Institute of Infection and Global Health

Department of Clinical Infection, Microbiology and Immunology

Natural Immunity to Pneumococcal Pilus-1 RrgA and RrgB Antigens, and Its Relationship with Pneumococcal Carriage

Thesis submitted in accordance with
the requirements of the University of Liverpool
for the degree of Doctor in Philosophy

by

Muhammad Shamsher Ahmed

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Abstract

Streptococcus pneumoniae (pneumococcus) is a leading cause of childhood morbidity and mortality around the world. The available polysaccharide-based vaccines are limited by either low efficacy in young children or narrow serotype coverage. Recent research has focused on developing protein vaccines. Pneumococcal pilus-1 proteins play an important role in pneumococcal adherence to host respiratory epithelium and subsequent bacterial colonization or invasion; and may therefore be an effective vaccine candidate. This PhD project investigated natural immunity in humans to pneumococcal pilus-1 proteins and its association with nasopharyngeal carriage of pneumococcus.

Nasopharyngeal carriage of pneumococcus was analysed by bacteriological culture of nasal swabs on blood agar. Pneumococci were identified based on their colony morphology and optochin sensitivity; and further confirmed by PCR detection of pneumolysin gene in the isolates. Pneumococcal carriage was found to be common in young children, which gradually decreased with advancing age. PCR detection of pilus islet 1 (PI-1) gene revealed that percentage of pilus-1 positivity was low among these carriage isolates. This low prevalence may be associated with the recent introduction of pneumococcal conjugate vaccination, which covers the common piliated serotypes.

ELISA based measurement of serum and salivary antibodies to pilus-1 proteins detected significant antibody levels to both RrgA and RrgB, presumably developed as a part of natural immune response in children and adults. An age-dependent increase in serum antibody levels to both RrgA and RrgB was also observed, and anti-RrgA appeared to develop earlier in childhood than anti-RrgB. Moreover, higher levels of antibody, especially anti-RrgA were found in children who were culture-negative than in those who were culture-positive for pneumococcus. It suggests that these naturally developed antibodies may contribute to the protection against pneumococcal carriage in humans.

Using an *in vitro* model of human NALT, the study revealed that adenotonsillar tissues are important induction sites for immune response to these antigens, by priming B cell memory. The induction of antibody secreting cells in NALT was enumerated by ELISpot assay; and the antibody production was measured by antigen-specific ELISA. *In vitro* stimulation with a wild type (TIGR4) pneumococcal culture supernatant (containing both RrgA and RrgB proteins) induced a significant memory B cell and antibody response in adenotonsillar cells. Current carriage *in vivo* enhanced the memory B cell response and antibody production.

Flowcytometric analysis of CFSE labelled adenotonsillar MNC suggested that pneumococcal pilus-1 proteins RrgA and RrgB were capable of stimulating $CD4^+$ T cell proliferative response in human NALT. Stimulation with pneumococcal CCS induced Th17 related cytokines production in both human adenotonsillar MNC and PBMC culture supernatant. Importantly, this i*n vitro* production of Th17 cytokines after stimulation with TIGR4wt CCS was significantly higher than that of CCS derived from its isogenic $RrgA^{-/-}$ and $RrgB^{-/-}$ mutants, indicating a contribution from both of these proteins. The ability of these pilus-1 proteins to stimulate $CD4^+$ T cell proliferation and Th17 response may contribute to the natural immunity to pneumococcus in humans.

This study has revealed that both of these pilus-1 proteins, RrgA and RrgB are immunogenic and are capable of priming for memory B and T cell response in human NALT. These findings aid to our understanding on the naturally developed immune response to pilus-1 proteins, and may inform future vaccination strategy with intranasal immunisation containing protein antigens against pneumococcal infection.

Index

Declaration

No part of the work referred to in this thesis has been submitted in support of an application for another degree or qualification at this or any other university, or other institution of learning. All laboratory experiments described here have been carried out by the author, in the Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool.

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Muhammad Shamsher Ahmed

October 2013

Table of Contents

List of tables

List of Figures

List of Abbreviations

ANOVA	Analysis of variance
AP	Alkaline phosphatase
APC	Antigen presenting cell
ASC	Antibody secreting cell
BA	Blood agar
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
CbpA	Choline binding protein A
CCS	Concentrated culture supernatants
CD	Cluster of differentiation
CDC	Centres for Disease Control and Prevention
CFSE	5-(6) Carboxyfluorescein diacetate succinimidyl ester
cfu	Colony forming units
CI	Confidence interval
cm	Centimetre
CO_2	Carbon dioxide
CPS	Capsular polysaccharide
DC	Dendritic cell
ddH_2O	Deionised distilled water
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetate
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunosorbent spot
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	Centrifugal force
GC	Germinal centre
gm	Gram
GMT	Geometric mean titre
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
hr	Hour
HRP	Horseradish peroxidase
ICAM-1	Intracellular adhesion molecule-1
IFN-γ	Interferon gamma
IgA	Immunoglobulin A

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
kb	Kilobase
l	Litre
LP	Lipoprotein
LPS	Lipopolysaccharide
LytA	Autolysin A
M	Molar
mA	Milliampere
mAb	Monoclonal antibody
MALT	Mucosal-associated lymphoid tissue
mg	Milligram
MHC	Major Histocompatibility Complex
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MNC	Mononuclear cell
MSCRAMM	Microbial surface component recognising adhesive matrix molecules
NALT	Nasal-associated lymphoid tissue
NCBI	National Centre for Biotechnology Information
NCSP	Non-classical surface proteins
NF-κB	Nuclear factor-kappa B
ng	Nanogram
NIST	National Institute of Standards and Technology
NICE	NIST's Integrated Colony Enumerator
NK cell	Natural killer cell
NLR	Nod-like receptors
OD	Optical density
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-0.05%Tween20
PCho	Phosphorylcholine
PCR	Polymerase chain reaction
PE	Phycoerythrin
pg	Picogram
Pht	Pneumococcal histidine triad proteins
PI	Pilus islet
Ply	Pneumolysin
PNPP	P-Nitrophenyl Phosphate
PRR	Pathogen recognition receptor
PsaA	Pneumococcal surface adhesin A

PspA	Pneumococcal surface protein A
rlrA	*rofA* like regulator
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
rpm	Revolutions per minute
RT	Room temperature
SC	Secretory component
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEB	*Staphylococcus aureus* Enterotoxin
sec	Second
SEM	Standard error of the mean
S-IgA	Secretory Immunoglobulin A
SP	Surfactant protein
Srt	Sortase
SSC	Side scatter
STGG	Skim milk, tryptone, glucose, glycerol transport medium
TBS	Tris buffered saline
TBS-T	Tris buffered saline-0.05%Tween20
TCR	T-cell receptor
TG	Triglyceride
Th	T helper
Th1	T helper 1 cell
Th17	T helper 17 cell
Th2	T helper 2 cell
THYB	Todd-Hewitt-Yeast broth
TI	T cell independent
TIGR4	The Institute for Genomic Research *Streptococcus pneumoniae* serotype 4
TIGR4wt	TIGR4 wild type
TLR	Toll like receptor
TNF-α	Tumour necrosis factor alpha
U	Units
v	Volts
WB	Western Blot
WCA	Whole cell antigen
WCV	Whole cell vaccine
WHO	World Health Organization
wt	wild type
yr	year
µg	Microgram
µl	Microlitre
µM	Micromolar

Chapter 1

General Introduction

1.1 Natural Immunity to *Streptococcus pneumoniae*

Streptococcus pneumoniae (pneumococcus), a Gram positive bacterium, is responsible for a wide spectrum of diseases in humans, ranging from asymptomatic colonisation of upper respiratory tract to life threatening infections. It is one of the leading causes of childhood morbidity and mortality around the globe. Infection rate is particularly high in developing countries (O'Brien et al. 2009). Pneumococcus is responsible for some common mucosal infections in childhood, such as otitis media. It also causes a number of invasive infections, such as bacteraemic pneumonia, septicaemia and meningitis (CDC 2000). It is responsible for over 50% of severe pneumonia cases, and probably a higher proportion of fatal cases (Hodge 2006). It is the leading cause of community acquired bacterial meningitis and septicaemia in childhood (Peltola 2001).

Pneumococci are transmitted by direct contact with respiratory secretions from patients or healthy carriers. They may remain as a part of normal microbial flora in the upper respiratory tract in 5-40% humans (Brooks and Carroll 2007). The primary site of colonisation is nasopharynx. This carriage is generally asymptomatic in immunocompetent individuals. However, prolonged nasopharyngeal carriage favours the escape of pneumococcus into the middle ear cavity and normally sterile parts of the respiratory tract, resulting in otitis media and pneumonia. The other severe forms of infection (septicaemia or meningitis) occur as a sequel of these mucosal infections. The persistence of carriage also serves as a potential source for spreading the infection to close contacts (Bogaert et al. 2004a).

The onset of colonisation varies in different part of the world. But it is believed that, almost every child is colonised by at least one of the pneumococcal serotypes before their second birthday (Käyhty et al. 2006). Pneumococcal carriage is potentially

immunogenic, and triggers both local mucosal and systemic immune response, conferring protection against re-colonisation or invasive pneumococcal disease (Ferreira et al. 2013). Pneumococcal carriage in the early childhood primes for immunological memory to pneumococcal antigens (Vukmanovic-Stejic et al. 2006) and that may lead to the development of protective immunity in later life (Zhang et al. 2006b).

Considering the huge burden of diseases caused by pneumococcus, development of vaccines against pneumococcus has been one of the priorities. Reports of the strong immunogenicity of pneumococcal capsular polysaccharides identified them as vaccine candidates (Heidelberger and Avery 1924). Based on the success of a preliminary trial (MacLeod et al. 1945), the first commercial production of a quadrivalent pneumococcal polysaccharide vaccine was started after the end of the Second World War. However, pneumococcal capsular polysaccharides are highly variable, which serve as the basis of its classification into more than 92 serotypes (Calix and Nahm 2010). Later efforts were concentrated more on increasing the serotype coverage and eventually a 23-valent polysaccharide vaccine (PPSV23) was introduced in 1977. However, the polysaccharide vaccines do not induce T cell dependent antibody and memory response. Hence it is not effective in young children (<2 years), who are the most vulnerable to pneumococcal colonisation and invasion (Bogaert et al. 2004b).

Conjugation of a protein carrier to capsular polysaccharide has overcome these limitations, but the coverage they offer is limited. In addition, invasive infections by serotypes not covered in these vaccines are posing increasing threats (Spratt and Greenwood 2000). Moreover, they are too expensive to be included in the universal immunisation schedule in the developing countries. For these reasons, efforts have

been made to develop protein vaccines, which will be effective against most serotypes. Also commercial production of protein vaccines is relatively easy and may be more cost effective (Bogaert et al. 2004b). Identification of suitable protein vaccines has therefore become a key target in the pneumococcal vaccine research. A number of surface proteins are being evaluated for this purpose.

1.2 Pneumococcus and Disease Burden

S. pneumoniae is the commonest aetiologic agent of community-acquired pneumonia (Welte et al. 2010). According to a WHO report in 2005, pneumonia was ranked as the top-most killer disease of under-five (<5 years) children, which was responsible for nearly one-fifth of the 10.6 million deaths around the world (Bryce et al. 2005). Nearly 156 million under-five children suffered from pneumonia in the year 2000; more than 95% of them were in developing countries. One out of 12 cases of pneumonia was severe enough to be hospitalised, with the death toll approaching 2 million per year. Most of those death occurred in Africa and South-East Asian countries (Rudan et al. 2008). The pneumococcal burden is high in the developed countries also, with remarkably increased vulnerability in elderly. Pneumococcus is responsible for nearly half of the cases of community-acquired pneumonia in adult population in the UK (Lim et al. 2001) and in Finland (Jokinen et al. 2001). In developed countries the incidence rate is higher among the underprivileged and indigenous population compared to the average national burden (Davidson et al. 1994; Torzillo 1997).

People of any age may be infected by pneumococcus, but those in extremes of age (<2 years and >65 years) are at particular risk. Chronic heart or lung disease, smoking and splenectomy increase the hazard (Örtqvist et al. 2005). Any preceding viral infection of the lungs (i.e. influenza) enhances the susceptibility and fatality of

pneumococcal infection (Brundage 2006). Another important risk factor is HIV infection (Hart et al. 2000). The risk of pneumococcal colonisation and infection is higher in densely populated communities (Dagan et al. 2000).

1.3 Microbiology of *Streptococcus pneumoniae*

Streptococcus pneumoniae are Gram-positive, encapsulated cocci. In Gram stained smears they often appear as elongated cocci, with outer curvatures somewhat pointed; giving them the shape resembling lancets. They usually occur in pairs (diplococci), may also appear in solitary or in short chains (Brooks and Carroll 2007). The average diameter of the bacterium varies from 0.5µm to 1.25µm. They are usually non-motile, non-sporeforming, facultative anaerobes. Like other *Streptococci*, they are catalase negative, but can be differentiated from the β-haemolytic group A *Streptococci* by their characteristic α-haemolytic (green) colonies in blood agar. On blood agar, they produce glistening raised colonies, measuring roughly 1mm in diameter. With progression of time, the colonies start to flatten and small depressions appear in the centre, owing to the activities of an autolytic enzyme. This typical plateau-shaped colony morphology, popularly termed as 'draughtsmen' colonies; together with some special biochemical tests are used to differentiate them from other α-haemolytic upper respiratory tract commensals. The properties of inulin hydrolysis, bile solubility and optochin (ethylhydrocupreine) sensitivity are used to distinguish *Streptococcus pneumoniae* from the other α-haemolytic (viridans) *Streptococci* (Ryan 2003).

Almost all pathogenic strains of *Streptococcus pneumoniae* have a polysaccharide capsule surrounding their cell wall (Mitchell and Mitchell 2010). These capsular polysaccharides react with the specific antisera, showing the 'Quellung' (swelling) reaction. Based on this property of capsular polysaccharides, pneumococci were

classified into 90 different serotypes (Henrichsen 1995). Later, two new serotypes 6C (Park et al. 2007) and 11E (Calix and Nahm 2010) were identified.

Like other gram positive bacteria, pneumococcal cell wall is composed of thick peptidoglycan layer, teichoic and lipotiechoic acid residues. Teichoic acid residues are bound to the N-acetylmuramic acid of peptidoglycan. They contain two forms of phosphorylcholine (PCho), free or protein bound. PCho are capable of triggering innate response by C-reactive proteins and lung surfactants. Also, the proteins anchored on them are important mediators of pneumococcal inflammation and tissue damage in the host (McCullers and Tuomanen 2001). The lipoteichoic acids are attached to the cell membrane by their lipid moiety. Recently, hair-like projection, also known as pili were found in the surface of many pneumococcal strains (Barocchi et al. 2006).

The complete genomic sequence of 'The Institute for Genomic Research' serotype 4 (TIGR4) strain of *Streptococcus pneumoniae,* revealed 2,160,837 base pairs in a single circular chromosome. The genome contains 2,236 predicted protein coding regions, 1440 of those was biologically functional (Tettelin et al. 2001). Some of these proteins are integrated in the cell wall. The others are membrane-associated lipoproteins of various structure and functions. Many of these proteins contribute to the virulence of pneumococcus; and some of them elicit protective responses, showing the prospect of being potential vaccine candidates.

1.4 Pathogenesis of Pneumococcal Diseases

From their ecological niche in the nasopharynx, pneumococci might spread endogenously to the carrier or exogenously to other individuals through respiratory droplets. The endogenous spread of pneumococci down to the respiratory tract,

paranasal sinuses, the middle ear cavity, CNS or blood stream causes inflammatory reaction in these organs leading to mucosal infection or invasive diseases. The pathogenic routes for pneumococcal infection are shown in the Figure (Bogaert et al. 2004a).

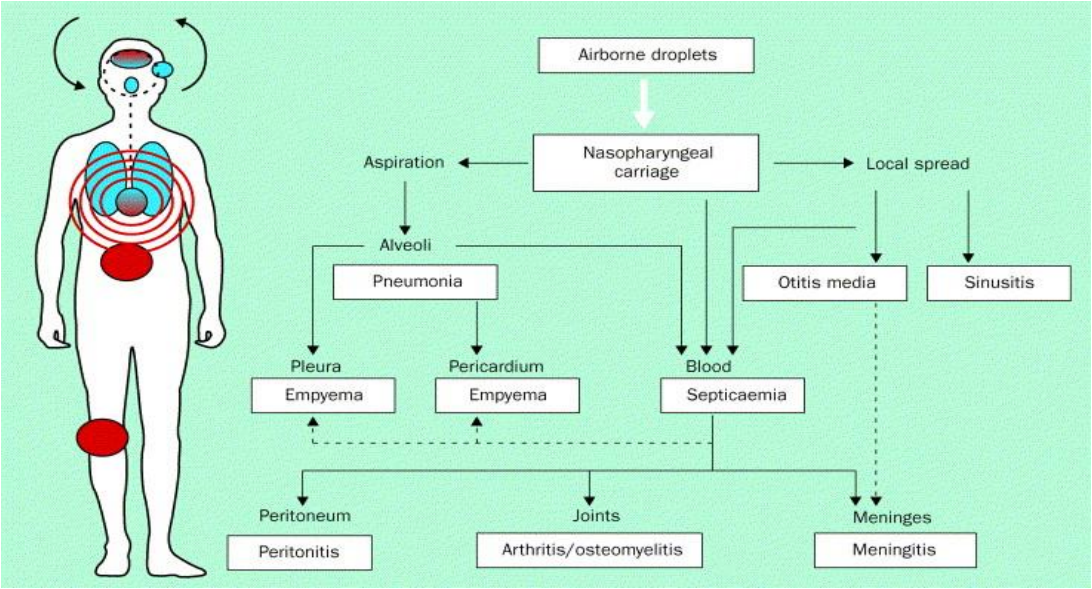


Figure-1.4: Pathogenic routes for *S. pneumoniae* infection. Organs in sky-blue colour get infected by airborne droplets; organs in red are invaded through haematogenous route (Bogaert et al. 2004a).

Otitis media: It is the commonest bacterial infection of children and most frequently caused by pneumococcus (Gray et al. 1980). The middle ear cavity is connected to the nasopharynx by the eustachian tube, through which the organism spreads from its colonisation site.

Acute bacterial pneumonia: *S. pneumoniae* may be transmitted down to the lower respiratory tract leading to suppurative infection in the lungs. Pneumococcal infection triggers release of fibrinous exudates and polymorphonuclear infiltration into the alveoli, resulting in lung consolidation (Johnston 1991). Bacteria are abundant in these exudates and may spread to the bloodstream via the lymphatic

drainage. The onset of pneumococcal pneumonia is usually sudden, with high fever, chills, sharp pleural pain and productive cough.

Bacteraemia and Septicaemia: Bacteraemia in the absence of a focus of infection is commonly caused by pneumococcus, especially in splenectomised individuals. Pneumococcal bacteraemia can lead to severe complications like meningitis, endocarditis, and septic arthritis.

Meningitis: *S. pneumoniae* is a common cause of bacterial meningitis. This disease has a high mortality rate, even when treated appropriately. Infection usually extends from the mastoid, also can follow pneumococcal sepsis.

1.5 Pneumococcal Virulence Factors

1.5.1 Polysaccharide Capsule

Pneumococcal polysaccharide capsule is anti-phagocytic, protecting the bacteria from polymorphonuclear leukocyte attack (Kadioglu et al. 2008). It acts as a defensive barrier around the bacteria, impeding attachment of complement components and immunoglobulins with bacterial cell wall. As a result, bacteria multiply freely before the appearance of anti-capsular antibodies. Capsules also prevent mechanical clearance of the bacteria by airway mucous (Mitchell and Mitchell 2010).

1.5.2. Pneumolysin

This pore-forming toxin is well conserved among pneumococcal isolates (Kadioglu et al. 2008). Pneumolysin (Ply) is located within the bacterial cytoplasm and released after autolytic degradation of bacteria (López et al. 1997). Pneumolysin mediates host cell damage by two mechanisms; pore formation and complement activation

(Gilbert et al. 1999). It is also involved with production of proinflammatory cytokines and chemokines (van der Poll and Opal 2009).

1.5.3. Pneumococcal Surface Proteins

Pneumococci express several proteins on their surface. They could be classified into five broad categories: choline binding proteins (CBP), lipoproteins, LPXTG proteins, histidine triad proteins and non-classical surface proteins (NCSP) (Pérez-Dorado et al. 2012; Adamou et al. 2001). Many of these proteins play specific roles in different stages of pneumococcal infection and disease process. The individual contribution of many proteins to the virulence are well-documented by this time. However, their effects are not mutually exclusive. Knocking-out of an individual protein compromises related virulence properties (adherence, colonisation, invasion, spreading), but does not necessarily make the bacteria totally avirulent. Understandably, the virulence of pneumococcus is multi-factorial; the essence of which is achieved by their coordinated interplay. Their cumulative effect favours for bacterial survival and thriving (Gámez and Hammerschmidt 2012). The roles of different groups of proteins are briefly discussed here.

1.5.3.1 Choline Binding Proteins (CBP)

Choline binding proteins (CBP), share a common structural organisation (Gosink et al. 2000). They are composed of a biologically active site projecting from the bacterial surface; and a choline binding site, anchoring the protein by a non-covalent attachment with phosphorylcholine residues of cell wall teichoic acids. The characteristic of this family is the choline binding motif, usually contains homologous repeats of 20 amino acid residues. So far 16 members of this family have been identified. Several of them, including PspA, CbpA and LytA are

implicated for significant contribution to pneumococcal virulence (Pérez-Dorado et al. 2012). Most of the CBPs are adhesins and exert their pathogenic role through host pathogen interactions. However some of them are associated with enzymatic functions (Frolet et al. 2010).

1.5.3.2 Pneumococcal Lipoproteins

Lipoproteins (LP) appear to be very crucial component for bacterial survival, as evident by their universal expression on bacterial surface. Genomic sequencing and informatics analyses identified 46 lipoproteins on the *Streptococcus pneumoniae* (TIGR4) genome (Tettelin et al. 2001); most of them contributing to its wellbeing and survival. Pneumococcal lipoproteins contributing to its virulence include; divalent metal ion binding ABC transporters (PsaA, PiaA, PiuA), sortases (SrtC, SrtD), and peptide isomerises (PpmA, SlrA) (Bergmann and Hammerschmidt 2006).

1.5.3.3 Pneumococcal LPXTG Proteins

This group of proteins share a common mechanism of covalently anchoring themselves to the cell-wall peptidoglycan. Genomic sequence analyse identified at least 18 LPXTG proteins in pneumococcal TIGR4 strain (Tettelin et al. 2001). Most of these proteins are associated with enzymatic or adhesin functions. Interaction of pneumococcal LPXTG adhesins (PavB, MucB, PclA, PsrP, PfbA and Spr1806) with host sugar or protein molecules allows them to colonise in nasopharyngeal mucosa (Löfling et al. 2011). The enzymatic proteins possess specific domains required for their homing to the target tissues, where they catalyse various substrate molecules. Pneumococcus has six polysaccharide lyasing glycosyl hydrolases; these are SrtH, NanA, BgaA, EndoD, SpuA and Eng. There are also four proteases; ZmpA, ZmpB, ZmpC and PtrA. These proteases hydrolyse host glycoproteins, glycosaminoglycans

and intracellular glycans; thus playing an important role in pneumococcal pathogenesis. The mechanism of pneumococcal adhesion and colonisation is shown in figure-1.5.3.1.

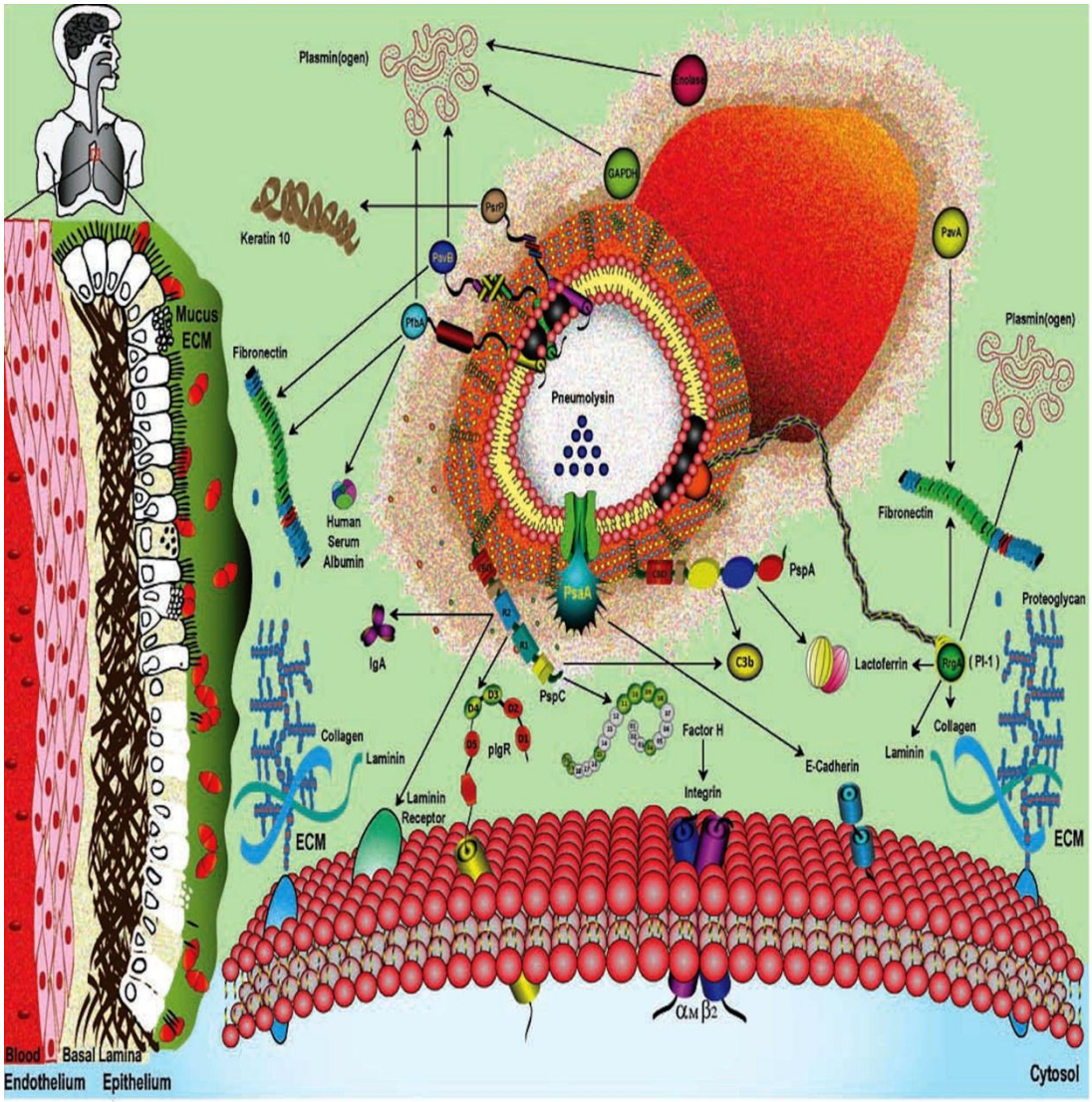


Figure-1.5.3.1: Pneumococcal adherence and colonisation. Pneumococcal adhesins interact directly with human ECM components, soluble plasma proteins and/or cellular receptors at the host epithelium. PavA, PavB, PfbA, RrgA, and PspC interact with ECM proteins such as fibronectin, laminin and collagen. PspA, PspC, PavB, PfbA, RrgA, enolase and GAPDH interact with the circulating plasma proteins like plasminogen, human serum albumin, complement regulator Factor H, C3b, IgA, and lactoferrin. Interactions of PspC with laminin receptor and pIgR, PsrP with Keratin 10, and PsaA with E-cadherin are also shown (Gámez and Hammerschmidt 2012).

1.5.3.4 Pneumococcal Histidine Triad Proteins

This group of proteins share one common thing, four to six repeating histidine triad motifs (HXXHXH) within their sequence (Adamou et al. 2001). These motifs bind to divalent cations, especially with the Zn^{2+} ions. Their interaction with this co-factor was found to have important regulatory effect on the catalytic activity of human phosphodiesterase enzyme, hydrolysing cAMP to AMP (Omburo et al. 1998).

1.5.3.5 Pneumococcal Non-classical Surface Proteins

Streptococcus pneumoniae express a number of proteins that do not show the classical features of other surface proteins. These proteins lacking conventional anchoring or secretory signals are known as non-classical surface proteins. They are cytoplasmic proteins with intracellular metabolic functions, not directly taking part in host–pathogen interactions. However, they could be translocated to the cell surface by mechanisms not yet specified. Some of them act as adhesins to different host molecules; and promotes pneumococcal invasion and the spread of the infection. This group includes six proteins, PavA, Eno, GAPDH, 6PGD, HtrA and PGK.

1.5.3.6 Pilus in Pneumococcus

1.5.3.6.1 Bacterial Pilus

A number of surface structures have been identified to facilitate bacterial adhesion on the host epithelial cells. One of these structures is known as pilus, which is a hair-like projection on bacterial surface; helps them to adhere with the host epithelium. Pilus was first described in Gram-negative bacteria by Charles Brinton Jr. in 1965 and in Gram-positive bacteria by Yanagawa and colleagues in 1968 (Telford et al. 2006).

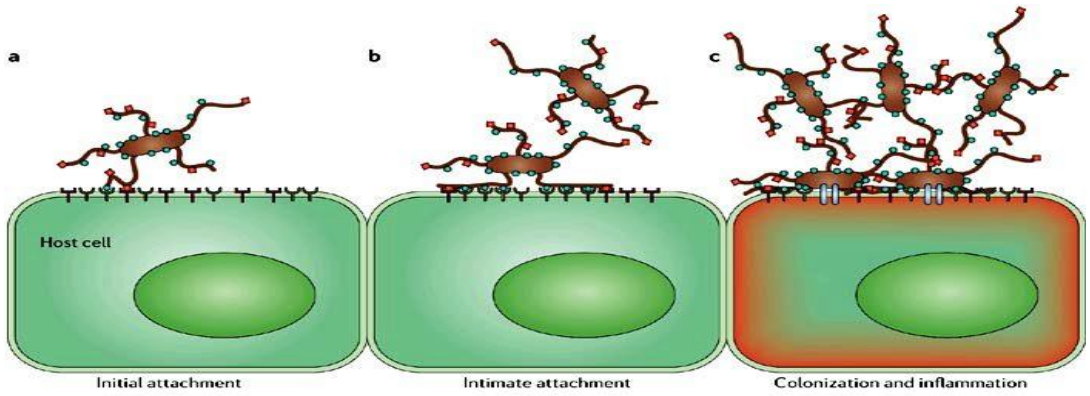


Figure 1.5.3.6.1: Pilus-mediated adherence of streptococcus to cell surfaces. (a) Bacteria initiate attachment to host cells by extending their pili towards the apical surface of host cells in a process involving a tip protein (red). (b) An intimate attachment follows, in which ancillary pilus proteins (green) mediate a zipper-like adhesion of pili to host cells. (c) This leads to colonisation of the apical surface of the host cell, in a process mediated by the expression of high-affinity surface adhesins (Telford et al. 2006).

Gram-positive pili differ from Gram-negative pili structures and assembly mechanisms. Gram-negative pili are composed of non-covalently linked protein subunits, whereas Gram-positive pili are formed by covalent linkage of protein subunits. Pilus in Gram-positive bacteria comprises three protein subunits; each containing an LPXTG motif. They are assembled by covalent linkage of their subunit proteins catalysed by the sortase enzymes. These sortases also attach them to the cell wall peptidoglycan layer by a transpeptidase reaction (Telford et al. 2006). These Gram-positive pili proteins act like microbial surface components recognizing adhesive matrix molecules (MSCRAMM). They bind to the extracellular matrix components (fibronectin) of the host mucosa (Schwarz-Linek et al. 2004). Apart from mediating their adhesion to host mucosa to initiate colonisation, pili also assist in bacterial aggregation to build a microbial community in the infected tissue. Bacterial colonisation particularly with this higher bacterial density leads to a heightened inflammatory reaction and robust immune response (Telford et al. 2006).

1.5.3.6.2 Pneumococcal Pilus Proteins

Barocchi et al. reported the presence of pilus (later designated as pilus type-1) in some of encapsulated strains (TIGR4 and ST162) of pneumococcus (Barocchi et al. 2006). Later on, a different type of pilus (pilus type-2) was demonstrated in some other strains belonging to serotype 1, 2, 7F and 19A (Bagnoli et al. 2008). Pilus-1 has been reported to be present in 30% of pneumococcal strains (Moschioni et al. 2010a); and 27% of the pneumococcal isolates responsible for invasive diseases (Aguiar et al. 2008). On the other hand, 7% of pneumococcal strains possess pilus-2 gene (Moschioni et al. 2010a); and among the invasive isolates its prevalence has been reported as high as 16% (Bagnoli et al. 2008). A recent study in Malawi has revealed relatively lower prevalence of pilus-1 (9%) and pilus-2 (6%) among the invasive pneumococcal isolates (Kulohoma et al. 2013). Prevalence of pilus genes has been reported to be higher in antibiotic resistant strains of pneumococcus (Moschioni et al. 2010a).

Cryo-electron microscopic analysis of purified type-1 pilus of TIGR4 has shown them as elongated structures with a maximum length of 1µm (Hilleringmann et al. 2008). TIGR4 pilus is composed of three subunits. The major structural component is the RrgB, from where subunit RrgA extends to the periphery and subunit RrgC remains attached to the bacterial cell wall (Hilleringmann et al. 2009). RrgA binds to the extracellular matrix (ECM) components; such as, collagen I, laminin, fibronectin, fibrinogen and lactoferrin, but not to vitronectin (Hilleringmann et al. 2008). Binding of RrgB and RrgC to these ECM components is negligible (Hilleringmann et al. 2008). In addition to that, RrgA binds to host respiratory epithelial cells (Nelson et al. 2007).

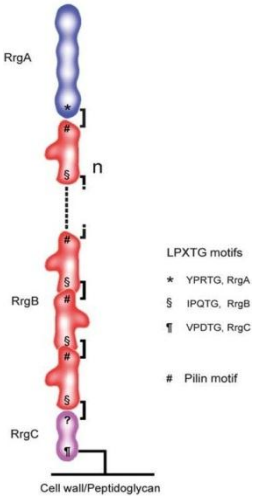


Figure-1.5.3.6.2: Schematic diagram of *S. pneumoniae* pilus structure. The TIGR4 pilus consists of a shaft composed of RrgB, with RrgA at its distal and RrgC at its proximal end. Sortase SrtC-1 mediates the polymerisation of RrgB via the LPXTG motif (§, IPQTG) and pilin motif (#) into a single string of monomers, covalently linked head-to-tail. Sortases also mediates the addition of the ancillary proteins RrgA and RrgC to the pilus shaft, and anchorage of RrgC to the peptidoglycan cell wall, using respective LPXTG motifs for RrgA (*, YPRTG) and RrgC (¶, VPDTG) (Hilleringmann et al. 2009).

Like other Gram-positive pili, these proteins possess C-terminal sorting signals, functioning as MSCRAMMs. The C-terminal sorting signals of TIGR4 pilus protein shows conserved LPXTG motifs and charged tails (Figure-1.5.3.2), except the leucine of LPXTG is substituted by tyrosine, isoleucine and valine in RrgA, RrgB and RrgC respectively (Hava and Camilli 2002).

Protein	C-terminal sequence
RrgA	YPRTGGIGMLPFYLIGCMMMGGVLLYTRKHP
RrgB	IPQTGGIGTIIFAVAGAAIMGIAVYAYVKNNKDEDQLA
RrgC	VPDTGEETLYILMLVAILLFGSGYYLTKKPNN

Figure-1.5.3.6.3: The C-terminal sorting signals of TIGR4 pilus proteins. Underlined portions denote hydrophobic regions (Hava and Camilli 2002).

Two functionally similar variants of RrgA has been reported, clade I (893 amino acids) and clade II (890 amino acids). Both variants have similar structural organizations with an N-terminal leader sequence and an LPXTG-like (YPRTG) C-terminal region (Moschioni et al. 2010b). Overall, they share 84% to 98% sequence identity; and the variability is mostly located in the distal D3 (head) domain, leaving a well conserved D1, D2, and D4 (stalk) domains (Moschioni et al. 2008; Moschioni et al. 2010b). RrgB, the major component of pilus-1 is also organised into four immunoglobulin-like domains; D1 (residues 1-184), D2 (residues 184–326), D3

(residues 326–446) and D4 (residues 446–627) (Gentile et al. 2011; Spraggon et al. 2010). Each of the D2, D3, and D4 domains is stabilized by one covalent intramolecular isopeptide bond, giving them a rigid molecular structure and protect them from proteolytic cleavage (Gentile et al. 2011). Among the three pilus-1 components, RrgB is the least conserved subunit protein, which exists in three different variants with 49% to 67% sequence homology (Moschioni et al. 2008). The most conserved subunit protein of pilus-1 is RrgC showing 98% to 99% sequence homology (Moschioni et al. 2008).

1.5.3.6.3 Pneumococcal Pilus Genes

The genes encoding the two types of pneumococcal pili reported so far are designated as pilus islet (PI) -1 and 2. The PI-1 genes are located in the *rlrA* pathogenicity islet of pneumococcal TIGR4 strain (Barocchi et al. 2006). This islet contains seven genes; namely *rlrA, rrgA, rrgB, rrgC, srtB, srtC,* and *srtD*. The *rlrA* is a regulatory gene*; rrgA, rrgB,* and *rrgC* encode pilus-1 proteins; others (*srtB*, *srtC,* and *srtD*) encode sortases, which are catalytic enzymes (Hava et al. 2003).

The PI-2 locus consists of five genes *pitA, sipA, pitB, srtG1*and *srtG2*. Two of them (*srtG1* and *srtG2)* are sortases; another two code for LPXTG-type pilus protein subunits (*pitB* and *pitA);* and the other one codes a signal peptidase-related product (*sipA*). PitB is the major structural subunit, which forms the backbone of type-2 pilus. SrtG1- and SipA- dependent polymerisation of PitB is the essential step for PI-2 pilus assembly on the bacterial surface. The other two genes *srtG2* and *pitA* are not essential for pilus assembly; and there is not much evidence on their ability to encode functional proteins.

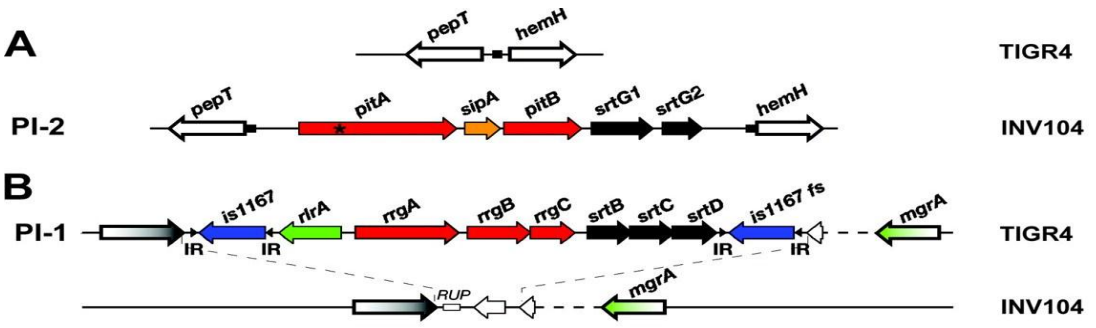


Figure-1.5.3.6.4: The genomic organization of pilus-encoding islets PI-2 (A) and PI-1 (B) in *S. pneumoniae*. Schematic representation of genomic regions in TIGR4 (positive for PI-1 and negative for PI-2); and INV104 strains (positive for PI-2 and negative for PI-1) are shown (Bagnoli et al. 2008).

1.6 Host Immunity to Pneumococcus

1.6.1 Innate Immune Response

The Human innate immune response to pneumococcus includes mucociliary clearance of bacteria in the upper respiratory tract; complement mediated lysis and opsonisation followed by phagocytosis. On recognition of the bacteria by various pathogen recognition receptors (PRR), host respiratory epithelium signals to recruit phagocytes, such as neutrophils and macrophages. Apart from phagocytising bacteria, these cells also release many inflammatory mediators, which contribute to the clearance of bacteria.

Apart from providing a protective barrier and mucociliary cushion, respiratory epithelium secretes many other protective molecules against pneumococcal infection. These include various cytokines, chemokines, anti-microbial peptides, lysozyme, lactoferrin, β-defensins, phospholipase A2, and surfactants (Bals and Hiemstra 2004). Breach of these innate defence results in an acute inflammatory response, marked by neutrophil influx at the site of invasion. Failure of this inflammatory response to clear the bacteria, results in their dissemination to the lungs (Kadioglu et al. 2008).

In the lungs, three types of cellular responses occur. The cell type which engages first is the resident alveolar macrophages (AM), which can contain the infection by phagocytosis if the bacterial load is low (Dockrell et al. 2003). If the bacterial load is high, the polymorphonuclear cells are recruited for phagocytosis, while alveolar macrophages play a scavenging role to remove apoptic inflammatory cells (Knapp et al. 2003). Finally the monocyte-derived macrophages (MDM) get involved in the phagocytosis process (Smith et al. 2011). Eventually all phagocytic cells undergo apoptic process to prevent spreading of bacteria in circulation (Dockrell et al. 2003).

Phagocytosis of pneumococcus is facilitated by complement mediated opsonisation. Activation of complement may be triggered by the bacteria itself, but a more robust activation occurs in the presence of a pre-existing serotype-specific antibody. The importance of complement in innate host defence against the pneumococcus is well documented. Complement deficient patients are vulnerable to more severe form of pneumococcal infections; usually in recurrent episodes throughout their life. The classical pathway seems to be the most important, as its defect is associated with pneumococcal septicaemia and other fatal consequences (Brown et al. 2002).

1.6.1.1 Pattern Recognition Receptors (PRR)

A number of PRR have been suggested to be involved in the recognition of pneumococcus. These include C-reactive protein (CRP), LPS binding proteins (LBP), toll-like receptors (TLR), specific ICAM-3 grabbing nonintegrin-related 1 (SIGN-R1), macrophage receptor with collagenous structure (MARCO) and intracellular nod-like receptors (NLR) (Paterson and Mitchell 2006). A schematic diagram of pneumococcal interaction with different PRR is shown in figure (Gámez and Hammerschmidt 2012).

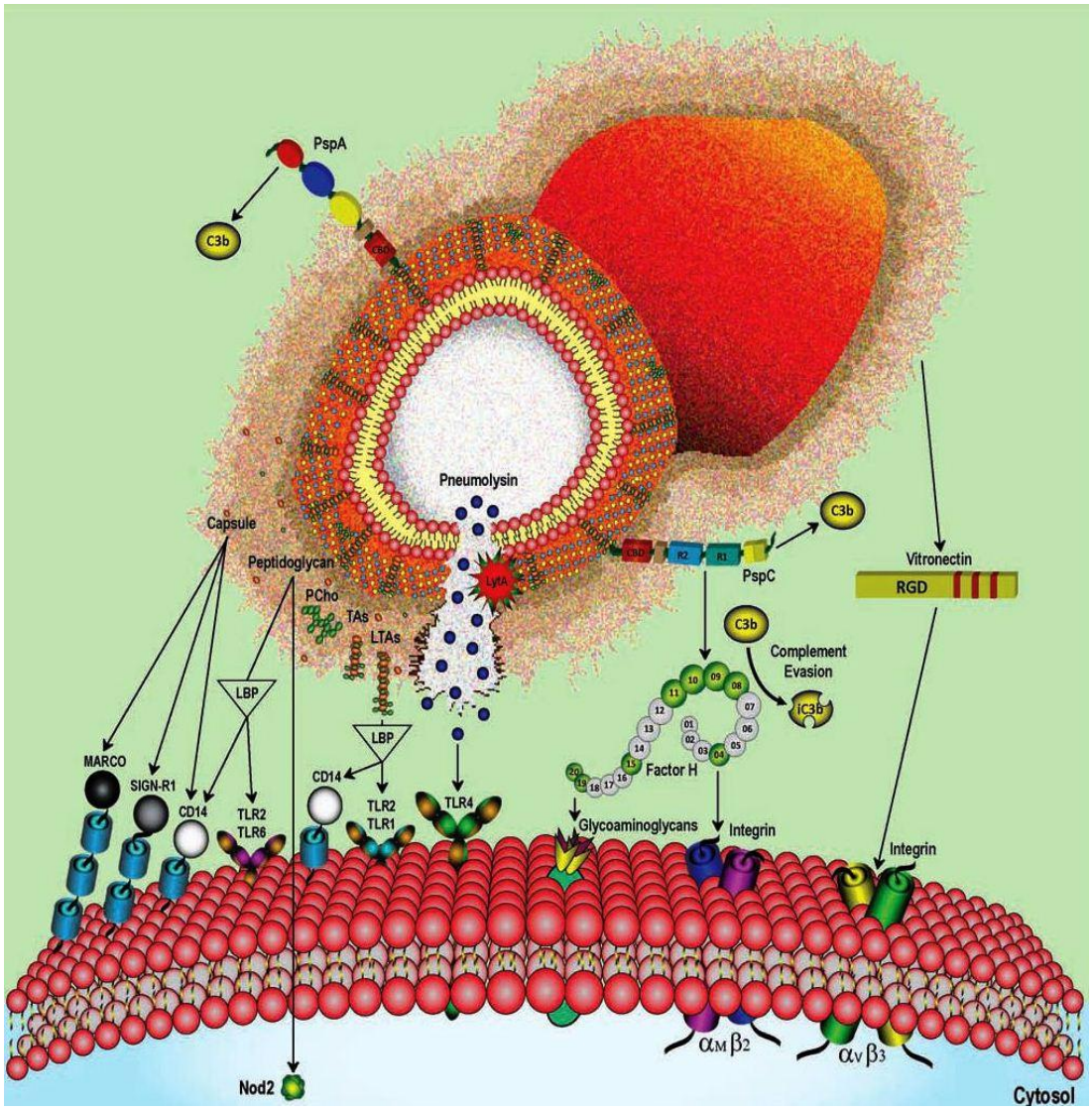


Figure-1.6.1: The PRR sensing pneumococci. The interaction of pneumolysin with TLR4, the recognition of LTAs, TAs and PCho by TLR2, TLR1, TLR6 and CD14; and the sensing of the pneumococcal capsule by MARCO, CD14 and SIGN-R1 are depicted. The interactions between pneumococci and integrin receptors ($\alpha_M\beta_2$ and $\alpha_V\beta_3$) together with their molecular bridges (factor H and vitronectin); complement protein C3b with PspA; and Factor H with PspC are also shown (Gámez and Hammerschmidt 2012).

1.6.2 Adaptive Immune Response

The adaptive immune response to pneumococcus develops following exposure to pneumococcal antigens during symptomatic infection or asymptomatic bacterial colonisation.

1.6.2.1 Antibody Response to Pneumococcus

As in other extracellular pathogens, the elimination of *Streptococcus pneumoniae* mostly depends on killing by various phagocytes including alveolar or tissue macrophages, and neutrophils. However, antibodies developed against capsular polysaccharides crucially contribute to this elimination process by opsonising the bacteria, and making them available for phagocytic killing or complement mediated lysis (Weinberger et al. 2008). Antibodies developed against non-capsular protein antigens also play an important role (Vukmanovic-Stejic et al. 2006). Patients suffering from various B cell defects with impaired antibody production were found to be more susceptible to recurrent and severe forms of pneumococcal infections (Picard et al. 2003). This suggests that antibodies are important component of host protective immunity against pneumococcus.

1.6.2.1.1 Antibodies to Capsular Polysaccharides

Production of antibodies against capsular polysaccharide is serotype-specific and associated with asymptomatic nasopharyngeal colonisation or symptomatic pneumococcal infection (Soininen et al. 2001). These antibodies confer serotype-specific and some cross protective immunity (Lipsitch et al. 2005). The protective efficacy of anti-polysaccharide antibodies have been well documented by the success of pneumococcal polysaccharide vaccines (Cornu et al. 2001), and polysaccharide conjugate vaccines (Gertz et al. 2003). The evidence of their protective function can be traced as early as 1938, in a paper (Lord and Heffron. Pneumonia and serum therapy), showing bacterial clearance with serum containing type-specific anti-capsular antibodies (Weinberger et al. 2008).

1.6.2.1.2 Antibodies to Non-capsular Antigens

Apart from inducing anti-capsular antibodies, nasopharyngeal carriage of pneumococcus also leads to generation of antibodies against other pneumococcal components, particularly surface-exposed proteins. An experimental carriage model in healthy adult human found that, pre-existing antibodies to pneumococcal surface protein A (PspA) was protective against pneumococcal colonisation (McCool et al. 2002). A study using a mouse model (Briles et al. 2000) showed that, a number of other proteins; pneumolysin (Ply), pneumococcal surface adhesion A (PsaA), pneumococcal surface protein C (PspC) were also able induce protective antibody responses. Subsequent studies have also validated the protective effect of responses to pneumococcal surface proteins, showing age dependent increase of antibody titre with a decreasing nasopharyngeal carriage; and a higher antibody levels to culture-negative children (Zhang et al. 2006b; Vukmanovic-Stejic et al. 2006). These antibodies appear early in the childhood, achieving a good titre before the second birthday; as evidenced in a Finnish (Rapola et al. 2000) and a Kenyan study (Ellis and Beaman 2004). Considering the fact that, the anti-capsular antibodies start to develop during the second and third year of life; antibodies to protein antigens could play a crucial protective role in young children (Lipsitch et al. 2005).

Serum analysis of convalescent children recovering from invasive infections showed high titre of antibodies to commonly expressed pneumococcal proteins. Sera from their parents, understandably exposed to the organism, were also analysed. High titres of antibody were observed in their parents also, which were possibly generated from their exposure to the bacteria. These antibodies seemingly had protective capacity, as none of them were colonised with pneumococcus (Giefing et al. 2008).

