study appointment schedule
Following participants consent to take part in the study, appointments were arranged for all other clinical visits. The first visit is a screening appointment for baseline research samples and safety check. It also involved a nasal wash to screen for natural colonisation of pneumococcus. The week following this screening appointment participants underwent pneumococcal exposure. All participants were followed up for 9 days after exposure session. Any participant who was still colonised with pneumococcus (6B) at the end of the study was given a three-day course of amoxicillin in an attempt to clear colonisation. Clearance of colonisation was not confirmed with repeat nasal wash following antibiotics.

3.2.6 Participant safety

Details of participant safety procedures, safety monitoring and symptom reporting are detailed in Chapter 2, sections 2.3.6 and 2.3.7.

There is a risk of unplanned pneumococcal environmental spread during the procedure of exposing participant's hands to the pneumococcus and while the participant attempts bacterial transmission into the nostrils. To reduce this risk, we gave the participants full body gowns and eye shields to wear and used a draped area for the exposure procedure followed by the area being decontaminated with anti-bacterial cleaning wipes following each participant.

To mitigate any potential risk of spreading pneumococcus to vulnerable groups in the community, we discussed these risks with our participants and excluded anyone with close physical contact with at risk individuals (children under 5 years of age, immunosuppressed adults, elderly, chronic ill health) during the trial period. We also reduced pneumococcal colonisation burden in all study participants who were still colonised with pneumococcus at the end of the study, by giving them oral amoxicillin 500mg three times daily for 3 days. Clearance of colonisation was not confirmed with repeat nasal wash following antibiotics.

3.2.7 Pneumococcal challenge: hand exposure and transmission

Clinical isolates of serotype 6B BHN418 (GenBank accession number ASHP00000000.1), a gift from Professor P Hermans, Radboud University Nijmegen, were used to create the 'parent 'stock for the Hand to Nose study. See Chapter 2, section 2.4.1 for details about how the 'parent' stock was made and for general aspects of pneumococcal challenge which are common to both studies in this thesis.

For the Hand to Nose study, challenge involved exposing an area of the participant's hand to pneumococcus, followed by the participant attempting to transmit the bacteria into their nose using one of two transmission methods (Figure 15). The pneumococcal stock preparation on the day of challenge and the exposure/transmission process is outlined in Chapter 2, sections 2.3.9 and 2.4.2.

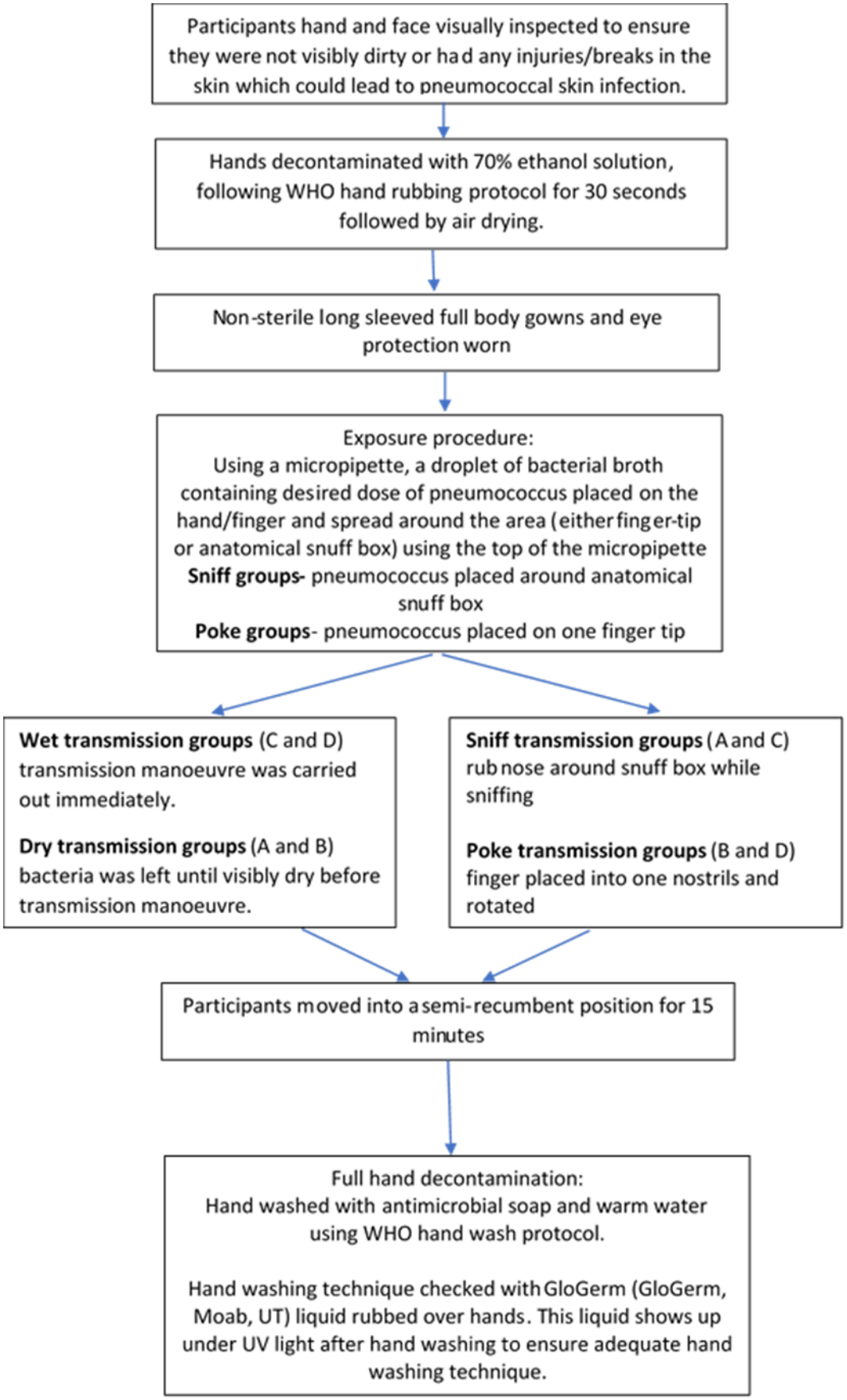

Figure 15: Pneumococcal exposure and transmission process for participants using WHO hand hygiene guidelines [145]

3.2.8 Clinical sampling processes

All participant visits were carried out at the CRU in RLBUHT, UK. Samples obtained from participants include; nasal wash, throat swab, blood sample and urine samples (only women of child bearing age). See Chapter 2, sections 2.3.10-2.3.12 for sample collection process. Participants' full blood count (taken as part of safety procedure) and viral throat swabs were analysed by RLBUHT, clinical laboratories. All other research samples were processed and stored at LSTM.

3.2.9 Sample analysis

Details of nasal wash sample processing can be found in Chapter 2, section 2.4.3. The process for detection of pneumococcal colonisation by culture and by qPCR can be found in Chapter 2 sections 2.4.4 and 2.4.5 respectively. The process used for detection of concurrent upper respiratory tract viral infection can be found in section 2.4.6.

3.2.10 Endpoints and objectives

The primary endpoint was experimental pneumococcal colonisation (detection of 6B in nasal wash) by classical culture methods at any time point (day 2, 6 or 9) following pneumococcal hands to nose transmission. This rate will be expressed as a percentage of participants colonised with pneumococcus over the total number exposed.

The secondary endpoints were (1) Determination of the duration and density of pneumococcal colonisation (2) Rates of pneumococcal nasopharyngeal colonisation following immediate attempted transmission (3) Rates of pneumococcal nasopharyngeal colonisation following attempted transmission following drying of bacteria (4) The detection of pneumococcal colonisation by qPCR at any time point.

3.2.11 Statistical methods

As this was a pilot study, with no previously published data pertaining to pneumococcal hand to nose transmission rates available, we used proportion estimates to ensure the number of participants used in the study was large enough to give a specified degree of precision.

Previous EHPC studies using nasal inoculation of *S. pneumoniae* bacteria found approximately 40% colonisation rates. If our study had the similar rates, 34 people would have given 95% confidence that the rate of colonisation lies within 18% on either side of this rate (22-58% colonisation). However, as this study does not involve nasal inoculation but adds a further step of hand exposure and attempted transmission, this rate is likely to be much lower. If we estimate we will get half the amount of colonisation observed in other studies (20%), then 34 people would give a 95% confidence that the rate of colonisation lies within 14% on either side of this (6-34%).

Taking into consideration the possibility of 10% of participants being natural carriers [12] and the possibility of 10% drop out/exclusion rate (taken from approximate

dropout rates from previous EHPC studies), it was concluded that we would need to recruit a maximum of 80 participants to complete 64 participants. This study was not powered to compare rates of colonisation between the four different transmission methods. The preliminary 40 participants were used to primarily investigate the feasibility of the transmission methods to decide which would be taken to the full cohort.

The Fisher's exact test and the Chi squared test were used to analyse colonisation rates. Graphing and statistical analysis were performed using GraphPad prism (California, USA). All P values were two-tailed and considered significant if $P \leq 0.05$. Differences in density were evaluated using one-way ANOVA test. Spearman's rank correlation coefficient was used to assess the correlation of qPCR and culture densities.

3.3 Results

3.3.1 Screening and recruitment

Between March and June 2017, 76 participants were recruited for the Hand to Nose study, with 63 participants completing all the study visits. 9 participants withdrew pre-screening due to personal reasons, 4 participants attended the screening visit; 2 were excluded due to natural carriage of pneumococcus, 1 had an abnormal full blood count and one withdrew for personal reasons (see Figure 16). All 63 participants were challenged with pneumococcus between April and May 2017, with the final participant's final visit on 1st June 2017.

The mean average age of volunteers was 22.6 years (range 18-45, median 21) and the male:female ratio was 23:40. When evaluating the first 40 participants who were randomly allocated to 4 different transmission groups, the mean average ages were similar (wet sniff: 23 [range19-28]; wet poke: 21.4 [range 20-24]; dry sniff: 22.5 [range 19-27]; dry poke: 23.3 [range 19-44]). There were no statistically differences when comparing gender ratios of all 4 groups using Chi-squared (P=0.19) or when comparing the wet and dry groups using the Fisher's exact test (P=0.08) (male:female ratios: wet sniff 5:5; wet poke 4:6; dry sniff 1:9; dry poke 2:8).

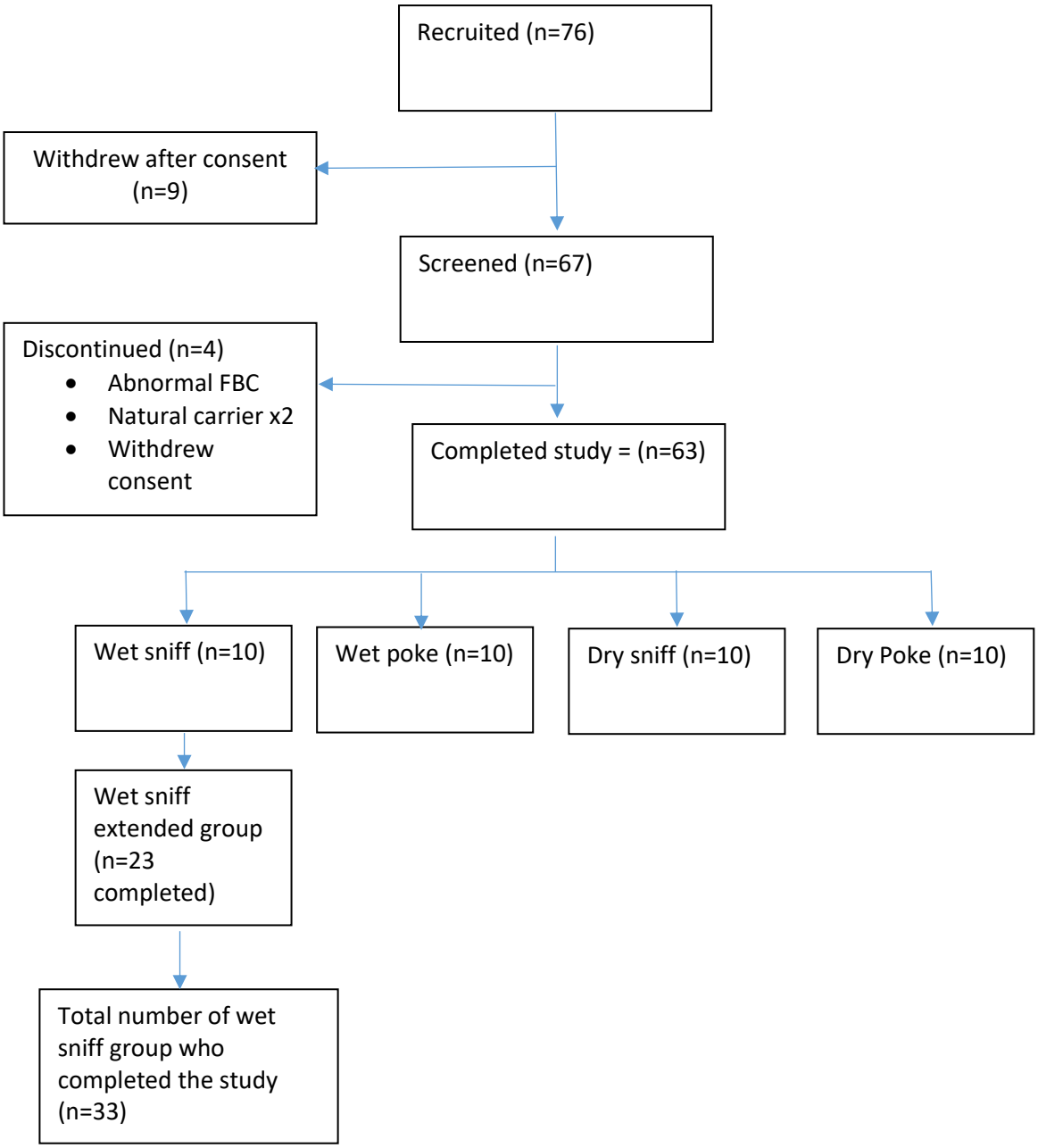

Figure 16: Consort flow diagram for Hand to Nose study

3.3.2 Inoculum doses were compliant with protocol

All inoculum doses were compliant with the protocol as they fell within half and did not exceed double the target amount ($3.2x10^6$ CFU; see Table 9). The average

pneumococcal dose participants were exposed to in each of the 4 different transmission groups were similar (wet sniff $3.4x10^6$ CFU; wet poke $3.3x10^6$ CFU; dry sniff $3.3x10^6$ CFU; dry poke $3.4x10^6$ CFU). To complete the full cohort, a total of 15 different inoculation sessions were conducted, on 5 different days (See Table 9). The first 3 challenge days, in which the first 40 participants were challenged, included exposure with the four different transmission methods. For the last 2 challenge days only the 'wet sniff' transmission method was used to complete the full cohort.

Table 9: Average pneumococcal challenge dose for all participants groups.

Date of Challenge	Number of participants	Number of challenge procedures	Average dose, CFU (SD)
24/04/2017	22	4	$3.50\ x10^6$ ($4.46\ x10^5$)
02/05/2017	15	3	$3.24\ x10^6$ ($2.00\ x10^5$)
03/05/2017	3	1	$2.78\ x10^6$ (0)
16/05/2017	12	4	$3.33\ x10^6$ ($1.00\ x10^5$)
23/05/2017	11	3	$3.15\ x10^6$ ($2.23\ x10^5$)

3.3.3 Hands were vectors for transmission of pneumococcus into the nasopharynx- classical culture results

Initially forty participants were allocated to four different transmission groups. Eight individuals (20%) were found to be colonised with 6B serotype pneumococcus at follow up visits by culture, with highest rates found in the 'wet poke' (4/10, 40%), and 'wet sniff' (3/10, 30%) groups. Drying of the bacteria on the skin before "sniff" or "poke" led to 1/10 (10%) and 0/10 participants becoming colonised respectively

(see Figure 17). The difference in colonisation rates between groups was not significant (first 40 participants P=0.10; Chi-square test).

Acquisition of colonisation following attempted transmission while the bacteria were wet was significantly higher than attempted transmission following drying of the bacteria (7/20 vs. 1/20 respectively, two-tailed P=0.04, Fisher's exact test). The only group with no acquisition of colonisation following attempted transmission was the 'dry poke' group. No difference was observed when comparing colonisation rates in the sniff groups versus poking groups (4/20 vs. 4/20 respectively, two-tailed P=1.00, Fisher's exact test).

The 'wet sniff' group was expanded to improve precision-estimates of rates. A total of 33 participants completed the study in this group. Of these, 6 participants became colonised (18.2%, 95% CI, 8.6%-34.4%). This was a lower overall colonisation rate than we expected following a colonisation rate of 30% in the initial 10 participants exposed. However, this fluctuation in colonisation rates week on week is not uncommonly seen in previous EHPC studies. For serotype 6B we have often had colonisation rates per group range from 10%-70% but with the overall study colonisation rate consistently between 45-55%. Overall experimental pneumococcal colonisation was found in 11/63 participants (17.5%, 95% CI, 10.0%-28.6%) at any time point, following hand to nose transmission of the pathogen.

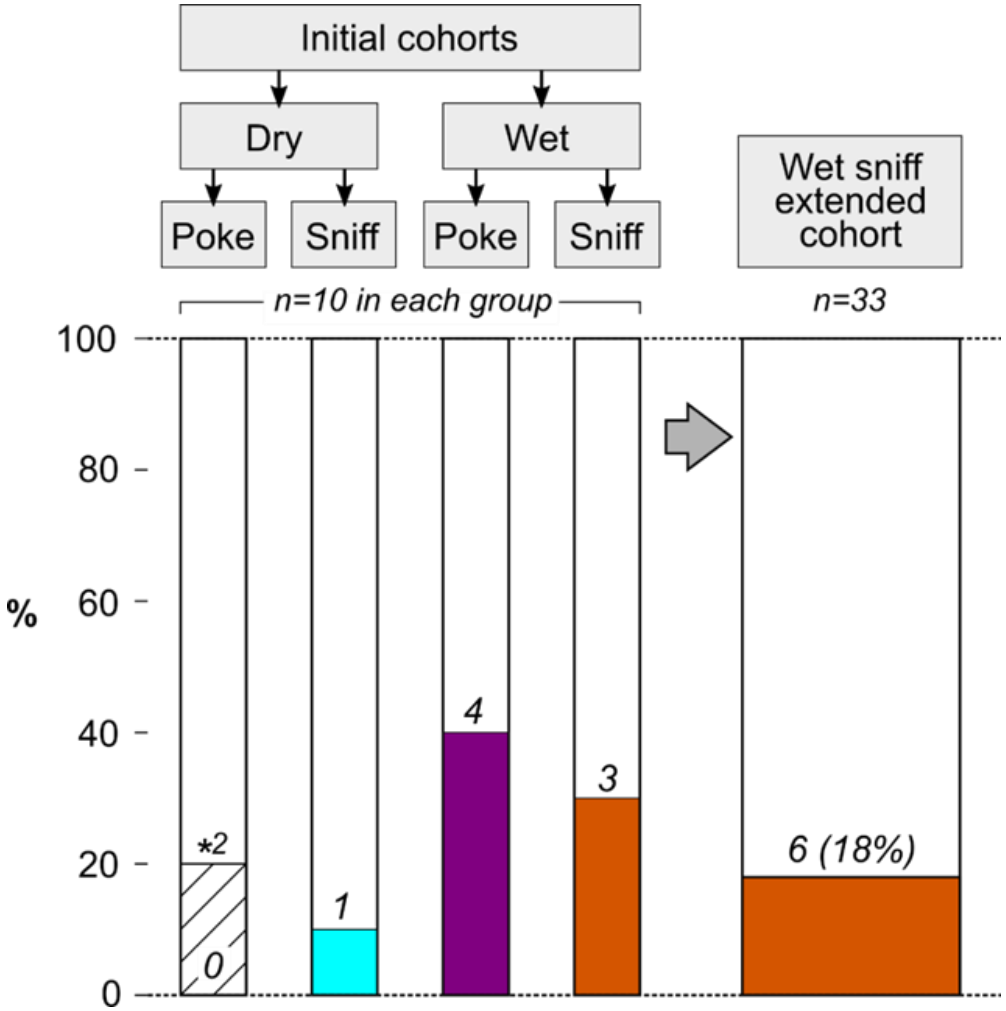

Figure 17: Colonisation rates following classical culture of nasal wash samples at any time point after exposure in each transmission group.

*No experimental carriers of serotype 6b were found in the dry poke group but 2 participants became natural carriers of pneumococci after baseline. 10 participants were exposed to pneumococcus in each of the 4 different transmission groups (dry poke, dry sniff, wet poke, wet sniff). Following discussion by the trial steering committee it was decided that the wet sniff group should be expanded to a total of 33 participants (the end graph includes participants from the initial cohort and extended cohort; 10 from initial cohort and 23 from extended). Percentage represents the number of participants in the group that were found to be experimentally colonised with pneumococcus at any time point following exposure.

3.3.4 Natural Pneumococcal Colonisation

Overall the natural colonisation rate in our cohort was 6% (4/67). Two participants were excluded at baseline due to natural carriage and two participants became natural carriers during the study (**Error! Reference source not found.**). No participants were co-colonised with 6B and the naturally acquired serotype when assessed using culture. However, 6A/B specific qPCR showed that one participant was co-colonised with naturally acquired serotype 19 and 6B at both day 2 and 6. No baseline nasal wash samples were positive for lytA qPCR confirming that all participants were negative for carriage at time of exposure.

