CORRELATION OF SALIVA AND SERUM ANTIBODY TITRE RESPONSE TO PNEUMOCOCCAL CAPSULAR POLYSACCHARIDES

Dissertation submitted for the degree

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Name of Student: Metsekae Richard Madimabe

Student Number: 20108141

Highest qualification of student: Baccalaureus Technologiae

Biotechnology

Supervisor: Dr C.J. Grobler

Co-supervisor: Dr P.V. Adrian

Approved date: October 2017

DECLARATION

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ABSTRACT

Background: Infants and young children under the age of 2 years are vulnerable to *Streptococcus pneumoniae* infections, especially those who are born in developing countries. Antibiotic therapy was an effective treatment until resistance to antibiotics emerged, and then vaccines were developed to assistant with the treatment. The first successful conjugate vaccine was the 7-valent pneumococcal conjugate which resulted in a decrease in vaccine serotype IPD in infants and children below the age of two years.

Objectives: The main aim of this study was to assess the saliva and serum antibody concentration response to pneumococcal capsular polysaccharides in children vaccinated and unvaccinated.

Design: This is a sub-study within a retrospective analysis of a prospective cohort study on the safety and immunogenicity of 7-valent pneumococcal polysaccharide-protein conjugate vaccine (PncCV) and the immunogenicity of a *H. influenzae* type b conjugate vaccine (HibCV).

Setting and participation: Infants aged between ≥ 4 and ≤ 10 weeks were enrolled, who only received BCG and Polio vaccine following birth. Infants were enrolled according to HIV status during routine antenatal screening in the obstetrics wards of the two hospitals, in Johannesburg and Cape Town.

Measurements: Saliva IgG and IgA concentrations against pneumococcal capsular polysaccharides serotype 4, 6B, 7F, 9V, 14, 18C, 19F and 23F were quantified a by multiplex bead-based assay using the Luminex technology. Serum IgG against polysaccharides serotype 4, 6B, 7F, 9V, 14, 18C, 19F and 23F were measured by a competitive Enzyme Linked Immuno-Sorbent Assay (ELISA).

Results: Post three primary vaccine doses, both serum IgG and saliva IgG and IgA antibody concentrations to vaccine serotypes were protective in children who received the vaccine. The antibody concentration in children whom did not receive the vaccine was much lower in comparison to the vaccine group in both serum and saliva to vaccine serotypes (P = 0.0001).

And also high positive correlation (>0.6) was observed of IgG in serum and in saliva following vaccination.

Conclusion: Pneumococcal conjugate vaccines induce pneumococcal capsular polysaccharide specific antibodies in both serum and saliva. However, there are differences between the vaccines' ability to induce mucosal immune response and there are also serotype specific differences in the antibody concentrations and in the proportion of positive samples after a series of vaccinations. The pneumococcal conjugate vaccine in this study was able to induce mucosal immune memory: the anti-pneumococcal IgA concentrations also increased with age in the saliva of unvaccinated children.

DEDICATION

To Kgosi, Mamotsumi, Pulane, Lakabane, I dedicate this written words for your everlasting souls, Rest with Peace.

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List of Abbreviations

°C	Degree Celsius
µg	Microgram
µg/mL	Microgram per millilitre
µL	Microliter
23vP	23-valent pneumococcal vaccine
ABC	Active Bacterial Core
AIDS	Acquired Immunodeficiency Syndrome
AOM	Acute Otitis Media
APC	Antigen Presenting Cells
ART	Anti-Retroviral Treatment
ATCC	American Type Culture Collection
BD	Becton Dickson
B-cell	B Lymphocytes
C3	Complement Component 3
C5	Complement Component 5
C9	Complement Component 9
CA	California
CASPER	The Calgary Area *Streptococcus pneumoniae* Research
CBP	Choline Binding Proteins
CbpA	Choline Binding Protein A
CbpG	Choline Binding Protein G
Cbps	Choline Binding Proteins
CD4+	(Cluster of differentiation 4) glycoprotein found on the surface of white cells
CD8+	(Cluster of differentiation 8) glycoprotein found on the surface of white cells
CDC	Centres for Disease Control and Prevention
ChoP	Phosphorylcholine
CI	Confidence Interval
CIPRA-ZA	Comprehensive International Programme for Research on AIDS South Africa
CPS	Capsular Polysaccharide
CpG	Cytosine-Phosphate-Guanosine
CR3	Complement Receptor 3

CSF	Cerebrospinal Fluid
CV	Coefficient of Variance
DAIDS	The Division of AIDS
DCs	Dendritic Cells
DMTMM	4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
DNA	Deoxyribonucleic Acid
DTwP	Diphtheria-Tetanus-Whole Cell Pertussis Vaccine
ECM	Extracellular Matrix
EIA	Enzyme Immuno-Assay
ELISA	Enzyme-linked Immuno-sorbent Assay
EPI-SA	Expanded Programme on Immunisation in South Africa
Exp Conc	Expected Concentration
Fcy	Fragment crystallizable-gamma
FI	Fluorescence Intensity
GE	General Electric
GMC	Geometric Mean Concentration
Hib	*Haemophilus influenzae* type b
HibCV	*Haemophilus influenzae* type b conjugate vaccine
HbIG	Hepatitis B Immune-globulin
HIV	Human Immunodeficiency Virus
HIV-/M-	HIV uninfected Child with HIV uninfected Mother
HIV-/M+	HIV uninfected Child with HIV infected Mother
HIV+/ART-	HIV infected Child on late Anti-Retroviral Therapy
HIV+/ART+	HIV infected Child on early Anti-Retroviral Therapy
HREC	Human Research Ethics Committee
Hyl	Hyaluronate Lyase
IL	Interleukin
IVIG	Intravenous Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Igs	Immunoglobulins
IPD	Invasive Pneumococcal Disease
kDa	Kilodalton

LLOD	Lower Limit of Detection
LLOQ	Lower Limit of Quantification
LOD	Limit of Detection
Lipid A/LPS	Lipid A/Lipoposaccharides
LTA	Lipoteichoic Acid
LytA	Autolysin
MAC	Membrane Attack Complex
MD	Maryland
MO	Missouri
mg/mL	Milligram per millilitre
MFI	Median Fluorescence Intensity
MHC	Major Histo-compatibility Complex
MI	Michigan
MIC	Minimum Inhibition Concentration
min	Minutes
mL	Milliliter
MRD	Minimum Required Dilution
mRNA	Messenger Ribonucleic Acid
N/A	Not Applicable
NALT	Nasal Associated Lymphoid Tissues
NanA	Neuraminidase A
NanB	Neuraminidase B
NanC	Neuraminidase C
NFAT	Nuclear Factor of Activated T-cells
NIH	National Institute of Health
nm	Nanometer
NY	New York
Obs Conc	Observed Concentration
OD	Optical Density
P	P-value
PBPs	Penicillin Binding Proteins
PBSF	Phosphate Buffer Solution with Fetal Bovine Serum
PBSFN	Phosphate Buffer Solution with Fetal Bovine Serum and Sodium Azide
PBST	Phosphate Buffer Solution with Tween 20

PBSTN	Phosphate Buffer Solution with Tween 20 and Sodium Azide
PCR	Polymerase Chain Reaction
PCV7	7-Valent Pneumococcal Conjugate Vaccine
PCV9	9-Valent Pneumococcal Conjugate Vaccine
pH	Potential of Hydrogen
Ply	Pneumolysin
PAMP	Pathogen-associated Molecular Pattern
PCV	Pneumococcal Conjugate Vaccine
Pnc	Pneumococcus/*Streptococcus pneumoniae*
PncCV	Pneumococcal Conjugate Vaccine
PncPS	Pneumococcal Polysaccharide
PRRs	Pattern Recognition Receptors
PspA	Pneumococcal Surface Protein A
PspC	Pneumococcal Surface Protein C
R	Pearson's Correlation Coefficient
r^2	Coefficient of Determination in Linear Regression
R-PE	R-phycoerythrin from red algae
rpm	Revolutions per Minute
SIgA	Secretory Immunoglobulin A
SpsA	Secretory Pneumococcal Surface Protein A
Stdev	Standard Deviation
T-cell	T Lymphocytes
TCR	T-cell Receptor
Th	T-helper Cell
TLR	Toll-like Receptors
TX	Texas
UK	United Kingdom
USA	United States of America
VAR	Vaccine Attributable Reduction
VZIG	Varicella Zoster Immune-globulin
WHO	World Health Organisation

List of Symbols

±	Plus, minus
-	Till
%	Percentage
/	or
+	Plus or more than
<	Smaller than
>	Greater than
≤	Equal and smaller than
≥	Equal and greater than
=	Equal to
√	Yes
x	Times
#	Hashtag

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CHAPTER 1

INTRODUCTION TO THE STUDY

1.1 INTRODUCTION

Streptococcus pneumoniae, also known as the pneumococcus, is an encapsulated gram positive bacterium that causes significant global morbidity and mortality (Lynch & Zhanel, 2010:217). Pneumococcus initiates an infection in the nasopharynx through colonisation (Muńoz-Elías et al., 2008:5049), which is largely asymptomatic (Serrano et al., 2006:1; Malley et al., 2007:928). Colonization then spreads and can progress to invasive disease. Invasion of the upper respiratory tract can result in infections of the middle-ear cavity called acute otitis media (AOM) (Murphy et al., 2009:S121; Mackenzie et al., 2009:1-11), sinuses causing sinusitis (Sousa et al., 2005:4696) and infection in the meninges causing meningitis (Lynch & Zhanel, 2010:217). Pneumococcus also invades the lower respiratory tract and spreads to the lungs, causing pneumonia (Klugman et al., 2008:S202) and into the bloodstream, causing bacteraemia (Isaacman et al., 2010:e197).

Pneumococcal disease occurs most often in children younger than five years and in the elderly, because their immune systems are either immature or unable to respond effectively to pneumococcal capsular polysaccharides (Shinefield, 2010:4335-4336). In addition, patients, especially children with certain chronic conditions like anatomic abnormalities, nephrotic syndrome, asthma, various cancers, those who underwent surgery such as cochlear implants and bone marrow transplantation, those with immunosuppressive conditions, including human immunodeficiency virus (HIV) infection and adult cigarette smokers have a greater risk of severe pneumococcal disease (Hjuler et al., 2008:e26; Kourtis et al., 2010:128; Herr et al., 2008:144).

1.2 PREVALENCE OF PNEUMOCOCCAL DISEASE

S. pneumoniae is a leading cause of bacterial pneumonia, meningitis, septicaemia and acute otitis media in children worldwide (O'Brien et al., 2009:893). It is widely known that many sovereign nations, especially developing countries, do not have available information on the

national estimates of the burden of disease. There are many reasons why the national burden of disease is not available and under-estimated; mainly is due to poor healthcare infrastructure or lack of surveillance and some are due to children who are not documented (O'Brien et al., 2009: 893-902; Lawn et al., 2005:891-900).

It is also believed that many babies who die in the poorest countries are unnamed and their births go unrecorded in which case their deaths are perceived as inevitable (Lawn et al., 2005:891-900). Children without access to healthcare facilities have greater case fatality rates compared to those receiving health care (O'Brien et al., 2009:894-902), and most infant deaths occur at home (Lawn et al., 2005:891-900).

To evaluate the seriousness of the bacterial infection caused by *S. pneumoniae* in children, monitoring surveillance is required to identify the prevalence of pneumococcal carriage and colonisation, prevalence of invasive pneumococcal disease (IPD) and burden of pneumococcal disease (Feikin et al., 2013:1-28).

According to Scott's paper, published in 2007, he states that the estimates of pneumococcal carriage, burden and prevalence of pneumococcal disease can be epidemiologically obtained in three ways. The first way of acquiring epidemiological data involves the population-based incidence estimates of IPD. This however, would bring about variations in estimating the incidence attributable to a difference in methodologies employed because studies involving young children (aged <2 years) will have a higher incidence than those studies including children in the first five years of life. As it has been shown in recent studies, the risk of IPD is greatest among young children in the first two years of life. Secondly, the data can be obtained by population-based incidence estimates of syndromes associated with pneumococcal disease such as meningitis or pneumonia that also can be missed due to passive hospital-based surveillance and the likelihood to underestimate IPD incidence compared to active referral from community clinics. Lastly, data can be obtained by morbidity and mortality reductions attributable to pneumococcal conjugate vaccine (PCV) analysis (Scott, 2007:2398-2405).

IPD as it is classified internationally, is defined clinically as infection and isolation of *S. pneumoniae* in sterile body sites, usually from blood samples, cerebrospinal fluids (CSF) and

other sterile fluid samples (Muñoz-Almagro et al., 2008:174-182; Scott, 2007:2398-2405; Whitney et al., 2006:1495-1502).

There are numerous factors involved in the IPD estimates, such as age of the study population, geographic area, race, economic status of a country, a study/surveillance conducted, site of infection with also underlying conditions of the patients; not to mention that the IPD-causing serotype prevalence may vary among different geographical areas, vaccine serotype coverage and the non-vaccine serotype emergence (Muñoz-Almagro et al., 2008:174-182).

The data that estimates the pneumococcal disease burden should be based on comprehensive and clear methods that are needed (O'Brien et al., 2009:894-902). There is however, a lack of global investment for child survival in Africa (Lawn et al., 2005:891). In developing countries, child survival programs tend to focus on vaccine preventable diseases attributable to *S. pneumoniae* by relying on pneumococcal conjugate vaccine efficacy trials. Findings from such studies, trials or surveillance provide necessary insight into the case specific pneumococcal burden estimates that would influence national policy makers to prioritise interventions to pneumococcal disease (Lawn et al., 2005:891-900; O'Brien et al., 2009:893).

1.2.1 Global Incidence of Invasive Pneumococcal Disease

The prevention of IPD incidence globally, has been well managed, especially in first world countries since the introduction of pneumococcal conjugate vaccine. According to World Health Organisation (WHO) report, disease burden and incidence are higher in developing countries than in highly industrialised countries (WHO, 2012).

In Spain, a prospective study was conducted in the city of Barcelona that was carried out for a period of ten years on children with reported IPD who were admitted to a hospital. The study was divided into two consecutive periods, the pre-vaccine period which took place from 1997 to 2001 and the vaccine period started from 2002 to 2006. The reported overall IPD cases per 100 000 population in children under the age of two years (P = 0.037), 2 to 4 years (P = 0.002) and over 5 years (P = 0.06) increased between the pre- and post-vaccine periods (Muñoz-Almagro et al., 2008:174-182).

Table 1: Global incidence of invasive pneumococcal disease[1]

Authors	Vaccine	Period of Study	Location	Infant Age	Pre-Vaccine Period			Post-Vaccine Period			P Value[2]		
					No. of IPD cases[1]			No. of IPD cases					
					All Serotypes	PnCV Serotypes	Non-PnCV Serotypes	All Serotypes	PnCV Serotypes	Non-PnCV Serotypes	All Serotypes	PnCV Serotypes	Non-PnCV Serotypes
Kellner et al., 2009	PCV7	1998-2007	Calgary, Alberta, Canada	<2 Years	77.7	66.4	11.3	18	9.0	8.0	<0.001	<0.001	0.61
				2-4 Years	22.7	17.9	4.8	12.6	7.3	5.2	0.03	0.006	>0.99
				>5 Years	3.3	2.5	0.7	2.4	1.3	1.1	0.41	0.15	0.57
Muñoz-Almagro et al., 2008	PCV7	1997-2006	Barcelona, Catalonia, Spain	<2 Years	32.4	26.8	5.6	51.3	16.1	35.2	0.037	0.095	<0.001
				2-4 Years	11.3	6.8	4.5	26.5	9.2	17.3	0.002	0.054	0.001
				>5 Years	2.1	0.5	1.7	4.0	0.3	3.7	0.06	0.68	0.03
Lehmann et al., 2010	PCV7	1997-2007	Australia[3]	<2 Years	73.8	61.2	9.1	24.2	6.6	13.9	-	-	-
				2-4 Years	21.2	17.9	2.5	9.9	4.2	4.2	-	-	-
				>5 Years	2.8	2.3	0.5	1.7	0.5	1.2	-	-	-
Vestrheim et al., 2008	PCV7	2002-2007	Norway	1 Years	66.9	53.7	9.7	43.3	24.3	17.3	0.06	<0.01	0.18
				2-4 Years	23.6	13.5	7.4	11.0	7.5	3.5	<0.01	0.07	0.1
				<5 Years	36.0	23.9	8.0	19.7	10.0	9.0	<0.01	<0.01	0.63
Lepoutre et al., 2008	PCV7	2001-2006	France	<2 Years	-	14.8	7.0	-	5.3	12.2	-	<0.001	<0.001
Rückinger et al., 2009	PCV7	1997-2007	Germany	0-1 Years	16.7	-	-	7.4	-	-	-	-	-
				2-4 Years	4.4	-	-	3.1	-	-	-	-	-

[1] No. IPD cases per 100, 000 population.
[2] Comparison between pre-vaccine and post-vaccine periods.
[3] Data from non-aboriginal Australians.

[1] Table 1 summarises the pre- and post- vaccine periods IPD incidences in the countries where pneumococcal conjugate vaccine was licensed for use in children. The incidence of IPD was defined as the number of confirmed cases per 100 000 population, of pneumococcal isolates from sterile fluids.

This increase is attributable to non-vaccine serotypes that increase IPD cases from 5.6 to 35.2 cases per 100 000 population (P<0.0001) in children under two years. IPD cases caused by vaccine serotypes decreased from 26.8 to 16.1 and 0.5 to 0.3 cases in children under the age of two years and over 5 years respectively. Only children 2 to 4 years of age had IPD cases increased from 6.8 to 9.2 cases per 100 000 population (Muñoz-Almagro et al., 2008:174-182).

The Germans also conducted a two period study for the surveillance of IPD in the pre-vaccine period running from 1997 to 2003 and vaccine period running through 2007 and 2008. The overall reported incidence of IPD was reduced significantly by the introduction of pneumococcal conjugate vaccine. Cases per 100 000 population were reduced from 16.7 to 7.4 and 4.4 to 3.1 cases in children 0 to 1 years and 2 to 4 years respectively (Rückinger et al., 2009:4136-4141).

The Institute of Public Health in Norway conducted a five year surveillance study in children 0 to 1 year, 2 to 4 years and children under 5 years of age from 2002 to 2007. IPD cases caused by all reported serotypes decreased non-significantly from 66.9 to 43.3 cases per 100 000 population in children 0 to 1 year (P = 0.06). However, a significant decrease in IPD cases caused by vaccine serotypes was observed from 53.7 to 24.3 (P <0.01) in children 0 to 1 year. Children 2 to 4 years and under 5 years had a significant drop in IPD cases caused by both vaccine and non-vaccine serotypes. IPD cases decreased from 23.6 to 11.0 (P<0.01) and 36.0 to 19.7 (P<0.01) in children 2 to 5 and under 5 years respectively (Vestrheim et al., 2008:3277-3281).