1.6.2.1.3 Antibody subclasses induced by pneumococcal antigens

Analyses of pre-vaccination antibodies against several pneumococcal serotypes (3, 24 and 18C) in adults revealed a predominance of IgM and IgG2 isotypes, although IgGl, IgG3, IgG4, and IgA were also detectable. However, after vaccination with pneumococcal polysaccharide vaccine (PPSV23), isotypes IgG1, IgG2, and IgA showed the strongest responses (Sarvas et al. 1989). Other studies also reported a predominant IgG1 and IgG2 responses following vaccination with polysaccharide antigens (Chudwin et al. 1987). In healthy children both IgG1 and IgG2 subclasses were found to be higher than in otitis prone children; although IgG1 seemed to be the predominant subclass (Freijd et al. 1984). In general, antibody response to pneumococcal polysaccharides in adults is mostly contributed by IgG2; and in children the predominant subclass is IgG1 (Bruyn et al. 1992).

On the other hand, microbial protein antigens induce an antibody response dominated with IgG1 and IgG3 subclasses (Hjelholt et al. 2013). This trend has been shown for pneumococcal proteins also (Zhang et al. 2006b). The ability of different IgG subclasses to activate complement varies. Generally, IgG3 is the most potent complement activator, followed by IgG1 than IgG2; and IgG4 has no complement activation property (Ram et al. 2010). Considering this, the pneumococcal protein antigens might be more efficient than the capsular polysaccharides in generating functional antibody responses.

1.6.2.2 Cellular Immunity to Pneumococcus

Antibody mediated protective responses were believed to be the key against pneumococcal infection, before evidence regarding involvement of adaptive cellular immunity became available. Mice, genetically rendered unresponsive to

polysaccharide antigens (CBA/N) or deficient of mature B cells (μMT), were able to clear experimental pneumococcal colonisation (McCool and Weiser 2004). The crucial role played by $CD4^+$ T cells in anti-pneumococcal immunity was immediately evidenced, with a study showing their migration towards the site of infection (Kadioglu et al. 2000); and higher susceptibility of MHCII-knockout mice to pneumococcal colonisation and invasion (Kadioglu et al. 2004).

The superiority of T cells over antibody mediated immunity was also documented, as nasopharyngeal colonisation of pneumococci was inhibited in experimental mice lacking antibody responses, not those lacking T lymphocytes. Evidently, this protective response was mediated by $CD4^+$ cells, rather than the $CD8^+$ subset (Malley et al. 2005). Experimental colonisation by live pneumococcus or killed whole-cell vaccine (WCV) confers protection in antibody deficient, but not in $CD4^+$ T cells deficient mice (Lipsitch et al. 2005). A number of pneumococcal proteins, including pneumolysin, PspA, CbpA, PsaA can also induce $CD4^+$ T cell mediated protective response (Kadioglu et al. 2004; Basset et al. 2007a; Zhang et al. 2007; Mureithi et al. 2009). This antigen-specific induction may occur as both primary and memory $CD4^+$ T cell response (Zhang et al. 2007; Trzciński et al. 2008).

1.6.2.2.1 Phenotypes of $CD4^+$ T cells Involved in Pneumococcal Immunity

By assessing the cytokine response triggered by stimulation from different pneumococcal antigens, involvement of all major phenotypes (Th1/Th2/Th17) of $CD4^+$ T cells has been reported. A number of Th1 cytokines take part in the host defence against pneumococcus, including IFN-γ, TNF-α, IL-2 and their inducers such as IL-12 and IL-18 (Khan et al. 2002). $CD4^+$ T cells migrating towards the site of pneumococcal challenge shows higher IFN-γ expression than the non-migrating population (Kadioglu et al. 2004). Appearance of TNF-α in the host serum is the

quickest cytokine response to pneumococcal lung infection. Together with another early response cytokine IL-1, it enhances neutrophil recruitment to induce a local pro-inflammatory response; and assist in bacterial elimination through NF-κB mediated pathway (Jones et al. 2005). IL-12, an IFN-γ inducer, also plays a protective role in pneumococcal lung infection. A murine study demonstrated that, intranasal administration of IL-12 was associated with decreased severity of pneumococcal pneumonia with a lower bacterial burden and a higher survival rate; seemed to be mediated by IFN-γ induced polymorphonuclear recruitment (Sun et al. 2007).

An up-regulated expression of IL-4, the signature cytokine of Th2 type response was also reported in $CD4^+$ T cells migrating towards the site of pneumococcal challenge (Kadioglu et al. 2004), suggesting the importance of Th2 support required for an effective antibody-mediated immune response against pneumococcus. Another Th2 related cytokine IL-10 contributes to pneumococcal immunity by suppressing Th1 type response and shifting the balance towards a potent antibody response (Zhang et al. 2006a).

Subsequently, a subset of $CD4^+$ T cells secreting IL-17A (Th17 cells) has been identified as an important effector for both innate and acquired immune response against pneumococcus. IL-17A production by cultured mice splenocytes has been shown to be up-regulated when stimulated with pneumococcal cell wall polysaccharides (Malley et al. 2006). Moreover, prior treatment of experimentally colonised mice with anti-IL-17A antibody was associated with marked reduction in the nasopharyngeal clearance of pneumococci (Malley et al. 2006). IL-17A recruits phagocytic cells in both primary and secondary pneumococcal colonisation (Zhang et al. 2009). Pneumococcal WCA stimulation has also been shown to induce a strong

IL-17A response in human adenotonsillar MNC culture. Also an enhanced phagocytosis of pneumococci by human polymorphonuclear cells has been reported after pre-incubation with IL-17A (Lu et al. 2008). Another Th17 related cytokine, IL-22 is has been shown to be induced by pneumococcal WCA and pneumolysoid (PdT) (Lundgren et al. 2012).

1.6.3 The Mucosal Immune System

Based on the location and function, the mucosal immune system can be subdivided into two components; the site of immune induction and the effector site (McGhee and Fujihashi 2012). The immune induction takes place in the organised lymphoid tissues in various organs, collectively known as mucosa-associated lympho-reticular tissues (MALT). These are the sites for activating antigen-specific B and T cells along with assignment of their homing signals. These activated lymphocytes then move to the mucosal effector sites in the lamina propria region for polymeric IgA synthesis and T cell response (Kiyono and Fukuyama 2004). MALTs are distributed widely along the various mucosal surfaces and are known as different names, such as; gut-associated lymphoid tissues (GALT), nasopharynx-associated lymphoid tissues (NALT), and some less defined lymphoid tissues in other mucosal sites (McGhee and Fujihashi 2012).

1.6.3.1 Nasopharynx Associated Lymphoid Tissue (NALT)

Human nasopharynx-associated lymphoid tissue (NALT) includes tonsils, adenoids, inducible bronchus-associated lymphoid tissues (iBALT), cervical lymph nodes and hilar lymph nodes (McGhee and Fujihashi 2012). The major contributor of the human NALT is the ‘Waldeyer’s ring’, an interrupted circle of lymphoid tissues at the junction of the nasopharynx and oropharynx, which is strategically positioned to

guard against both airborne and ingested pathogens. Besides, playing a crucial role in immune induction, they also contribute as an effector site for local systemic and secretory mucosal immunity (Brandtzaeg 2011).

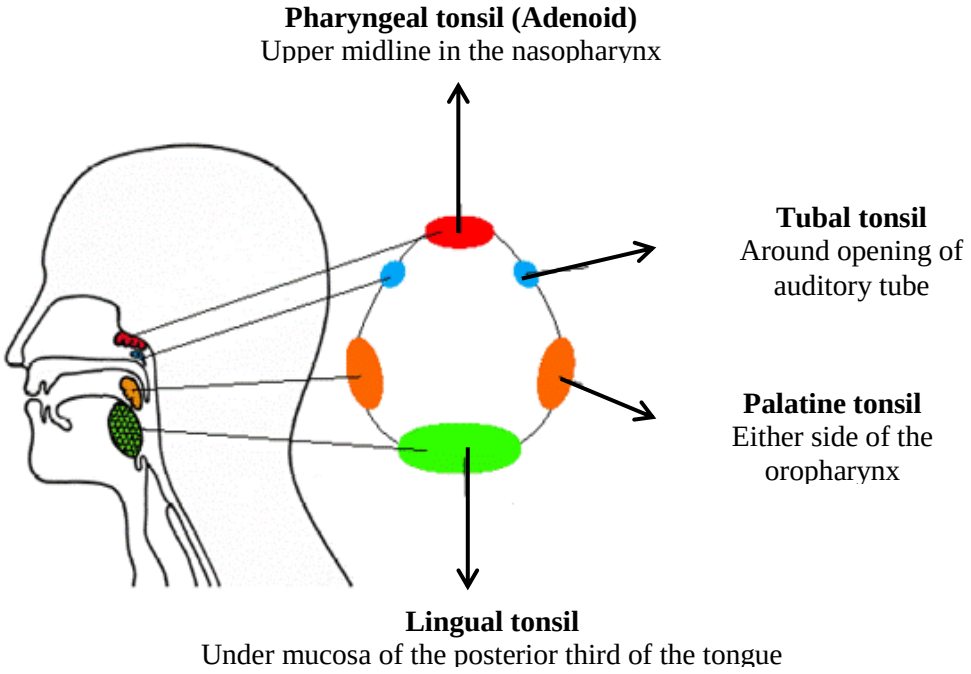


Figure-1.6.3.1: The Waldeyer's ring, formed by the adenoid, tubal tonsils, palatine tonsils and lingual tonsil

Structurally, adenoids and tonsils resemble lymph nodes, except they do not have the afferent lymphatics to carry the antigens into them. Rather they encounter antigens which are trapped into the numerous crypts containing microfold or 'M' cells. This trapping expose the luminal antigens to local antigen presenting cells (APC) (Brandtzaeg 2003). Apart from the abundance of B cells, adenotonsillar tissues are also rich in macrophages and dendritic cells for presenting antigens, which help them to develop both Th1 and Th2 types of responses to these antigens (Boyaka et al. 2000). Adenotonsillar MNC, contains various types of lymphocyte populations, including naive ($CD45RA^{+}$), memory ($CD45RO^{+}$) and recently activated ($CD25^{+}$) cells, and are capable of inducing both primary and secondary immune responses (Brandtzaeg 2011).

Anatomically human adenoid and tonsils are divided into four specialized lymphoid compartments; the reticular crypt epithelium, the extra-follicular area, the mantle zones of lymphoid follicles, and the follicular germinal centres (GC) (Brandtzaeg 2003). Adenotonsillar GCs are formed by activated B cells upon interaction with exogenous antigens. This process initiates shortly after birth as a part of T cell-dependent B-cell responses (Brandtzaeg 2003). The extra-follicular region contains both T and B lymphocytes. The latter is also found in the germinal centers of the lymphoid follicles, the mantle zone and the reticular sites of the tonsillar crypts (Boyaka et al. 2000). Unlike the peripheral blood compartment, where T cells are the predominant lymphocyte population, adenotonsillar tissues are rich in B cells. $CD4^+$ T cells were found to be the predominant T cell subtypes in adenotonsillar tissues, with a low percentage of $CD8^+$ cytotoxic T cells (Passàli et al. 2003). IgG is the predominant antibody isotype of B cells in adenotonsillar tissues and the frequency of IgM and IgA isotypes are low (Passàli et al. 2003).

1.6.3.2 Effect of Adenotonsillectomy

In this study adenotonsillar tissues were collected from patients underwent routine tonsillectomy and/or adenoidectomy. Common indications for surgical removal of these tissues include hypertrophy associated with obstructive respiratory symptoms (like mouth breathing, sleep disturbance) and recurrent infections (Mattila et al. 2010; van Staaij et al. 2005).

The effect of adenotonsillectomy on the immune system, especially on the upper respiratory mucosal immune response remains questionable. One of the common indications for their surgical removal is to get rid of chronic infections. Yet there were reports of an increased risk of nasopharyngeal colonisation by respiratory pathogens after adenotonsillectomy (Mattila et al. 2010). A meta-analysis on the

effect of adenotonsillectomy on upper respiratory tract infections showed only a small reduction in episodes of sore throat and associated school absence, and upper respiratory infections (van Staaij et al. 2005).

Following adenotonsillectomy, immunoglobulin levels seem to fall transiently. IgA titre is the most affected isotype, although IgG1 and IgG2 levels also decrease (Østergaard 1977). However, the antibodies often rise to the original level within one year or so, particularly when repeated infections are encountered (van den Akker et al. 2006). This suggests that a compensatory mechanism exists, where the remaining mucosal lymphoid tissues compensate for the initial loss of antibody responses after surgical removal of adenotonsillar tissues.

NALT are important induction and effectors site for immunity against respiratory pathogens. Adenotonsillectomy seems to increase the likelihood of pneumococcal carriage especially when performed in very early age, and decrease the pneumococcal polysaccharide-specific antibody response (Mattila et al. 2010). This suggests that adenotonsillar tissues are crucially involved in the immune response to pneumococcus in children.

1.7 Pneumococcal Vaccines

1.7.1 Historical Perspective of Pneumococcal Vaccine Research

The first successful vaccination attempt was reported a century earlier in 1911, when Wright and his colleagues developed a pneumococcal crude whole cell vaccine and immunised gold mine workers in South Africa (Butler et al. 1999). Subsequently, Heidelberger and Avery reported the polysaccharides nature of pneumococcal capsule (Heidelberger and Avery 1923) and suggested of their immunogenic potential (Heidelberger and Avery 1924). In 1930, researchers (Tillett and Francis

1930) observed capsular polysaccharide induced specific antibody response in humans. Inspired by these findings, efforts to develop vaccines from pneumococcal capsular polysaccharides went on with development of a quadrivalent vaccine from capsular polysaccharides of serotype 1, 2, 5 and 7 (MacLeod et al. 1945). Commercial production of this pneumococcal polysaccharide vaccine started after the end of the World War II. In the meantime, with the discovery of penicillin and other antibiotics; the attention was diverted from preventive vaccines to curative approach. The researches on vaccine development lost momentum and by the middle of the last century pneumococcal vaccines disappeared from the market (Butler et al. 1999).

1.7.2 Pneumococcal Polysaccharides Vaccines (PPSV)

With the emergence of antibiotic resistance, the need for pneumococcal vaccine was raised again (Austrian and Gold 1964). Austrian and co-workers pioneered the revival of pneumococcal vaccine research and reported the safety and high efficacy (78.5%-82.3%) of polyvalent vaccines, prepared from purified capsular polysaccharides in three different trials (Austrian et al. 1976). Subsequently, a 14-valent PPSV was approved for general use in 1977, which consisted of 50µg purified capsular polysaccharides from each of the following serotypes; 1, 2, 3, 4, 6, 8, 9, 12, 14, 19, 23, 25, 51 and 56 (Hilleman et al. 1978). Soon after that, the serotype coverage was increased in a 23-valent polysaccharide vaccine (PPSV23), which was licensed in 1983 (Lee et al. 1991). PPSV23 consists of 25µg capsular polysaccharides from serotypes; 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F (CDC 1997). PPSV23 covers against most of the invasive pneumococcal serotypes, with a very good efficacy in immunocompetent adults and older children (Shapiro et al. 1991).

Selection of these serotypes was based on the worldwide prevalence, which varied among different regions. The prevalence of invasive pneumococcal diseases by these vaccine serotypes ranged from 72% to 95% in different part of the world (Huss et al. 2009). In the developed countries of Europe and America PPSV23 covers serotypes responsible for nearly 90% of invasive pneumococcal infections (Butler et al. 1993). In Britain the 80-90% of the severe pneumococcal infections occurred by the vaccine serotypes (Noah 1988). A study in Scotland revealed that, nearly 99% non-invasive isolates in <5 children and 96% isolates in >65 adults were covered with PPSV23 (Kyaw et al. 2002). However, some of the developing countries reported lower coverage. A Malaysian study showed that, 62.7% of total pneumococcal infections and 71% of invasive diseases occurred with these 23 serotypes (Rohani et al. 1999). A study in Bangladesh found that, these vaccine serotypes are responsible for about 70% of the pneumococcal infections (Saha et al. 1997).

1.7.3 Pneumococcal Conjugate Vaccines (PCV)

Soon after the introduction of PPSV23 for routine vaccination it was apparent that it is poorly immunogenic in younger children, particularly in those aged less than 2 years (Borgoño et al. 1978), who are the most vulnerable group for pneumococcal infection. Therefore, development of a vaccine effective in young children and infant was much needed. In the meantime, the utility of conjugation of carrier protein with polysaccharide antigens was evident on the meningococcal and *Haemophilus* conjugate vaccines. Several carrier proteins were tested for conjugation with pneumococcal capsular polysaccharides.

At first, a tetra-valent conjugate vaccine was prepared with outer membrane protein complex of *Neisseria meningitides;* which showed good immunogenicity in 31 toddlers as well as 62 infants in Finland (Käyhty et al. 1995). Another tetravalent

vaccine conjugated with diphtheria toxoid also found to induce a good memory response upon boosting (Åhman et al. 1998). Subsequently, a penta-valent (6B,14,18,19F,23F) (Ahman et al. 1996) and a hepta-valent (4,6B,9V,14,18C,19F and 23F) polysaccharide vaccine (Shinefield et al. 1999) conjugated to a nontoxic form of diphtheria toxin CRM_{197} were found to induce a good antibody response in infant and toddlers.

A large scale safety and efficacy trial of the hepta-valent vaccine (PCV7) conjugated to CRM_{197} was carried out in Northern California, USA; which included 37,868 children in a randomly selected double blinded trial (Black et al. 2000). The safety and efficacy of that vaccine was reported to be excellent. It revealed that, 3 doses of the vaccine in two months interval, starting from the 2 months age; followed by a booster dose on the second years, had successfully protected the children from pneumococcal diseases (Black et al. 2000). With the success of this trial, PCV7 was approved for the routine immunisation schedule of children in USA in the year 2000. Since then, it has been routinely used in children younger than 2 years of age, administered in a 3 doses schedule, followed by a booster at 12-15 months (CDC 2000). A contemporary Finnish trial also reported a good safety and efficacy profile of the PCV7 (Eskola et al. 2001). As a result, the polysaccharide vaccine was replaced with PCV7 in the European countries; and by the year 2007, PCV7 had been licensed in more than 70 developed countries in the world (WHO 2007).

After its inclusion in the routine childhood immunisation programme, PCV7 successfully reduced the rates of invasive pneumococcal disease among in USA; from 97 to 24 cases per 100,000 of population. Invasion by vaccine-type strains fell from 80 to 4.6 cases per 100,000 populations (Mahon et al. 2006). Possibly through generation of herd immunity, PCV7 significantly decreased the incidence of

pneumococcal disease among unvaccinated individuals in USA (Whitney et al. 2003). Another study suggested that, introduction of PCV in USA reduced the incidence of invasive diseases and hospitalizations in elderly by 22.6%, 30.2% and 40.6% in the year 2002-01, 2001-02 and 2002-03 respectively; compared to the baseline in 1996-97 (McBean et al. 2005). An evaluation carried out after 7 years of the introduction of PCV7 has shown 45% reduction of invasive infection caused by all serotypes of pneumococci. Whereas, the incidence of invasive pneumococcal diseases caused by serotypes included in the PCV7 had declined by 94% (Pilishvili et al. 2010). PCV7 has also been shown to confer very good protection (74% efficacy) in HIV-infected adults from the vaccine serotypes and a vaccine related serotype, 6A (French et al. 2010).

PCV7 was designed for the markets in the industrialised countries. The serotypes incorporated in the vaccine were the most prevalent in USA. Some of the common invasive serotypes (like 1 and 5), prevalent in developing countries are not included into it. Another important drawback of PCV7 was evident in countries, where it was introduced for routine vaccination. The initial magical success of PCV7 had been overshadowed by the reports of replacement diseases with serotypes not included in the vaccine (O'Brien et al. 2007b; Singleton et al. 2007).

Thus, the serotype replacement and emergence of newer invasive serotypes has become the matter of new concern. One of the possible solutions could be broadening of the serotype coverage. It is not an easy task to incorporate more serotypes; as the serotype distribution around the world is quite diverse. Moreover, conjugation of additional serotypes in the PCV is technically challenging. Nevertheless, up-gradation of pneumococcal conjugate vaccine with incorporation of additional serotypes continued.

At first, a 9-valent formulation including serotypes 1 and 5 with PCV7 was developed (Mbelle et al. 1999). This vaccine was found to be effective in reduction of overall pneumococcal burden, although there was still an indication of increasing infection by non-vaccine serotypes (Klugman et al. 2003). Later, trial with an 11-valent conjugate vaccine adding two more serotypes 3 and 7F showed promising prospect (Prymula et al. 2008). Relentless efforts to broaden the serotype coverage of the conjugate vaccine went on to develop a 13-valent PCV including two extra serotypes 6A and 19A. After a successful safety and efficacy trial of this 13-valent conjugate vaccine (PCV13) (Dinleyici and Yargic 2009); it was licensed for routine immunisation of children in the USA in February 2010 (CDC 2010b). Shortly after that, PCV13 has been introduced in many other developed countries including UK, replacing PCV7 for routine immunisation (van Hoek et al. 2012). The PCV13 formulation (Prevnar 13, Wyeth Pharmaceuticals Inc.) contains capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F; all of them individually conjugated to CRM_{197} carrier protein (CDC 2010b). A study in USA carried out before the licensure revealed that 64% of invasive pneumococcal diseases (IPD) was caused by serotypes included in PCV13; the additional six serotypes (than PCV7) accounted for 95% (60% of total) of them (CDC 2010a).

1.7.4 Cellular Immune Mechanisms of PPSV and PCV

The pneumococcal polysaccharide vaccine induces type-specific antibodies, which activate complement complex and promote bacterial opsonisation and phagocytosis (CDC 1997). Pneumococcal capsular polysaccharides are large, complex molecules composed of repeating immunogenic epitopes. They are recognised by the B cell receptors (BCR) and trigger the signal transduction for clonal expansion of serotype-

specific B cells. The activated B cells are converted to plasma cells secreting type specific antibodies. These antigens are capable of stimulating the B lymphocytes directly, without getting broken-down and presented by the antigen presenting cells (APCs). As these antigens are recognised without the intervention of helper T cells, they are termed as T-independent (TI) antigens (Käyhty et al. 1984).

Multivalent pneumococcal conjugate vaccines (PCV) are prepared by the covalent coupling of the capsular polysaccharides to a protein carrier. The protein conjugated polysaccharides also stimulate the BCR in an identical way. Additionally, the conjugated carrier proteins are processed by the antigen presenting cells (APC); and presented to the $CD4^+$ helper T cells. These T cells offer so-stimulatory signals for an enhanced primary antibody response. They also initiate a memory B cell and T cell response resulting in a quicker and stronger secondary response (Mazmanian and Kasper 2006).

The immune response induced by pneumococcal conjugate vaccine is stronger than that of polysaccharide vaccine. Particularly, it is highly effective in infants, young children, the elderly and immunodeficient persons. Moreover, the conjugate proteins prime memory B cells and T cells, which results in immune enhancement on a booster dose or subsequent exposure to infection (Mazmanian and Kasper 2006).

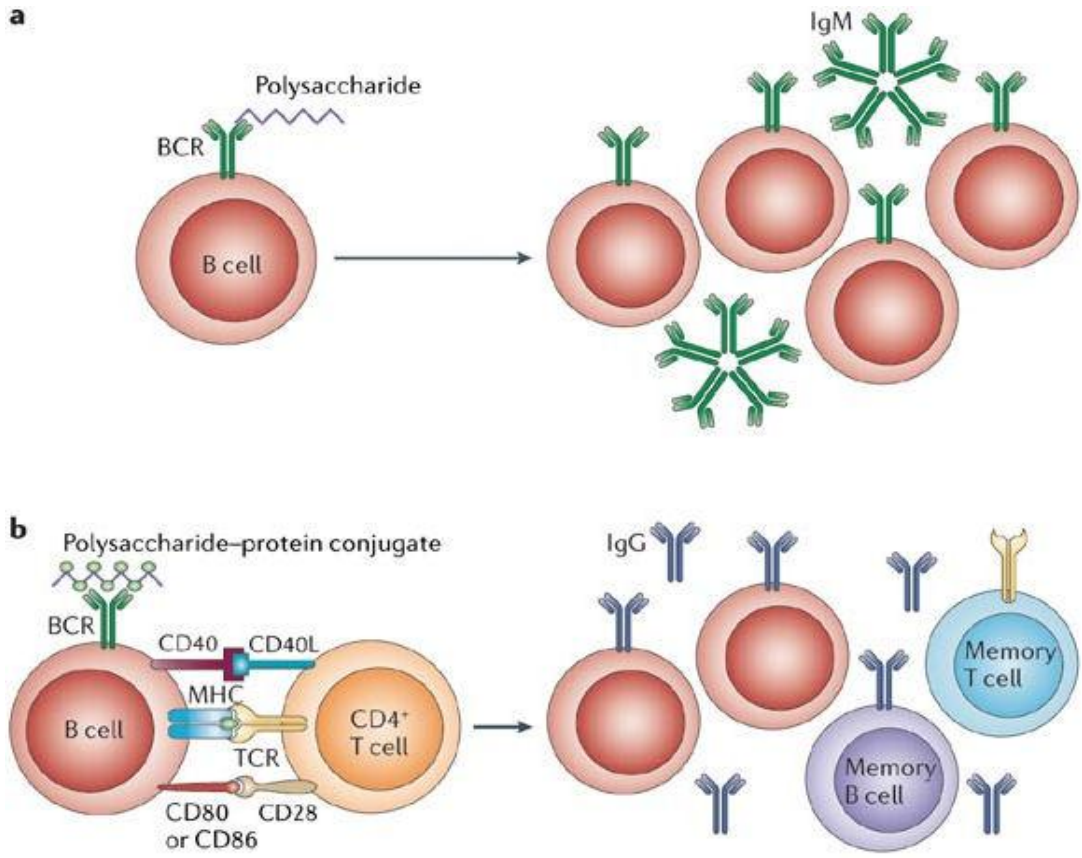


Figure-1.7.4: The immune responses to (a) polysaccharide only vaccine and (b) conjugate vaccines (Mazmanian and Kasper 2006).

1.7.5 Advantages and Disadvantages of PPSV and PCV

Despite a huge success in controlling pneumococcal diseases, the PPSV23 failed to become a universal choice, due its poor immunogenicity in infant and young children. The B cells of new-born children and infants respond poorly to these polysaccharide antigens (Barrett et al. 1984). With further maturation of immune system, antibody response start to develop slowly after the first year but protective efficacy remains unsatisfactory in children younger than 2 years. Moreover, these antigens do not induce a memory response; hence there is no immune enhancement on revaccination (Dintzis 1992). As there is no memory, periodic revaccination with polysaccharide vaccine is necessary. But there is evidence that, pneumococcal polysaccharide vaccine develops immune tolerance on repeated exposures (O'Brien

et al. 2007a). PPSV23 does not provide protection against mucosal infection, and thus has little effect on reducing nasopharyngeal carriage of pneumococci.

The immune response induced by pneumococcal conjugate vaccine is stronger than that of polysaccharide vaccine. Particularly, it is effective in infants, young children, the elderly and immunodeficient persons. Moreover, the conjugate vaccine can induce mucosal immunity, which plays an important role in the protection against extracellular bacteria. PCV induced production of antibodies (by NALT) in the nasopharyngeal mucosa can prevent pneumococcal colonisation (Nieminen et al. 1999). This ability to inhibit nasopharyngeal colonisation is another key advantage of conjugate vaccine over the polysaccharide vaccine (Dagan et al. 1996). The protection against pneumococcal carriage not only limits its progression to an overt infection in that individual, but also reduces the possibility of transmission of the organism to others. Indeed, vaccination of children with a conjugate vaccine has been shown to reduce pneumococcal transmission in adults also, thus creating 'herd immunity' in a community (Millar et al. 2008; Hammitt et al. 2006).

PPSV23 includes more serotypes than that covered by PCV13. These additional serotypes account for 23% of the invasive pneumococcal diseases (IPD) among high-risk children of 6–18 years (CDC 2013). It is evident that, even after introduction of a PCV with relatively extended serotype coverage (PCV13), protection against the full spectrum of invasive pneumococcal serotypes is not achievable. While the burden of invasive infections has been dramatically reduced with the introduction of PCV, their impact on non-invasive diseases is difficult to assess, due to lack of confirmatory diagnosis and/or reporting. Moreover, the licensing of PCV13 was based on immunogenicity studies, not on clinical efficacy

trials (CDC 2010a). It remains to be seen the full effect of PCV13 on pneumococcal disease burden and on serotype replacement.

Another disadvantage of conjugate vaccine is their high price, which makes them impracticable for global use. The expense of inclusion of PCV13 (multiple doses) in the national immunisation programme is far beyond the affordability of the countries, which harbour the highest pneumococcal burden. There are some other relevant issues to consider, such as; standardisation of dose schedule, duration of protective response, justification of a booster dose and its timing. Nevertheless, PCV13 is the best available option in the market so far, especially for the children immunisation.

Vaccination with a conjugate vaccine, followed by a polysaccharide vaccine could provide an enduring immunity against pneumococcal disease. It has been shown to maintain a protective level of antibody with broader serotype coverage (de Roux et al. 2008). Immunisation with a conjugate vaccine induces a T cell-dependent memory response which is boosted by a dose of polysaccharide vaccine. Boosting may also happen in response to a subsequent natural exposure. Priming of the immune system with PCV13 followed by a single dose of PPSV23 seems to be an effective option (Paradiso 2012). In many countries, combined use of PCV13 and PPSV23 has been recommended for high risk population to overcome the shortcoming of each other. In the USA, one dose of PPSV23 is recommended for following-up after PCV13 vaccination in older children (6-18 years) and adults (19-64 years) with increased risk of pneumococcal infection; and for all senior citizens (≥65 years) (CDC 2013; CDC 2012).

1.7.6 Development of Protein Based Vaccines for Pneumococcus

Pneumococcal infections remain on the top of the fatal diseases list. Limitations of the capsular polysaccharides and conjugate vaccines have driven the initiatives for finding alternative vaccination strategies. Not surprisingly, development of effective protein vaccines has been one of the priorities in pneumococcal vaccine research. Protein antigens induce a T cell-dependent response and induce memory. If a protein antigen is found to be effective and safe, production would be relatively simple. Commercial production of such a vaccine by recombinant DNA technology would be efficient.

The essence of vaccinology relies on the production of antibodies, which can either inhibit the virulence factors by direct neutralisation or promote opsonophagocytosis by host immune cells. Understandably, pneumococcal virulence factors, if sufficiently immunogenic, could potentially be suitable vaccine candidates. That is why the pneumococcal proteins involved in disease pathogenesis, especially those acting as adhesins are being thoroughly investigated. The efficacy of a protein vaccine depends on its ability to induce a strong antibody response together with a good immunological memory, and be protective against colonisation and/or infection.

1.7.7 Current Development of Protein-based Vaccines

Several pneumococcal surface proteins are being evaluated as vaccine candidate. Among the adhesins, pneumococcal LPXTG proteins (PavB, PfbA, PclA, PsrP, RrgA, RrgB, RrgC, PitA and PitB), pneumococcal choline-binding proteins (PspA, CbpA, CbpD, LytA, Pce), lipoproteins (PsaA, PiaA, PiuA and SlrA), pneumococcal histidine triad proteins (PhtA, PhtB, PhtC, PhtD and PhpA), and other surface-

exposed proteins (PavA, PppA, PcsB and StkP) were shown to possess good immunogenicity (Gámez and Hammerschmidt 2012). Apart from these adhesins, pneumococcal virulence factors (pneumolysin and/or its derivatives) were also found to be highly immunogenic (Briles et al. 2003; Zhang et al. 2006b). Some of these proteins are well conserved among pneumococcal serotypes. Hence, the vaccines developed with these are expected to confer serotype-independent protection (Tai 2006; Gámez and Hammerschmidt 2012).

Pneumococcal choline-binding proteins are extensively studied for immunogenicity. Subcutaneous vaccination with pneumococcal surface protein A (PspA) protected experimental mice from pneumonia (Briles et al. 2003). Pneumococcal choline binding protein A (CbpA or PspC) was also found to be protective against nasopharyngeal carriage and fatal sepsis (Glover et al. 2008), whereas pneumococcal surface adhesin A (PsaA) showed protective response against carriage only (Briles et al. 2000). Another lipoprotein, the putative proteinase maturation protein A (PpmA), was found to be immunogenic in humans, and this immune response was protective in mice (Ling et al. 2004).

Among the LPXTG adhesins, immunogenicity of pneumococcal adherence and virulence factor B (PavB) was assumed by detection of PavB-specific antibodies in human convalescent sera (Jensch et al. 2010). Another protein of this group, the pneumococcal serine-rich repeat protein (PsrP) also showed protective immune response in experimental mice following intranasal challenge (Shivshankar et al. 2009). Immunisation with pneumococcal pilus-1 subunit proteins (RrgA and RrgB) protected experimental mice; and increased their survival following lethal intra-peritoneal challenge (Gianfaldoni et al. 2007). The immunogenicity of two other

LPXTG proteins PrtA (Pneumococcal protease A) (Bethe et al. 2001), and SpuA (surface-located pullulanase) was also documented (Bongaerts et al. 2000).

Two other pneumococcal surface proteins, PcsB (protein for cell wall separation of group B streptococcus) and StkP (serine-threonine protein kinase), identified by genomic scale antigenic fingerprinting of pneumococcus were found to elicit a strong functional antibody response in human (Giefing et al. 2008). Pneumococcal protective protein A (PppA) was shown to induce sufficient antibody responses to inhibit pneumococcal carriage in mice nasopharynx (Green et al. 2005).

Another important group of pneumococcal proteins capable of inducing immune response are the pneumococcal histidine triad proteins: PhtA, PhtB and PhtD. These proteins are well conserved; especially PhtD appears to be present in all of the currently identified serotypes of *S. pneumoniae* (Hamel et al. 2004). The protective capacity of these histidine triad proteins against pneumococcal sepsis were documented in experimental mice (Adamou et al. 2001). Safety and efficacy of a vaccine produced with PhtD was promising (Seiberling et al. 2012). A combination of CbpA and PhtD also showed a good safety and efficacy profile in human (Bologa et al. 2012). In another phase I randomized study with a detoxified form of pneumolysin (PlyD1), it was found to be quite safe and highly immunogenic in human (Kamtchoua et al. 2013).

It is clear from the above discussion that, pneumococcal toxins and several surface-expressed proteins are strongly immunogenic. Combination of different protein antigens resulted in a more robust response; and their permutation-combination yielded better options in achieving specific targets, such as protection against invasive infection, nasopharyngeal carriage, development of herd immunity (Briles et al. 2000; Ogunniyi et al. 2007).

1.7.8 Prospect of a Mucosal Protein Vaccine for Pneumococcus

All available pneumococcal vaccines are designed for intramuscular administration. These vaccines predominantly induce systemic immunity to prevent invasive infections. Nasopharyngeal colonisation is the prerequisite for pneumococcal diseases. Therefore, local immunisation via nasal spray could be an effective alternative vaccination strategy. This could potentially be protective against local nasopharyngeal carriage and also be effective against invasive disease. Intranasal immunisation against influenza in humans was shown to be safe and very effective (Belshe et al. 2004). Unlike injectable vaccines, the immune response induced by mucosal vaccines closely resembles natural immunity (Cox et al. 2004). Studies in mice using intranasal immunisation of pneumococcal protein antigens (PsaA) has shown to induce a strong immune response in both mucosal and systemic compartment, which successfully protected against nasopharyngeal colonisation (Oliveira et al. 2006; Xu et al. 2011). Intranasal vaccination with another immunogenic protein (PspA) has been shown to protect mice against invasive pneumococcal infection (Ferreira et al. 2009).

1.8 Pilus Protein Antigens as Vaccine Candidate – Current Data

All of the three pilus-1 subunit proteins (RrgA, RrgB and RrgC) have been shown to induce protective immune response in murine model (Gianfaldoni et al. 2007). Mice intraperitoneally immunised with recombinant RrgA, RrgB and RrgC produced substantial amount of antibodies against them, which were sufficient to protect them on subsequent challenge with a pilus-1 expressing strain. Intraperitoneal administration of a previously tested lethal dose of TIGR4wt strain (expressing RrgA, RrgB and RrgC) resulted in a lower level of bacteraemia and higher survival

rates in immunised mice than controls. Protection was also conferred upon passive immunisation with antibodies specific to these pilus-1 antigens. Transfer of mouse antisera raised against Recombinant RrgA, RrgB and RrgC protected naive mice form pneumococcal TIGR4wt challenge. Anti-RrgA showed the strongest response with only one mice out of eight (1/8) became bacteraemic and all (8/8) of them survived following intraperitoneal bacterial challenge. With the transfer of anti-RrgB, two mice (2/8) became bacteraemic and all (8/8) of them survived. Three out of eight (3/8) mice had bacteraemia and one (1/8) died after passive transfer of anti-RrgC serum. These results suggested that these pilus-1 subunit proteins are capable of inducing an efficient antibody-mediated protective response against pneumococcus (Gianfaldoni et al. 2007).

RrgA is an adhesin, which mediates pneumococcal attachment to host respiratory epithelium (Nelson et al. 2007). Using human embryonic kidney cell (HEK) line, researchers have shown that a TLR2-dependent interaction of RrgA triggers a robust inflammatory response (Basset et al. 2013). During an episode of colonisation by a piliated strain of pneumococcus, interaction of RrgA with host immune system generates a protective antibody response (Nelson et al. 2007). RrgA has also been shown to be associated with rapid dissemination of *S. pneumoniae* in circulation by its interaction with complement receptor 3 (Orrskog et al. 2013). RrgA has two variants (clade I and II), based on the sequence variation in its terminal end. Both variants possess similar binding capacity to ECM components; like collagen, laminin and fibronectin. Importantly, both of them are able to produce cross protective immunity. Mice passively-immunised with antibodies developed against either of these variants has shown to be protected in subsequent challenge with pneumococcal strains expressing both variants (Moschioni et al. 2010b).

The major structural component, RrgB has also shown to induce a strong antibody response in mice. Passive transfer of anti-RrgB antibody in experimental mice efficiently protected them from invasive infection and increased their survival upon pneumococcal challenge. Molecular structural analysis has revealed three variants of RrgB (Clade I, II and III) (Moschioni et al. 2008). Unlike, anti-RrgA, passive immunisation of experimental mice with antisera raised against each of these RrgB variants showed clade-specific protection. However, a fusion of these three variants (RrgB321) exhibited efficient opsonophagocytic killing of piliated pneumococcal strains (Harfouche et al. 2012).

The other ancillary component, RrgC is reported to be the most conserved protein of pneumococcal pilus-1 structure (Moschioni et al. 2008). Antigenic properties of RrgC have also been documented, although its protective efficacy seems to be inferior to both RrgA and RrgB (Gianfaldoni et al. 2007). RrgC is located in the basal part of the pilus shaft; and might not interact sufficiently with the host immune system to generate a robust antibody response (Nelson et al. 2007). Possibly for this reason, immunogenic potential of RrgC has been the least studied, despite being highly conserved among pilus-1 expressing pneumococci.

Pneumococcal type-1 pilus subunits RrgA and RrgB have been shown to be strongly immunogenic in experimental mice. Immunisation with both of these proteins has shown to protect mice from subsequent challenge from pilus-1 expressing strains of pneumococcus. However, their immunogenic potential in human remains unexplored. So far, there is not much information regarding the role of pilus protein-specific antibodies in protective immunity against pneumococcal carriage. A good understanding of the natural immune response induced by these pilus-1 proteins in humans and its relationship with pneumococcal carriage is necessary to evaluate

their protective efficacy. The currently offered conjugate vaccination with PCV13 is expected to protect against both pilus-1 and pilus-2 expressing serotypes (Kulohoma et al. 2013). Yet information regarding the immunogenicity of pilus-1 components in human could be valuable for planning future strategies.

1.9 Hypothesis

We hypothesize that pilus-1 subunit RrgA, as an adhesin might interact extensively with host respiratory mucosa during an episode of colonisation. Therefore, natural colonisation or infection with a pilus-1 expressing pneumococcus is likely to induce a strong mucosal immune response against it. Similarly, the major structural unit of pilus-1, RrgB is also expected to have a considerable interaction with the host mucosa to induce a substantial immune response. Considering their ability to induce a protective antibody response in mice, it would be valuable to study whether these two proteins induce antibody and Th17-mediated immune response in humans and whether the immune responses to RrgA or RrgB can protect against colonisation/carriage.

1.10 Aims/Objectives of the Thesis

The aims of this thesis are

1. To investigate the prevalence of pilus-1 genes among pneumococcal isolates colonising a cohort of healthy children in the UK by culturing nasal swab samples in blood agar plates and conventional PCR for detection of pilus-1 gene among the isolates.
2. To evaluate the naturally developed immune response against pneumococcal type-1 pilus proteins RrgA and RrgB by analysing circulating antibodies to these proteins in serum samples.
3. To study whether previous colonisation/infection primes for immunological memory to pneumococcal RrgA and RrgB antigens in NALT (adenotonsillar MNC) by B cell ELISpot and *in vitro* antibody production assay.
4. To examine the ability of these antigens to induce $CD4^+$ T cell and Th17 cell-mediated immune responses in peripheral blood and NALT by (CFSE labelled) cell proliferation assay and cytokine ELISA.
5. To investigate whether the naturally developed antibody and/or cell mediated immune response to these proteins are protective against pneumococcal carriage, and
6. To evaluate the potential of these proteins (RrgA and RrgB) as mucosal vaccine candidate in children.

Chapter 2

Materials and Methods

2.1 Subjects and samples

2.1.1 Subjects

The subjects included in this study were patients who underwent tonsillectomy and adenoidectomy due to upper airway obstruction or tonsillitis from Alder Hey Children's Hospital and Royal Liverpool and Broadgreen University Hospitals. Informed written consent was obtained in all cases from the patients or their legal guardians. Patients who received antibiotics or systemic steroids within 3 weeks of surgery, or who had any known immunodeficiency were excluded from the study.

2.1.2 Patients Samples

2.1.2.1 Adenotonsillar Tissues

Immediately after surgical removal, adenotonsillar tissues were transferred into a 25ml universal tube, containing 10ml Hank's Balanced Salt Solution (HBSS) (Sigma Aldrich, UK) supplemented with 10µg/ml gentamycin (Sigma) and 1% L-glutamine (Sigma). The tissues were then transported to the laboratory for processing.

2.1.2.2 Peripheral Blood

Before operation, a 2-5ml peripheral blood sample was collected into a 25ml universal tube containing anticoagulant (heparin 100µl, LEO Pharma, UK); and transported to the laboratory.

2.1.2.3 Nasopharyngeal Swabs

Nasopharyngeal swabs were collected into a sterile vial containing 1ml Skim milk-Tryptone-Glucose-Glycerol (STGG) broth (Appendix-I) as described previously (O'Brien et al. 2001). Briefly, immediately before the operation (while patient is under general anaesthesia), the nasopharyngeal swab was collected from patients

using a sterile Dryswab™ (Medical Wire & Equipment, UK). The swab was then inoculated to a vial containing STGG, and the shaft was cut off at the top level of the vial. Then the screw-cap of the vial was tightened and transported into the laboratory, where it was stored in a -80°C freezer before analysis.

2.1.2.4 Saliva

Before operation, a sample of unstimulated saliva was collected with a sterile 'Oracol' sponge swab (Malvern Medical Developments Limited, UK). The absorbent sponge swab was inserted into the oral cavity and left at the side of mouth for about 1 minute to soak with saliva. Once saturated, the saliva swab was then replaced into the test tube and transported to the laboratory; where the saliva samples were squeezed out into 1.5ml eppendorf tubes by the help of 20ml plastic syringes and immediately stored in a -80°C freezer.

2.2 Processing and/or Storage of Samples

2.2.1 Isolation of mononuclear cells (MNC) from adenotonsillar tissues

Adenotonsillar tissues were processed within a class-II Bio-safety cabinet following methods as previously described (Zhang et al. 2006b). At first, tissue samples were rinsed with 10ml of fresh HBSS and transferred into petri-dishes. With the help of a sterile pair of forceps, the tissues were minced by a scalpel to release cells into the medium. With a sterile Pasteur pipette, the cell suspension was filtered through a 70μm cell strainer (BD biosciences, USA). The cells were then layered carefully on to 15ml Ficoll-Paque (GE Healthcare Life Sciences, UK) and isolated by gradient centrifugation (400×g for 30 min). After washing with sterile PBS (400x*g* for 10 min), the cells were resuspended in 5ml RPMI (Sigma) supplemented with 10μg/ml

gentamycin (Sigma), 1% L-glutamine (Sigma) and 10% FBS (Sigma); and counted in an automated cell counter (BioRad, UK).

2.2.2 Collection of serum and isolation of peripheral blood mononuclear cells (PBMC)

After centrifugation of a peripheral blood sample (at 400x*g* for 10 min), serum was collected into a 1.5ml eppendorf tube and stored in a -80°C freezer. For isolation of PBMC, the peripheral blood sample was diluted with HBSS and layered onto 10ml Ficoll-Paque (GE Healthcare Life Sciences, UK). The mononuclear cells were isolated by gradient centrifugation (400×*g* for 30 min); and washed with sterile PBS (400x*g* for 10 min). The cells were resuspended in 1ml RPMI-1640 (supplemented as above); and counted in an automated cell counter (BioRad, UK).

2.3 Bacteriological Culture of Nasopharyngeal Swabs

Nasopharyngeal swabs were cultured on blood agar (BA) plates (Oxoid, UK) following a standard procedure as described previously (O'Brien and Nohynek 2003). Briefly, 40µl of broth from the swab containing vials was pipetted into a blood agar plate, and streaked with a sterile 5µl plastic loop (Technical Service Consultants Ltd, UK) using standard inoculating procedure. The inoculated plates were incubated overnight at 37°C, in 5% CO_2. On the following day, the plates were observed for typical α-haemolytic colonies. Greenish α-haemolytic colonies with typical central depression were identified as possible *Streptococcus pneumoniae* (Pneumococcus). Each suspected α-haemolytic growth was subcultured and confirmed by standard optochin disc inhibition test, before being confirmed as pneumococcus. The density of bacterial load was measured semi-quantitatively following the methods described in (O'Brien and Nohynek 2003). The first

inoculation on the plate was considered as Quadrant 1, from where it was streaked into all four quadrants. The growth was termed as 4+ if >10 colonies were in Quadrant 4; growth was 3+ if <10 colonies were in Quadrant 4 and >10 colonies in Quadrant 3; growth was labelled 2+ if there were <10 colonies in Quadrant 3 and >10 in Quadrant 2; finally, 1+ growth was noted if there were <10 in Quadrant 2 with growth in Quadrant 1.

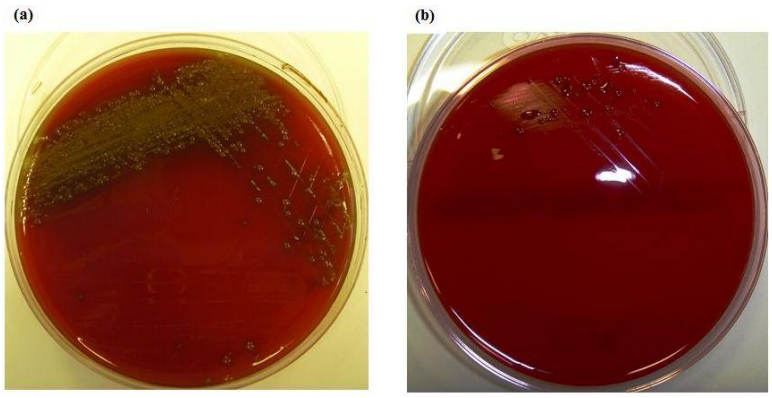


Figure-2.3: Growth of *Streptococcus pneumoniae* on blood agar plates; (a) typical 'Draughtsman's' colonies of pneumococci with 3+ growth, (b) mucoid α-haemolytic colonies (1+ growth).

2.3.1 Optochin Susceptibility Test

Typical pneumococcal colonies were picked up very carefully and streaked on BA plates in confluent lines with 5µl plastic loops. Using a pair of steel forceps (sterilized with red flame) 5µg optochin disk with 6mm diameter (Oxoid, UK) were placed on to the streaked area, at the intersection of first and second streaking. These plates were then cultured overnight (at 37°C, 5% CO_2), and observed for optochin disk inhibition (Gardam and Miller 1998). The organism was identified as optochin susceptible if the zone of inhibition was ≥14 mm (Chandler et al. 2000). Isolates susceptible to optochin were confirmed as *Streptococcus pneumoniae*.

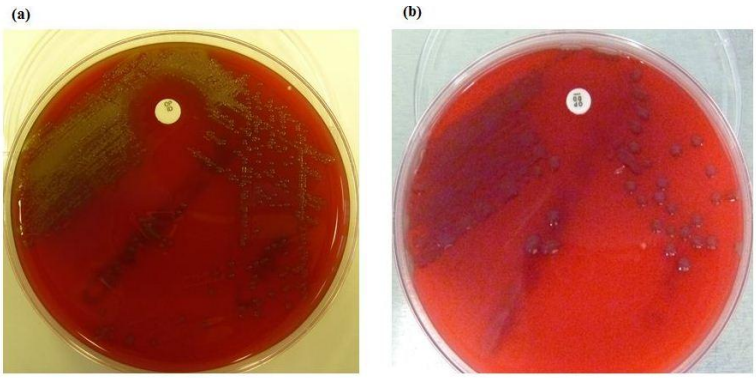


Figure 2.3.1: Optochin susceptibility tests of pneumococcal isolates; (a) on a typical 'Draughtsman's' colony; and (b) on a mucoid α-haemolytic colony.

2.3.2 Storage of Streptococcus pneumoniae Isolates

The confirmed isolates of *Streptococcus pneumoniae* were subcultured on blood agar plates at 37°C, in 5% CO_2 overnight. A loop-full of organism from each pure culture was picked with a sterile 5µl plastic loop, and inoculated into screw capped Protect Plus bacterial preservative vials (Technical Service Consultants Ltd, UK), by rubbing gently against the hollow beads within those vials. The vials were then stored in -80°C for future analysis.