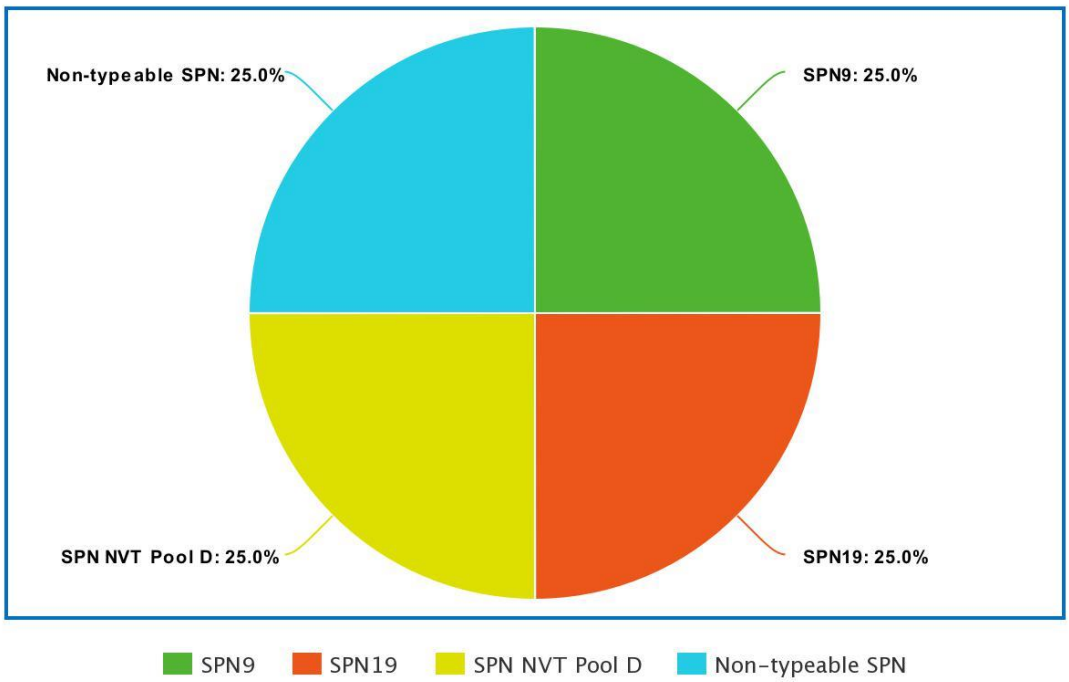

Figure 18: Natural pneumococcal colonisation serotypes as found by culture in 4 individuals
NVT: non-vaccine type, serotypes identified by Statens Serum latex kit; SPN: *Streptococcus pneumoniae*. Pool D NVT serotypes: 16(16F, 16A), 36 and 37.

3.3.5 Pneumococcal colonisation densities were similar in each transmission group- classical culture results

Median post-exposure colonisation densities (CFU/ml of nasal wash) of the 'wet sniff', 'wet poke' and 'dry sniff' groups were $5.6x10^{1}$ (range $4.3x10^{-1}$-$3.7x10^{6}$), 4.72 (range $4.5x10^{-1}$-$1.25x10^{2}$) and 2.42 (range 1.16-9.55) respectively. See Figure 19for densities at each time point.

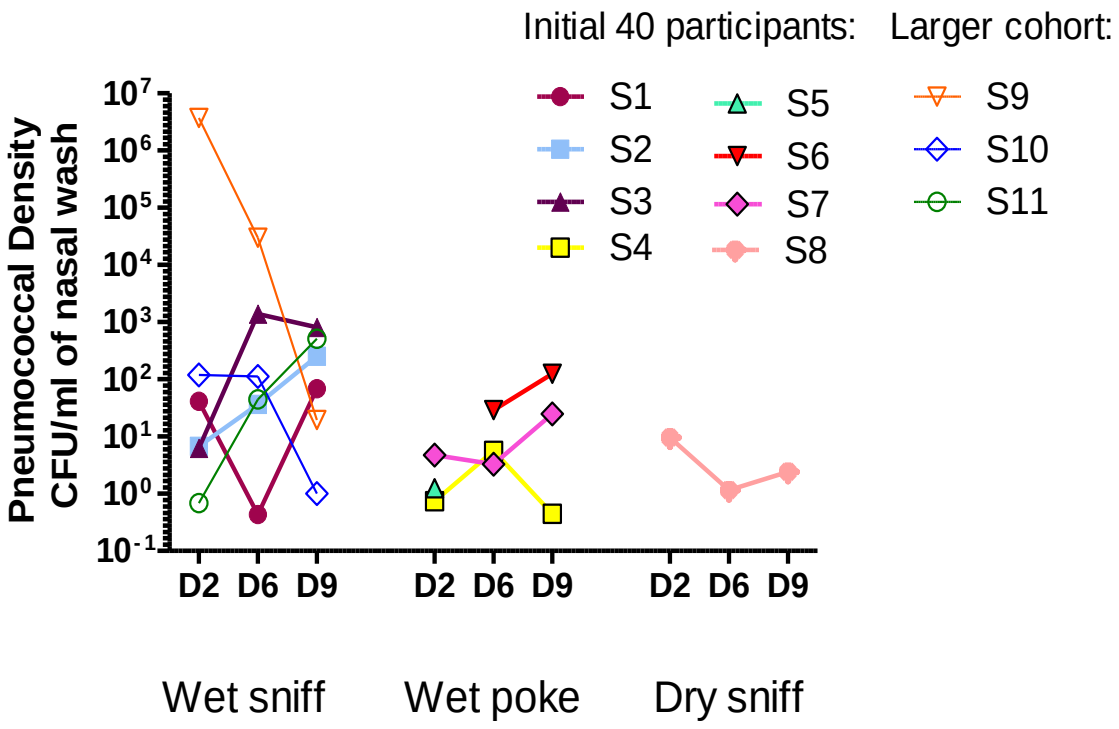

Figure 19: Pneumococcal colonisation densities, using classical culture method of pneumococcal identification, at each time point post exposure to pneumococcus.
Results from the first 40 participants/subjects are in filled points and participants positive from the second part of the wet sniff group are in open points. Nasal washes were performed on day 2, 6 and 9 post exposure to determine carriage status and density. Density is reported as CFU/ml of nasal wash returned.

Differences in densities between groups, when comparing the colonisation positive participants from the initial cohort (n=8), were not statistically different using a one-way ANOVA test at any time point. Overall density over time for each participant was calculated using area under the curve (AUC). Comparison of AUC of the different transmission groups was also not statistically significantly different.

When the wet sniff group was extended, the AUC appeared statistically significantly different between the wet sniff, wet poke and dry sniff groups (one-way ANOVA test) (Table 10). However, when applying Dunn's multiple comparison test, no significant differences were observed (Table 11).

Table 10: Comparison of transmission groups (full cohort) colonisation densities from culture. One-way ANOVA test with a P value of <0.05 considered significant. All densities log transformed prior to analysis.

	Wet Sniff Mean Densities, Log transformed (Standard error) N=6 total	Wet Poke Mean Densities, Log transformed (Standard error) N=4 total	Dry Sniff Mean Densities, Log transformed N=1 total	P value (one-way ANOVA test)
D2	1.96 (0.97) N=6	0.21 (0.24) N=3	0.98 N=1	0.23
D6	2.09 (0.67) N=6	0.91 (0.28) N=3	0.06 N=1	0.20
D9	1.86 (0.44) N=6	1.05 (0.73) N=3	0.38 N=1	0.41
AUC	14.57 (3.52) N=6	4.62 (1.62) N=4	4.02 N=1	0.04*

Table 11: Dunn's multiple comparison test results from AUC densities from culture of different transmission groups

Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?
Wet Sniff AUC vs Wet Poke AUC	5.083	No
Wet Sniff AUC vs Dry Sniff AUC	5.333	No
Wet Poke AUC vs Dry Sniff AUC	0.250	No

3.3.6 lytA qPCR detected pneumococcal DNA in more samples than classical culture

252 nasal wash samples were collected from the 63 participants who completed the study; all were tested for the presence of *S. pneumoniae* by microbiological culture and retrospectively using lytA qPCR. The proportion of samples positive for colonisation by qPCR was significantly more than the proportion of samples positive for carriage by culture (23.8% vs. 13.1%, P<0.0001, Fisher's exact test) (Table 12). There were no samples where pneumococcus was detected by culture but not lytA qPCR but in 10.7% of samples pneumococcus was only detected by lytA qPCR.

Table 12: Comparison of microbiological culture and qPCR in detection of pneumococcus in nasal washes.

**3 samples culture positive for natural carriage of pneumococcus after baseline included*

	Culture Positive (%)	Culture Negative (%)	Total
qPCR Positive (%)	33 * (13.1%)	27 (10.7%)	60 (23.8%)
qPCR Negative (%)	0 (0%)	192 (76.2%)	192 (76.2%)
Total	33* (13.1%)	219 (86.9%)	252(100%)

3.3.7 qPCR detected significantly higher rates of pneumococcal colonisation compared to classical culture

Using molecular methods (lytA qPCR) resulted in higher colonisation detection rates compared with culture (35/63 [56%] vs 13/63 [23%] respectively, P=0.0001, Fisher's exact test) (Table 13).

Table 13: Comparison of microbiological culture and qPCR in detection of participants colonised with pneumococcus.
* 2 participants who were natural carriers of pneumococcus after baseline included

	Culture Positive (%)	Culture Negative (%)	Total
qPCR Positive (%)	13* (20.6%)	20 (31.7%)	33 (52.4%)
qPCR Negative (%)	0 (0%)	30 (47.6%)	30 (47.6%)
Total	13* (20.6%)	50 (79.4%)	63 (100%)

qPCR detected a larger proportion of colonisation positive participants in every transmission group and at every time point except for day 9 in the dry sniff group compared to culture (Figure 20). The difference was most apparent in dry poke group, where 7/10 (70%) volunteers were experimentally colonised at any time point, with detectable pneumococcal DNA in nasal washes at 9 days after exposure.

Of the 24 participants who were only determined as experimental colonisers of 6B using qPCR, 5 participants were colonised for more than one time point and 8 further participants were found to be colonisers at day 6 or day 9 nasal washes.

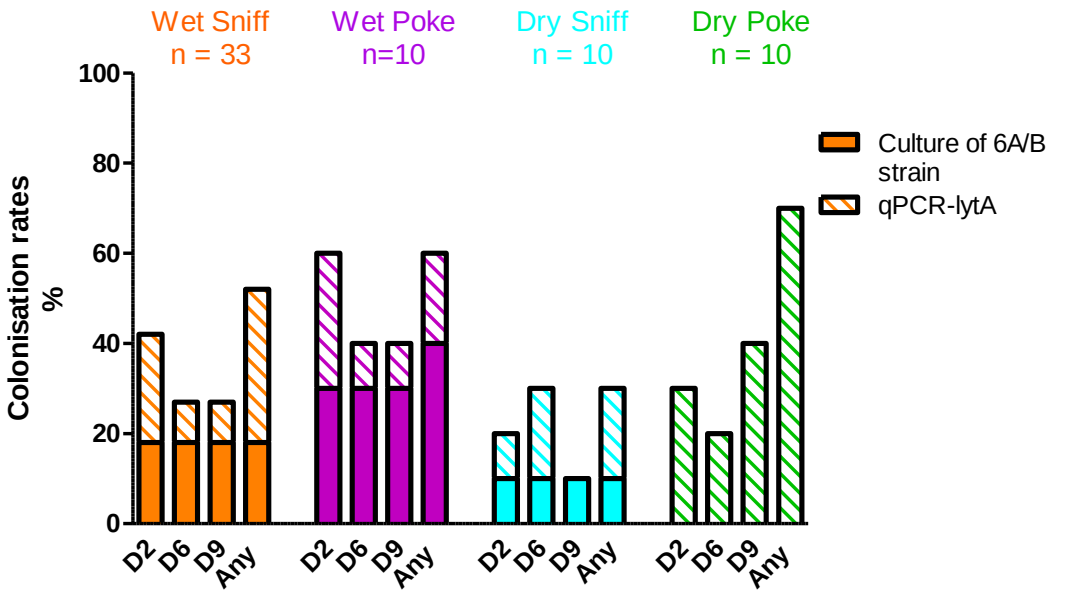

Figure 20: Comparison of culture (6A/B serotype only) and lytA qPCR results for different transmission methods

Nasal washes performed on day 2, 6- and 9-days post exposure to pneumococcus to determine colonisation status. Nasal washes were plated on blood agar plates in real time and read the following day. Those participants with confirmed serotype 6B in nasal washes were deemed colonisation positive. Filled bar charts represent colonisation rates at each time point for each transmission method. At the end of the study all samples were re-run using lytA qPCR method of detecting pneumococcus. Striped bars are the additional participants found to be colonised with this molecular method. No samples were culture positive and qPCR negative for pneumococcus.

3.3.8 All transmission groups had similar densities of colonisation when assessed using qPCR

Differences in densities between groups were not statistically different at any time point or AUC analysis for either the first 40 participants or the full data set (Table 14). Samples which were only positive with qPCR tended to have lower densities compared to samples which were both qPCR and culture positive (Figure 21).

Table 14: Comparison of transmission groups (full cohort) colonisation densities from lytA qPCR.
One-way ANOVA test with a P value of <0.05 were considered significant. All densities were log transformed prior to analysis.

	Wet Sniff Mean Densities, Log transformed (Standard error) N=17 total colonised	Wet Poke Mean Densities, Log transformed (Standard error) N=6 total colonised	Dry Sniff Mean Densities, Log transformed (Standard error) N=3 total colonised	Dry Poke Mean Densities, Log transformed (Standard error) N=7 total colonised	P value (one-way ANOVA test)
Day 2	1.97 (0.39) N=14	1.13 (0.07) N=4	2.41 (0.40) N=2	1.56 (0.26) N=2	0.34
Day 6	2.99 (0.61) N=9	2.09 (0.32) N=6	1.32 (0.72) N=2	1.65 (0.56) N=3	0.42
Day 9	2.64 (0.58) N=9	2.29 (0.41) N=4	2.75 (NA only one positive sample) N=1	1.08 (0.28) N=3	0.49
Area Under Curve	10.9 (2.90) N=17	10.4 (2.45) N=6	7.78 (4.58) N=3	3.74 (1.31) N=7	0.41

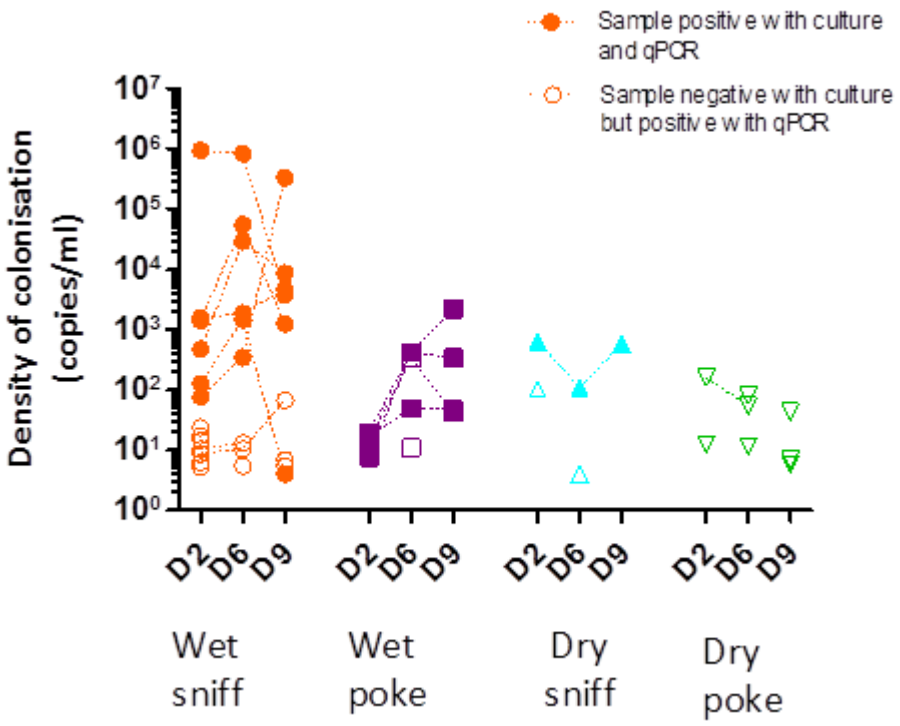

Figure 21: lytA qPCR densities for each transmission group
Nasal washes were performed at days 2, 6 and 9 post pneumococcal exposure. At the end of the study all nasal wash samples underwent DNA extraction and lytA qPCR for detection of pneumococcal DNA. Densities expressed as copies/ml of nasal wash. Graph shows each participant who was found to be colonisation positive following qPCR for each transmission group. Participants who were positive at more than one-time point are linked with a line. Open circles represent those samples which were only found to be positive using qPCR, filled circles are those samples that were positive with both culture and qPCR.

When the difference between pneumococcal detection was stratified by qPCR density it was clear that qPCR detects more carriers colonised at $<10^1$ CFU/ml density than classical culture method (Table 15). However, at qPCR densities 10^3 copies/ml and higher there was 100% concordance between qPCR and culture results.

Table 15: Detection of pneumococci in nasal wash by bacterial culture and qPCR (categorised according to qPCR density)

Density by QPCR (copies/ml)	Number culture positive/number qPCR positive (%)
<10	2/12 (17%)
10^1	8/29 (28%)
10^2	10/13 (77%)
10^3	9/9 (100%)
10^4	1/1 (100%)
10^5	3/3(100%)

3.3.9 Density of colonisation reported by qPCR correlated with density reported by culture

The correlation between pneumococcal density found by microbiological culture and qPCR was determined for 33 samples. There was a positive correlation between density of pneumococcus by culture and qPCR (r_s=0.77, P<0.0001) (Figure 22).

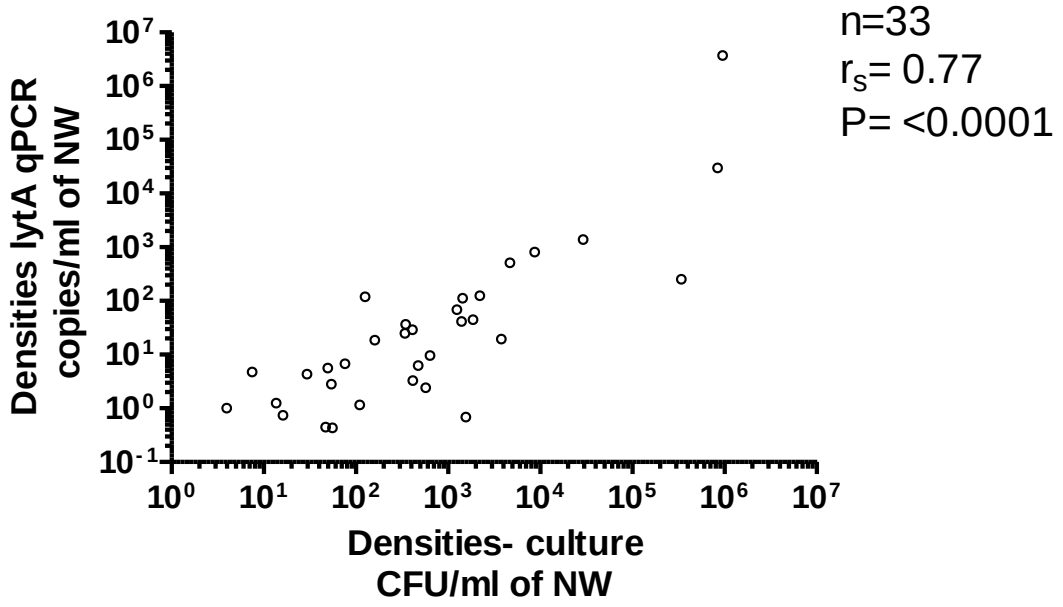

Figure 22: Correlation between bacterial culture and qPCR in quantifying pneumococci in nasal wash.
Quantification of pneumococci by culture and qPCR were positively correlated. Spearman's rank correlation coefficient for samples positive by both qPCR and culture is 0.77. P<0.05 was considered significant.

3.3.10 No difference in length of time colonised was found between transmission groups

Mean duration of carriage was not significantly different between transmission groups by classical microbiology nor by molecular methods of pneumococcal detection (1-way ANOVA culture results, P=0.42, qPCR results, P=0.82). Also, no significant difference was observed when comparing length of time colonised between wet and dry groups (Mann Whitney test culture results, P=1.0, qPCR results, P=0.59) or the poke vs sniff transmission methods (Mann Whitney test culture results, P=0.36, qPCR results, P=0.67) (Table 16).