A five year pneumococcal surveillance was conducted in France by two surveillance networks from pre-vaccine period (2001-2002) through to post-vaccine period (2006). IPD cases in children under two years of age were ascertained to have been caused by both vaccine and non-vaccine serotypes. IPD cases caused by vaccine serotypes were reduced from 14.8 to 5.3 (P<0.001) cases per 100 000 population, while IPD caused by non-vaccine serotypes increased from 7.0 to 12.2 (P<0.001) cases per 100 000 population (Lepoutre et al., 2008:367-372).

The Calgary Area *S. pneumoniae* Research (CASPER) group conducted surveillance that continuously monitored the IPD in the city of Calgary and surrounding communities from

January 1 1998 through to December 31 2007. The IPD cases were monitored between 2003 and 2007 (post-vaccine period) with a baseline in 1998 to 2001 (pre-vaccine period). IPD data obtained in year 2002 was excluded in the analysis because that was the year that 7-valent pneumococcal conjugate vaccine (PCV7) was introduced in the country. There was a significant decrease in IPD cases in children under 2 years old, 2 to 4 years and over the age of 5. Children under 2 years' IPD cases decreased from 77.7 to 18.0 (P<0.001) cases per 100 000 population cause by both vaccine and non-vaccine serotypes. However, IPD cases caused by vaccine serotypes dropped significantly from 66.4 to 9.0 (P<0.001) cases compared to non-vaccine serotypes: from 11.3 to 8.0 (P = 0.61) cases in children younger than 2 years. Furthermore, IPD obtained from children 2 to 4 years and over 5 years of age decreased in vaccine serotypes (17.9 to 7.3; P = 0.006) and (2.5 to 1.3; P = 0.41) and increased in non-vaccine serotypes (4.8 to 5.2; P>0.99) and (0.7 to 1.1; P = 0.57) respectively (Kellner et al., 2009:205-212).

The Australian IPD surveillance system reported the observed cases during a pre-vaccine period (1997-2001) and post-vaccine period (2005-2007) in children 2 years younger, 2 to 4 years and over 5 years of age. Overall IPD reduction caused by all serotypes was observed with a reduction of 73.8 to 24.2 (<2 years), 21.2 to 9.9 (2-4 years) and 2.8 to 1.7 (>5 years) cases per 100 000 population. Significant reduction in IPD cases caused by vaccine serotypes was recorded from 61.2 to 6.6 (<2 years), 17.9 to 4.2 (2-4 years) and 2.3 to 0.5 (>5 years) cases per 100 000 population. However, IPD cases caused by non-vaccine serotypes increased from 9.1 to 13.9 (<2 years), 2.5 to 4.2 (2-4 years) and 0.5 to 1.2 (>5 years) cases per 100 000 population (Lehmann et al., 2010:1477-1486).

1.2.2 Prevalence of Pneumococcus in Sub-Saharan Africa

Many studies conducted in Africa indicate that children born in rural areas and villages in Africa have the probability of about 30 to 50 percent of dying before the age of five years (Mulholland & Adegbola, 2005:75). It is also estimated that about 4 million of 15 million deaths worldwide each year of children younger than five years, is attributable to bacterial pneumonia which is caused by *S. pneumoniae* and *Haemophilus influenzae* (Adegbola et al., 1994:975-982). It is also believed and understood that these deaths are the result of poor or absence of medical care (Mulholland & Adegbola, 2005:75), with also the combined effects of poverty and malnutrition (Adegbola et al., 1994; Cashat-Cruz et al., 2005:84).

About three decades ago, the scourge of pneumococcal disease mortality and morbidity has decreased, but African countries still report a death rate for children under the age of five years of more than 20 percent. This figure remains very high by both national and international standards (Mulholland & Adegbola, 2005:75-76).

In a study conducted in Gambia, the evaluation of the efficacy of pneumococcal conjugate vaccine was assessed in children aged 2 to 29 months. The burden and prevalence of IPD was assessed in children who received pneumococcal vaccine and those who had not received the vaccine. The incidence of IPD in children between 2 to 29 months who had not receive the vaccine was 453 (95% CI 356, 569) cases per 100 000 population and 256 (95% CI 185, 347) cases per 100 000 population in children who received the vaccine. The pneumococcal incidence was reduced by the vaccine (Cutts et al., 2005:1139-1146; Enwere et al., 2006:700-705; Antonio et al., 2008:81-91).

In a rural district of Manhiça of southern Mozambique, a hospital based surveillance to determine the burden of IPD among children under the age of 5 years was conducted. The surveillance was carried out over a 2 year surveillance period from June 2001 to May 2003, where sterile body fluids (Blood and CSF) were collected and tested. The estimate of IPD cases in infants younger than 3 months was 779 (95% CI 359, 1557), children between 3 to 24 months was 677 (95% CI 506, 907) and children between 24 to 60 months was 217 (95%CI 144,327) per 100 000 population (Roca et al., 2006:1422-1431).

There were two studies conducted in Kenya at the Kilifi district hospital where children admitted to a hospital were recruited (Berkley et al., 2005:39-47; Brent et al., 2006:482-488).

In a study by Berkley and his team, they recruited children under the age of 13 years between August 1998 and July 2002 to determine the prevalence and incidence of invasive bacterial infection among children in sub-Saharan Africa. The incidence of IPD was defined as the number of episodes or cases per 100 000 population. The annual community acquired IPD incidence was estimated at 241 cases among children under 1 year of age, 213 case among children under 2 years of age and 111 cases in children under 5 years of age (Berkley et al., 2005:39-47).

In another study by Brent and his colleagues, they presented data on children under 5 years of age for the incidence of IPD that is also defined as number of cases per 100 000 population. They reported the incidence of IPD at 597 (95% CI 416,775) cases per 100 000 population (Brent et al., 2006:482-488).

1.2.3 Prevalence of IPD in South Africa

A study involving 39 000 recruited infants, randomised to receive the study vaccine or placebo, began on March 2 1998 and ended on October 30 2000, in an effort to evaluate the efficacy of a 9-valent pneumococcal conjugate vaccine (PCV9). Before the trial began, the incidence of vaccine specific-serotype IPD was estimated to be at 112 cases per 100 000 population. The findings of this study proved that the vaccine reduced incidence of vaccine serotype IPD to 33 cases per 100 000 population and unvaccinated children remained at 109 cases per 100 000 population. The study was conducted on HIV-1-infected and HIV-1-uninfected children (Klugman et al., 2003:1341-1348).

In a study conducted in Soweto at Chris Hani Baragwaneth Hospital where the vaccine efficacy was evaluated, blood culture-confirmed pneumococcal infections were used to estimate the burden of pneumococcal infection prevented by vaccines. The vaccine attributable reduction (VAR) rates was estimated at 410 cases per 100 000 population (Madhi et al., 2005:1511-1518).

1.3 BACKGROUND OF THE STUDY

The purpose of the study is to evaluate the effect of a PCV7 on the T-independent immune system that elicits a memory B-cell antibody response. The antibody response that is provoked (induced) by the protein-conjugated polysaccharide antigen in the vaccine can mimic the presence of natural polysaccharides presented by *S. pneumoniae* located on the surface of the bacterium. We are looking at two distinct antibody responses: the salivary antibody response of the mucosal system and the serum antibody response in the humoral system. The salivary antibody response to bacterial carriage and colonisation on the mucosal areas and the serum antibody response once the bacteria have crossed into the blood stream. By measuring the antibody concentrations of the two systems induced by the polysaccharides, we can have an idea of how each system functions during infection.

1.4 THE SIGNIFICANCE OF THE STUDY

The significance of the study is that very little information is known about the impact of PCV7 on IgA production in saliva. Very little is also known about salivary IgG whether it originated at the mucosal site or leaked from the blood. Is there a relationship between IgG correlates of protection in serum and saliva, and also between salivary IgG and IgA? These are questions with unknown answers, and may help to resolve questions such as why people with certain types of immune deficiencies are protected and others not. Or why high serum IgG concentrations can protect against pneumococcal acquisition, and not initiate clearance of existing pneumococcal colonization. In this study, we attempt to answer the questions and plug gaps in literature and relate this to prevention of pneumococcal disease.

1.5 AIM AND OBJECTIVES

1.5.1 Aim

The main aim of this study is to correlate saliva and serum antibody concentration in response to pneumococcal capsular polysaccharides. To achieve this aim the following objectives were carried out:

1.5.2 Objectives

1. To Optimize the Luminex assay for the detection of saliva antibodies concentrations.
2. To measure serum and saliva IgG antibodies using conventional and bead-based Enzyme Immuno-Assay (EIA) respectively in vaccinated and unvaccinated children.
3. To compare and correlate salivary IgG concentrations to serum IgG concentrations.
4. To measure the saliva IgA and IgG concentrations in vaccinated and unvaccinated HIV uninfected children.
5. To assess trends of maturing saliva antibody response in the first two years of life.
6. To compare the saliva IgA and IgG immune response to vaccination with respect to HIV status and compared to HIV uninfected children as the control group.

1.6 STRUCTURE OF THE DISSERTATION

1.6.1 Chapter 1 – Introduction

This chapter introduces and provides an overview of the research that is to be undertaken.

1.6.2 Chapter 2 – Literature Review

The literature review is used to draw information from the known from which to guide an idea for substantive information for research.

1.6.3 Chapter 3 – Methods and Materials

The methods are the procedures used to acquire experimental evidence with a purpose of answering the study problem statement.

1.6.4 Chapter 4 – Results and Discussion

The results are generally reported in this section and also interpreted.

1.6.5 Chapter 5 – Conclusion and Recommendations

This is a section of the study in which meaning is given to the results on literature, research, policies.

1.6.6 References

In accordance with the regulation and guidelines for dissertation submission, the bibliographies of literature used for this study are outlined in the reference section.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

S. pneumoniae is one of the many competing microbial flora inhabiting the upper respiratory tract of the invaded host, colonising particularly in this case the human nasopharynx. Although colonisation of the human upper respiratory tract with pneumococcus is asymptomatic, it may however become symptomatic when it escapes the nasopharyngeal niche and invades the sterile sites (Murphy et al., 2009:S121; Hammerschmidt et al., 2005:4653). Colonisation is the initial phase of pneumococcal mucosal pathogenesis, in which the successful progression to an invasive state would result in transmigration through tissue barriers and initiation of invasive disease to different host niches (Hammerschmidt et al., 2005:4653). For pneumococcal invasive disease to take place, various pathogenic mechanisms are initiated which involves virulence factors synthesised by the pneumococcus.

2.2 HISTORY OF STREPTOCOCCUS PNEUMONIAE

The first description of the pneumococcus was made around 1875 by a microbiologist Klebs. At the time Klebs was examining the fluid from the lungs of a pneumonia patient with a microscope when he came across a non-motile group-linked organism (White, 1938:2). After the first discovery of the pneumococcal organism, years went by when two scientists working independently around the same year in 1881, Stenberg in USA and Pasteur in France, inoculated healthy rabbits with saliva fluids. Stenberg used his own saliva and Pasteur used saliva from a patient who had died of rabies to show the pathogenicity of the organism. Not only did Pasteur manage to be the first person to report his findings of the organism's pathogenicity, he was able to isolate the organism from the infected rabbits by cultivation (White, 1938:2-3; Watson et al., 1993:913).

The technique of identification was established in the same decade by Christian Gram during the 1880's. He was experimenting on the visualisation of bacteria in pathological specimens when he stumbled upon the technique of what we now know as Gram Stain. Gram was

examining the portion of lung tissue of a patient who had died of pneumonia, when he exposed the specimen to aniline-gentian violet solution. He observed the specimen retaining the dark aniline-gentian violet stain and being what is now called Gram positive. Pneumococcus was one of the first pathological organisms observed during the development of the Gram stain technique (Watson et al., 1993:914). Figure 1 shows the Gram stained appearance of the pneumococcus as an oval shaped, Gram positive coccus, typically in singles, pairs and short chains.

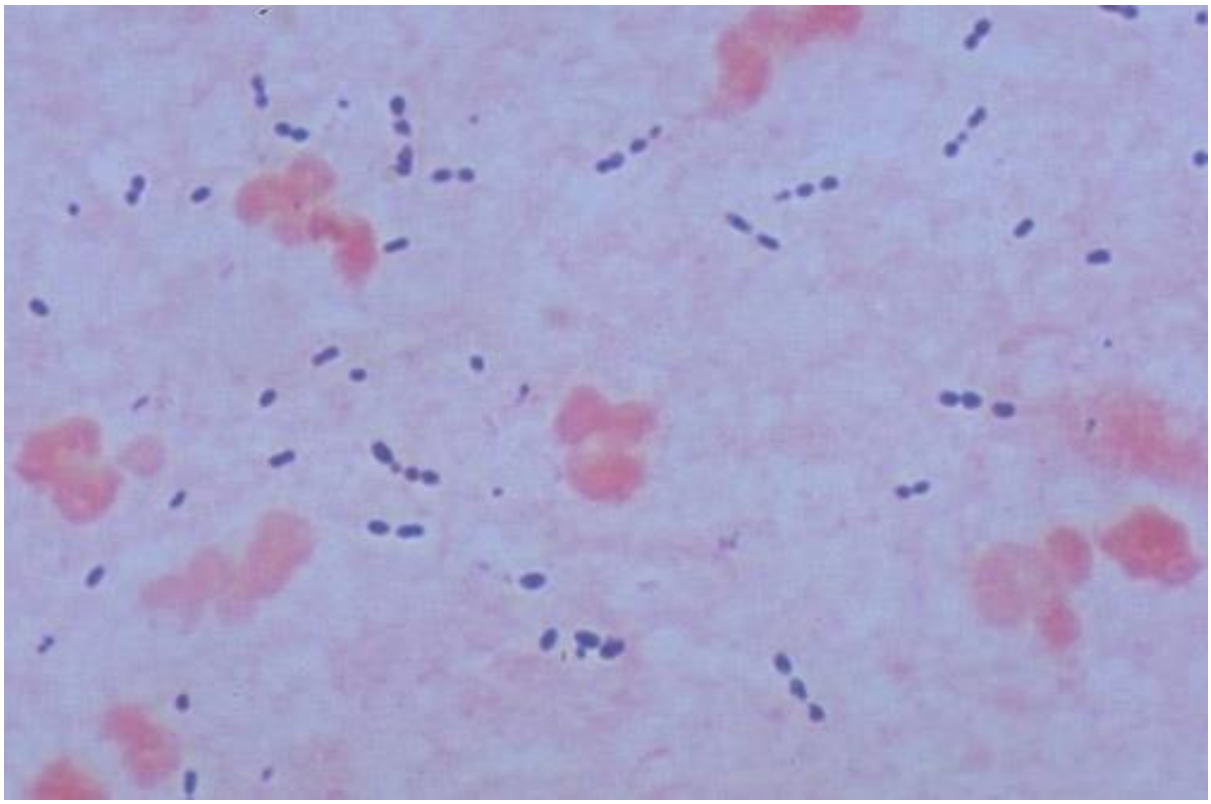

Figure 1: A gram-positive *S. pneumoniae* in pairs and chains on a Gram stain.
(http://library.tcmedc.org/webpath/microbio/microbe/strep-pn.htm)

This early discovery sparked a whole new adventure into pneumococcal research which was about the time animal studies were taken up front in a quest to determine the infectious nature of the pneumococcus. This led to discoveries of different groups or types associated with pneumococcus, and also the immunity to pneumococcal infection when rabbits were protected from pneumococcal infection with serum from rabbits that were inoculated with heat killed pneumococcal isolates of the same serotype (Watson et al., 1993:914-915). Pneumococcal microscopic agglutination was observed when specific antiserum was added to

a pneumococcal suspension which showed bacterial clumping, a simple method of serotyping. Credit must be given to Fred Neufeld for his remarkable contribution to the identification and characterising of pneumococcus which he differentiated from other bacteria. He introduced a test that resulted in the swelling of the properties of the pneumococcal capsule when exposed to homologous antibodies; that is called capsular precipitation reaction. The technique was introduced as a primary test for typing *S. pneumoniae* (López, 2006:180). This came about when the infectious material of pneumococci during early stages of growth was found free in circulating blood and the urine; also it diffused into the culture medium (Dochez & Avery, 1917:480-481).

2.3 PHYSICAL CHARACTERISTICS OF STREPTOCOCCUS PNEUMONIAE

The nature of pneumococcus is to bind avidly to cells of the upper and lower respiratory tract. Early attachment does not involve fimbriae or invoke actin polymerization as has been seen for other invasive bacteria. Rather, adherence is a process enabling carriage for several weeks without invoking an inflammatory response, and yet invoking serotype-specific immunity is based on the structure of the capsular polysaccharide of the infecting strain (Tuomanen, 1997:254). In the nasopharynx, pneumococcus initially binds to *N*-acetylated galactosamine residues on host cells. Xylitol, a carbohydrate, competitively inhibits the interaction of the bacteria with the *N*-acetylated galactosamine residues subsequently inhibiting colonization and cellular invasion (Orihuela & Tuomanen, 2006:69). *S. pneumoniae* is a Gram-positive bacterium with a profound cell-envelope structure different to that of Gram-negative bacteria. Just as in other Gram-positive bacteria, the pneumococcus lacks the normal outer membrane of Gram-negative organisms, and the cell wall is usually much thicker. The cell wall is also surrounded by a protective layer of capsular polysaccharide (Weidenmaier & Peschel, 2008:276). *S. pneumoniae* has an anaerobic metabolism which lacks the genes that encode functions of the tricarboxylic acid cycle; therefore, its energetic metabolism relies on glycolysis and fermentation (Martín-Galiano et al., 2004:2313). In otitis media, *S. pneumoniae* is suspected to be present in bio-films on the mucosal surface of the middle ear, and possibly also during asymptomatic colonization of the nasopharynx. Physiological changes due to the bio-film mode of growth may include a decreased pH (Martín-Galiano et al., 2005:3935).

2.3.1 Pneumococcus Capsular Polysaccharide and Cell Wall

Capsular polysaccharides are usually composed of repeating units of simple sugars covalently attached to the outer surface of the bacterial cell wall peptidoglycan, making a thick layer of approximately 200-400 nm surrounding the bacterial cell wall peptidoglycan (Kadioglu et al., 2008:296). The capsule of the pneumococcus is considered to be an anionic matrix as it contains a charged site which is highly hydrated (Hammerschmidt et al., 2005:4656). There are about 90 structurally and serologically distinct capsule types synthesised by *S. pneumoniae* strains providing a variety of and extended virulence from highly effective to non-virulence existence (Kadioglu et al., 2008:296).

Some strains of pneumococci are deficient in their ability to produce capsular polysaccharides and are referred to as non-typeable pneumococci (Park et al., 2012:1-11). The pneumococcal cell wall consists of more than a dozen structurally different peptides of a glycan backbone. Phosphorylcholine is an important component of the pneumococcal cell wall attached to teichoic acid that is linked directly to the peptidoglycan; while on the other hand another component of the pneumococcal cell wall, lipoteichoic is anchored through its fatty acid to the plasma cell membrane. Both teichoic acid and lipoteichoic acid consist of extended repeating carbohydrates differing only in their attachment to the pneumococcal cell wall surface (McCullers & Tuomanen, 2001:878).