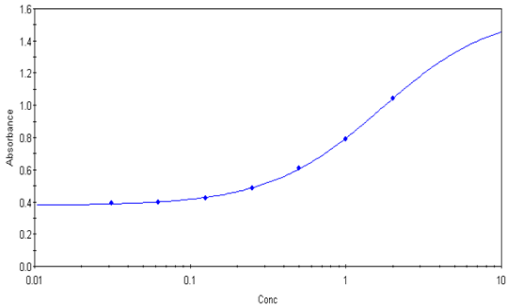
2.4 Preparation of Pneumococcal CCS

Pneumococcal strains used in this study included a pilus expressing strain of *Streptococcus pneumoniae* serotype 4 (TIGR4) (Tettelin et al. 2001); and its isogenic $RrgA^{-/-}$ and $RrgB^{-/-}$ mutant strains (Hilleringmann et al. 2009). Concentrated culture supernatants (CCS) from these strains were prepared using a procedure as described previously (Zhang et al. 2002). Briefly, these pneumococcal strains were cultured (37°C, 5% CO_2) overnight on blood agar plates (Fisher Scientific, Loughborough, UK). On the following day, typical pneumococcal colonies from each strain were cultured (37°C, 5% CO_2) again in Todd-Hewitt broth (Oxoid, Basingstoke, UK) with 0.5% yeast extract (THYB) (appendix-I). The cultures were continued until it

reached the exponential phase (OD 0.4-0.5 at 620nm, approximately 10^8cfu/ml) of growth. They were then centrifuged at 3000×g for 30 min; and the supernatants were filtered through a 0.45mm sterile filter. The filtrate was filtered again through a 0.2mm sterile filter; before concentrated by centrifugation (3000×g for 30 min) in Vivaspin15 (MWCO5000) concentrator (Sartorius Stedim Biotech, Germany). The centrifugation process was repeated 2/3 times until a ten-fold concentration of the supernatants was achieved. These CCS were then aliquoted into 1.5ml microcentrifuge tubes and stored in -80°C freezer.

2.4.1 Measurement of protein concentration in pneumococcal CCS

The protein concentrations of these CCS were measured by Bradford protein assay, using Bradford protein dye reagent (Sigma), following the manufacturer's instructions. The protein standard was prepared from bovine serum albumin (BSA) by seven serial (double) dilutions, starting with 2mg/ml. CCS solutions were assayed in duplicate and in three different concentrations (neat, 1:10 and 1:100). Firstly, 250µl of dye reagent was dispensed into a 96-well Costar plate. Thereafter, 5µl of standards and samples were added to them and mixed thoroughly. The plate was incubated at room temperature in dark for 5 min. Absorbance was measured at 595nm wavelength using a microtiter plate reader (Opsys MR, Thermo labsystems, UK). A standard curve was produced from the absorbance of BSA standards and the concentration (mg/ml) of each sample was calculated against the standard curve, with the help of microplate analysis software DeltasoftPC (Biometallics Inc., USA).



BSA Standard	Concentration (mg/mL)	Mean OD
1	0.031	0.393
2	0.063	0.399
3	0.125	0.423
4	0.25	0.486
5	0.5	0.612
6	1	0.79
7	2	1.043

Figure-2.4.1: **Standard curve of Bradford protein assay**

Table-2.4.1: Concentration of the pneumococcal CCS measured by protein assay

Pneumococcal CCS	Conc. (mg/ml)	Conc. used to stimulate cells
TIGR4wt	0.571	1µg/ml
RrgB$^{-/-}$	0.587	1µg/ml
RrgA$^{-/-}$	0.527	1µg/ml

2.5 Western Blotting

2.5.1 Principle of the assay

In this method, proteins are separated electrophoretically on sodium dodecyl phosphate- polyacrylamide gels (SDS-PAGE), according to their molecular weight. Then these proteins are electrphoretically transferred into a nitrocellulose or PVDF membrane. After that, non-specific binding capacity on the membrane is blocked with bovine milk (skimmed) proteins. Thereafter, the target protein is probed with a specific primary antibody, which is then detected by chemiluminescence with an enzyme conjugated secondary antibody allowing it to react with its substrate.

2.5.2 Western Blotting of Pneumococcal CCS

Immunostaining of RrgA and RrgB proteins in pneumococcal CCS was performed following Western blotting protein transfer using rabbit anti-RrgA and anti-RrgB antisera (Novartis vaccine, Sienna, Italy) to confirm the presence of RrgA and RrgB

pilus proteins in the TIGR4wt CCS and the absence of RrgA protein in RrgA$^{-/-}$ mutant strain and RrgB protein in RrgB$^{-/-}$ mutant strain.

2.5.2.1 Gel Electrophoresis

Samples were prepared in Laemmli reducing Buffer (1:2 dilutions) and then heated on a heat block at 100°C for 5 min. After that, 20µl of CCS (derived from TIGR4wt, RrgB$^{-/-}$ or RrgA$^{-/-}$ strain) and 5µl of precision plus protein kaleidoscope ladder (Bio-Rad, UK) were loaded into a 12% mini protean precast TGX^{TM} gels (BioRad). The gel was run for 1 hr at a constant 250v in 50mA.

2.5.2.2 Protein Transfer by Western blotting

Western blotting transfer of protein was performed using a Transblot Turbo^{TM} transfer system (BioRad) into a 0.2µm nitrocellulose membrane (Transblot turbo transfer pack). After gel electrophoresis, the gel was carefully placed onto a nitrocellulose membrane and then placed on to the bottom ion reservoir (anode) stack. The top ion reservoir (cathode) stack was then laid over the gel, and run for 10 min at a constant 25v in 1000mA.

2.5.2.3 Detection of RrgA and RrgB proteins in pneumococcal CCS

Following Western blot transfer, the nitrocellulose membrane was blocked with 5% skimmed milk in TBS containing 0.05% Tween20 (TBS-T) (appendix I) for 2 hr at room temperature (RT). After washing 3 times in TBS-T, rabbit anti-RrgA and anti-RrgB antiserum, diluted 1:10,000 in blocking solution were added. The membrane was incubated in room temperature for 2 hr at RT. After, washing for 5 times in TBS-T, the membrane was incubated with secondary antibody (donkey anti-rabbit IgG-HRP) for 1 hr at RT. The secondary antibody was prepared by adding 2µl donkey anti-rabbit IgG-HRP (1:30,000) (Santa Cruz Biotechnology, Germany), to

visualise the ladder 2µl Streptactin-HRP (1:30,000) (Bio-Rad) and to reduce background 30µl normal donkey serum (Sigma) into 60 ml blocking solution. The membrane was washed 5 times in TBS-T and then in PBS, before the addition of substrate to the membranes. Western Blot substrate was prepared by adding equal volume (1:1) of Immun-star WesternC chemiluminescence reagent A and B (BioRad). The membrane was incubated with the prepared substrate solution for 5 min in the dark. Finally, the membranes were imaged in the Chemi-DocXRS system (BioRad), after removal of excess substrate with PBS.

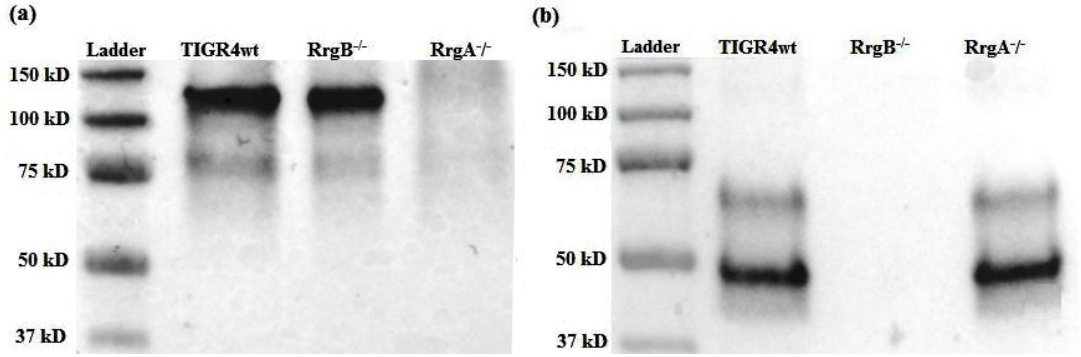


Figure 2.5.2.3: Western blot analysis of pneumococcal CCS (TIGR4wt, RrgB$^{-/-}$ and RrgA$^{-/-}$). (a) Nitrocellulose membrane blotted with pneumococcal CCS were immunostained with rabbit anti-RrgA antiserum showing that, RrgA band is present in TIGR4wt and RrgB$^{-/-}$ CCS but absent in RrgA$^{-/-}$ mutant CCS. RrgA band shows a higher molecular mass (~120 kDa) than predicted from its sequence (92.7 kDa) (LeMieux et al. 2008). (b) Blotted with rabbit anti-RrgB antiserum showing that, RrgB band (~50 kDa) is present in TIGR4wt and RrgA$^{-/-}$ CCS but absent in RrgB$^{-/-}$ mutant CCS.

2.5.2.4 Western blotting of recombinant pilus proteins (rRrgA and rRrgB)

Western blotting and immunostaining of recombinant pilus (rRrgA and rRrgB) proteins (Gianfaldoni et al. 2007) were also performed with rabbit anti-RrgA and anti-RrgB antisera (Novartis vaccine, Sienna, Italy). This was later used as the standard control in the detection of pilus-specific antibodies in serum samples using Western blotting. Samples for this purpose was prepared by adding 5µl of

recombinant proteins (rRrgA and rRrgB) to 95µl of Laemmli reducing Buffer (appendix-I), to make a 1:20 dilutions. After heating on a heat block at 100°C for 5 min, 5µl of these mixtures were loaded into the gel. The rest of the Western blot procedure was the same as that of the pneumococcal CCS, as described earlier.

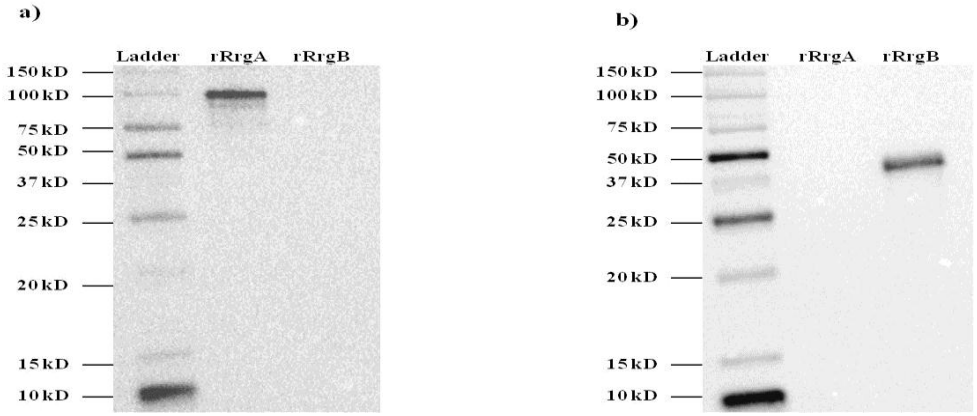


Figure 2.5.2.4: Western blotting of recombinant RrgA (rRrgA) and RrgB (rRrgB) proteins. (a) Probing with rabbit anti-RrgA antiserum showing the (~100 kDa) RrgA band. (b) Probing with rabbit anti-RrgB antiserum showing the (~ 50 kDa) RrgB band.

2.5.2.5 Serum Western Blotting for detection of pilus-specific antibodies

Serum anti-RrgA and anti-RrgB antibodies were also analysed by Western blotting. Recombinant pilus proteins (rRrgA and rRrgB) were mixed with Laemmli reducing buffer (1:20) and heated at 100°C for 5 min. The mixtures were loaded into a mini protean TGX gel (BioRad, UK), and run for 1 hr at a constant 250v in 50mA. Transfer of protein to a 0.2µm nitrocellulose membrane was performed with a Transblot turbo transfer system (BioRad). The membrane was then blocked with 5% skimmed milk TBS-T for 2 hr. The membrane was then probed with serum samples (1:10,000 in blocking solution) for 2 hr. After washing, murine anti-human IgG-HRP (Sigma) and Streptactin-HRP (BioRad), both diluted (1:10,000) in blocking solution were added and incubated for 1 hr. The membrane was imaged on Chemi-DocXRS system (BioRad), 5 min after the addition of 1:1 mixture of Immun-star WesternC chemiluminescence reagent A and B (BioRad).

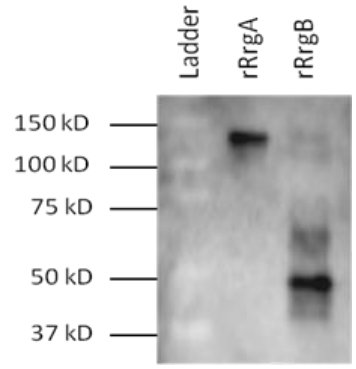


Figure 2.5.2.5: Western blotting of recombinant RrgA and RrgB proteins with patients' sera. Figure shows the MW marker ladder in lane 1, positivity of anti-RrgA in lane 2 and positivity of anti-RrgB in lane 3 in a representative serum sample.

2.6 Measurement of *In vitro* Antibody Production in NALT

2.6.1 Preparation of adenotonsillar MNC for culture

Mononuclear cells isolated from adenotonsillar tissue were resuspended at a concentration of 4×10^6/ml, in RPMI-1640 medium supplemented with HEPES (Sigma), 10µg/ml gentamycin (Sigma), 1% L-glutamine (Sigma), 10% FBS (Sigma) and 2.5µg/ml amphotericin B (Sigma).

2.6.2 Stimulation of adenotonsillar MNCs

1ml of diluted (4×10^6) cells were stimulated with (1µg/ml) of pneumococcal CCS (TIGR4 wt, $RrgA^{-/-}$, $RrgB^{-/-}$). As a positive control, some cells were stimulated with a polyclonal B cell activator CpG-ODN (1µg/ml) (InvivoGen, USA). An unstimulated (only the RPMI culture medium) control was included in each experiment. To exclude any non-specific stimulation from components of Todd-Hewitt-Yeast broth (THYB) media, some cells were co-incubated with this media; concentrated following the same procedure as in pneumococcal CCS preparation. Some samples were also stimulated with recombinant pilus (rRrgA and rRrgB) antigens alone or in combination with the respective mutant ($RrgA^{-/-}$, $RrgB^{-/-}$) CCS.

2.6.3 Culture of adenotonsillar MNCs

After stimulation, the cells were added into a 96-well culture plate (Corning Inc., USA). The plate was then incubated in a CO_2 incubator (at 37°C, 5% CO_2). Culture supernatants were collected on day 7 and day 14 of the incubation from each well. They were then centrifuged at 5,000x*g* for 5 min, and the supernatants were stored at -80°C until further analysis by ELISA.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

2.7.1 Detection of serum pilus-specific IgG antibodies by ELISA

Pilus specific antibodies (anti-RrgA and anti-RrgB) were detected in serum by ELISA using the following protocol. Coating solutions (1µg/ml) were prepared from recombinant RrgA (2.7mg/ml) and RrgB (2.7mg/ml) stock solutions, by adding 3.7µl of antigens into 10ml of sterile PBS. A 96-well flat-bottom, high binding Costar EIA plate (Corning) was coated with 100µl of coating solution per well. The plate was then incubated overnight in 4°C. On the following morning, the plate was washed 5 times with PBS containing 0.05% Tween20 (PBS-T). The plate was then blocked (150µl/well) with blocking buffer (10% FBS in PBS) at 37°C for 1 hr. In the meantime, samples and standards were prepared by diluting them (1:100) in blocking buffer. Human immunoglobulin (sandoglobulin, Sandoz, UK), containing high IgG antibody titers to pilus antigens, was used as a reference standard for measurement of IgG antibodies; and the dilution standard series was constructed by 8 serial dilutions, starting with 1:50. Diluted samples and standards (50µl in each well) were added to the plate in duplicate; and incubated at RT for 2 hr. At the end of this incubation, the plate was washed 5 times with PBS-T; and 50µl of alkaline phosphatase-conjugated mouse anti-human IgG (1:2000 dilutions) (Sigma) was added into each well. Then the plate was incubated at room temperature for 2 hr. Thereafter, the plate had been

washed 5 times with PBS-T, before 50µl of freshly prepared PNPP substrate was added into every well (appendix-I). Then the plate was incubated at RT for 15 min protecting it from direct light. Finally, the absorbance (OD) was measured using a microtiter plate reader (Opsys MR, Thermo labsystems, UK) at 405nm. The concentration (units/ml) of each sample was calculated against the standard curve constructed with serial dilution of Sandoglobulin, with the help of microplate analysis software DeltasoftPC (Biometallics, Inc., USA).

Before starting the ELISA analysis of the test samples, the antibody titre of the reference standard (sandoglobulin) for both RrgA and RrgB was determined by the following method. Twelve dilutions (1:50, 1:100, 1:200, 1:400, 1:600, 1:800, 1:1000, 1:1200, 1:1400, 1:1600, 1:1800, and 1:2000) of the standard was analysed. The plates were coated with rRrgA (1µg/ml) and rRrgB (1µg/ml) as usual; and the ELISA procedure was followed as described previously. The optical density (OD at 405 nm) was measured after 30 min of adding the substrate solutions. The anti-RrgA and anti-RrgB titre of sandoglobulin was assigned as the reciprocal dilutions at which their corresponding OD reached to 1.00 at that time point. Based on this, the stock solution of Sandoglobulin was assigned to an antibody titre of 1000 units/ml for anti-RrgA and 1400 units/ml for anti-RrgB.

2.7.1.1 Specificity of Serum Pilus-specific Antibody ELISA

The specificity of the ELISA measuring anti-pilus antibodies was confirmed by antigen-specific inhibition ELISA assays. Each recombinant protein (rRrgA and rRrgB) was added in following concentrations (0.3125, 0.625, 1.25, 2.50, 5.0 and 10.0 µg/ml) to the serum sample. They were then incubated for 30 min at RT to adsorb the specific antibodies present in each sample. To measure both anti-RrgA and anti-RrgB antibody titres in these samples, ELISA assays were carried out, following the above mentioned procedures.

2.7.1.2 Detection of pilus-specific IgG subclasses in serum

Serum IgG subclasses (IgG1, IgG2, IgG3 and IgG4) to RrgA and RrgB were also measured by ELISA. The above mentioned protocol was followed for IgG subclasses, except alkaline phosphatase-conjugated mouse monoclonal anti-human IgG1 (Southern Biotech, UK), IgG2 (Southern Biotech), IgG3 (Southern Biotech) or IgG4 (Southern Biotech), all diluted 1:500 in 10% FBS-PBS were added as conjugates respectively. Also, after adding PNPP substrate, the plates were incubated for 3 hr at RT before reading the OD at 405nm.

2.7.1.3 Detection of pilus-specific IgM antibodies in serum

Serum IgM antibodies to RrgA and RrgB were also measured by ELISA following a procedure similar to serum IgG measurement, except alkaline phosphatase-conjugated mouse anti-human IgM (1:1000 dilution) (Sigma) was used as conjugate. In the absence of a reference standard, results were expressed as the optical densities (OD at 405nm) 30 min after the addition of substrate solution.

2.7.2 Measurement of pilus-specific antibodies in cell culture supernatants

Pilus specific (anti-RrgA and anti-RrgB) antibodies were detected in cell culture supernatants by ELISA using the same procedure followed for serum antibody measurement; except samples (cell culture supernatants) were used in 1:10 dilution and 8 serial dilutions of standard (sandoglobulin) was used starting from 1:200 on the top. Also, after adding PNPP substrate, the plates were incubated for 2 hr at RT (protecting it from direct light) before reading the OD at 405nm.

2.7.3 Measurement of total IgG, IgM and IgA in cell culture supernatants

Production of total IgG, IgM and IgA by adenotonsillar MNC was measured in cell culture supernatants by ELISA using the following protocol. Cell culture supernatants from pneumococcal TIGR4wt CCS stimulated and unstimulated (medium control) samples were analysed for this purpose. Culture supernatants stimulated with a polyclonal B cell activator CpG-ODN (InvivoGen, USA) were used as positive control. The ELISA procedure was similar to the previously described procedure for pilus-specific antibody detection, except for the coating antigens, standards and conjugate preparations described below.

2.7.3.1 Measurement of total IgG

For total IgG measurement, coating solution was prepared using Fab specific anti-human IgG (Sigma) which was diluted at 1:1,000 in PBS. The standard for this total IgG assay was purified IgG from human serum (Sigma). The concentration of the stock solution was 4.8mg/ml. A concentration of 1µg/ml was obtained by adding 1µl of the stock solution to 4.8ml of blocking buffer (10% FBS-PBS). The series of standards were constructed by 8 serial dilutions; starting with 0.5µg/ml. Samples for this assay were prepared as 1:100 dilutions of the cell culture supernatants in blocking buffer. Following an incubation of 2 hr in RT, 50µl of alkaline phosphatase-conjugated mouse anti-human IgG (1:2000 dilution) (Sigma) was added into each well of the plate and incubated for 2 hr at RT. After final washes, PNPP substrate was added and absorbance was measured after 5 min by a microtiter plate reader (Opsys MR), at 405nm. The concentration (µg/ml) of each sample was calculated against the standard curve, with the help of microplate analysis software DeltasoftPC (Biometallics).

2.7.3.2 Measurement of total IgM

For total IgM measurement, coating solution was prepared with polyclonal rabbit anti-human-IgM (Dako, Denmark) which was diluted at 1:1,000 in PBS. The standard for this total IgM assay was purified human serum IgM (Sigma), with a stock solution concentration of 0.9mg/ml. A concentration of 1µg/ml was obtained by adding 1µl of the stock solution to 900µl of blocking buffer (10% FBS-PBS). Rest of the ELISA procedure was exactly same as the total IgG measurement, described earlier, except 50µl of alkaline phosphatase-conjugated mouse anti-human IgM (1:1000 dilution) (Sigma) was used as conjugate.

2.7.3.3 Measurement of total IgA

For total IgA measurement, coating solution was prepared with polyclonal rabbit anti-human-IgA (Dako) which was diluted 1:1,000 in PBS. The standard for this total IgA assay was prepared from human colostrum IgA (Sigma). The concentration of the stock solution was 1.6mg/ml. A concentration of 1µg/ml was prepared by adding 1µl of the stock solution to 1.6ml of blocking buffer (10% FBS-PBS). Rest of the ELISA procedure was exactly same as the total IgG measurement, described earlier, except 50µl of alkaline phosphatase-conjugated mouse anti-human IgA (1:1000 dilution) (Sigma) was used as conjugate.

2.7.4 Measurement of salivary IgG and IgA antibodies

For salivary ELISA, saliva samples were prepared as follows. After thawing in RT for 15-20 min, they were centrifuged at 10,000x*g* for 10 min. The supernatants were collected into appropriately labelled microcentrifuge tubes; and used for the subsequent analysis.

2.7.4.1 Measurement pilus-specific IgG antibody in saliva

Pilus specific IgG antibodies were detected in saliva using the following an ELISA procedure similar to that used for the detection of RrgA- and RrgB-specific IgG in adenotonsillar cell culture supernatants described previously, except for the saliva samples were used in a dilution of 1:50, and measurement of absorbance was done after 1 hr incubation with the PNPP substrate.

2.7.4.2 Measurement pilus-specific secretory component (S-IgA) in saliva

Pilus specific secretory component (S-IgA) was measured in saliva using the following procedures. Plates were coated with rRrgA or rRrgB antigen at 1µg/ml, incubated overnight in 4°C and then blocked at 37°C for 1 hour as described previously. After that, 50µl of samples (1:50 in blocking buffer) was added to each well in duplicate in the plate, and incubated for 2 hr at RT. After washing, 50µl of murine monoclonal anti-human IgA secretory component (1:1,000) (Sigma) was added into each well and incubated for 2 hr at RT. 50µl of (1:1,000) alkaline phosphatase conjugated goat anti-mouse IgG (Sigma) as then added into each well and incubated overnight at RT. The subsequent procedure was the same as the IgG assay as described above.

2.7.4.3 Measurement of total IgG and IgA in saliva

Measurement of total IgG and IgA in saliva was performed using the same procedure as above for the measurement of total IgG and IgA in the cell-culture supernatants, except that saliva samples were diluted 1:200 for these assays.

2.7.5 Measurement of cytokine production in cell culture supernatants

2.7.5.1 Measurement of IL-17A

In vitro IL-17A production in adenotonsillar MNC and PBMC cultures was measured with human IL-17A ELISA Ready-Set-Go® set (eBioscience, UK), following manufacturer's instructions. 96-well Costar plates were coated with the capture antibody which was prepared by adding 44µl of purified anti-human IL-17A into 11ml carbonate buffer (1:250 dilutions). 100µl of the capture antibody solution was added into each well of the plates and incubated overnight at 4°C. In the following morning, the plate was washed 5 times with PBS-T. After blocking with 1x assay diluent (200µl/well), the plate was incubated at RT for 1 hr. Then the plate was washed 5 times with PBS-T. In the meantime, the top concentration (500pg/ml) of the standard was prepared by adding 5µl human IL-17A recombinant protein into 10ml of 1x assay diluent. The samples were also prepared (1:20 dilution) in 1x assay diluent. After blocking for 1 hr, the plate was washed 5 times with PBS-T. Thereafter, the standards and samples (100µl/well) were added to the plate. A total of 8 standards were prepared by 2-fold serial dilutions from the top standard. The plate was then incubated at RT for 2 hr. In the meantime, detection antibody was prepared by adding 44µl of purified anti-human IL-17A Biotin into 11ml 1x assay diluent (1:250 dilutions). At the end of the incubation, the plate had been washed 5 times with PBS-T, before adding 100µl of detection antibody to each well. Then the plate was incubated at RT for 1 hr, and then washed 5 times with PBS-T. Thereafter, 100µl of Avidin-HRP (diluted 1:250) was added to each well; and incubated at RT for 30 min. After washing 5 times with PBS-T, 100µl of TMB substrate solution was added to each well, and incubated at RT for 15 min in dark. Then, 50µl of stop solution (1M H_2SO_4) was added to each well. Finally, the plate was read at 450nm,

and the concentration (pg/ml) of each sample was calculated against the standard curve, with the help of DeltasoftPC software (Biometallics).

2.7.5.2 Measurement of IL-17F

In vitro IL-17F production in adenotonsillar MNC and PBMC cultures was measured with human IL-17F ELISA Ready-Set-Go® set (eBioscience), following manufacturer's instructions. The capture antibody was prepared by adding 44µl of purified anti-human IL-17F into 11ml coating buffer (1:250 dilutions). The top concentration (500pg/ml) of the standard was prepared by adding 5µl human IL-17F recombinant protein into 10ml of 1x assay diluent. The detection antibody was prepared by adding 44µl of anti-human IL-17F Biotin into 11ml 1x assay diluent (1:250 dilutions). The rest of the procedure was the same as described for IL-17A measurement.

2.7.5.3 Measurement of IL-22

In vitro IL-22 production in adenotonsillar MNC cultures and PBMC was measured with human IL-22 ELISA Ready-Set-Go® set (eBioscience), following manufacturer's instructions. The capture antibody was prepared by adding 44µl of purified anti-human IL-22 into 11ml coating buffer (1:250 dilutions). The top concentration (500pg/ml) of the standard was prepared by adding 5µl human IL-22 recombinant protein into 10ml of 1x assay diluent. The detection antibody was prepared by adding 44µl of Biotin conjugated anti-human IL-22 into 11 ml 1x assay diluent (1:250 dilutions). The rest of the procedure was the same as described for IL-17A measurement.

2.8 Enzyme Linked Immuno Spot (ELISpot) Assay

2.8.1 Principle of the test

A solid phase (membrane) is pre-coated with an antigen of interest. Then B Lymphocytes are incubated with that membrane. Upon stimulation, B cells produce antigen-specific antibodies that bind to the antigen in the vicinity of the cells secreting them. These captured antibodies are visualised by a secondary antibody-enzyme conjugate and its reaction with the substrate, which results in the development of visible spots. Each spot represents an antibody secreting cell. In the current project, ELISPOT assay was used to measure the frequencies of memory B cells specific to pneumococcal pilus-1 proteins, RrgA and RrgB. Following preparation of freshly isolated adenotonsillar MNC, they were stimulated with pneumococcal TIGR4wt CCS (1μg/ml). Pilus-1 antigen-specific memory B cells were activated to differentiate into plasma cells and produce specific antibodies; which were then enumerated by ELISpot assay.

2.8.2 Culture of adenotonsillar MNCs

Mononuclear cells isolated from adenotonsillar tissue were resuspended in RPMI-1640 medium (4×10^6/ml) and stimulated with TIGR4wt CCS (1μg/ml) and CpG-ODN (1μg/ml) (InvivoGen, USA) as described previously (Zhang et al. 2006b) for antibody production. Equal volume of unstimulated cells was incubated as medium control. The cells were incubated in a 24-well cell culture plate (Corning Inc., USA) at 37°C, in 5% CO_2 for 5 days. Thereafter, the cells were harvested and washed with sterile PBS with 1% BSA followed by centrifugation at 400x*g* for 10 min. The pellets were then resuspended in RPMI-1640 medium to a final cell concentration of 4×10^6/ml.

2.8.3 Plate coating

One day before the cells were harvested, a filtered 96 well ELISpot plate (Millipore, UK) were coated with different antigens. For detecting pneumococcal pilus-1 specific memory B cells, plates were coated with recombinant RrgA and RrgB antigens. Coating solutions were prepared by adding 3.7µl of RrgA (2.7mg/ml) and RrgB (2.7mg/ml) antigens into 5ml of PBS to get 2µg/ml concentrations. For enumeration of total IgG, IgA and IgM spots as positive controls, the plate was also coated with capture anti-IgG, -IgM and -IgA antibody in 1:1000 dilutions. Fab specific anti-human IgG (Sigma), polyclonal rabbit anti-human-IgM (Dako) and polyclonal rabbit anti-human-IgA (Dako) were used for this purpose. Before coating, each well was pre-wet with 15µl of 35% ethanol for 1 min. The wells were washed three times with 150µl of PBS. After coating, the plate was incubated overnight at 4°C.

2.8.4 Incubation of cells in the ELISpot plate

On the following morning, the plate was washed 3 times with PBS-T. Then the plate was blocked with 150µl of RPMI-1640 media with 10% FCS for 1 hr at RT. In the meantime, adenotonsillar cells were harvested from the incubation as described earlier. 100µl of cell suspension was added into each of the designated wells in triplicate. Cell concentration used for different antigens were: $4x10^6$/ml (stock suspension) for RrgA and RrgB; $4x10^5$/ml for total IgM and IgA (1:10 dilution of stock) and $2x10^5$/ml for total IgG (1:20 dilution of stock). Only RPMI-1640 media (no cell) was added into the wells designated as negative controls (at least one well for each antigen). The plate was then incubated overnight at 37°C in 5% CO_2 incubator.

2.8.5 ELISpot assay procedure

The plate was washed 3 times with PBS-T. 50µl of biotin conjugated anti-human antibodies was then added into the designated wells followed by incubation for 30 min at RT. Goat anti-human IgG (H+L) biotin (Invitrogen, USA), Goat anti-human IgM $(Fab)_2$ biotin (Invitrogen) and Goat anti-human IgA $(Fab)_2$ biotin (Invitrogen) were used in 1:2,000 dilutions respectively. At the end of incubation, the plate was washed 3 times with PBS-T, followed by the addition of 50µl of (1:10,000 dilutions) Horseradish peroxidase Avidin D (Vector laboratories Inc., USA). The plate was then incubated for 30 min at RT. After washing 3 times with PBS-T, 50µl of freshly prepared AEC substrate was added into each well. The substrate was prepared with 0.5 ml 3-amino-9 ethylcarbazole into 9.5 ml acetate buffer (appendix-I); adding 25 µl of H_2O_2 immediately before use. The plate was then incubated in dark for 15-20 min at RT. The reaction was stopped with cold water and both sides of the plate were washed with running tap water. Finally, the plate was blotted gently with adsorbent paper to remove the excess water and allowed to dry for 2-3 hr.

2.8.6 Counting of ELISpots

The spots were imaged by Chemi-doc XRS system, and analysed with NIST's Integrated Colony Enumerator (NICE) software (Version 1.2.1, National Institute of Standards and Technology, USA). The result was expressed as number of ASC/million of lymphocytes. For antigen-specific ASC (RrgA and RrgB), the average spot counts were multiplied by 2.5 to get this result. This calculation was obtained by dividing $1x10^6$ (one million) by $4x10^5$ that was the number of cells in 100µl of ($4x10^6$) cell suspension, which was put into each wells. Similarly, for total IgM and IgA the result was obtained by multiplying the spot counts with 25; and for total IgG 50 time multiplication of the average spot count was performed.

2.9 Flow Cytometric Analysis of Cells

2.9.1 Principle of the test

The flow cytometry analyses the optical properties of the fluorocrome-labelled cells passing through a focused laser beam. In this process the cells disrupt and scatter the laser signals as the forward and side scattered light. Forward scatter (FSC) is related to cell size, distributing the larger cells more scattered in its axis. Whereas, side scatter (FSC) is an indicator of cells internal complexity or granularity, distributing the more granular cells more scattered. A combination of FSC and SSC characteristically distribute different subsets of cells in sample containing a mixed population. The FSC is also used to distinguish the dead cells (with low FSC distribution) from the viable cells. Apart from these scatters, flow cytometry also measures fluorescence parameters to detect other structural and functional properties of cellular subsets (Goetzman 1993).

Flow Cytometric analyses in this project were carried out on BD FACS Calibre (BD Biosciences) and BD AccuriV6 flow cytometers (BD Biosciences). Different subsets of lymphocyte were defined with multiple parameters, including FSC and SSC based gating. Fluorescence emissions of different fluorocromes were measured at different wavelengths; FITC or Alexafluor488 in FL1 at 519nm, PE in FL2 at 578nm, PerCPCy5.5, PECy5 or PECy7 in FL3 at 695nm, and APC or Alexafluor660 in FL4 at 660nm. For BD FACS Calibre, data were acquired with Cell Quest software (BD Biosciences) and analysed with WinMDI 2.9 software. For BD AccuriV6, CFlow plus software (BD Biosciences) was used for both flow cytometer data acquisition and analysis.

2.10 T cell Proliferation Assay

2.10.1 Principle of the Assay

Carboxyfluorescein diacetate succinimidyl ester (CFDASE), a fluorescein derivative is initially non-fluorescent and highly permeable across the cell membrane. After it's uptake into the cell the acetate groups are cleaved by cellular esterase to form carboxyfluorescein succinimidyl ester (CFSE). The removal of these acetate groups makes the CFSE molecule highly fluorescent and impermeable to plasma membrane. The succinimidyl group reacts with amino groups, forming a highly stable covalent bond. Although a small amount of these fluorescent conjugates decays or leaks out of the cells, most of them are retained within the cell for a long time. These remaining dyes are evenly distributed among the dividing daughter cells. This dilution of fluorescence in the offspring cells is used to measure the cell proliferation by flow cytometry. Other cellular properties of the dividing cells can be detected by using other fluorocromes, which are compatible with fluorescein (Quah et al. 2007).

2.10.2 Labelling of adenotonsillar MNCs and PBMCs with CFSE

At first, the adenotonsillar MNC and/or PBMC were washed with PBS at 400x*g* for 10 min, to get rid of protein containing media (RPMI); and the cell pellet was dissolved in 3ml sterile PBS. In the meantime, a working concentration of CFSE (50µM) was prepared by adding 5µl of CFSE to 10ml of sterile PBS. 3 ml of this freshly prepared CFSE solution was added to the cell suspension; and mixed thoroughly by vortexing at high speed. The cells were then incubated at 37°C for 8 min. Then, the reaction was quenched by adding 15ml of ice-cold RPMI-1640 media. Thereafter, the cells were centrifuged at 400x*g* for 10 min; and the pellet was dissolved in RPMI-1640 medium supplemented with HEPES (Sigma), 10µg/ml gentamycin (Sigma), 1% L-glutamine (Sigma) and 10% FBS (Sigma). For

adenotonsillar MNC, $4x10^6$cells/ml suspension was prepared; and for PBMC, $2x10^6$cells/ml suspension was used. 500µl of these cell suspensions was stimulated with different concentrations of recombinant pilus (rRrgA and rRrgB) antigens and pneumococcal CCS (TIGR4wt, $RrgA^{-/-}$, $RrgB^{-/-}$). Three controls were used with each set of samples; two without any stimulation (one for unstained and one for CD4-PE stained negative control), and a positive control stimulated with anti-CD3 antibody (4µl/ml). The cells were then added into a 96-well cell culture plate (Corning); and incubated at 37°C in 5% CO_2 for 4-5 days.

2.10.3 Staining of the CFSE labelled cells for FACS analysis

At the end of incubation, the cells were harvested and washed with FACS staining buffer (PBS with 0.02% BSA), by centrifuging at 500x*g* for 8 min. The pellets were resuspended and 2.5µl of CD4-PE (BD bioscience) was added into all the tubes except the unstained negative control. Then the tubes were vortexed and incubated in dark at 4°C for 30 min. Thereafter, the tubes were washed (500x*g* for 8 min) with 1ml of FACS staining buffer. The pellets were resuspended in 400µl of FACS staining buffer; and the tubes were kept in dark at 4°C, until analysed in the BD FACS Calibre (BD bioscience).

2.11 Intracellular Cytokine Staining for Th1/Th2/Th17 Phenotyping

Phenotyping of different T helper cell subsets were performed by intracellular cytokine staining with Human Th1/Th2/Th17 Phenotyping kit (BD Bioscience, UK), following the manufacturer's instruction.

2.11.1 Cell stimulation

For cell stimulation 500µl of cell suspension ($4X10^6$/ml) was incubated at 37°C for 24hr in 5% CO_2, for each antigen and a medium control. A positive control was also stimulated with Ionomycin (1µg/ml) and PMA (20ng/ml). After 24hr, 0.5µl

(1.0µl/ml) of a protein transport inhibitor, BD GolgiStop™ (monensin) was added to each samples; and incubated at 37°C in 5% CO_2 for 6hr. After that, the incubation was stopped, wrapped with cellophane and kept at 4°C for overnight.

2.11.2 Cell harvest, fixation and permeabilization

In the following morning cells were harvested and washed with FACS staining buffer (0.02% PBS-BSA) by centrifugation (500x*g* for 8 min). The pellets were resuspended and fixed with 1ml cold BD Cytofix™ (4% paraformaldehyde in PBS). The cells were then incubated in dark, at RT for 20 min; after washing (500x*g* for 8 min), the pellets were resuspended with 1ml of 1x Perm/Wash™. The cells were then incubated in dark, at RT for 20 min.

2.11.3 Intracellular cytokine staining

After washing (500x*g* for 8 min), the pellets were resuspended with 50µl of 1x Perm/Wash™. Then 20µl fluorochrome-labeled antibody cocktail for detection of intracellular cytokines was added to each samples; and incubated in the dark at RT for 30 min. After that, 1ml of Perm/Wash™ was added to each tube. After washing (500x*g* for 8 min), the stained cells were resuspended in 400µl of FACS staining buffer; and analysed on a flow cytometer.

2.12 Depletion of adenotonsillar MNCs for obtaining T helper cell subsets

To evaluate the proliferative and cytokine production response of different subsets of T helper (Th) cell repertoire, effector memory ($CD45RO^+$) and activated ($CD69^+$) cells were depleted from the adenotonsillar MNC with the help of magnetic beads (Miltenyi Biotech, Germany), magnetic column (Miltenyi Biotech), and a MACS magnet (Miltenyi Biotech). Usually the PBMC sample volumes were small; hence depletion of PBMC was not done.

2.12.1 Principle of the MACS separation

Firstly, the cells to be depleted ($CD45RO^+$ or $CD69^+$) are magnetically labelled with the specific micro-beads binding with the target surface molecules (CD45RO or CD69). Thereafter, the cell suspension is allowed to pass through a MACS LD column, placed within the magnetic field of a MACS separator. The unlabelled cells (not expressing the target molecules) run through the column. These depleted cells are collected in a tube placed underneath the column. The process is known as negative selection. The magnetically labelled cells (those expressing the target molecules) are retained within the column.

2.12.2 Depletion of $CD45RO^+$ Cells

CD45RO is expressed on memory $CD4^+$ and $CD8^+$ T cells. It is also present on $CD4^+$ effector T cells, monocytes, macrophages and granulocytes. Depletion of $CD45RO^+$ cells with CD45RO microbeads yields a naive T cell population, from which naive $CD4^+$ cells can be analysed by flow cytometry.

2.12.2.1 Magnetic labelling and depletion

Approximately, $50x10^6$ cells were resuspended in 400µl (80µl/10^7cells) of depletion buffer (0.5% BSA in PBS); and 100µl (20µl/10^7cells) of CD45RO magnetic beads (Miltenyi Biotech) was added into them. After thorough vortexing, the cells were incubated at 4˚C for 15 min. After that, the cells were washed with 10ml (2ml/10^7cells) of depletion buffer (400x*g* for 10 min). The cell pellet was resuspended in 250µl (50µl/10^7cells) of depletion buffer; and magnetic separation was carried out in a class-II bio-safety cabinet. The MACS LD column (Miltenyi Biotech) was placed inside the magnet groove; and primed by adding 2ml buffer avoiding any bubbles. Then the cell suspension was added to the column and

allowed to pass through. After that, 2ml buffer was added to the column and allowed to pass through. The naive ($CD45RO^+$ depleted) T cells, obtained by this process were resuspended into RPMI medium to a concentration of $4x10^6$cells/ml. CFSE labelling, stimulation with antigens and FACS analysis were done following the same techniques, described previously for undepleted lymphocytes.

2.12.3 Depletion of $CD69^+$ Cells

CD69, a member of C-type lectin family is expressed on activated T cells, B cells, NK cells, macrophages, neutrophils, eosinophils and platelets. It is the earliest inducible cell surface marker of lymphoid activation. It acts as a signal transmitting receptor for cellular activation (Santis et al. 1994). Depletion of $CD69^+$ cells with CD69 microbeads gets rid of already activated T cells in a population, from which the resting $CD4^+$ cells can be analysed by flow cytometry.

2.12.3.1 Magnetic labelling and depletion

Approximately, $50x10^6$ cells were taken for this depletion. The cells were washed with PBS (400x*g* for 10 min); and the cells were resuspended in 200µl (40µl/10^7cells) of depletion buffer (0.5% BSA in PBS). After that, 50µl (10µl/10^7cells) of CD69-biotin (Miltenyi Biotech) was added into the tube and vortexed thoroughly. Then the cells were incubated at 4˚C for 15 min. At the end of the incubation, 100µl (20µl/10^7cells) of CD69 magnetic beads (Miltenyi Biotech), and 150µl (30µl/10^7cells) of depletion buffer were added into the cells. After vortexing thoroughly, the cells were incubated again at 4˚C for 15 min. After that the cells were washed with 10ml (2ml/10^7cells) buffer solution at 400x*g* for 10 min. Finally, the pellet was resuspended into 250µl (50µl/10^7cells) buffer solution. Magnetic separation of these cells was carried out in a class-II bio-safety cabinet as

described above for CD45RO depletion. The resting ($CD69^+$ depleted) T cell populations, obtained by this process were resuspended into RPMI medium to a concentration of $4x10^6$cells/ml. CFSE labelling, stimulation with antigens and FACS analysis were done following the same techniques, described previously for undepleted lymphocytes.

2.13 PCR of Pilus 1 Islet and Pneumolysin Genes

2.13.1 Principles of the Assay

Polymerase Chain Reaction occurs in three major steps. First step is the denaturation, when the double stranded DNA is split into two single strands at 94°C. The second step, annealing occurs at 56°C, when two (forward and reverse) primers are attached with each of the strands. The final step is the extension of the DNA templates at 72°C, producing two double stranded DNAs. After that, the first cycle is completed. Repeating of such cycles many (30 or 40) times, produces more than a billion copies of the template DNA. The amplified DNA can be detected by various methods, after electrophoresis in agarose gel.

2.13.2 PCR of pilus 1 islet and pneumolysin genes in pneumococcal isolates

The primers were designed to amplify the three genes in the rlrA pathogenecity islet (PI-1) that encode proteins constituting the pneumococcal pilus-1 (Basset et al. 2007b). As a positive control for pneumococcus, amplification of ply gene (encoding pneumolysin) was done in each of the isolates with primers described in previous studies (Regev-Yochay et al. 2010, Whatmore et al. 1999). All the primers were validated by NCBI primer BLAST. Also the size of each amplicon was determined by aligning the forward and reverse primer sequences with that of each respective gene (*rrgA, rrgB, rrgC* and *ply*) in the *Streptococcus pneumoniae* (TIGR4) genome

(ftp://ftp.tigr.org/pub/data/Microbial_Genomes/s_pneumoniae_tigr4/annotation_dbs/s_pneumoniae_tigr4.seq). The amplicon sizes were calculated as; *rrgA* 2550 Kb, *rrgB* 1850 Kb, *rrgC* 1056 Kb and *ply* 1191 Kb. Detailed information of the primers (ordered from Sigma Aldrich, UK) are mentioned in table-2.13.2.

Table-2.13.2: Primers used for the PCR detection of PI-1 and *Ply* genes in pneumococcal isolates

Oligo Primer	**Primer sequence (5′-3′)**	**Tm°**	**nmol**	**GC%**	**Reference**
rrgA Forward	AAGATATTTCAGAAGGCAGTTGCA	64.7	47.3	37.5	(Basset et al. 2007b)
rrgA Reverse	TTCTCTCTTTGGAGGAATAGGTTC	62.6	37.4	41.6	(Basset et al. 2007b)374
rrgB Forward	CTTGCTGCCTTATTACTGA	55.6	39.5	42.1	(Basset et al. 2007b)
rrgB Reverse	GATAGTGATTTTTTTGTTGAC	52.9	105.3	28.5	(Basset et al. 2007b)
rrgC Forward	GCTCTGTGTTTTTCTCTTGTATGG	62.8	57.9	41.6	(Regev-Yochay et al. 2010)
rrgC Reverse	ATCAATCCGTGGTCGCTTGTTATTTTTA	69.8	49.5	35.7	(Regev-Yochay et al. 2010)
ply Forward	TTGTTGTTATCGAAAGAAAGAAGCGGA	69.7	44.0	37.0	(Whatmore et al. 1999)
ply Reverse	AAACCGTACGCCACCATTCCCA	72.9	46.2	54.5	(Whatmore et al. 1999)

2.13.3 DNA extraction

The *S. pneumoniae* isolates were streaked on blood agar plates and then incubated overnight at 37°C and 5% CO_2. Then 8-10 pure colonies of the bacteria were picked-up with a sterile loop and dissolved in a sterile 1.5ml eppendorf tube containing 500µl sterile PBS. They were centrifuged at 1000x*g* for 10 min; and the supernatants were aspirated carefully. The pellets were resuspended into 100µl of 1x ThermoPol buffer (New England Biolabs, USA); and boiled for 10 min at 94°C. Then they were centrifuged at 1000x*g* for 5 min; and cooled on ice. The DNA concentration and purity were measured with a nanodrop apparatus using 2µl supernatants from these boilates.

2.13.4 PCR amplification

1µl of the supernatant of the boiled bacteria was used as DNA template. A final volume of 25µl was prepared by adding 12.5µl BioMix Red reaction mix (Bioline, UK), 2.5µl of each forward and reverse primer (conc. 0.3µM) and 6.5µl ddH_2O. In each set of PCR assay a sample of TIGR4wt pneumococcus was run as a positive control; and a sample of ddH_2O was run as a negative control. For detection of *ply* (pneumolysin) and *rrgC* genes; reaction conditions consisted of 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, followed by 5 min at 72°C. Reaction conditions for detection of *rrgA* and *rrgB* genes consisted of 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min, followed by 5 min at 72°C.

2.13.5. Gel running and visualisation

Before loading into a 0.75% agarose gel, 1µl Ultrasafe Blue^{TM} (Syngene, UK) was added to 10µl of each PCR products and a 1 kb DNA ladder (Invitrogen, UK). The gel was run in 1x TBE buffer at constant 100v for 1 hr. It was then visualised in a ChemiDoc XR system (BioRad, UK), over a blue filter tray.

2.14 Statistical Analysis

Data were analysed using SPSS statistics version 20 and GraphPad Prism version 5. At first the data were tested for normality with D'Agostino and Pearson omnibus normality test. Further analyses of the data that did not pass the normality test were done by nonparametric test; and the data that pass the normality test were analysed by parametric test. In some cases, log transformation of data was done before analysing by parametric statistical methods. Differences between two groups (*i.e.*, carrier and non-carriers) were analysed by Student's t (parametric) or Mann-Whitney (non-parametric) test. Differences between CCS stimulated and unstimulated samples in the same group of subjects were analysed by paired t (parametric) or Wilcoxon matched-pairs signed rank (non-parametric) test. One way analysis of variance (ANOVA) was performed to evaluate the effects of age and nasopharyngeal carriage status on the antibody titre. Association between two factors was analysed by Chi square (χ^2) test. Correlation between two factors was analysed by Pearson's (parametric) or Spearman's (non-parametric) correlation test. A p value of <0.05 was taken as a level of statistical significance.

Chapter 3

Nasopharyngeal Carriage of *Streptococcus pneumoniae* and Prevalence of Pilus-1 Gene among The Carriage Isolates

3.1 Introduction

Human nasopharynx is the ecologic niche for a number of mucosal pathogens, from where they escape into the adjacent sites or spread to close contacts via droplet and respiratory secretions (Hill and Virji 2003). *Streptococcus pneumoniae* is one of the important members of this microbial flora. Despite being a very common event in early childhood, pneumococcal carriage remains asymptomatic most of the time. Occasionally, the bacteria disseminate to the sterile sites causing overt infections (Austrian 1986).