Table 16: Mean and median days of colonisation for each transmission group, detected by both culture and qPCR methods

Group (number colonised culture and qPCR)	Median – culture (days with interquartile range)	Median-qPCR (days with interquartile range)	Mean- culture (days with standard deviation)	Mean -qPCR (days with standard deviation)
Wet sniff n=33 (culture n=6, qPCR n=17)	9.0 (9.0-9.0)	9.0 (2.0-9.0)	9.0 (0.0)	6.2 (3.3)
Wet poke (culture n=4, qPCR n=6)	9.0 (3.8-9.0)	9.0 (5.0-9.0)	7.3 (3.5)	7.3 (2.9)
Dry sniff (culture n=1, qPCR n=3)	9.0 (9.0-9.0)	6.0 (2.0-9.0)	9.0 (0.0)	5.7 (3.5)
Dry poke (culture n=0, qPCR n=7)	NA	6.0 (2.0-9.0)	NA	6.1 (3.1)
Full wet group (culture n=10, qPCR n=23)	9.0 (9.0-9.0)	9.0 (2.0-9.0)	8.3 (2.2)	6.5 (3.2)
Full dry group (culture n=1, qPCR n=10)	9.0 (9.0-9.0)	6.0 (2.0-9.0)	9.0 (0.0)	6.0 (3.1)
Full sniff group (culture n= 7, qPCR n=20)	9.0 (9.0-9.0)	7.5 (2.0-9.0)	9.0 (0.0)	6.1 (3.3)
Full poke group (culture n= 4, qPCR n=15)	9.0 (5.5-9.0)	9.0 (4.0-9.0)	9.0 (3.1)	6.7 (3.0)

3.3.11 No participants had asymptomatic viral infection at baseline

We wanted to investigate whether the presence of asymptomatic upper respiratory tract (URT) viral infection increases the susceptibility to experimental pneumococcal colonisation following hand to nose transmission of the pathogen. Oropharyngeal swabs were taken between 4-7 days prior to pneumococcal exposure for all participants. At the end of the study we retrospectively analysed swabs from carriage

positive participants and negative controls (matched by date of exposure) by PCR for the presence of URT viruses. 22 samples were analysed in total due to funding constraints. No viral co-infections were found in any samples (tested for Influenza A, Influenza B, Parainfluenza 1-4, Adenovirus, Human Metapneumovirus (hMPV), Respiratory Syncytial Virus (RSV), Human Rhinovirus (hRV), Coronaviruses OC43, NL63, 229E, HKU1).

3.4 Discussion

This novel use of a human challenge model allowed for the study of pneumococcal colonisation in a controlled environment evaluating transmission of pneumococcus from hands into the nose. The study shows, for the first time, that the hands can be a vehicle for transmission of pneumococcus leading to acquisition of nasopharyngeal colonisation.

3.4.1 Success in the model expansion; hands were vehicles for transmission of pneumococcus

We were able to successfully modify the Experimental Human Pneumococcal Challenge model [146] to allow for the study of transmission dynamics from the hands to the nose. Experimental pneumococcal colonisation was established in 17.4% of participants (11/63) when assessed using microbiological culture.

The EHPC model was established using nasal inoculation of the pneumococcal bacteria. Previous EHPC studies in Liverpool showed an overall colonisation rate of 63% using this inoculation method (colonisation rates ranged from 55%-70% depending on dose of bacterial stock inoculated with) [139]. A further study found colonisation acquisition was dose dependent with carriage rates ranging from 10%-60% when inoculated with dose between $1x10^4$-$1.6x10^5$, with carriage rates stabilising around 50% above $4x10^4$ CFU/naris inoculation dose [10]. Lower rates observed in this study may be due to the sniffing transmission method causing movement of the pathogen out of the nasopharynx. During the inoculation process, the inoculum is instilled around the anterior naris and participants are instructed not to sniff or blow. Another possible explanation for lower colonisation rates is death of the pneumococcal bacteria during the drying process on the hands.

Epidemiological studies of nasopharyngeal colonisation rates with matched hand contamination rates support the findings of this study and suggest that hands could be vectors for direct and in-direct transmission of pneumococcus. One study reported simultaneous nasopharyngeal colonisation of pneumococcus and hand contamination rate of 22%, with serotype concordance of 85% (17/20) [74]. A further study found hand contamination with pneumococcus is an indicator for risk of otitis media in populations at risk for tympanic membrane perforation (relative risk 8.4; 95% CI 4.6-15.2) [42].

An association between frequent sharing of drinking glasses or bottles and pneumococcal colonisation has also been reported. A significant correlation between

colonisation prevalence and self-reported frequency of sharing a drinking glass/bottle with 33% of frequent sharing group (sharing a glass/bottle always or usually) colonised with pneumococcus versus 16% of infrequent sharing group (sharing a glass/bottle half of the time, occasionally or never) [44]. The authors suggested that these findings indicate pneumococcal transmission through saliva is a highly plausible route in young adults. However, the possibility that the hands could be vectors for this transmission was not considered or investigated. Following the results of this study, it is plausible that hand contamination and hand-to-nose transmission could be implicated in the higher rates of colonisation observed in the 'frequently sharing group'.

It is widely known that hand washing is an effective means of preventing gastroenteric infections, especially in the developing world and it is a cost effective prevention strategy[147] [148] [70]. The effect of hand washing for preventing acute respiratory tract infections (ARIs) is less well understood. A systematic review of 8 studies considering this question found a consistent impact of hand washing on ARIs with reduction of risk of ARIs by 16% (95% confident interval 11-21%)[70]. This reduction in risk is lower than that observed with the effect of hand washing on gastroenteric infection (47% reduction in risk of diarrhoeal illness) [147], but supports the results of this study that contaminated hands likely aid in the transmission of ARIs. It supports the view that hand washing could be a cost effective and feasible option for helping prevent ARIs and other contagious illnesses.

3.4.2 Factors affecting transmission

When assessing colonisation rates using microbiological culture, results suggested that transmission of pneumococcus resulting in colonisation was more likely when the bacteria were transmitted while still wet. However, interestingly when evaluating colonisation rates using molecular methods (qPCR) this difference disappeared with both the wet and dry groups having approximately 50% colonisation rates.

Drying of the bacteria tended to lead to lower density of colonisation compared to transmission while the bacteria were still wet. No significant differences were observed between the two different transmission methods; sniffing versus poking. Participants who attempted transmission using the sniffing technique tended to have higher colonisation densities. This difference is likely due to the dispersion of the bacteria into both nostrils and to a larger area of the nasopharynx compared to the poking method.

Little is known or understood about the survival of pneumococcus on the hands and the role of contaminated hands and fomites in pneumococcal transmission. One study reported a significant drop in pneumococcal counts at 3 minutes post hand exposure, but did recover some viable bacteria 3 hours post exposure [68]. Interestingly, there was significant inter-volunteer variation in pneumococcal survival, with the greatest difference (4% recovery to 79% recovery rate at 3 minutes) observed between two volunteers exposed to 435 CFU of pneumococcus suspended in Serum broth (10% horse serum in Brain Heart Infusion Broth, Oxoid) [68]. A limitation of this study was that the researchers were unable to assess whether the numbers

of pneumococci recovered were sufficient to lead to colonisation or infection. Our study confirms that pneumococcus is still viable following drying and can cause nasopharyngeal colonisation, albeit at lower densities.

Transmission models using infant mice have previously been able to give some insights into transmission dynamics. Most transmission models have depended on influenza A virus co-infection. This has been shown to greatly enhance the pneumococcal shedding and leads to more acquisition of colonisation in new hosts [48 49]. Direct contact between pups was not needed for transmission and acquisition of colonisation to occur; this suggests that an environmental reservoir may be important for the spread of this pathogen [50]. When evaluating *Streptococcus pneumoniae* mono-infection similar to all participants in this human challenge study, one murine model reported bacterial shedding was highest over the first 4 days post challenge [50]. This suggests that host-to-host transmission may peak immediately following acquisition of pneumococcal colonisation. Epidemiological studies support this. A study investigating spread of pneumococcus within families showed rapid spread between family members if a new serotype was introduced to the family with 17/25 transmission episodes took place within 2 weeks of a new serotype entering the household [52].

Transmission in the same murine model was found to be increased when there was a high ratio of colonised pups to uncolonised pups in a litter [50]. This supports previous evidence to suggest close contact increases in transmission of pneumococcus. Pneumococcal outbreaks have been reported in nursing homes [58] and prisons [60] and

colonisation rates were found to be significant higher at the end of the Hajj where close contact and overcrowding is common [53].

Host and pathogen factors are also likely to impact transmission and the potential for the acquisition of pneumococcal colonisation. Murine models have shown that transmission is increased with high levels of bacterial shedding [48-50]. Shedding is increased with acute inflammation in the nasopharynx and is also observed with co-infection with influenza A virus [48-50]. It has also been shown that nasopharyngeal inflammation in contact mice lowers their threshold for acquisition of pneumococcal colonisation [48]. High bacterial load in index mice and capsular type were also found to affect transmission [48] [69]. One study used neutrophil depletion in index mice (experimentally colonised with pneumococcus) to investigate this. They found a small but significant increase in pneumococcal nasopharyngeal load can increase transmission [48]. A further study showed that capsular type is correlated with transmission rates; capsule types which are better at escaping mucus entrapment show increased transmission [69].

3.4.3 qPCR detected a higher rate of pneumococcal colonisation compared with culture

The proportion of nasal wash samples positive for colonisation by qPCR was significantly higher than the number detected by culture. This is in keeping with a previous EHPC study which also found significantly higher colonisation rates using qPCR (42.6% vs 27.5%, P<0.0001)[149].

Culture is considered the gold standard method for detection of upper respiratory colonisation of pneumococcus and is recommended by the WHO Pneumococcal Carriage Working Group [150]. The main advantage of culture is that further tests can determine serotype of pneumococcus recovered. Molecular methods of pneumococcal detection are increasingly being used either to complement culture or on their own to detect pneumococcal DNA. The WHO Pneumococcal Carriage Working Group state that there is no current gold standard molecular method of pneumococcal detection; they support the lytA PCR assay as widely used and species specific. The specificity of molecular methods to detect *S. pneumoniae* have been questioned due to similarities in gene profile of other streptococcal species [151-154]. A study looking at pneumococcus in cerebrospinal fluid assessed specificity of lytA qPCR and reported a specificity of 70% [155]. Many studies have showed that qPCR is more sensitive at detecting colonisation compared to culture [155-157], with increased sensitivity shown when an enrichment step was used [158]. However, when the density of pneumococcal carriage is of interest, as in our study, this extra step cannot be utilised. The most thorough study methodology would utilise both of these methods; an initial qPCR could be run with the raw samples on which density could be measured then a second qPCR could be run following enrichment.

The main concern about using qPCR is that this technique cannot confirm the viability of pneumococcal DNA detected; a positive PCR result which is culture-negative could represent either live bacteria causing low-density colonisation or non-viable/dead pneumococcal debris. It has been suggested that using an enrichment step in the qPCR method can increase the sensitivity of the method by reducing the impact of

pneumococcal DNA signal originating from possible non-viable bacteria [159]. A previous study supports our findings; they reported significantly higher rates of pneumococcus in saliva using qPCR compared to culture (44/50 [88%] vs. 2/50 [4%] respectively) [39]. In this study, higher quantities of pneumococcal-specific genes were detected in culture-enriched saliva samples. This study also used sham-inoculated samples with pneumococcal DNA. These samples were found to be negative with lytA qPCR which supports the hypothesis that qPCR detects low density colonisation of live bacteria rather than non-viable bacterial DNA [39]. A further study reported that pneumococci were recovered following re-culturing from the majority of samples that were qPCR positive and initially culture negative, further supporting the validity of the method [159].

The results of this study support the hypothesis that qPCR can detect subclinical or low-density colonisation rather than detecting non-viable pneumococcal debris. In 20% of new qPCR colonisers pneumococcus was found at more than one-time point. In addition, 33% were found to be colonisers at day 6 or day 9 nasal washes. It is unlikely that non-viable or dead bacterial DNA would be still in the nasopharynx >6days following initial exposure or be detectable at multiple time points, supporting that qPCR likely detects low density colonisation. Using a novel home sampling method, the EHPC team have recently examined bacterial movement after experimental pneumococcal challenge [160]. In this study, the kinetics of pneumococcal colonisation or clearance during the first 48 hours following challenge by using self-collected saliva and nasal lining fluid samples [160]. The study showed that at 48 hours less than one fifth of non-colonised participants had detectable levels of

pneumococcus from nasal lining fluid samples (4/21 (19%) [160]. The results suggest two distinct profiles of those protected against colonisation;

1. Saliva clearers; these participants demonstrated a fast-initial movement of pneumococcus into the bacteria. With 40% having complete clearance of pneumococcus from the nose by 8 hours [160].
2. Nasal clearers; these participants show a rapid and strong neutrophil baseline activity [160].

This study supports the hypothesis that dead bacteria do not stay in the nasopharynx for prolonged periods of time, with the majority of clearance happening within 24 hours of exposure or inoculation.

3.4.4 Culture and qPCR methods used together improved sensitivity of pneumococcal detection

Our data suggest that using both culture and qPCR to determine pneumococcal colonisation is beneficial. By using both methods the chance of missing pneumococcal colonisation is likely to be reduced. Higher colonisation rates were found using qPCR, but pneumococcal densities measured with both methods were positively correlated. All samples that were culture-positive for pneumococcus were also positive for lytA in qPCR.

We found that culture positivity rate decreased with decreasing densities measured by qPCR, suggesting that qPCR may be more appropriate for detecting low density colonisation episodes. All the 27 samples that were culture negative and qPCR positive had qPCR densities below 10^3 copies/ml. It has previously been shown that culture detected fewer carriers of pneumococcus and *S. aureus* and *H. influenzae* at bacterial densities of $<10^5$ CFU/ml (measured by qPCR) (P<0.0001) [157].

qPCR has also been shown to be superior at detecting multiple pneumococcal serotypes at low carriage densities. One study found 28.7% of participants were co-colonised with multiple pneumococcal serotypes when using qPCR and only 4.5% of these were found to be co-colonised using only culture (P<0.001) [156]. The majority of additional serotypes only detected by qPCR had a density of $<10^4$ CFU/ml [156]. This correlates with the findings of this study. We found that one participant who was determined to be colonised with only serotype 19 using culture, was co-colonised with 6B using molecular methods of detection.

3.4.5 Asymptomatic viral carriage and acquisition of colonisation

We were unable to investigate the relationship between colonisation acquisition and concurrent viral infection due to the absence of viral infection in our participants. The lack of any concurrent viral infection is likely due to the season in which the study was carried out (Spring; April-May)[161]. Previous EHPC studies, conducted during winter months, found that asymptomatic viral co-infection increased odds of experimental pneumococcal colonisation (15/20, 75% virus positive volunteers became colonised vs. 37/81, 46% virus-negative, P=0.02) [162]. In addition, a recent

randomised control trial investigated the effect of viral upper respiratory tract infections in pneumococcal colonisation using the live attenuated influenza vaccine (LAIV) as a surrogate [45]. No change in prevalence of colonisation was observed but there was evidence of increasing density following vaccination [45].

Results from murine models have been able to show that viral co-infection can increase transmission and colonisation acquisition. One study inoculated index mice with pneumococcus and 8 days later inoculated index and contact mice with Influenza A; 47% of the contact mice acquired colonisation at day 14. In contrast, there were no colonisation episodes detected in any contact mice in the control group (no influenza inoculation) [49]. Epidemiological studies have also found that viral co-infection, such as rhinovirus, facilitate acquisition and transmission of pneumococcus between individuals [163].

Our ability to prevent pneumococcal transmission is limited by the lack of understanding about transmission dynamics of pneumococcus and which factors promote acquisition of nasopharyngeal colonisation. We have shown that an experimental human carriage model can be used to investigate modes of transmission. The study shows, for the first time, that the hands can be a vehicle for transmission of pneumococcus and lead to acquisition of nasopharyngeal colonisation. It also shows that pneumococcus continues to be viable following drying. The data suggest that using both culture and qPCR methods for pneumococcal detection is important to ensure that low density colonisation

episodes are not missed. This is important for further transmission studies where carriage density may be an endpoint.

4 The Effect of Different Serotypes of Pneumococcus on Colonisation in Healthy Participants (New Serotypes)

4.1 Introduction

Epidemiological studies have shown that rates of pneumococcal colonisation and disease are dependent on multiple factors. Many of these are host specific including age, geographic area, socio-economic factors and viral or bacterial co-infection [14 164]. The host's innate and adaptive immune responses have also been shown to effect rates of colonisation and disease. Mucosal and systemic antibodies to pneumococcal capsular polysaccharides (IgG antibodies) are thought to play a role in pneumococcal immunity but the degree of protection against pneumococcal colonisation and disease is unclear.

Current evidence suggests different serotypes may induce different sizes of IgG antibody responses to the pneumococcal capsular polysaccharide. During an outbreak of pneumococcal pneumonia caused by serotype 1 at a military training camp, 27.8% of the men who did not develop pneumonia had serotype-specific IgG antibodies detected in sera (systemic blood) compared to 3.6% of controls (3/83, controls used were a comparable population of soldiers at a training base with no outbreak of pneumococcal pneumonia) [165]. A later study showed that for some serotypes (9V, 14, 18C, 19F and 23F) a colonisation episode can lead to a significant increase in levels of serotype-specific anticapsular IgG [12]. However, serotype 6B failed to induce the same immune response [12]. For serotype 14, higher levels of anticapsular IgG at the beginning of the study was also associated with reduced chance of colonisation during the study [12]. In contrast, in this study antibody responses to pneumococcal proteins (PsaA, PsaA and Ply) did not correlate with

protection against colonisation. The role of antibodies against the polysaccharide capsule and towards proteins in the protection against pneumococcus remain unclear. The data suggest that these responses may vary depending on serotype. However, more information is needed before this conclusion is made.

Pneumococcal serotype has also been shown to play a major role in acquisition of colonisation or disease. There are over 90 serotypes of *Streptococcus pneumoniae*. Serotypes are defined by biologically different polysaccharide capsules. Some serotypes are more invasive and cause significant disease whereas others mainly cause colonisation episodes and are rarely recovered in disease cases [133]. A recent pneumococcal colonisation surveillance study in the UK showed that the most prevalent serotypes causing colonisation are 15B/C, 11A, 23B and 10A in 2015/2016southern). In addition, serotypes 8, 12F, 3 and 9N caused approximately 50% of all invasive pneumococcal disease cases in England/Wales during 2016/2017 [166].

Capsular polysaccharides are understood to play a key part in bacterial physiology and are vital in determining the host's immune response to the pneumococcus. Due to differences in the capsular polysaccharides between serotypes, capsule structure has been implicated in the varying clearance and acquisition rates observed between serotypes [167 168]. Specific characteristics of the capsular polysaccharide which affect this process are yet to be fully understood. In addition to different capsular polysaccharide, multiple other proteins have been suggested to be important in the

colonisation process, such as PspC and ChoP [167]. The relative contribution of these factors is also unknown [167].

Better understanding of the dynamics of colonisation and systemic immunity for different pneumococcal serotypes is vital. This study expanded the current EHPC model by re-testing a modified 23F serotype and for the first time tested a non-vaccine serotype 15B. Investigating the varying propensity of pneumococcal serotypes to establish nasopharyngeal colonisation will allow for better understanding of transmission dynamics and disease.

Serotype 6B is currently used in the EHPC model. This serotype consistently colonises 40-50% of participants. The model is currently limited in generalisability, only having one serotype that colonised consistently at a high rate, which is already covered by the current PCV (serotype 6B). Expanding the model to include non-vaccine serotypes is important for vaccine testing. As colonisation is a likely a pre-requisite for invasive infection, it could be used as a surrogate marker of disease risk. Protection against colonisation therefore could be used in a vaccine trial to predict the protective effect against invasive pneumococcal disease [101]. The model has previously been used to test the effectiveness of PCV13. A sample size of 100 was needed in the study to give power to detect 50% reduction in colonisation rates compared to control [169]. New serotypes that are able to colonise at similar or higher rates than 6B in this EHPC model would allow for further vaccine testing trials.

To develop a reproducible model of colonisation with smallest numbers necessary, we examined colonisation rates at increasing doses until a colonisation rate of greater than 40% was achieved. Following this, to improve the precision of our estimates of colonisation potential, one dose was tested in a larger number of participants.

This chapter addresses the following question: What is the capacity of 23F and 15B serotypes to cause experimental pneumococcal colonisation in healthy adults?

4.2 Methods

4.2.1 Study set up

New Serotypes study set up procedures and approvals obtained can be found in Chapter 2, section 2.2.

4.2.2 Trial design

Non-specific EHPC trial design information is described in Chapter 2, section 2.3.1 and ethical principles which were considered prior to the study initiation are outlined in section 2.3.2.

The New Serotypes study experimentally exposed human participants to *Streptococcus pneumoniae*:

Dose-ranging:

- **Group 1:** participants were entered into cohorts which were all inoculated with the same dose of serotype 23F (P1121). Successive cohorts had increasing does of the serotype inoculated if desired colonisation rates were not obtained (see Figure 23;n = 33-67).
- **Group 2:** participants were entered into cohorts which were all inoculated with the same dose of serotype 15B (non-vaccine serotype). Successive

cohorts had increasing does of the serotype inoculated if desired colonisation rates were not obtained (see Figure 23;n = 33-67).

- **Extended Cohort:** To enhance the precision of the estimated colonisation rate, a dose and serotype that lead to more than 40% of carriage in the dose ranging part of the study was tested in a larger group of volunteers.

We hypothesised a dose-dependent relationship in probability of colonisation. We used the dose escalation method to test this hypothesis, to estimate the optimum inoculation dose for these new serotypes and to minimise unnecessary exposure of volunteers. Also, to ensure safety of participants, by inoculating small groups we monitored for any adverse events before continuing to higher doses and to larger groups.