2.3.2 Pneumococcus Adhesion Proteins

2.3.2.1 Choline Binding Proteins (Cbps)

There are about 10 to 15 choline binding proteins (Cbps) synthesised by *S. pneumoniae* that include pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC) and autolysin (LytA) that are crucial virulence factors in colonisation (Kadioglu et al., 2008:294-295). The proteins are anchored to the surface of the pneumococcal cell wall teichoic acid and membrane-bound lipoteichoic acids by a phosphorylcholine (ChoP) molecule (Kadioglu et al., 2008:294-295; Jedrzejas, 2001:188-191). These proteins have highly conserved repeating sequences of 20 amino acids that mediate a non-covalent attachment to the cell surface through phosphorylcholine (Kadioglu et al., 2008:294-295; Jedrzejas, 2001:188-191). Cbps are multifunctional proteins that are expressed during colonization and invasive

diseases in both saliva and the bloodstream. In particular choline binding protein G (CbpG) that plays a role in virulence of *S. pneumoniae* during colonization and invasive diseases that makes it a better candidate of new conjugate vaccines (Mann et al., 2006:821-829). The Cbps motif has also been found among the surface proteins of other bacteria, like *Clostridium acetobutylicum*, *Clostridium difficile*, *Streptococcus mutans*, and *Streptococcus downei* (Jedrzejas, 2001:191). The following proteins will be discussed in detail.

1. PNEUMOCOCCAL SURFACE PROTEIN A (PspA)

PspA is located on the cell wall of the pneumococcus and it is found on all *S. pneumoniae* strains. PspA is a choline surface protein with variable molecular size ranging from 67 to 99 kDa (Jedrzejas, 2001:188). PspA has three distinct structural domains: a surface exposed N-terminal region with a highly electro-negatively charged α-helical region (288 amino acids) which able to inhibit complement binding, a C-terminal choline-binding region that anchors PspA to pneumococcal surface and a proline-rich region, that lies between the N- and the C-terminal region bearing highly conserved 20-amino-acid repeats of up to 83 amino acids. The N-terminal end extends from the cell wall and protrudes outside the capsule (Jedrzejas, 2001:188; Kadioglu et al., 2008:294).

2. PNEUMOCOCCAL SURFACE PROTEIN C (PspC)

Pneumococcal surface protein C (PspC), usually known as Choline-binding protein A (CbpA), was the first choline-binding protein to be identified on the surface of *S. pneumoniae* (Jedrzejas, 2001:201) and was the predominant entity to be purified by ChoP-affinity chromatography. PspC is a highly conserved cell-surface choline-binding protein that is known for several of its different activities (Kadioglu et al., 2008:295).

2.3.3 Neuraminidase

Neuraminidases are yet another virulence factor of *S. pneumoniae* that are present on all strains of pneumococcus sequenced to date. *S. pneumoniae* synthesises three forms of neuraminidase enzymes viz. neuraminidase A (NanA), neuraminidase B (NanB) and neuraminidase C (NanC) with NanA having a molecular mass of 108 kDa, NanB having a mass of 75 kDa (Jedrzejas, 2001:201) and NanC that is present in only 56% of pneumococcus

strains (Gualdi et al., 2012:200-211). These proteins, particularly NanA which is expressed significantly in elevated numbers in the nasopharynx, play an important supporting role in pneumococcal colonisation by promoting the clearance of competing bacteria (LeMessurier et al., 2006:205-311).

2.4 PATHOGENESIS OF STREPTOCOCCUS PNEUMONIAE

According to Musher (1992:802-803) the pathogenesis of pneumococcus from a first point of infection in the mucosal area to invasive disease in the bloodstream, is outlined in the following schematics (Figure 2).

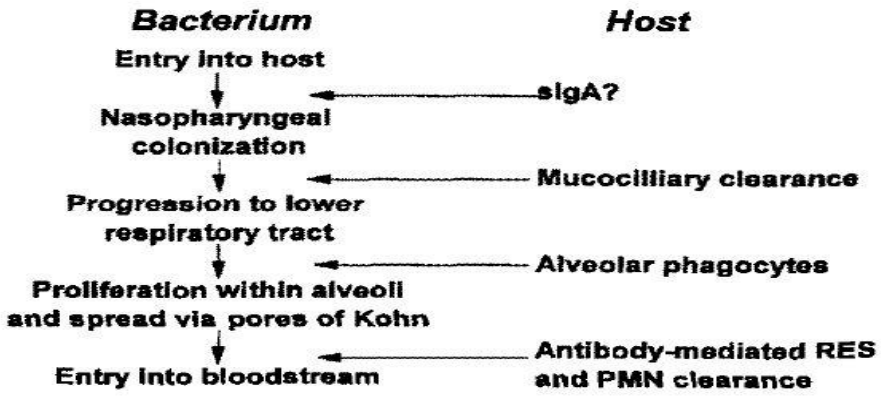

Figure 2: Progression of infection by *S. pneumoniae.*
(Watson et al., 1995:482)

Pneumococcal pathogenesis in humans and animal models is believed to be caused by the virulence factors synthesised by *S. pneumoniae*, a process of multifunctional activities that are highly regulated (Hammerschmidt et al., 2005:4653). Of all the virulence factors that pneumococcus synthesises, the external polysaccharide capsule is the most strongly associated with this process. It is followed by the proteins synthesised by the bacteria such as pneumolysin, which in rat models was capable of reproducing all of the symptoms of pneumonia. Pneumolysin is capable of eliciting a host immune response due to its contribution to multiple stages in the pneumococcal pathogenic process (LeMessurier et al., 2006:309).

There are a number of proteins produced, which are involved during the host invasion, that are capable of maintaining pneumococcal infection in the host, with the likes of PspA, PspC and protease enzymes. They assist with the attachment of the bacterium to the mucosal epithelial surface and the cleaving of active host IgA1 antibodies to an inactive form respectively. It is very crucial that the bacterium carry necessary virulent components in order to initiate invasive disease (Orihuela et al., 2004:1661-1669). Other proteins are responsible for the transition of pneumococcus from one site to another and are only specific to the area involved and in prolonging infection. Once the bacterium has moved from the previous site, proteins which had no effect previously, take up effect (Orihuela et al., 2004:1661). Pneumococcus can initiate a programmed cell death, where cells die by an active process characterised by nuclear fragmentation and cell shrinkage while maintaining cell membrane integrity (Marriott & Dockrell, 2006:1848).

During infection autolysin activity stimulates release of pneumococcal cell wall components and pneumolysin. Pneumococcal virulence factors implicated in nasopharyngeal colonisation include neuraminidase, pneumococcal surface antigen A, choline binding protein A, pneumococcal surface protein A and pyruvate oxidase, that produces microbiocidal hydrogen peroxidase. The binding of choline binding protein A to the polymeric immunoglobulin receptor or to the platelet activating factor receptor contributes to tissue invasion (Marriott & Dockrell, 2006:1848-1854). The capsular polysaccharide and the pneumococcus virulence proteins have different properties and roles in host disease development that will be outlined individually.

According to the literature, pneumococcal colonisation and adherence to host cells has been suggested to be a two-step process. The first step involves colonisation of the host nasopharynx that leads to attachment of the host surface glyco-molecules on respiratory epithelial and endothelial cells. The following step is the induction and activation of cytokines, the expression of glycans on the surface of activated cells and increased pneumococcal adherence. The successful progression of the two steps into the host nasopharynx and mucosal sites, leads to pneumococcal invasive disease. *S. pneumoniae*'s adherence to the host epithelial cells involves an array of protein molecules expressed on its surface. In addition to adherence to the host epithelial cells, the pneumococcus has developed other ways to interact with the host and its tissues. Such interactions involve a variety of host cells, tissues and tissue components, and pneumococcus utilizes an array of its surface and

cytoplasmic molecules. These proteins are involved in interactions with the host complement system, lysis of cholesterol containing membranes, degradation of peptidoglycan layers of the pneumococcus and inflammatory degradation products of the cell wall. The functions of all the proteins facilitate significant aspects of pneumococcal colonization and invasion; compromising these functions leads to compromised pathogenicity of *S. pneumoniae* (Jedrzejas, 2001:193-204).

2.4.1 Pneumococcus Capsular Polysaccharide

The capsule (figure 3) is a crucial contributor of colonisation, invasion, dissemination and survival of the pneumococcus in the host (van der Poll & Opal, 2009:1546). Pneumococcal strains with transparent capsule were associated with high epithelial attachment and host colonisation, and colonisation was not observed in opaque strains with thick capsules. Furthermore phase variation reversible switching (transparent to opaque and opaque to transparent) in some phenotype strains, were observed in a large number of colonies (Weiser et al., 1994:2586).

However, it is believed that the capsule type is responsible for enabling a long duration with high density of colonisation of pneumococcus in the respiratory tract, also allowing a short duration with low density of colonisation of pneumococcus to necessitate invasion into the sterile sites. The short duration with low colonisation density allows for the invasion to take place in order to reach the nutritionally rich environment for the survival of the strain (Hathaway et al., 2012:1-12).

The capsule prevents mechanical clearance by mucous secretion (van der Poll & Opal, 2009:1546), in which it converts it into an apathogenic state where the reduced capsule helps in evading host immune system (Hammerschmidt et al., 2005:4661). The capsular polysaccharide is highly negatively charged and it is strongly anti-phagocytic in non-immune hosts (van der Poll & Opal, 2009:1546; Kadioglu et al., 2008:296). The capsule is capable of inhibiting the interaction between complement receptor 3 (CR3) receptors of phagocytic cells to complement component 3 (iC3b) and fragment crystallizable-gamma (Fcy) receptors of phagocytic cells to the Fc component of the IgG (van der Poll & Opal, 2009:1546; Kadioglu et al., 2008:296).

Although capsular polysaccharide does not play a role in adherence, invasion or inflammation in the host (McCullers & Tuomanen, 2001:878), its virulence is associated with the adhesion's molecule recognition and the protection of the key infection proteins from circulating antibodies (Watson et al., 1993:914-915), involved in the initiation of bacterial colonisation on the host pharynx (López, 2006:186).

The capsule also functions by completely isolating the bacterial cells from the host immune system (García & López, 1997:1-2; Watson et al., 1993:915) by preventing the complement-mediated opsonisation and/or interaction of bound complement with receptors on phagocytes (Kadioglu et al., 2008:296; McCullers & Tuomanen, 2001:878; Watson et al., 1993:915), thereby reducing the total amount of complement that is deposited on the bacterial surface (Kadioglu et al., 2008:296).

Capsules provide resistance to autolysis and antibiotic-induced autolysis that reduces exposure to several antibiotics (Marriott & Dockrell, 2006:1851; Kadioglu et al., 2008:296), that vary between capsular serotypes (Kadioglu et al., 2008:297). The degree of pneumococcal capsule effect on virulence appears to be dependent on the composition of the capsule and the underlying cell wall components rather than its thickness (McCullers & Tuomanen, 2001:878).

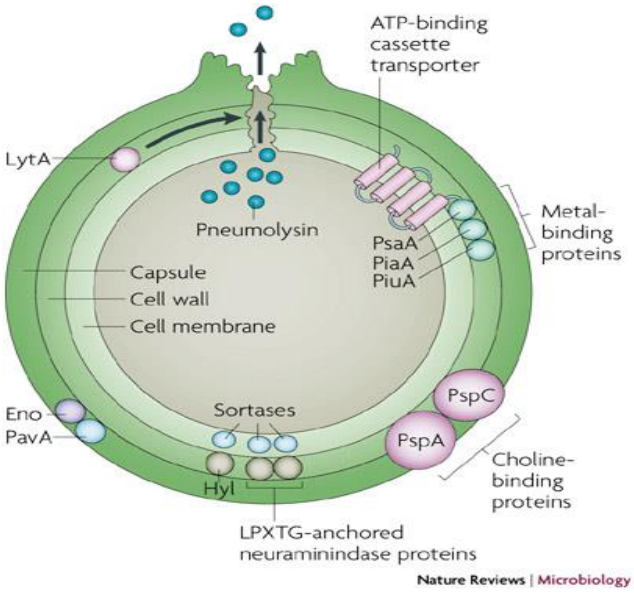

Figure 3: *Streptococcus pneumoniae* virulent molecules.
(http://www.nature.com/nrmicro/journal/v6/n4/fig_tab/nrmicro1871_F2.html)

2.4.2 Pneumococcus Adhesion Proteins

2.4.2.1 Choline Binding Proteins (Cbps)

1. PNEUMOCOCCAL SURFACE PROTEIN A (PspA)

PspA is attached to the surface of *S. pneumoniae* by non-covalent binding to the choline of both lipoteichoic and teichoic acids via its C-terminal end of the cell wall (Figure 3). PspA is not the only choline binding protein (CBP) present on the surface of pneumococcus. Examples of other pneumococcal CBPs are the major cell wall hydrolase LytA and an adhesin choline binding protein A (CbpA), also called pneumococcal surface protein C (PspC) (Jedrzejas, 2001:191).

The function of PspA is to protect against the host complement system. PspA interferes with the fixation of complement component 3 (C3) on the pneumococcal cell surface, and thus inhibits complement-mediated opsonisation, that reduces the complement mediated clearance by phagocytosis of *S. pneumoniae* (Kadioglu et al., 2008:294; Jedrzejas, 2001:190). Iron is essential for pneumococcal growth and survival, as it is for other pathogenic bacteria. PspA has been shown to bind lactoferrin, an iron-sequestering glycoprotein, at respiratory mucosal sites (LeMessurier et al., 2006:309). The binding of lactoferrin to pneumococcus is thought to protect the bacterium from the bactericidal activity of apolactoferrin and host immune functions by aiding in the acquisition of iron at the site of infection. PspA-knockout mutants are more sensitive to killing by apolactoferrin, and anti-PspA antibodies enhance the bactericidal activity of apolactoferrin (Kadioglu et al., 2008:294; LeMessurier et al., 2006:309).

The results from *in vivo* experiments conducted by LeMessurier and colleagues (2006:309), suggest that the enhanced levels of PspA in the nasopharynx compared to the lungs indicate that PspA is important for colonization of the nasopharynx. The study also demonstrated a higher level of PspA transcript in bacteria recovered from the blood of infected mice than from the lungs, consistent with the second function of PspA in preventing complement-mediated opsonisation of blood-borne pneumococcus (LeMessurier et al., 2006:309).

This is also echoed by the study done that suggests PspA producing strains are capable of binding more free complement components in blood that will see an increased disappearance of antigenic C3 from the circulation (Jedrzejas, 2001:190). Similar results have been obtained by other groups using microarray analysis that has shown that PspA transcription appears to be up-regulated both in the blood and in Detroit cells (LeMessurier et al., 2006:309). PspA also functions in stabilising the capsule charge through its electro positive end of the molecule, resulting in prevention of complement activation.

2. PNEUMOCOCCAL SURFACE PROTEIN C (PspC)

PspC (Figure 3) is thought to be important in mediating adherence to the nasopharyngeal epithelium, activated lung epithelial and endothelial cells (LeMessurier et al., 2006:309). A PspC-knockout mutant binds less well to epithelial cells and sialic acid *in vitro*, and shows reduced nasopharyngeal colonization compared with the wild type (Kadioglu et al., 2008:295; LeMessurier et al., 2006:309). PspC also binds the polymeric immunoglobulin receptor that normally transports secretory IgA; hence it is also called secretory pneumococcal surface protein A (SpsA). This activity could be the first stage of translocation across the respiratory epithelium, which is consistent with the reduced virulence of a PspC mutant in a mouse model of pneumonia. An additional property of PspC is its ability to bind to factor H83 that has been shown to prevent formation of C3b though the alternative complement pathway, and thus preventing pneumococcal opsonisation (Kadioglu et al., 2008:295).

Data from a study conducted by LeMessurier et al. (2006:309) indicate that *cbpA* messenger ribonucleic acid (mRNA) was present at elevated levels in the nasopharynx and lungs as compared to the bloodstream. This suggests that CbpA is not required for the entry of pneumococcus into the bloodstream from the lungs and for survival in the blood.

3. PNEUMOCOCCAL AUTOLYSIN (LytA)

Pneumococcal autolysin (LytA) is an amidase protein, a widely distributed group of enzymes that degrade the peptidoglycan backbone of bacterial organisms and its function in the separation of daughter cells during cell division. LytA cleaves the *N*-acetylmuramoyl-l-alanine bond of pneumococcal cell wall peptidoglycan. LytA is located in the cell envelope

and is responsible for the lysis of *S. pneumoniae* that leads directly to cell death. LytA also participates in cell wall growth and turnover that operates in the release of pneumolysin (Figure 3) (Jedrzejas, 2001:198; Kadioglu et al., 2008:295).

Other studies of LytA also have shown that this amidase induces a protective response in mice to streptococci when inoculated into the lungs. One of the direct implications is the release of the components of cell wall shown to be highly inflammatory in some animals (Jedrzejas, 2001:198).

2.4.3 Neuraminidase

NanA is the most prominent first-in-line virulence factor for sialic acid removal, followed by NanB that is responsible for the metabolic use of sialic acid and lastly NanC that is responsible for the production of metabolic molecules functioning as sialic inhibitor (Gualdi et al., 2012:200-212). Neuraminidase proteins are also known as sialidases that cleave terminal sialic acid residues from glycoproteins, glycolipids and cell-surface oligosaccharides (Kadioglu et al., 2008:296) with significant damage to host cell glycans as well as to the host (Jedrzejas, 2001:201).

NanA has been shown to have a role in facilitating bacterial adherence by removing terminal sialic acid residues from glyco-conjugates (LeMessurier et al., 2006:305-309). A recent study showed that neuraminidases can remove sialic acid from soluble proteins, such as lactoferrin, IgA2 and secretory component while NanB is important for pneumococcal survival in the respiratory tract and bloodstream (Kadioglu et al., 2008:296). This action changes the glycosylation patterns of the host and probably exposes more of the host cell surface, which may reveal surface receptors for possible interaction with pneumococci, contributing to increased adhesion and other processes (Jedrzejas, 2001:203; Kadioglu et al., 2008:290).

2.4.4 Hyaluronate Lyase

Hyaluronate lyase (Hyl) is another major surface protein of *S. pneumoniae* with antigenic variables that are essential for full pneumococcal virulence (Figure 3). Hyaluronate lyase is part of a broader group of enzymes called hyaluronidases. The hyaluronidase enzyme facilitates the host tissue invasion by breaking down the extracellular matrix (ECM)

components (Jedrzejas, 2001:191-192). Binding to extracellular matrix components could also promote bacterial adherence once the organism has gained access to basement membranes (Kadioglu et al., 2008:296).

Increased tissue permeability caused by the action of hyaluronidase on the ECM facilitates bacterial spread through a matrix of the hyaluronan (Kadioglu et al., 2008:290), and appears to play a role in wound infections, pneumonia, and other sepses such as bacteremia and meningitis (Jedrzejas, 2001:192). Bacterial hyaluronate lyases use β-elimination to degrade hyaluronan and often yield disaccharides (Jedrzejas, 2001:192; Kadioglu et al., 2008:296). *S. pneumoniae* hyaluronate lyase cleaves the 1, 4-glycosidic linkage between *N*-acetyl-b-D-glucosamine and D-glucuronic acid residues in hyaluronan and catalyzes the release of unsaturated polysaccharides, with the disaccharide unit 2-acetamido-2-deoxy-3-*O*-(b-D-gluco-4-enepyranosyluronic acid)-D-glucose being the main end product (Jedrzejas, 2001:192).