Usually, pneumococcal acquisition in the human nasopharynx occurs in the first year of life (O'Brien and Nohynek 2003). The onset of this acquisition shows wide geographical variation; which usually occurs at an earlier age in the developing countries than in the developed world. A longitudinal study in Papua New Guinea showed over 60% of the neonates were colonised by 1 month of age (Gratten et al. 1986). In a study in Bangladesh, 50% of the new-born children were reported to experience at least one episode of pneumococcal colonisation within 8 weeks, which surpassed 90% within 21 weeks (Granat et al. 2007). A similar trend was observed in neighbouring India, where 50% of the new-borns were colonised by pneumococcus before the age of 2 months and 80% before 6 months (Coles et al. 2001). A South African study showed a comparatively delayed onset of acquisition; which was 30% in 6 weeks, 44% in 10 weeks, 51% in 14 weeks and 61% in 9 months of age (Mbelle et al. 1999). In developed countries, pneumococcal acquisition starts around 6 months of age, which peaks on the following 6 months (WHO 2009). In USA, the onset of carriage varied between 4 days to 18 months, with a mean age of about 6 months (Gray et al. 1980). A study in Swedish children showed a much slower acquisition, 12% at the age of 2 months and 30% at the 6 months (Aniansson et al. 1992).

The high carriage rate in the early childhood gradually declines with increasing age (Bogaert et al. 2004b). A study carried out in the UK before the introduction of conjugate vaccination showed that the average prevalence of pneumococcal carriage rates dropped from over 50% in children <2 years to less than 10% in adults (Hussain et al. 2005). Not only the frequency of pneumococcal carriage, but also the incidence of diseases caused by them shows a decreasing trend as the children grow older. This decline is thought to be mediated by the naturally acquired or vaccine induced immunity (O'Brien and Nohynek 2003).

Geographical variations in the serotypes colonising human nasopharynx is relatively small (Bogaert et al. 2004a), and the most commonly isolated carriage serotypes are 6, 14, 19 and 23 (Ghaffar et al. 1999). Risk factors for higher pneumococcal carriage include ethnicity, over-crowding, surrounding environment, and socioeconomic conditions (Bogaert et al. 2004a). Indigenous people in the developed countries, such as Apache and Navajo (USA), Eskimo (Alaska), Aborigines (Australia) and African American are among the highly susceptible ethnic groups for pneumococcal colonisation and diseases (Bogaert et al. 2004a; Mackenzie et al. 2010). Most important socioeconomic condition affecting pneumococcal carriage is the number of family members (older siblings in particular) (Petrosillo et al. 2002). Environmental factors potentiating colonisation include overcrowding and smoking (Ghaffar et al. 1999). Higher carriage was reported in children attending day-care centres (Bogaert et al. 2001) and living in orphanages (Raymond et al. 2000). Apart from these, carriage rate increases in winter. This may be associated with a preceding viral infection, and closer contact with other people or sharing warm clothing (Ghaffar et al. 1999).

Several factors are involved in facilitating pneumococcal colonisation and invasion. Biochemical structure of polysaccharide capsules is an important factor affecting pneumococcal carriage, and serotypes producing capsules are more likely to colonise and survive neutrophilic killing in the nasopharynx (Weinberger et al. 2009). Colonisation by a particular pneumococcal serotype also differs owing to its phase variation, yielding either transparent or opaque phenotypes of colonies (Weiser et al. 1994). Transparent varieties, with thinner polysaccharide capsules are more likely to colonise the nasopharynx. On the other hand, the opaque varieties having thicker capsules are more resistant to phagocytosis and more likely to cause invasive infections (Kim et al. 1999).

Capsulated bacteria express adhesins on their surface to augment their attachment in the respiratory mucosa (Carbonnelle et al. 2009). Pneumococci are decorated with a number of adhesins, which help them to colonise the nasopharynx. They include, phosphorylcholine (ChoP), choline binding protein A (CbpA), neuraminidase (NanA), β-galactosidase (BgaA), β-N-acetylglucosaminidase (StrH), hyaluronate lyase (Hyl), pneumococcal adhesion and virulence factor A (PavA), and enolase (Eno) (Kadioglu et al. 2008). Some pneumococcal strains express another important ECM-binding adhesin on their surface, known as pilus (Barocchi et al. 2006).

Bacterial pili are hair-like surface projections, helping them to adhere with the host epithelium (Schwarz-Linek et al. 2004). Besides facilitating adhesion to host mucosal surface, Gram-negative pili have diverse biological functions; as for example, meningococcal pili contribute to bacterial motility and facilitate uptake of genetic material (Hill et al. 2010). On the contrary, Gram-positive pili are thinner and primarily involved in cell attachment (Proft and Baker 2009). Their adhesive function is mediated by interaction with adhesive matrix molecules (MSCRAMM) in the host mucosa (Schwarz-Linek et al. 2004).

Streptococcus pneumoniae expresses two types of pili. Type-1 pilus is composed of three subunit proteins; RrgA, RrgB and RrgC. The backbone of pilus shaft is formed by RrgB, anchoring RrgA at the tip and RrgC at the base (Hilleringmann et al. 2009). On the other hand, the type-2 pilus is composed of only a core structural protein, PitB (Bagnoli et al. 2008). Compared to their non-piliated variants, pilus-expressing pneumococci are more efficient to colonise and cause invasive infections; and associated with an enhanced host inflammatory response in experimental mice (Barocchi et al. 2006). Genes encoding pneumococcal type-1 pilus (known as pilus islet 1 or PI-1), are located in the *rlrA* pathogenicity islet. This islet is composed of seven genes; a regulatory gene *rlrA,* three structural genes (*rrgA, rrgB, rrgC*) encoding subunit proteins, and three sortase genes (*srtB*, *srtC*, and *srtD*) encoding catalytic enzymes (Hava et al. 2003).

Type-1 pilus islet is clonally inherited by pneumococcal strains and exists in three variants, clade I, II and III (De Angelis et al. 2011). Not all pneumococcal strains containing PI-1 islet necessarily express pilus. Biphasic expression of pilus-1 has been observed even in a single clonal population of pneumococci; showing both high pilus expression (HPE) and low pilus expression (LPE) phenotypes (Basset et al. 2011; De Angelis et al. 2011). This variability of pilus-1 has been reported to be positively regulated by an endogenous promoter (*rlrA*) and negatively regulated by one of the pilus-1 subunit (RrgA) proteins (Basset et al. 2011). A complex regulatory mechanism of pilus-1 expression has been described by studies identifying some other negative regulators (MgrA, HK343, MerR, CbpS, TCS08, mntE, PsaR) (Hemsley et al. 2003; Rosch et al. 2008; Song et al. 2009).

Based on the sequence variation in the terminal end, RrgA has two clades (clade I and II) (Moschioni et al. 2010b); and RrgB has three variants (clade I, II and III) corresponding the clonal variation of pilus-1 operon (Moschioni et al. 2008). Although definite function of RrgC is yet to be established (Hilleringmann et al. 2009); *rrgC* has been shown to be the most conserved at the DNA level (Moschioni et al. 2008). For this reason detection of *rrgC* gene is considered as the marker of positivity for pilus-1 positivity (Basset et al. 2007b; Regev-Yochay et al. 2010).

Prevalence of pilus islets (PI) in pneumococcal strains is not very common. PI-1 has been reported to be present in 30-50% of the pneumococcal strains. Importantly, their prevalence is higher among antibiotic resistant strains of vaccine serotypes 4, 6B, 9V, 14, 19F and 23F (Aguiar et al. 2008, Moschioni et al. 2010a, Moschioni et al. 2010b, Regev-Yochay et al. 2010). PI-1 has also been detected in some vaccine-related (6A and 23A/B) and non-vaccine serotypes (11A and 17F) (Regev-Yochay et al. 2009). Prevalence of PI-2 is even rarer than PI-1; and mostly associated with emerging serotypes (1, 2, 7F and 19A). Co-existence of both pilus islets has been reported in some of the pneumococcal strains (Bagnoli et al. 2008).

This study focuses on the nasopharyngeal carriage of pneumococci in a cohort (patients undergoing routine adenoidectomy/tonsillectomy) of UK population. We also investigated the prevalence of PI-1 genes in these carriage isolates. Studies in animal model have shown that pneumococcal pili facilitate their adherence to the respiratory epithelium (Barocchi et al. 2006). So far, there is no information regarding their contribution to nasopharyngeal colonisation in humans.

3.2 Aims of Study

To investigate pneumococcal colonisation rate in children and adults; to isolate the carriage strains and analyse the prevalence of pilus-1 genes among these isolates.

3.3 Experimental Design

1. Bacteriological culture of nasopharyngeal swab samples was done for detection of pneumococcal carriage.

2. *Streptococcus pneumoniae* was isolated from these cultures and stored for further analyses.

3. PCR amplification of genes (*rrgA, rrgB* and *rrgC*) in the PI-1 operon was performed to detect the prevalence of type-1 pilus among these isolates.

4. Data were analysed to see whether there is any association between PI-1 genes and density of pneumococcal growth in the nasopharyngeal swab culture.

3.3.1 Culture of nasal swabs for *Streptococcus pneumoniae*

Detailed procedure of culture for pneumococcus from nasopharyngeal swabs is described in the methods chapter-2. Briefly, swabs were collected in vials containing 1ml Skim milk-Trypone-Glucose-Glycerine (STGG) broth, and stored at -70°C. After thawing and vortexing 40µl of broth were inoculated on Columbia blood-agar plates (Oxoid, UK), following a standard technique (O'Brien and Nohynek 2003). The plates were incubated overnight at 37°C, in 5% CO_2; and observed for α-haemolytic colonies of pneumococcus, which were then confirmed by optochin disc inhibition tests (Chandler et al. 2000). The density of bacterial load was measured semi-quantitatively following a standard method (O'Brien and Nohynek 2003). The first inoculation on the plate was considered as Quadrant 1, from where it was streaked into all four quadrants. The growth was termed as 3+ if <10 colonies were in Quadrant 4 and >10 colonies in Quadrant 3; growth was labelled 2+ if there were <10 colonies in Quadrant 3 and >10 in Quadrant 2; finally, 1+ growth was noted if there were <10 in Quadrant 2 with growth in Quadrant 1.

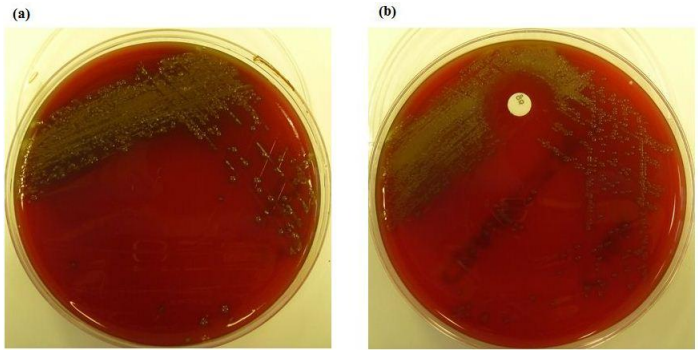


Figure 3.3.1: Growth of *Streptococcus pneumoniae* on blood agar plates; (a) Pneumococcal colonies (3+ growth) and (b) their Optochin susceptibility.

3.3.2. PCR for detection of pilus islet 1 (PI-1) in pneumococcal isolates

Detailed procedure for detection of pilus-1 operon (PI-1) by PCR in pneumococcal isolates from nasopharyngeal swabs is described in the methods chapter-2. Briefly, DNA was extracted by boiling the bacterial cells (10 min at 94°C) in 1× Thermo Pol buffer (New England Biolabs, USA). For the PCR reaction, 1µl of the DNA template was used in a final volume of 25µl reaction mix, prepared by adding 12.5µl BioMix Red reaction mix (Bioline, UK), 2.5µl of each forward and reverse primers (0.3µM) (obtained from Sigma Aldrich, UK) and 6.5µl ddH_2O. The reactions were carried out in a Techne Flexigene Thermal Cycler (Techne, UK). The reaction conditions for *rrgC* and *ply* consisted of 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 min, followed by 5 min at 72°C. For *rrgA* and *rrgB* same conditions were applied, except a longer extension phase 72°C for 2 min. Primer sets used for these reactions are listed in table 3.3.2 with references. After the amplification 10µl of each PCR products were run into 1.0% agarose gel with 1µl Ultrasafe Blue (Syngene), and then visualised in a ChemiDoc XR (BioRad), over a blue filter tray. Pneumococcal isolates carrying *rrgC* gene was considered as pilus positive, since it is highly conserved within the PI-1 islet (Moschioni et al. 2008; Basset et al. 2007b). Presence of pneumolysin (*ply*) confirmed the isolate as pneumococcus (Regev-Yochay et al. 2010).

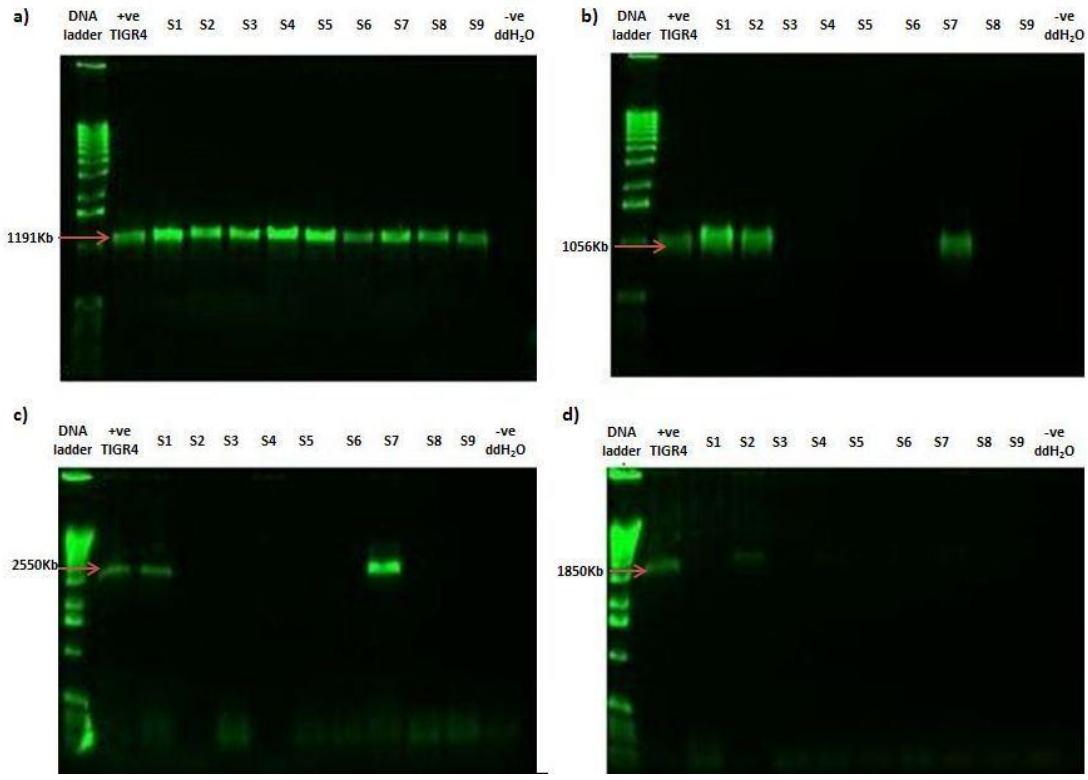


Figure-3.3.2: Detection of PI-1 and pneumolysin (*ply*) genes in pneumococcal isolates. Figure shows the a) *ply* (1191 Kb), b) *rrgC* (1056 Kb), c) *rrgA* (2550 Kb) and d) *rrgB* (1850) genes.

3.3.3 Statistical analysis

Data were analysed using IBM SPSS statistics (version 20.0). Mean age differences between PI-1 positive and negative groups was analysed by Student's t-test. Association of PI-1 gene positivity in the pneumococcal isolates and the density of growth in nasal swab culture was analysed by Chi square (χ^2) test. A *p* value of <0.05 was taken as a level of statistical significance.

3.4 Results

3.4.1 Pneumococcal carriage rate declines with increasing age

A total of 295 subjects were included in the study among whom 195 were children (age <16 years, Mean±SEM = 6.04±0.259), and 100 were young adults (age 16-30 years, Mean±SEM = 21.72±0.39). Pneumococcal carriage was assessed by culturing nasopharyngeal swabs. As expected, there was an age-related decrease in carriage rate, with more than 50% in <3 years old children to 3% in young adults (table-1). There was no significant difference between carriage rates in male and female patients.

Table-3.4.1: Demography of the study subjects and nasopharyngeal carriage of pneumococcus

	n (% colonised)		
Age group	**Male**	**Female**	**Total**
1-3 yrs.	32 (56.3)	24 (54.2)	56 (55.4)
4-6 yrs.	40 (37.5)	40 (32.5)	80 (35.0)
7-9 yrs.	11 (27.3)	13 (38.5)	24 (33.3)
10-15 yrs.	9 (22.2)	26 (15.4)	35 (17.1)
16-30 yrs.	24 (4.2)	76 (2.6)	100 (3.0)

3.4.2 Prevalence of pilus-1 gene (PI-1) among the carriage isolates

To investigate the prevalence of pilus-1 genes among the pneumococcal isolates obtained from nasal swab culture of study subjects, PCR amplification was performed for *rrgA*, *rrgB* and *rrgC* genes. Among the total 76 isolates, 8 were positive for *rrgC* gene. Only 2 of these strains were positive for *rrgA* and *rrgB* genes. All of these strains were positive for *ply* gene, which was amplified to confirm them as pneumococci. The isolates carrying the *rrgC* gene was considered as pilus-1 positive, which has been reported to be highly conserved within the pilus islet 1 (PI-1). Based on *rrgC* positivity, the overall of prevalence PI-1 among the isolates in our study was about 10.6% (figure 3.4.2). Colonisation by pilus-1 positive strains was highest (19.4%) in children younger than 3 years, followed by 7.1% in the 3-6 years age group. None of older children and adults was colonised with a pilus 1 positive strain (Figure-3.4.2).

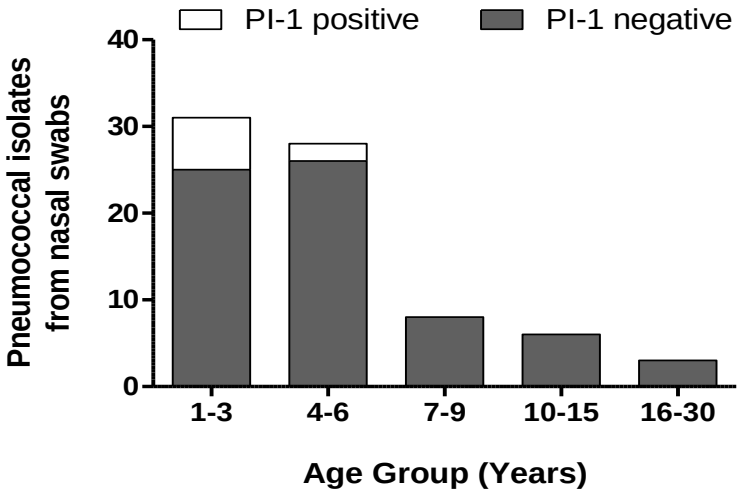


Figure-3.4.2: Prevalence of PI-1 genes among the pneumococcal isolates from nasopharyngeal swab samples in different age groups. Figure shows the total number of pneumococcal isolates recovered from nasal swab culture (bar diagram) with the relative share of PI-1 positive (blank area) and negative (shaded area) strains.

The mean age (2.88±0.29) of children colonised with PI-1 positive strains was significantly lower than that of (5.1±0.36) children colonised by PI-1 negative strains (Figure-3.4.3, p=0.0383).

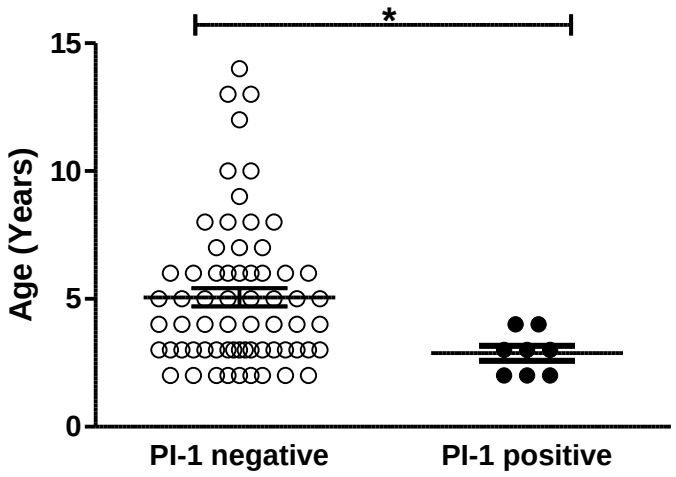


Figure-3.4.3: Mean age of children colonised by PI-1 negative and positive pneumococcal strains. The scatter plot shows the age distribution of children (n=73) colonised by PI-1 negative (blank circles) and positive strains (filled circles) of pneumococcus, with mean age ± SEM (error bars) and significance level *p<0.05.

3.4.4 Relationship of pneumococcal density on nasal swab and patients' ages

Semi-quantitative estimation of the density of the pneumococcal growth in the nasal swab culture was performed, which roughly represented the bacterial load in the nasopharynx. As a whole, the distribution of pneumococcal growth densities (1+, 2+ and 3+) was (43%, 32% and 25% respectively) (Figure-3.4.4). These densities were analysed in relation to different age groups of the colonised subjects. More than two-thirds (71%) of the <3 years children yielded higher pneumococcal growth density (2+ and 3+). In the 4-6 years age group, this percentage was 53%, which dropped to 35% in older subjects (Figure-3.4.4).

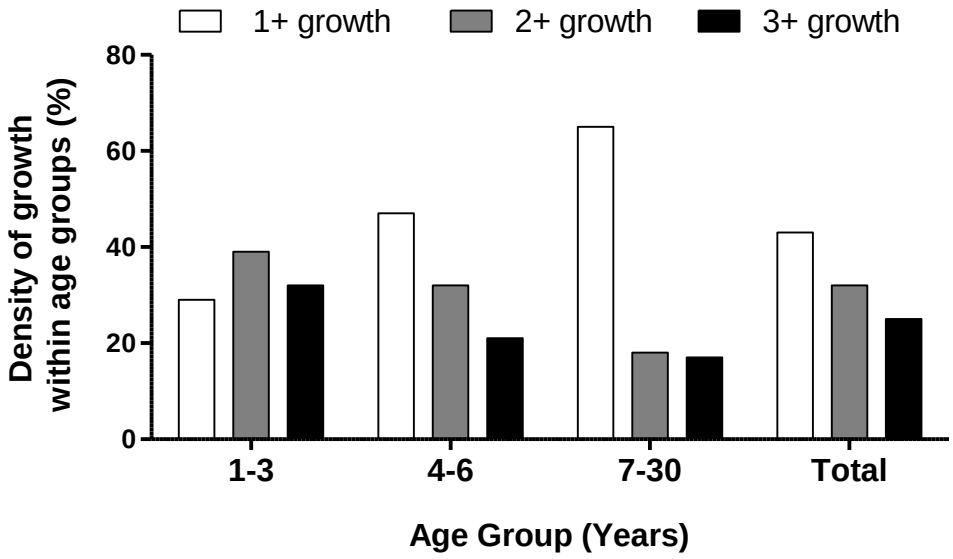


Figure-3.4.4: Density of pneumococcal growth on nasal swab culture in different age groups of colonised subjects. Figure shows the density of pneumococcal growth among different age groups in colonised subjects (n=76). Result represents 1+ growth (blank bars), 2+ growth (shaded bars) and 3+ growth (black bars) of pneumococcus during nasal swab culture on blood agar.

3.4.5 PI-1 positive pneumococcal strains are associated with higher density of pneumococcal growth in nasal swab culture

To investigate whether there was any association between bacterial growth density and the pilus-1 positivity in pneumococcal isolates; Chi-square (χ^2) test was performed (table-3.4.5); which showed that presence of PI-1 gene was associated with a higher pneumococcal growth density on nasal swab culture (n=76, Pearson χ^2=9.01, DF=2, *p*=0.011).

Table-3.4.5: Association of PI-1 positivity with density of pneumococcal growth in the nasal swabs from the carriers

	Density of pneumococcal growth			
PI-1 gene	**1+**	**2+**	**3+**	**Total**
Negative	33	21	14	68
Positive	0	3	5	8
Total	33	24	19	76

3.5 Discussion

In this study, nearly half of the children aged less than six years were found to be colonised by pneumococcus. This finding was consistent with the colonisation rate among UK children of this age group, as reported in other studies (Roche et al. 2007). As expected, pneumococcal colonisation rate showed an age-dependent decline in our study population (table-3.4.1). This finding was in agreement with the results of a previous study among a similar cohort of UK children showing that carriage rates peaked in 2 years then declined steadily and reached to ~30% beyond 3 years (Zhang et al. 2006b). The pattern is similar among the children of other European countries. A carriage study in healthy Dutch children reported peak incidence (55%) at the age of 3 years, then declined gradually to around 10% beyond age of 10 years (Bogaert et al. 2006). Whereas, in Switzerland colonisation rate in children aged <4 years was reported to be 48%, which then gradually declined throughout the childhood (Mühlemann et al. 2003).

Children at their early childhood are not only at greater risk of pneumococcal colonisation, but also more likely to carry them for longer duration of time (Högberg et al. 2007). This might be the reason for higher density of bacterial growth in younger children on nasal swab culture as observed in our study. We observed an age-dependent decrease in the percentage of children with higher bacterial growth density on nasal swab culture (figure-3.4.4). This may be due to the natural immunity developed during previous exposures.

Serotyping of the colonizing isolates in our study was not performed. Therefore, we are unable to comment about serotype prevalence among the population. However, we have analysed the prevalence of pilus-1 positive strains among these isolates. Only 10.6% of the isolates in our study population was positive for *rrgC* gene. The

positivity of *rrgA* and *rrgB* genes, which encode the other two pilus-1 component proteins was even lower (2.6%). This might be due to the fact that RrgA and RrgB has different clades (two and three respectively).

The positivity rate of PI-1 (*rrgC*) genes among the isolates in our study population was lower than previous studies, which showed that 25% to 50% of pneumococcal strains possess PI-1 genes (Aguiar et al. 2008; Moschioni et al. 2010a; Basset et al. 2007b); and the prevalence was similar in both human carriage and invasive strains (Basset et al. 2007b). This low positive rate of PI-1 genes in our study is likely to be associated with the introduction of pneumococcal conjugate vaccines in the UK, as it happened in the years immediately after introduction of PCV7 in USA (Regev-Yochay et al. 2010). PCV7 was introduced for routine vaccination in the UK in 2006, which was replaced by PCV13 in 2010. Serotypes included in the pneumococcal conjugate vaccines are more likely to carry the *rrgC* gene than the non-vaccine serotypes (Basset et al. 2007b). As shown in a study on children (<7 years) in USA, the prevalence of pilus-1 positive strains among nasopharyngeal isolates dropped from ~25% to 15% after 3 years of introduction of PCV7. Interestingly, they observed the prevalence to rise again in seven years (~26%) matching that of the pre-vaccination era; which was most likely due to colonisation by non-vaccine serotypes (Regev-Yochay et al. 2010). However, the contribution of vaccination to this low prevalence of pilus-1 gene positive isolates in our study was unknown, as the percentages of vaccine and non-vaccine serotypes were not analysed.

In this study, PI-1 gene could be detected only in a small fraction of strains isolated from young children (<6 years); not in the isolates recovered from the older children or adults. The study subjects included in our study were individuals undergoing routine elective adenotonsillectomy and they were otherwise healthy and not

immunodeficient. It is assumed that pneumococcal colonisation rate and pilus-1 positivity rate among the isolates would be similar in normal healthy population. The mean age of children colonised with a PI-1 positive strain was significantly lower than that of children colonised with a PI-1 negative strain (Figure-3.4.3, p=0.0383). It has been shown previously that pneumococcal colonisation is a dynamic process, with most young children (<2 years) colonised serially with single or even multiple serotypes of the pneumococcus (Syrjänen et al. 2001). So it is possible that older children or adults included in this study had been exposed to pilus-1 expressing strains previously. Such case of a colonisation event is likely to induce a natural immune response to the pilus-1 components, which are known to be potently immunogenic in animal models (Gianfaldoni et al. 2007). This natural immunity resulting from a previous exposure to one of the pilus-1 expressing stains might also contribute to their overall low prevalence, particularly in the older children and adults (Regev-Yochay et al. 2010).

Pneumococcal colonisation triggers a natural immunising process conferring protection against future colonisation and diseases. This protection is not strictly confined to that particular colonizing serotype; and has been shown to cover other serotypes as well (Ferreira et al. 2013). It is possible that children, who were colonised by piliated strains of pneumococci, developed a protective immune response. They were therefore protected from any subsequent carriage episode by these strains. As many protein antigens in the piliated strains are shared with other pneumococcal strains, there is a high possibility of developing cross-protective immunity. In general, colonisation by any pneumococcal strains in the early childhood reduces the likelihood of colonisation by the piliated strains in later life. While this explains the absence of pilus positive pneumococci among the carriage isolates from older children and adults, their prevalence among the strains colonizing

<1 year children remains unknown, as our study population did not include any children of this age group. These children may carry pneumococci, with a higher percentage of pilus-1 gene positivity, as expected by the trend we observed in our study (figure-3.4.2).

Studies in animal models suggest pneumococcal pili contribute to mucosal colonisation (Barocchi et al. 2006). So far, there is no information regarding their role in colonising human nasopharynx. To establish human carriage models for this purpose is beyond the scope of our study. However, analysis of culture densities of piliated and non-piliated pneumococci in nasal swabs may indicate their capacity to colonise. Our findings suggest that PI-1 positive isolates were associated with higher colonisation densities than those of the PI-1 negative strains (table-3.4.5).

It can be debated whether the higher bacterial density yielded in the nasal swab culture of piliated strains is attributable to pilus expression only, as it could also be related to the younger age of the children. Our findings suggest that both the pneumococcal strains (piliated or not) and the age of the carrier could contribute to the higher bacterial load in the nasopharynx, similar to their effect on the duration of carriage (Högberg et al. 2007). The younger children are less likely to develop a strong immune response to clear the colonizing bacteria early (Granat et al. 2009); and therefore may carry a higher bacterial load. On the other hand, expression of pili is likely to facilitate pneumococcal adhesion to nasopharynx (Nelson et al. 2007); and may contribute to the higher density of pneumococcal growth on nasal swab culture.

The pneumococcal genome is highly diverse with more than 50% of it is composed of accessory genetic regions varying in different strains (Hiller et al. 2007). Expression of these genes affects the virulence of pneumococcal strains from

different genetic background. Pilus islet-1 (PI-1) is one of these accessory genetic components, likely to be acquired for facilitating pneumococcal adherence and survival on mucosal surfaces (Henriques-Normark et al. 2008). PI-1 is present in a limited number of pneumococcal clones, and can be divided into three different clades, mostly contributed by the main pilus subunit RrgB (Moschioni et al. 2008). Pneumococcal pili enhance bacterial adhesion to human respiratory epithelium, promote colonisation in mice and facilitate *in vitro* biofilm formation (Nelson et al. 2007; Barocchi et al. 2006; Muñoz-Elías et al. 2008). Despite these advantages of piliated strains in colonisation and invasion, acquisition of both pilus islets (PI-1 and PI-2) in pneumococcal strains is not very common. One of the reasons limiting a widespread selection of the pilus-1 genes in pneumococci could be the strong immunogenicity of its components (Gianfaldoni et al. 2007), which is likely to induce a protective response against colonisation.

PI-1 positive pneumococci exist in two phenotypically distinct (pilus-expressing and non-expressing) forms (Basset et al. 2011; De Angelis et al. 2011), which probably helps them to evade the host immune response. These phases are interchangeable, with reversion of pilus-high to pilus-low expression state and vice versa. A complex interaction between RlrA (positive regulator) and a number of negative regulators determines the fraction of cells in each phenotype (Basset et al. 2012). Bacterial phase variation can be considered as their evolutionary survival mechanism in the face of the host immune response. Phase variation is more common among the structures expressed on bacterial surface and therefore exposed to the immune system (van der Woude and Bäumler 2004). Variation of pilus expression has been reported in meningococci as well (Virji et al. 1992), which affects their interaction with host respiratory epithelium (Hill et al. 2010).

Several mechanisms are accounted for bacterial variable protein expression. There might be changes at genetic level due to genetic rearrangement or DNA modification; or it might result from epigenetic phenotypic variation without affecting the DNA sequence (van der Woude and Bäumler 2004). The biphasic expression of pneumococcal pilus-1 has been reported to be mediated by the later cause. Comparison of PI-1 islet from both pilus-high and pilus-low expression populations of three different strains has revealed no difference in their genomic sequence. There was no indication that their biphasic expression pattern resulted from genetic mutation or recombination events PI-1 operon (De Angelis et al. 2011). While evidence suggesting that this phenotypic variability in the pneumococcal pilus is not due to change at genetic level, it is likely to be controlled by the positive-feedback from RlrA, which is also encoded within the same PI-1 islet (Basset et al. 2012, De Angelis et al. 2011).

Prevalence of pneumococcal piliated strains drastically dropped immediately after the introduction of the pneumococcal conjugate vaccine in USA, which went up again to the pre-vaccination level after a short interval (Regev-Yochay et al. 2010). This re-emergence of pilus-1 in pneumococcal populations suggests that it can offer significant selective advantages in bacterial colonisation and invasion. The biphasic expression pattern pneumococcal pilus-1 enables them to survive under different challenging conditions. Considering the fact that the regulators of the expression are located within the same islet encoding the pilus-1 genes, acquisition of the PI-1 islet would easily spread them to non-piliated pneumococcal strains. The association of pilus-1 and antibiotic resistance could be a matter of great concern in this regard (Moschioni et al. 2010a; Selva et al. 2012).

In summary, pneumococcal carriage is common in young children, which gradually decreases with advancing age. The presence of type-1 pilus is rare among these carriage strains in our population. This low prevalence could be associated with the introduction of pneumococcal conjugate vaccine, which covers the common piliated serotypes. It might also be contributed by the acquisition of natural immunity developed during colonisation episodes by piliated or non-piliated (sharing other common epitopes) strains. It will be interesting to investigate whether a re-emergence of piliated pneumococcal strains follows this initial decline in our population as it happened in USA. Although the presence of pilus-1 genes is not widespread among pneumococcal strains, its association with antibiotic resistance and relatively higher prevalence among carriage isolates in younger children may have clinical implications.

Chapter 4

Naturally Developed Serum and Salivary Antibodies to Pneumococcal RrgA and RrgB Antigens in Children and Adults

4.1 Introduction

The naturally developed antibody responses to polysaccharide capsules are induced by the exposure to the pneumococcus during nasopharyngeal carriage which is common in younger children (Regev-Yochay et al. 2004). Natural antibody responses also develop against other pneumococcal targets, particularly to the surface expressed proteins (Jódar et al. 2003). Pneumococcal colonisations induce antibody responses to both capsular and non-capsular antigens in the local mucosal sites also. Following an exposure to pneumococcus, salivary IgA antibodies to the capsular polysaccharides develops more rapidly than the serum IgG (Simell et al. 2002). Local production of salivary IgA antibodies to pneumococcal surface proteins (PsaA, Ply, and PspA), following pneumococcal carriage or episodes of acute otitis media was also reported (Simell et al. 2001).

Several pneumococcal cell-surface or secreted proteins have been identified, which induce substantial antibody responses. Antibodies to some of these protein antigens showed good protective efficacy in experimental mice models (McCool et al. 2002; Briles et al. 2000). A combination of these antigens enhanced their antibody responses. For achieving specific targets; such as protection against invasive infection, nasopharyngeal carriage, development of herd immunity, a permutation-combination of these antigens was found to be useful (Briles et al. 2000).

Subsequent studies in humans also suggested the possible protective role of these non-polysaccharide antibodies. An experimental carriage model in healthy adults showed pre-existing antibodies to pneumococcal surface protein A (PspA) was protective against pneumococcal colonisation (McCool et al. 2002). These antibodies appear quite early in childhood, achieving a good titre before the second birthday, as evidenced in a Finnish study (Rapola et al. 2000) and a Kenyan study (Ellis and Beaman 2004). Considering the fact that, anti-capsular antibodies start to develop

during the second and third year of life; antibodies to protein antigens could play a crucial protective role in young children (Lipsitch et al. 2005).

The antibody levels to these proteins in humans increase with age, while pneumococcal carriage rates decrease. Moreover, higher antibody levels to certain protein antigens were observed in culture-negative children than those who were colonised (Zhang et al. 2006b, Vukmanovic-Stejic et al. 2006). The inverse relationship between the magnitude of immune responses and pneumococcal carriage, suggests that naturally developed immunity to these protein antigens might be protective against pneumococcal carriage.

All three subunits of pneumococcal pilus-1 were found to be immunogenic in experimental mice (Gianfaldoni et al. 2007). Intraperitoneal immunisation of mice with recombinant pilus subunits (RrgA, RrgB and RrgC) and heat-inactivated pilus-1 expressing strain (TIGR4) produced specific serum antibodies detectable by ELISA. The antibody responses to all three proteins were sufficient to protect these immunised mice on subsequent intraperitoneal challenge with a TIGR4 strain in a lethal dose, showing lower level of bacteraemia and higher survival rates than controls. Moreover, passive transfer of mouse antisera raised against recombinant pilus antigens was able to protect previously unexposed mice (Gianfaldoni et al. 2007).

RrgA mediates pneumococcal adhesion to host respiratory epithelium. During colonisation with a piliated strain RrgA interacts with the host immune system, and elicits a strong host immune response (Nelson et al. 2007). Based on the sequence variation in the terminal end, two variants of RrgA (Clade I and II) were reported. Passively-immunised mice with antibodies developed against either of these variants showed cross-protection in subsequent challenge with pneumococcus (Moschioni et al. 2010b). Based on the molecular structural analysis, three variants of RrgB (Clade

I, II and III) were reported (Moschioni et al. 2008). Passive immunisation of experimental mice with antisera raised against each of these variants showed clade-specific protection; but a fusion of these three variants (RrgB321) has been shown to be protective against piliated pneumococcal strains (Harfouche et al. 2012).

Data on the immunogenicity of pneumococcal pilus-1 proteins in humans are limited. Little information is available with regard to the role of pilus-1 specific antibodies in protective immunity against pneumococcal carriage. Understanding the naturally developed immunity to pilus-1 proteins in humans and its relationship with carriage may provide valuable information to future vaccination strategy against pneumococcal infection. We hypothesize that, RrgA being an adhesin plays an important role in the bacterial interaction with host respiratory mucosa. Therefore, natural colonisation and/or infection with pilus-1 expressing pneumococci, is expected to induce systemic and mucosal antibody response against it. RrgB, the major structural unit of pneumococcal type-1 pilus may also induce protective antibody response. If these antibody responses are sufficiently strong, they might protect against subsequent colonisation and infection.

4.2 Aims of Study

To study naturally developed pneumococcal RrgA and RrgB antigen-specific antibodies in serum and saliva samples; and their relationship to nasopharyngeal carriage of pneumococcus in children and adults.

4.3 Experimental Design

1. An ELISA based method was established to measure pilus-1 antigen (RrgA and RrgB) specific antibodies in serum and saliva. The specificity of this method was ascertained by antigen-specific inhibition ELISA.

3. Serum and salivary antibodies to RrgA and RrgB was measured in children and adults by ELISA and Western blot.

4. The relationship between the antibody levels and nasopharyngeal carriage of pneumococcus was analysed.

4.3.1 Recombinant RrgA and RrgB Antigens

Purified His-tagged recombinant RrgA and RrgB proteins from *Streptococcus pneumoniae* serotype 4 TIGR4 strain; obtained from Novartis Vaccines, Sienna, Italy (Gianfaldoni et al. 2007) were used for detection of anti-RrgA and -RrgB antibodies by ELISA and Western Blot.

4.3.2 ELISA Assay

ELISA assay was performed as described in methods (chapter 2). In brief, ELISA plates were coated with individual recombinant RrgA and RrgB antigens, and incubated overnight at 4°C. After washing, plates were blocked with 10% FBS-PBS followed by incubation of serum and saliva samples. Thereafter, alkaline phosphatase conjugated anti-human IgG (Sigma) was incubated before addition of PNPP substrate. Optical density was measured at 405 nm and data were analysed using DeltasoftPC microplate analysis software (BioMetallics) (for details see materials and methods chapter 2).

Pooled human immunoglobulin (Sandoglobulin, Sandoz, UK) was used as the reference standard. The antibody titre of sandoglobulin for both RrgA and RrgB was determined by using a serial dilution of the immunoglobulin followed by the same ELISA procedure as described previously. The anti-RrgA (1000 units/ml) and anti-RrgB (1400 unit/ml) titre of sandoglobulin was assigned as the reciprocal dilutions (1:1000 and 1:1400 respectively) at which their OD (at 405 nm) reached to 1.00, 30 minutes after adding the substrate solutions.

4.3.3. Western Blot detection of anti-RrgA and anti-RrgB

Western Blot detection of anti-RrgA and -RrgB antibodies are described in details in the methods chapter 2. Briefly, recombinant proteins (rRrgA and rRrgB), mixed with Laemmli reducing buffer were loaded and run into a mini protean TGX^{TM} gel (BioRad). Thereafter, the protein bands were transferred to a 0.2µm nitrocellulose membrane by a Transblot Turbo^{TM} transfer system (BioRad). After blocking with 5% skimmed milk in 0.1% TBS-Tween20 for 2 hr, the membrane was blotted with serum samples (1:10,000 in blocking solution) for 2 hr. After washing, murine anti-human IgG-HRP (Sigma) and Streptactin-HRP (BioRad), were added and incubated for 1 hr. The membrane was imaged on Chemi-DocXRS system (BioRad), 5 minutes after adding Immun-Star WesternC Chemiluminescence reagent A and B (BioRad). The densities of positive bands were measured semiquantitively using Image Lab software version 4.0.1 (BioRad). For details procedures please see the materials and methods in chapter 2.

4.3.4 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 20). Log transformation of data was done before analysing by parametric statistical methods. Differences in antibody titres between groups were analysed by analysis of variance and Student's t-test. One way analysis of variance (ANOVA) was performed to evaluate the effects of age and nasopharyngeal carriage status on the antibody titre. Association between two factors was analysed by Spearman's correlation. A *p* value of <0.05 was considered statistically significant.

4.4 Results

4.4.1 Establishment of ELISA for measuring RrgA and RrgB Specific antibodies

ELISA assay was designed and established to measure pneumococcal RrgA and RrgB -specific antibodies. Each set of experiment was performed after optimisation for antigen coating concentration, sample dilution, conjugated-antibody concentration and incubation conditions. Standard curves were constructed for anti-RrgA and anti-RrgB titres of test samples based on a reference standard sandoglobulin. As shown in figure (4.4.1) standard curve was obtained to calculate concentration of antigen-specific antibody levels. The optical densities (OD) of test samples within the standard curve were analysed. Samples with OD higher than the topmost standard were repeated with higher dilution.

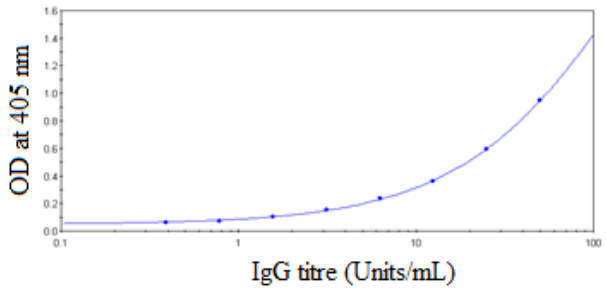


Figure 4.4.1: Establishment of standard curve after performing ELISA. Following the ELISA analysis of the reference standard and samples, standard curves were constructed based on the optical densities (OD at 405nm) and the antibody titres assigned to the serially diluted standard, using the DeltasoftPC microplate analysis software (BioMetallics, USA).

4.4.2 Specificity of pneumococcal pilus-1 specific antibody ELISAs

The specificity of the ELISA measuring anti-pilus antibodies was confirmed by antigen-specific inhibition ELISA assays adopted from (Zhang et al. 2000) with some modifications (details see chapter 2 materials and methods).

4.4.2.1 Specificity of anti-RrgA ELISA

Three serum samples with high anti-RrgA titres were used and prepared (1:100 dilutions). Recombinant RrgA were co-incubated with the serum samples in the following concentrations (0.3125, 0.625, 1.25, 2.50, 5.0 and 10.0) µg/ml, to adsorb the antibodies present in the serum. The percentage of inhibition was 20% at 0.2µg/ml of RrgA antigen, and reached to about 100% with 2.5µg/ml rRrgA antigen. The mean concentration of the RrgA corresponding 50% inhibition of antibody activity was 0.3125µg/ml. The inhibition curve with increasing RrgA concentrations ascertains the specificity of anti-RrgA ELISA (Figure-4.4.2.1a). The failure of inhibition by adsorption by a heterologous antigen, recombinant RrgB, also supported the specificity of this assay (figure 4.4.2.1b).

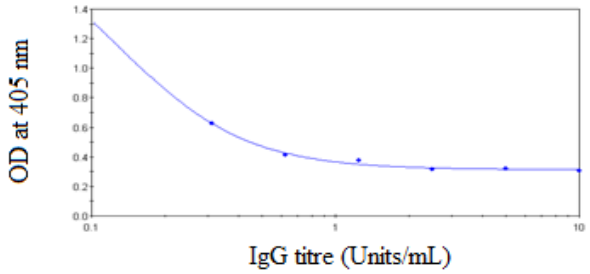


Figure-4.4.2.1a: Inhibition of Anti-RrgA IgG ELISA by recombinant RrgA. Anti-RrgA titre in the serum decreased with increasing concentration of the adsorbent recombinant RrgA antigen in a dose dependent manner confirming the specificity of the ELISA assay (one of three representative samples is shown).

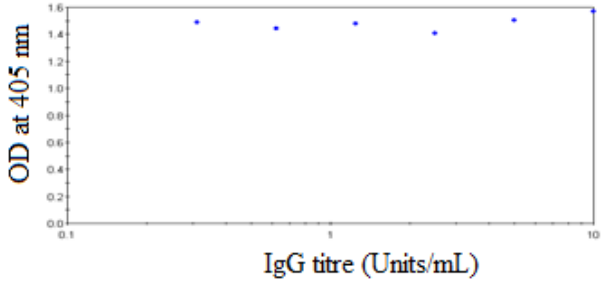


Figure 4.4.2.1b: Failure of inhibition of anti-RrgA IgG ELISA by recombinant RrgB. Serum samples were adsorbed by recombinant RrgB antigen showing no inhibition on the anti-RrgA titres in the serum samples (one of three representative samples is shown).

4.4.2.1 Specificity of anti-RrgB ELISA

Three serum samples with high anti-RrgB titres were used and prepared (1:100 dilutions). Recombinant RrgB were co-incubated with the serum samples in the following concentrations (0.3125, 0.625, 1.25, 2.50, 5.0 and 10.0) µg/ml, to adsorb the antibodies present in the serum, before ELISA assay for anti-RrgB antibody. The mean concentration of the RrgB corresponding to 50% inhibition of antibody activity was 2.5µg/ml. The inhibition curve of anti-RrgB antibody with increasing RrgB concentration (Figure-4.4.2.1c), and the failure of inhibition by a heterologous antigen, recombinant RrgA, supported the specificity of this assay (Figure-4.4.2.1d).

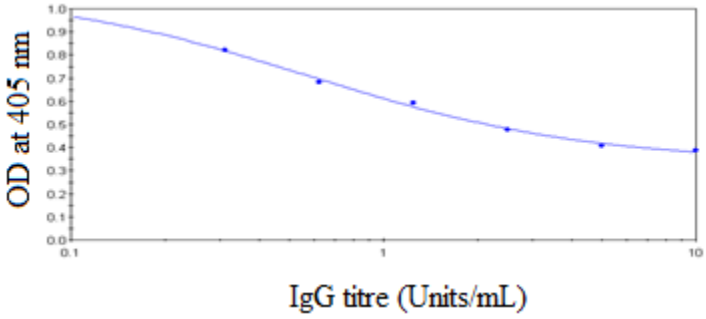


Figure-4.4.2.1c: Inhibition of Anti-RrgB IgG ELISA by recombinant RrgB. Anti-RrgB titres detected in the serum sample decclined with increasing concentration of the adsorbent recombinant RrgB antigen in a dose dependent manner supports the specificity of the ELISA assay (one of three representative samples is shown).

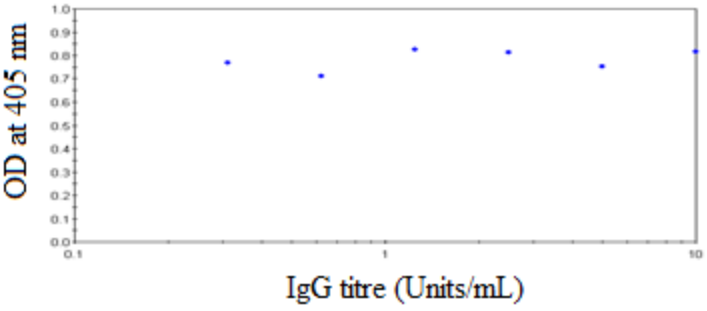


Figure 4.4.2.1d: Failure of inhibition of anti-RrgB IgG ELISA by recombinant RrgA. Serum samples adsorbed by recombinant RrgA antigens show no inhibition on anti-RrgB titres of the samples (one of three representative samples is shown).

4.4.3 Association of serum anti-RrgA and anti-RrgB IgG titres with age

To investigate if there is any association between RrgA and RrgB -specific IgG antibody titres and age, serum samples from 195 children (<16 years) and 109 adults (>16 years) were measured for these antibodies using ELISA and analysed in association with age. Both anti-RrgA and –RrgB antibody titres were higher in adults than in children (Figure-4.4.3a, p<0.0001).