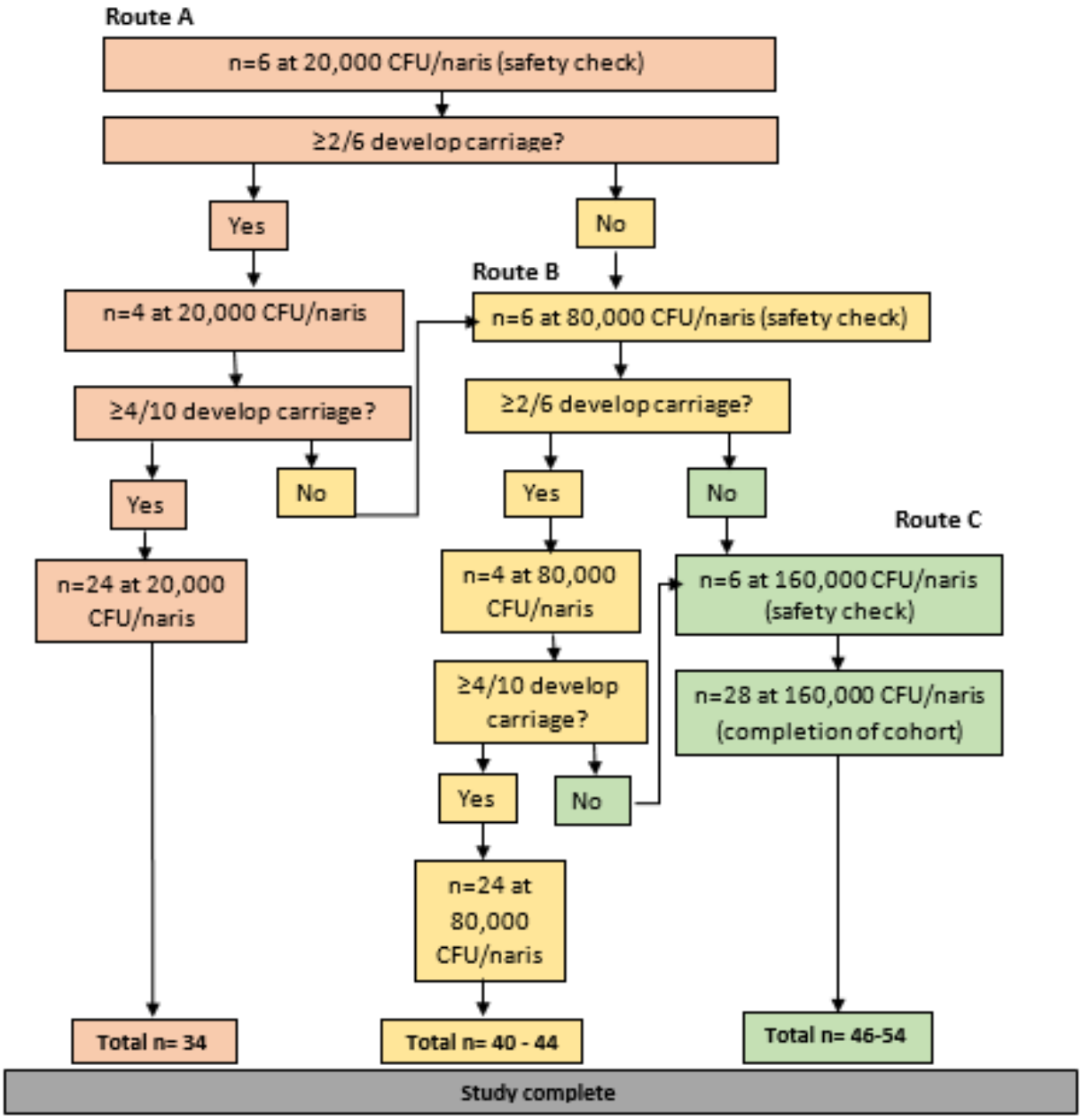

Figure 23: Dose escalation study design for New Serotypes study.
Used for both 15B and 23F serotypes; methodology adapted from Waddington et al [170].

4.2.3 Trial procedures

General details of trial procedures common to both studies outlined in this thesis are described in Chapter 2 Sections pertaining to different trial procedures are outlined below in Table 17.

Table 17: Generic trial procedures outlined in Chapter 2: Methods.

Trial procedure	Section
Recruitment strategies	Section 2.3.3
Prevention of over-volunteering process	Section 2.3.3
Inclusion and exclusion criteria	Section 2.3.4
Study schedule	Section 2.3.5
Participant safety procedures	Sections 2.3.6, 2.3.7 and 2.3.8

In the New Serotypes study following pneumococcal inoculation, participants were followed up for 3 further visits on day 2, 7 and 14 post inoculation. These follow up visits involved a nasal wash sample at each visit and bloods for all participants at day 14. See Figure 24 for full appointment schedule.

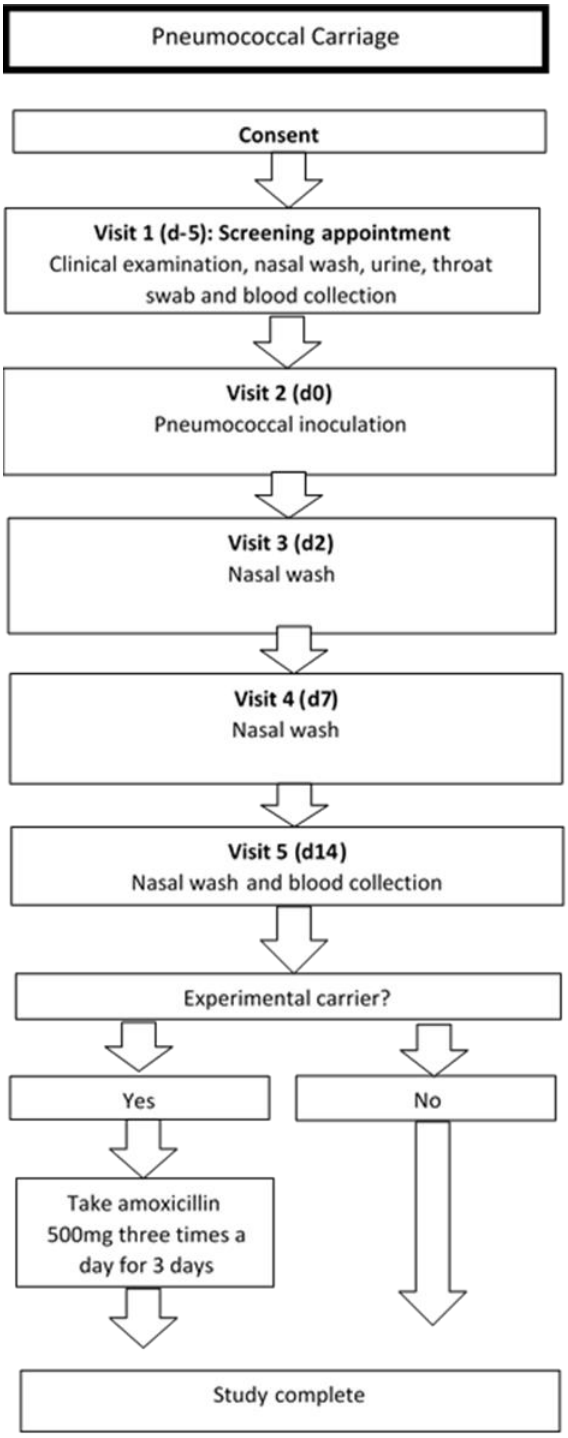

Figure 24: Flow chart of New Serotypes study participant appointments
Following participants' consent to take part in the study, appointments were arranged for all other clinical visits. The first visit is a screening appointment for baseline research samples and safety check. The week following this screening appointment, participants underwent pneumococcal exposure. All participants were followed up for 14 days after exposure session. Any participants who were still colonised with pneumococcus (6B) at the end of the study were given a three-day course of amoxicillin in an attempt to clear colonisation. Longer follow up was decided for this study compared to the Hand to Nose study because we were investigating immunological responses. Previous EHPC studies found that serotype 6B induced significant immune response at day 14 post inoculation[10 171].

4.2.1 Symptom reporting

To examine whether experimental colonisation with pneumococcus causes symptoms, all volunteers filled out a daily symptom log on inoculation day and for each of the following 6 days. For the 23F study a 7-point Likert-type scale was used for symptom reporting (nasal and non-nasal symptoms) as published by Spector et al [172] (recommended to evaluate severity of rhinitis). This scoring system was slightly modified with the removal of 'mental function' as a possible non-nasal symptom (see Appendix B: Daily Symptom Logs)

For the 15B study, a different daily symptom log was used to cover a larger number of potential non-nasal symptoms as a further safety strategy and to ensure colonisation did not cause systemic symptoms that we previously did not enquire about. A further benefit of this was also to move to a generic symptom log for all EHPC studies to allow better comparison of results (see Appendix B: Daily Symptom Logs). The numeric scoring system was replaced with descriptive options based on participant feedback. Participants felt that the lack of explanation of what constituted a score of 2, 4 and 6 made it difficult to use. The study team also believed that descriptive options would reduce the subjective variation between participants reporting on a numbered scale.

4.2.2 Bacterial Serotypes

4.2.2.1 23F Serotype

In 2001 a human challenge experiment was performed in the USA using serotype 23F P833.This achieved colonisation rates of 43% (6/14 participants) [29]. This serotype was transferred to the EHPC group. A repeat study found 23F serotype had a reduced ability to colonise humans, maximum colonisation rate of 10%. Upon discussions with the USA team it was discovered that the 23F inoculum contained two different cell populations, one wild-type for the *amiC* gene and the other contained an *amiC* frameshift mutation (Dalia and Weiser, personal communication with Ferreira). It was confirmed that the inoculum used for the EHPC study containing the *amiC* frameshift mutation. Both genetic serotypes (P833 and P1123) were sequenced using an Illumina Hi-Seq and frameshift mutation was determined by PCR amplification and sequencing (described in J. Gritzfeld PhD thesis [173]).

AmiC is a pneumococcal transmembrane protein that has been shown to play a role in adherence and oligopeptide transport [174 175]. Following this discovery, a murine model assessed the effect of this mutation on nasopharyngeal colonisation. The 23F serotype with *amiC* mutation did not establish any colonisation, while a 23F serotype with a full length *amiC* gene was able to establish colonisation and is not further considered in this thesis (unpublished work, M.de Jonge).

In this study we tested a natural derivative isolate of 23F serotype P833; P1121, containing a wild-type *amiC* gene, with no frameshift mutation on *amiC* gene. Using the wild type serotype, we expected to be able to reproduce findings from the USA group which established carriage in 75% participants using 17,000 CFU/naris [29]. This penicillin-sensitive isolate was obtained from a child with otitis media by tympanocentesis, gifted from University of Pennsylvania as described below.

4.2.2.2 Non-Vaccine Serotype

We also wanted to investigate a pneumococcal serotype not included in the PCV13 vaccine; no human infection model has tested any such serotype previously. Multiple serotypes were assessed using laboratory testing prior to choosing the best candidate. 15B P1262 was chosen based on superior pneumococcal survival in presence of human blood neutrophils and adherence to human nasal cells compared to 6B serotype (Pojar, unpublished work). This penicillin-sensitive isolate was obtained from a child during a colonisation episode, gifted from Southampton. Collection as described below.

4.2.3 Pneumococcal inoculation

Clinical isolates of serotype 23F serotype P1121 (a gift from Professor JN Weiser, University of Pennsylvania. European Nucleotide Archive accession number: ERS1072059) and serotype 15B serotype P1262 (a gift from Dr D. Cleary, University of Southampton. European Nucleotide Archive accession number: ERS2632437),

were used to create two 'parent' stocks for the New Serotypes Study. See Chapter 2, section 2.4.1 for details about how the 'parent' stock was made and for general aspects of pneumococcal challenge which are common to both studies in this thesis. Prior to human challenge, a sample of the bacterial stock was sent to an independent reference laboratory (Public Health England, UK) to confirm bacterial stock purity, serotype and antibiotic sensitivity.

Intra-nasal inoculation with either 15B or 23F serotypes of *S. pneumoniae* was carried out by a research nurse or clinical fellow, trained in the inoculation procedure. Participants were sat in a semi-recumbent position with their head tilted back. Using a P200 micropipette 0.1ml broth containing the desired dose of pneumococcus was inserted into each nostril Figure 25. The pipette tip never touched the nasal mucosa during the procedure to ensure the integrity of the epithelium was not disrupted. Following inoculation, the participant remained in this position for up to 15 minutes without sniffing or blowing the nose.

Figure 25: Inoculation of the nasal mucosa procedure
Participants sat in a semi-recumbent position. Inoculation of the nasal mucosa with pneumococcus was undertaken using a P200 pipette. 100µl of the bacterial inoculum was instilled into the anterior nasal cavity in a circular motion.

4.2.4 Clinical sampling processes and sample analysis

All participant visits were carried out at the Clinical Research Facility in the RLBUHT. Samples obtained from participants include; nasal wash, blood samples and urine sample (only women of child bearing age). Sample collection and processing can be found in Chapter 2 (see below for sections in Table 18). Participants' full blood count were analysed at RLBUHT, clinical laboratories. All other research samples were processed and stored at LSTM.

Table 18: Generic clinical sampling process and sample analysis is outlined in Chapter 2

Process	Section
Nasal wash sampling method	Section 2.3.10
Blood sampling method	Section 2.3.12
Pneumococcal stock preparation (batch)	Section 2.4.1
Preparation of pneumococcal stock on day of challenge	Section 2.4.2
Nasal wash sample processing	Section 2.4.3
Detection of pneumococcal colonisation by culture	Section 2.4.4
Detection of pneumococcal colonisation by q PCR	Section 2.4.4

4.2.4.1 Measurement of anti-pneumococcal polysaccharide antibodies by enzyme-linked immunosorbent assay (ELISA)

We measured the levels of serotype 15B specific polysaccharide antibody in volunteers' serum. Initially a 96-well plate was coated with 10µl purified 15B capsular polysaccharide (Statens Serum Institute Diagnostica, Denmark) in 10ml of phosphate-buffered saline (concentration 5µg/ml) then incubated at 4°C overnight. Human anti-pneumococcal capsule reference serum lot 007sp (National Institute for

Biological Standards and Control, UK) was used to make a standard curve. The standard was serially diluted from a concentration of 271.04ng/ml to 4.235ng/ml.

In a separate 96-well plate 2.5 µl of serum sample was mixed with 100µl of dilution buffer (2ml of 10% heat-inactivated foetal bovine serum blocker in 20ml PBS), 10µg/ml of cell wall polysaccharide (CPWS Multi, Statens Serum Institute Diagnostica, Denmark) was added and incubated at room temperature for 30 minutes. Following these dilution and absorption steps samples were transferred to the pre-coated plates and incubated at room temperature for 2 hours. Bound antibodies were detected using goat anti-human IgG (Sigma Life Sciences, UK), 2.5µl in 10ml phosphate-buffered saline and incubated at room temperature for 1 hour 30 minutes. Finally, 100µl of p-nitrophenylphosphate (5mg added to 40ml of distilled water) was added to plates and left to develop in the dark for 15-20 minutes, following this absorbance was measured at 405nm using FLUOstar Omega plate reader (BMG Labtech, Germany). All samples and standard curve were measured in duplicate. Between all steps described above plates were washed with PBS-Tween 0.005% using a microplate washer (ELx50™, BioTek™, USA).

4.2.5 Endpoints and objectives

The primary endpoint was the detection of experimental pneumococcal colonisation (serotype 23F or 15B) in nasal wash by classical culture methods at any time point (day 2, 7 or 14) following pneumococcal challenge.

The secondary endpoints were: (1) Determination of the duration and density of pneumococcal colonisation with 23F and 15B serotypes; (2) Rates of colonisation at different inoculum doses for both serotypes; (3) The detection of pneumococcal colonisation by qPCR at any time point; (4) Quantification of a systemic humoral immune response to nasopharyngeal carriage (capsular specific IgG concentration in serum).

4.2.6 Statistical methods

Previous EHPC studies using serotype 6B *S. pneumoniae* bacteria attained colonisation rates of approximately 45%. We used this as the basis of our sample size calculation; to estimate colonisation rates with 95% confidence (+/-5%) 34 participants completing the study with a single inoculation dose ($2.0x10^4$, $8.0x10^4$ or $1.6x10^5$ CFU/naris), using 45% colonisation as an estimate for the 'real rate'. To allow for an estimated drop-out rate of 10% and natural colonisation of 10% (who will be excluded from primary endpoint analysis) between 34 and 67 participants was required for each serotype.

The Fisher's exact test and Chi squared test were used to analyse colonisation rates. We compared colonisation rates seen between serotype 23F and 15B. In addition, comparison was made between colonisation rates found at different inoculation doses. Differences in density was evaluated using one-way ANOVA test. Graphical and statistical analysis were performed using GraphPad prism (GraphPad Software, CA, USA). All P values were two-tailed and considered significant if $P<0.05$.

4.3 Results

4.3.1 Screening and recruitment

4.3.1.1 23F study

During September 2016, 18 participants were recruited for the 23F group, 16 participants completed all the study visits. This study was suspended early due to poor results, further discussed in section 4.3.3. Two participants were excluded following screening both due to abnormal full blood counts (see Figure 26). All 16 participants were inoculated with pneumococcus in September 2016, with final participant, final visit on 11th October 2016. The mean average age of volunteers was 30.2 years (range 25-47, median 29) and the male:female ratio was 5:11.

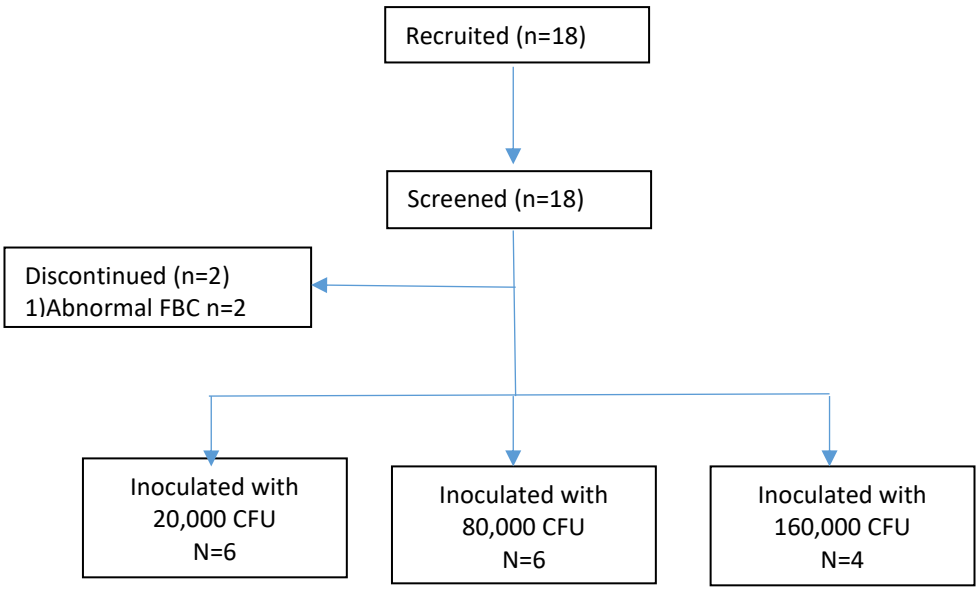

Figure 26:Consort flow diagram for 23F group New Serotypes study

4.3.1.2 15B study

Between August-November 2017, 72 participants were recruited to the 15B group, with 54 participants completing all study visits. 10 participants withdrew pre-screening, 7 excluded or withdrew post screening visit and 1 was excluded at day 1 post inoculation due to starting antibiotics for a non-study related issue (see

Figure 27). All 54 participants who completed the study were challenged with pneumococcus between September-November 2017, with the final participants, final visits on 13th December 2017. The mean average age of volunteers was 24.6 years (range 18-49, median 21) and the male:female ratio was 29:25.

Sequential groups were inoculated with higher doses of bacterial inoculum if optimal rates of colonisation were not achieved by day 7 (>40% colonisation rates). This was a safety feature to ensure that we were taking forward to an extended cohort the lowest inoculum dose possible to achieve adequate colonisation rates. Participants were recruited into groups depending on their availability. The inoculation dose which achieved the optimal colonisation rates was extended to complete a full cohort. This extended cohort would give us statistical confidence to estimate the colonisation rate of this serotype.