2.4.5 Intracellular Released Toxic Protein

2.4.5.1 Pneumolysin (Ply)

Pneumolysin (Ply) is a potent and wide ranging virulence factor of *S. pneumoniae* that penetrates the physical defences of the host, and is produced in most clinical pneumococcal isolates. Unlike other pneumococcal protein antigens, pneumolysin is not surface exposed. Pneumolysin is a member of the family of cholesterol-dependent cytolysins that are synthesized by Gram-positive bacteria (Jedrzejas, 2001:197; Kadioglu et al., 2008:293). Pneumolysin is a cytoplasmic enzyme produced as a 52 kDa soluble protein that oligomerizes in the membrane of target cells to form a large ring-shaped transmembrane pore (Kadioglu et al., 2008:293), that is released due to the action of surface pneumococcal autolysin (Jedrzejas, 2001:198). Once the pores are formed, the targeted cell undergoes lysis. These virulence properties of pneumolysin are therefore directly dependent on the action of autolysin. Pneumolysin is required for the progression of pneumococcal infection from nasopharyngeal colonisation, down to lung infection and ultimately into the bloodstream.

Pneumolysin has several distinct functions, especially in the early pathogenesis of pneumococcal infection. These functions include but are not limited to, inhibition of ciliary

beating in human respiratory epithelium, thus augmenting the migration of bacteria to the lungs (Jedrzejas, 2001:197; Kadioglu et al., 2008:293; LeMessurier et al., 2006:309; Marriott & Dockrell, 2006:1851). It also acts by disrupting tight junctions between epithelial cells that may provide an alternative pathway by which the pneumococcus infiltrates the bloodstream (Jedrzejas, 2001:197; LeMessurier et al., 2006:309). Even in low concentrations, pneumolysin is able to inhibit human neutrophil and phagocyte respiratory bursts and induction of cytokine synthesis and CD4+ T-cell activation and chemotaxis (Kadioglu et al., 2008:293; LeMessurier et al., 2006:309). The cytotoxic effects of pneumolysin can directly inhibit phagocyte and immune cell function, which leads to suppression of the host inflammatory and immune responses that limit production of lymphokines and immunoglobulins (Jedrzejas, 2001:197; Marriott & Dockrell, 2006:1851).

2.5 IMMUNE RESPONSE TO PNEUMOCOCCAL INFECTION

2.5.1 Innate Immunity during Colonisation

Innate immune defence is the most crucial immune system for survival of infants in the first days and weeks of life. At birth, new-borns are confronted with a vast array of potentially pathogenic microorganisms that they were protected against and not encountered in utero. At this age, cellular components of the adaptive immune system are in a delayed state and are slow to respond (Firth et al., 2005:143; Bogaert et al., 2009:1613-1614). The innate immune system has been identified as a collection of factors, both cell-associated and cell-free, that comprises an effective and well-organized immune system that is capable of immediate recognition of microbes and microbial components and activation of adaptive immune system (Fleer & Krediet, 2007:145).

Innate immune cells such as macrophages, neutrophils and dendritic cells (DCs) directly kill the invading microorganism through phagocytosis and/or through the induction of the cytokines, which aids in the elimination of the pathogens (Kumar et al., 2009:1-12; Iwasaki & Medzhitov, 2010: 291-295). However, the protection that infants are provided with are antibodies received from the maternal immune system that are essential for defence, but represent a finite and dwindling resource. There are components of the innate immune system that are ready from the maternal influence, which are soluble, especially in the breast milk (Firth et al., 2005:150).

It is worth mentioning that maternal breast milk contains free floating oligosaccharides, lipids, mucins, lactoferrins, lactoperoxidases and toll-like receptors (TLR), proteins that provide protection to infants against a vast array of infectious pathogens as pattern recognition receptors (PRRs). Oligosaccharides are recognized to provide protection against a variety of viral and toxic agents. It is believed that oligosaccharides inhibit adhesion of pathogens by mimicking receptors present on host cells, preventing contact with the target cell surface (Morrow et al., 2005:1305). Free fatty acids and monoglycerides are lipids, which are products of digestion of milk triglycerides that occurs in the upper digestive tract of infants, with activity against some viruses (especially enveloped viruses), bacteria and protozoa that collectively inhibit growth of pathogens (Isaacs, 2005:1286; Newburg, 2005:1310). Human breast milk contains highly glycosylated mucin proteins that have been shown to inhibit replication and attachment of respiratory pathogens to epithelial cells (Newburg, 1999:120-125).

Yet another human innate defence component is none other than lactoferrin proteins, found in colostrum, milk, tears, saliva, bronchial mucus, intestinal secretions and in granules of neutrophils. Lactoferrin has been shown to have antimicrobial properties in which it binds directly to lipid A/lipopolysaccharides (LPS) and is synergistic with lysozyme in inducing membrane lesions that cleave invading pathogenic bacteria (Caccavo et al., 2002:403-412). Lactoferrin poses additional antimicrobial properties against a range of viruses infecting the gastrointestinal tract and upper respiratory tract.

According to Seganti et al. (2004:295-299), lactoferrin is capable of blocking attachment of microbes to mucosal epithelial cells and transport of zinc ions into the cytoplasm that varies with microorganisms. Another protein in human breast milk is lactoperoxidase enzyme that mediates the synthesis of a hypothiocyanate compound that is toxic to Gram-positive bacteria such as *S. pneumoniae* and also Gram-negative bacteria (Lönnerdal, 2003:1540S).

Last but not least, are the toll-like receptors that are the most crucial and important component of the innate immune defence system. TLRs can detect a wide range of exogenous and endogenous ligands such as bacterial surface molecules like peptidoglycans, lipoproteins, LPS, flagellins and intracellular bacterial deoxyribonucleic acid (DNA) (unmethylated cytosine-phosphate-guanosine (CpG)) (Takeda & Akira, 2005:1-14). Human

breast milk contains soluble forms of TLR that is (sTLR2) capable of mediating neonatal inflammatory responses during crucial initiation stages of bacterial colonisation (LeBouder et al., 2003:6686-6688).

According to Fleer and Krediet (2007:147), the mammalian family of TLRs was identified and estimated to identify at least 10-15 separate receptor proteins. It is also shown that the innate immune sensing and recognition system strength of the TLRs is specifically aimed at recognising highly conserved microbial components such as peptidoglycan, lipopolysaccharide, bacterial lipopeptides and lipoteichoic acids (LTA) (Fleer & Krediet, 2007:148).

The innate immune system is capable of initiating the complement system, in which three pathways are activated, namely the lectin, classical and the alternative complement system. These important complement systems generate the complement effector molecules that are the C3b opsonin, chemo-attractants and the C5-C9 membrane attack complex (MAC). The complement system has evolved into a multifunctional effector system, not only providing opsonins and chemo-attractants, but also cooperating with TLRs in the modulation of adaptive immune responses (Hawlisch & Köhl, 2006:13-19; Dunkelberger & Song, 2010:35-45).

2.5.2 Adaptive Immunity during Colonisation

The innate immune response pattern recognition receptors (PRRs) are somewhat responsible for the activation of transcription factors NF-kB, IRF, or nuclear factor of activated T-cells (NFAT) that are sufficient to induce both T- and B-cell responses (Figure 4). Furthermore the TLRs are capable of the activation of adaptive immune responses of several effector classes, including immunoglobulin M (IgM), IgG, and IgA antibody responses; T-helper cell 1 (TH1) and TH17 CD4+ T-cell responses; and CD8+ T-cell responses (Iwasaki & Medzhitov, 2010:291-295; Levy, 2007:379-390). The adaptive immune system consists of B- and T-cells that provide pathogen specific immunity to the host through somatic rearrangement of antigen receptor genes. B-cells produce pathogen-specific antibodies to neutralize toxins produced by pathogens, whereas T-cells provide the cytokine milieu to clear pathogen-infected cells through their cytotoxic effects or via signals to B-cells (Kumar et al., 2009:379-388).

The respiratory tract houses a collective number of tissues called Waldeyer's ring, that are also called Nasal Associated Lymphoid Tissues (NALT). This collective tissue ring is thought to contain B- and T-cells that are important in adaptive immune responses to respiratory flora, including *S. pneumoniae* (Matthias et al., 2009:6246-6247). Between 1 and 3 days after the initiation of colonization in mice, there is an influx of neutrophils due to inflammation response and uptake of pneumococcal cells in nasal spaces (Nelson et al., 2007:83-90).

The same influx of neutrophils was also observed in murine models (Matthias et al., 2009:6246). However, the inflammation response by release of neutrophils at the sterile site due to pneumococcal colonisation is short lived as it is ineffective at clearing the colonising infection.

Pneumococcus would persist with epithelial surface colonisation, even after the neutrophils have been induced at the infection site. An important inducer of the inflammatory response to the pneumococcal colonisation is pneumolysin, a member of a family of thiolactivated, cholesterol-binding cytotoxins expressed by many Gram-positive bacteria. Pneumolysin has been associated with the stimulation of neutrophil recruitment and T-cell responses. These effects of pneumolysin on the epithelial surface suggest that pneumococcus might have to promote the inflammation by increasing host response that accelerates the eventual clearance of the pathogen (Kadioglu et al., 2008:292; Matthias et al., 2009:6246).

Neutrophil interaction with the pore-forming toxin pneumolysin leads to delivery of pneumococcus to the NALT by an increase in chemokines that attract more neutrophils and correlates with more rapid clearance of *S. pneumoniae* from the nasopharynx. These results demonstrate the importance of the acute inflammatory response in pneumococcal clearance, not as a direct means of controlling infection, but rather as an enhancer of adaptive immunity (Matthias et al., 2009:6246). The complete killing of pathogenic *S. pneumoniae* by phagocytes requires opsonisation of pneumococcus by a serotype-specific antibody-complement complex. According to Malley et al., (2005:4850-4851), mice that are TLR2 deficient that initiate the inflammation response and lack expressing major histo-compatibility complex class II (MHC), exhibit delayed pneumococcal clearance and prolonged carriage respectively.

In the adaptive immune response, the antimicrobial defence mechanisms are activated indirectly by T-cells and antibodies in an antigen-specific manner. Adaptive immunity responds to pneumococcus colonising infection through antigen-specific clonal expansion of a selected number of lymphocytes whose antigen receptors bind pneumococcus antigens in the context of MHC. Naive T-cells differentiate into CD4+ T_H cells, which recruit and activate effector phagocytic cells that cooperate in pathogen clearance, and provide help for differentiation of antigen-specific B-cells into antibody-producing plasma cells and memory B-cells. Some naive T-cells also differentiate into memory cells that trigger future defence against repeated attacks by the same pathogen (Silva, 2010:805). Inflammation response on mucosal surface is reduced due to the pneumococcal expressed protease enzyme that specifically targets human IgA1.

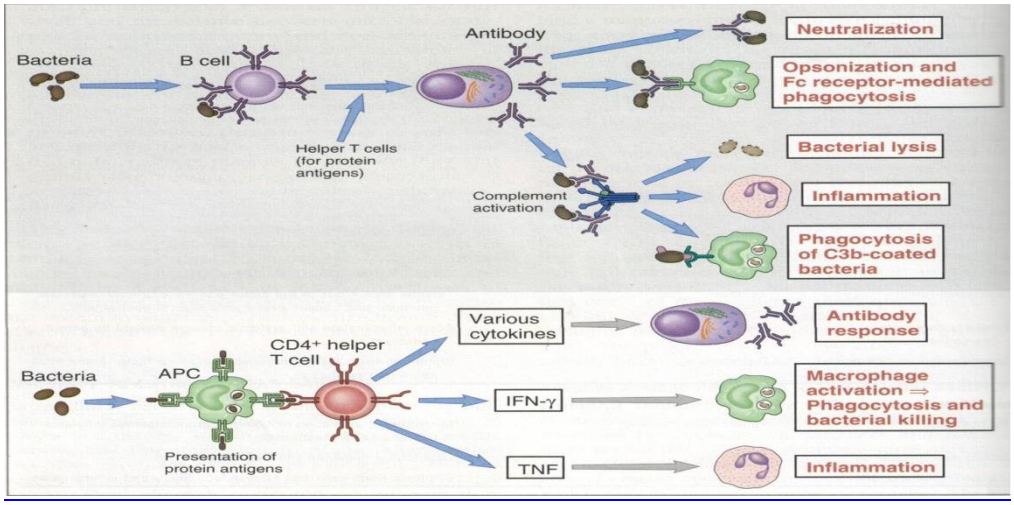

Figure 4: Adaptive immune response to extracellular microorganisms (Bacteria)
(http://thrissyguntoro.blogspot.com/2010_11_01_archive.html)

Because of the activity of the IgA1 protease, antibody mediated clearance might occur only after sufficient amounts of other classes and subclasses of specific antibody have been generated, especially humoral IgG that is leaked through the epithelial cell which is not targeted by proteases, following the use of pneumococcal conjugate vaccine and through normal contact with colonising pneumococci (Kadioglu et al., 2008:292).

2.6 PREVENTION AND CONTROL

2.6.1 Antibiotic Therapy

S. pneumoniae resistance to antibiotic therapy was discovered in Australia and New Guinea and reported by Hansman and his colleagues in 1967. Since the first reported incidence, emergence of resistant clinical pneumococcal strains was soon reported elsewhere in the world. The first antibiotic resistance reported in *S. pneumoniae* was to penicillin, which was followed by other classes of β-lactam antibiotics**,** erythromycin, clindamycin, tetracycline and chloramphenicol. Soon after 1974, penicillin resistant pneumococcus was reported worldwide. In 1977, the first case of a clinical pneumococcus strain resistant to penicillin was reported in Durban, in patients suffering from meningitis, bacteremia and pneumonia. This was later reported to have also appeared in Johannesburg (Klugman, 1990:179; Appelbaum, 1992:77). About the same year an outbreak of pneumococcal disease was reported in South African hospitals due to multidrug resistant pneumococcal clinical strains (Tomasz, 1997:S85).

2.6.1.1 Antimicrobial Therapy against Pneumococcal Infections

In the 1940s a vaccine against *S. pneumoniae* virulent serotypes was being developed from purified polysaccharide. Despite this, the vaccine development was held back due to the discovery and use of antibiotics. Even though the vaccines were still being manufactured, the use of an antibiotic as antimicrobial therapy was preferred over the vaccine. It was later reported that many people still succumbed to the colonisation and invasive disease caused by *S. pneumoniae* and died from its infections (Peltola et al., 2004:509-511). Through a lot of research, scientists discovered that *S. pneumoniae* was gaining resistance to antibiotics, especially to the over-used penicillin.

There are levels of pneumococcal resistance and the clinical impact is dependent on the definition of the resistance through the minimum inhibition concentration (MIC), but also on the pharmacodynamics of the drugs. Table 2 provides the pneumococcal resistance criteria set by the Clinical and Laboratory Standards Institute (Dagan & Klugman, 2008:785-795).

Table 2: Epidemiological breakpoints by drug[3]

Antibiotics	Susceptible[1]	Intermediate[1]	Resistance[1]
Penicillin	≤0.06	0.12-1.0	≥2.0
Amoxicillin	≤2.0	4.0	≥8.0
Cefotaxime or cetriaxon	≤2.0	2.0	≥4.0
Macrolides	≤0.25	0.5	≥1.0
Tetracycline	≤2.0	4.0	≥8.0
Levofloxacin	≤2.0	4.0	≥8.0
Co-trimoxazole[2]	≤0.5 and ≤9.5	1.0-2.0 and 19.0-38.0	≥4.0 and ≥76.0

[1]Data are minimum inhibitory concentration (MIC) (µg/mL)

[2]Co-trimoxazole is combination of trimethoprim and sulfamethoxazole with different pharmacokinetics and pharmacodynamics hence two ranges (Brown, 2014:13-21.

[3](Dagan & Klugman, 2008:785-795)

2.6.1.2 Pneumococcal Mechanisms of Antimicrobial Resistance

β-lactam antimicrobial agents are widely used in the fight against infectious microorganisms, and recently *S. pneumoniae* have become resistant to members of this class of antimicrobial agents. The cause of resistance to β-lactam antimicrobial agents is attributed to alteration of the target genes that participate in the peptidoglycan biosynthesis pathway. Since β-lactam antimicrobial agents are involved in inhibiting the biosynthesis of the cell wall, alteration of these genes increases pneumococcal resistance (Davies & Davies, 2010:418; Chewapreecha et al., 2014:4).

The interactive molecules that allow *S. pneumoniae* to bind to penicillin are Penicillin Binding Proteins (PBPs). PBPs are natural constituents of many bacteria; they are cytoplasmic membrane associated, have some essential functions, and are the primary targets of β-lactam antibiotics (Reinert, 2009:7). However, PBPs in penicillin resistant pneumococcus strains have undergone a dramatic reduction in affinity to penicillin (Tomasz, 1997:S85). The pneumococcus synthesises 6 distinct penicillin binding proteins that are: la, lb, 2x, 2a, 2b, and 3 proteins.

The mechanism of penicillin resistance in *S. pneumoniae* is based on the alterations of one or more of the PBP proteins (Arias & Murray, 2009:439-443; Davies & Davies, 2010:419), that restrict the binding of penicillin to PBPs. The acquisition of genes which encode altered penicillin-binding proteins, has facilitated resistance to β-lactam antibiotics, which is now a

common problem in the treatment of pneumococcal infections (Kadioglu et al., 2008:292). The ability of the pneumococcus to take up DNA fragments and incorporate homologous sequences into its genome is also observed in other microorganisms (Jenkins & Farrell, 2009:1260-1264).

2.6.2 Vaccines

The history of vaccines dates back as far as the 1700s, when infections caused by pathogens were treated by traditional medication. The development of vaccines came about when a physician, Edward Jenner, administered the compound he believed to have cured the disease caused by the vaccinia virus. This vaccine administration was the first to be labelled and known world-wide as a clinical trial. It was however later discovered that this vaccinia virus was in fact a strain of horsepox virus that was discovered to eradicate smallpox (Plotkin & Plotkin, 2011:889).

Since this discovery, the world of vaccine development was opened and different kinds of vaccines were developed, from bacterial to viral. Vaccines licensed for human use range from live vaccines, whole cell vaccines, component vaccines, recombinant vaccines and conjugate vaccines (Bonanni & Santos, 2011:1-24).

Live vaccines are predominantly active viruses and bacteria with their virulence weakened, hence the name live attenuated vaccines. Because attenuated vaccines contain a live organism, it is able to replicate and stimulate immune response without causing disease (Minor, 2015:379-392).

Whole cell vaccines are disease causing viruses and bacteria with their virulence inactivated and unable to replicate. However, since the vaccine antigen is inactivated it may not be affected by circulating antibodies, the way live antigens are. Furthermore, whole cell vaccines may stimulate a weaker immune response that requires several doses to stimulate sufficient immune response (Keenan & Jaffee, 2012:276-286).