(a)

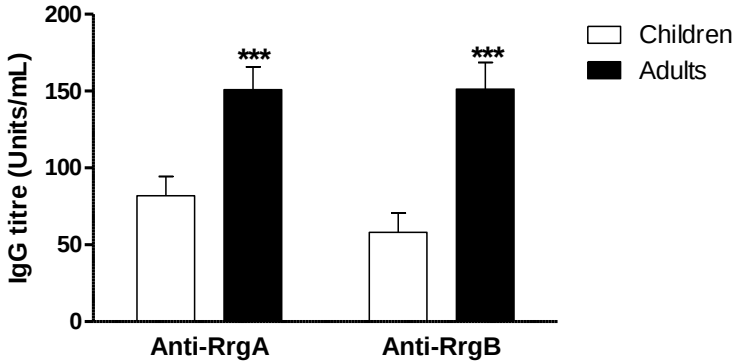


Figure-4.4.3a: Serum anti-RrgA and anti-RrgB IgG titre in children and adults. Serum antibody titre was measured by ELISA in 195 children (n=195) and 109 adults (n=109). The results expressed as the geometric mean titre (GMT) ± 95% confidence interval (CI). Significance level (****p*<0.0001) was compared between adult and children in student's t test.

The age-related increase of anti-RrgA antibody is presented in figure 4.4.3b. As shown in the figure, anti-RrgA antibody could be detected in early childhood before the age of three years old. The antibody titre increased with age until around the age 15 years, followed by a drop of in those aged between 16 and 30 years. One way ANOVA showed significant difference of anti-RrgA titre among the age groups ($F=12.19$, $p<0.0001$). In post hoc tests, the antibody titre in the age group of 10-15 years was found to be significantly higher than that of 1-3, 4-6 and 16-30 years group. The titre of age group 16-30 years was higher than that of 1-3 and 4-6 years age groups (Figure 4.4.3b).

(b)

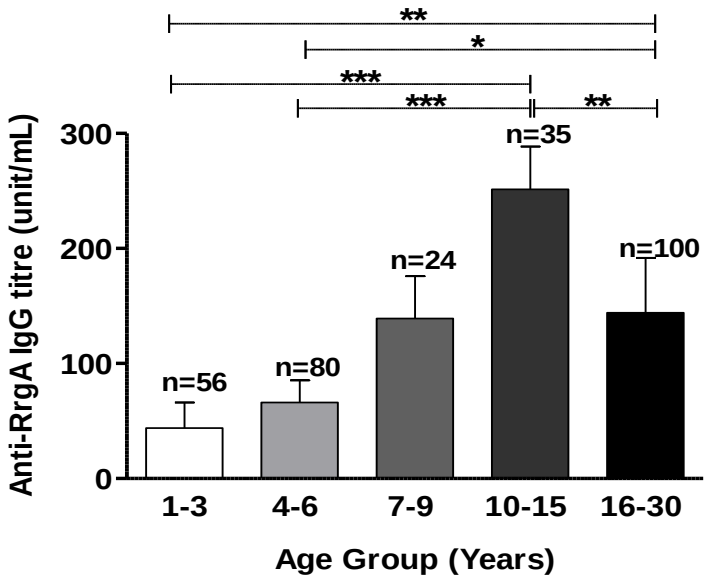


Figure-4.4.3b: Serum anti-RrgA IgG titre in different age groups. Serum anti-RrgA titres at different age groups are shown. A total of 295 samples (n=295) are analysed including: 1-3 years (n=56), 4-6 years (n=80), 7-9 years (n=24), 10-15 years (n=35) and 16-30 years group (n=100). The results are expressed as the geometric mean titre (GMT) ± 95% confidence interval (CI). Statistical differences (***$p<0.0001$, **$p<0.01$, and *$p<0.05$) between different groups were calculated by one way ANOVA with post hoc test.

The age-related increase in anti-RrgB antibody titres is presented in figure 4.4.3c. Anti-RrgB antibody titres were low in early childhood, which appeared to rise after the age of 6 years. Similar to anti-RrgA antibody, anti-RrgB antibody increased with age until around 15 years and then there was a decline in those aged 16-30 years. One way ANOVA showed difference in anti-RrgB titres among the age groups ($F=12.71$, $p<0.0001$). In post hoc tests, the antibody titre in the age groups of 7-9, 10-15 and 16-30 years were higher than that of 1-3 and 4-6 years group (Figure 4.4.3c).

(c)

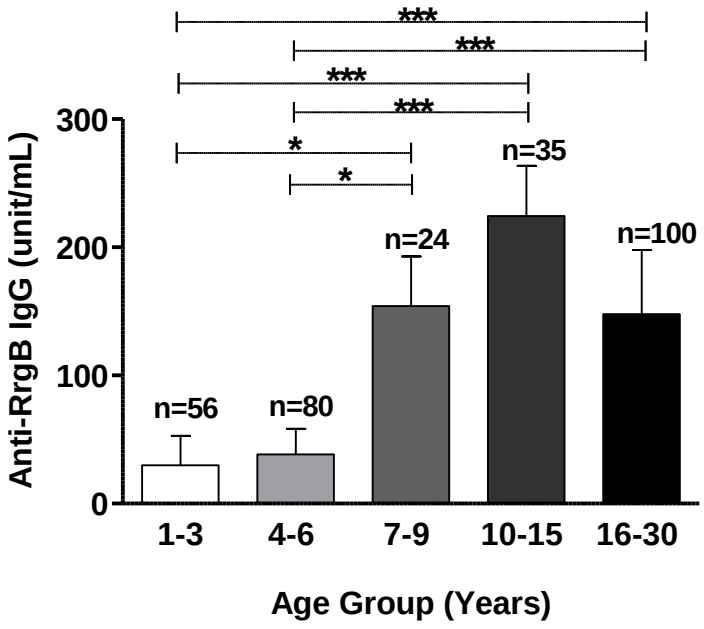


Figure-4.4.3c: Serum anti-RrgB IgG titre in different age groups. Serum anti-RrgB titres at different age groups are shown. A total of 295 samples (n=295) are analysed including age groups, 1-3 years (n=56), 4-6 years (n=80), 7-9 years (n=24), 10-15 years (n=35) and 16-30 years group (n=100). The results are expressed as the geometric mean titres (GMT) ± 95% confidence intervals (CI). Statistical differences (***$p<0.0001$ and *$p<0.05$) between different groups were calculated by one way ANOVA with post hoc test.

4.4.4 IgG subclasses of serum anti-RrgA and anti-RrgB antibodies

To investigate which types of IgG subclasses are contributing to the antibody responses against the pilus-1 proteins, serum anti-RrgA and anti-RrgB IgG subclasses were also measured by ELISA. A total of 129 samples were analysed, of whom, 64 were children and 65 were adults. The predominant subclass for both antibodies was found to be IgG1 followed by IgG3 then IgG2, and IgG4 was the lowest. One way ANOVA showed significantly higher level of IgG1 for both anti-RrgA and -RrgB than all three other subclasses (Figure-4.1.2.1, a+b). In case of anti-RrgB, a significant difference between IgG3 and IgG4 was also observed (Figure-4.4.4, a+b).

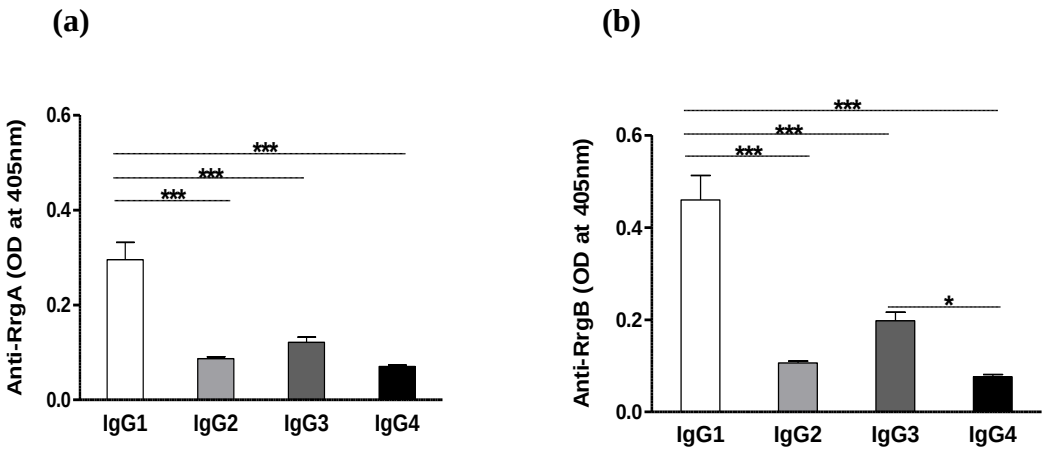


Figure-4.4.4a+b: IgG subclasses of serum anti-RrgA and anti-RrgB antibodies. IgG subclasses of antibodies to RrgA and RrgB were analysed. Result shows OD (at 405 nm) of IgG subclasses for (a) anti-RrgA and (b) anti-RrgB antibodies. Statistical differences (***p<0.0001 and *p<0.05) between different subclasses were calculated by one way ANOVA with post hoc test.

4.4.5 Detection of serum anti-RrgA and anti-RrgB antibodies by Western blotting

To confirm the presence or absence of RrgA- and RrgB-specific antibodies in serum samples, Western blot assay was also performed by immunoblotting serum samples against recombinant RrgA (~120kD) and RrgB (~50kD) proteins (Figure-4.4.5a). A total of 33 randomly selected serum samples (16 adults, 17 children) were tested, among them 19 were anti-RrgA positive and 24 were anti-RrgB positive. The detection of anti-RrgA and -RrgB antibodies in the serum samples by Western blotting confirm the presence of the antibodies detected by ELISA. The results from the Western blot analysis was applied to construct receiver operating characteristic (ROC) curves to set arbitrary cut-off values for positivity of serum anti-RrgA and -RrgB titres (measured by ELISA).

(a)

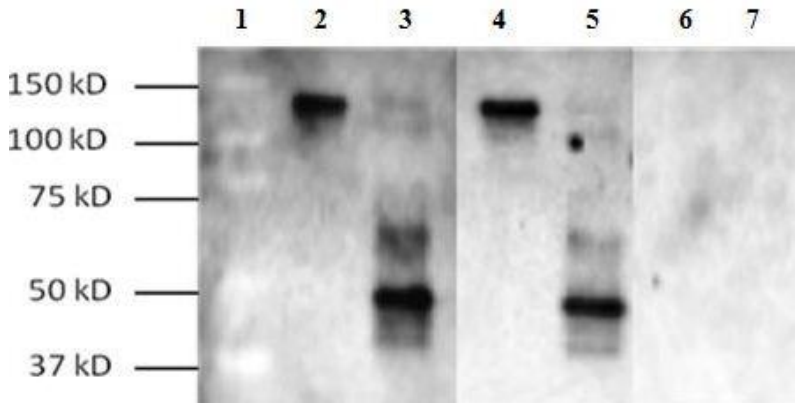


Figure-4.4.5a: Detection of serum anti-RrgA and -RrgB antibodies by Western blotting. The presence or absence of anti-RrgA (lane 2, 4 and 6) and anti-RrgB (lane 3, 5 and 7) in serum samples was detected by western blot technique. Blotting of three different serum samples were shown, first (lane 2, 3) and second (lane 4, 5) were positive for both RrgA and RrgB, third (lane 6, 7) was double negative. Lane 1 shows the molecular weight marker.

The densities of western blot bands were measured semiquantitively with Image Lab, version 4.0.1 (BioRad, UK). These relative densities analysed by Western blotting were shown to be well correlated with the antibody titres measured by ELISA for both anti-RrgA ($p<0.05$) and -RrgB ($p<0.05$) (Figure-4.4.5b+c).

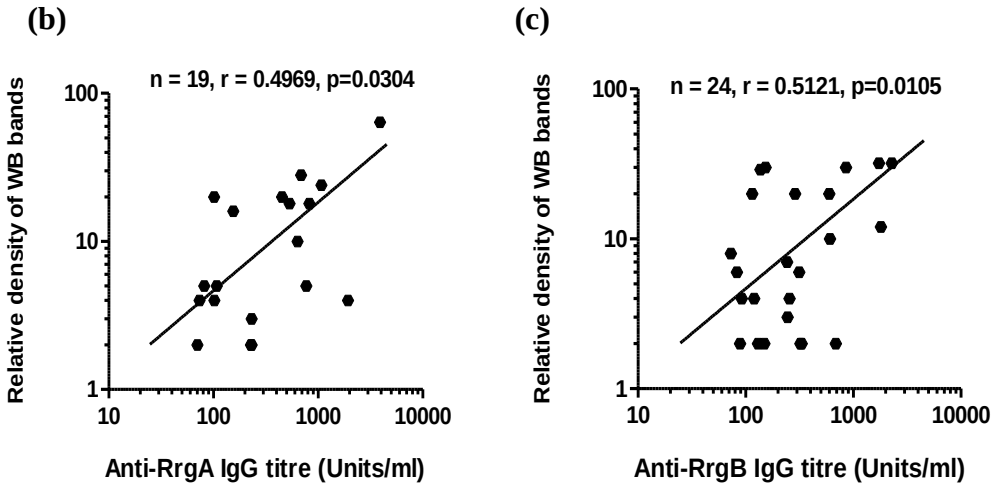


Figure-4.4.5b+c: Correlations of serum antibodies measured by ELISA and Western Blotting. Correlation between the densities of antibody bands by Western blotting and the serum antibody titres measured by ELISA are shown. (b) Anti-RrgA (n=19) measured by these two methods were correlated (*$p<0.05$) in Pearson's correlation analysis; (c) anti-RrgB (n=24) measured by these two methods were also well correlated (*$p<0.05$) in Pearson's test.

4.4.5.1 Calculation of a cut off value for serum anti-RrgA positivity measured by ELISA

Based on the antibody positivity by western blot analysis, a receiver operating characteristic (ROC) curve was constructed for setting an arbitrary cut-off value of serum anti-RrgA titre (Figure-4.4.5.1). The area under the curve (±SE) was 0.959±0.031. By the help of this ROC curve, the cut-off value for serum anti-RrgA was set as 100units/ml, with sensitivity of 84.2% and specificity of 92.9%. Using this cut-off, among the 304 patients studied, 61.5% of serum samples (56.9% of children and 69.7% of adults) were considered as serum positive for anti-RrgA.

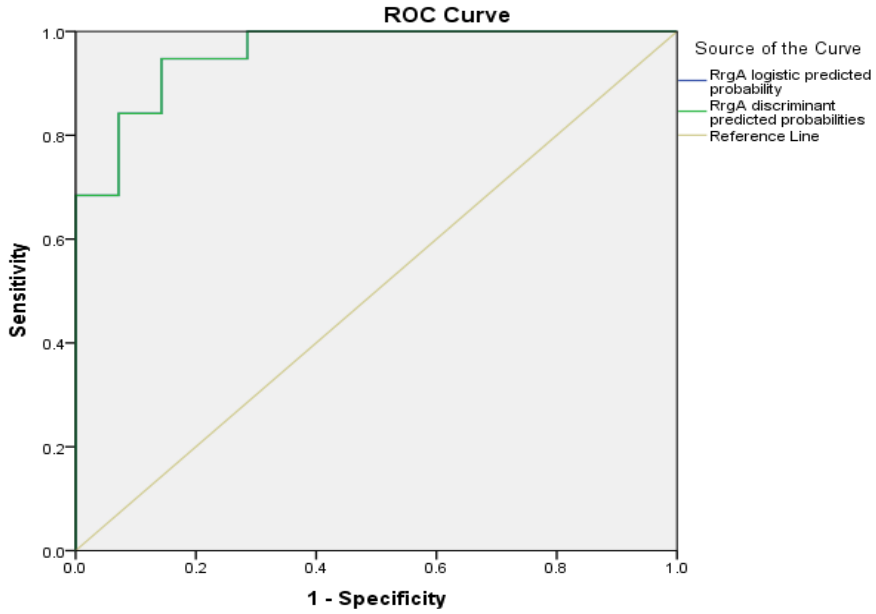


Figure-4.4.5.1: The ROC curve for setting anti-RrgA cut-off. The ROC curve for determining the cut-off value for positivity of serum anti-RrgA titre was constructed by the western blot analysis of 33 samples (n=33) of which 19 showed positive bands and 14 negative. The area under curve (±SE) calculated by both logistic predicted probability and discriminant predicted a probability was 0.959 (±0.031), and the 95% CI (lower bound-upper bound) was 0.899 -1.00 with asymptomatic significance ***p<0.0001.

4.4.5.2 Calculation of a cut off value for serum anti-RrgB positivity measured by ELISA

Based on the antibody positivity by western blot analysis, a ROC curve was also constructed for setting an arbitrary cut-off value of serum anti-RrgB (Figure 4.4.5.2). The area under the curve (±SE) was 0.921 (±0.054). With the help of this ROC curve, the cut-off value for serum anti-RrgB was set at 100units/ml and that gave sensitivity of 87.5% and specificity of 77.7%. Among the 304 patients studied, 58.2% of serum samples (51.8% of children and 69.7% of adults) were considered as serum positive for anti-RrgB antibody.

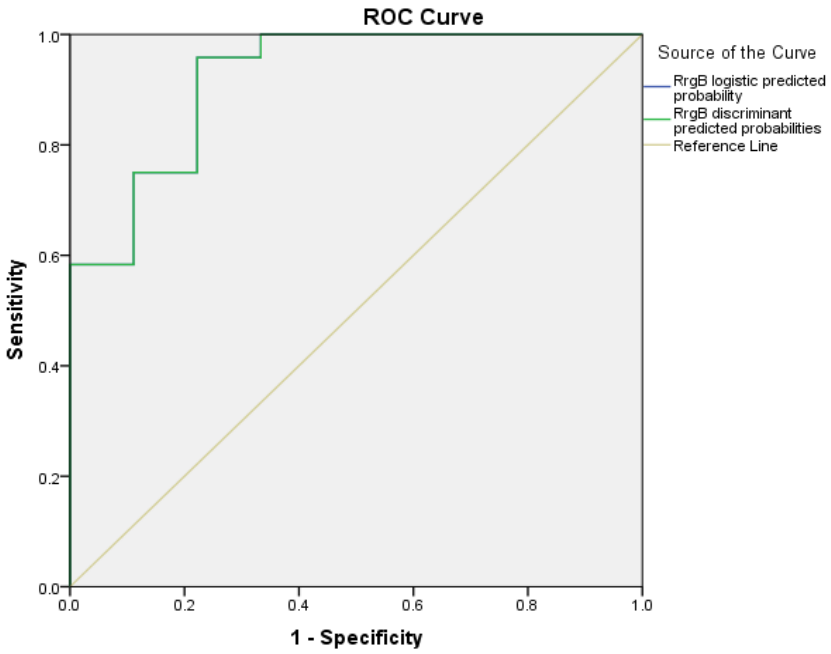


Figure-4.4.5.2: The ROC curve for setting anti-RrgB cut-off. The ROC curve for determining the cut-off value for positivity of serum anti-RrgB titre was constructed by the western blot analysis of 33 samples (n=33), of which 24 showed positive bands and 9 negative. The area under curve (±SE) calculated by both logistic predicted probability and discriminant predicted a probability was 0.921 (±0.054); and the 95% CI (lower bound-upper bound) was 0.816 -1.00 with asymptomatic significance ***p<0.0001.

4.4.5.3 Antibody positivity by Western blot in different age groups

The antibody positivity determined by the cut-off values calculated from Western blot analysis also showed an age-dependent increase. Both anti-RrgA and -RrgB positivity was less than 50% in 1-3 years children. Anti-RrgA positivity appeared to be higher in early childhood (<6 years), but anti-RrgB seemingly predominated in older children (>7 years) and adults. Positivity for both anti-RrgA and -RrgB peaked (>80%) at the age group of 10-15 years, In 16-30 years group it was around 70% (Figure 4.4.5.3).

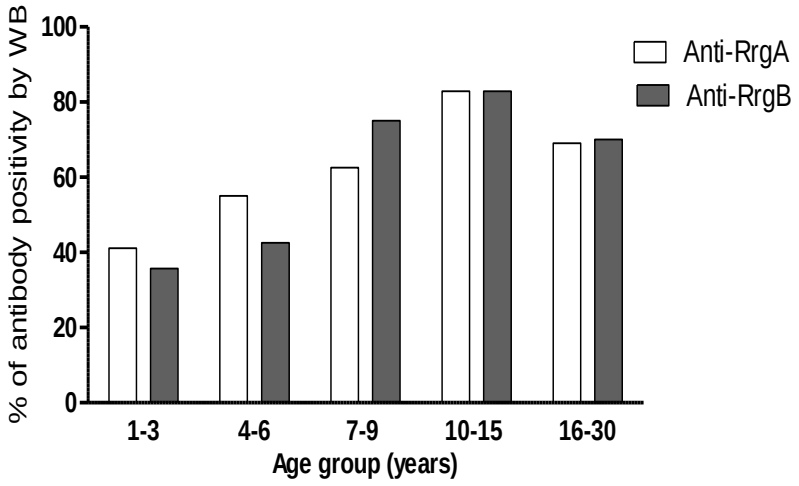


Figure-4.4.5.3: Antibody positivity by Western blot in different age groups. The antibody positivity rates (determined by the cut-off values of 100units/ml based on Western blotting) in different age groups are shown. A total of 295 samples (n=295) were analysed including 1-3 years (n=56), 4-6 years (n=80), 7-9 years (n=24), 10-15 years (n=35) and 16-30 years group (n=100). The results are expressed as the percentage (%) of antibody positivity.

4.4.6 Association of serum anti-RrgA and anti-RrgB IgM titres with age

Pilus -1 (RrgA and RrgB) antigen-specific IgM antibodies in serum were analysed in 70 children (<16 years) and 66 adults (>16 years) by ELISA. In the absence of a reference standard, results were expressed as the optical densities (OD at 405nm) 30 min after the addition of substrate solution. The mean OD for both anti-RrgA and -RrgB IgM were significantly higher in children compared to those in the adults in independent sample t test (Figure-4.4.6, p=0.006 and p=0.008 respectively).

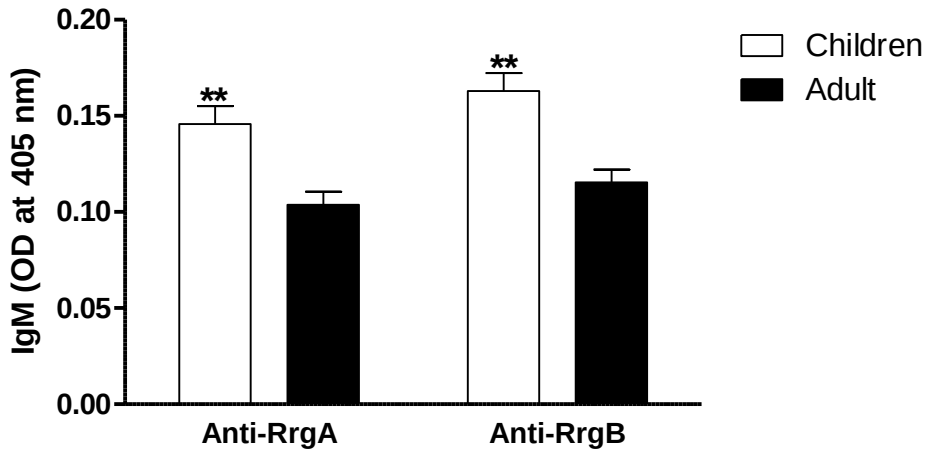


Figure-4.4.6: Serum anti-RrgA and anti-RrgB IgM titre in children and adults. Serum IgM titre was measured by ELISA in children (n=70) and adults (n=66). The results are shown as the mean OD ± 95% confidence interval (CI). Significance (***p*<0.001) compared with adults in Student's t test.

4.4.7 Association of serum antibody titres with pneumococcal carriage

To investigate whether there is any relationship between antibody levels and pneumococcal carriage, serum anti-RrgA and anti-RrgB IgG antibody titres were analysed in association with the presence/absence of nasopharyngeal carriage of pneumococcus. When all children and adults samples were included in the analysis, both anti-RrgA and anti-RrgB antibody titres were found to be higher in pneumococcal culture-negative patients than in culture-positives (figure-4.4.7, $p<0.0001$).

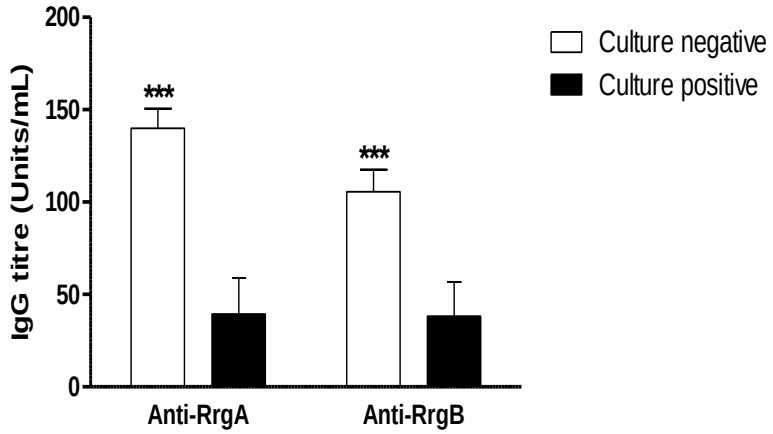


Figure-4.4.7: Serum anti-RrgA and -RrgB titres in relation to pneumococcal carriage Mean concentration (units/ml) of antibodies to RrgA and RrgB antigens were analysed in culture negative and colonised subjects. Significance (****p*<0.0001) were compared with colonised by Student's t test. Results represent GMT ± 95% CI (error bars) of 304 study subjects, of whom 76 were colonised and 228 were negative for pneumococcal carriage.

4.4.7.1 Association of serum anti-RrgA titres with pneumococcal carriage in different age groups

As the antibody levels were shown to be age-dependent, we analysed the individual effects of carriage and age on antibody levels using a general linear model of analysis of variance (GLM-ANOVA). It was observed that the effect of carriage status on serum anti-RrgA antibody levels was independent of age, i.e. both carriage status and age were determinant factors of anti-RrgA antibody titres ($p<0.007$ and $p=0.034$ respectively). The geometric mean titres of anti-RrgA IgG antibodies in culture-negative patients were significantly higher than those of culture-positives in all age groups (p = 0.0048, <0.0001, <0.0001, <0.0001 and 0.0049 respectively, Figure-4.4.7.1). The number of culture-negative and colonised patients in 1-3 years groups were 25 and 31; in 4-6 years 52 and 28; in 7-9 years 16 and 8; in 10-15 years 29 and 6; and in 16-30 years were 97 and 3.

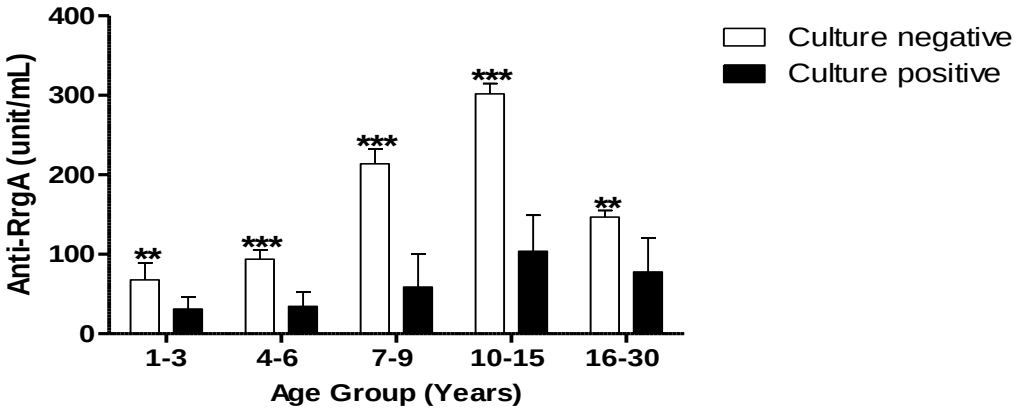


Figure-4.4.7.1: Serum anti-RrgA titre in different age groups and pneumococcal carriage. Anti-RrgA titres were analysed in relation to nasopharyngeal carriage of pneumococcus in different age groups. Results represent GMT ± 95% CI (error bars) of anti-RrgA (units/ml) in culture negative and colonised subjects. Statistical difference (***p<0.0001, **p<0.01) was compared with colonised in Student's t test.

4.4.7.2 Association of serum anti-RrgB titres with pneumococcal carriage in different age groups

Unlike anti-RrgA, anti-RrgB titres were very low children younger than 6 years, and no difference was found between culture- and culture+ patients in these children. However, there appeared to be a differences between culture – and culture+ subjects in 7-9 and 10-15 years age groups ($p<0.0001$, Figure-4.4.7.2). Figure 4.4.7.2 shows the geometric mean titres of anti-RrgB IgG antibodies in culture-negative and culture-positive patients in different age groups. However, GLM-ANOVA analysis showed no independent effect of carriage status on anti-RrgB antibodies, as it was for age ($p=0.065$ and $p=0.028$ respectively). The number of culture-negative and colonised patients in 1-3 years groups were 25 and 31; in 4-6 years 52 and 28; in 7-9 years 16 and 8; in 10-15 years 29 and 6; and in 16-30 years were 97 and 3.

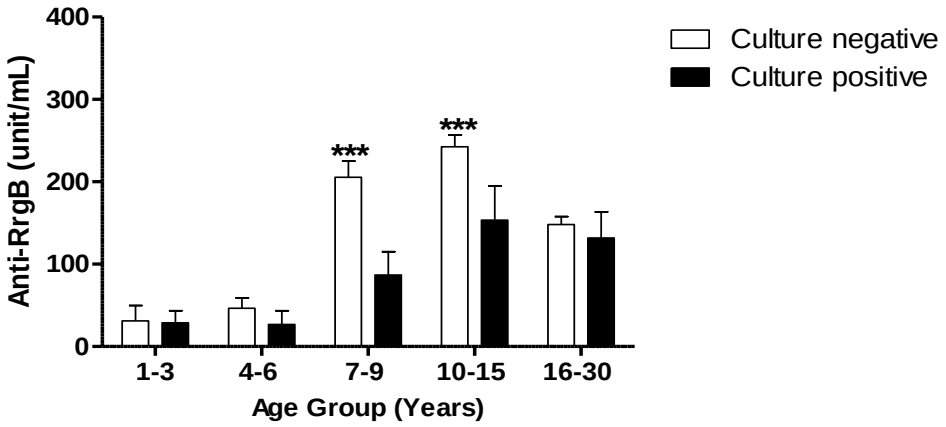


Figure-4.4.7.2: Serum anti-RrgB titre in different age groups and pneumococcal carriage. Anti-RrgB titres were analysed in relation to nasopharyngeal carriage of pneumococcus in different age groups. Results represent GMT ± 95%CI (error bars) of anti-RrgB (units/ml) in culture- and culture+ subjects. Statistical difference (***$p<0.0001$) was compared with colonised in Student's t test.

4.4.7.3 Association of IgG subclasses with pneumococcal carriage

Anti-RrgA and -RrgB IgG subclasses were also analysed in relation to nasopharyngeal carriage of pneumococcus. The titre of IgG1 subclass for both antibodies was found to be significantly higher in pneumococcal culture-negative patients than in culture-positives (Figure 4.4.7.3a+b, p=0.022 and <0.001 respectively). No significant difference was found in other subclasses (IgG2, IgG3 and IgG4) for both anti-RrgA and -RrgB. (Figure 4.4.7.3a+b, p>0.05).

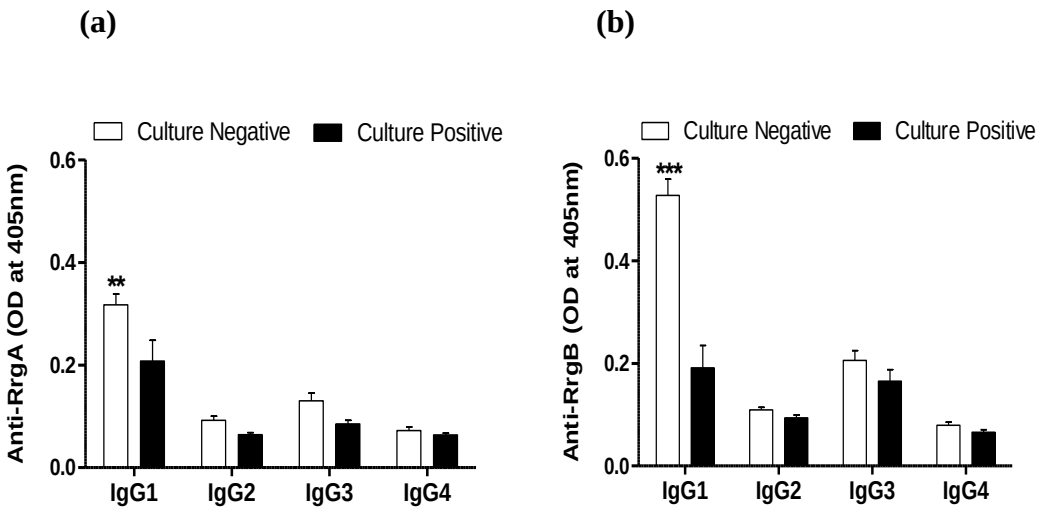


Figure-4.4.7.3a+b: IgG subclasses and pneumococcal carriage. The IgG subclasses for (a) anti-RrgA and (b) anti-RrgB antibodies were analysed in relation to pneumococcal carriage. Significance levels (**p<0.0001 and ** p<0.01) for the particular subclass were compared between culture-negative (n=103) and culture-positive (n=26) patients in Student's t test.

4.4.7.4 Association of serum IgM titres with pneumococcal carriage

Serum anti-RrgA and anti-RrgB IgM titres in children were also analysed in association with the presence/absence of nasopharyngeal carriage of pneumococcus. As the IgM titres were low in the adults, who also had a lower carriage rates, only children samples were analysed for association of carriage and IgM titre. Both anti-RrgA and anti-RrgB antibody titres were found to be higher in pneumococcal culture-positive than in culture-negative children (Figure-4.4.7.4, p=0.002 and p<0.0001 respectively).

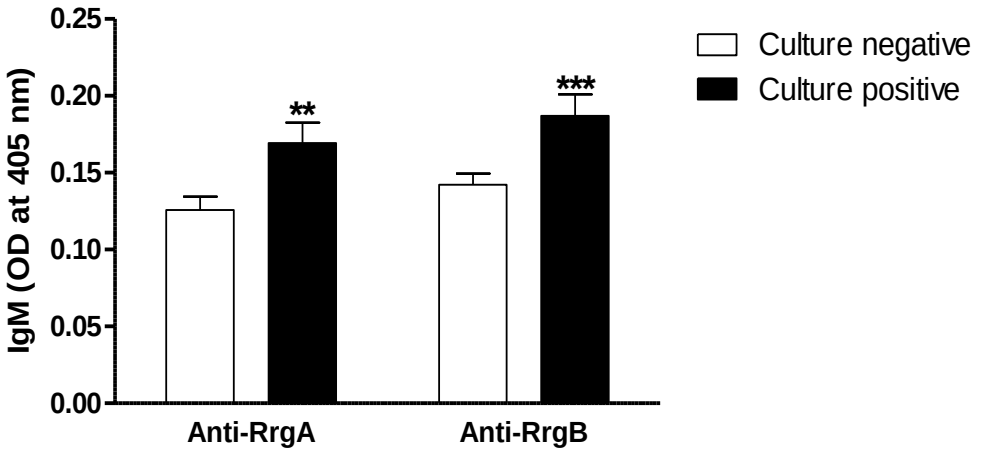


Figure-4.4.7.4: Serum IgM in children in relation to pneumococcal carriage. IgM antibodies to RrgA and RrgB were analysed in culture negative and colonised children. Statistical significance (**p<0.01, ***p<0.0001) was compared with colonised in Student's t test. Results represent mean OD ± 95% CI (error bars) of 70 children, of whom 34 were colonised and 36 were negative for pneumococcal carriage.

4.4.8 Salivary IgG and secretory IgA antibodies to RrgA and RrgB antigens

Salivary anti-RrgA and anti-RrgB IgG antibody levels were measured by antigen-specific ELISA. For the antigen-specific salivary IgG assays, pooled human serum immunoglobulin (Sandoglobulin) was used as the reference standard in a similar way it was used for serum IgG assays; and the results were expressed as units/ml. The salivary IgG antibodies to RrgA and RrgB antigens were analysed in relation to the corresponding serum IgG titres. A good correlation was found between serum and salivary IgG antibody titres to both RrgA and RrgB (Figure-4.6.2a+b, *n=84,* r=0.6121, *P<0.0001; and n=84, r*=0.7900, *p<0.0001* respectively) in nonparametric (Spearman's) correlation test.

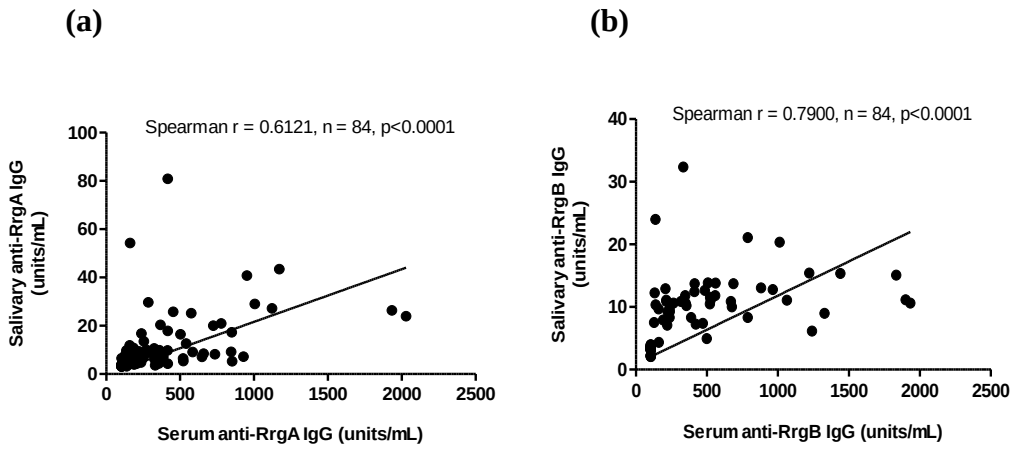


Figure-4.4.8a+b: Correlation of serum and salivary IgG antibodies to RrgA and RrgB. Correlation between serum and salivary IgG antibodies to RrgA and RrgB in 84 samples (n=84); showing significant correlation for both (a) Serum and salivary anti-RrgA IgG and (b) Serum and salivary anti-RrgB IgG.

To analyse the secretory IgA in saliva samples, anti-RrgA and anti-RrgB secretory component that represents secretory IgA (S-IgA) was also measured by antigen-specific ELISA. In the absence of reference standard, the optical density (OD at 405nm) 30 min after the addition of substrate solution was analysed.

4.4.8.1 Salivary RrgA and RrgB specific IgG in relation to pneumococcal carriage

Salivary anti-RrgA and anti-RrgB IgG titre were analysed in relation to nasopharyngeal carriage of pneumococcus. Salivary antibody was measured in a total of 85 patients among them 47 were children and 38 were adults. None of the adults (n=38) were colonised. For this reason only children's saliva samples (n=47) were included in the comparative analysis to investigate whether there was any association or relation to pneumococcal carriage with the antibody titres.

Both salivary anti-RrgA and -RrgB IgG levels was found to be higher in culture-negative children than in those who were culture-positive (Figure 4.4.8.1, p=0.0081 and $p=0.0478$ respectively).

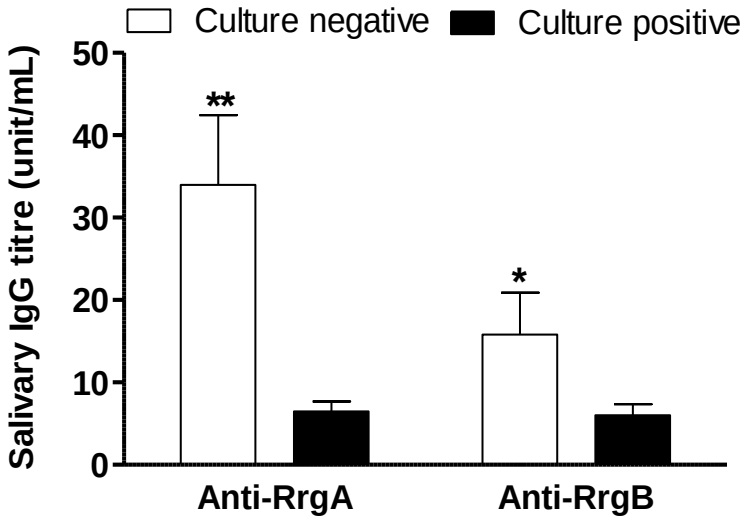


Figure-4.4.8.1: Relationship between levels of salivary IgG antibodies and pneumococcal carriage. Salivary anti-RrgA and anti-RrgB IgG were analysed in culture negative (n=27) and culture positive (n=20) children. Results expressed as the geometric mean ± 95% confidence interval (CI) of antigen (RrgA and RrgB) specific IgG (units/ml). Statistical significance (*p<0.05 and **p<0.01) was compared with culture-positive children in Student's t test.

4.4.8.2 Salivary anti-RrgA and RrgB S-IgA antibodies in relation to pneumococcal carriage

Salivary anti-RrgA and anti-RrgB secretory IgA (S-IgA) levels were analysed in relation to nasopharyngeal carriage of pneumococcus. As described previously, only children's saliva samples were included in this analysis.

Both salivary anti-RrgA and anti-RrgB S-IgA levels were lower in the culture-negative children than those who were colonised (Figure-4.4.8.2, p=0.0042 and $p=0.0228$).

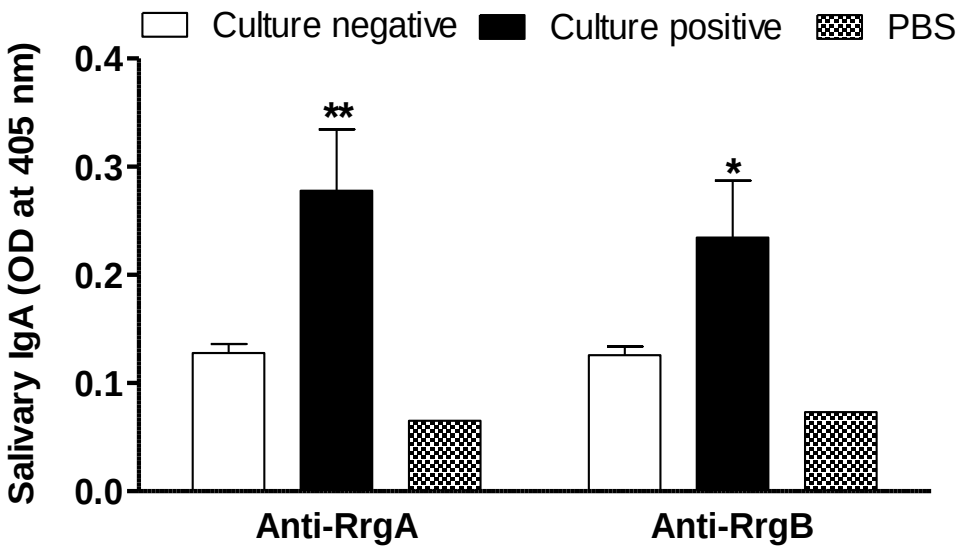


Figure-4.4.8.2: Relationship between salivary S-IgA antibodies and pneumococcal carriage. Salivary anti-RrgA and anti-RrgB S-IgA were analysed in culture negative (n=27) and culture positive (n=20) children. Results expressed as the geometric mean ± 95% confidence interval (CI) of antigen (RrgA and RrgB) specific S-IgA (OD at 405 nm). Statistical significance (**p<0.01 and *p<0.05) was compared to culture-negative children in Student's t test.

4.5 Discussion

Pneumococcus is a major human pathogen often colonises the human nasopharynx asymptomatically (Bogaert et al. 2004a). Pneumococcal infection is particularly common in young children and a major contributor of high mortality for children under five years (Obaro 2009). The incidence of invasive pneumococcal diseases gradually decreases with progression of age (Jódar et al. 2003). Nasopharyngeal carriage also declines in an age-dependent manner (Bogaert et al. 2004a). This decreased susceptibility to colonisation or infection is likely to be associated with the development of natural immunity to pneumococcus. Development of antibodies to pneumococcal capsular polysaccharides and surface proteins is a very important component of natural immune response to pneumococcus. Antibodies to capsular polysaccharides efficiently protect against subsequent carriage or infection by the corresponding serotypes (Shapiro et al. 1991). Similarly, antibodies against protein antigens also possess protective efficacy (Kamtchoua et al. 2013).

Measurement of antibodies in serum (or in other body fluids, such as saliva, bronchoalveolar lavage) is an important parameter of assessing naturally or vaccine-induced immune response to pneumococcal antigens. The most commonly used technique for measuring these antibodies is ELISA. ELISA based detection of anti-pneumococcal capsular polysaccharide-specific antibodies became the choice of techniques in earlier 1980s, replacing the previously practiced radioimmunoassay (RIA). ELISA provided several advantages over RIA, including requirement of less sample volumes and avoidance of handling radioactive substances. However, earlier generation of ELISA lacked specificities, and after several modifications a third generation of ELISA had been standardised, which was universally adopted in a meeting of experts held at WHO headquarter in 2000 (Wernette et al. 2003).

ELISA based techniques are widely used for detection of antibodies to pneumococcal protein antigens also. Gianfaldoni et al, (2007) used an ELISA technique to measure antibodies to pilus-1 (RrgA, RrgB and RrgC) subunit proteins in mouse serum (Gianfaldoni et al. 2007). A modification of that ELISA procedure for analysis of RrgA- and RrgB-specific antibodies in human serum was applied in this study. The solution used for blocking ELISA plates, and diluting samples, standards and conjugates was 10% FCS in PBS. The presence of fibronectin in the FCS, which might bind to the adhesin RrgA, could potentially lead to non-specific background in the ELISA assay. However, this effect may be minimal, if present, as the negative control wells showed low background colour development. Another limitation of this ELISA was the use of a recombinant RrgB antigen from a single clade (clade-1) of pilus-1, which might result in a lower than estimated prevalence of serum anti-RrgB positivity

Specificities of these assays were confirmed by inhibition ELISA. Anti-RrgA ELISA was inhibited by adsorption of the serum antibodies after incubating with recombinant RrgA, but not with recombinant RrgB. Vice versa, anti-RrgB ELISA was inhibited by adsorption of the serum antibodies after incubating with recombinant RrgB, but not with recombinant RrgA.

An in house modification of Western blot technique was also developed to further confirm the presence of pilus-1 specific antibodies. Indeed, Western blotting confirmed the presence of these antibodies in serum samples with antibody titres above certain threshold levels. This has provided supportive evidence of specificity for the antibodies detected by ELISA. It has enabled us to determine detection limits for the antibody positivity from antibody titres measured by ELISA.

In this study, we detected significant levels of serum anti-RrgA and RrgB antibodies in large number of patients (both children and adults), which were likely to be derived from previous colonisation of pneumococcus expressing pilus. To the best of our knowledge, this is the first report of naturally developed antibodies to pneumococcal RrgA and RrgB antigens in humans. In general, the antibody titres to both RrgA and RrgB were higher in older children and adults than in children (Figure-4.4.3a). This may be a common phenomenon seen in antibodies to other of pneumococcal protein antigens including pneumolysin, PsaA, CbpA and PspA (Brandileone et al. 2004). Based on the cut-off value (100units/ml) calculated by the ROC curves constructed from western blot analysis, 61.5% of total serum samples were estimated to be positive for anti-RrgA, and 58.2% estimated to be positive for anti-RrgB antibodies.

RrgA is the major contributor to the interaction of pneumococcal pilus-1 with host immune system (Nelson et al. 2007). This could be the reason why antibodies to RrgA appeared to develop earlier in childhood than that to RrgB, as shown in our study. Although an age-dependent increase in antibody levels for both serum anti-RrgA (Figure-4.4.3b) and anti-RrgB (Figure-4.4.3b) IgG titres was observed, the titre of anti-RrgB was very low in general before the age of 6 years. However, anti-RrgB titres elevated rapidly in children older than 6 years and reached similar levels to anti-RrgA antibody in older children (Figure-4.4.3c). The titres of both these antibodies were highest in adolescents and appeared to decline to some extent in the 16-30 years old subjects (Figure-4.4.3b and 4.4.3c).

The development of RrgA- and RrgB-specific antibodies in the serum could be considered as part of natural immune response in humans, induced by exposure to these proteins during colonisation or infection. Detection of anti-RrgA and -RrgB in

a large proportion of sera (in both children and adults) suggests that pilus-1 expressing pneumococcal strains may frequently colonise children during early childhood, despite the prevalence of pilus-expressing strains has been estimated to be around only 30% (Barocchi et al. 2006). It has been shown previously that pneumococcal colonisation is a dynamic process; and most young children (<2 years) are colonised serially with single or even multiple serotypes of the pneumococcus (Syrjänen et al. 2001). So it is possible that children in this study had been exposed to pilus-expressing strains previously.

As shown previously, antibodies to other pneumococcal proteins including CbpA and pneumolysin (Zhang et al. 2006b), IgG1 was the predominant subclass to RrgA and RrgB antigens (Figure-4.4.4, a+b). This was typical for antibody responses to protein antigens, with the predominance of IgG1 followed by IgG3 and with minimal levels of IgG2 and IgG4 (Hjelholt et al. 2013). Both IgG1 and IgG3 are potent complement activators (Ram et al. 2010). Therefore, the predominant generation of these two isotypes of antibodies to RrgA and RrgB protein antigens may be advantageous for their opsonophagocytic functionality, which contributes to the complement mediated phagocytosis of pneumococcus.

When the antibody titres were analysed in all the patients samples in relation to pneumococcal carriage, both serum anti-RrgA and anti-RrgB IgG titres appeared to be higher in culture-negative patients than in those who were colonised (Figure-4.4.7a). Detailed analysis in different age groups showed a consistently higher titre of anti-RrgA IgG antibodies in culture negative than in culture positive subjects in all age groups studied. This suggests that anti-RrgA antibodies may contribute to protection against pneumococcal carriage in humans. For anti-RrgB antibody, there appeared to be a difference between culture-negative and culture-positive subjects in

older children (7-15 years) (Figure-4.4.7.2) when the antibody titre reached higher level. This suggests that anti-RrgB antibody may also contribute to protection against carriage in older children and adults.