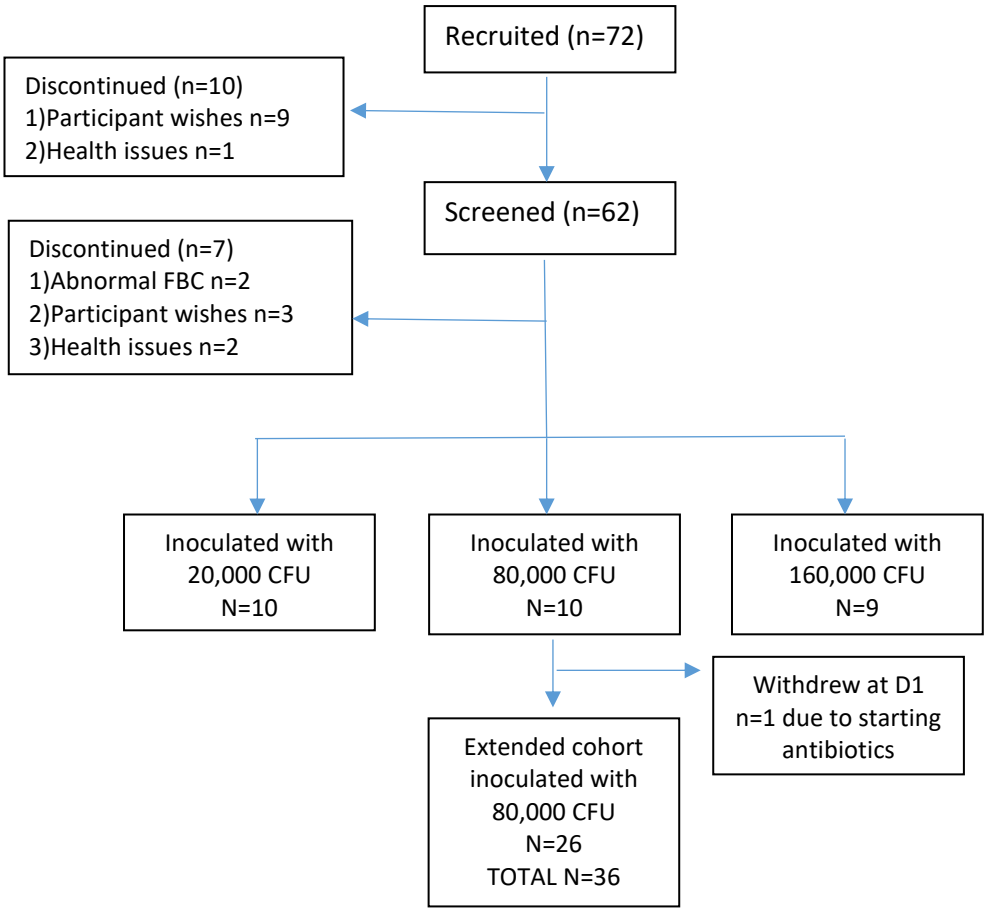

Figure 27: Consort diagram for 15B group of New Serotypes study

4.3.2 Inoculum doses were within target range

All inoculation doses were compliant with protocol as they fell within half or double of the target amount. The average inoculation doses per group can be seen below (Table 19).

Table 19: Average pneumococcal challenge dose for all participant groups

Dose Group Target inoculation dose per naris	Serotype 23F Average dose (CFU/naris)	Serotype 15B Average dose (CFU/naris)
$2x10^4$	$1.7x10^4$ (SD 0)	$1.9x10^4$ (SD $9.9 x10^2$)
$8x10^4$	$7.5x10^4$ (SD 0)	$8.9x10^4$ (SD $1.3x10^4$)
$1.6x10^5$	$1.7 x10^5$ (SD 0)	$1.9x10^5$ (SD 0)

4.3.3 Dose-ranging study: Serotype 15B lead to more acquisition of colonisation compared to 23F serotype

Naturally colonised participants at baseline were excluded from analysis (see Table 20). There was no significant difference in colonisation rates between participants inoculated with 15B and those receiving 23F (P=0.29, Fisher's exact test).

Table 20: Number of participants in each dose-ranging group for each serotype tested, with number of naturally colonised participant as baseline who were excluded from primary analysis of data.

Dose Group	23F group Number of participants	23F Group Naturally Colonised at baseline	15B Group Number of participants	15B Group Naturally Colonised at baseline
$2x10^4$	6	0	10	0
$8x10^4$	6	1	10	1
$1.6x10^5$	4	0	9	1

Overall the colonisation rate with 23F was 13% (2/15; excluding participant naturally colonised at baseline). The highest colonisation rates were observed in the group inoculated with $8x10^4$ CFU/naris (20%, 1/5) and no colonisation events were detected in the $1.6x10^5$ dose group (Figure 28). Due to poor colonisation rates with serotype 23F in the dose-ranging portion of the study, this serotype was not taken forward to be completed in an extended cohort.

The 15B serotype led to acquisition of colonisation in each dose group. Initially colonisation rates were dose dependent with more participants in the $8x10^4$ group becoming colonised compared to the $2x10^4$ group (4/9, 44% vs. 3/10, 30%

respectively). However, rates fell from 44% at $8x10^4$ CFU/naris to 13% (1/8) when the dose rose to $1.6x10^5$ CFU/naris. This difference was not statistically different (P=0.29) (Figure 28).

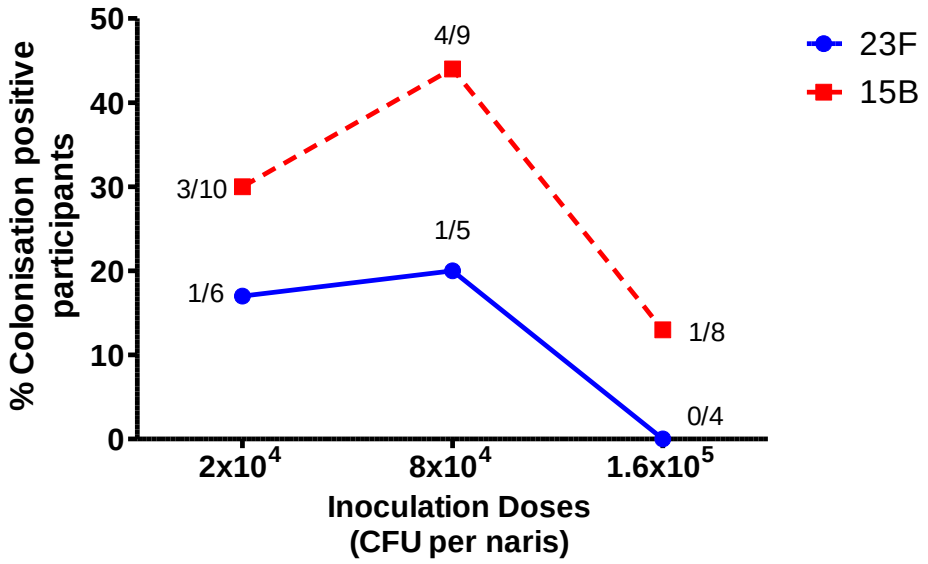

Figure 28: Colonisation rates (%) for 23F and 15B during dose range portion of the study.
Participants who were naturally colonised with pneumococcus at baseline were removed prior to analysis. Participants were intranasally inoculated with either serotype 23F (blue circle) or 15B (red squares) over a range of doses, starting with $2x10^4$ CFU/naris to $1.6x10^5$ CFU/naris. Y axis shows percentage of participants experimentally colonised at each dose.

4.3.4 Dose-ranging study: Density of colonisation and inoculum dose

Colonisation rates were not found to rise significantly with increasing inoculum dose for either serotype. We wanted to investigate whether colonisation density may increase in parallel with this inoculum dose increase. Figure 29 shows the colonisation density recovered from nasal washes at each time point (day 2, 7 and 14) for both 23F and 15B dose-ranging participants. Due to low colonisation rates in

the 23F group we cannot confidently analyse differences in colonisation density at increasing inoculum doses.

Participants inoculated with 15B with an inoculation dose of $8x10^4$ CFU/naris had significantly higher colonisation density at day 2 compared to those inoculated with $2x10^4$ (P=0.047, t test). This difference did not continue at later follow up time points (day 7 and 14).

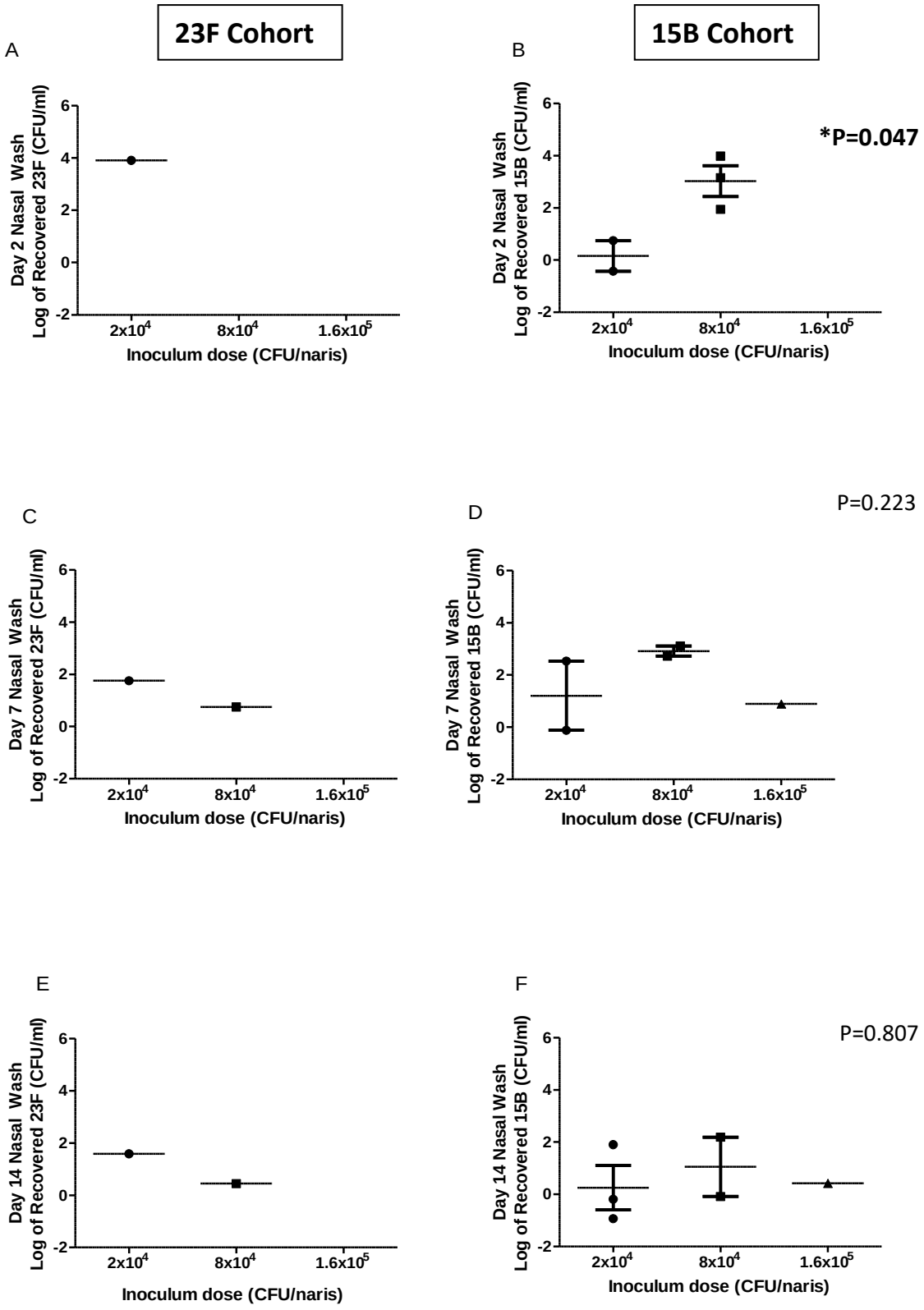

Figure 29: Density of colonisation for both 23F and 15B groups at each time point tested.
Results from nasal washes performed at day 2 (A&B), day 7 (C&D) and day 14 (E&F). Density is reported as CFU/ml of nasal wash recovered. Data bars represent mean and standard deviation. We were unable to compare inoculation groups for 23F as too few were colonised. For the 15B cohort, t-test was used to compare groups at day 2 as only two inoculation doses lead to colonisation. One-way anova was used to compare groups at day 7 and 14. P value of <0.05 considered significant.

When evaluating the area under the curve (AUC) of densities for each serotype, no differences were observed between 15B and 23F either when looking at all positive participants or when this was broken down into inoculum doses (Figure 30). Overall colonisation density during the 14 days follow up period was not a function of inoculation dose.

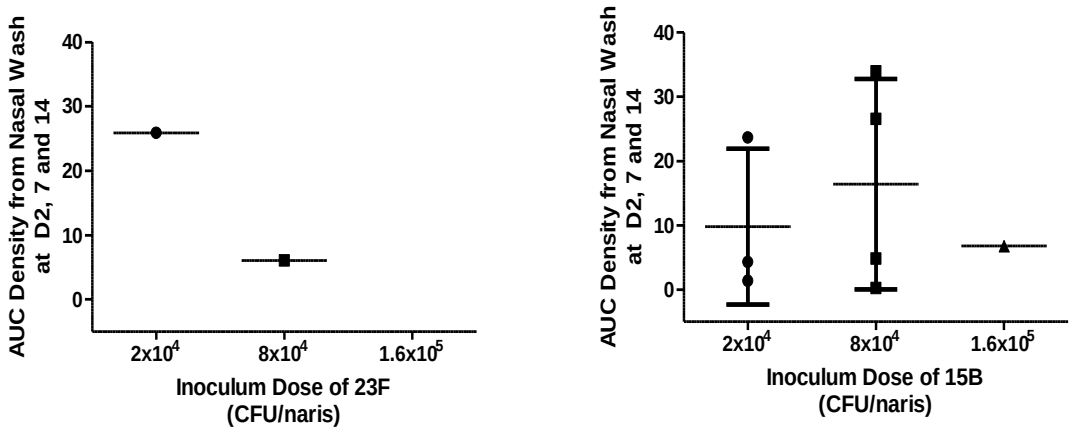

Figure 30: Area under the curve densities. For 23F and 15B groups at each inoculum dose
Nasal washes were performed at day 2, 7 and 14 for all participants. Pneumococcus was detected in nasal washes using classical culture techniques. Area under the curve was calculated from the density of colonisation calculated at each positive time point. Density reported as CFU/ml of nasal wash returned. Data bars represent mean ± standard deviation.

4.3.5 Dose-ranging study: Majority of the participants colonised up to day 14

Both colonised participants with 23F continued to be positive to day 14. The majority (75%, 6/8) of participants colonised with 15B during the dose-ranging portion of the study also continued to be positive to day 14. All participants inoculated with $2x10^4$ and $1.6x10^5$ CFU/naris were colonised up to day 14, but in the $8x10^4$ CFU/naris group 50% (2/4) cleared colonisation prior to day 14. Those participants who were found to

be colonised at either day 7 or 14 received antibiotics at the end of the study to clear colonisation.

4.3.6 Extended Cohort: Precision of estimated colonisation rate with 15B improved with extension of cohort to 33 participants

During the dose-ranging portion of this study, 15B was the only serotype to reach colonisation rates above the desired 40% attack rate. Colonisation rates of 44% were observed at the $8x10^4$ inoculation dose group; therefore, this was taken forward into the reproducibility portion of the study.

Twenty-five further participants were inoculated with 15B at $8x10^4$ inoculation dose in this part of the study. One participant was naturally colonised with pneumococcus at baseline; they were removed from the population prior to analysis of the data for the primary outcome (modified intention-to-treat). Following challenge, 29% (7/24) of participants were experimentally colonised with 15B by microbiological culture. Analysis including all participants who were inoculated with $8x10^4$ CFU/naris of serotype 15B, indicated an overall colonisation rates were 33% (11/33) (See Figure 31).

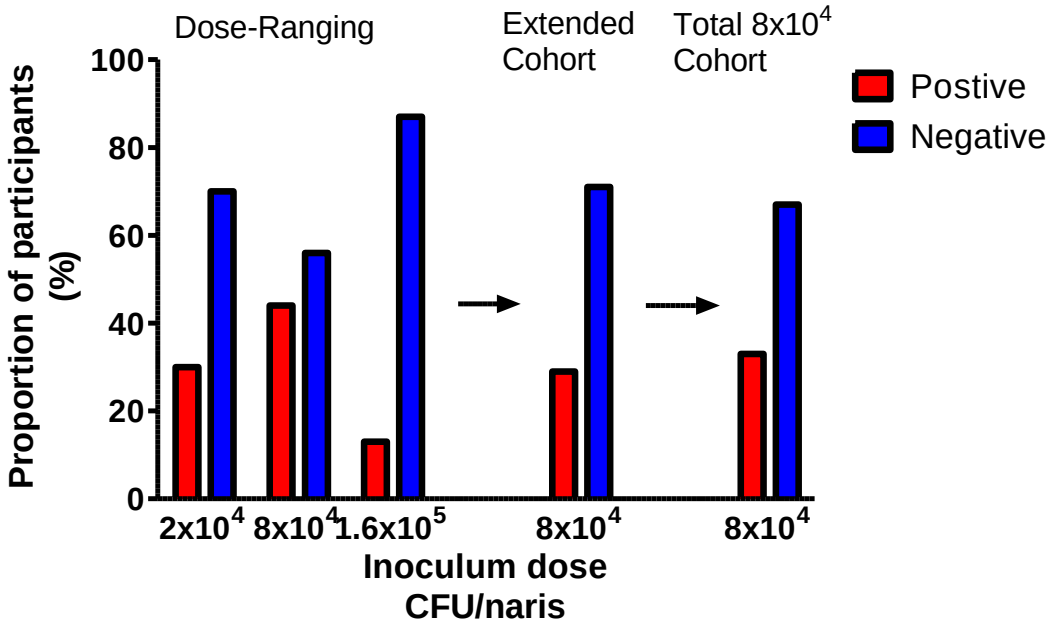

Figure 31: Proportion of colonisation positive and negative participants detected by classical culture
Participants who were naturally colonised with pneumococcus at baseline were removed from analysis. Participants were intranasally inoculated in groups with serotype 15B over a range of doses; $2x10^4$ (10 participants), $8x10^4$ (9 participants) and $1.6x10^5$ (8 participants). We extended the $8x10^4$ group as this had the best colonisation rates. The extended cohort shows results of a further 24 participants inoculated with this dose.

4.3.7 Extended cohort: Half of participants had cleared colonisation by day 14

Of the 11 participants colonised following inoculation with $8x10^4$ CFU/naris of 15B serotype, over half cleared colonisation prior to the final visit at day 14 (55%, 6/11), see

Figure 32. This was similar to results found in the dose ranging part of the study where 50% of participants (2/4) in the 8x10^4 CFU/naris group cleared colonisation prior to day 14.

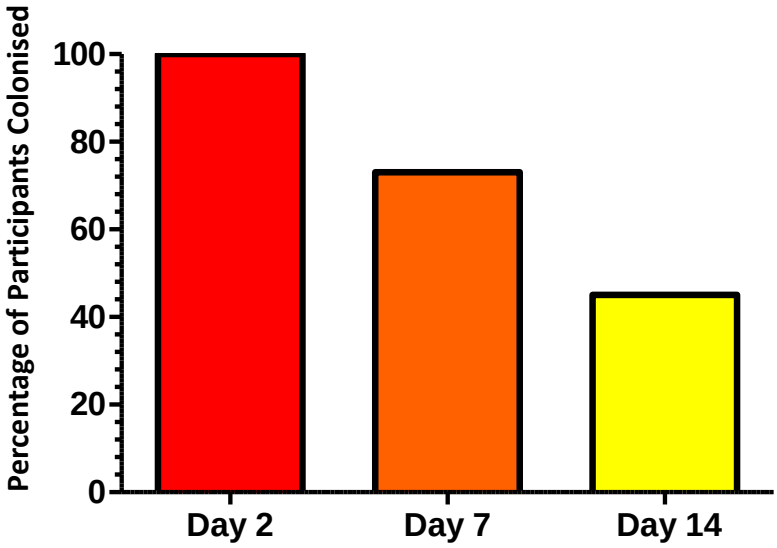

Figure 32: Proportion of participants colonised at each time point following inoculation for participants inoculated with 8x10^{4}CFU/naris.
Clearance of colonisation was considered as the first negative nasal wash found by classical culture after which all further nasal washes were also negative. All participants in this group who were colonisation positive during the study had pneumococcus detected at day 2. By the end of the study (day 14) only less than half of participants were still colonised. Any participant that did not have 2 negative nasal wash results after day 14 visit were given 3 days of amoxicillin in an attempt to clear colonisation.

4.3.8 Complete 15B Cohort: No difference found between colonisation rates when using lytA qPCR compared to culture

128 nasal wash samples were collected from 32 participants in the full 15B 8x10^4 CFU/naris inoculum group. These were tested for the presence of *S. pneumoniae* by

microbiological culture and retrospectively by lytA qPCR at the end of the study. For this analysis, natural carriers (3 participants) were excluded to remove the interaction of more than one prominent colonising serotype which might introduce wider unexplained variation in responses.

The proportion of samples positive for carriage by qPCR was higher than by culture, but this difference was not statistically significant (29/128 [23%] vs. 21/128 [16%] respectively, P=0.27, Fisher's exact test). LytA qPCR improved detection of colonisation and therefore colonisation rate but this was also not statistically different from the culture colonisation rate (16/32 [50%] vs. 11/32 [34%] respectively, P=0.31, Fisher's exact test) (Figure 33). Six participants were only colonisation positive using qPCR. Of these 5/6 only had pneumococcus detected from nasal wash at day 2 post inoculation (Figure 33). In 1.6% samples (2/124), pneumococcus was detected by culture but not qPCR (one participant at 2-time points), but in 8% of samples (10/128) pneumococci were only detected by qPCR. Table 21 shows concordance rates between the two methods of pneumococcal

detection.