Conjugate vaccines are in most cases an outer coating of bacteria, a capsule which is a polysaccharide conjugated to an immune provoking protein. Capsular polysaccharide is not immunogenic but a highly virulent component of bacteria that provides protection to the cell

from circulating antibodies (Soininen et al., 1998:561). Conjugate vaccines do not cause disease the same as whole cell vaccines; however the difference between the two is that conjugate vaccines contain only a subunit of the microorganism chemically linked to a carrier protein (Goldblatt, 2000:1).

One benefit of conjugate vaccines that was realised during disease protection was that it interrupts the circulation of the organism through reduction of colonisation; this resulted in herd immunity with protection of non-vaccinated individuals and near elimination of the pathogen (Rappuoli et al., 2011:361).

2.6.2.1 Pneumococcal Vaccine Types

It is believed that the fight against infectious bacteria, especially encapsulated ones, could be prevented by vaccines containing capsular polysaccharide. The reason this notion is widely believed so much is due to the fact that capsular polysaccharide is the most virulent component of the encapsulated bacteria (Weller et al., 2005:85). Wright and his colleagues in 1914 reported the results of the prevention of pneumococcal pneumonia and death in black South African miners by injecting them with killed whole cell *S. pneumoniae* (Austrian, 1999:2; French, 2003:78).

In the 1970's, during antibiotic resistance by meningococcal and pneumococcal infections, the development of both meningococcal and pneumococcal vaccines, plain and purified capsular polysaccharides were used. The vaccines were effective in inducing an immune response to infections that saw a decrease of infections associated with vaccine serotypes and the vaccine immunogenicity was established. The problem with this was that the immunogenicity of plain capsular pneumococcal vaccine was very high in adults as opposed to children, especially the vulnerable infants under 24 months (French, 2003:78-79; Zepp, 2010:C14-C24).

There are two types of licensed pneumococcal vaccine viz. the plain polysaccharide vaccine and the polysaccharide conjugated (chemically linked) to a protein carrier (Peltola et al., 2004:509-511). These vaccines are based on the recognition of different and multiple serotypes of *S. pneumoniae* (French, 2003:78-80). The plain pneumococcal vaccines contain purified capsular polysaccharides of 23 pneumococcal serotypes marketed as 23-valent

pneumococcal vaccine (23vP) (Moffitt & Malley, 2011:407). The 23vP provides effective immunity against most prevalent and infectious serotypes the pneumococcus. The first successful conjugate vaccine was the PCV7 that saw the decrease in vaccine serotype infections in infants and children below the age of two years (Pletz et al., 2008:199).

It is believed that children under two years of age, who are below required minimum pre-vaccination serotype-specific antibody concentrations, find the immune benefit from conjugate vaccines such as PCV7 after post-vaccines antibody measurements; while those that carry above the minimum pre-vaccination serotype-specific antibody concentrations may struggle to induce higher post-vaccination serotype-specific antibody concentrations (Paris & Sorensen, 2007:462-464).

2.6.4 Immune Response to Vaccines

It is important that the type of antigen that makes up the vaccine mimics the antigen presented by the pathogen that is causing the infection since the vaccine's aim is to elicit response towards the immunogenic and virulent molecules that assist the pathogens to initiate an infection.

A study conducted by Clutterbuck and workers (2012:1408) described how 23vP was unable to induce attenuated splenic marginal zone B-cell response in children under 24 months of age. They demonstrated how the pneumococcal capsular antigen was incapable of inducing a T-cell dependent response (Clutterbuck et al., 2012:1408-1409). T-cell dependent immune response is required in infants and children under two years of age as it induces immunologic memory (Balmer & Borrow, 2004:77). See figure 5 for picture illustration.

2.7 IMMUNE RESPONSE MEASUREMENT

To assess the quality of immune response, whether natural or vaccine induced, laboratory correlates of protection are most important and useful in determining the immunogenicity. It is the best practice to measure the concentration of antibodies synthesised in response to an immunogenic antigen borne naturally and induced through vaccination (Balmer & Borrow, 2004:78). The assessment of immune response is interpreted by the measurement of serotype-specific antibody concentration, measured by enzyme-linked immuno-sorbent assay (ELISA).

The principle of ELISA is to mimic the interactions that occur in nature (*in vivo*) whereby the antigen is bound by a complementing antibody, forming an antigen-antibody complex. This is the step in which the antigen is presented to phagocytes for opsonisation. ELISA however, has been the method used in quantifying to the antigen-antibody complex. It is capable of measuring the amount of specific antibody synthesised by the individual immune response to specific infectious substance or molecule (Gan & Patel, 2013:1-3).

The use of differing competitive ELISA assays provides specific but different immunoglobulin measurements to pneumococcal serotype antigens. The WHO standard ELISA methods were developed for world-wide use to measure antibodies against pneumococcal serotype antigen, so that the laboratories results are standard and comparable (Balloch et al., 2010:1333-1334).

The WHO ELISA assay incorporates the absorption of pneumococcal capsular polysaccharides (CPS) and a capsular polysaccharide 22F that increases the assay specificity and improving the assay correlation with functional antibody measurements. This WHO assays provide a specific, reproducible, accurate and standardized pneumococcal antibody assay with acceptable inter- and intra-assay applications that allow for comparison of results between laboratories to be advocated internationally (Balloch et al., 2010:1334; Balloch et al., 2013:335-336).

Parallel to the ELISA assays there are new technologies that enable the detection and measurement of pneumococcal serotype-specific antibodies in a multiplex format. Multiplex technologies such as Luminex® bead-based assay provides for simultaneous measurement of serotype-specific immunoglobulin with high efficiency in a single sample, requiring a small sample volume that gives high dynamic range and increased throughput (Goldblatt et al., 2011:1744-1751).

It has also been reported that the use of Luminex® multiplex bead-based assay has very good inter- and intra assay accuracy, precision and reliability when evaluated (Balloch et al., 2013:336). The multiplex assay is similar to the WHO ELISA method in that the CPS and 22F pneumococcal antigens are also used to absorb cross-reactive epitopes (Borgers et al., 2010:198-199).

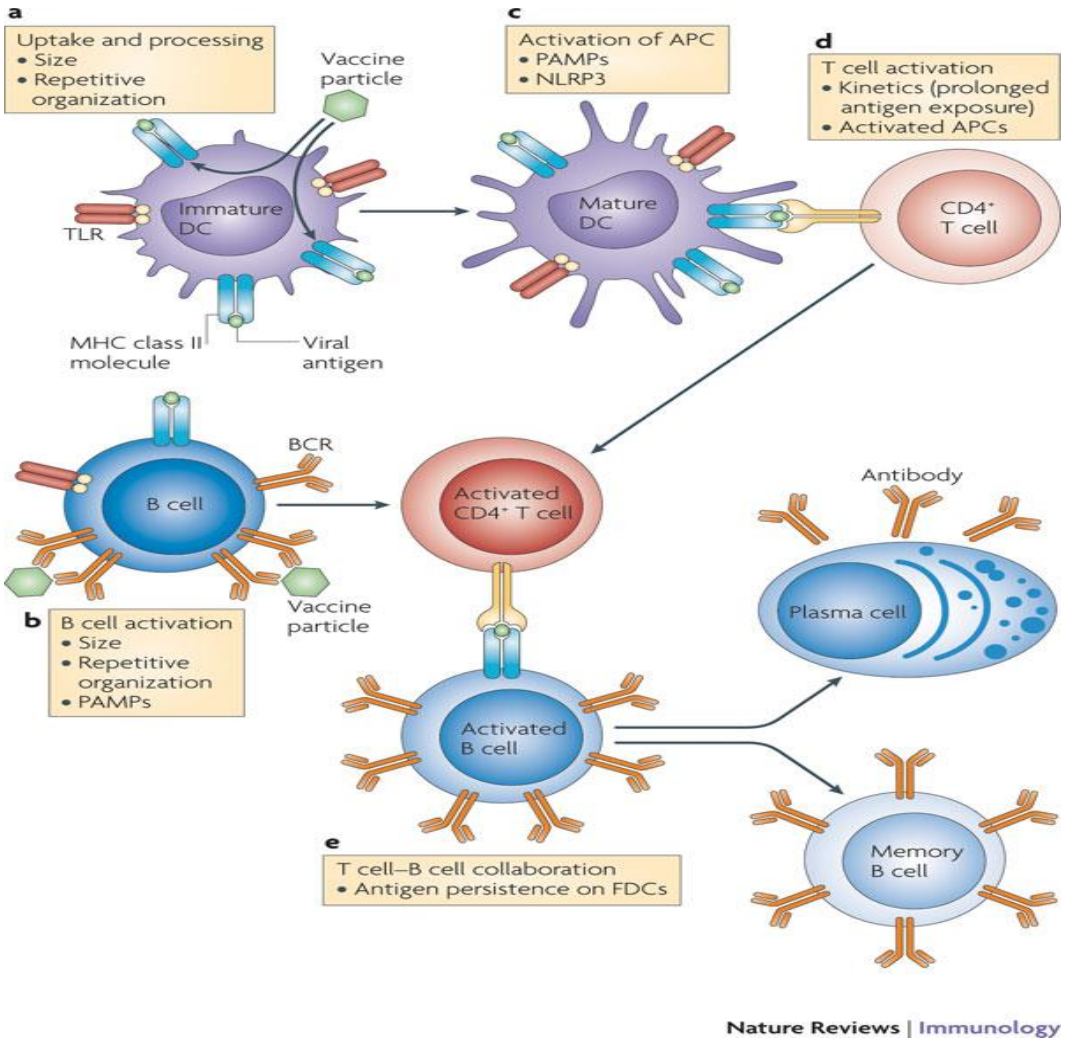

Figure 5: Immune response to vaccination.

(http://www.nature.com/nri/journal/v10/n11/images/nri2868-f1.jpg)

An antigen presenting cell (APC) is the link between innate and adaptive immune responses. After engulfment, a pathogen is processed and parts of it are presented by the major histocompatibility complex (MHC) to the T-cell receptor (TCR). A pathogen-associated molecular pattern (PAMP) is recognised by a Toll-like receptor (TLR). Other co-stimulatory signals are also expressed by the APC through intracellular signalling. Cytokines like interleukin (IL) are secreted and play an important role in the further proliferation of the T-cells and the specific ratio of T-helper cells (Th1,Th2,fTh,Th17).The profile of activated T-helper cells guides the gradient of subsequent humoral and cell mediated responses

2.8 CONCLUSION

S. pneumoniae is a Gram-positive bacterium that normally inhabits the human nasopharynx as normal flora. It usually colonises the upper respiratory tract and spreads to other parts of the body to cause disease, especially in the lungs, the blood and the meninges. There are about 90 distinct serotypes that are differentiated by the makeup of the capsular polysaccharide, a very virulent substance of the pneumococcus. The capsular polysaccharide is capable of evading a host immune response with the aid of surface virulence proteins. However, a matured immune system of a host can defend against the disease initiation by responding with specific antibodies to the capsule and the proteins. This mechanism of defence is only effective in adults and children over the age of 2 years. An infant's antibody response is incapable of responding to capsular polysaccharide infection, without help from a conjugate vaccine. Pneumococcal conjugate vaccine is manufactured to assist with priming the infants' immune system to elicit a T-cell dependent immune response to capsular polysaccharide. This also assists in the generation of memory B-cells' immune response for the continuous encounter of the same capsular polysaccharide. The current study was established to evaluate the mucosal and humoral antibody response to capsular polysaccharide of the 7-valent vaccine serotypes.

CHAPTER 3

METHODS AND MATERIALS

3.1 INTRODUCTION

The content of this dissertation forms part of a major study sponsored by the National Institute of Health (NIH) The Division of Acquired Immunodeficiency Syndrome (DAIDS) programme, Comprehensive International Programme for Research on AIDS project 4 (CIPRA-ZA 004) entitled "Evaluation of quantitative and qualitative antibody response to *S. pneumoniae* and *H. influenzae* type b conjugate vaccine amongst HIV-1-exposed-infected children that are receiving vs. those not receiving antiretroviral therapy, as well as among HIV-1-exposed-uninfected children and HIV-1-unexposed-uninfected children". The study was conducted at Chris Hani Baragwanath Hospital in Johannesburg and at Tygerberg Hospital in Cape Town between 2005 and 2012. In addition to the main study, several sub studies were added to the parent study that involved the collection of saliva from infants and their mothers, including additional sampling points for pneumococcal colonization.

3.2 ETHICAL CONSIDERATIONS

During enrolment, the parent or the legal guardian of the infant (the participant in this study) received information about the study and they provided the written informed consent for the child's participation in the study. The study protocol (CIPRA-ZA 004 Human Research Ethics Committee (HREC) # 040704) and informed consent document was reviewed and approved by Institutional Review Board (IRB) of the Human Research and Ethics Committee of the University of the Witwatersrand in Johannesburg and the Committee for Pharmaceutical Trials of the University of Stellenbosch in Tygerberg, South Africa, prior to the enrolment of the study participant.

3.3 STUDY DESIGN AND POPULATION

3.3.1 Study Design

This study falls within a retrospective analysis of a prospective cohort study on the safety and immunogenicity of PCV7 and the immunogenicity of a *H. influenzae* type b conjugate vaccine (HibCV). It consisted of 563 enrolled children, enrolled within the Expanded Programme on Immunisation in South Africa (EPI-SA). The vaccination schedule is based on the World Health Organization (WHO) and South African Department of Health guidelines. In addition, qualitative and quantitative antibody responses to pneumococcal conjugate vaccine was assessed. Following three doses of PCV7 and HibCV, administered at approximately 6 (6-12), 10 (9-18) and 14 (12-24) weeks of age, the children were randomized within each group to receive either a booster dose of PCV7 or HibCV between 64 and 76 weeks of age. Antibody response to the study vaccines was assessed on HIV-1-uninfected (exposed and unexposed) and HIV-1-infected children stratified by antiretroviral therapy status, who were born and/or receiving care at the Chris Hani Baragwanath Hospital in Johannesburg, and at the Tygerberg Hospital in Cape Town, South Africa (Table 3).

3.3.2 Study Population

The enrolment age of infants was between ≥ 4 and ≤ 10 weeks with a birth weight of at least 2000 grams and these children were enrolled according to HIV status as follows: HIV-1-infected, HIV-1-exposed uninfected and HIV-1-unexposed uninfected children. HIV-1 uninfected and unexposed participants were identified at the time of delivery as part of the established routine antenatal screening in the obstetrics wards of the two hospitals. The HIV-1 uninfected, exposed participants were recruited from children whose HIV-1 PCR tests were negative during screening or through any other programme that screens for HIV infection status at 4-10 weeks of age. A total number of 665 subjects were enrolled; due to an estimated attrition rate of 20% (for any reason), the number of “evaluable” subjects was expected to be 563.

Additionally there was data used from a group of unvaccinated children from a mother to child study where sampling was done which is similar and used in this study to compare the vaccinated from the unvaccinated.

3.3.2.1 Inclusion Criteria

- The parent or legal guardian of the infant must be able and willing to provide written informed consent and comply with all study requirements, including the ability to complete the Vaccine Reaction Diary.
- The parent or legal guardian of the infant must indicate the intention to remain in the study area for the duration of the trial.
- Documentation of the mother's HIV status after 24 weeks of gestation of the child if the child is HIV uninfected. Prior source documentation of HIV infection in the mother, at any time, is considered adequate documentation of HIV status. Documentation of the infant's HIV infection is considered adequate documentation of the mother's HIV status.
- A breastfed child may be enrolled in the study.
- Antiretroviral naïve except for receipt of antiretroviral drugs used to prevent mother-to-infant transmission.

3.3.2.2 Exclusion Criteria

- Receipt of any blood products (e.g., Intravenous Immunoglobulin (IVIG), Varicella Zoster Immune-globulin (VZIG), Hepatitis B Immune-globulin (HbIG), transfusion, etc.) prior to study entry.
- Receipt of any vaccine, with the exception of BCG and oral polio vaccine, prior to study entry, if HIV uninfected; HIV infected children with prior vaccinations will be allowed to participate.
- Receipt of any immunosuppressant agent for more than 2 weeks, within one week of study entry.
- Inability to tolerate oral medications.
- Presence of any major congenital anomalies that are life threatening.
- Infants with an acute, moderate to severe, inter-current illness or fever of >38°C within 72 hours prior to immunization that requires hospitalization. Note that those children subject to this exclusion may be re-screened for the study at a later time after symptoms resolve.
- Parent/legal guardian unable or unwilling to attend regularly scheduled study visits.

- Use of the following medications: 1. All antiretroviral therapies other than the regimens described in CIPRA-ZA, 2. All investigational drugs, 3. Systemic cytotoxic chemotherapy and 4. Interleukin or other immune modulators.

3.3.3 Stratification of Study Groups

The Study participants were stratified into four groups, namely group 1, group 2, group 3 and group 4. Study participants in group 1 were children who are HIV-1-uninfected born to HIV-1-uninfected mothers. Group 2 study participants from CIPRA-ZA project 2, were HIV-1-infected with functional CD4+ T-lymphocytes of ≥25% who were on delayed Anti-Retroviral Treatment (ART) therapy.[2]

Table 3: The four stratified groups

Group	HIV-1 Status[1]	Available Sample Size	CDC Disease Classification (CD4 T-lymphocyte %)	ART Status
1	HIV-1-uninfected, unexposed	124	Not Applicable	Not Applicable
2	HIV-1-infected	104	Category 1 (CD4 T-Cell % ≥25%)	Delayed ART[2]
3	HIV-1-infected	210	Category 1 (CD4 T-Cell % ≥25%)	Receiving ART from age 6 to 12 weeks onwards[3]
4	HIV-1-uninfected, exposed	125	Not Applicable	Not Applicable[4]

[1] HIV infection has been identified through HIV-1DNA/RNA PCR test performed between 4 and 10 weeks of age and by a second HIV-1 RNA PCR test (>10 000 copies/ml) within that window period, before study entry and receipt of the first dose of PCV7 and HibCV.

[2] Infants randomised to the "delayed therapy[3]" arm (ART-delayed).

[3] Infants randomised to the "early therapy[4]" arms (ART-40W) and (ART-96W) – ART continued until their first and second birthday, respectively.

[4] HIV-uninfected exposed infants have been identified through a non-reactive HIV DNA PCR test performed at 4 and 10 weeks of age before entry into the study. Repeat PCR testing was performed amongst children in this group at 6 months of age (qualitative HIV DNA PCR assay) and again at 18 months of age (HIV ELISA).

[2] Infants with a baseline CD4 percentage (CD4%) of at least 25% and HIV infection diagnosed between 6 and 12 weeks of age were randomly assigned to deferred ART treatment strategy arm (ART-delayed). Those who were initially deferred treatment in ART-delayed were reassessed for initiation of first-line, continuous ART.

[3] Participant received study ART later in the study, unless CD4+ cells falls below 25% then they immediately received ART.

[4] Participant received study ART in the bigining of the study, wether CD4+ cells above or below 25%.

Group 3 study participants from CIPRA-ZA project 2, are HIV-1-infected with functional CD4+ T-lymphocytes of ≥25% who were on early ART therapy respectively.[5] And lastly group 4 study participants who are HIV-1-exposed born to HIV-1-infected mothers, presumed uninfected children (Cotton et al., 2013:1555-1563). See Table 3 below for the stratification and sample size.