Salivary IgG antibodies to both RrgA and RrgB were also detected in the study subjects. There was a significant difference in salivary anti-RrgA and -RrgB IgG titres between pneumococcal culture-negative and culture-positive patients, with higher titres in the former (Figure-4.4.8.1a). The higher salivary IgG in non-colonised patients supports our hypothesis that it might significantly contribute in the protection against pneumococcal colonisation at the mucosal sites (figure-4.4.8.1a).

There was a good correlation between the serum and salivary IgG antibodies for both anti-RrgA and anti-RrgB (Figure-4.4.8.1c+d), suggesting that the salivary IgG antibodies were mostly derived from serum leakage (Zhang et al. 2000), although local mucosal immune tissue NALT could also contribute to this local pool of antibodies (Ivarsson et al. 2004; Zhang et al. 2006b).

On the other hand, the salivary secretory IgA (S-IgA) to both RrgA and RrgB were relatively higher in the colonised children compared to the non-colonised (Figure-4.4.8.2). This increase of S-IgA in colonised patients could be explained by the fact that they are secreted from the local mucosal immune system (NALT), induced by recent colonisation. These locally produced S-IgA may contribute to protection against local invasion of pneumococcus to cause invasive disease, but may not be effective to clear pneumococcal carriage in the absence of an effective level of IgG.

The primary antibody response to pathogens characteristically shows an early production of IgM antibodies, which later undergo affinity maturation and class-switching to produce other antibody isotypes (Boes 2000). Following a recent

colonisation, the IgM antibodies rise quickly. That may explain the higher RrgA- and RrgB-specific IgM titres in colonised than in the non-colonised children (Figure-4.4.7.4).

One limitation of this study is the interpretation of association between antibody titre and carriage on the basis of a cross sectional data. A longitudinal follow-up of colonised patients to observe any subsequent rise of IgG titre and clearance of carriage would be helpful. Our cross sectional data suggests that recent pneumococcal colonisation induces a secretory IgA (in local mucosal sites, like saliva) and a systemic IgM (in serum) antibody responses to pneumococcal RrgA and RrgB proteins. These initial responses might not be sufficient to clear the nasopharyngeal carriage until a strong systemic IgG response develops, which potentially contributes to the eradication of carriage. This explanation is supported by the findings of a recent study where experimental human carriage induced strong mucosal and systemic antibody responses, which were protective against re-colonisation or invasive pneumococcal disease (Ferreira et al. 2013).

In summary, we show significant antibody levels to pilus RrgA and RrgB proteins developed from natural immunity in children and adults. There was an age-dependent increase in serum antibody levels to both RrgA and RrgB. Notably, anti-RrgA antibody starts to develop earlier in childhood than anti-RrgB antibody. Also, higher antibody levels, anti-RrgA antibodies in particular, were found in children who were culture-negative than in those who were culture-positive for pneumococcus in nasopharynx. These results suggest that naturally developed antibodies to pneumococcal pilus-1 proteins, particularly anti-RrgA might have a significant contribution in the protection against pneumococcal carriage in humans.

Chapter 5

B cell Response to Pneumococcal RrgA and RrgB Antigens in Human Nasal Associated Lymphoid Tissues (NALT)

5.1 Introduction

Streptococcus pneumoniae is a mucosal pathogen which frequently colonises the human nasopharynx. Although regarded as a precondition for disease causation, pneumococcal carriage can also be considered as a natural immunisation process (Ferreira et al. 2013). Pneumococcal carriage rates are highest in early childhood, which decline gradually in older children (Bogaert et al. 2004b). Priming of immunological memory to pneumococcal antigens occurs during the episodes of carriage in childhood, which may lead to the development of protective immunity in later life (Zhang et al. 2006b; Vukmanovic-Stejic et al. 2006). The nasal associated lymphoid tissues (NALT) play a crucial role in the induction of local mucosal immune response to pneumococcal protein antigens (Zhang et al. 2006b).

Adenoids and palatine tonsils are major contributor of the human NALT (Brandtzaeg 2003). These are the store houses for pathogen specific memory B and T cells (Passàli et al. 2003). Surgically removed adenotonsillar tissues were found to be rich in memory cells specific to pneumococcal protein antigens (Zhang et al. 2002). These memory cells are likely to be induced by previous colonisation by pneumococcus. Subsequent carriage enhances the magnitude of antigen specific antibody responses in these tissues, possibly by recalling these memory cells. Activation of these memory cells may mount a rapid and robust antibody response against pneumococcus (Zhang et al. 2002).

Human adenoids and tonsils have structural and functional similarities. Unlike those in the other mucosal sites, adenotonsillar B cells are more of IgG secreting than of IgA. The IgA subclass produced in these tissues are mostly of IgA1 which is vulnerable to the degradation of IgA1 protease secreted by many respiratory pathogens, including pneumococcus. For this reason the IgG antibodies play a major

role in the protective response in these tissues (Boyaka et al. 2000). The abundance of B cells, together with the relative predominance of $CD4^+$ T cells over $CD8^+$ cells (Passàli et al. 2003) supports the crucial role of the adenotonsillar tissues in the development of antigen-specific antibody response (Zhang et al. 2002). Pneumococcal protein antigens are capable of inducing antibody secreting cells (ASC) of different isotypes (IgG, IgM and IgA), with the predominance of IgG response in adenotonsillar tissues (Zhang et al. 2002). Adenotonsillar B cells express phenotypic properties of both mucosal and systemic compartments (Brandtzaeg 2003). The mucosal phenotypes migrate preferentially in and around the upper respiratory tract (Johansen et al. 2005). Pneumococcal protein antigens stimulate both mucosal (expressing J chains) and systemic phenotypes of B cells in adenotonsillar MNC (Zhang et al. 2006b).

Pneumococcal type-1 pilus subunits RrgA and RrgB showed good immunogenicity and protective capacity in experimental mice (Gianfaldoni et al. 2007). The subunit RrgA is an adhesin, which mediates the attachment of the bacteria and facilitates nasopharyngeal colonisation. It interacts extensively with the host mucosal cell adhesion molecules, and induces antibody responses in colonised children during this process (Nelson et al. 2007). Antibody response to subunit RrgB, the major structural component of pilus-1 was also reported. However, no data is available whether these proteins induce the local mucosal immunity in the human NALT.

This study have investigated the antibody responses to RrgA and RrgB in the adenotonsillar MNC after *in vitro* stimulation with pneumococcal concentrated culture supernatant (CCS), containing both RrgA and RrgB proteins. We examined the B cell responses to these RrgA and RrgB antigens in human NALT after stimulation with pneumococcal CCS or a polyclonal B cell activator CpG-ODN.

5.2 Aims of Study

To investigate the B cell responses to pneumococcal RrgA and RrgB antigens in human nasal associated lymphoid tissues (NALT)

5.3 Experimental Design

1. Adenotonsillar MNC were stimulated with pneumococcal CCS or a polyclonal B cell activator (CpG-ODN) to induce B cell responses and *in vitro* production of antigen-specific or total antibodies.
2. The B cell responses was analysed by enumerating RrgA- and RrgB-specific antibody secreting cells (ASC) by ELISpot assay.
3. *In vitro* production of antigen-specific or total antibodies was measured by ELISA.
4. Analyses were done to investigate whether current nasopharyngeal carriage of pneumococcus influences the *in vitro* antibody production or memory B cell responses to RrgA and RrgB.

5.3.1 Patients and samples

Surgically removed adenotonsillar tissues were obtained from both children and adults (1–30 years). A nasopharyngeal swab from each patient was obtained on the same day of operation. Patients who were known to be immunocompromised or had received antibiotics or steroids two weeks before surgery were excluded. The study was approved by the local ethics committee (Liverpool Paediatric Research Ethics Committee). An informed written consent was obtained from each patient/guardian as appropriate.

5.3.2 Pneumococcal Concentrated Culture Supernatant (CCS)

Concentrated pneumococcal culture supernatant (CCS) used for *in vitro* stimulation of adenotonsillar mononuclear cells in this study was produced from a pilus-1 expressing *Streptococcus pneumoniae* serotype 4 (TIGR4) strain (Tettelin et al. 2001) following a previously described method (Zhang et al. 2006b). Detailed procedures for preparation of CCS from the TIGR4 wild type and its isogenic RrgA^{-}/$^{-}$ and RrgB^{-}/$^{-}$ mutants (Hilleringmann et al. 2009) was discussed in methods chapter-2. Briefly, the CCS was prepared by concentrating the supernatants of pneumococcal broth cultures grown up to the exponential phase. The supernatant was removed following centrifugation (3000×g for 30 min). To get rid of any residual bacteria, the supernatant was filtered through a 0.2-mm sterile filter, and then concentrated 10-fold before aliquoted for storage in the -80°C freezer. The protein concentrations of the CCS were measured by a Bio-Rad protein assay following the manufacturer's instructions (Sigma). Western blots of the CCS were performed with rabbit anti-RrgA and anti-RrgB antisera to confirm the presence or absence of RrgA and RrgB proteins in the CCS from wild type TIGR4, RrgA^{-}/$^{-}$ and RrgB^{-}/$^{-}$ mutant strains.

The choice of pneumococcal CCS for cell stimulations offers several advantages. First of all, it obviates the need for using live bacteria, therefore safe for handling. Secondly, the chance of cytotoxicity is less compared to live cells. Finally, it allows investigating the stimulatory effects of soluble proteins in their native form, excluding other structural components of pneumococcal cells. The effect of an individual protein may be assessed by comparing effect induced by CCS from the wild-type with the isogenic strains depleted of a particular protein.

5.3.3 Recombinant RrgA and RrgB Antigens

Purified His-tagged recombinant RrgA and RrgB proteins from *Streptococcus pneumoniae* serotype 4 TIGR4 strain (obtained from Novartis Vaccines, Sienna, Italy) (Gianfaldoni et al. 2007) were used for detection of RrgA and RrgB-specific B cell response by ELISpot assay, and for measuring anti-RrgA and -RrgB antibodies by ELISA.

5.3.4 Cell culture and stimulation by pneumococcal CCS

Adenotonsillar MNC was cultured at $4x10^6$/ml in RPMI-1640 with or without pneumococcal CCS (1µg/ml). For enumerating the frequency of antibody secreting cells (ASC) by ELISpot assay, cells were incubated (at 37°C, in 5% CO_2) for 5 days before being transferred to ELISpot plates. To analyse the *in vitro* antibody production after CCS stimulation, cell culture supernatants were collected at day 14 and stored at –70°C until the antibody production was measured by ELISA.

5.3.5 Measurement of B cell response by ELISpot assay

B cell responses to pneumococcal RrgA and RrgB antigens were analysed using ELISpot assay. The detailed procedures are described in the methods chapter-2. Briefly, ELISpot plates (Millipore) were coated overnight with recombinant RrgA and RrgB antigens (5µg/ml). After blocking with RPMI (containing 10% FBS) at 37°C for 2 hr, adenotonsillar MNC were added and incubated overnight at 37°C. After washing, biotinylated anti-human IgG antibodies (Invitrogen) were added and incubated for 30 min at RT. Thereafter, Avidin D-HRP conjugate (Vector Laboratories) was added and incubated for 30 min at RT. Coloured spots developed after addition of AEC substrate, which were imaged by Chemi-Doc XRS system, and analysed with NIST's Integrated Colony Enumerator (NICE) software.

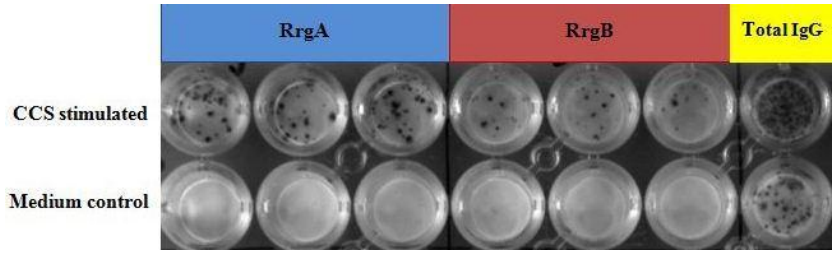


Figure-5.3.5: ELISpot assay to enumerate antibody secreting B cell response showing number of RrgA- and RrgB-specific antibody secreting cells (ASCs) in TIGR4wt CCS stimulated samples compared to the corresponding medium controls. Total numbers of IgG secreting cells are shown on the right hand side column for each sample.

5.3.6 ELISA Assay

ELISA assay was performed as described in methods (chapter 2). In brief, ELISA plates were coated with individual recombinant RrgA and RrgB antigens (1µg/ml); and incubated overnight at 4°C. After washing, plates were blocked with 10% FBS-PBS followed by incubation of cell culture supernatants. Thereafter, alkaline phosphatase conjugated antibodies were added and incubated before addition of p-Nitrophenyl phosphate substrate. Optical density was measured at 405nm and data were analysed using DeltaSoft microplate analysis software (BioMetallics) (for details see materials and methods chapter 2).

5.3.7 Statistical analysis

Differences in B cell response or antibody titres between different groups were analysed by analysis of variance and Student's t-test. Association between two factors was analysed by Pearson's correlation. A p value of <0.05 was considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics (version 20).

5.4 Results

5.4.1 Optimisation of pneumococcal CCS concentration for in vitro stimulation of adenotonsillar MNC

The optimal concentration of pneumococcal CCS required for induction of antibody production to pilus-1 antigens in adenotonsillar MNC culture was obtained by stimulation of 5 samples with different concentrations (0.5, 1.0, 2.0 and 5.0µg/ml) of TIGR4wt CCS. One way ANOVA shows significant induction of both anti-RrgA and -RrgB production with all four concentrations of CCS compared to the unstimulated (0 µg/ml) controls (Figure-5.4.1). Antibody production to both antigens was highest when stimulated at CCS concentrations ≥1µg/ml (figure-5.4.1). This concentration (1µg/ml) was used to stimulate adenotonsillar MNC by all three CCS (TIGR4wt, $RrgB^{-/-}$ and $RrgA^{-/-}$) in subsequent experiments.

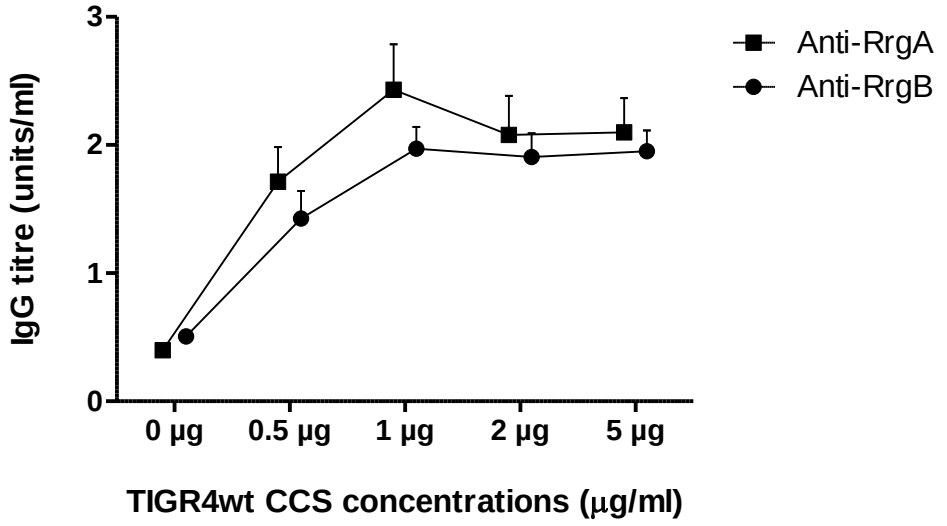


Figure-5.4.1: Optimisation of TIGR4 CCS concentration for *in vitro* stimulation of adenotonsillar MNC for specific antibody response. Dose response of TIGR4wt CCS in the induction of RrgA- and RrgB-specific IgG production in adenotonsillar MNC cultures in 5 patients are shown. Results are expressed as mean IgG titre (units/ml) + SEM (error bars).

5.4.2 Optimisation of the duration of adenotonsillar cell culture for specific antibody production

Time-course of antibody production to pilus-1 antigens in adenotonsillar MNC culture following pneumococcal CCS stimulation was optimised. MNC culture supernatants were collected at different time points (1, 5, 7, 10 and 14 days) following stimulation with TIGR4wt CCS in 6 patients. Production of anti-RrgA was higher after 5 days of stimulation and onwards, when compared to day 1; whereas, anti-RrgB production was significant after 7 days of stimulation and onwards (Figure-5.4.2). The highest level was observed for both antibodies at day 14 (Figure-5.4.2). In subsequent experiments, culture supernatants were collected at day 14 day of stimulation for measurement of antibody production by ELISA.

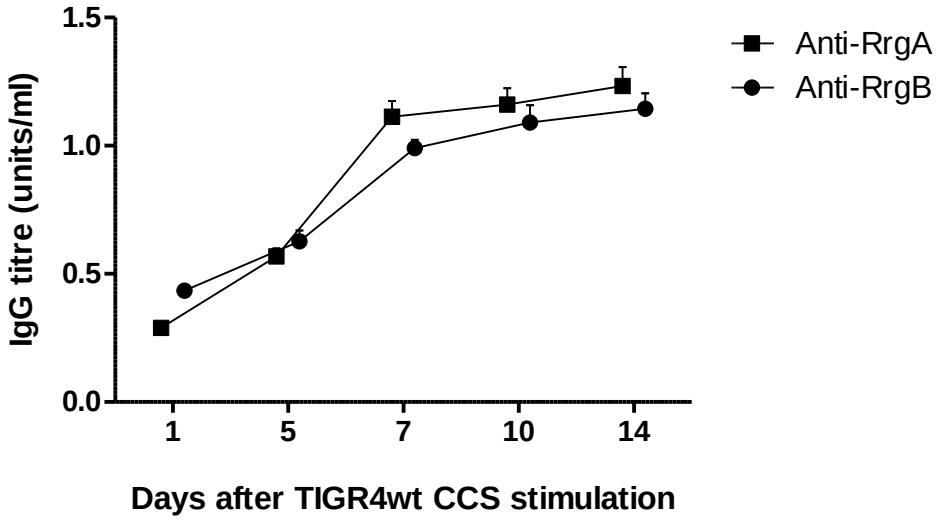


Figure-5.4.2: Time course of antibody production after stimulation with pneumococcal CCS. Production of RrgA- and RrgB-specific IgG in adenotonsillar MNC culture was observed after 5-7 days of stimulation with TIGR4wt CCS and continues to increase until day 14. Results are expressed as mean IgG titre (units/ml) + SEM (error bars) of 6 patient samples.

5.4.3 Pneumococcal CCS stimulation induces anti-RrgA and anti-RrgB antibody responses in adenotonsillar MNC

To investigate the *in vitro* production of antibodies to RrgA and RrgB by adenotonsillar MNC, antibody titres were measured in the cell culture supernatants. Cells were stimulated with an optimum concentration (1.0µg/ml) of pneumococcal CCS derived from TIGR4 wild type and its isogenic mutants ($RrgA^{-/-}$ and $RrgB^{-/-}$) for 14 days. Production of anti-RrgA and anti-RrgB antibodies in the MNC culture supernatants (n=58) was measured by ELISA. Stimulation with CCS derived from wild type pneumococcus (TIGR4wt) induced both anti-RrgA and anti-RrgB IgG antibody production (figure-5.4.3a+b, $p<0.001$ and $P<0.01$ respectively) compared to medium control. The CCS derived from $RrgB^{-/-}$ mutant of TIGR4 strain induced anti-RrgA IgG production, but there was no induction of anti-RrgB IgG production (figure-5.4.3a+b, $p=0.0036$ and $p>0.05$ respectively). The CCS derived from $RrgA^{-/-}$ mutant of TIGR4 strain failed to induce anti-RrgA production; but it was able to induce production of anti-RrgB (figure-5.4.3a+b, $p>0.05$ and $p=0.0032$ respectively).

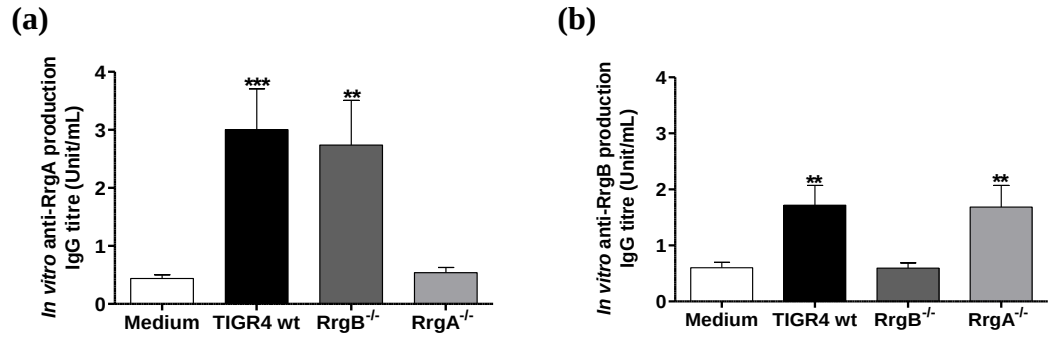


Figure-5.4.3a+b: Induction of anti-RrgA and -RrgB antibody responses in adenotonsillar cells after pneumococcal CCS stimulation. (a) Anti-RrgA IgG (b) Anti-RrgB IgG. IgG antibody response to RrgA and RrgB in adenotonsillar MNC cultures in 58 patients with or without stimulation by CCS derived from three different pneumococcal strains (TIGR4wt, $RrgB^{-/-}$ and $RrgA^{-/-}$) are shown. Results are expressed as mean titre (units/ml) ± SEM (error bars). Significance (***$p<0.001$, ** $p<0.001$) was compared with the medium control in paired t test.

It has been shown that the protein-specific memory B cell antibody production is dependent on memory ($CD45RO^+$) Th cells (Crotty and Ahmed 2004). To determine whether the pilus-1 specific antibody response was of memory type, *in vitro* antibody production in $CD45RO^+$ cell depleted MNC were analysed and compared with unfractionated whole MNC in 4 samples. Production of both anti-RrgA and -RrgB IgG antibodies following TIGR4wt CCS stimulation were markedly decreased in $CD45RO^+$ cell-depleted MNC compared to unfractionated MNC (Figure, 5.4.3c+d, p=0.0094 and 0.0288 respectively). This suggests that the antigen specific antibody levels detected were most likely to derive from memory B cells.

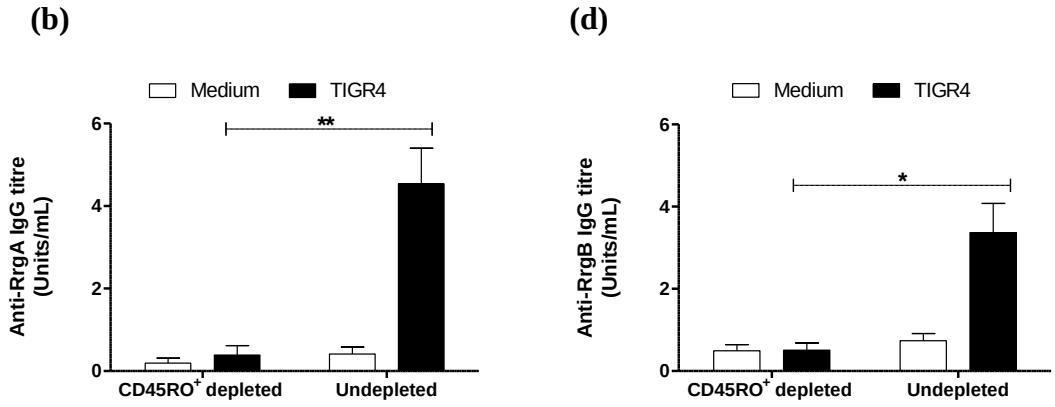


Figure-5.4.3c+d: Effect of $CD45RO^+$ cell depletion on the *in vitro* antibody production. Comparison of (c) anti-RrgA and (d) anti-RrgB IgG production by $CD45RO^+$ cell-depleted MNC and unfractionated adenotonsillar MNC are shown (n=4). Antibody production in $CD45RO^+$ cell-depleted MNC was much lower than that of unfractionated whole MNC after stimulation with TIGR4wt CCS. Results are expressed as mean titre (units/ml) ± SEM (error bars); *p<0.05 **p<0.01 as compared between naïve ($CD45RO^+$ depleted) and undepleted cells in paired t test.

5.4.4 Induction of antibody responses by Pneumococcal CCS is not a general polyclonal but antigen-specific

To investigate whether the induction of *in vitro* antibody production by pneumococcal CCS results from a general polyclonal activation of adenotonsillar memory B cells or from a response specific to pneumococcal antigens, the total IgG, IgA and IgM antibodies production by adenotonsillar MNC were measured. The MNC were stimulated with a polyclonal B cell activator (CpG-ODN) and pneumococcal TIGR4wt CCS, and the cell culture supernatants collected after 8 days were analysed for total IgG, IgA and IgM production by ELISA. IgG titres following both stimulations were compared with unstimulated medium control in 24 patients (n=24). There was no increase in total IgG, IgA and IgM production in adenotonsillar MNC following stimulation by CCS. In contrast, CpG-ODN stimulation induced a marked increase of all 3 isotypes of immunoglobulins, i.e. total IgM, IgG and IgA (Figure-5.4.4, ***p<0.001, *p<0.05 and *p<0.05 respectively).

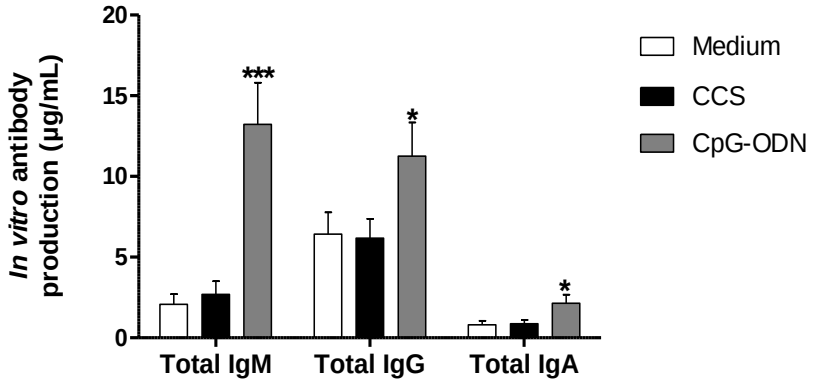


Figure-5.4.4: Induction of total antibody production by pneumococcal TIGR4wt CCS and CpG-ODN. Pneumococcal CCS stimulation did not affect the total IgM, IgG and IgA concentration in adenotonsillar MNC culture, whereas significant increase in total immunoglobulin production (in all three isotypes) was observed following CpG-ODN stimulation. Results are expressed as mean antibody titre (units/ml) ± SEM (error bars) for total IgM, IgG and IgA in 24 patients (n=24); significance (*p<0.05 and ***p<0.001) was compared with the medium control in paired t tests.

5.4.5 Culture medium (THBY) alone does not induce antibody production

Pneumococcal culture supernatants were produced by growing the organism in Todd-Hewitt Broth with 0.5% Yeast extract (THBY) medium. To determine whether there was any contribution of the THBY medium in the induction of the antibody responses by pneumococcal CCS, tonsillar MNC (n=21) were stimulated with this culture medium alone (concentrated in the same way as in the CCS) as a negative control. For both anti-RrgA and -RrgB IgG, addition of THYB culture medium alone did not induce any antibody response (Figure, 5.4.5a+b, $p>0.05$); whereas, all three CCS (TIGR4wt, $RrgB^{-/-}$ and $RrgA^{-/-}$) induced specific antibody responses. CCS derived from $RrgB^{-/-}$ induced anti-RrgA, CCS from $RrgA^{-/-}$ induced anti-RrgB IgG and CCS from TIGR4wt induced both (Figure, 5.4.5a+b, ***$p<0.001$).

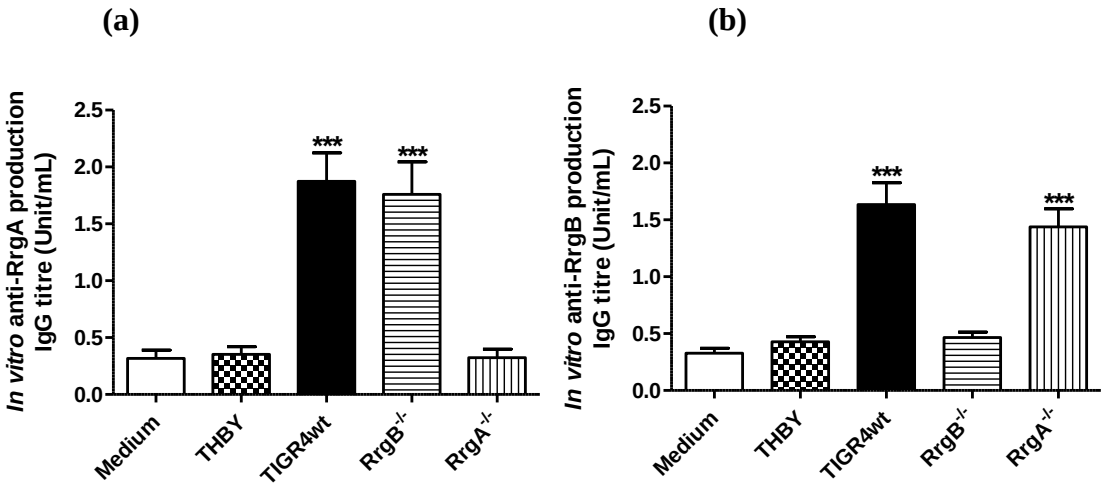


Figure-5.4.5a+b: Stimulation of adenotonsillar MNC with THYB medium; for (a) Anti-RrgA IgG (b) Anti-RrgB IgG production. Addition of THYB medium alone is not sufficient to induce RrgA- and RrgB-specific antibodies. TIGR4wt CCS induces both anti-RrgA and -RrgB, $RrgB^{-/-}$ CCS induces anti-RrgA and $RrgA^{-/-}$ CCS induces anti-RrgB IgG in adenotonsillar MNC cultures (n=21). Results showing mean IgG titre (units/ml) ± SEM (error bars); significance (***$p<0.0001$) was compared with the medium control in paired t test.

5.4.6 Recombinant antigen alone does not induce specific antibody response

To investigate whether recombinant RrgA (rRrgA) or RrgB (rRrgB) protein induces antibody production in adenotonsillar MNC culture, tonsillar MNC (n=9) were co-incubated with rRrgA (1μg/ml) or rRrgB (1μg/ml). Addition of either recombinant protein did not induce any significant antibody response (Figure, 5.4.6a+b, $p>0.05$). However, co-incubation of recombinant RrgA or RrgB with $RrgA^{-/-}$ CCS or $RrgB^{-/-}$ CCS appeared to induce some anti-RrgA or -RrgB antibody production in adenotonsillar MNC culture (Figure, 5.4.6a+b, $^{**}p<0.01$ and $^{*}p<0.05$), although the magnitude of the antibody response was lower than that induced by TIGR4wt CCS.

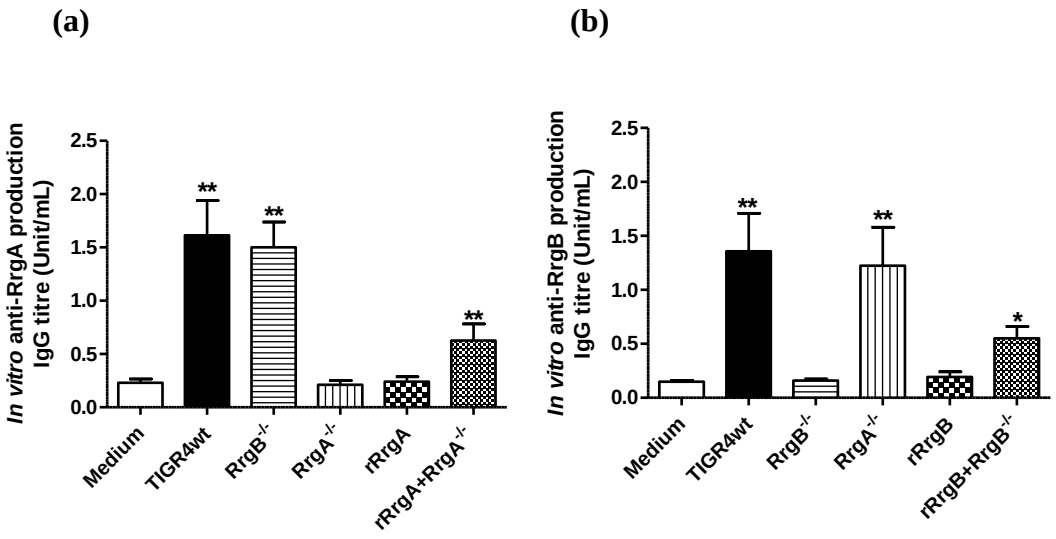


Figure-5.4.6: Stimulation of adenotonsillar MNC with recombinant RrgA and RrgB antigens. Production of (a) anti-RrgA IgG and (b) anti-RrgB IgG after stimulation with CCS derived from TIGR4wt and isogenic mutant strains ($RrgB^{-/-}$ and $RrgA^{-/-}$); rRrgA or rRrgB alone (1.0μg/ml), or recombinant RrgA or RrgB together with $RrgA^{-/-}$CCS or $RrgB^{-/-}$CCS (rRrgA+$RrgA^{-/-}$ and rRrgB+$RrgB^{-/-}$) were analysed and compared with medium (unstimulated) control in 9 patient samples. Results showing mean IgG titre (units/ml)± SEM (error bars); significance ($^{**}p<0.01$ and $^{**}p<0.05$) was compared with the medium control in paired t test.

5.4.7 Detection of antibody secreting cells (ASC) following stimulation by CpG-ODN and pneumococcal CCS.

To analyse pilus-1 antigen specific B cell response in adenotonsillar MNC, ELISpot assay was performed. Firstly, memory B cell frequencies to RrgA and RrgB were enumerated after stimulation with a polyclonal B cell activator CpG-ODN. Significant memory B cell response (numbers of IgG ASC) were observed following CpG stimulation compared to the medium control (Figure-5.4.7a, ***p<0.001). Number of RrgA-specific IgG ASC (mean±SEM, 29.95±2.97) appeared to be higher than that of RrgB (mean±SEM, 16.08±5.7) (Figure-5.4.7a, *p<0.05).

(a)

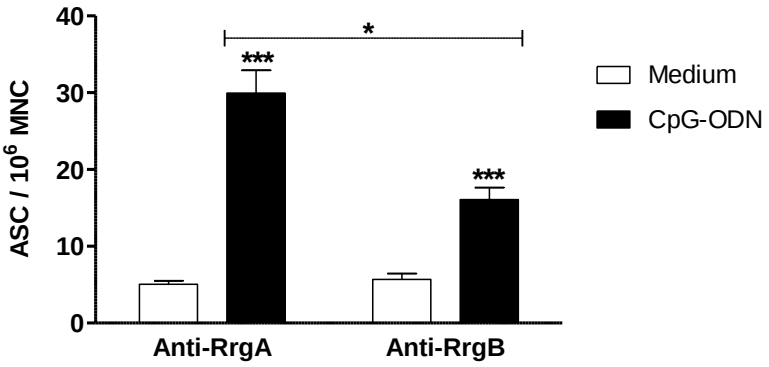


Figure-5.4.7a: Induction of RrgA and RrgB-specific B cell responses in NALT by CpG-ODN. Numbers of RrgA- and RrgB-specific memory IgG cells ASC in adenotonsillar MNC were enumerated by ELISpot assay following CpG-ODN stimulation. Significant numbers of IgG ASC to RrgA and RrgB (****p*<0.001) were observed after CpG-ODN stimulation (filled bars) compared with medium controls (open bars) paired t test. Data represent the mean±SEM (error bars) of number of antigen-specific ASC/10^6 MNC (n=24). Also the frequency of RrgA-specific IgG ASC after CpG-ODN stimulation was significantly higher (*p<0.05) than that of RrgB in paired t test.

ELISpot assay was also performed to enumerate the numbers of RrgA- and RrgB-specific B cell responses following stimulation by pneumococcal TIGR4wt CCS. Enumeration of antigen-specific ASC in adenotonsillar cells was done with or without TIGR4wt CCS stimulation in 58 patients (n=58). Significant numbers of IgG ASC to both RrgA and RrgB antigens were observed following CCS stimulation (mean±SEM, 20.55±2.67 and 13.56±1.69 respectively) compared to the medium control (mean±SEM, 5.39±0.69 and 5.09±0.60 respectively) (Figure-5.4.7b, ****p<0.001*). Number of RrgA ASC appeared to be higher than that of RrgB ASC (Figure-5.4.7b, **p=0.0291*)

(b)

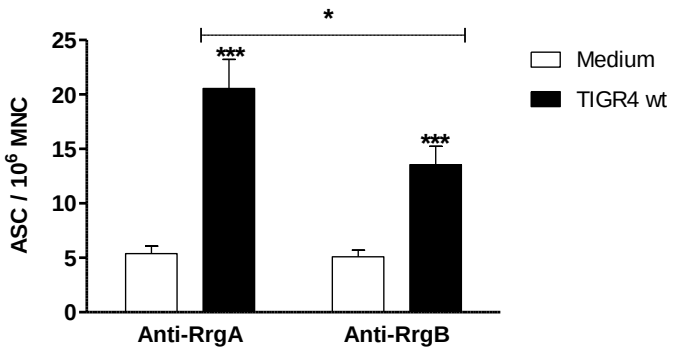


Figure-5.4.7b: Induction of RrgA and RrgB-specific B cell responses in NALT by TIGR4wt. Numbers of RrgA- and RrgB-specific IgG ASC in adenotonsillar MNC following TIGR4wt CCS stimulation (filled bars) were enumerated by ELISpot assay. Results expressed as the mean±SEM (error bar) of frequency of antigen-specific ASC/10^6 MNC (n=58); significance (*p<0.001) was compared with medium control (blank bars) in paired t test. Also the frequency of RrgA-specific IgG ASC after TIGR4wt stimulation was significantly higher (*p<0.05) than that of RrgB in paired t test.

The association between *in vitro* production of antibodies to pilus-1 antigens in adenotonsillar MNC culture supernatant and numbers of IgG ASC following stimulation by TIGR4wt CCS was examined in 33 patient's samples. There was a good correlation between the two as shown in figure-5.4.7c+d.

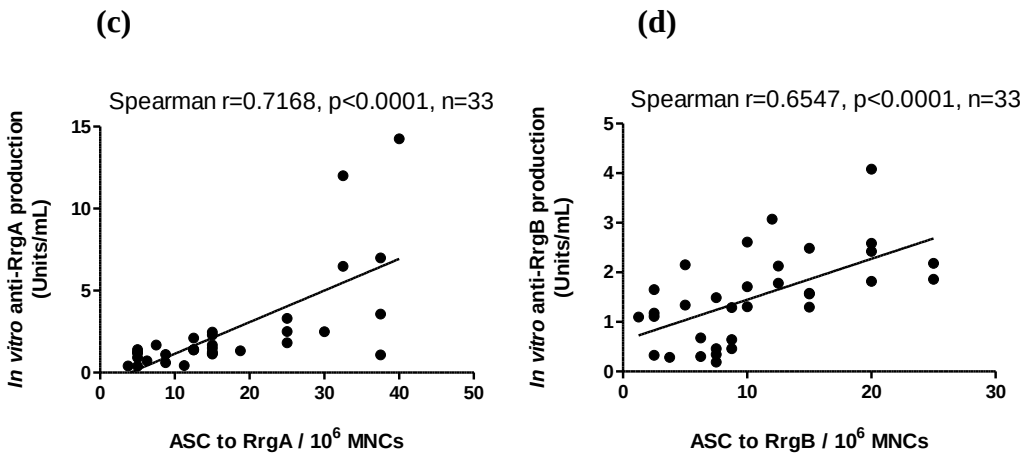


Figure-5.4.7c+d: Correlation between antibody levels in cell culture supernatant and numbers of ASC in adenotonsillar MNC after TIGR4wt CCS stimulation; (c) antibodies to RrgA, (d) antibodies to RrgB. *In vitro* antibody production was well correlated to the frequency of IgG ASC to both RrgA and RrgB (n=33) in Spearman's correlation test; significance (***p<0.001).

Frequency of memory B cells is traditionally measured by enumerating numbers of antigen-specific ASC following *in vitro* polyclonal stimulation of B cells by agents such as *Staphylococcus aureus* Cowan strain (SAC) and/or CpG-ODN (Crotty and Ahmed 2004). In this study, numbers of IgG ASC in adenotonsillar MNC following stimulation by both CpG-ODN and TIGR4wt CCS were enumerated (n=6) (figure-5.4.7e+f). Both stimulations elicited significant numbers of IgG ASC in unfractionated MNC, whereas the numbers of ASCs to both RrgA and RrgB were markedly reduced in $CD45RO^+$ cell-depleted MNC than in unfractionated whole MNC (Figure-5.4.7e+f). This suggests that the ASC detected in adenotonsillar MNC following pneumococcal CCS stimulation were predominantly antigen-specific memory B cells.

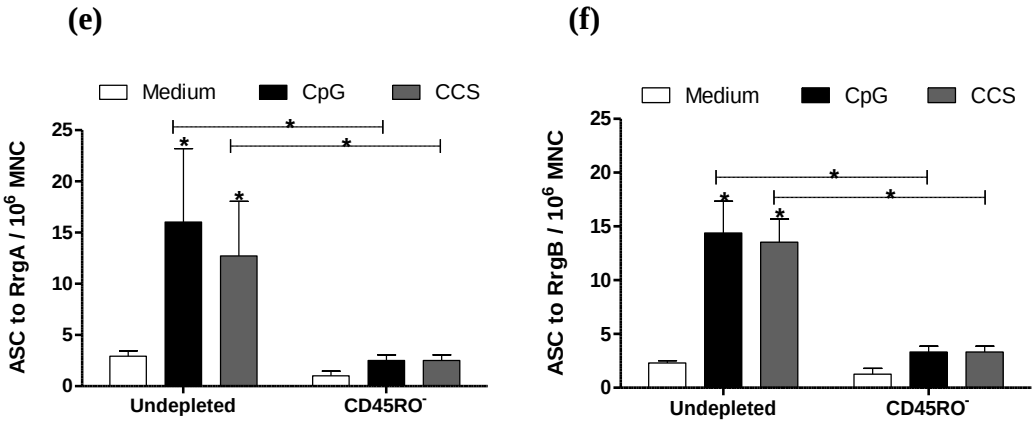


Figure-5.4.7e+f: Numbers of IgG ASC in undepleted and memory ($CD45RO^+$ depleted) adenotonsillar MNC following stimulation by CpG-ODN and TIGR4wt CCS; (e) ASCs to RrgA, (f) ASCs to RrgB. Results expressed as the mean±SEM (error bar) of numbers of antigen-specific ASC/10^6 MNC (n=6). Both CpG-ODN and TIGR4wt CCS significantly induced pilus-1 (RrgA and RrgB) specific IgG ASC in unfractionated adenotonsillar MNC; significance (*p<0.05) was compared to unstimulated (medium control) in paired t test. Frequencies of ASC to both RrgA and RrgB after stimulation with CpG-ODN and TIGR4wt CCS were significantly reduced in $CD45RO^+$ cell-depleted MNC; significance (*p<0.05) was compared to $CD45RO^+$ depleted MNC in paired t test.

5.4.8 Association of *in vitro* tonsillar MNC antibody production and pneumococcal colonisation

To examine whether there is any relationship between pneumococcal carriage and the *in vitro* B cell antibody response following stimulation, antibody production by adenotonsillar MNC from colonised and non-colonised patients were compared. Production of anti-RrgA in adenotonsillar MNC culture supernatants following *in vitro* stimulation with pneumococcal TIGR4wt CCS were found to be higher in patients currently colonised with pneumococcus than in those non-colonised (Figure 5.4.8a, $p<0.01$). Similarly, anti-RrgB production in culture-positive patients was also shown to be higher than in culture-negative patients (Figure 5.4.8b, $p<0.01$).

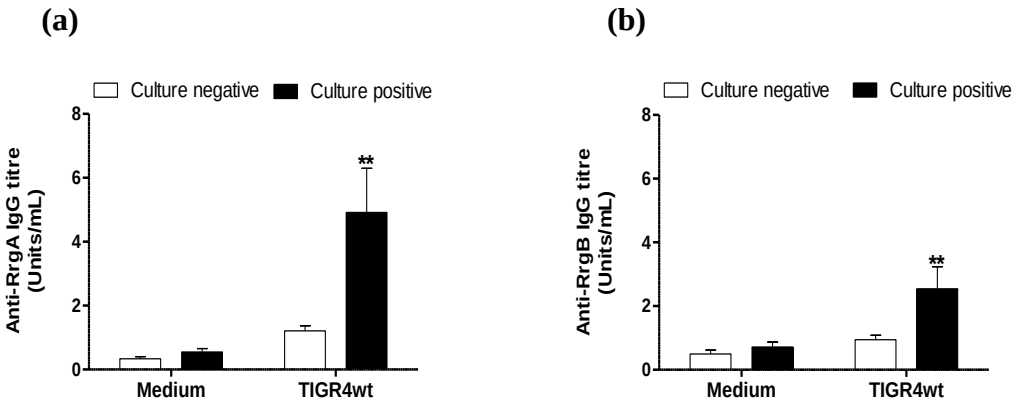


Figure-5.4.8a+b: Relationship between pneumococcal colonisation status and antibody response to pilus-1 antigens following CCS stimulation. (a) Anti-RrgA IgG (b) Anti-RrgB IgG. Data showing means± SEM (error bars) of IgG titre in colonised (n=28) and non-colonised (n=30) children. Significance level (**$p<0.01$) was compared with culture negative subjects in unpaired student's t test.

Numbers of antibody secreting cells in adenotonsillar MNC following pneumococcal CCS stimulation were also compared in colonised and non-colonised patients. Numbers of IgG ASC to both RrgA and RrgB following stimulation by TIGR4wt CCS were also shown to be higher in colonised than in non-colonised patients (Figure 5.4.8c+d, *p<0.05).

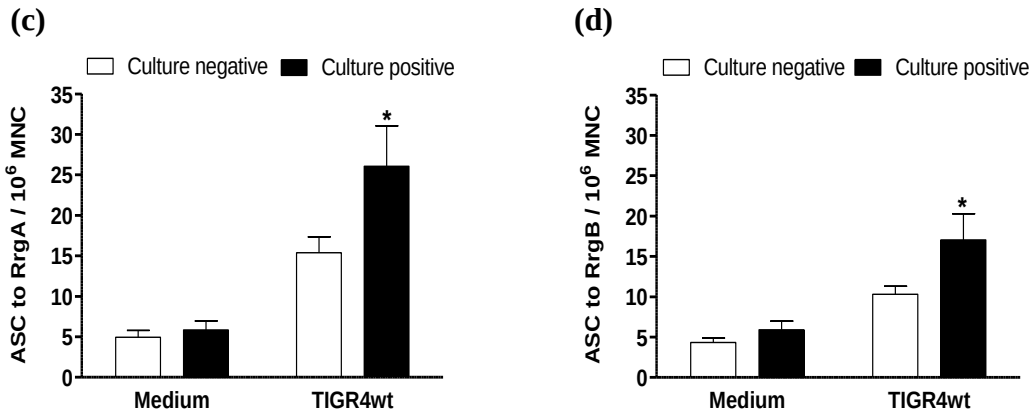


Figure-5.4.8c+d: relationship between pneumococcal colonisation status and memory B cell response to pilus antigens; (c) ASC to RrgA (d) ASC to RrgB. Data showing antibody secreting cell/million ± SEM (error bars) in colonised (n=28) and non-colonised (n=30) children. Significance level (*p<0.05) compared with culture negative subjects unpaired student's t test.

5.5 Discussion

Pneumococcus commonly colonises the mucosal surfaces of human nasopharynx. Although carriage can be regarded as a pre-requisite for pneumococcal disease process, it also induces natural immunity to the organism. Younger children are particularly susceptible to pneumococcal colonisation and infection. As the age progresses, both carriage and infection rates gradually decline, possibly due to the development of natural immunity and maturation of the immune system (Bogaert et al. 2004b).

Natural immunity to pneumococcus develops during the episodes of colonisation and/or infection. Carriage induces protective immune response to pneumococcal capsular polysaccharides and protein antigens in both local mucosal and systemic compartments (Ferreira et al. 2013). The nasal associated lymphoid tissues (NALT) plays a crucial role in development of local mucosal immunity to pneumococcus. Human NALT is important induction and effector site for natural immune response to pneumococcal protein antigens (Zhang et al. 2006b). They harbour significant numbers of antibody-secreting cells against those proteins (Zhang et al. 2002). The B cell response following *in vitro* pneumococcal stimulation was shown to be higher in tonsillar MNC from colonised patients. This may suggest that recent colonisation/carriage boosted memory B cell numbers.

The ability of pneumococcal protein antigens to induce local mucosal immunity may have important implication in future vaccination strategy against pneumococcus. Currently available vaccines are formulated as intramuscular injections to induce a predominantly systemic immunity for protection against invasive infection. Activation of mucosal immune system with these vaccines is minimal. A vaccine preparation applied intranasally may induce local mucosal immunity that could

protect against invasive diseases as well as carriage (Brandtzaeg 2007). A number of pneumococcal proteins were shown to induce protective response against both invasive infection and colonisation with intranasal application in murine models (Briles et al. 2000).

Expression of pilus-1 in pneumococcus is associated with enhanced colonisation and invasiveness of the bacteria; which is associated with an intensified inflammatory response in the host (Barocchi et al. 2006). This gave an early indication that the pilus-1 components might possess immunogenic properties, which could be exploited for vaccination. Indeed recombinant pilus-1 proteins, RrgA, RrgB and RrgC were shown to induce protective antibody response against invasive pneumococcal infection in experimental mice model (Gianfaldoni et al. 2007). So far, data regarding their immunogenicity in humans is limited. It remains unclear whether these proteins prime mucosal B cell memory in humans.