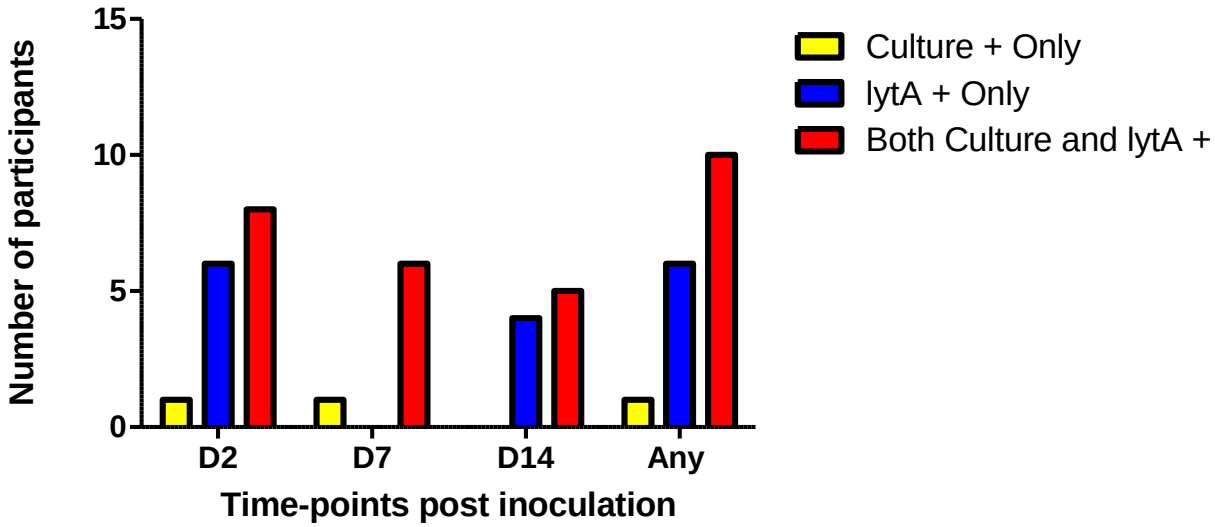

Figure 33: Number of participants colonised at each time point detected by culture and lytA qPCR for 15B full cohort.
For detection by classical culture techniques, nasal wash samples were plated on blood agar. For lytA qPCR detection, nasal wash was added to RNA protect and frozen on day of sample collection, DNA extraction process was completed retrospectively following completion of the study. Samples with a Ct value of <40 were considered qPCR positive. Samples from 32 participants were analysed (naturally pneumococcal colonised participants were excluded from analysis). Blocks represent participants who were culture positive only (culture +), lytA qPCR positive only (lytA +) and those that were found to be colonised by both culture and lytA qPCR. All time points post inoculation are shown and results from any time point (any).

Table 21: Concordance between microbiological culture and qPCR in detection of pneumococcus in nasal washes.
3 participants who were natural carriers of pneumococcus either at baseline or during the study were excluded from this analysis (modified intention-to treat analysis).
[a] Denominator is total number of samples or participants which are positive with either culture or lytA qPCR and numerator is number of samples positive by both methods. [b] Denominator is total number of samples or participants which are positive with either culture or lytA qPCR and numerator is number of samples negative by both methods.

	Nasal Wash Samples	Number of Participants
Concordance of Positive Results	63% (19/30) [a]	59% (10/17) [a]
Concordance of Negative Results	89% (97/109) [b]	68% (15/22) [b]

4.3.9 Natural carriers of pneumococcus

Overall natural colonisation rates in the 23F group was 1/18 (5.5%) and 15B cohort was 6/62 (9.6%). All serotypes found were non-vaccine serotypes (see Figure 34). No participants were co-colonised with 23F or 15B and the naturally acquired serotype when assessed using culture.

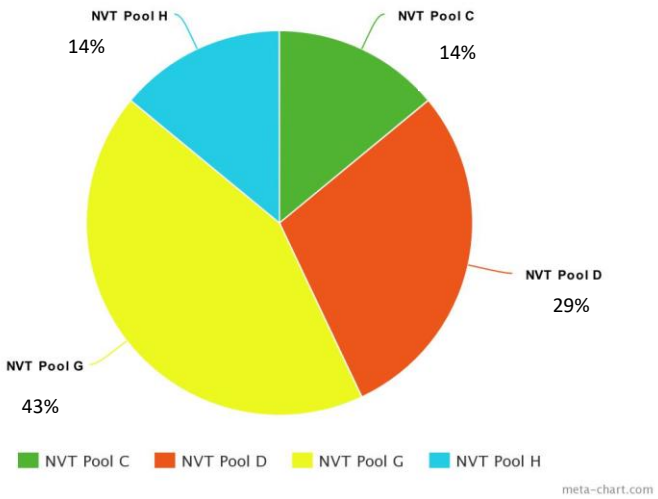

Figure 34: Natural pneumococcal colonisation serotypes as found by culture.
NVT: non-vaccine type, serotypes identified by Statens Serum latex kit. Pool C NVT serotypes: 24 (24F, 24A, 24B), 31, 40. Pool D NVT serotypes: 16 (16F, 16A), 36, 37. Pool G NVT serotypes: 29, 34, 35 (35F, 35A, 35B, 35C), 42, 47 (47F, 47A). Pool H NVT serotypes: 13, 28 (28F, 28A).

4.3.10 Levels of polysaccharide 15B (PS15B) IgG in serum at baseline were not associated with protection against colonisation acquisition.

We measured pneumococcal antibody levels in serum samples from 34 participants before inoculation with $8x10^4$CFU/naris of serotype 15B to assess whether levels correlated with protection against colonisation. Levels of PS15B IgG at baseline were similar between colonisation positive (those who acquired colonisation following

inoculation) and colonisation negative participants (those who did not acquire colonisation) (Figure 35). Median 15B IgG levels in colonisation positive volunteers was 3787ng/ml (interquartile range [IQR] 1607-6465) compared to 3681ng/ml (IQR 1927-6008) in colonisation negative participants (P=0.78, unpaired t test).

In addition, no association was observed between baseline PS 15B IgG levels and colonisation density (Figure 36) (R_S= -0.21 Spearman's rank correlation coefficient, P=0.54). Baseline levels of PS 15B IgG were therefore not predictive of colonisation status nor associated with colonisation density.

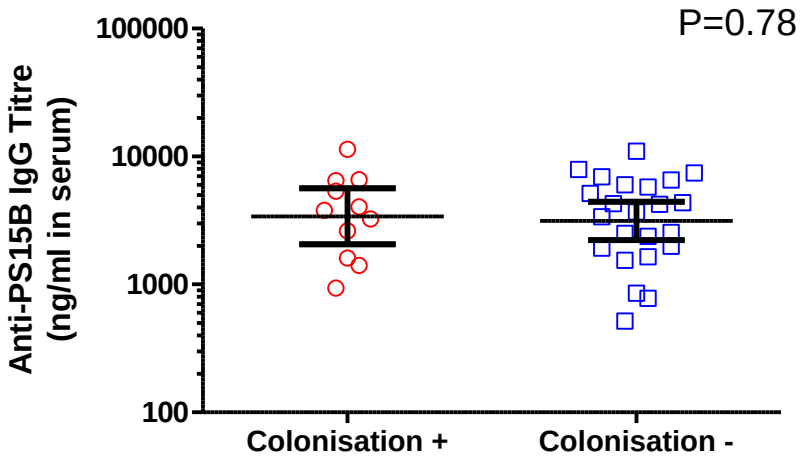

Figure 35: Baseline polysaccharide15B (PS15B) IgG levels prior to experimental human pneumococcal challenge.
IgG specific ELISA's in serum were performed against pneumococcal PS15B. levels of antibody were determined from colonisation-positive participants (colonisation+ n=11) and colonisation negative participants (colonisation – n=23). Serum samples were taken at screening visit (day -5). Horizonal bars represents geometric mean and error bars represents the 95% confident intervals. Comparison of colonisation positive and negative participants carried out using unpaired t test. P value of <0.05 where considered significant.

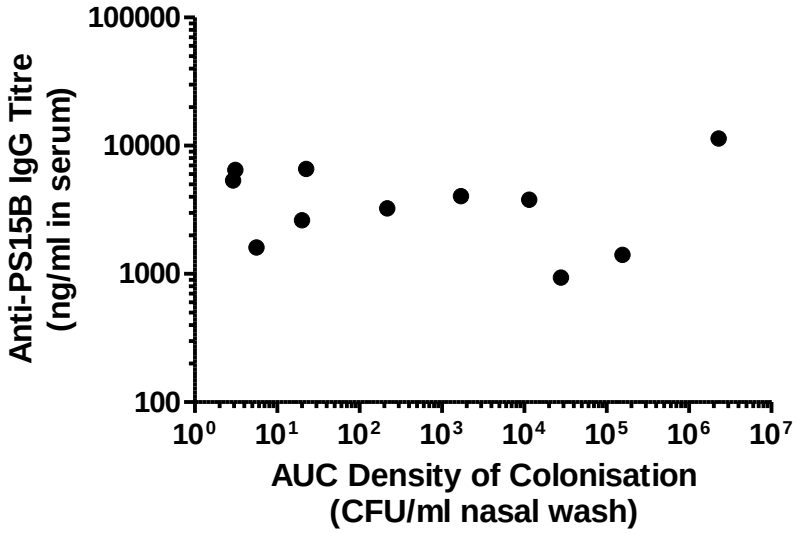

Figure 36: Correlation between baseline anti-PS 15B IgG levels and AUC density of colonisation positive participants.
Spearman's rank correlation coefficient is -0.21 with P value of 0.54.

4.3.10.1 PS 15B IgG levels are increased post inoculation in colonisation positive participants

Paired baseline and final follow up (day 14) serum samples from 33/34 participants from this cohort were evaluated. In colonisation positive participants we observed a significant rise in levels of PS 15B IgG at day 14 post inoculation compared to baseline (mean 4693ng/ml at baseline vs 18164ng/ml at day 14, P=0.0005 paired t-test). In contrast, colonisation negative participants levels of PS 15B IgG at day 14 were comparable to those at baseline (mean 4059ng/ml at baseline vs. 3866ng/ml at day 14, P= 0.31 paired t test). See Figure 37.

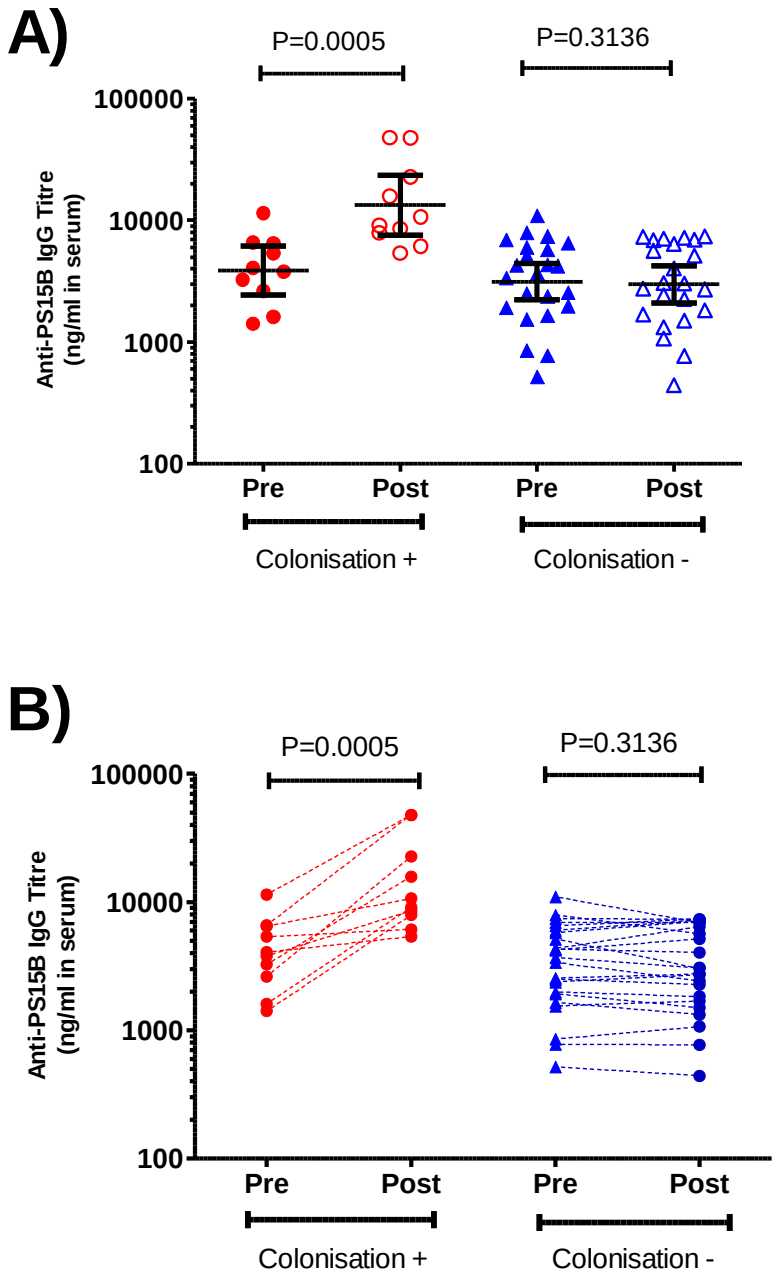

Figure 37: Levels of polysaccharide 15B (PS 15B) IgG before and after pneumococcal inoculation. IgG specific ELISA in serum were performed against pneumococcal PS15B. Levels of antibody were determined from colonisation positive participants (colonisation +) and colonisation negative participants (colonisation -). Serum samples taken at screening visit (day -5) labelled as 'Pre', serum samples taken on final follow up visit (day 14) labelled as 'Post'. A) Measurement of PS 15B IgG levels was carried out in all samples as duplicate. Horizonal bars represents geometric mean and error bars represents the 95% confidence intervals. B) Pre and Post PS 15B IgG levels are linked for each subject. Comparison of antibody levels 'Pre' and 'Post' exposure was carried out using paired t test. P value of <0.05 was considered significant.

4.3.11 Active symptom reporting

All participants in the 23F study group completed the daily symptom logs (16/16), and 98% (53/54) participants in the 15B study group completed the daily symptom logs. Within the 23F study, two participants became experimentally colonised with pneumococcus. One participant was naturally colonised with pneumococcus at baseline and throughout follow up visits. For the 15B study, 15 participants became experimentally colonised and a further 4 participants who were either naturally colonised at baseline or during the study. The only participant who did not complete the daily symptom log, was not colonised with pneumococcus throughout the study.

In both scoring systems, a score of 1-2 (no symptoms-occasional limited symptom) was considered asymptomatic. This was to limit the impact of participants reporting an isolated event of short duration such as one sneezing episode. Participants who reported symptoms prior to inoculation where only considered symptomatic if they reported a score which was higher than the baseline score.

4.3.11.1 23F group symptom analysis

There were 5 categories relating to nasal symptoms (sneezing, runny nose, congestion/stuffiness, itchy nose and post-nasal drip) and 5 categories relating to non-nasal symptoms (eye symptoms, throat symptoms, cough, ear symptoms and headache) (see Appendix B: Daily Symptom Logs). Overall 19% (3/16) of participants reported either nasal or non-nasal symptoms (score ≥3) during the 7 days following

inoculation. These symptoms consisted of one participant reporting each of the following; sneezing, nasal congestion and throat symptoms. All were reported on day 7 post inoculation and no symptoms were scored as 4 or more (mild steady symptoms).

Due to small number of positively colonised participants we are unable to definitively determine whether colonisation with 23F is symptomatic. However, of the 3 participants who reported symptoms, none were experimentally colonised with 23F, and one was a natural carrier of pneumococcus at baseline and up to day 7.

4.3.11.2 Colonisation with 15B does not increase likelihood of nasal symptoms but may cause coughing

There were 5 categories related to nasal symptoms (sneezing, runny nose, congestion/stuffiness, itchy nose and post-nasal drip) and 15 categories related to non-nasal symptoms (cough, chest pain, breathlessness, coughing up phlegm, sweating, chills, headache, nausea/vomiting, muscle pain, anorexia, trouble concentrating, fatigue, trouble sleeping due to breathlessness, waking at night due to breathlessness and wheeze) (see Appendix B: Daily Symptom Logs). Naturally colonised participants were included in the colonisation positive group for this analysis.

Overall 25% (13/53) of participants reported nasal symptoms (score ≥3) during the 7 days following inoculation and 13% (7/53) reported non-nasal symptoms. Similar

rates of colonisation positive participants reported nasal symptoms (26%, 5/19) compared to colonisation negative participants (24% 8/34). For non-nasal symptoms 21% (4/19) of colonisation positive participant reported symptoms compared to 9% (3/34) of negative participants. No statistically significant difference was observed between number of participants who reported symptoms in colonisation positive or negative groups (nasal symptoms P=1.00, non-nasal symptoms P=0.23, Fisher's exact test). When evaluating the number of episodes of each symptom being reported (number of times over 7-day period), the only statistically significant difference found was that colonisation positive participants reported more episodes of cough (9/19 colonisation positive participants reported cough vs 0/34 non-colonised, P<0.0001, Fisher's exact test), see Figure 38.

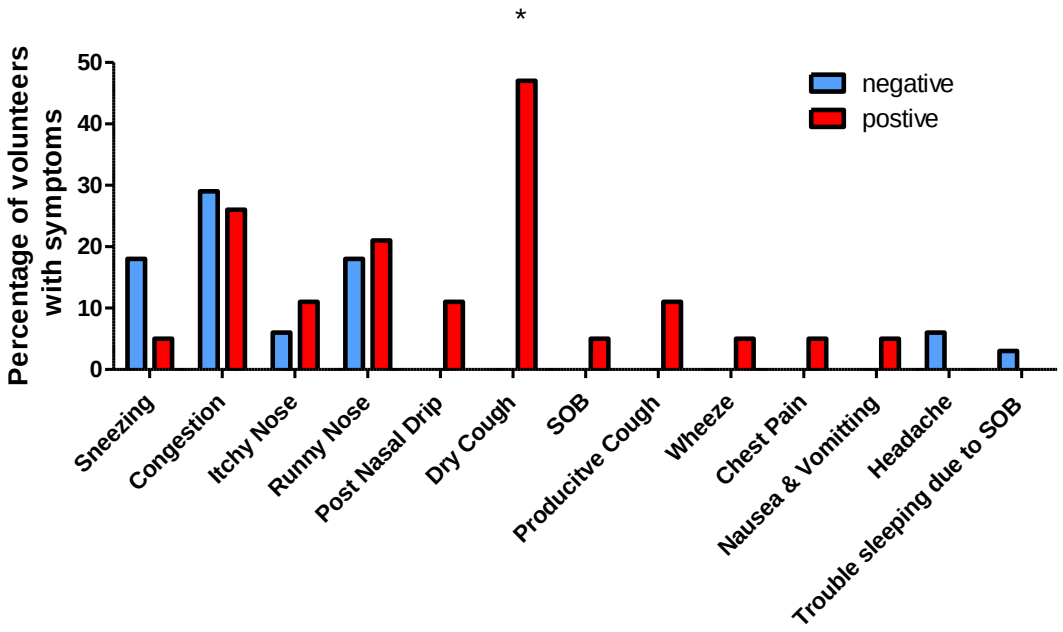

Figure 38: Number of symptom episodes reported over 7/7 period post inoculation.
Only symptoms that have been reported as ≥3 score on the daily symptom log are shown. *Cough is the only symptom in which colonisation positive participants reported significantly more than non-colonised participants (9/19 vs 0/34 P<0.0001, Fisher's exact test).

Five participants scored symptoms 4 or 5 (“moderately” or “quite a bit” bothered with symptom). Three of which were colonisation positive, all from the $8x10^4$ dose group; all complained of cough and one complained of wheeze. Of the two non-colonised participants both from the $1.6x10^4$ dose group; one complained of sneezing and one complained of runny nose, sneezing and congestion. During the study no participants had any serious adverse events due to any symptoms reported. This included no hospital admission, no extra visits for clinical review and no participants were advised to take antibiotics due to clinical need.

4.4 Discussion

The use of the experimental human pneumococcal challenge model allowed us to directly study the propensity of two pneumococcal serotypes to cause colonisation. The study successfully determined which inoculation dose of 15B, a non-vaccine serotype, achieved colonisation rates of >40% and tested the precision of estimated colonisation rates at the chosen dose.

4.4.1 Success in the model expansion; experimental colonisation of non-vaccine type was successful and reproducible

Colonisation dynamics of a non-vaccine type pneumococcal serotype (15B) was evaluated. Experimental pneumococcal colonisation was established in 40% (4/10) when inoculated with $8x10^4$ CFU/naris and was shown to be reproducible in the extended cohort where colonisation rates were found to be 30% (7/23, overall colonisation rate of 33%, 11/33).

Despite the small number inoculated, the data suggest that a non-linear increase exists in colonisation acquisition rates for serotype 15B with increasing inoculum doses. The EHPC model has previously used serotype 6B for experimental challenge; with this serotype colonisation rates of 40-50% have been achieved in multiple studies [139]. We hypothesised that colonisation rates would be higher than this for the 15B serotype following promising *in vivo* testing which showed superior survival in the presence of human blood neutrophils and adherence to human nasal cells

compared to the 6B serotype (not discussed further in this thesis). We expected to see a dose dependent increase in colonisation rates; in previous EHPC studies following inoculation with serotype 6B, colonisation rates were found to be dose dependent between a dose of $1x10^4$-$4x10^4$ CFU/naris. Following this the colonisation rates plateaued after $4x10^4$ up to a dose of $3.2x10^5$ CFU/naris. However, in this study following inoculation with serotype 15B, colonisation rates dropped after $8x10^4$, with lowest colonisation rates of 13% observed with $1.6x10^5$ CFU/naris dose.