3.4 STUDY VACCINE ADMINISTRATION

A primary series of three doses of PCV7 were administered at the beginning of enrolment at 6-24 weeks of age, followed by a booster dose at 64-76 after initial 3 doses. The first dose of PCV7 was administered at 6-12 weeks of age (Study Visit 2). Two subsequent doses of PCV7 were administered at 3-6 weeks after receipt of the proceeding dose, i.e. at 9-18 (Study Visit 3) and 12-24 weeks of age (Study Visit 4). A booster dose of PCV7 was administered to children randomised to receive either the PCV7 and /or combined diphtheria-tetanus-whole cell pertussis (DTwP) and *H. influenzae* type b (Hib) conjugate vaccine (CombAct-Hib®, Wyeth) at 64-76 weeks of age (Study Visit 7).

Table 4: Study and routine paediatric vaccination schedule

Vaccination	**Dose 1**	**Dose 2**	**Dose 3**	**Booster**
Study Visit No.	**2**	**3**	**4**	**7**
Age in Weeks	**6-12**	**9-18**	**12-24**	**64-76**
PCV7 (Prevnar®)[1]	√	√	√	√[2]
HibCV-DTwP (CombAct-Hib®)[3]	√	√	√	√[2]

[1] PCV7 is the study vaccine. All other vaccines administered through this study are routine paediatric vaccines that are recommended and administered through the EPI in South Africa. All routinely administered paediatric vaccines were accessed from the Expanded Program for Immunisation in South Africa. Each of the sites maintained vaccine accountability log in accordance with Expanded Program for Immunisation in South Africa.

[2] Within each Group, subjects were randomised 1:1 to receive either a booster dose of PCV7 or HibCV at 64-76 weeks of age. Although HibCV booster is not part of the EPI, it is approved and is given routinely in the private sector. It is not part of the EPI due to cost concerns.

[3] HibCV-DTwP (Hib PRP-tetenus toxoid conjugate vaccine, reconstituted wit DTwP) is administered as a combination vaccine, CombAct-Hib® (HibCV + diphtheria toxoid + tetanus toxoid + whole cell pertussis).

[5] Infants with a baseline CD4 percentage (CD4%) of at least 25% and HIV infection diagnosed between 6 and 12 weeks of age will be randomly assigned to two ART treatment strategy arms. Infants will receive ART for approximately 40 weeks until their first birthday (ART-40W) and infants will receive ART for approximately 96 weeks until their second birthday (ART-96W). Treatment in both arms will begin with first-line, continuous treatment of zidovudine, lamivudine, and lopinavir/ritonavir.

The schedule for vaccination is listed in Table 4, which illustrates the timing of administration of the study vaccine and routinely administered paediatric vaccines recommended by the Expanded Programme on Immunisation in South Africa (EPI-SA).

3.5 VACCINES

3.5.1 Pneumococcal Vaccine Formulation

In this study, Prevnar® (Wyeth, West Henrietta, New York (NY)), PCV7 is considered the study vaccine which was introduced into South Africa's Expanded Programme on Immunisation schedule in 2008, although it has been licensed for use since May 2003. Prevnar® contains a sterile solution of polysaccharide of capsular antigen of *S. pneumoniae* serotype 4, 6B, 9V, 14, 18C, 19F and 23F, that are individually conjugated to diphtheria CRM_{197} protein. Each 0.5 mL dose is formulated to contain 2 μg of polysaccharide for serotype 4, 6B, 9V, 14, 18C, 19F and 23F, and 4 μg of serotype 6B per dose (16 μg of total polysaccharide) and approximately 20 μg of CRM_{197} carrier protein. The vaccine also contains aluminium phosphate as an adjuvant. It is presented as a white, homogeneous suspension.

3.5.2 Other Vaccines

Children in this study also received the normal/routine childhood vaccine that is included in South Africa's EPI schedule (Baker, 2010:18-21). They received a combined diphtheria-tetanus-whole cell pertussis (DTwP) and *H. influenzae* type b (Hib) conjugate vaccine (CombAct-Hib®, Wyeth), at 6, 10 and 14 weeks of age, then followed by randomised booster dose of combined diphtheria-tetenus-whole cell pertussis (DTwP) and *H. influenzae* type b (Hib) conjugate vaccine (CombAct-Hib®, Wyeth) at 68 weeks of age.

3.6 SAMPLE COLLECTION

3.6.1 Serum Samples

Serum samples from children were collected into a 4 mL red capped blood collection vial, with clot activator (Becton Dickson (BD) Vacutainer® cat# 367812, Franklin Lakes, United States of America (USA)) by a study doctor or phlebotomist. The blood collected was allowed to clot in the tube for about 30 to 60 min. The collection tube containing clotted blood was centrifuged at 4000 rpm for 10 min, and the serum was divided into two equal volumes and aliquoted into cryotubes with a sterile 1 mL pipette tip and stored in a -70°C freezer until analysis. Table 5 provides the detailed schedules for the sample collection, evaluation and immunisation.

Table 5: Sample collection schedule, evaluation and vaccination

Study Visit Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Study Visit Code		**01.0**	**03.0**	**04.0**	**05.0**	**10.0**	**17.0**	**18.0**	**28.0**
Age in Weeks	**4-10**	**6-12**	**9-18**	**12-24**	**15-30**	**38-42**	**64-76**	**65-78**	**102-116**
Signed Consent[1]	√	√							
Counseling for HIV Status[2]	√	√							
Vaccination[3]		**1st Dose**	**2nd Dose**	**3rd Dose**	**3-6 weeks after study visit 4**		**Boosters**	**1-2 weeks after study visit 7**	
Serum Antibody Conc[4]		√	√	√	√	√	√	√	√
Saliva Antibody Conc[5]					√	√	√	√	√
NP Swab for Pnc Carriage		√	√	√	√	√	√	√	√

[1]Signed consent was obtained from the parent /legal guardian of the child prior to any study procedures being performed.

[2]All parents/legal guardians were offered pre- and post-test counselling regarding evaluating their children for HIV-1 infection. For HIV exposed children, this was done as part of the enrolment screening procedure for inclusion into CIPRA Project 2 or through other programs that screen the child for HIV infection at 4-10 weeks of age.

[3]The following vaccines were provided: Prevnar® (PCV7); CombAct-Hib® (HibCV-DTwP); Hepatitis B; Trivalent® Oral Polio Vaccine; Primary Measles Vaccine.

[4]Blood was drawn for Pnc concentration evaluation at visit number 2 (before administration of the first dose of vaccine) for baseline concentration evaluation. Antibody assays were performed for all 7 serotypes included in PCV7 and a control serotype 7F not included in PCV7.

[5]Saliva was first collected at visit number 5 then continued till visit number 9. Antibody assays were performed for all 7 serotypes included in PCV7 and a control serotype 7F not included in PCV7.

3.6.2 Saliva Samples

Saliva samples from children were collected with an Oracol® device (Malvern Medical Developments Ltd., Worcester, United Kingdom (UK)) which consists of a cylindrical plastic sponge mounted on a short plastic stick. The tongue, gums and teeth were brushed by the Oracol sponge for 60 seconds to absorb saliva. The Oracol sponge was then placed into an empty Oracol tube, centrifuged at 4000 rpm for 5 min, to extract the saliva from the sponge into the Oracol tube. The collected saliva sample was divided into two equal volumes and aliquoted into cryotubes with a sterile 1 mL pipette tip and stored in a -70°C freezer until analysis.

3.7 IMMUNOLOGICAL LABORATORY ANALYSIS

3.7.1 Enzyme-Linked Immunosorbent Assay (ELISA)

3.7.1.1 ELISA Principle

The ELISA (ENZYME-LINKED IMMUNOSORBENT ASSAY) is a widely used biochemical technique for the detection of an antibody in a sample. An inhibition indirect ELISA is applied when an antigen is diluted in a buffer that contains no other antigens that might compete with the target antigen for passive attachment to the plastic solid phase during a period of incubation. A sample with target antibody is diluted in a buffer to allow attachment to antigen adsorbed on solid phase. An enzyme labelled antibody is then diluted in a buffer containing detergent at low concentration, which inhibits passive adsorption (blocking) of non-target proteins, but that still allows immunological binding. The substrate is then added to allow development of a colour reaction through catalysis of the enzyme conjugated to the antibody. After a period of time the reaction is stopped and the colour is quantified by the use of a spectrophotometer reading at the appropriate wavelength.

3.7.1.2 ELISA Procedure

Serum IgG concentrations against pneumococcal capsular polysaccharides serotypes 4, 6B, 9V, 14, 18C, 19F and 23F were measured by a competitive ELISA. Optimal coating concentrations were determined for each antigen and ranged between 5 μg/mL and 10

μg/mL. 96 well microtiter plates (MaxiSorp^{TM}, Nunc, Roskilde, Denmark) were coated with 100 μL per well of the pneumococcal capsular polysaccharides (PncPSs) in 1x phosphate buffer solution (PBS) pH 7.2 (Each serotype for 1 microtiter plate). The plates were either incubated for five hours at 37°C or overnight at room temperature. After incubation the plates were washed four times with 1x phosphate buffer solution with Tween 20 (PBST) on a microtiter plate washer (SkanWasher 400, Titertek) and stored at 4°C and used within 20 working days.

Frozen serum samples were allowed to thaw at room temperature and tested at 1:100 and then further diluted if required. Human Serum 89-SF was used as a reference serum for the pneumococcal capsular polysaccharide antibody concentration measurement of IgG. Serum samples were diluted in 1x phosphate buffer solution with Fetal Bovine Serum (PBSF) containing 10 μg/mL CPS and 30 μg/mL 22F[6] to absorb and remove the anti-CPS and poly-reactive antibodies, while the reference serum was only neutralised with 10 μg/mL CPS in 1x PBSF. The samples were incubated at room temperature for 30 min to allow the neutralising effect to take place. The samples were assayed at four serial three-fold dilutions in duplicate, the reference serum at seven serial two fold dilutions in duplicate. Each Pnc serotype plate was blocked with 1x PBSF and incubated for one hour at 37°C. After incubation plates were emptied without washing.

Samples, controls and reference serum were aliquoted 100 μL/well and incubated for two hours at 37°C. After the incubation, the plates were washed four times with 1x (PBST). Alkali phosphatase conjugated goat anti-human IgG (A-3188, Sigma, St. Louis, Missouri (MO)) was diluted (1:1 000) in 1x PBSF, aliquoted 100 μL/well and incubated for two hours in 37°C. After the incubation, the plates were washed three times with 1x PBST and twice with deionised water. Substrate, p-nitrophenyl phosphate disodium Salt Hexahydrate (71768, Sigma, St. Louis, MO) 1 mg/mL in carbonate buffer pH 9.8, aliquoted 100 μL/well and incubated for one hour at room temperature. Immediately after the incubation, plates were measured on the microtiter plate reader (Multiskan plus II) at an absorbance at 405 nm and results expressed as optical densities (OD). Controls, specifically high and low controls as

[6] Poly-reactive antibodies are prevalent in serum of some individuals and recognise pneumococcal polysaccharides of some different serotypes. Inhibiting cross-reactivity and antibody binding, using soluble polysaccharides of serotype 22F removes these poly-reactive antibodies which improves specificity.

positive controls, were run with every plate along with plain phosphate buffer solution with Fetal Bovine Serum and sodium azide (PBSFN) as a blank (negative control).

3.7.1.3 ELISA Data Interpretation

For the ELISA analysis, data acquisition was performed using ELISA software provided by the Centres for Disease Control and Prevention (CDC). For each analyte, optical density at a wavelength of 405 nm was converted to µg/mL by interpolation from a 4-parameter logistic reference curve. Lower limit of quantification (LLOQ) was determined for each Pnc serotype, and for statistical analysis, concentrations below the LLOQ were assigned half the LLOQ.

3.7.2 Luminex

3.7.2.1 Luminex Principle

Luminex technology is built on proven, existing flow cytometry, microspheres, lasers, digital signal processing and traditional chemistry that have been combined in a unique way. Featuring a flexible, open-architecture design, Luminex technology can be configured to perform a wide variety of bioassays quickly, cost-effectively and accurately.

Firstly, Luminex color-codes tiny beads, called microspheres, into 100 distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. Within the Luminex compact analyser, lasers excite the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. Many readings are made on each bead set, further validating the results. In this way, Luminex technology allows multiplexing of up to 100 unique assays within a single sample, both rapidly and precisely.

3.7.2.2 Luminex Procedure

The Bio-Rad beads (microsphere) were coupled to purified PncPSs (American Type Culture Collection (ATCC), Rockville, Maryland (MD), USA)) (1 bead colour for each Pnc serotype) with cross-linking agent 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM**) (**Aldrich, Buchs, Switzerland) as follows: 40 mg of DMTMM was added

into 2.7 mL of 1 mg/mL PncPSs, vortexed and then incubated at room temperature on a rotating shaker for 60 minutes. A desalting column, Sephadex G25 PD-10 (General Electric (GE) Healthcare, Buckinghamshire, UK) was used to separate the PncPS-DMTMM complex from free DMTMM by eluting 2.5 mL of PncPS-DMTMM with 3.5 mL 1x PBS pH 7.2. Half a millilitre of PncPS-DMTMM was added to a 1.5 mL microcentrifuge tube containing 1.25 x 10^7 beads/mL. The microcentrifuge tube was covered with foil and incubated overnight at room temperature on a rotating shaker at 500 rpm. After incubation, the bead-DMTMM-PncPS complex was washed twice with washing buffer 1x Phosphate Buffer Solution with Tween 20 and Sodium Azide (PBSTN) and stored in 1mL of 1x assay buffer (PBSFN) at 4°C in the dark (foiled). A haemocytometer slide was used to count the beads after coupling.

Saliva samples were allowed to thaw at room temperature and the saliva assay was run in a multiplex format. Human Serum 89-SF from the U.S. Food and Drug Administration, (Bethesda, MD, USA) was used as reference serum for the pneumococcal capsular polysaccharide antibody concentration measurement of IgA and IgG (Quataert et al., 1995: 590-597). Seven 4-fold serial dilutions starting at 1:100 of reference serum 89-SF were prepared in PBSFN pH 7.2 and 10 μg/mL CPS antigen was added to neutralise the CPS antibodies. Saliva samples were diluted in 1x PBSFN containing 10 μg/mL CPS (Statens Serum Institute, Copenhagen, Denmark) and 30 μg/mL 22F (ATCC, Manassas, Canada) to neutralise the CPS and poly-reactive antibodies, with a minimum required dilution (MRD) of 1:10 and if they were above the limit of detection (LOD), they were further diluted.

Each dilution of the reference serum and the sample (50 μL) was mixed with an equal volume of coupled microspheres (3500 bead/region/well) in a 96-well AcroPrep™ filter plate (Pall Life Sciences, Ann Arbor, Michigan (MI)) prepared in PBSFN. The filter plates were incubated at room temperature on a plate shaker at 500 rpm. After incubation, the beads in the filter plate were washed three times with 100 μL PBSTN by filtration with a vacuum manifold. To each well, 50 μL of 1:100 dilution of R-phycoerythrin (R-PE) Goat anti human serum IgA (Cat#109-155-011) for IgA measurement and R-PE Goat anti human IgG (Cat#109-155-098) for IgG measurement (Jackson's Immuno Research Laboratories, West Grove, Pennsylvania (PA)) in PBSTN were added and the plate was incubated for 30 min at room temperature on the plate shaker at 500 rpm. After the second incubation, the beads in the filter plate were washed three times with 100 μL PBSTN, the beads were then

resuspended in 130 µL PBSTN and transferred to a round bottomed 96 well reading plate (Sterilin Ltd., United Kingdom) before analysis with a Bio-Plex 200 in combination with Bio-Plex Manager Software version 5.0 (Bio-Rad Laboratories, Hercules, California (CA)). High and low control samples and a blank consisting of buffer without sample were used as positive and negative controls, and were run with every plate for quality control purposes.

3.7.2.3 Luminex Data Interpretation

The Bio-Plex manager software version 5.0 was used to determine the IgG and IgA concentrations for each analyte (antigen). The program uses the median fluorescent intensity (MFI) of the duplicate samples and converts it to µg/mL by interpolation from a 5-parameter logistic reference curve (log(x)-linear(y)) for every bead region/standard.

3.8 STATISTICAL DATA ANALYSIS

Differences in log-concentrations between groups were tested using Student's T test, linear regression and also using the correlation test. The analysis was conducted with the Stata program (Statacorp, College Station, Texas (TX)).

The results were given as geometric mean antibody concentrations (GMC) with 95% confidence intervals (CI) in micrograms per milliliter (µg/mL). Non-parametric statistical methods were used, because of the non-normality of salivary antibody data. Antibody concentrations at different ages were compared using the Wilcoxon signed ranks test. Differences in antibody concentrations between vaccinated groups were analysed with the Kruskal-Wallis and Mann-Whitney tests. Log-transformed salivary IgG and IgA and concentrations were compared by Pearson's correlation analysis. Differences were considered statistically significant when the p-value was <0.05.

3.9 CONCLUSION

This is a sub-study to a main study that looked at the evaluation of quantitative and qualitative antibody response to *S. pneumoniae* and *H. influenzae* type b conjugate vaccine amongst HIV-1-exposed-infected children who received vs. those did not receive antiretroviral therapy, as well as among HIV-1-exposed-uninfected children and HIV-1-unexposed-

uninfected children. Pneumococcal conjugate vaccine was administered in three primary doses to four groups of HIV-1-uninfected unexposed, HIV-1-uninfected exposed and HIV-1-infected children on early and delayed ART therapy. A booster dose vaccine was administered randomly to half of the study participants after their first birthday. Serum and saliva samples were collected at various sample-points to determine the antibody concentration levels to vaccine serotypes. Two immunoassays were employed to measure the antibody concentration levels, IgG ELISA assay for serum samples and IgA and IgG Luminex multiplex assay for saliva samples. Data were collected as OD for ELISA and FI for Luminex that was converted to concentration and analysed statistically.

CHAPTER 4

RESULTS

4.1 INTRODUCTION

This chapter of the dissertation describes the data obtained in response to the objectives. Data from the first objective is obtained from the measured calibration, optimisation and validation with control samples. The data provided insight on assay performance and reproducibility that offered it trustworthy for the measurement of target samples. Data obtained from the remaining objectives is explained in detail by tables and figures, and also described in full for the benefit of giving life to aim and objectives of this study.

4.2 GENERAL CHARACTERISTICS OF THE STUDY POPULATION

The samples were collected from five sample points as described in the methods section. At each of the five sample points, saliva and serum samples and their assay measurement were recorded and later analysed statistically. Each sample point represents mean age in weeks of children enrolled in the study where the sample was collected. Table 6 provide the mean age in weeks with 95% Confidence Interval (CI) for each sample point. HIV status of each group is provided in Table 7.