In this study we have shown significant antigen-specific anti-RrgA and -RrgB antibody production in adenotonsillar cells following stimulation by pneumococcal CCS that contain secreted native RrgA and RrgB proteins. Using an ELISpot assay, we also detected significant numbers of IgG ASC upon stimulation by pneumococcal CCS and CpG-ODN. The frequency of IgG ASC was predominant in adenotonsillar MNC and depletion of memory T cells by CD45RO microbeads significantly reduced the numbers of IgG ASC elicited by either pneumococcal CCS or CpG-ODN. It suggests that memory B cells are abundant in the NALT tissue, which are likely to be primed *in vivo* by previous colonisations. It is known that protein antigen-specific antibody production is T cell-dependent and memory T cells are crucial in memory B cell response (Crotty and Ahmed 2004).

In vitro production of antibodies to both RrgA and RrgB proteins was shown upon stimulation with pneumococcal CCS (Figure-5.4.3a+b). Stimulation of adenotonsillar MNC with TIGR4wt CCS (containing both RrgA and RrgB proteins) induced a significant (~6.8 fold-increase) anti-RrgA antibody response. Stimulation with the $RrgB^{-/-}$ mutant CCS also induced a similar (~6.2 fold-increase) response; whereas, CCS lacking RrgA ($RrgA^{-/-}$) did not induce any (Figure-5.4.3a). Significant induction of anti-RrgB antibody response following TIGR4wt stimulation was also observed, although the magnitude was somewhat lower (~2.9 fold increase). Stimulation with the $RrgA^{-/-}$ mutant CCS induced an anti-RrgB response comparable (~2.8 fold-increase) to that induced by the wild type, whereas the $RrgB^{-/-}$ mutant CCS failed to induce any response (Figure-5.4.3b). Production of these antigen-specific antibodies were mostly contributed by the memory ($CD45RO^{+}$) B cell, as their depletion markedly reduced both anti-RrgA and -RrgB levels (Figure-5.4.3c+d).

Considering that RrgA acts as a pneumococcal adhesin, it may be a good mucosal immunogen and induce local immune response in the nasopharynx. A relatively stronger response to RrgA compared to the response to RrgB following the CCS stimulation may suggest a stronger immunogenicity of RrgA antigen at the mucosal level. It is possible that RrgA interacts with the mucosal immune system through its adherence property and induce more significant immunity especially in young children. In the previous chapter, it was shown that serum anti-RrgA antibodies appeared to develop earlier in childhood than anti-RrgB antibodies.

The failure of $RrgA^{-/-}$ and $RrgB^{-/-}$ mutant CCS to induce anti-RrgA and -RrgB production respectively suggests that *in vitro* stimulations by CCS induce antigen-specific antibody production, not by a non-specific polyclonal stimulation. This was

supported by the finding that total immunoglobulin productions in TIGR4wt CCS-stimulated cell culture supernatants were not increased; whereas stimulation with a polyclonal B cell activator (CpG-ODN) resulted in a significant production of total IgM, IgG and IgA (Figure-5.4.4).

Stimulation of the adenotonsillar MNC with concentrated culture medium (THBY) alone, which was used to prepare pneumococcal CCS did not induce any antibody response. It excluded any non-specific contribution from the components of pneumococcal culture medium in the production of RrgA- and RrgB specific antibodies with CCS stimulations (Figure-5.4.5a+b). This finding was in accordance with the previous reports that stimulation of adenotonsillar MNC with THYB medium does not enhance *in vitro* antibody response to tetanus toxoid; which is known to be a potent immunogen (Zhang et al. 2006b).

Stimulation with either recombinant RrgA (rRrgA) or RrgB (rRrgB) alone did not induce an antibody response. However, their ability to induce specific antibody production was shown to be restored to some extent, when they were co-incubated with CCS derived from their corresponding mutant strains (Figure-5.4.6a+b). This phenomenon was also observed with other antigenic proteins of pneumococcus (Zhang et al. 2006b). It is possible that pneumococcal CCS contains something which has adjuvant effect for regaining of immunogenicity in recombinant proteins. It might well be the putative contribution by the mixture of antigenic proteins, inducing a stronger response to all of the components than what could have been generated by a single antigen *per se*.

This study demonstrates a significant B cell response to pneumococcal pilus-1 proteins in human NALT, upon stimulation by pneumococcal CCS. For enumerating antigen-specific memory B cell response we performed an ELISpot analysis,

following the methods developed by Shane Crotty and colleagues with some adaptation (Crotty et al. 2004). Adenotonsillar MNC was stimulated either with a polyclonal B cell activator CpG-ODN or pneumococcal TIGR4wt CCS (containing pilus-1 antigens RrgA and RrgB, confirmed by Western immunoblotting). Stimulation with both CpG and TIGR4wt CCS elicited significant numbers of RrgA- and RrgB-specific antibody secreting cells in adenotonsillar MNC. These antigen-specific antibody secreting cells mostly represent the memory B cells in the adenotonsillar MNC, as evidenced by the significant reduction of their number when the memory T ($CD45RO^{+}$) cells were depleted (Figure-5.4.7e+f).

Antigen-specific memory B cells, primed during an earlier exposure are readily activated in subsequent antigenic challenges (Crotty and Ahmed 2004). Accordingly, we believe that the source of the IgG ASCs to RrgA and RrgB is from the IgG memory B cell repertoire in the human NALT (Figure 5.4.7e+f). This pool of antigen-specific IgG cells is likely to be primed by previous episodes of carriage and/or infection by pilus-1 expressing pneumococcal strains. The abundance of these IgG memory cells in the NALT suggests the role of NALT in the induction of antibody responses to pneumococcal proteins antigens. The predominance of IgG immunocytes is one of the distinguishing characteristics of NALT from other mucosal inductive sites such as Payer's patches in GI tract where IgA immunocytes are predominant. IgG antibodies may contribute significantly to the local mucosal immune response against respiratory pathogens (Boyaka et al. 2000).

In vitro stimulation of adenotonsillar cells with pneumococcal culture supernatants induced a higher anti-RrgA and anti-RrgB antibody response in culture-positive than culture-negative patients. This result is consistent with the previous findings of higher *in vitro* antibody production to some other protein antigens (CbpA and

pneumolysin) in culture-positive children (Zhang et al. 2006b), indicating an enhanced memory response upon antigen re-exposure (Figure-5.4.8a+b). This suggests that recent pneumococcal colonisation or carriage in vivo may boost the antigen-specific memory B cell pool. This is supported by the enhancement in the number of antigen-specific ASCs in adenotonsillar cells of colonised patients (Figure-5.4.8c+d). In the previous chapter, we showed higher serum antibody levels to pilus proteins in non-colonised than colonised patients. Both systemic and local mucosal responses may contribute to the regulation of acquisition and elimination of pneumococcal carriage in the nasopharynx, and may exert their effects independently. High antecedent levels of antibodies in serum may help prevent colonisation, and enhanced memory-type local immune responses in NALT following re-exposure may help terminate it (Zhang et al. 2006b).

Since pneumococcus frequently colonises nasopharyngeal mucosa, NALT is likely to be the major induction and effector site for B cell response to pneumococcal proteins. Our results suggest that previous episodes of colonisation and/or infection by pilus-1 expressing strains of pneumococcus prime the local mucosal immune system in NALT and resulted in the generation of a memory B cell repertoire.

This study have presented evidences that both of these RrgA and RrgB are immunogenic and, and are capable of priming for memory B cell response in human NALT. Among these two pilus-1 antigens, the mucosal adhesin RrgA appeared to induce a relatively stronger immune response than the core structural protein RrgB. These findings provide supporting evidence that, pilus proteins especially RrgA could be exploited as component of mucosal pneumococcal vaccines.

Chapter 6

$CD4^+$ T cell Response to Pneumococcal RrgA and RrgB Antigens in Human NALT and Peripheral Blood

6.1 Introduction

For many years antibody production was considered to be the most important host protective immune responses to pneumococcal infection. However, recent studies suggest cellular immunity may also be important. Mice, genetically rendered unresponsive to polysaccharide antigens (CBA/N) or deficient of mature B cells (μMT), showed no remarkable decline in clearance of experimental pneumococcal colonisation (McCool and Weiser 2004). A new dimension of acquired immunity to pneumococcus is revealed, showing the limitations of antibodies to eliminate pneumococcal infection.

In a murine model, it was revealed that both innate and $CD4^+$ T cell mediated immunity were more important than antibody mediated immunity against pneumococcus (van Rossum et al. 2005). *In vivo* migration of $CD4^+$ T cells towards the site of infection was documented in a study, which also revealed that MHC-II deficient mice (low $CD4^+$ cell) were more vulnerable to pneumococcal colonisation and invasion (Kadioglu et al. 2004). Importance of $CD4^+$ T cell mediated immunity to pneumococcal infection was demonstrated when experimental mice lacking antibody responses inhibited nasopharyngeal colonisation, but mice lacking T lymphocytes failed to do so (Malley et al. 2005). This cellular protective response was found to be mediated by $CD4^+$ cells, not by the $CD8^+$ subset (Malley et al. 2005). A number of pneumococcal proteins, including pneumolysin, PspA, CbpA, PsaA can trigger this $CD4^+$ T cell mediated protective response (Basset et al. 2007a, Mureithi et al. 2009, Zhang et al. 2007).

More recently, $CD4^+$ T cells producing IL-17 (Th17 cells) have been suggested to be an important immune effector in pneumococcal defence. Nasopharyngeal clearance of pneumococcus in a high dose challenge model of mice was found to be

significantly reduced when treated with anti-IL-17A antibody (Malley et al. 2006). Pneumococcal cell wall polysaccharides up-regulated IL-17A production by cultured splenocytes of mice, previously vaccinated with these antigens (Malley et al. 2006). IL-17A recruits phagocytic cells in primary and secondary bacterial colonisation in the respiratory tract (Zhang et al. 2009). As shown in a mouse infection model, influx of neutrophils as part of an acute inflammatory response might not be sufficient to clear the bacterial load in case of primary pneumococcal colonisation. A TLR-2 dependent recruitment of other phagocytes (monocyte or macrophages) mediated by IL-17A was required to accomplish the task. However, on subsequent exposures, Th17 memory cell response was found to be associated with a quicker and vigorous influx of neutrophils resulting in an earlier clearance of carriage (Zhang et al. 2009).

IL-17A mediated recruitment of neutrophil may play an important role in the elimination of pneumococcal carriage from nasopharynx (Lu et al. 2008). Clearance of pneumococcal carriage in mice vaccinated with a whole cell vaccine was associated with a heightened IL-17A response. Significant induction of IL-17A secretion was also observed in human adenotonsillar MNC culture following pneumococcal stimulation. Moreover, bacterial phagocytosis by human polymorphonuclear cells was enhanced when pre-incubated with IL-17A (Lu et al. 2008). Patients suffering from a genetic disorder (autosomal dominant hyper IgE syndrome or HIES), with impaired IL-17 response are more susceptible to mucosal infections by several extracellular bacteria including *Streptococcus pneumoniae (Milner et al. 2008).* These suggest a crucial role by Th17 cells in natural immunity to pneumococci.

Th17 cells secrete a number of cytokines including IL-17A, IL-17F, IL-21 and IL-22. IL-22 has been described as an important mediator of host mucosal immunity to invading pathogens. It plays a crucial role on maintaining the integrity of epithelial barriers at different mucosal sites (Aujla and Kolls 2009). Mice treated with anti-IL-22 antibodies showed decreased survival during bacterial infection of respiratory tract (Aujla et al. 2008). Although it is a member of Th17 cytokine family, it can act independently and may not co-express with IL-17A (Lundgren et al. 2012). Pneumococcal whole cell antigen (WCA) and pneumolysoid (PdT) have been shown to induce both IL-17A and IL-22, which seems to be originated from the effector/memory ($CD45RO^{+}$) T cells in a MHC-II dependent mechanism (Lundgren et al. 2012). Besides pneumococcal WCA and PdT, several other pneumococcal proteins; such as, PcsB (protein required for cell separation in group B streptococci), StkP (serine/threonine protein kinase) and PsaA (pneumococcal surface adhesin A) have also been shown to stimulate Th17 immunity in humans (Schmid et al. 2011).

Pneumococcal pilus-1 proteins play an important role in virulence by facilitating adherence to host respiratory epithelium (Barocchi et al. 2006). It has been reported that presence of pilus locus is associated with pneumococcal invasiveness (Aguiar et al. 2008). Expression of pneumococcal pilus-1 (RrgA and RrgB) proteins by pneumococcus was shown to be associated with a heightened host inflammatory response (Barocchi et al. 2006). We have observed higher serum antibody levels to RrgA and RrgB in pneumococcal culture-negative children than in those who were culture-positives, indicating that these antibodies may contribute to protection against carriage.

Pneumococcus frequently colonises human nasopharynx, but evasion of local mucosal immune system is relatively rare. An episode of colonisation, either

transient or persistent, is likely to prime local mucosal immune system (NALT) in the nasopharynx. Adenotonsillar tissues, as important components of human NALT, may play an important role in protection against pneumococcal carriage. Human adenotonsillar MNCs contain a large numbers of naturally developed T and B cells to pneumococcal protein antigens (Zhang et al. 2007, Zhang et al. 2006b). In this study, we have investigated $CD4^+$ T cell proliferative responses and Th17 related cytokine production in both adenotonsillar mononuclear cells (MNC) and peripheral blood mononuclear cells (PBMC) after stimulation with recombinant RrgA and RrgB proteins and pneumococcal CCS (containing or lacking these proteins).

6.2 Aims of Study

To investigate the $CD4^+$ T cell and Th17 Cell responses to pneumococcal RrgA and RrgB antigens in human adenotonsillar MNC and PBMC.

6.3 Experimental Design

1. Adenotonsillar MNC and PBMC were stimulated by purified recombinant RrgA and RrgB antigens followed by analysis of T cell proliferation. These cells were also stimulated by pneumococcal culture supernatants derived from TIGR4 wild type pneumococcus (containing RrgA and RrgB) or the isogenic mutants lacking RrgA or RrgB followed by analysis of T cell proliferation.
2. Th17 cell response following stimulation with pneumococcal CCS derived from TIGR4wt and its isogenic $RrgB^{-/-}$ and $RrgA^{-/-}$ mutant strains was evaluated by measurement of IL-17A, IL-17F and IL-22 by ELISA.

6.3.1 Patients and samples

Surgically removed adenotonsillar tissues were obtained from both children and adults (1–30 years). A nasopharyngeal swab from each patient was obtained on the same day of operation. Patients who were known to be immunocompromised or had

received antibiotics or steroids three weeks before surgery were excluded. The study was approved by the local ethics committee (Liverpool Paediatric Research Ethics Committee). An informed written consent was obtained from each patient/guardian as appropriate.

6.3.2 Recombinant RrgA and RrgB Antigens

Purified His-tagged recombinant RrgA and RrgB proteins from *Streptococcus pneumoniae* serotype 4 TIGR4 strain (obtained from Novartis Vaccines, Sienna, Italy) (Gianfaldoni et al. 2007) were used for stimulation of adenotonsillar MNC and PBMC.

6.3.3 Pneumococcal Culture Supernatant

Pneumococcal CCS were produced from a pilus expressing *Streptococcus pneumoniae* serotype 4 (TIGR4) wild type (Tettelin et al. 2001) and its isogenic $RrgA^{-/-}$ and $RrgB^{-/-}$ mutants (Hilleringmann et al. 2009) following methods discussed in chapter-2. Both adenotonsillar MNC and PBMC were stimulated with these CCS to estimate the $CD4^{+}$ T cell proliferative response and Th17 cytokine responses. Use of pneumococcal CCS to stimulate cell proliferation and cytokine induction has a major limitation. Although, the presence or absence of pilus-1 (RrgA and RrgB) proteins was documented by Western Blot analysis, these CCS contain other components, some of which might also be immunogenic. The effects of those components on the cell proliferation index and cytokine production remain undetermined. Therefore, the interpretation of the data obtained with pneumococcal CCS stimulations has to take this limitation into considerations and the inferences drawn from this would be less conclusive when compared to the T cell proliferation and cytokine responses after stimulations with purified recombinant proteins.

6.3.4 Cell culture and stimulation by recombinant pilus-1 antigens and pneumococcal CCS

Adenotonsillar MNC ($4x10^6$/ml) and PBMC ($2x10^6$/ml) were cultured at in RPMI-1640 culture medium with or without recombinant (RrgA and RrgB) proteins (0.5µg/ml) and pneumococcal (TIGR4wt, $RrgB^{-/-}$ and $RrgA^{-/-}$) CCS (1µg/ml). For analysing $CD4^+$ T cell proliferative responses, cells were incubated (at 37°C, in 5% CO_2) for 5 days before FACS analysis. To measure the Th17 cytokine production with *in vitro* CCS stimulation, cell culture supernatants were collected after 72 hours and stored at –70°C before analysed by ELISA.

6.3.5 Analysis of $CD4^+$ T cell proliferation

T cell proliferation was analysed by CFSE staining followed by flow cytometry. Memory and naive T cell responses were analysed following naïve ($CD45RA^+$) T cell depletion and memory ($CD45RO^+$) T cell depletion respectively from tonsillar MNC using magnetic (MACS) cell sorting described in details in chapter-2. Briefly, adenotonsillar MNC and PBMC were labelled with CFSE (at 37°C, for 8 min) and the reaction was quenched with ice cold media. After washing, cells were resuspended in RPMI 1640 media and stimulated with recombinant pilus-1 antigens or pneumococcal CCS, and incubated (at 37°C, in 5% CO_2) for 5 days. Then the cells were harvested, washed and resuspended in 0.02% BSA in PBS for surface staining with phycoerythrin (PE) conjugated anti-CD4 antibody. $CD4^+$ T cell proliferation index was analysed by flow cytometry (BD FACS Calibre; Becton Dickinson). The data was analysed using WinMDI software version2.9. The gating strategy for $CD4^+$ T cell proliferation index is presented in figure 6.3.5.

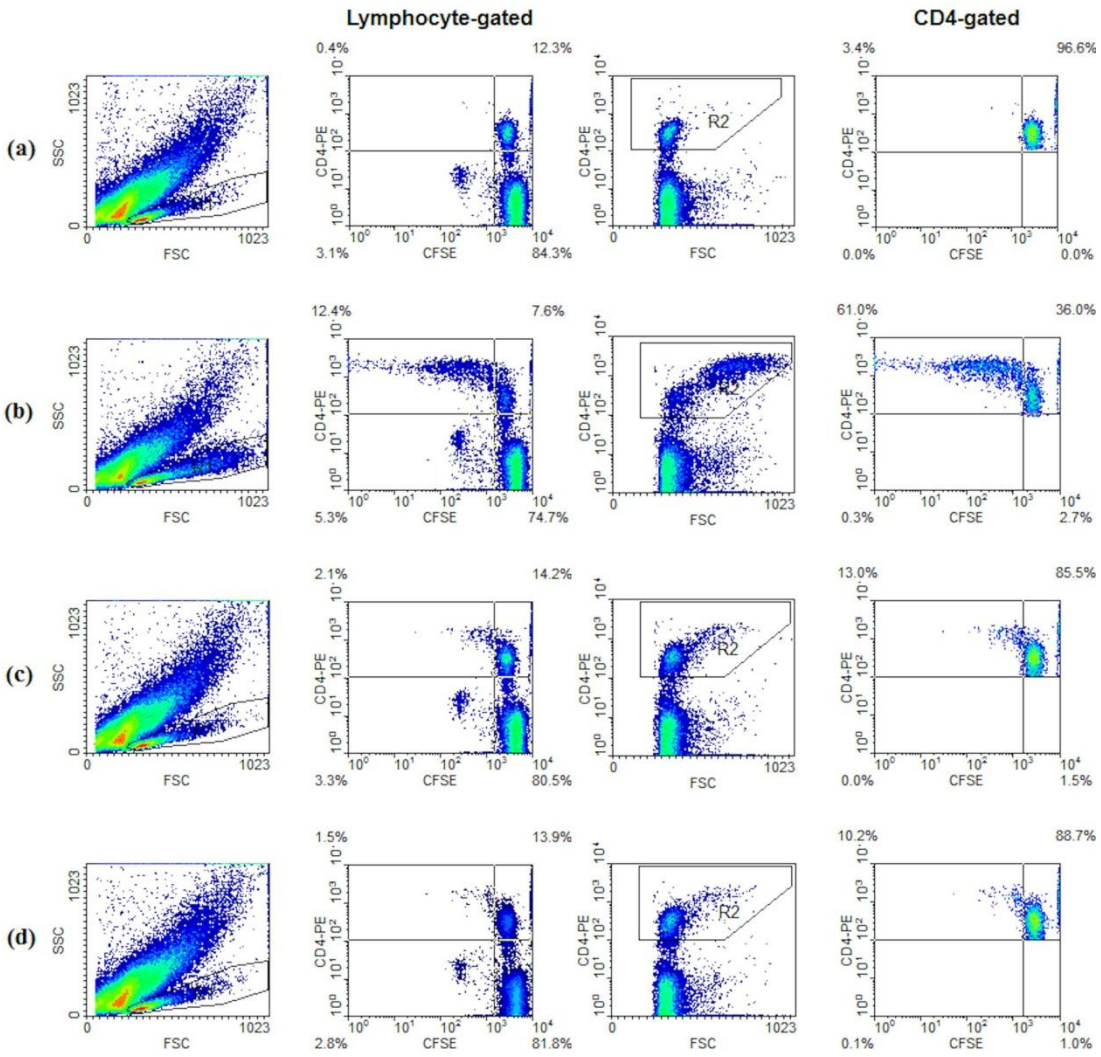


Figure-6.3.5: The gating strategy for CD4$^+$ T cell proliferation index. Lymphocyte and CD4$^+$ T cell gating were used for this analysis. Lymphocytes were gated on the basis of their forward scatter (FSC) and side scatter (SSC) properties (size and granularity). Lymphocytes were gated on region 1 (R1) (Figure-6.3.5). Within R1, CD4$^+$ T cells were gated on region 2 (R2) (Figure 6.3.5) based on CD4-PE staining plotted against the forward scatter. Finally, R2-gated CD4$^+$ T cells plotted as CD4-PE staining on y axis against CFSE-labelling on x axis. The CD4$^+$ T cell proliferative index was calculated as the percentage of proliferating CD4$^+$ T cell of total CD4$^+$ T cells. Figure shows an example of CD4$^+$ T cell proliferative index in (a) unstimulated medium control (b) anti-CD3 antibody stimulated positive control (c) stimulated with recombinant RrgA and (d) and RrgB antigens.

6.3.6 Phenotyping of CD4$^+$ T helper (Th1/Th2/Th17) cells

Phenotyping of different $CD4^+$ T helper cell subsets induced by recombinant pilus-1 antigens (rRrgA and rRrgB) and pneumococcal TIGR4wt CCS in PBMC and adenotonsillar MNC were performed with Human Th1/Th2/Th17 Phenotyping kit (BD Bioscience, UK), by a method described in chapter-2. Briefly, cell suspensions were incubated at 37°C for 24 hours in 5% CO_2. A protein transport inhibitor 'monensin' (BD GolgiStop™) was then added and incubated for another 6 hours. The cells were harvested, washed and fixed with 4% paraformaldehyde (BD Cytofix™). The cells were permeabilised with (Perm/Wash™) before the addition of 20µl fluorochrome-labeled antibody cocktail (CD4 PerCP-cy5.5, IL-17A PE, IFN-γ FITC and IL-4 APC) for surface staining of $CD4^+$ T cells and for detection of intracellular cytokine production, followed by flowcytometry (BD Biosciences) using CFlow sampler software.

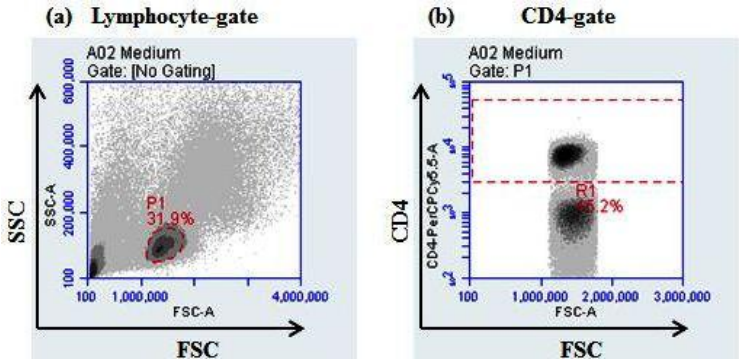


Figure-6.3.6a+b: The gating strategy for $CD4^+$ T cells. (a) Lymphocytes were gated on the basis of their forward scatter (FSC) and side scatter (SSC) properties on plot 1 (P1); (b) $CD4^+$ T cells were gated on region 1 (R1) based on the properties of CD4 PerCP-Cy5.5 staining plotted against the forward scatter.

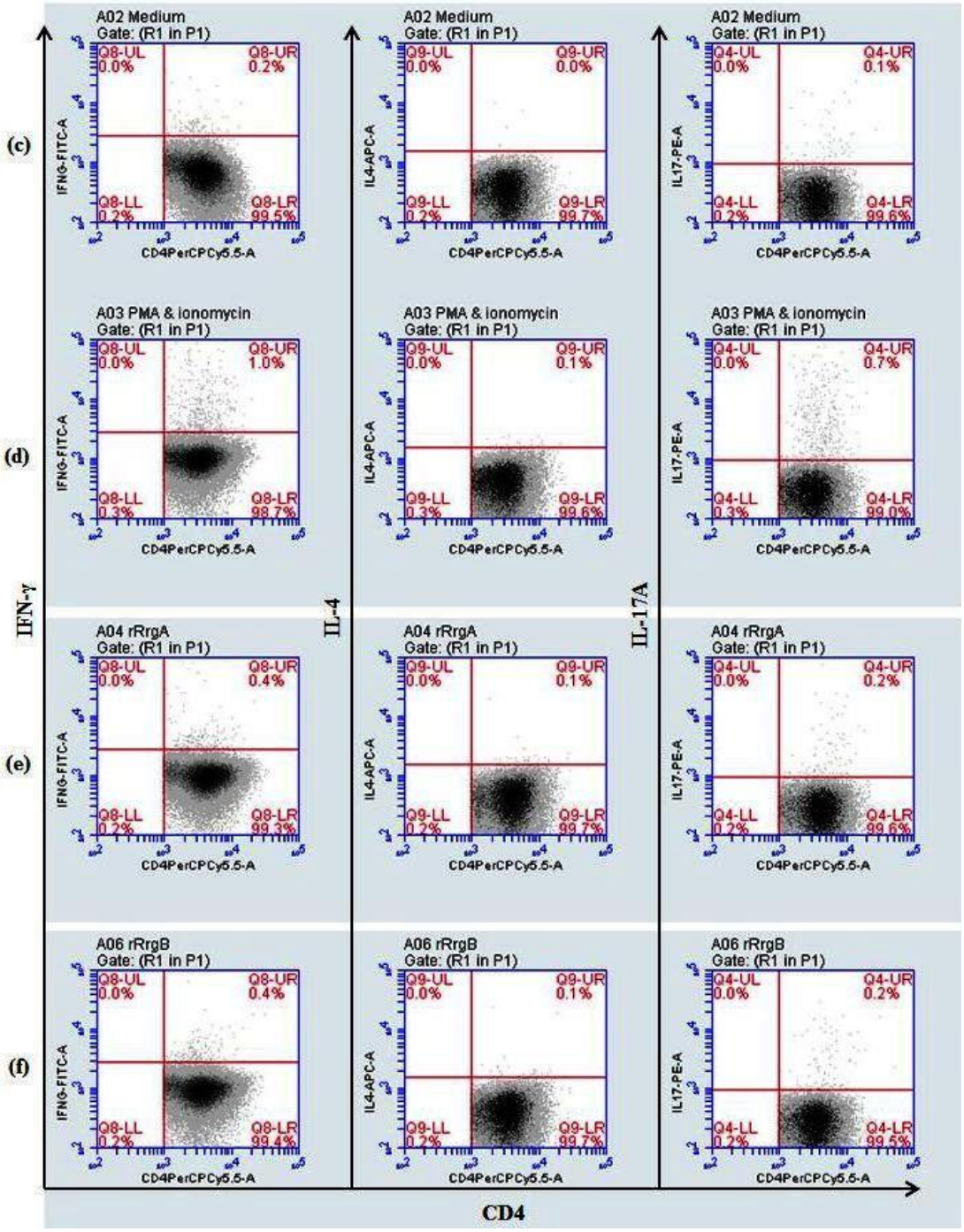


Figure-6.3.6c-f: Intracellular cytokine staining for IFN-γ, IL-4 and IL-17**.** R1-gated $CD4^+$ T cells plotted as CD4-PerCP-Cy5.5 staining on the 'X' axis and IFN-γ FITC, IL-4 APC, IL-17A PE staining on the 'Y' axis. The Th1/Th2/Th17 phenotyping of the $CD4^+$ T cells were calculated as the percentage of IFN-γ, IL-4 and IL-17A expressing cells (in the upper right quadrants) in the CD4 gated (R1 in figure-6.3.6b) population of T cells. Figure shows example of a representative adenotonsillar sample in (c) unstimulated medium control, and stimulated with (d) PMA and ionomycin (e) recombinant RrgA (f) RrgB antigens.

6.3.7 Measurement of Th17 (IL-17A, IL-17F and IL-22) cytokines in the adenotonsillar MNC and PBMC culture supernatants by ELISA

In vitro production of Th17 cytokines (IL-17A, IL-17F and IL-22) in adenotonsillar MNC and PBMC culture supernatants were measured by Human IL-17A, IL-17F and IL-22 Ready-Set-Go® ELISA sets (eBioscience, UK), following manufacturer's instructions. The detailed procedures are described in chapter 2. Briefly, ELISA plates were coated with the capture antibody for overnight incubation. After blocking, standards (total of 8, starting from 500 pg/ml by 2-fold serial dilutions) and diluted samples (1:20) were added and incubated for 2h at RT. After washing, detection antibody was added and incubated for 1h at RT. Avidin-HRP was then added and incubated for 30min at RT, followed by the addition of TMB substrate solution for 15min at RT in dark. The reaction was stopped with 1M H_2SO_4 before the absorbance was measured using a microtiter plate reader (Opsys MR, Thermo labsystems, UK) at 450nm. The cytokine concentration (pg/ml) was calculated against the standard curve, with the help of microplate analysis software DeltasoftPC (Biometallics, Inc., USA).

6.3.8 Statistical analysis

At first the data were tested for normality with D'Agostino and Pearson omnibus normality test. Further analyses of the data that did not pass the normality test were done by nonparametric (Wilcoxon matched-pairs signed rank) test; and the data that pass the normality test were analysed by parametric (paired t) test, when the responses between different stimulations were compared. A p value of <0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism (version 5).

6.4 Results

6.4.1 Optimum dose of recombinant pilus-1 antigens and TIGR4wt CCS for $CD4^+$ T cell stimulation

Doses of pneumococcal recombinant pilus-1 antigens (rRrgA and rRrgB) and TIGR4wt CCS were optimised for analysis of $CD4^+$ T cell proliferative response in adenotonsillar MNC (n=6). Four doses (0.1, 0.5, 1.0 and 2.0 µg/ml) of recombinant antigens and three doses (0.1, 0.5 and 1.0 µg/ml) of TIGR4wt CCS were used. The optimum response for rRrgA was at 0.5 µg/ml stimulation, and for rRrgB which was (0.1µg/ml). The strongest response with TIGR4wt CCS showed observed at a dose of 1.0 µg/ml (Figure-6.4.1).

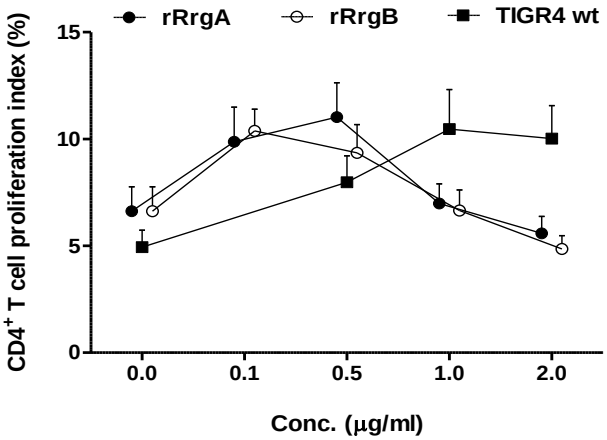


Figure-6.4.1: Dose-response curve for $CD4^+$ T cell stimulation with pneumococcal TIGR4wt CCS and recombinant RrgA and RrgB antigens in NALT (n=6). Optimum concentration for rRrgA (filled circle) was 0.5 µg/ml and for rRrgB (open circle) was 0.1 µg/ml. Pneumococcal TIGR4wt CCS (filled square) showed strongest response at 1.0 µg/ml dose. The medium control is designated as 0 µg/ml stimulation.

6.4.2 Induction of CD4$^+$ T cell proliferation with pneumococcal recombinant pilus-1 antigens

To investigate whether pneumococcal pilus-1 antigens induce CD4$^+$ T cell proliferation in human adenotonsillar tissue, CFSE-labelled tonsillar cells were stimulated with recombinant RrgA (rRrgA) and RrgB (rRrgB) antigens and analysed by FACS. Possibly due to some pre-activated lymphocytes in human NALT, a high background proliferation was observed in some unstimulated MNC. For this reason, we depleted pre-activated (CD69$^+$) T cells from the adenotonsillar MNC followed by cell stimulation. This cell population retained CD45RO$^+$ (memory) T cells representing a memory type of response in the adenotonsillar MNC. A significant CD4$^+$ T cell proliferative response was observed in the CD69$^+$ depleted adenotonsillar cells, following stimulation with both rRrgA and rRrgB (figure-6.4.2a: n=11, p=0.0020 and 0.0262 respectively, in Wilcoxon matched-pairs signed rank test).

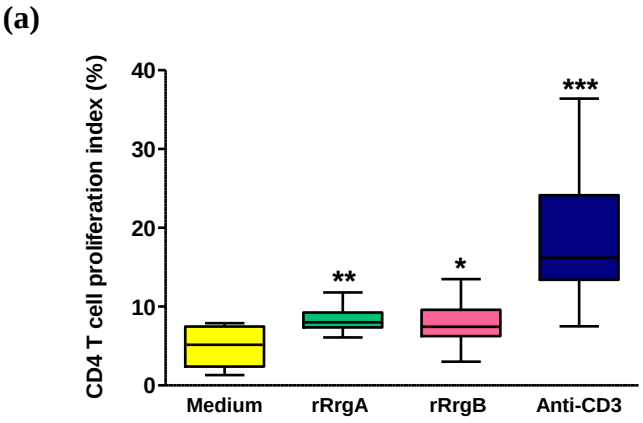


Figure-6.4.2a: CD4$^+$ T cell proliferative response in the CD69$^+$ depleted adenotonsillar MNC after stimulation with recombinant pilus-1 (rRrgA and rRrgB) antigens (n=11). Data represent the median with interquartile range for percentage of CD4$^+$ T cell proliferation. Anti-CD3 stimulations used as positive control. Significant induction (*p<0.05, **p<0.01 and ***p<0.001) with different stimulations was compared with medium controls (yellow bars).

To investigate the naive $CD4^+$ T cell response adenotonsillar MNC, samples were analysed after magnetic depletion of memory ($CD45RO^+$) T cell population. Stimulation of these memory ($CD45RO^+$) T cell-depleted MNC with recombinant rRrgA and rRrgB also induced a T cell proliferative response (figure-6.4.2b: n=21, p=0.0023 and 0.0066 respectively, in Wilcoxon matched-pairs signed rank test).

(b)

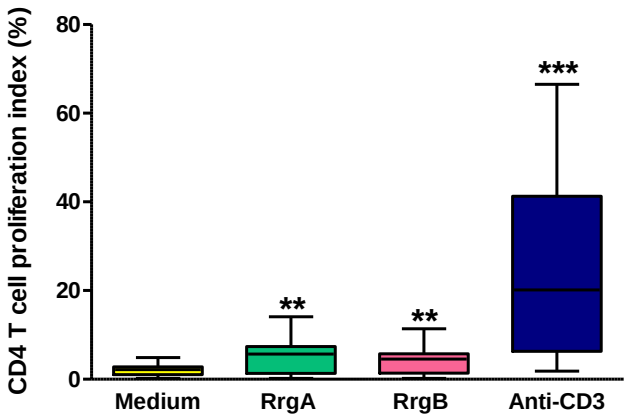


Figure-6.4.2b: Proliferative response in the naïve ($CD45RO^+$ depleted) $CD4^+$ T cell populations in adenotonsillar MNC after stimulation with recombinant pilus-1 (rRrgA and rRrgB) antigens (n=21). Data represent the median with interquartile range for percentage of $CD4^+$ T cell proliferation. Significant induction (**p<0.01) with different stimulations was compared with medium controls (yellow bars).

6.4.3 CD4$^+$ T cell proliferation with pneumococcal CCS stimulation

Stimulation with pneumococcal CCS derived from TIGR4wt and its isogenic (RrgB$^{-/-}$ and RrgA$^{-/-}$) mutants induced a significant CD4$^+$ T cell proliferation in the undepleted adenotonsillar MNC (figure-6.4.3: n=24, p<0.0001, 0.0002 and 0.0039 in Wilcoxon matched-pairs signed rank test). The CD4$^+$ T cell proliferative response elicited by CCS derived from the RrgB$^{-/-}$ and RrgA$^{-/-}$ mutants appeared to be lower than that by TIGR4wt CCS stimulation (figure-6.4.3: n=24, p=0.0009 and <0.0001 respectively, in Wilcoxon matched-pairs signed rank test).

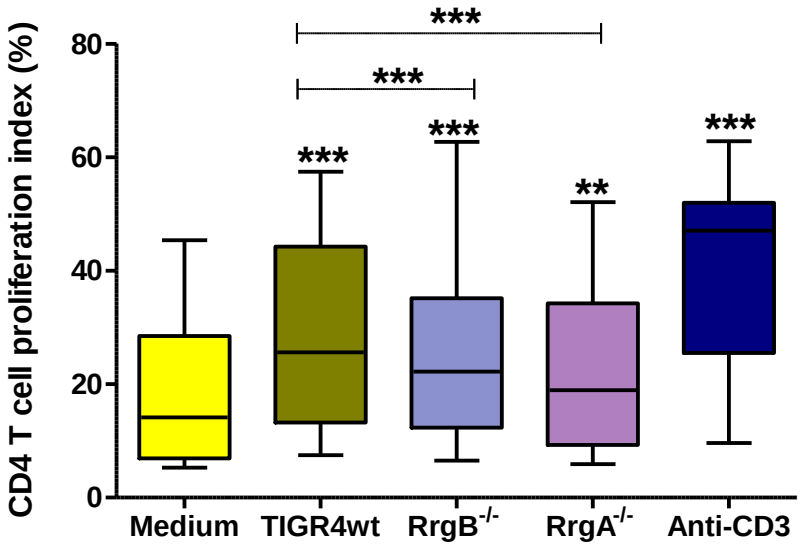


Figure-6.4.3: CD4$^+$ T cell proliferative response in the adenotonsillar MNC after stimulation with pneumococcal (TIGR4wt, RrgB$^{-/-}$ and RrgA$^{-/-}$) CCS (n=24). Data represent the median with interquartile range for percentage of CD4$^+$ T cell proliferation. Significant induction (**p<0.01 and ***p<0.001) with different stimulations was compared with medium controls (yellow bars). Significantly higher proliferation (***p<0.001) was also observed in samples stimulated with TIGR4wt CCS compared to the stimulations with mutant (RrgB$^{-/-}$ and RrgA$^{-/-}$) CCS.

6.4.4 Recombinant pilus-1 antigens do not induce CD4$^+$ T cell proliferation in human PBMC

To investigate whether pneumococcal recombinant pilus-1 antigens could induce proliferative response in CD4$^+$ T cell in human peripheral blood, we analysed some PBMC samples (n=12) after rRrgA and rRrgB stimulation. No significant induction of CD4$^+$ T cell proliferation was found following either rRrgA or rRrgB stimulation (figure-6.4.3: n=12, *p*=0.0977 and 0.3394 respectively in Wilcoxon matched-pairs signed rank test).

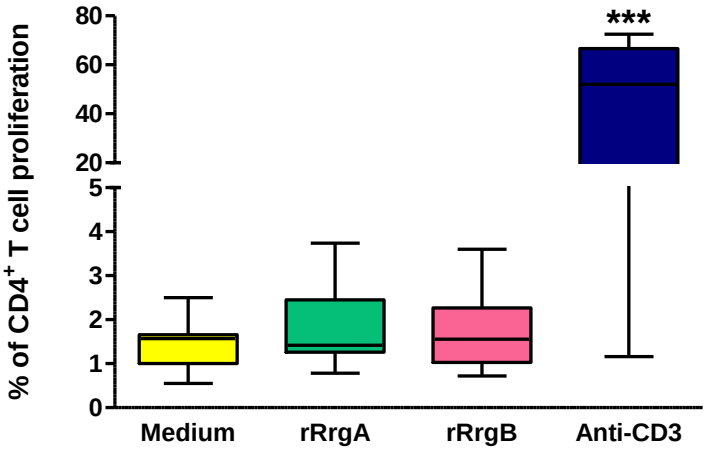


Figure-6.4.4: CD4$^+$ T cell proliferative response in the peripheral blood mononuclear cells (PBMC) after stimulation with recombinant pilus-1 (rRrgA and rRrgB) antigens (n=12). Data represent the median with interquartile range for percentage of CD4$^+$ T cell proliferation. Induction of proliferative response with different stimulations was compared with medium control (yellow bars). No significant induction (p>0.05) was observed with either rRrgA or rRrgB stimulations.

6.4.5 Phenotyping (Th1/Th2/Th17) of adenotonsillar CD4$^+$ T cells stimulated by pneumococcal pilus-1 antigens and CCS

To investigate which phenotypes of CD4$^+$ T cells were induced in adenotonsillar MNC by pneumococcal pilus-1 antigens, intracellular cytokine staining of IFN-γ, IL-4 and IL-17A was performed following rRrgA and rRrgB stimulation. There was no marked induction of individual cytokine expressing (IFN-γ+, IL-4+ and IL-17A+) CD4$^+$ T cells after stimulation with the recombinant antigens compared to the medium control (figure-6.4.5a: n=9, p=0.177, 0.1336 and 0.0978 for rRrgA and p=0.1052, 0.1995 and 0.1078 for rRrgB respectively in paired t test).

(a)

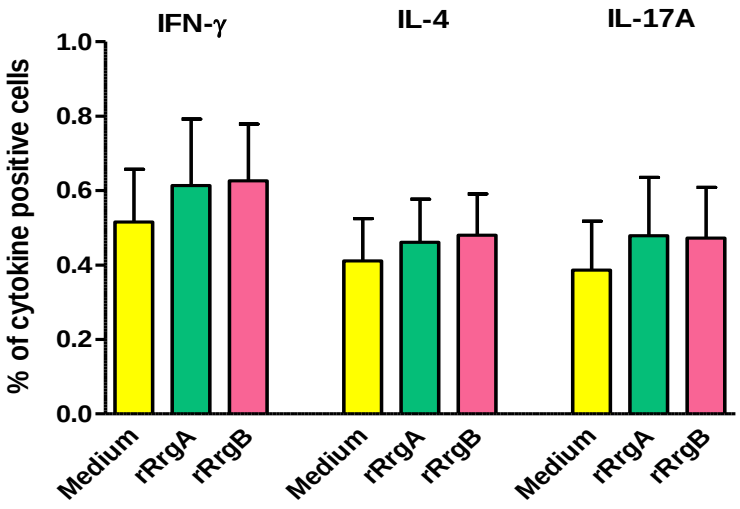


Figure-6.4.5 a: Phenotyping (Th1/Th2/Th17) of CD4$^+$ T cells based on the expression of their signature cytokines (IFN-γ, IL-4 and IL-17A) after stimulation with recombinant rRrgA and rRrgB antigens in human adenotonsillar MNC. Data represent the mean±SEM (error bars) of percentage of CD4$^+$ T cells (n=9) expressing theses cytokines. No significant induction (p>0.05) was observed for either rRrgA or rRrgB stimulations when compared to medium control (yellow bars).

However, stimulation with pneumococcal TIGR4wt CCS markedly induced IFN-γ+, IL-4^{+} and IL-17A^{+} cells compared to unstimulated control (figure-6.4.5b: n=9, *p*=0.0129, 0.0152 and 0.0199 respectively in paired t test). For this reason, the subsequent experiments for cytokine induction were carried out with TIGR4wt and its isogenic RrgB$^{-/-}$ and RrgA$^{-/-}$ mutant strains.

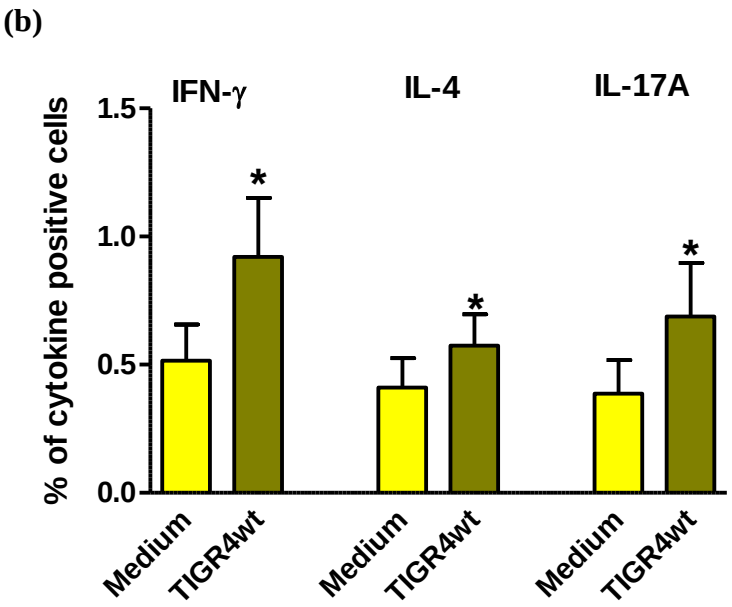


Figure-6.4.5b: Phenotyping (Th1/Th2/Th17) of CD4^{+} T cells based on the expression of their signature cytokines (IFN-γ, IL-4 and IL-17A) after TIGR4wt CCS stimulation in human adenotonsillar MNC. Data represent the mean±SEM (error bars) of percentage of CD4^{+} T cells expressing theses cytokines. Significant induction (*p<0.05) was observed with CCS stimulation compared to the medium control (yellow bars).

6.4.6 Time-course of Th17 cytokines production in adenotonsillar MNC culture

Induction of *in vitro* production of Th17 cytokines (IL-17A, IL-17F and IL-22) in human adenotonsillar MNC culture after stimulation with pneumococcal TIGR4wt CCS was analysed in culture supernatants collected at different time points(6, 24, 48, 72 and 96 hours interval) to standardise the optimum duration of stimulation before collection of culture supernatants. For all of the three Th17 cytokines (IL-17A, IL-17F and IL-22) the maximum concentration of cytokine was measured after 72 hours stimulation (figure-6.4.8). For all of the subsequent experiments, supernatants from adenotonsillar MNC and PBMC culture were collected after 72 hours of stimulation.

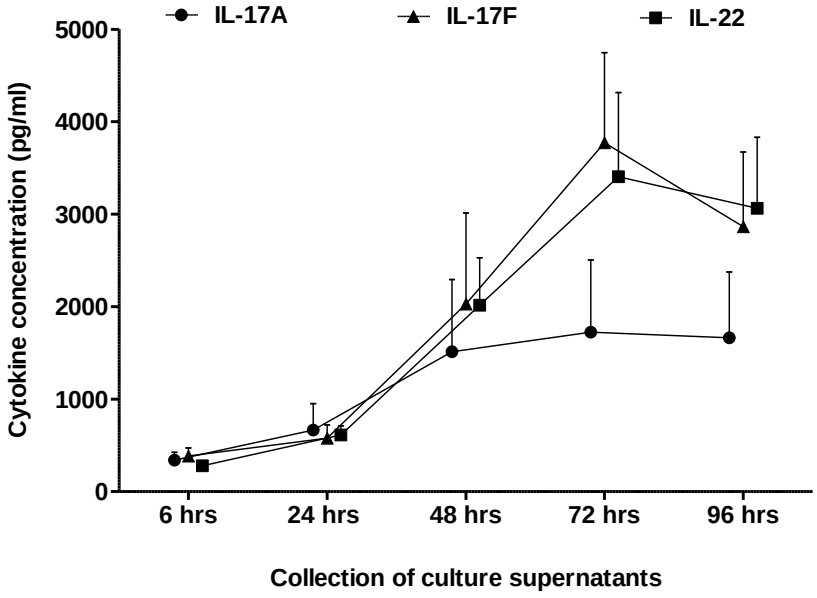


Figure-6.4.6: Time course of *in vitro* Th17 cytokines (IL-17A, IL-17F and IL-22) production in TIGR4wt CCS stimulated adenotonsillar MNC culture supernatants. Data represent the mean±SEM (error bars) of cytokine levels (pg/ml) measured by ELISA. All three cytokines showed maximum concentration after 72 hours of incubation

6.4.7 Induction of Th17 cytokines in adenotonsillar MNC with pneumococcal TIGR4wt CCS stimulation is stronger than stimulation with CCS derived from RrgB$^{-/-}$ and RrgA$^{-/-}$ mutants

Induction of Th17 cytokines (IL-17A, IL-17F and IL-22) was analysed in adenotonsillar MNC culture supernatants, 72 hours after stimulation with pneumococcal TIGR4wt and its isogenic (RrgB$^{-/-}$ and RrgA$^{-/-}$) mutants CCS.

Production of IL-17A was shown to be higher with TIGR4wt CCS stimulation than that induced by CCS derived from its isogenic RrgB$^{-/-}$ or RrgA$^{-/-}$ mutant strains (figure-6.4.7a: n=22, p=0.0007 and <0.0001 respectively, in Wilcoxon matched-pairs signed rank test).