Better understanding of the dynamics of colonisation of non-vaccine pneumococcal serotypes is of growing importance with recent epidemiologic evidence of significant serotype replacement following the introduction of PCV13. A cross-sectional study in the USA analysed serotype data from children under 7 who were colonised with pneumococcus between 2004 and 2014 [176]. They found that 15B emerged as the most common colonisation serotype in 2014 overtaking 19A which was the predominant serotype in 2004 but substantially reduced following the introduction of PCV13 [176]. Two UK studies further evaluated the impact of the PCV on pneumococcal colonisation and invasive disease. A cross sectional study of nasopharyngeal colonisation rates in children under 5 between 2006-2011 found that the overall prevalence of pneumococcal colonisation remained stable following introduction of PCV7 in 2006 and PCV13 in 2010 [177]. Vaccine efficacy was good with a significant reduction in vaccine serotypes but with a simultaneous increase in non-vaccine serotypes [177]. A further UK observational cohort study reported a 37% reduction in overall invasive pneumococcal disease incidence in 2016/17 compared

to pre-PCV7 incidence, however a concurrent doubling of invasive disease due to non-PCV13 serotypes was observed with an acceleration since 2013/14 [166].

4.4.2 No difference in colonisation rates found with lytA qPCR compared to classical culture

The proportion of participants who were positive for colonisation and the number of positive samples were apparently higher using lytA qPCR for pneumococcal detection compared to culture. But these differences were not statistically significantly different. When evaluating the whole cohort, irrespective of inoculation dose, 8 participants (8/37 previously colonisation negative participants, 22%) were found to be new colonisers with lytA qPCR only. The majority of these were only found to be colonised at day 2 post inoculation (7/8, 88%, one participant found to be positive only at day 14). As previously discussed, higher rates of colonisation using qPCR method could be explained by this method detecting more low-density colonisation episodes.

Turner et al [178] suggested that the World Health Organisation's suggested methodology of culture and serotyping with the use of latex agglutination (one colony used for latex agglutination test) can significantly underestimate multiple-serotype colonisation. This study found that using sweep serotyping (a sweep of colonies are taken from the culture plate and serotyped using latex agglutination) identified 1.4 times the number of pneumococci compared to culture and *S. pneumoniae* molecular-serotyping microarray found 1.6 times the number [178]. qPCR has also been identified as a method of improving pneumococcal detection during

co-colonisation episodes with one study reporting a co-colonisation rate of 28.7% with qPCR compared to 4.5% detected by culture alone [156]. It has previously been suggested that non-vaccine serotypes such as 15B, may have been more prevalent, prior to introduction of PCV than previously described. These non-vaccine serotypes may be low density colonisers which were not detected if co-colonised with a vaccine type serotype (likely high-density colonisers). Following the introduction of conjugate vaccines, the prevalence of vaccine types reduced and true rates of non-vaccine serotypes may have been unmasked with the possibility of no real increase in non-vaccine type colonisation rates [105].

4.4.3 Baseline levels of PS 15B IgG in serum were not associated with protection against colonisation acquisition

We evaluated levels of PS 15B IgG in serum of participants in the 15B extended cohort who were inoculated with pneumococcus ($8x10^4$ CFU/naris). All participants had measurable levels of IgG to 15B capsular PS at screening. No relationship was found between levels of serum IgG at screening and colonisation outcome after intranasal inoculation. This result is in keeping with previous EHPC studies assessing experimental colonisation with 6B serotype. Two studies found no association between pre-exposure 6B specific polysaccharide IgG levels and protection against colonisation acquisition [10 171].

The protective effect of pneumococcal capsular-specific antibodies against disease has been well established. As early as 1938 published data showed that serotype-specific anti-pneumococcal serum passively transferred to patients with

pneumococcal pneumonia could improve survival rates [179]. In addition, the ability of vaccine-induced serotype specific antibodies to protect against pneumococcal disease has been well established. It is still unclear what amount of circulating serum antibodies are sufficient to protect against disease. Research suggests that generally higher levels are needed for protection against mucosal endpoints in comparison to invasive pneumococcal disease and that there is a degree of variation between serotypes [111 180 181]. Being able to estimate the minimum antibody concentrations needed for protection against pneumococcal disease is of critical importance for assessing new vaccine efficacy. A meta-analysis pooled the data from three double blinded controlled trials which evaluated the immunogenicity of the PCV immunisation [182]. The protective effect of the PCV immunisation is mediated exclusively or primarily by antibodies, therefore, the authors sought to find the specific concentration of antibody which is estimated to lead to protection against pneumococcal disease. This correlate of protection level was reported as an antibody level of 350ng/ml [107 182-185]. The results of this study showed baseline levels much higher than this; median of 3787ng/ml in participants who went on to become colonisation positive and 3681ng/ml in those who were protected against colonisation.

4.4.4 Nasopharyngeal colonisation with 15B found to boost immunity by day 14

Murine models and experimental human challenge models have improved the understanding of immune responses to pneumococcal colonisation episodes;

colonisation protects against re-colonisation with homologous serotypes and against disease [26 186]. The role of antibody-mediated immune responses targeting serotype-specific pneumococcal polysaccharides has been well researched. It is believed that protection against pneumococcal disease involves antibody mediated immune responses which target serotype-specific capsular polysaccharides [187]. We have demonstrated that intranasal exposure to 15B serotype, without acquisition of colonisation, did not boost serum IgG levels to the 15B capsular PS. Conversely in participants who acquired experimental pneumococcal colonisation (with serotype 15B) during the study a significant increase in serum IgG levels to 15B capsular PS was observed.

Our data are consistent with previous EHPC studies, in which inoculation with serotype 6B was not associated with a change in 6B specific IgG levels in serum [24]. One study showed that nasopharyngeal pneumococcal exposure alone may be immunising; capsular specific IgG levels were found to significantly increase in nasal wash and broncho-alveolar lavage samples following intranasal inoculation without colonisation [24]. The EHPC team have previously demonstrated colonisation infers 100% protection against re-acquisition of a homologous serotype, up to 11 months following the first colonisation episode [10]. In this study during the initial colonisation episode a significant rise in serum anti-6BPS IgG levels were observed which is consistent with the finding for serotype 15B demonstrated in this thesis [10].

4.4.5 Low colonisation rates observed with 23F serotype

We were unsuccessful in expanding the EHPC model with a further vaccine type serotype. Colonisation rates remained low with escalating inoculum doses between $2x10^4$-$1.6x10^5$ CFU/naris, with highest colonisation rates observed of 20% in $8x10^5$ CFU/naris inoculation group. No participants became colonised following inoculation with $1.6x10^5$ CFU/naris dose. This serotype (P833, inoculum doses ranging from $2x10^4$- $3.2x10^5$ CFU/naris) had previously been tested by our group, poor colonisation rates were observed with a maximal colonisation rate of 10%. Following investigation of the serotype used in this study (23F P833), an *amiC* frameshift mutation was found and investigated in a murine pneumococcal challenge, results suggesting this mutation was the cause of the poor colonisation rates.

For the current study we ensured the isolate used, 23F P1121, contained a wild-type form of the *amiC* gene. This isolate change did not lead to significantly better colonisation rates. The *amiC* gene may have contributed to poor colonisation rates in the initial study but it is clear now that either another factor or multiple factors may be implicated in a serotype's ability to colonise the host. As previously discussed, host factors may affect colonisation rates. Epidemiological data suggest that 23F serotype was highly prevalent in UK prior to the introduction of PCV7 into the childhood immunisation schedule [15]. Recent studies suggest that rates of 23F serotype have significantly fallen over the last decade [176]. Previous colonisation with this serotype may have induced immunity and impacted on colonisation rates observed. Using the experimental human pneumococcal carriage model, it has been

shown that protection from colonisation (with 6B serotype) was associated with a high number of circulating 6B specific IgG-secreting memory B cells at baseline [171]. Alternatively, other bacteriological differences observed between 23F and 6B (known to have high experimental colonisation rates) may have impacted on this serotypes ability to colonise the nasopharynx.

Poor colonisation rates with the 23F serotype are likely multifactorial and may be due to a combination of serotype and host factors. Pneumococcus is a pathogen that grows in chains, it has been suggested that serotypes grow in long-chain formation may be more successful in colonising the nasopharynx [188]. *In vitro* and *in vivo* analyses have shown that pneumococcal adherence is increased proportionally to the average size of the chain [188]. The ability for serotypes or specific isolates to grow in chains and the average length of chains may be an important predictor for determining successful colonisation. Longer chains may improve adherence of pneumococcus to the mucosal service and therefore give an advantage in colonisation through a larger number of possible adhesive events per particle. Assessing chain formation of any future serotypes prior to experimental inoculation may be beneficial for any future experimental pneumococcal human challenge studies.

It has also been suggested that the biochemical structure of the capsular polysaccharide of different serotypes of pneumococcus may impact on their success at colonising the nasopharynx. *In vitro* assays have shown an association between resistance to neutrophil-mediated killing and colonisation prevalence; serotypes which were more heavily encapsulated were more resistant to this neutrophil-

mediated killing [189]. Further research showed that surface charge of *Streptococcus pneumoniae* may affect colonisation potential. It has been shown that serotypes which are more negatively charged also have a higher resistance to killing by neutrophils *in vitro* [190]. Using previous epidemiological studies, it was also shown that the more negatively charged serotypes were associated with higher colonisation rates in human populations [190]. Understanding better the factors which may explain why serotypes have varying colonisation potential is important for predicting serotype replacement and can support the identification of serotypes to be used in future human challenge models.

4.4.6 Experimental colonisation does not cause nasal symptoms but increased cough

We actively sought symptom data using a daily symptom log on the day of inoculation and 7 days following. We were unable to compare symptoms reported in the 23F group due to small numbers of colonisation positive participants. In the 15B group, due to small numbers in individual inoculation dose groups, we combined all colonisation positive and negative participants. The number of participants reporting nasal symptoms in both groups were similar, suggesting that colonisation is an asymptomatic process in adults. Interestingly of the non-nasal symptoms reported cough was found to be more common in colonisation positive group, in terms of both number of participants reporting the symptom and the frequency with which it was reported over a 7-day period.

Nasopharyngeal colonisation is generally considered an asymptomatic event in adults. However, there is little definitive research looking at this question. Cross-sectional studies in children report a strong association between a history of upper respiratory tract infection (URTI) symptoms, cough or coryza (in the weeks preceding sampling) and the detection of nasopharyngeal pneumococcal colonisation [164 191]. Unfortunately, due to this study design the causality of symptoms is not clear. Pneumococcal colonisation may cause URTI symptoms or coryza may increase the rate of pneumococcal colonisation acquisition or there may be a third factor that both colonisation and viral URTIs are independently associated with.

One study did report that for serotype 19F, colonisation was strongly associated with children who complained of symptoms such as coryza, sneezing, cough and expectoration [192]. These children were recruited from the emergency room of a local paediatric hospital. The study did not report on the diagnosis given to these patients therefore an independent cause is possible for the symptoms reported [192]. The same study also recruited participants from a day care centre; for this group the results of symptom reporting was not published [192].

5 General Discussion

We investigated factors affecting nasopharyngeal pneumococcal colonisation using an experimental human carriage model.

The two main aims were:

Aim 1: To investigate if the hands can be a vector for transmission of *S. pneumoniae* from hands into the nasopharynx, leading to colonisation.

Aim 2: To investigate the propensity of serotypes 23F and 15B to cause colonisation following experimental human challenge.

These projects used the Experimental Human Pneumococcal Carriage model to investigate these research questions. This model offers a novel and feasible method of investigating pneumococcal transmission in a controlled environment. The expansion of this model during this project to include a non-pneumococcal vaccine serotype (15B), further supports the model's potential for use in vaccine development.

A discussion of the main findings was provided at the end of each chapter; therefore, the aim of this general discussion is to provide an overall summary of the findings of these research studies. It also outlines some limitations of the research studies which may impact on interpretation of the results. Finally, it details implications of this work and how future work could provide deeper insights.

5.1 Main findings

5.1.1 Chapter 3- Hands were vectors for hand to nose transmission of pneumococcus

Chapter 3 reports the results from a pilot study we conducted to investigate pneumococcal transmission using a human challenge model [146]. It is generally thought that transmission of *S. pneumoniae* occurs primarily by indirect contact via inhalation of infected airborne droplets. Previously it has been unclear if hands or fomites have a role in pneumococcal transmission. We found that the hands can be vectors for transmission of pneumococcus leading to acquisition of colonisation. When using a combination of microbiological culture and lytA qPCR for pneumococcal detection, colonisation rates following hand to nose transmission are comparable to rates observed following intranasal pneumococcal inoculation in previous studies [149].

We demonstrated that pneumococcus can be transmitted leading to colonisation even after drying on the skin. Molecular methods of detecting pneumococcus showed similar rates of transmission when the bacteria were wet and immediately after visual drying on skin (50%). In addition, significant direct contact with the nasal mucosa (such as picking/poking the nose) is not needed for colonisation to occur following pneumococcal transmission. Overall rates of colonisation following transmission using the rubbing and sniffing method were comparable to the poking nose method. Direct contact with the mucosa was an important factor in enhancing

acquisition of colonisation in the dry bacteria sub-groups. Dry poke group had an overall colonisation rate of 70% (7/10) compared to 30% (3/10) in the dry sniff group.

Streptococcus pneumoniae is a leading bacterial cause of infections worldwide; transmission, colonisation and invasion are the key stages in the pathogenesis of this bacteria. Our understanding of the process of transmission has profited recently by new models of transmission in rodents. In addition, experimental pneumococcal human challenge studies have greatly improved our understanding of the colonisation process. The Hand to Nose study described in Chapter 3 sought to build upon previous work by using this human challenge model to study pneumococcal transmission for the first time in humans. The results are in keeping with murine models which suggest that an environmental reservoir of pneumococcus may be an important source of transmission [47 50]. Pneumococci have been shown to be able to survive days outside the human host, in nutrient-sufficient conditions, and have been cultured from common objects [193 194]. It has been proposed previously that pneumococcus could be acquired from fomites and that bio-film growth characterises may be a key factor in length of time it remains infectious in the environment [193]. Transmission during colonisation episodes is crucial to the infectious life cycle of pneumococcus. Future use of human infection studies investigating mediators of pneumococcal transmission could identify interesting targets for prevention of pneumococcal spread.

Finally, the data from this chapter suggest that using both microbiological culture and a molecular method such as lytA qPCR for the detection of pneumococcal

colonisation can reduce the chance of missing low-density pneumococcal colonisation episodes. We found that all culture-positive samples were also qPCR positive. Interestingly the culture positivity rate decreased with decreasing densities measured by qPCR. All samples that were culture negative but qPCR positive had qPCR densities below 10^3 copies/ml. This suggests that qPCR may be more appropriate for detecting low density colonisation episodes.

This study adds to the growing body of evidence suggesting that traditional culture methods may be suboptimal for detecting low density pneumococcal colonisation episodes. Our findings were in line with Olwagen et al [156] who found that qPCR was more sensitive in detecting concurrent colonisation with multiple pneumococcal serotypes. They reported that the majority of additional serotypes detected by the molecular method had bacteria load of $<10^4$ CFU/ml[156]. The use of qPCR method in addition to classical culture for detection of pneumococcal colonisation could help improve our understanding of the process and the effect of interventions such as PCV immunisation. Evaluation of PCV13 immunisation using the experimental human pneumococcal challenge model suggested that the success of this vaccine lies in reduction of colonisation acquisition [169]. Using culture methods of detection, a 78% reduction in colonisation rates was found using PCV13 compared to control [169]. Reduction of on average three logs in colonisation density in volunteers vaccinated with PCV13 was also observed. These results suggest the efficacy of PCV vaccine may be partially mediated by controlling pneumococcal colonisation density rather than solely stopping acquisition of colonisation.

5.1.2 Chapter 4 – The EHPC model was successfully expanded to include non-pneumococcal vaccine serotype

Chapter 4 demonstrated the propensity of serotypes 23F and 15B to cause nasopharyngeal colonisation in health adults. Colonisation rates for serotype 23F remained at or below 20%, regardless of dose. For serotype 15B acquisition of colonisation was dose-dependent until $8.0x10^4$ CFU/naris, at this dose the highest colonisation rates were observed. An extended group of participants were inoculated with this dose to give a better precision of estimated colonisation rates (overall colonisation rate of 33% [11/33]). At the higher dose of $1.6x10^5$ CFU/naris lower colonisation rates were observed. A previous human challenge study using serotype 6B found that colonisation rates plateaued at inoculum doses higher than $8.0x10^4$ CFU/naris [139]. This adds to previous data from human challenge studies and murine models which have showed a lack of relationship between dose of inoculated pneumococcus and colonisation densities [139 195].

Pneumococcal serotype affects pneumococcal pathogenesis and the bacteria's ability to colonise the host. It has been shown that serotypes differ in their prevalence, tendency to cause disease or outbreaks and their age distribution [89 133 196]. The New Serotypes study did not investigate possible bacterial factors which may have impacted on colonisation potential.

This chapter also reported that nasopharyngeal colonisation does not cause nasal symptoms when symptom data are actively sought from participants for 7 days post inoculation. However, the data suggest that participants who are colonised are more

likely to develop a cough during a colonisation episode. Cross-sectional studies in children have reported that upper respiratory tract symptoms can be a significant risk factor for pneumococcal colonisation [164 191]. It is unknown from this type of study design if colonisation causes symptoms or if concurrent symptomatic viral infection increases risk of colonisation.

Chapter 4 demonstrated that for serotype 15B pre-exposure levels of anti-capsular antibodies were not associated with protection against colonisation acquisition. This was in keeping with previous experimental human challenge study results where baseline levels of 6B specific IgG levels were similar in all participants and did not predict colonisation status following exposure to serotype 6B [10 171]. The role of these antibodies and levels needed to protect against colonisation are still unclear. Murine models have shown that passive transfer of anti-capsular polysaccharide IgG to contacts pups could stop acquisition of colonisation [197]. Pre-exposure PS 15B IgG levels of all participants were ten times higher than the previously established and accepted threshold antibody level which is thought to protect against IPD of 350ng/ml. Protection against mucosal colonisation may be better associated with IgG or IgA which are produced by B cells at the mucosal surface rather than serum levels [171].

This study corroborated observations from previous experimental human challenge studies, demonstrating that a colonisation episode increases serum capsular-specific antibody levels [10 171]. We did not assess the function of the anti-PS responses observed here. However, previous research found that colonisation induced a

significant increase in opsonophagocytic killing activity [10]. In addition, a significant increase in mucosal IgG to PspA levels was observed 28 days following exposure in colonisation positive participants [10]. These results suggest that colonisation may be beneficial and could lead to immune boosting to provide protection against subsequent pneumococcal disease and colonisation. This protection is likely to be serotype-specific as shown by two experimental human challenge studies; one showed 100% protection when participants were re-challenged with a homologous serotype[10]. Whereas the second study demonstrated that heterologous challenge lead to 50% colonisation rates which comparable to conisation rates observed in previous experimental challenge studies using the same serotype (6B) [171].

5.2 Methodological criticisms

One area which could impact the generalisability of results from both studies lies within the recruitment of individuals. All participants were healthy young adults. It is unknown if the rates of results from the two studies described in this thesis would be predictive of results in children, the elderly, or the immunocompromised. Understanding of the drivers of pneumococcal colonisation in these cohorts is important for prevention strategies as they are known to be high risk for pneumococcal infection. Due to concerns about safety, using a human infection model in children is likely unethical and its use in immunocompromised adults or adults with chronic medical complaints would need significant consideration and enhanced safety protocols. Controlled human infection model (CHIM) studies in these groups would allow for a unique opportunity for the investigation of host-pathogen interactions in these groups which are high risk for disease. We could also investigate how differences in the microbiome of these groups effects the dynamics of mucosal colonisation of pneumococcus.

In children higher rates of colonisation would be expected in human infection model studies. Previous studies have shown that the highest rates of pneumococcal colonisation are observed in infants and that rates of colonisation fall with increasing age. Evidence also suggests shorter duration of colonisation episodes occur with increasing age but that children are colonised at high densities [198]. Children are also believed to be the primary reservoirs for community pneumococcal transmission. Therefore it could be hypothesised that the Hand to Nose study may have shown

higher rates of transmission and colonisation in a paediatric population [199]. Multiple epidemiological studies have shown that in family transmission was a key source of new episode of nasopharyngeal colonisation; results suggest that children tend to initiate this by bringing pneumococcus into the household [15 198 200]. Higher densities of colonisation observed in children would likely impact on transmission, this may lessen the impact or need for direct contact for transmission to occur.

Conversely in the elderly many studies have reported low rates of pneumococcal colonisation [201]. One study that sampled over 3000 participants, with a mean age of 74, reported a colonisation rate of just 1.8% [202]. Due to this we would hypothesise that experimental colonisation would also be lower with increasing age. Paradoxically, this reduction in colonisation coincides with an increase in the prevalence of pneumococcal disease in this age group with causes significant morbidity and mortality [201]. The mechanisms for this are unclear. One hypothesis is that in the elderly, increased levels of proinflammatory cytokines may lead to clearance of colonisation before a natural boosting of pre-existing immunity can take place. A further hypothesis is that the elderly have lower density pneumococcal colonisation episodes and therefore colonisation is undetected but is highly prevalent in this age group.