Table 6: Sample points

Age[1]	15-30 Weeks	38-42 Weeks	64-76 Weeks	65-78 Weeks	102-116 Weeks
Vaccinated	19.5 (19.4-19.6)	39.6 (39.4-39.8)	67.8 (67.3-68.4)	69.9 (69.4-70.4)	111.3 (110.4-112.2)
Unvaccinated	19.2 (19.0-19.5)	40.7 (40.5-41.0)	53.5 (53.2-53.8)	69.4 (69.0-69.8)	104.5 (104.2-104.9)

[1]Age (mean weeks, 95% CI)

Table 7: HIV status codes for each group

Group 1	HIV Negative, Unexposed	HIV-/M-
Group 2	HIV Positive, Delayed ART Therapy	HIV+/ART-
Group 3	HIV Positive, Early ART Therapy	HIV+/ART+
Group 4	HIV Negative, Exposed	HIV-/M+

4.3 THE OPTIMISATION AND VALIDATION OF LUMINEX

The appropriateness of the optimisation and validation of the assay depends mainly on the objectives and the goals of the study. The validation assessed in this study included the required and necessary parameters and procedures that demonstrated the ability of this optimised assay to quantitatively measure IgA and IgG antibody concentrations in saliva accurately. The validation protocol had the following parameters assessed to address the acceptance levels. Determination of limit of detection and quantification (quantification ranges) was the first parameter to be assessed which was followed by specificity. Precision was also looked at to address the reproducibility of an assay with the ability to measure linearity and parallelism of sample serial dilution against a standard reference curve.

4.3.1 Determination of Limit of Detection and Quantification

The pneumococcal standard reference serum (89-SF) used in this study that generated the standard curve was quantitatively validated to have contained purified human IgA, immunoglobulin D, IgG and IgM against most pneumococcal serotype polysaccharides (Quataert et al., 1995:590-597). The 89-SF reference serum was used to assess IgA and IgG for eight pneumococcal serotype polysaccharide antigens to determine the quantification ranges. Target serotypes assessed were serotype 4, 6B, 9V, 14, 18C, 19F and 23F and a control serotype 7F that is not a vaccine type.

Table 8: Saliva IgA reference serum concentration comparison

Serotype	N[1]	Exp Conc (µg/mL)	Obs Conc Mean (µg/mL)	STDV	CV (%)	Recovery (%)
Pnc 4	15	0.012	0.012	0.0002	1.31	101.00
Pnc 6B	15	0.015	0.015	0.0004	2.35	101.88
Pnc 9V	15	0.017	0.018	0.0008	4.64	106.33
Pnc 14	15	0.019	0.019	0.0005	2.63	99.62
Pnc 18C	15	0.008	0.008	0.0004	4.78	106.13
Pnc 19F	15	0.020	0.020	0.0004	1.90	101.34
Pnc 23F	15	0.013	0.013	0.0004	2.77	102.46
Pnc 7F	15	0.011	0.011	0.0002	2.11	101.54

[1]The number of plate runs
[2]IgA concentrations to each pneumococcal serotype at 1:100 dilution factor (first standard curve point), with mean of expected concentration (Exp Conc) in microgram per millilitre (µg/mL) versus mean of observed concentration (Obs Conc) in µg/mL.

The reference serum was run and measured 15 times for determination of IgA and IgG concentration levels in saliva against all serotypes. When the expected concentration was compared to the observed concentration against vaccine serotypes and a control serotype, the

coefficient of variance (CV) was below 5% (Table 8 and 9). The highest CV recorded was 4.7% and 4.64% for serotype 18C and 9V respectively for IgA and 11.79% and 4.98% for serotype 14 and 18C respectively for IgG.

The percentage recovery for the observed concentration against the expected concentration for all serotypes was between 95% and 110% (Table 8 and 9), which is close to 100% recovery and between the set standard of 80% and 120%. A serotype with an observed concentration level which came close to expected concentration level, was serotype 4 and 19F for IgA (Table 8), and serotype 6B and 7F for IgG (Table 9).

Table 9: Saliva IgG reference serum concentration comparison

Serotype	N[1]	Exp Conc (µg/mL)	Obs Conc Mean (µg/mL)	STDEV	CV (%)	Recovery (%)
Pnc 4	15	0.041	0.042	0.0017	4.06	101.94
Pnc 6B	15	0.169	0.167	0.0031	1.85	99.00
Pnc 9V	15	0.069	0.070	0.0044	6.27	101.62
Pnc 14	15	0.278	0.251	0.0296	11.79	90.24
Pnc 18C	15	0.045	0.046	0.0023	4.98	102.78
Pnc 19F	15	0.130	0.131	0.0027	2.10	100.59
Pnc 23F	15	0.081	0.083	0.0029	3.47	102.88
Pnc 7F	15	0.052	0.052	0.0008	1.60	100.37

[1]The number of plate runs
[2]IgG concentration to each pneumococcal serotype at 1:100 dilution factor (first standard curve point), with mean of expected concentration (Exp Conc) in microgram per millilitre (µg/mL) versus mean of observed concentration (Obs Conc) in µg/mL.

In order to determine the lower limit of quantification (LLOQ), the lower limit of detection (LLOD) was first measured and determined. Standard reference serum (Table 10) and a blank sample (Table 11) were used to assess the LLOD and LLOQ concentrations against vaccine serotypes and a control serotype 7F in IgA and IgG. In reference serum (Table 10), serotype 9V, 14 and 19F had high LLOD concentrations of 0.00002 µg/mL, 0.000021 µg/mL and 0.000024 µg/mL respectively for IgA.

For IgG, serotype 6B, 14 and 19F had high LLOD concentrations of 0.000044 µg/mL, 0.000073 µg/mL and 0.000036 µg/mL respectively. The LLOQ concentrations were set in acceptable three time's higher levels to the LLOD, to exclude any possibility of emerging background. The same assessment was done on the blank sample (Table 11), where Fluorescence Intensity (FI) values for IgA and IgG LLOD for all serotypes were recorded at 2.64 to 6.10 FI and 3.67 to 7.34 FI respectively. The LLOQ for IgA on all the serotypes was 7.91 to 18.30 FI and LLOQ for IgG on all serotypes was 11.35 to 22.01 FI.

Table 10: Saliva IgA and IgG lower limit of detection and quantified concentrations

	Saliva IgA (89SF Reference Serum)		Saliva IgG (89SF Reference Serum)	
Serotype	LLOD (µg/mL)	LLOQ (µg/mL)	LLOD (µg/mL)	LLOQ (µg/mL)
Pnc 4	0.000011	0.000034	0.000011	0.000032
Pnc 6B	0.000011	0.000034	0.000044	0.000131
Pnc 9V	0.000020	0.000059	0.000018	0.000054
Pnc 14	0.000021	0.000062	0.000073	0.000220
Pnc 18C	0.000010	0.000029	0.000012	0.000036
Pnc 19F	0.000024	0.000072	0.000036	0.000108
Pnc 23F	0.000012	0.000035	0.000022	0.000065
Pnc 7F	0.000011	0.000033	0.000014	0.000043

Table 11: Saliva IgA and IgG lower limit of detection and quantified fluorescence

	Saliva IgA (Blank Samples)		Saliva IgG (Blank Samples)	
Serotype	LLOD (FI)	LLOQ (FI)	LLOD (FI)	LLOQ (FI)
Pnc 4	2.64	7.91	5.91	17.74
Pnc 6B	3.72	11.17	5.11	15.32
Pnc 9V	3.71	11.14	6.61	19.83
Pnc 14	3.11	9.32	3.79	11.36
Pnc 18C	3.05	9.14	3.67	11.02
Pnc 19F	3.98	11.93	7.34	22.01
Pnc 23F	6.10	18.30	6.15	18.45
Pnc 7F	2.64	7.93	3.78	11.35

4.3.2 Specificity and Inhibition Parameters

Specificity is referred to as the binding ability of an assay specifically to a target antigen/antibody and to measure and quantify the analyte of interest respectively (Schwenk et al., 2007:125-132). This is however, done by the assay isolating the target analyte in a sample that contains more than one analyte that is closely related to the target analyte structurally or otherwise with low cross-reactivity. For specificity, the absorption assay is performed to determine the inhibition effect in a multiplex format to determine the cross-reactivity of specific antigens to the target antibody. IgA and IgG antibody concentration levels were measured for target serotypes in saliva, including the fluorescent intensity of each serotype.

Table 12: Reference serum inhibition assay

	Saliva IgA Reference Serum Inhibition Values in %		Saliva IgG Reference Serum Inhibition Values in %	
Serotype	FI	Concentrations (µg/mL)	FI	Concentrations (µg/mL)
Pnc 4	99.94	99.95	99.74	99.90
Pnc 6B	99.89	99.94	99.93	99.98
Pnc 9V	99.84	99.90	99.83	99.95
Pnc 14	99.88	99.93	99.88	99.98
Pnc 18C	99.91	99.98	99.91	99.96
Pnc 19F	99.81	99.86	99.85	99.94
Pnc 23F	99.78	99.90	99.87	99.96
Pnc 7F	99.82	99.87	99.77	99.92

Inhibition assay by absorbing reference serum with 100 µg/mL of each pneumococcal serotype polysaccharide antigen for both IgA and IgG

IgA and IgG in reference serum were absorbed with 100 µg/mL polysaccharide concentration from pneumococcal target serotypes and control serotype. The removal of antibody specific to these serotypes resulted in the reduced measurement of each serotype that validates the specificity of an assay. The absorption of antibodies to all serotypes resulted in over 90% inhibition measurements (Table 12) both in fluorescent intensity and concentrations. The recommended inhibition is set at 80% and above for specificity.

4.3.3 Assay Precision

One of the key performance attributes of a clinical assay is to maintain the precision that was evaluated during the optimisation, with the same conditions in place. We can assess the precision by measuring the average coefficient of variance on high and low controls and random saliva samples from healthy individuals. Concentration measurements for inter-assay precision on high and low controls are markers that the assay is running optimally and that there will be little variance between batches. Mean of means was statistically analysed along with standard deviation (Stdev) of means and percentage coefficient of variance.

Table 13: Inter-Assay CV: Saliva IgA average coefficient of variance

		High Control			Low Control		
Serotype	N[1]	Mean of Means	Stdev of Means	%CV of Means	Mean of Means	Stdev of Means	%CV of Means
Pnc 4	10	0.0131	0.0021	16.1	0.0006	0.0001	22.9
Pnc 6B	10	0.0305	0.0032	10.5	0.0037	0.0009	23.3
Pnc 9V	10	0.0179	0.0023	12.9	0.0067	0.0009	13.5
Pnc 14	10	0.0269	0.0030	11.0	0.0060	0.0009	14.2
Pnc 18C	10	0.0114	0.0020	17.1	0.0005	0.0001	13.2
Pnc 19F	10	0.0351	0.0036	10.2	0.0014	0.0002	13.8
Pnc 23F	10	0.0163	0.0019	11.7	0.0002	0.0001	27.1
Pnc 7F	10	0.0080	0.0007	9.3	0.0203	0.0010	4.8

[1]The number of plate runs

Control samples that have high and low antibody concentration levels to target serotypes were measured and analysed for inter-assay precision for IgA and IgG (Table 13 and 14). The control samples were run ten times to ascertain the mean concentration levels for each control. The concentration readings for IgG antibodies to all target serotypes were much higher than the IgA antibodies.

The batch to batch IgA (Table 13) mean concentration comparison for high control had a CV value below 18%. The highest CV recorded was for 18C with 17.1% and the lowest recorded CV was 9.3%. Although the recorded CV for the low control was slightly higher than that of high control, they were still within the recommended CV range of 30%. The highest CV at 27% was for serotype 23F and the lowest was 4.8% for control serotype 7F. The higher CVs recorded from the low control are most likely due to the fact that these measurements are closer to the lower limit of detection.

Table 14: Inter-Assay CV: Saliva IgG average coefficient of variance

		High Control			Low Control		
Serotype	N[1]	Mean of Means	Stvd of Means	%CV of Means	Mean of Means	Stvd of Means	%CV of Means
Pnc 4	10	0.650	0.137	21.1	0.015	0.003	18.6
Pnc 6B	10	3.128	0.434	13.9	0.063	0.019	29.7
Pnc 9V	10	2.561	0.403	15.7	0.083	0.016	19.6
Pnc 14	10	13.64	0.876	6.40	0.052	0.013	23.9
Pnc 18C	10	4.341	1.094	25.2	0.063	0.023	37.1
Pnc 19F	10	5.946	0.677	11.4	0.045	0.005	11.2
Pnc 23F	10	3.666	0.888	24.2	0.050	0.011	22.1
Pnc 7F	10	3.776	0.245	6.50	0.185	0.012	6.60

[1]The number of plate runs

Absolute IgG antibody concentrations of high and low controls were around ten-fold higher than for IgA. The IgG concentration (Table 14) against serotype 14 was high, reaching 13.64 µg/mL and had the lowest concentration in the high control of 0.65 µg/mL serotype 4. Low control antibody concentration against serotype 23F was the lowest with 0.05 µg/mL and serotype 7F had the highest with 0.188 µg/mL. CV values for both high and low controls ranged from 6.5% to 37%. One serotype, 18C, failed to fall within the acceptance threshold of 30% for the low control sample only.

Table 15: Intra-Assay CV: Average coefficient of variation for saliva IgA duplicates

Serotype	N[1]	Mean Conc Group 1	Mean Conc Group 2	Mean of Means	Stvd of Means	%CV of Means
Pnc 4	40	0.0142	0.0154	0.0148	0.0017	10
Pnc 6B	40	0.0225	0.0228	0.0227	0.0012	6.6
Pnc 9V	40	0.0121	0.0129	0.0125	0.0011	7.6
Pnc 14	40	0.1550	0.1420	0.1485	0.0131	9.1
Pnc 18C	40	0.0116	0.0137	0.0127	0.0018	8.6
Pnc 19F	40	0.0168	0.0179	0.0174	0.0014	7.3
Pnc 23F	40	0.0222	0.0246	0.0234	0.0024	7.5
Pnc 7F	40	0.0039	0.0042	0.0040	0.0004	8.8

[1]The number of plate runs

Table 16: Intra-Assay CV: Average coefficient of variation for saliva IgG duplicates

Serotype	N[1]	Mean Conc Group 1	Mean Conc Group 2	Mean of Means	Stvd of Means	%CV of Means
Pnc 4	40	0.0350	0.0342	0.0346	0.0017	8.2
Pnc 6B	40	0.1087	0.1039	0.1063	0.0048	8.0
Pnc 9V	40	0.0784	0.0841	0.0813	0.0083	8.5
Pnc 14	40	0.0415	0.0402	0.0408	0.0040	9.5
Pnc 18C	40	0.0289	0.0262	0.0275	0.0034	8.3
Pnc 19F	40	0.0378	0.0343	0.0361	0.0035	8.9
Pnc 23F	40	0.1063	0.1020	0.1041	0.0046	10.6
Pnc 7F	40	0.1248	0.1251	0.1249	0.0035	8.3

[1]The number of plate runs

For intra-assay comparisons, duplicate samples were compared 40 times for IgA and IgG. The concentrations were analysed pairwise, and the mean CV was recorded to be below 10% for IgA (Table 15) and for IgG it was recorded to be below 11% (Table 16). The values show that the intra-assay repeatability is good.

4.4 IMPACT OF PCV VACCINATION ON IGG RESPONSES IN SERUM AND SALIVA

At age 15-30 weeks (Table 17) post three primary vaccine doses, both serum and saliva IgG antibody concentrations to vaccine serotypes were high in children who received the vaccine. The highest observed IgG concentration in the vaccinated group was 7.37 µg/mL in serum and 0.0173 µg/mL in saliva to both 19F and 23F serotypes respectively. The antibody concentration of children who did not receive the vaccine was much lower in comparison to the vaccinated group in both serum and saliva to vaccine serotypes ($P < 0.0001$). The highest observed antibody concentration in the unvaccinated group was 0.089 µg/mL (serotype 14) and 0.0029 µg/mL (serotype 19F) in serum and saliva respectively. A response to non-

vaccine serotype 7F was measured only in saliva, and no significant difference in antibody concentration observed in both vaccinated and unvaccinated group (P = 0.2239).

Table 17: Serum and saliva IgG GMC's (µg/mL) with 95% CI for age 15-30 weeks

Age 15-30 Weeks							
		Vaccinated		Unvaccinated			
Serotypes	N	Serum IgG	Saliva IgG	Serum IgG	Saliva IgG	P value[1]	P value[2]
Pnc 4	190-480	6.44 (5.90-7.03)	0.0095 (0.0081-0.0112)	0.006 (0.005-0.006)	0.0009 (0.0008-0.0011)	0.0001	0.0001
Pnc 6B	134-482	2.25 (1.97-2.57)	0.0061 (0.0050-0.0074)	0.012 (0.010-0.014)	0.0012 (0.0010-0.0015)	0.0001	0.0001
Pnc 9V	163-481	5.50 (5.07-5.97)	0.0157 (0.0134-0.0185)	0.012 (0.011-0.015)	0.0011 (0.0009-0.0013)	0.0001	0.0001
Pnc 14	147-480	4.61 (4.16-5.10)	0.0069 (0.0058-0.0081)	0.089 (0.073-0.108)	0.0021 (0.0017-0.0025)	0.0001	0.0001
Pnc 18C	185-480	6.20 (5.68-6.77)	0.0152 (0.0130-0.0178)	0.011 (0.009-0.013)	0.0007 (0.0006-0.0009)	0.0001	0.0001
Pnc 19F	122-478	7.37 (6.78-8.01)	0.0166 (0.0138-0.0200)	0.032 (0.027-0.038)	0.0029 (0.0022-0.0037)	0.0001	0.0001
Pnc 23F	176-480	5.15 (4.62-5.74)	0.0173 (0.0145-0.0207)	0.013 (0.011-0.016)	0.0010 (0.0008-0.0013)	0.0001	0.0001
Pnc 7F	173-267	N/A	0.0015 (0.0013-0.0019)	N/A	0.0013 (0.0010-0.0016)	N/A	0.2239

[1]P-value: Serum IgG (vaccinated children) vs. Serum IgG (unvaccinated children), log transformed

[2]P-value: Saliva IgG (vaccinated children) vs. Saliva IgG (unvaccinated children), log transformed

The serum and saliva IgG antibody concentrations for most vaccine serotypes had decreased over time at age 38-42 weeks (Table 18) compared to the previous sampling collected 3-6 weeks after the three primary vaccine series (Table 17) for both vaccinated and unvaccinated groups. Children in the vaccinated group had significantly higher serum and salivary antibody concentration against all serotypes (P < 0.0001) than children in the unvaccinated group. The one exception was serotype 14 that had high serum and saliva IgG concentrations of 7.17 µg/mL and 0.0098 µg/mL respectively (vaccinated group), compared to serotype 14 at age 15-30 weeks (Table 17). The highest GMC antibody in the unvaccinated group were 0.021 µg/mL in serum and 0.0022 µg/mL in saliva at age 38-42 weeks (Table 18).