Similarly, stronger IL-17F production was observed after stimulation with TIGR4wt CCS compared to stimulations with either RrgB$^{-/-}$ or RrgA$^{-/-}$ mutant strains (figure-6.4.7b: n=22, p=0.0008 and 0.0074 respectively, in Wilcoxon matched-pairs signed rank test).

TIGR4wt CCS also induced a higher IL-22 production in adenotonsillar MNC culture supernatants than RrgB$^{-/-}$ or RrgA$^{-/-}$ mutant strains (figure-6.4.7c: n=22, p=0.0123 and 0.0054 respectively, in Wilcoxon matched-pairs signed rank test).

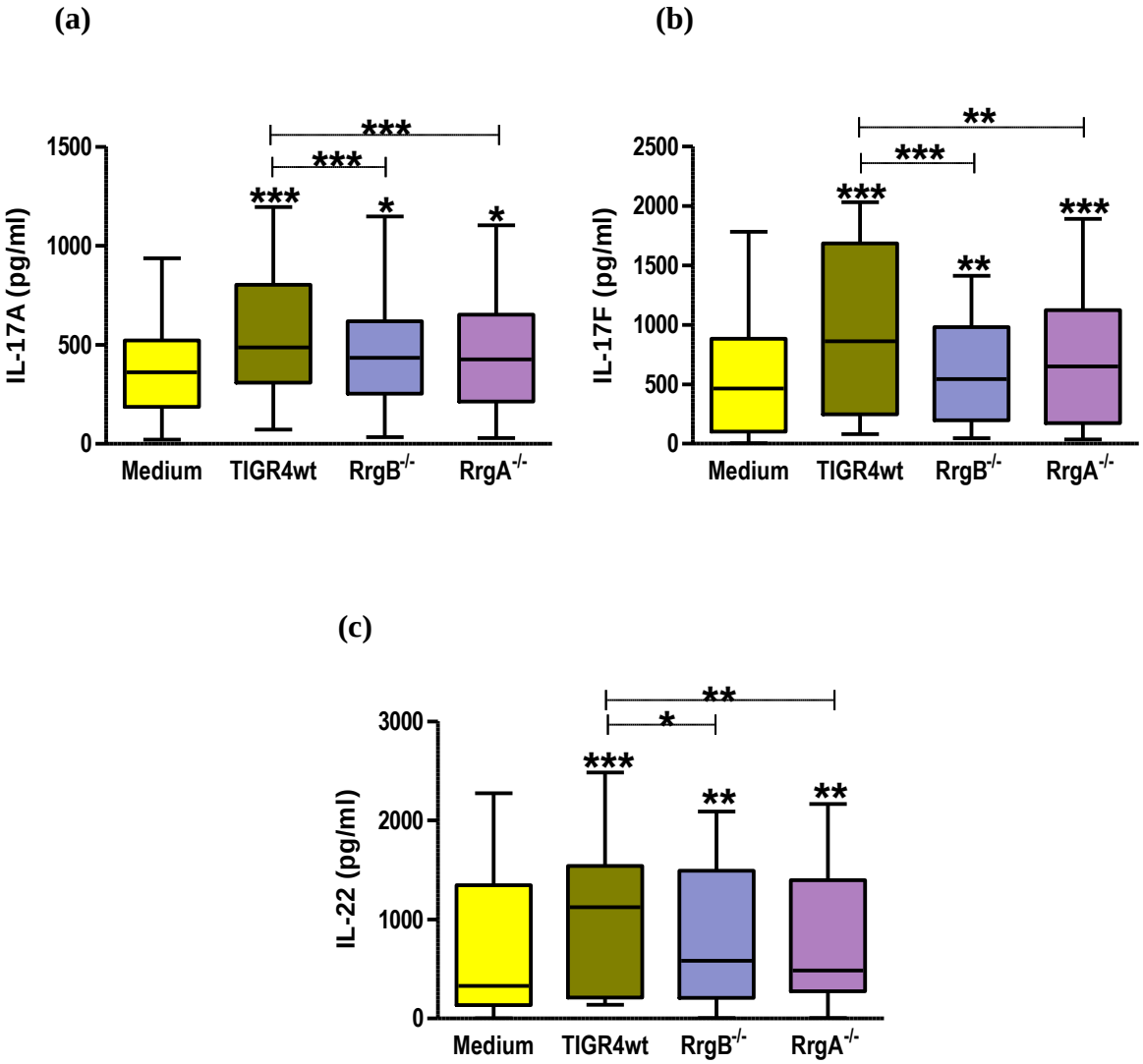


Figure-6.4.7 a-c: Induction of Th17 cytokines in adenotonsillar MNC culture supernatants; (a) production of IL-17A, (b) production of IL-17F and (c) production of IL-22 72 hours after stimulation with TIGR4wt, RrgB$^{-/-}$ and RrgA$^{-/-}$ CCS. Data represent the median with interquartile range of cytokine levels (pg/ml) measured by ELISA. Significant induction of cytokine production (*p<0.05, **p<0.01, ***p<0.001) with different stimulations was compared with medium control (open bars). Also the cytokine production after stimulation with CCS derived from TIGR4wt was compared with that of RrgB$^{-/-}$ and RrgA$^{-/-}$ mutants and the significant differences (*p<0.05, **p<0.01, ***p<0.001) are shown in the graphs.

6.4.8 Induction of Th17 cytokines in PBMC with pneumococcal TIGR4wt CCS stimulation is stronger than stimulation with CCS derived from RrgB$^{-/-}$ and RrgA$^{-/-}$ mutants

Induction of Th17 cytokines (IL-17A, IL-17F and IL-22) was analysed in the culture supernatants of PBMC, stimulated for 72 hours with pneumococcal TIGR4wt and its isogenic (RrgB$^{-/-}$ and RrgA$^{-/-}$) mutants CCS.

Production of IL-17A was shown to be higher with TIGR4wt CCS stimulation than that induced by CCS derived from its isogenic RrgB$^{-/-}$ or RrgA$^{-/-}$ mutant strains (figure-6.4.8a: n=21, p=0.0178 and 0.0054 respectively, in Wilcoxon matched-pairs signed rank test).

Similarly, stronger IL-17F production was observed after stimulation with TIGR4wt CCS compared to stimulations with either RrgB$^{-/-}$ or RrgA$^{-/-}$ mutant strains (figure-6.4.8b: n=21, p=0.0130 and 0.0145 respectively, in Wilcoxon matched-pairs signed rank test).

TIGR4wt CCS also induced a higher IL-22 production in culture supernatants of PBMC than RrgB$^{-/-}$ or RrgA$^{-/-}$ mutant strains (figure-6.4.8c: n=22, p=0.0136 and 0.0181 respectively, in Wilcoxon matched-pairs signed rank test).

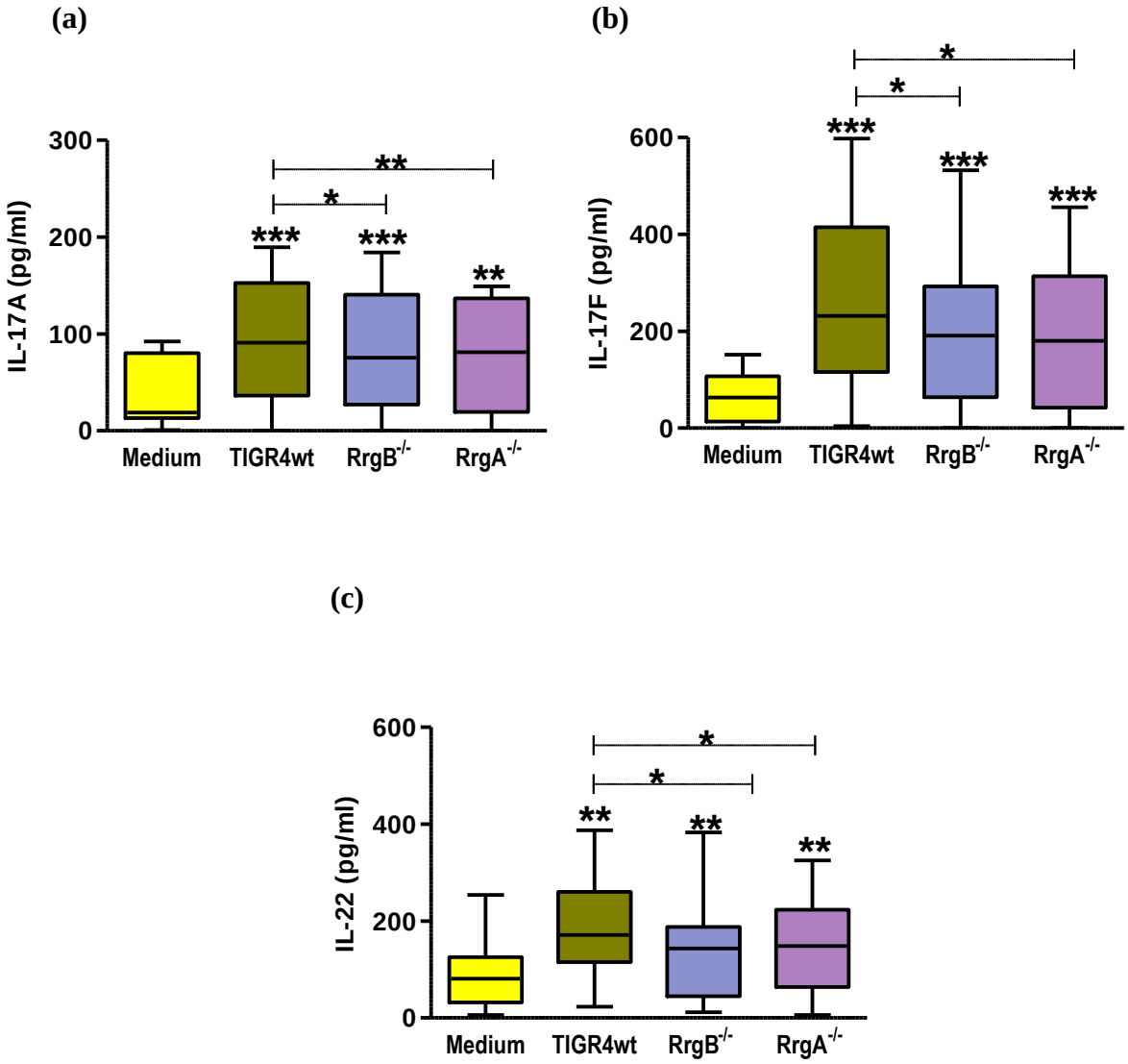


Figure-6.4.8 a-c: Induction of Th17 cytokines in PBMC culture supernatants; (a) production of IL-17A, (b) IL-17F and (c) IL-22 72 hours after stimulation with TIGR4wt, RrgB$^{-/-}$ and RrgA$^{-/-}$ CCS. Data represent the median with interquartile range of cytokine levels (pg/ml) measured by ELISA. Significant induction of cytokine production (**p<0.01 and ***p<0.001) with different stimulations was compared with medium control (open bars). Also the cytokine production after stimulation with CCS derived from TIGR4wt was compared with that of RrgB$^{-/-}$ and RrgA$^{-/-}$ mutants and the significant differences (*p<0.05 and **p<0.01) are shown in the graphs.

6.5 Discussion

The present study has revealed that pneumococcal pilus-1 proteins, RrgA and RrgB are able to induce $CD4^+$ T cell proliferative response in human adenotonsillar MNCs. We have previously shown pneumococcal CCS containing RrgA and RrgB induces *in vitro* antibody production in human NALT. The ability to induce a $CD4^+$ T cell response may augment the B cell antibody production in NALT. Our results also suggest that native form of these pilus-1 antigens may induce Th17 related cytokine response in human adenotonsillar MNCs.

Previous studies showed several pneumococcal protein antigens including pneumolysin, PspA, CbpA, PsaA are capable of inducing $CD4^+$ T cellular immune response in human (Basset et al. 2007a, Mureithi et al. 2009, Zhang et al. 2007). Among them, pneumolysin have been shown to induce both primary and memory $CD4^+$ T proliferative response in human NALT and PBMC, which may modulate nasopharyngeal colonisation of pneumococcus (Zhang et al. 2007). This study revealed that both RrgA and RrgB antigens were also able to stimulate a $CD4^+$ T cell proliferative response in adenotonsillar MNC. A small $CD4^+$ T cell response was observed in $CD45RO^+$ cell depleted adenotonsillar MNC following stimulation with both rRrgA and rRrgB (figure-6.4.2b). No significant response was observed following stimulation of PBMC (figure-6.4.4). However, we have observed a stronger proliferative response of $CD4^+$ T cells in adenotonsillar MNC following stimulation with pneumococcal CCS. The finding that CCS derived from isogenic of $RrgB^{-/-}$ and $RrgA^{-/-}$ mutant strains induced a smaller response than with the wild type (TIGR4wt) suggests that these two antigens in native form may induce significant activation of $CD4^+$ T cells that help antibody production by B cells

In human nasopharynx, pneumococcal colonisation may prime for antigen-specific $CD4^+$ T cell memory to pneumococcal proteins leading to the development of a protective T cell immunity against carriage (Zhang et al. 2007). Colonisation by a pilus-1 expressing strain of pneumococci is likely to prime for RrgA- and RrgB-specific memory $CD4^+$ T cells in human adenotonsillar tissues, which would be activated by subsequent antigenic stimulation. This could help to explain the $CD4^+$ T cell response in tonsillar MNC observed following stimulation with recombinant rRrgA and rRrgB antigens, which is likely to represent a memory response.

Recently, immune response mediated by IL-17A secreted by Th17 cells has been implicated as the key protective mechanism against pneumococcal infections (Malley et al. 2006). IL-17A mediated recruitment of phagocytic cells plays an important role in the clearance of both primary and secondary bacterial colonisation in the nasopharynx (Zhang et al. 2009). A number of pneumococcal proteins have been identified, which are capable of inducing Th17 mediated protective responses (Moffitt et al. 2012). Apart from IL-17A, Th17 cells secrete a number of other cytokines, like IL-17F and IL-22, which also play an important role in the mucosal immune response against extracellular bacteria like pneumococcus (Aujla and Kolls 2009).

This study has revealed that TIGR4wt CCS (containing RrgA and RrgB) induced significant production of Th17-related cytokines including IL-17A, IL-17F and IL-22. The finding that CCS derived from $RrgA^{-/-}$ and $RrgB^{-/-}$ mutants induced less production of these cytokines suggests that these pilus proteins may contribute to the induction of Th17 related cytokines in both adenotonsillar MNC and PBMC. A similar pattern of response was observed for both IL-17F and IL-22. The protective role of IL-22 at the different mucosal sites including respiratory tract is well

documented (Aujla and Kolls 2009). Significant production of IL-22 following stimulation with pneumococcal CCS supports previous report that pneumococcal protein antigens are capable of inducing this cytokine (Lundgren et al. 2012).

Significant production of IL-17F in adenotonsillar cells following stimulation by pneumococcal CCS was also observed. IL-17F is a structural homologue of IL-17A, which has also been reported to contribute on the protective responses against invading microbial pathogens. Like IL-17A, it recruits neutrophils and induce secretion of anti-microbial peptides (Kawaguchi et al. 2004). However, it may have lesser contribution than IL-17A into the pathogenesis of autoimmune diseases (Ishigame et al. 2009). This may be explained by their differential receptor (IL-17RA or IL-17RC) distribution pattern, and the diversity of gene expression programs of the immune effector cells (Dubin and Kolls 2009).

Relatively higher production of IL-17F and IL-22 than IL-17A was observed in both NALT and peripheral blood following stimulations with pneumococcal CCS. So far, no information is available regarding their relative importance in the protection against pneumococcal infection. Induction of IL-22 has been reported to be independent of the IL-17A induction (Lundgren et al. 2012). It has been reported that APCs, like dendritic cells and macrophages also secretes IL-22 (Dubin and Kolls 2008). It is possible that the higher level of IL-22 in both NALT and PBMC might result from a contribution by other cell types than $CD4^+$ T cells. Both IL-17A and IL-17F have been shown to contribute onto the mucosal immune response against extracellular bacteria. However, IL-17A has been reported to be more critically involved in the development of autoimmune reactions (Dubin and Kolls 2009).

While several reports of IL-17A mediated protection against pneumococcal infection are available (Malley et al. 2006, Lu et al. 2008, Moffitt et al. 2012); there is limited data regarding the specific protective role played by IL-17F. Both IL-17A and IL-17F have shown to induce IL-6, CCL3 and G-CSF by peritoneal macrophages. In addition, IL-17A was shown to induce several proinflammatory cytokines such as IL-1β, IL-12p70, and GM-CSF. In $CD4^+$ T cells, IL-17A have been shown to induce CCL2, CCL3, GM-CSF, IL-1β, and IL-9, which were not induced by IL-17F (Dubin and Kolls 2009). This suggests that production of different Th17 related cytokines (IL-17A or IL-17F) may be linked to their post-secretion cytokine activation profile.

The magnitude of Th17 cytokine response in NALT was higher than that observed in PBMC. Th17 related cytokine response following pneumococcal stimulation has been shown to be originating from the effector/memory T cell population (Lundgren et al. 2012). The adenotonsillar cells possess higher number of memory cell than the peripheral blood, and therefore generate a strong Th17 (IL-17A, IL-17F, IL-22) cytokine response.

In conclusion, our results suggest that pneumococcal pilus proteins RrgA and RrgB proteins are capable of activating $CD4^+$ T cells including Th17 cells in human nasopharynx-associated lymphoid tissue (NALT). *In vitro* production of Th-17 cytokines in cell culture supernatants after stimulation with pneumococcal CCS containing both of these proteins (TIGR4wt) was significantly higher than that with isogenic ($RrgA^{-/-}$ and $RrgB^{-/-}$) mutants, suggesting a contribution from these proteins. The ability of these pilus-1 proteins to induce a $CD4^+$ T cell response including Th17 response may contribute to the natural immunity developed with age in humans.

Chapter 7

General Discussion and Conclusion

7.1 Discussion

Streptococcus pneumoniae is an important human pathogen that accounts for significant morbidity and mortality around the world. The disease burden is particularly high in the developing countries (O'Brien et al. 2009). The vulnerability is highest in children younger than five years and in the elderly over sixty five (Örtqvist et al. 2005). Natural immunity to pneumococcus was believed to be mediated by the antibody responses to the capsular polysaccharides; which were targeted for development of vaccine against pneumococcus (Weinberger et al. 2008).

So far, the available vaccines are prepared from capsular polysaccharides. The efficacy of these vaccines is limited to the serotypes included in the formulation. These polysaccharide vaccines induce a T cell independent antibody response and therefore, not effective in young children less than 2 years (Bogaert et al. 2004b). Although conjugate polysaccharide vaccines are effective in these children, the serotype coverage is limited (Singleton et al. 2007). Also these conjugate vaccines are too expensive to be affordable for routine use in the developing countries, where they are needed most. For these reasons, development of suitable protein vaccines remains a priority in pneumococcal vaccine research (Tai 2006). A number of proteins expressed by pneumococci have been identified as potential vaccine candidate, and studies in animal models and humans have revealed their protective efficacy (Gámez and Hammerschmidt 2012; Bologa et al. 2012; Kamtchoua et al. 2013).

Studies in experimental mice have shown that pneumococcal pilus-1 proteins, RrgA and RrgB are good immunogens (Gianfaldoni et al. 2007; Nelson et al. 2007; Harfouche et al. 2012). This PhD study investigated the immunogenicity of these two antigens in humans. Significant levels of serum anti-RrgA and RrgB antibodies

were detected in both children and adults (figure-4.4.3a), which presumably derived from previous colonization by *S. pneumoniae*. Furthermore, serum anti-RrgA antibody titres was found to be higher in children who were culture-negative than in those who were culture-positive for *S. pneumoniae* in their nasopharynx (figure-4.4.7.1). Similarly, anti-RrgB antibody titres were also higher in culture-negative than in culture-positive children older than 6 years (figure-4.4.7.2). These results suggest a possible protective immune response by pneumococcal pilus-1 proteins against pneumococcal carriage or new colonization in humans.

S. pneumoniae is a frequent colonizer of the mucosa in the nasopharynx of young children, as shown in this study that over 50% of children aged under 3 years were carrying *S. pneumoniae* (table-3.4.1). Colonization of the nasopharynx by this bacterium is considered to be an essential first step for pneumococcal infection. The high carriage rate in young children may explain why they are prone to some common infections such as pneumonia. As is the case for many mucosal pathogens, colonization may not only precede pneumococcal infections but also serves as a prerequisite for the spread of the organism within the community (Bogaert et al. 2004a). Reduction of carriage through vaccination may therefore be an effective way of preventing pneumococcal disease.

Serum anti-RrgA IgG antibody levels were found to rise earlier in young children than anti-RrgB antibody, which suggests that RrgA protein may be a strong immunogen in early childhood (figure-4.4.3a-c). It is possible that RrgA interacts more closely with the mucosal immune system while facilitating the adhesion of *S. pneumoniae* to nasopharyngeal mucosa. A stronger *in vitro* production of anti-RrgA compared to anti-RrgB antibodies in adenotonsillar MNC following pneumococcal stimulation (figure-5.4.3a+b) further supports the notion that RrgA protein may be a strong immunogen in children.

The presence or absence of anti-RrgA and -RrgB antibodies shown by ELISA was further supported and confirmed by Western blotting, and there was a good correlation in the antibody levels detected by the two methods (figure-4.4.5a-c). Based on both methods, about 50-60% of the serum samples in this study were positive for anti-RrgA and -RrgB antibodies (figure-4.4.5.3). This suggests that pilus-expressing pneumococcal strains may frequently colonize children during childhood. Among the strains isolated from this study, all of the pilus-I expressing isolates were from young children <6 years. The overall percentage of pilus-1 expressing pneumococcal carriage isolates in this study was 11% (figure-3.4.2), which was lower than that reported in previous studies (25% to 50%) (Aguiar et al. 2008; Moschioni et al. 2010a; Basset et al. 2007b). This lower percentage of pilus-expressing strains may be associated with the recent introduction of pneumococcal conjugate vaccines in the UK, as reported previously in the US (Regev-Yochay et al. 2010). The conjugate vaccine serotypes were reported more likely to carry *rrgC* gene than the non-vaccine serotypes (Basset et al. 2007b). It was reported that the prevalence of pilus-1 positive strains dropped from 25% to 15% after 3 years of introduction of conjugate vaccination, although it was shown to rise again after 7 years which was attributed to replacement by non-vaccine serotypes (Regev-Yochay et al. 2010). It was shown previously that pneumococcal colonization could be a dynamic process, with most young children (<2 years) colonized serially with single or multiple serotypes of *S. pneumoniae* (Syrjänen et al. 2001). So it is possible that many children in this study had been exposed to pilus-expressing strains previously.

We observed that both serum anti-RrgA and anti-RrgB antibody levels increased with age in children until they reach adolescence (figure-4.4.3b+c). This trend was seen in antibodies to a number of pneumococcal protein antigens including pneumolysin and choline-binding proteins (Laine et al. 2004). Using a general linear

model of analysis of variance, we showed that anti-RrgA antibody titres appeared to have an age-independent association with pneumococcal carriage, in that the difference in antibody titres between carriage negative and culture positive children existed in all age groups, although for anti-RrgB antibody this difference was only shown in older children.

As shown previously from studies of immune response to other pneumococcal proteins including CbpA and pneumolysin (Zhang et al. 2006b), IgG1 was the predominant immunoglobulin subclass of anti-RrgA and -RrgB antibodies (figure-4.4.4a+b). This is typical of antibody responses to protein antigens, with a predominance of IgG1 followed by IgG3 and minimal levels of IgG2 and IgG4. Both IgG1 and IgG3 are known to be capable of complement activation and opsonisation of *S. pneumoniae*, potentially leading to its clearance from nasopharynx.

Salivary IgG antibodies to both RrgA and RrgB antigens were also detected, and again, there was a difference in these salivary IgG antibody levels between pneumococcal culture-negative and culture-positive patients, with higher titres in the former (figure 4.4.8.1). The higher salivary IgG titres in non-colonised patients support our hypothesis that it may contribute to protection against pneumococcal colonization at the mucosal level. A good correlation between the serum and salivary IgG antibodies for both anti-RrgA and anti-RrgB (figure 4.4.8a+b) suggests that the salivary IgG antibodies were mostly derived from serum leakage (Zhang et al. 2000), although local mucosal immune tissue NALT could also contribute to this local pool of antibodies(Zhang et al. 2006b, Ivarsson et al. 2004).

On the other hand, salivary secretory IgA antibodies to both RrgA and RrgB were shown to be relatively higher in culture-positive than culture-negative children

(figure 4.4.8.2). This increase of IgA in colonised patients could be explained by the fact that they were secreted locally induced by the current colonisation. These locally produced secretory IgA antibodies may contribute to protection against epithelial penetration by *S. pneumoniae* to cause invasive disease.

Considering that RrgA acts as a pneumococcal adhesin, it may be a good mucosal immunogen and induce local immune response in the nasopharynx. In this study, we show that stimulation with a wild type (TIGR4) pneumococcal culture supernatant (containing both RrgA and RrgB proteins) induced a significant anti-RrgA antibody response (~6.8 fold-increase) in adenotonsillar cells (figure 5.4.3a). An anti-RrgB antibody response was also induced following stimulation, although at a lower (~2.9 fold) magnitude (figure 5.4.3b). Stimulation with CCS derived from isogenic mutant ($RrgB^{-/-}$ and $RrgA^{-/-}$) strains failed to induce antibody responses against respective (lacking) antigens; but retained the ability to induce antibody production against the other proteins (RrgA and RrgB respectively). Production of these antigen-specific antibodies were mostly contributed by the memory ($CD45RO^{+}$) B cell, as their depletion markedly reduced both anti-RrgA and -RrgB levels (Figure-5.4.3c+d).

This *in vitro* stimulation of adenotonsillar cells was shown to induce a higher anti-RrgA and anti-RrgB antibody response in culture-positive than culture-negative patients (figure 5.4.8a+b). This result is consistent with our previous findings of higher *in vitro* antibody production to some other protein antigens (CbpA and pneumolysin) in culture-positive children (Zhang et al. 2006b). It is possible that a recent or current colonization in the host boosted the antigen-specific memory B cell pool in the NALT, and upon antigen stimulation *in vitro*, an enhanced memory response was elicited. Both systemic and local mucosal responses may contribute to the regulation of acquisition and elimination of pneumococcal carriage in the nasopharynx, and may exert their effects independently. High antecedent levels of

antibodies in serum may help prevent colonization, and enhanced memory-type local immune responses in NALT following re-exposure may help terminate it (Zhang et al. 2006b).

It has been shown that $CD4^+$ T cells mediated immunity is important for clearance of pneumococcus from the nasopharynx, which is the natural colonisation site (Kadioglu et al. 2004, Malley et al. 2005). Several pneumococcal proteins have been shown to induce $CD4^+$ T cellular immune response in humans (Basset et al. 2007a, Mureithi et al. 2009, Zhang et al. 2007). Pneumolysin was able to induce both primary and memory $CD4^+$ T proliferative response in human NALT and PBMC (Zhang et al. 2007). Here, we observed that both RrgA and RrgB antigens were also able to stimulate a $CD4^+$ T cell proliferative response in adenotonsillar MNC (figure-6.4.2a); but no significant response was observed following stimulation of PBMC (figure-6.4.4). We also observed a stronger proliferative response of $CD4^+$ T cells in adenotonsillar MNC following stimulation with pneumococcal TIGR4wt CCS compared to that of CCS derived from its isogenic $RrgB^{-/-}$ and $RrgA^{-/-}$ mutant strains (figure-6.4.3). It suggests that the native form of both RrgA and RrgB may aid in the antibody production by activating $CD4^+$ helper T cells.

Th17 cells contribute to the innate and adaptive cellular immunity against pneumococci by recruiting phagocytes (Malley et al. 2006; Zhang et al. 2009; Lu et al. 2008). Th17 cells secrete a number of cytokines, including IL-17A, IL-17F and IL-22. These cytokines have been shown to play a crucial role in the mucosal immune response against extracellular bacteria like pneumococcus (Aujla and Kolls 2009). Several pneumococcal proteins have been shown to generate Th17 mediated protective responses (Moffitt et al. 2012).

In this study, significant production of Th17-related cytokines (IL-17A, IL-17F and IL-22) was observed following stimulation with pneumococcal CCS (figure-6.4.7a-c and figure-6.4.8a-c). Notably, stimulation with TIGR4wt CCS (containing RrgA and RrgB) induces significantly higher production of Th17 cytokines compared to that of CCS derived from $RrgA^{-/-}$ and $RrgB^{-/-}$ mutants. This suggests that these pilus-1 proteins may contribute to the induction of Th17 related cytokines in both adenotonsillar MNC and PBMC.

Production of IL-17F and IL-22 following stimulations with pneumococcal CCS was relatively higher than IL-17A production in both NALT and peripheral blood (figure-6.4.7a-c and figure-6.4.8a-c). There are several reports of IL-17A mediated protection against pneumococcal infection (Malley et al. 2006, Lu et al. 2008, Moffitt et al. 2012). But limited information is available regarding the relative importance of different Th17 related cytokines (like IL-17A, IL-17F and Il-22) in the protection against pneumococcal infection. Induction of IL-22 production following stimulation with pneumococcal antigens has been shown (Lundgren et al. 2012). The contribution of IL-17F in the mucosal immune response against other extracellular bacteria has also been reported (Dubin and Kolls 2009).

This study has revealed that both of these pilus-1 proteins, RrgA and RrgB are immunogenic and, and are capable of priming for memory B and T cell response in human NALT. Immunogenicity of the adhesin RrgA has been found to be developing earlier in the childhood. Moreover, antibodies to one of the clades of RrgA have been reported to be cross- protective against both clades (Moschioni et al. 2010). On the other hand, lack of cross-protection by antibodies to different clades of RrgB might be overcome by using a fusion of three clades (RrgB321) (Harfouche et al. 2012). However, considering the low prevalence of pilus-1 among the colonising pneumococcal isolates, the inclusion of these RrgA or RrgB proteins as a vaccine

component needs to be critically evaluated. Particularly, with the availability of PCV13 which seems to cover all of the serotypes associated with pilus-1 and pilus-2 (Kulohoma et al. 2013), a vaccine formulation with their component proteins is unlikely to offer any extra advantage. Moreover, due to strong competition in the development of pneumococcal protein vaccines, pilus-1 components would likely to become underdog compared to some more conserved and better immunogenic proteins, such as pneumolysin, PspA, CbpA and PhtD (Bologa et al. 2012; Darrieux et al. 2013; Kamtchoua et al. 2013; Seiberling et al. 2012). However, in the context of rapidly changing perspectives in this field the information regarding the immunogenicity of RrgA and RrgB should always be valuable.

7.2 Conclusion

In the present study we detected significant level of serum antibodies to pilus RrgA and RrgB proteins in children and adults. An important finding of this work was higher antibody levels in carriage-negative than in carriage-positive children. These results suggest pneumococcal pilus antigens, especially RrgA are capable of inducing significant antibody response in children that may contribute to protection against pneumococcal carriage. The study also revealed that pneumococcal pilus-1 proteins, RrgA and RrgB are able to induce CD4+ T cell proliferative response in human adenotonsillar MNCs, which may augment the antibody production in NALT. Our results also suggest that native form of these pilus-1 antigens may induce Th17 related cytokine response in human adenotonsillar MNCs. In conclusion, these findings add significant information to our understanding on the natural immunity to pilus-1 proteins.

Chapter 8

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Chapter 9

Appendices

Appendix –I

Preparation of different media and buffers

Skim milk, Tryptone, Glucose, Glycerol Transport Medium (STGG)

Skim-milk tryptone glucose glycerol (STGG) transport medium was prepared using the following procedure.

1. In a sterile 100 ml glass bottle, 40 ml 10% milk was prepared by adding 4 gram skimmed milk powder (Oxoid, UK) into 40 ml distilled water.
2. The bottle was autoclaved in 121°C for 5 minutes with the cap loosened one turn.
3. After autoclaving, the cap was tightened and taken into a bio-safety cabinet.
4. In another sterile 500 ml glass bottle, 6 ml Tryptone soya broth (Oxoid, UK), 2.2 ml 45% D-Glucose solution (Sigma Aldrich, UK), 20 ml Glycerol (Sigma Aldrich, UK) were taken.
5. 131.8 ml distilled water was added into the bottle to make the final volume 160 ml.
6. The bottle was shaken gently to dissolve all ingredients.
7. Then bottle was autoclaved in 121°C for 10 minutes with the cap loosened one turn.
8. After that, the cap was tightened and taken into a bio-safety cabinet.
9. Then the previously prepared sterile 10% skimmed milk (40 ml) was added into the 500 ml bottle to make 200 ml 2% skimmed milk medium.
10. The milk was mixed thoroughly with the other contents by gentle shaking.
11. Aliquot of 1.0 ml media was prepared into 1.5 ml screw-capped vials.
12. The tubes were then kept in a refrigerator (2-8°C) until used for nasal swab inoculation.
13. Quality control test for sterility of the STGG medium was performed periodically by plating a full loop of a homogenized vial from each lot onto a blood agar plate, then incubating the plate at 37°C overnight.
14. The lot was discarded if growth of any organism was suspected.

Todd-Hewitt-Yeast Broth (THYB)

Todd-Hewitt Broth with 0.5% yeast extract was prepared using the following procedure.

1. 36.4 gram of Todd-Hewitt Broth (Oxoid, Basingstoke, UK) and 5 gram of yeast extract were taken into a sterile 1 litre glass bottle.
2. 1000 ml of distilled water was added to the bottle and mixed thoroughly, until all components were dissolved completely.
3. The bottle was autoclaved at 115°C for 10 minutes with the cap loosened one turn.
4. After autoclaving, the bottle cap was closed tightly and then kept in the cold room until used for growing bacteria.

Laemmli Reducing Buffer

10.0 ml Laemmli reducing Buffer for western Blot sample dilution was prepared using following recipe-

0.5 M Tris pH 6.8	1.25 ml
10% (w/v) SDS	2.0 ml
0.5% Bromphenol Blue	0.20 ml
Glycerol	2.50 ml
Deionized H_2O	3.55 ml
β-mercaptoethanol	0.50 ml (50 µl for each 0.95 ml)

10x Tris Buffered Saline (TBS) p^H 7.4

1. 1 Litre 10x TBS was prepared in a sterile 1 litre glass bottle, using following recipe-

Tris	30 gm
NaCl	80 gm
KCl	2 gm
Deionized H_2O	900 ml

2. Then p^H was adjusted to 7.4 by adding concentrated HCl drop by drop, with gentle shaking of the bottle and simultaneous measurement with a p^H meter. When the p^H is adjusted then the bottle was topped-up to 1 litre by adding deionized water.

Western Blot Washing Buffer (TBS-T)

1 litre of Western Blot washing buffer (1xTBS with 0.05% Tween20) was prepared in a sterile glass bottle, using following recipe-

10x TBS	100 ml
Deionized H_2O	900 ml
Tween-20	0.5 ml

Western Blot Blocking Buffer (5% skimmed milk in TBS-T)

1. 500 ml of Western Blot blocking buffer (5% skimmed milk in TBS-T) was prepared in a sterile glass bottle, with a magnetic stirrer bead inside, using following recipe-

Skimmed milk	25 gram
10x TBS	50 ml
Deionized H_2O	450 ml
Tween-20	0.25 ml

2. The bottle was kept on a magnetic stirrer for allowing the proper dissolving of the milk.

10x Phosphate Buffered Saline (PBS) p^H 7.4

1. 1 litre of 10x PBS was prepared in a sterile 1 litre glass bottle, using following recipe-

Na_2HPO_4	14.4 gm
KH_2PO_4	2.4 gm
NaCl	80 gm
KCl	2 gm
Deionized H_2O	900 ml

2. Then p^H was adjusted to 7.4 by adding concentrated HCl drop by drop, with gentle shaking of the bottle and simultaneous measurement with a p^H meter. When the p^H is adjusted then the bottle was topped-up to 1 litre by adding deionized water.

ELISA/ELISpot Washing Buffer (PBS-T)

1 litre of ELISA/ELISpot washing buffer (1xPBS with 0.05% Tween20) was prepared in a sterile glass bottle, using following recipe-

10x PBS	100 ml
Deionized H_2O	900 ml
Tween-20	0.5 ml

P-Nitrophenyl Phosphate (PNPP) Substrate for ELISA

1. 1 litre of p-nitrophenyl phosphate (PNPP) substrate (1M diethanolamine) buffer (p^H 9.8) was prepared in a sterile glass bottle, using following recipe-

Diethanolamine	97 ml
Deionized H_2O	800 ml
$MgCl_2$	100mg

2. Then the bottle was placed on a magnetic stirrer with a magnetic flea inside. Whilst stirring the p^H was measured and adjusted to 9.8 by adding 10M hydrochloric acid.
3. Once the pH is correctly adjusted, the buffer was transferred to a measuring cylinder and distilled water was added to give a final volume of 1000 ml.
4. Thereafter, the buffer was transferred back to the bottle and stored at 4˚C.
5. Finally, the substrate was prepared by dissolving p-nitrophenyl phosphate disodium salt (5mg) tablet into 5 ml of substrate buffer to give a PNPP concentration of 1 mg/ml.

Acetate buffer for AEC Substrate

1. 1 litre of 50 mM acetate buffer (p^H 5.0) was prepared in a sterile glass bottle, using following recipe-
2. 0.2M Acetic acid solution was prepared by adding 1.16 ml glacial acetic acid into 100 ml deionized H_2O.
3. 0.2M Sodium acetate solution was prepared by adding 6.8 gm sodium acetate into 250 ml deionized H_2O.
4. Then, the acetate buffer was prepared by adding 74 ml of 0.2M acetic acid solution, 176 ml of 0.2M sodium acetate solution and 700 ml of deionized H_2O.
5. Thereafter, the bottle was placed on a magnetic stirrer with a magnetic flea inside. Whilst stirring the p^H was measured and adjusted to 5.0 by adding glacial acetic acid.
6. Once the pH is correctly adjusted, the buffer was transferred to a measuring cylinder and distilled water was added to give a final volume of 1000 ml.
7. Finally, the buffer was transferred back to the bottle and stored at 4˚C.

3-Amino-9-ethylcarbazole (AEC) Substrate for ELISpot

1. AEC Substrate was prepared using following recipe-
2. 2.5 gm of 3-Amino-9-ethylcarbazole was added into 250 ml of dimethyl formamide (DMF) solution and kept in dark at 4°C (refrigerator).
3. Finally, the working substrate solution for the ELISpot assay was prepared by adding 0.5 ml of AEC substrate into 9.5 ml of acetate buffer; and 25 µl of H_2O_2 immediately before dispensing into the plate.

Appendix –II

List of antibodies/chemicals/reagents/consumables

A. Antibodies/kits	**Manufacturer**	**Catalogue no**	**Lot/batch no**
Anti-human IgA-Alkaline phosphatase (α chain specific, produced in goat)	Sigma Aldrich, UK	A9669	048K4869
Anti-human IgG $(Fab)_2$ (produced in goat)	Sigma Aldrich, UK	I3266	109K4786
Anti-human IgG1 (hinge)-AP (clone: 4E3, produced in mouse)	Southern Biotech, USA	9052-04	J3809-S520B
Anti-human IgG2 (FC)-AP (clone: 317-4, produced in mouse)	Southern Biotech, USA	9060-04	J3709-ZC09C
Anti-human IgG3 -AP (clone: HP6050, produced in mouse)	Southern Biotech, USA	9210-04	D610-PM40B
Anti-human IgG4-AP (clone: HP6025, produced in mouse)	Southern Biotech, USA	9200-04	C8010-QK20C
Anti-human IgG-Alkaline phosphatase (clone: GG-5, produced in mouse)	Sigma Aldrich, UK	A2064	011M4757
Anti-human IgM-Alkaline phosphatase (clone: MB-11, produced in mouse)	Sigma Aldrich, UK	A2189	107K4861
Anti-human secretory component (IgA) (clone: GA-1, produced in mouse)	Sigma Aldrich, UK	I6635	101M4772
Anti-mouse IgG-Alkaline phosphatase (produced in goat)	Sigma Aldrich, UK	A3562	079K6034
Anti-rabbit IgG FITC conjugate (produced in goat)	Sigma Aldrich, UK	F0382	060M6052
Anti-rabbit IgG-HRP (produced in donkey)	Santa Cruz Biotech, Germany	SC2077	K0810
CD45RO microbeads (mouse anti-human IgG2a)	Myltenyi Biotech, Germany	130-046-001	5130527257
CD69 microbead kit II Anti-human CD69-Biotin (isotype: mouse IgG1) Anti-Biotin microbeads	Myltenyi Biotech, Germany	130-092-355	5110704223
Goat anti-human IgA $(Fab)_2$ Biotin	Invitrogen, USA	AHI 1109	816042A
Goat anti-human IgG (H+L) Biotin	Invitrogen, USA	81-7140	730149A

Goat anti-human IgM $(Fab)_2$ Biotin	Invitrogen, USA	AHI 1609	445320A
Human CD-4 PE (clone: RPAT4)	BD Biosciences, UK	555347	2219543
Human CD-4 PE-Cy7 (clone: SK3)	BD Biosciences, UK	557852	2237922
Human IFN-γ AlexaFluor488 (clone:B27)	BD Biosciences, UK	557718	42448
Human IL-17A PE (clone: N49-653)	BD Biosciences, UK	560486	41725
Human IL-17F AlexaFluor488 (clone:O33-782)	BD Biosciences, UK	561331	2251561
Human IL-17F AlexaFluor647 (clone:O33-782)	BD Biosciences, UK	561333	21883
Human IL-22 AlexaFluor660 (clone:22URTI)	eBioscience, UK	50-7229-42	E12178-1630
Human IL-4 APC (clone: 8D4-8)	BD Biosciences, UK	560671	26947
Human Th1/Th2/Th17 phenotyping Kit CD4 PerCP-Cy5.5 (clone: SK3) IFN-γ FITC (clone:B27) IL-17A PE (clone: N49-653) IL-4 APC (clone: MP4-25D2)	BD Biosciences, UK	51-9006615	2178901
Human total IgA standard (purified IgA from human colostrum)	Sigma Aldrich, UK	I2636	021M4831
Human total IgG standard (purified IgG from normal sera)	Sigma Aldrich, UK	I2511	100M4830
Human total IgM standard (purified IgM from normal sera)	Sigma Aldrich, UK	I8260	050M4751
Polyclonal rabbit anti-human IgA	Dako, Denmark	A0262	00054730
Polyclonal rabbit anti-human IgM	Dako, Denmark	A0425	00055979
Ready set go ELISA kit Human IL-17A (homodimer)	eBioscience, UK	88-7176-88	E09433-1635
Ready set go ELISA kit Human IL-17F (homodimer)	eBioscience, UK	88-7478-88	E11356-1633
Ready set go ELISA kit Human IL-22 (homodimer)	eBioscience, UK	88-7522-88	E11112-1634

B. Chemicals/reagents	**Manufacturer**	**Catalogue no.**
3-Amino-9-ethylcarbazole (AEC)	Sigma Aldrich, UK	A5754
Agar	Sigma Aldrich, UK	A1296
Agarose	Sigma Aldrich, UK	A9539
Amphotericin B	Sigma Aldrich, UK	A2942
Biomix red (PCR reaction mixture)	Bioline Ltd, UK	BIO-25005
Blood agar base	Oxoid Ltd, UK	CM0055B
Bovine serum albumin (BSA)	Sigma Aldrich, UK	A8327
Bradford protein dye reagent	Sigma Aldrich, UK	B6916
Brefeldin A	eBioscience, UK	00-4506-51
Celltrace CFSE cell proliferation kit	Molecular Probe, USA	C34554
CpG oligonucleotide (Human TLR9 ligand)	InvivoGen, USA	tlrl-2006
Cytofix fixation buffer	BD Biosciences, UK	51-9006613
Deionised water (ddH_2O), DNase/RNase free	InvitroGen, USA	10977-035
Diethanolamine	Sigma Aldrich, UK	D8885
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, UK	D2650
Dulbecco's phosphate buffered saline (10L power)	Sigma Aldrich, UK	D5652
Ficoll-paque premium	GE Healthcare Life Sciences, UK	17-5442-03
Foetal bovine serum (FBS)	Sigma Aldrich, UK	F7524
Gentamycin	Sigma Aldrich, UK	G1272
Glucose	Sigma Aldrich, UK	G8769
Glycerol	Sigma Aldrich, UK	G5516
Golgistop protein transport inhibitor (Monensin)	BD Biosciences, UK	51-2092KZ
Hank's balanced salt solution (HBSS)	Sigma Aldrich, UK	H9269

Heparin sodium (mucous injection BP)	LEO Pharma, UK	DE0248
Horse blood (defibrinated)	Oxoid Ltd, UK	SR0050C
Horseradish peroxidase Avidin D	Vector Lab. Inc., USA	A-2004
Hydrogen peroxide (H_2O_2)	Sigma Aldrich, UK	H1009
IC fixation buffer	eBioscience, UK	00-8222-49
Immun-Star WesternC Chemiluminescent kit	Bio-Rad, UK	170-5070
L-glutamine	Sigma Aldrich, UK	G7513
NN Dimethylformamide (DMF)	Sigma Aldrich, UK	D4551
Normal donkey serum	Sigma Aldrich, UK	D9663
Optochin disk	Oxoid Ltd, UK	DD0001B
Penicillin/streptomycin	Sigma Aldrich, UK	P0781
Perm/wash buffer	BD Biosciences, UK	51-2091KE
Permeabilization buffer (10x)	eBioscience, UK	00-8333
p-Nitrophenyl phosphate disodium salt tablet	Sigma Aldrich, UK	N9389
Quick-load DNA ladder (1kb)	New England Biolabs, USA	N3232L
RPMI 1640 media	Sigma Aldrich, UK	R5886
Skim milk powder	Oxoid Ltd, UK	LP0031B
Sodium acetate trihydrate	Sigma Aldrich, UK	S8625
Streptactin-HRP	Bio-Rad, UK	61-0381
Thermopol reaction buffer (10x)	New England Biolabs, USA	B9004S
Tris Borate EDTA (TBE) buffer (10x)	National Diagnostics, USA	EC-860
Tris/Glycine (10x)	National Diagnostics, USA	EC-880
Tris/Glycine/SDS (10x)	National Diagnostics, USA	EC-870
Tryptone soya broth	Oxoid Ltd, UK	BO0369E

Tween-20	Sigma Aldrich, UK	P1379
Ultrasafe blue	Syngene, UK	USB0112
Yeast extract	Sigma Aldrich, UK	Y1625

C. Consumables	**Manufacturer**	**Catalogue No.**
BD Falcon cell strainer (70µm, nylon)	BD Biosciences, UK	352350
Columbia blood agar plate	Oxoid Ltd, UK	OXPB 0199A
Dryswab™ in peel pouch	Medical Wire & Equipment, UK	MW113
ELISA plates (96 well, flat, high protein binding EIA/RIA plate)	Corning, USA via Appleton Woods, UK	CC679
ELISpot plates (Multiscreen ^{HTS} filter, 0.45µm hydrophobic, high protein binding membrane)	Millipore, USA	MSIPN4550
MACS cell separation LD columns	Myltenyi Biotech Ltd, Germany	130-042-901
Mini Protean precast TGX gel (12%)	Bio-Rad, UK	456-1044
Oracol saliva collection system	Malvern Medical Dev. Ltd, UK	510
Transblot turbo mini transfer pack	Bio-Rad, UK	170-4156
Vivaspin 15R hydrosart (MWCO: 5000)	Sartorius Stedim, Germany	VS15RH11

Appendix –III

List of publications

Accepted journal article

Muhammad S Ahmed, Stephen Derbyshire, Brian Flanagan, Christopher Loh, Max McCormick, Michele Barocchi, Vega Masignani, Adam Finn and Qibo Zhang. Immune responses to pneumococcal pilus RrgA and RrgB antigens and their relationship with pneumococcal carriage in humans. Accepted in Journal of Infection, February 2014.

Manuscript under preparation

Muhammad S Ahmed, Stephen Derbyshire, Anand Kasbekar, Max McCormick, Michele Barocchi, Vega Masignani, Brian Flanagan and Qibo Zhang. Association of Th17 related cytokine response with pneumococcal pilus proteins in human nasopharynx associated lymphoid tissues.

Accepted abstracts

M.S. Ahmed, S. Derbyshire, A. Kasbekar, M. McCormick, M. Barocchi, V. Masignani, B. Flanagan and Q. Zhang (2013). 'TH17 related cytokine response with pneumococcal pilus proteins in human nasopharynx associated lymphoid tissues'. *Abstracts of the Annual Congress of the British Society for Immunology, 2-5 December 2013, Liverpool, UK*. Poster 0137, Page 103. Immunology, 140 (Suppl.1): 39-184; available at http://onlinelibrary.wiley.com/doi/10.1111/imm.12184/pdf

M. S. Ahmed, A. Kasbekar, C. Loh, S. Leong, M. McCormick, M. Barocchi, V. Masignani, B. Flanagan and Q. Zhang (2012). B cell response to pneumococcal pilus RrgA and RrgB antigens and its relationship with pneumococcal carriage in children and adults. *Abstracts of the European Congress of Immunology, 5-8 September 2012, Glasgow, Scotland*. Poster P0432, Page 328. Immunology 137 (Suppl.1): 185-772; available at http://onlinelibrary.wiley.com/doi/10.1111/imm.12002/pdf

M. S. Ahmed, C. Loh, S. Leong, M. McCormick, M. Barocchi, V. Masignani, B Flanagan and Q. Zhang (2011). Natural immunity to pneumococcal pilus proteins and its protective role against pneumococcal carriage in humans. *Abstracts of the Annual Congress of the British Society for Immunology, 5-8 December 2011, Liverpool, UK*. Poster 0514, Page 68. Immunology 135 (Suppl.1): 52-212; available at http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2567.2011.03534.x/pdf