There are some general limitations of the model which may impact on the studies described in this thesis. The bacteria stock used for exposure is derived from culture media and is mid-log phase, these experimental conditions may influence pneumococcal viability and colonisation potential. In addition, it is unknown if the

dose we expose participants to is similar to what the nasopharynx may be exposed to naturally. Due to this our experimental carriage model may overestimate or underestimate the potential for hand to nose transmission of pneumococcus or the potential for 23F and 15B to colonise the nasopharynx. For both studies described in this thesis we investigate the dynamics of mucosal colonisation with only one serotype of pneumococcus. In the community it is likely that we are exposed to multiple pneumococcal serotypes simultaneously and there is evidence to suggest that pneumococcal co-colonisation is not uncommon. One study found that 28.7% of participants who were known to be colonised with pneumococcus using classical culture, were co-colonised with multiple pneumococcal serotypes when using qPCR to detect the serotype [156].

One possible methodological criticism could be the use of nasal washes for detection of nasopharyngeal colonisation. This method has been validated by our team previously; it was found that nasal wash detected significantly more pathogens compared to nasopharyngeal swab and was more comfortable for volunteers [132]. More recently, it has been suggested that for adults trans-oral sampling may be superior for detecting colonisation [159]. No trans-oral samples were taken in either study described in this thesis, using a combination of nasal wash and oropharyngeal swab may have increased detection of pneumococcal colonisation further [159].

Another possible methodological criticism may be the cycle threshold (CT) value of 40 that was used for the qPCR experiments. Many of the culture-negative samples, that were found to be positive for pneumococcal colonisation using qPCR, had an

average Ct value >38, which is very close to the assay limit of 40. This amplification of samples that are culture negative has also been reported previously [155 203-205]. There is no consensus about these borderline positive results; we could attribute these to the superior sensitivity of PCR over culture or it could be attributed to detection bacterial DNA from dead organisms or contamination [151 203-205]. In these studies, our strict laboratory procedures regarding qPCR reduces the possibility that these results are due to contamination by PCR amplicons. In addition, as our samples are from non-sterile sites and the known difficulties of pneumococcal culture, the increased rates of pneumococcal colonisation using qPCR is not unexpected and have been seen in previous studies [149 151].

A further methodological criticism may be that patients were followed up for a maximum of 14 days post pneumococcal exposure and colonisation positive participants completed a course of antibiotics after this final visit to clear colonisation. This limits the ability for us to comment on duration of colonisation episodes in either study. The short follow up period of participants limits our ability to assess how duration of colonisation and different transmission methods affect longevity of colonisation. To confidently answer these questions participants would need to be followed up at regular intervals until they naturally cleared the pneumococcus.

For the Hand to Nose study one possible criticism may be the investigation of only potential transmission of pneumococcus by self-inoculation. There was also no investigation of pneumococcal shedding from a colonisation episode. Recent murine

models have highlighted the importance of understanding pneumococcal shedding to understand transmission, we need to better understand the process of pneumococcal exit from the colonised host [20] [49]. Examining the relative risk of pneumococcal spread via aerosolised bacteria, direct contact or indirect contact would be beneficial similar to previous studies investigating transmission of respiratory viruses [206-209]. One study described multiple experiments to investigate the transmission of rhinovirus using laboratory infected participants (donors) and susceptible participants (recipients) [206]. These experiments included:

- **Investigation of spread by aerosol**. The infection rate of recipients who were not allowed to touch their faces during contact with donors was analysed.
- **Investigation of a combination of spread by aerosol, indirect contact and direct contact.** Infection rate of recipients who were in contact with donors with no restrictions.
- **Investigation of possible transmission through infected fomites.** The only contact between donors and recipients in this experiment was potential fomites/objects which had been heavily used by donors for 12 hours.

Finally, neither study investigated the potential effects of the URT microbiome on pneumococcal colonisation. To successfully colonise the nasopharynx, the pneumococcus must compete with many other micro-organisms including *Staphylococcus aureus* and coagulase-negative staphylococci. It is still unclear why some individuals are at lower risk of pneumococcal colonisation and subsequent disease. It is hypothesised that competition and co-operation between the

pneumococcus and other bacteria which colonise the nasopharynx likely influences the incidence of colonisation episodes [210] [211]. Investigating and comparing the microbiome of participants in our model who become colonised and those who do not could provide potential new strategies to limit pathogenesis.

5.3 Implications and future work

5.3.1 Chapter 3: Hand to Nose

The hands have been implicated as vehicles for transmission of many pathogens and viruses. The results from Chapter 3 suggest that pneumococci can be transmitted by a similar process. Due to the high burden of disease caused by pneumococcus and the rise in antibiotic resistance, strategies to prevent pneumococcal infection are more important than ever. Better understanding of how pneumococcus is transmitted from person-person is crucial and can inform more effective prevention strategies. There is clear evidence that handwashing reduces transmission of many bacterial and viral pathogens and can save lives. This study adds to this knowledge base by specifically looking at hand transmission of pneumococcus. Unlike many other pathogens, the person colonised with pneumococcus is generally clinically well and asymptomatic which often relates to lower rates of hand washing. Our results suggest that regular hand washing, and cleaning of potential fomites could be an important approach to reducing the transmission of pneumococci via these routes. This is particularly important in care setting such as child care centres and hospitals where transmission and colonisation have a higher risk of leading to disease. These findings can be useful in health education campaigns and in the event of further epidemics of pneumococcal disease can inform containment strategies. Gavi, the Vaccine Alliance, in 2017, partnered with Unilever to promote handwashing with soap and immunisation together as they believe that promoting these two cost-effective child survival interventions together could save many lives [212].

We have developed a human infection model which can investigate transmission dynamics in a controlled environment. Following this pilot study, the international consumer goods company Unilever have funded the use of this modification of the EHPC model. Our group has recently concluded a randomised control trial to investigate the effectiveness of their anti-bacterial soap, Lifebuoy. In future the model could be in the product development phase of new hand cleaning products to ensure reduction in transmission of this important bacterial pathogen. On the other hand, future work could show that soap universally stops pneumococcal transmission, irrespective of formulation which could advise public health interventions in the developing world.

This chapter's results also add to the growing amount of research suggesting that molecular methods of pneumococcal detection have an important role to play in the detection of colonisation episodes. Culture is considered the gold standard method for detection of upper respiratory colonisation of pneumococcus and is recommended by the WHO Pneumococcal Carriage Working Group [150]. We have demonstrated that using microbiological culture and lytA qPCR methods are complementary. Simultaneous use improves detection rates of pneumococcus, especially in low density colonisation episodes and improves detection of simultaneous colonisation with multiple pneumococcal serotypes. This is important for any future transmission studies and for vaccine efficacy studies using experimental colonisation as a primary endpoint. If the endpoint used in future studies is a reduction in colonisation rates or density, the sensitivity of methods used to accurately detect these changes becomes increasingly important. The results from

our study suggest that using culture only may overestimate or underestimate the effect of any intervention if low density colonisation episodes are missed. From the current research, the significance and natural history of low density colonisation episodes is unclear. Future research investigating the dynamics of mucosal colonisation in participants who are qPCR positive culture negative would be beneficial to further inform this issue.

One way in which this research could be taken forward is to investigate how long pneumococcus continues to be viable at high enough numbers to cause colonisation following drying on the hands. In addition, better understanding of pneumococcal survival duration in nasal secretions would help to understand the dynamics of pneumococcal transmission. Pneumococcal shedding in humans during a colonisation episode has yet to be researched. Understanding how the pneumococcus exits the colonised host and which factors increase this process is also important for prevention of transmission.

5.3.2 Chapter 4: New Serotypes

Chapter 4 increases the scope of the EHPC model, this study showed the success of a non-pneumococcal vaccine type serotype for the first time in a human infection model. The results of this study improves current understanding of colonisation dynamics of serotype 15B, which is one of the most prevalent serotypes causing invasive pneumococcal disease currently in England and Wales [166]. Understanding of non- vaccine serotypes is increasingly important. The widespread use of pneumococcal conjugate vaccines has significantly reduced invasive pneumococcal

disease caused by serotypes covered by the vaccine. It has been estimated that nearly 40,000 invasive pneumococcal disease cases have been prevented in England and Wales since PCV was introduced [166]. However, the rapid increase in some non-vaccine serotypes highlights the importance of improved understanding of these non-vaccine serotypes. Using the EHPC model we can investigate colonisation dynamics of different pneumococcal serotypes which represent a variety of capsular types and protein expression profiles. Evaluating how serotype specific factors effect colonisation and immune responses, especially for serotypes not included currently in vaccines, may help aid in the development of new vaccines. This MD project focuses predominantly on the clinical aspects of the model, a number of other analyses and mechanistic work were undertaken by the wider EHPC team but are not discussed in this thesis.

The success in developing the EHPC model to include this non-vaccine serotype further supports its use for the testing of novel vaccines. Colonisation is a prerequisite for invasive disease and is the primary reservoir for pneumococcal transmission in the community. Herd immunity has been observed following the introduction of PCV into childhood immunisation programmes; control of nasopharyngeal colonisation has been suggested as the mechanism for this phenomenon. Due to this, it has been suggested that the EHPC model can use prevention of colonisation, or reduction in colonisation density as a surrogate of vaccine-induced immunity and potential for herd immunity. If this is true, it would allow for a cost-effective method of down-selecting pneumococcal vaccine candidates early in development. Using the controlled human infection model

(CHIM) early in vaccine development timeline, allows for preliminary efficacy testing to be carried out in a small number of participants. Early results suggesting poor efficacy can minimise the risks of a late clinical failure in following expensive phase 2 and 3 clinical trials [128].

Extending this model to investigate other serotypes and testing of a mixed serotype inoculum may provide important information about interspecies competition and how different serotypes with different capsules and expressed proteins effect colonisation. A recent study which used both standard culture methods and qPCR for pneumococcal detection found that culture alone significantly underestimated the rate of simultaneous colonisation of multiple serotypes [156]. This study highlighted the need for better understanding of the dynamics of interspecies competition and simultaneous colonisation. This may help to identify steps during transmission, colonisation or infection, where pneumococci must pass through a microbiological bottleneck and may help understanding of vaccine escape mechanisms. A study in which healthy adult participants are inoculated with a mixed inoculum could investigate this, either multiple serotypes, or similar serotypes with different gene expression, genome sequencing could be used to support identification of serotype recovery.

5.4 Overall considerations

The work conducted for this thesis adds to the current understanding of what drives nasopharyngeal pneumococcal colonisation. Results from the Hand to Nose project

uncovered the role of the hands as vehicles for the transmission of pneumococcus and acquisition of colonisation. This has never been shown in humans before, this modification of the human challenge model can in the future build upon recently developed murine models investigating pneumococcal transmission dynamics. Future studies should aim to investigate bacterial shedding during colonisation episodes and identify potential factors that promote shedding. Identification of factors that must be inhibited in order to effectively reduce pneumococcal transmission is key in prevention of pneumococcal disease.

The growing prevalence of serotypes not included in the PCV immunisation is a major concern. In children serotype 15B/C was a common coloniser prior to the introduction of PCV (2001) and its prevalence has continued to expand since the vaccine was introduced into many childhood immunisation programmes worldwide [176]. The use of the novel human challenge model used in this project has allowed for better understanding of this serotype and how host and bacterial factors can affect pneumococcal colonisation. A major challenge of new potential vaccine candidates is the huge costs involved in large scale phase II and III studies. The successful development of the EHPC model to include serotype 15B, which PCV 13 immunisation does not protect against, would support the models use in future vaccine testing, aiming at down-selecting candidates that failure to show an impact on colonisation. An area which needs further study prior to this is the question of how we measure vaccine efficacy with colonisation; what level reduction in acquisition is needed for protection, or can a reduction in colonisation density suffice to infer protection and herd immunity by reducing transmission?

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7 Appendices

7.1 Appendix A: Safety information leaflets

Hand to nose transmission of streptococcus pneumoniae in healthy participants – pilot study

Safety Information Sheet

If you are very unwell	Contact your GP or Emergency Department Simultaneously inform the research team tel: 07912 053 981
If you have mild or moderate symptoms	Contact the Respiratory Research team Land line: 0151 706 3381 Mobile: 07912 053 981 Royal Liverpool Hospital 0151 706 2000
If you are well	For safety monitoring we ask you to text us daily for the first 7 days before 12.00 (inc weekends) Tel: 07912 053 981

If you are unwell:

Pneumococcus bacteria may cause infection. Although this is very unlikely we ask you to familiarise yourself with early symptoms or signs that may indicate infection to make sure they are recognised and treated. Please contact the research team who are available day and night if you have any symptoms as follows:

If you have these symptoms Contact the research team:	Fever (temp>37.5 ˚C) (if below 36 ˚C please recheck) Shivering Headache New rash Drowsiness Cough Earache Signs of skin infection: pimples, blisters or raised red itchy bumps with redness or swelling

If you are very unwell or concerned about your health *Caution:* it is possible that you may be unwell for another reason not related to these bacteria.	Seek urgent care from your GP or hospital Start taking the antibiotic (one tablet of amoxicillin three times a day) Tell the doctor: You were exposed to l*ive Streptococcus pneumoniae on your hand and attempted to transmit this into your nose. It is sensitive to amoxicillin* *Your GP records say you have no history of allergy to this antibiotic.*

What if I am not near a phone?

If you are unwell and you are unable to make contact with the research team, we would advise that you start taking your antibiotics straight away. If you have any concerns we recommend you attend your nearest GP, Walk in centre or Emergency Department.

What do I tell the doctor?

If, for any reason you have to attend your doctor or the hospital you need to inform them that you have had live Streptococcus pneumoniae inoculated into your nose which is can be treated by amoxicillin as it is sensitive to this antibiotic). Please contact us as soon as you can.

What if I have a general cough or cold?

Please contact us and we can advise you whether we would like to assess you in the clinic. In some cases, we may ask you to have a throat swab to confirm if you have an infection or advise you to take the amoxicillin antibiotic or advise you to see your GP.

Safety monitoring

To ensure you are not experiencing any problems we ask you to contact us daily after we have put bacteria in your nose for week and to inform us at any time in the study if you are unwell for any reason.

For the first 7 days please:
Set an alarm on your phone
Take your temperature each morning
Text your temperature and if you are well any time before 12.00
If we do not hear from you, we will text then phone you.

Provide contact details of someone that can contact you if we have not heard from you (housemate etc)

Explain to them that you are in this research and that we may contact them if we are unable to contact you...

Record symptoms daily on the diary provided and return this at your clinic appointment
Keep your antibiotics and our contact details with you at all times during the study (return the antibiotic if unused on your last visit).

At any time in the study:
If you feel unwell, please contact us to let us know we are available day and night

Inoculation LEAFLET
Experimental Human Pneumococcal Carriage (EHPC) Model Using New Serotypes

Information Sheet
EMERGENCY RESEARCH TEAM
07912053981 24 hours/ day
OR CALL
0151 706 2000
Hospital Switchboard
Ask for Respiratory Research

Do you have any of the following?

Fever (>37.5 ˚C)
Headache
Rash
Drowsiness
Cough
Earache
Generally Unwell

YES

NO

Call the Emergency Research Team on 07912 053 981 24 hours/ day
Or Call 0151 706 2000 and Ask for Respiratory Research

If you are unable to get to a telephone

start taking your antibiotics

immediately

Contact Research Team by 12 noon every day for first 7 days on **07912 053 981**

What should I do?

If you have any of the above symptoms we would ask that you should contact the research team on the following numbers without delay 07912 053 981 24 Hours/ Day
0151 706 2000 Hospital switchboard - ask for Respiratory Research. The Consultant will be available by telephone 24 hours a day for advice.

What if I feel very unwell?

In the unlikely event you feel very unwell we advise you to start taking the antibiotics immediately and phone the emergency research team (xxxxx xxx xxx). In the unlikely situation that you are unable to make contact with the team we recommend you attend your nearest Emergency department.

What if I am not near a phone?

If for any reason you are unable to make contact with the emergency research team, we would advise that you start taking your antibiotics straight away. This is one tablet (500mg) of AMOXICILLIN to be taken three times per day. If you have any concerns we recommend you attend your nearest Emergency Department.

What do I tell the doctor?

If, for any reason you have to attend your doctor or the hospital you need to inform them that: You have had live Streptococcus pneumoniae type _____ inoculated into your nose on ___/___/_____ as part of a randomised control trial into nasal carriage and vaccination. The bacteria you carry are fully sensitive to amoxicillin and you have no history of allergy to this antibiotic.

Do I need to do anything if I feel well?

We ask that for the first 7 days you text or phone the research nurse by 12noon every day on the following number: 07912 053 981
This is to ensure that you are not experiencing any problems. If we do not hear from you by 12noon we will contact you to make sure you are not experiencing any problems. In the event that we cannot contact you, your next of kin will be contacted.

Things you should know.....Following inoculation with pneumococcus

After the pneumococcus is put into your nose it is possible that it may cause an infection. Although this is very unlikely it is sensible that you familiarise yourself with symptoms or signs that may indicate infection to make sure they are recognised and treated early. Keep your thermometer, antibiotics and contact numbers with you at all times during the study.

WHAT SHOULD I LOOK OUT FOR?
If you feel generally unwell or have any of the following:
Fever (temp>37.5 ˚C)
Shivering
Headache
New rash
Drowsiness
Cough
Earache

If you have any of the symptoms or signs marked in bold please call the emergency number immediately.

07912 053 981 24 hours/ Day
OR
Phone 0151 706 2000 and ask for Respiratory Research

7.2 Appendix B: Daily Symptom Logs

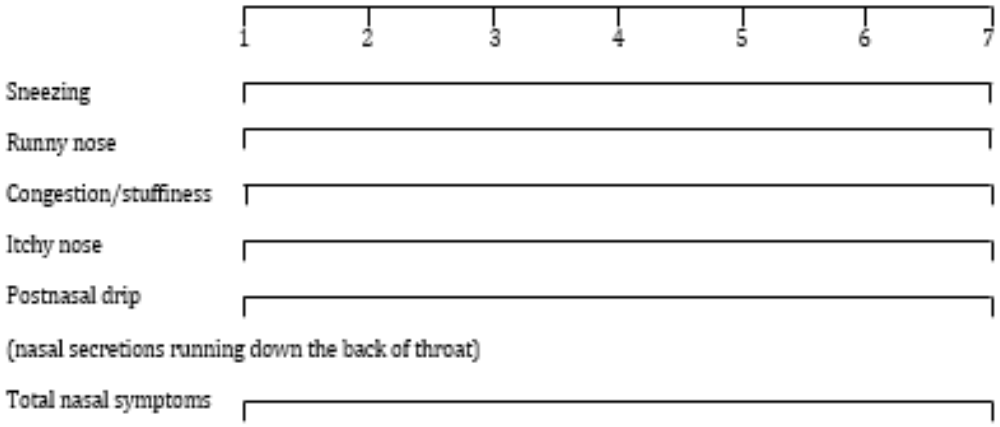
Daily symptom log used for all participants inoculated with 23F serotype. Adapted from modified Likert Score from Spector et al [172].

Day 0 Inoculation

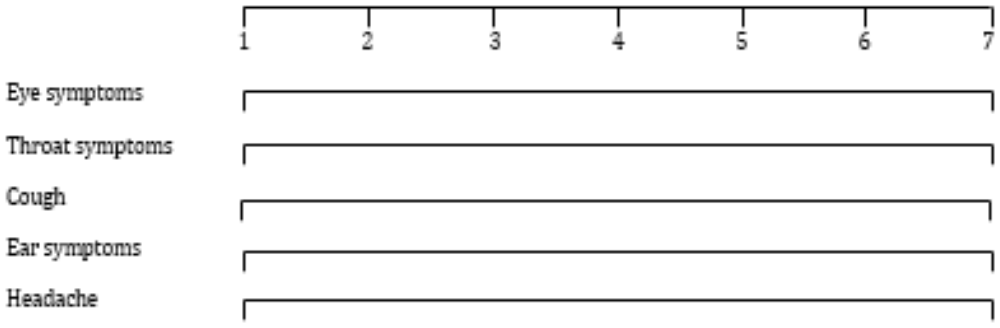
Key to symptoms

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



Assessment of non-nasal symptom severity



Overall assessment of both nasal and non-nasal symptom severity

Daily symptom log for all participants inoculated with 15B serotype

Daily symptom log: Day 0: Inoculation day in the following table, please mark one circle in each row according how much your symptoms have bothered you in the last 24 hours. ***We expect that the majority of our patients will not experience any of these symptoms.***

In the past 24 hours, how much have you been bothered by:	Did not have symptom	Had symptom, but not bothered	A little bothered	Moderately bothered	Quite a bit bothered	Extremely bothered
Sneezing	○	○	○	○	○	○
Runny nose	○	○	○	○	○	○
Congestion/stuffiness	○	○	○	○	○	○
Itchy nose	○	○	○	○	○	○
Post-nasal drip (nasal secretions running down back of throat)	○	○	○	○	○	○
Overall nasal symptoms	○	○	○	○	○	○
Coughing	○	○	○	○	○	○
Chest pains	○	○	○	○	○	○
Shortness of breath	○	○	○	○	○	○
Coughing up phlegm	○	○	○	○	○	○
Sweating	○	○	○	○	○	○
Chills	○	○	○	○	○	○
Headache	○	○	○	○	○	○
Nausea and/or vomiting	○	○	○	○	○	○
Muscle pain	○	○	○	○	○	○
Lack of appetite	○	○	○	○	○	○
Trouble thinking or concentrating	○	○	○	○	○	○
Fatigue	○	○	○	○	○	○
Trouble getting to sleep due to difficulty breathing	○	○	○	○	○	○
Wakening due to breathing difficulty	○	○	○	○	○	○
Wheeze	○	○	○	○	○	○
Overall general symptoms	○	○	○	○	○	○