Table 18: Serum and saliva IgG GMC's (µg/mL) with 95% CI for age 38-42 weeks

Age 38-42 Weeks							
		Vaccinated		Unvaccinated			
Serotypes	N	Serum IgG	Saliva IgG	Serum IgG	Saliva IgG	P value[1]	P value[2]
Pnc 4	101-422	1.65 (1.53-1.79)	0.0041 (0.0037-0.0047)	0.005 (0.005-0.006)	0.0006 (0.0005-0.0008)	0.0001	0.0001
Pnc 6B	53-421	1.39 (1.25-1.55)	0.0058 (0.0051-0.0067)	0.009 (0.008-0.010)	0.0009 (0.0007-0.0011)	0.0001	0.0001
Pnc 9V	78-421	1.68 (1.54-1.84)	0.0060 (0.0054-0.0067)	0.009 (0.008-0.011)	0.0009 (0.0008-0.0012)	0.0001	0.0001
Pnc 14	60-421	7.17 (6.46-7.95)	0.0098 (0.0086-0.0112)	0.021 (0.018-0.025)	0.0016 (0.0013-0.0019)	0.0001	0.0001
Pnc 18C	79-421	1.15 (1.05-1.25)	0.0035 (0.0031-0.0040)	0.005 (0.005-0.006)	0.0005 (0.0004-0.0006)	0.0001	0.0001
Pnc 19F	65-421	1.93 (1.75-2.14)	0.0055 (0.0048-0.0062)	0.020 (0.017-0.023)	0.0022 (0.0018-0.0027)	0.0001	0.0001
Pnc 23F	78-421	1.30 (1.18-1.43)	0.0040 (0.0035-0.0046)	0.008 (0.007-0.009)	0.0007 (0.0006-0.0009)	0.0001	0.0001
Pnc 7F	83-354	N/A	0.0013 (0.0011-0.0014)	N/A	0.0009 (0.0008-0.0011)	N/A	0.0072

[1]P-value: Serum IgG (vaccinated children) vs. Serum IgG (unvaccinated children), log transformed

[2]P-value: Saliva IgG (vaccinated children) vs. Saliva IgG (unvaccinated children), log transformed

The booster vaccine dose was administered at age 64-76 weeks (Table 19) before the blood was drawn and pre-booster baseline concentrations 40 weeks after the three primary vaccine doses. The vaccinated group was randomised to receive a booster dose of either PCV7 or Hib conjugate vaccine. As expected the serum and saliva antibody concentrations continued to fall against all serotypes in both vaccinated and unvaccinated groups. Vaccinated groups had significantly higher serum and salivary antibody concentrations against some serotypes (P < 0.0001) than the unvaccinated group. With exception to serotype 14 and 19F that had comparable serum antibody concentrations (P = 0.1706) in serum and antibody concentration (P = 0.7273) in saliva respectively (Table 19). Antibody concentrations in saliva were significantly higher (P < 0.0001) in the unvaccinated group at this time point (Table 19).

Table 18: Serum and saliva IgG GMC's (µg/mL) with 95% CI for age 64-76 weeks

Age 64-76 Weeks							
		Vaccinated		Unvaccinated			
Serotypes	N	Serum IgG	Saliva IgG	Serum IgG	Saliva IgG	P value[1]	P value[2]
Pnc 4	93-426	0.65 (0.59-0.70)	0.0015 (0.0013-0.0017)	0.103 (0.086-0.124)	0.0007 (0.0006-0.0009)	0.0001	0.0001
Pnc 6B	91-425	0.92 (0.81-1.04)	0.0048 (0.0041-0.0055)	0.401 (0.323-0.498)	0.0014 (0.0012-0.0018)	0.0001	0.0001
Pnc 9V	59-425	0.71 (0.65-0.78)	0.0028 (0.0024-0.0031)	0.394 (0.321-0.484)	0.0010 (0.0008-0.0013)	0.0001	0.0001
Pnc 14	105-424	2.63 (2.38-2.90)	0.0047 (0.0041-0.0054)	3.083 (2.508-3.789)	0.0032 (0.0025-0.0042)	0.1706	0.0125
Pnc 18C	79-422	0.48 (0.44-0.52)	0.0013 (0.0011-0.0015)	0.292 (0.236-0.362)	0.0006 (0.0005-0.0008)	0.0001	0.0001
Pnc 19F	104-425	1.35 (1.19-1.54)	0.0038 (0.0033-0.0045)	0.899 (0.721-1.121)	0.0037 (0.0030-0.0045)	0.0001	0.7273
Pnc 23F	98-427	0.64 (0.57-0.71)	0.0021 (0.0018-0.0024)	0.373 (0.296-0.468)	0.0010 (0.0008-0.0012)	0.0001	0.0001
Pnc 7F	105-387	N/A	0.0007 (0.0006-0.0008)	N/A	0.0013 (0.0011-0.0015)	N/A	0.0001

[1]P-value: Serum IgG (vaccinated children) vs. Serum IgG (unvaccinated children), log transformed

[2]P-value: Saliva IgG (vaccinated children) vs. Saliva IgG (unvaccinated children), log transformed

The serum and saliva IgG antibody concentrations for all vaccine serotypes increased 2 weeks after the booster dose at age 65-78 weeks (Table 20). Children in the vaccinated group had significantly higher serum and salivary antibody concentrations against all serotypes (P < 0.0001) than children in the unvaccinated group. Also the vaccinated group had a high saliva IgG concentration against the control serotype 7F compared to unvaccinated group (P < 0.0001). Serum antibody measurement achieved concentrations above the protective threshold of 0.35 µg/mL and 2.0 µg/mL against all vaccine serotypes. In the vaccinated group the highest observed concentration was 8.84 µg/mL in serum and 0.0169 µg/mL in saliva to both 14 and 6B serotypes respectively. However in the unvaccinated group only serotype 19F had a high antibody concentration of 0.023 µg/mL and 0.0021 µg/mL in serum and saliva respectively.

Table 19: Serum and saliva IgG GMC's (µg/mL) with 95% CI for age 65-78 weeks

Age 65-78 Weeks							
		Vaccinated		Unvaccinated			
Serotypes	N	Serum IgG	Saliva IgG	Serum IgG	Saliva IgG	P value[1]	P value[2]
Pnc 4	64-427	3.45 (2.87-4.13)	0.0085 (0.0068-0.0106)	0.006 (0.005-0.007)	0.0003 (0.0003-0.0004)	0.0001	0.0001
Pnc 6B	54-427	4.97 (4.08-6.04)	0.0169 (0.0133-0.0214)	0.008 (0.007-0.008)	0.0006 (0.0005-0.0008)	0.0001	0.0001
Pnc 9V	21-427	3.26 (2.72-3.90)	0.0136 (0.0110-0.0168)	0.014 (0.011-0.017)	0.0005 (0.0003-0.0008)	0.0001	0.0001
Pnc 14	75-426	8.84 (7.52-10.4)	0.0148 (0.0123-0.0178)	0.014 (0.012-0.017)	0.0015 (0.0012-0.0019)	0.0001	0.0001
Pnc 18C	47-426	2.44 (2.02-2.95)	0.0076 (0.0060-0.0096)	0.006 (0.005-0.008)	0.0003 (0.0002-0.0004)	0.0001	0.0001
Pnc 19F	74-425	4.29 (3.64-5.06)	0.0078 (0.0063-0.0097)	0.023 (0.020-0.028)	0.0021 (0.0017-0.0026)	0.0001	0.0001
Pnc 23F	59-426	3.32 (2.74-4.02)	0.0140 (0.0112-0.0174)	0.007 (0.006-0.008)	0.0005 (0.0004-0.0006)	0.0001	0.0001
Pnc 7F	79-395	N/A	0.0004 (0.0004-0.0005)	N/A	0.0008 (0.0007-0.0010)	N/A	0.0001

[1]P-value: Serum IgG (vaccinated children) vs. Serum IgG (unvaccinated children), log transformed

[2]P-value: Saliva IgG (vaccinated children) vs. Saliva IgG (unvaccinated children), log transformed

The serum and salivary antibody response post booster vaccine dose at age 102-116 weeks (Table 21) for the vaccinated group fell below the 2.0 µg/mL threshold against some serotypes. The exception was serotype 14 that had a high serum IgG concentrations above the 2.0 µg/mL threshold. The vaccine group had high serum and saliva IgG concentrations compared to unvaccinated group ($P < 0.0001$), however saliva IgG concentrations between both groups against serotype 7F was comparable ($P < 0.1035$). The unvaccinated group's antibody concentration was much higher at age 102-116 weeks (Table 21) when compared to the concentrations of previous sampling at age 65-78 (Table 20) weeks for both serum and saliva. The highest observed concentration in the vaccinated group was 3.47 µg/mL in serum and 0.0056 µg/mL in saliva against serotype 14 and serotype 6B respectively. Although the unvaccinated group antibody response was low, the highest concentration in serum was 0.059 µg/mL and 0.0010 µg/mL in saliva against serotype 19F (Table 21).

Table 20: Serum and saliva IgG GMC's (µg/mL) with 95% CI for age 102-116 weeks

Age 102-116 Weeks							
		Vaccinated		Unvaccinated			
Serotypes	N	Serum IgG	Saliva IgG	Serum IgG	Saliva IgG	P value[1]	P value[2]
Pnc 4	155-356	0.79 (0.70-0.90)	0.0013 (0.0011-0.0015)	0.015 (0.011-0.019)	0.0003 (0.0002-0.0003)	0.0001	0.0001
Pnc 6B	194-356	1.66 (1.44-1.92)	0.0056 (0.0047-0.0065)	0.013 (0.011-0.016)	0.0004 (0.0004-0.0005)	0.0001	0.0001
Pnc 9V	162-356	0.81 (0.72-0.92)	0.0028 (0.0024-0.0032)	0.033 (0.025-0.044)	0.0004 (0.0004-0.0005)	0.0001	0.0001
Pnc 14	194-356	3.47 (3.07-3.92)	0.0033 (0.0029-0.0038)	0.031 (0.024-0.039)	0.0007 (0.0007-0.0008)	0.0001	0.0001
Pnc 18C	160-355	0.55 (0.48-0.64)	0.0013 (0.0011-0.0016)	0.013 (0.009-0.017)	0.0003 (0.0002-0.0003)	0.0001	0.0001
Pnc 19F	136-356	1.93 (1.68-2.23)	0.0034 (0.0029-0.0040)	0.059 (0.047-0.074)	0.0010 (0.0009-0.0012)	0.0001	0.0001
Pnc 23F	60-356	0.91 (0.79-1.05)	0.0030 (0.0025-0.0035)	0.017 (0.013-0.022)	0.0005 (0.0004-0.0007)	0.0001	0.0001
Pnc 7F	154-356	N/A	0.0005 (0.0004-0.0005)	N/A	0.0005 (0.0005-0.0006)	N/A	0.1035

[1]P-value: Serum IgG (vaccinated children) vs. Serum IgG (unvaccinated children), log transformed

[2]P-value: Saliva IgG (vaccinated children) vs. Saliva IgG (unvaccinated children), log transformed

4.5 COMPARISON AND CORRELATION OF SALIVA AND SERUM IGG CONCENTRATIONS

The correlation coefficients (Pearson's R) were determined between the serum IgG and saliva IgG for all serotypes, within the vaccinated group. In the unvaccinated group, low positive correlation coefficient (0.3 to 0.5) for most vaccine serotypes was observed throughout the ages of study participants (graphs not shown). The focus of this section is to report the difference in saliva and serum IgG correlation before and after pneumococcal booster vaccine through scatter plots. Low positive correlation coefficient for most serotypes was observed before the booster vaccine at age 64-76 weeks; however serotype 6B and 23F were the only serotypes with moderate positive correlation (0.5 to 0.7).

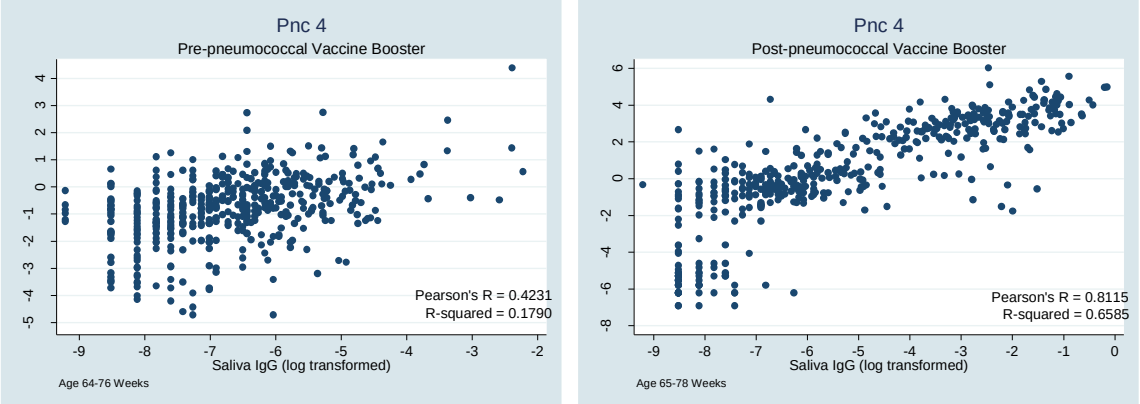

Figure 6: Serotype 4 pre and post booster scatter plots.

Serotype 4 had a low positive correlation coefficient of 0.4231 at age 64-76 weeks before the booster vaccine administration that significantly increased to 0.8115 after the booster vaccine at age 65-78 weeks. The scatter plots of the serum IgG versus saliva IgG correlation of serotype 4 is shown in Figure 6.

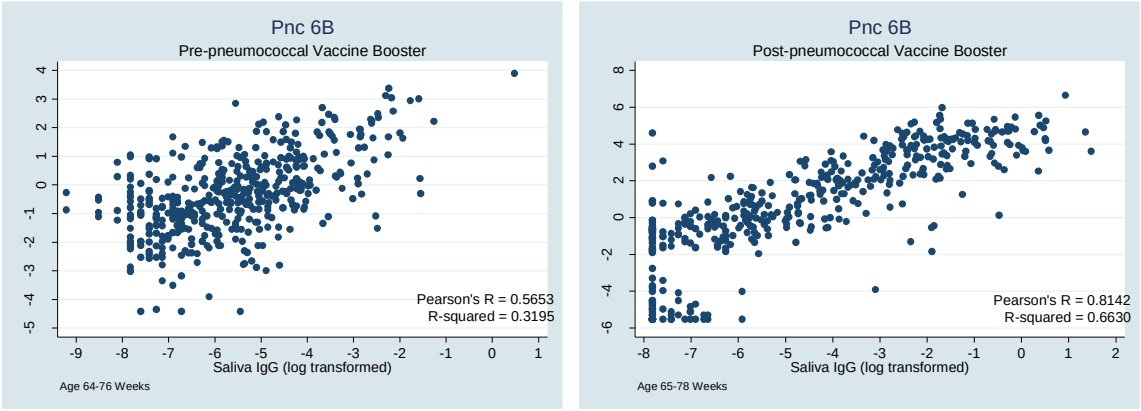

Figure 7: Serotype 6B pre and post booster scatter plots.

Post primary vaccine doses increased the antibody response, especially for serotype 6B with a moderate positive correlation of 0.5653 observed before administration of a booster vaccine (Figure 7). After the booster vaccine, the correlation coefficient for serotype 6B increased to 0.8142 at age 65-78 weeks.

Figure 8: Serotype 9V pre and post booster scatter plots.

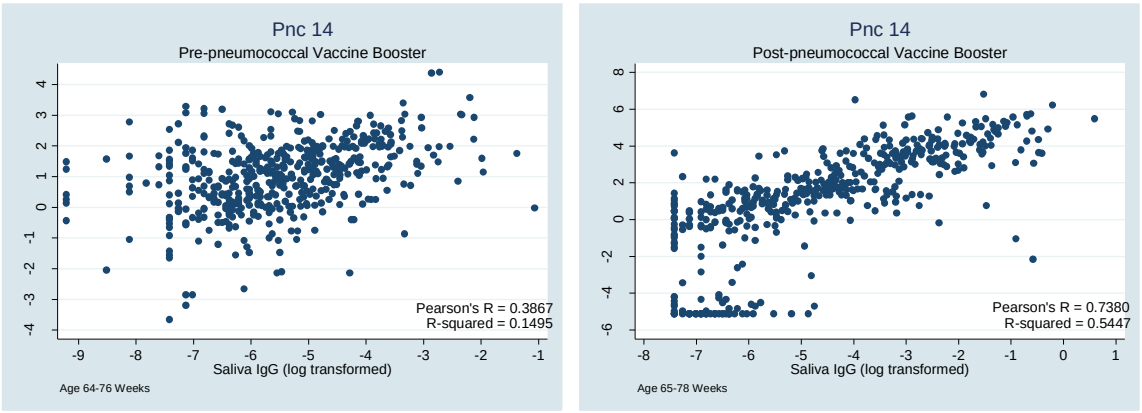

Figure 9: Serotype 14 pre and post booster scatter plots.

Serotype 9V (Figure 8) and 14 (Figure 9) had low positive saliva and serum IgG correlations before the booster vaccine with correlation coefficients of 0.3374 and 0.3867 respectively. The linear association of both serotypes was also low before the booster vaccine that increased to moderate and high association following the booster. Correlation coefficient and linear association after the booster vaccine for serotype 9V was 0.7858 and 0.6176 respectively and serotype 14 was 0.7380 and 0.5447 respectively.

Figure 10: Serotype 18C pre and post booster scatter plots.

Saliva and serum IgG linear association for serotype 18C (Figure 10) was low (r^2 = 0.1804) before booster vaccination, with low positive correlation (R = 0.4248) observed. However, after booster vaccination the saliva and serum IgG correlation increased to a high positive correlation (R = 0.8210) with a high linear association (r^2 = 0.6740).

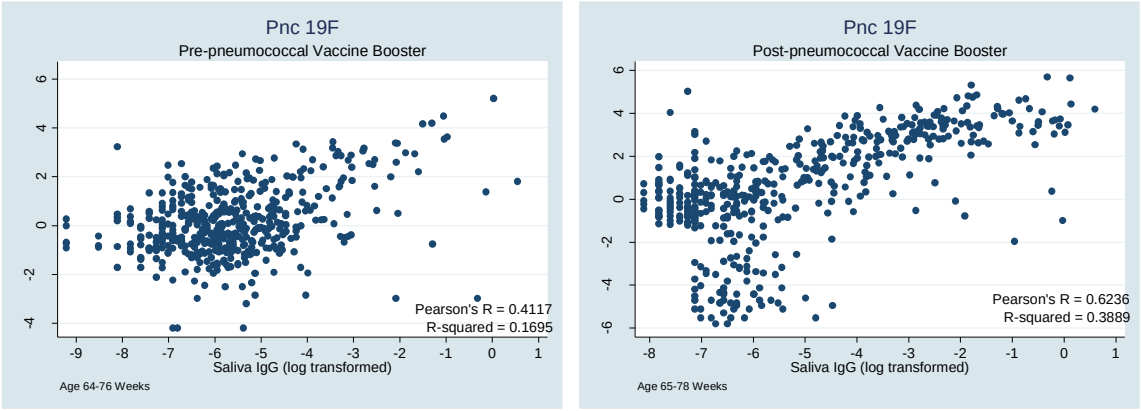

Figure 11: Serotype 19F pre and post booster scatter plots.

Serotype 19F had a low positive correlation coefficient (R = 0.4117) before the booster vaccine which increased to a moderate correlation (R = 0.6236) post booster vaccine administration. The linear association before (r^2 = 0.1695) and after (r^2 = 0.3889) booster vaccine increased from a low to a moderate association (Figure 11).

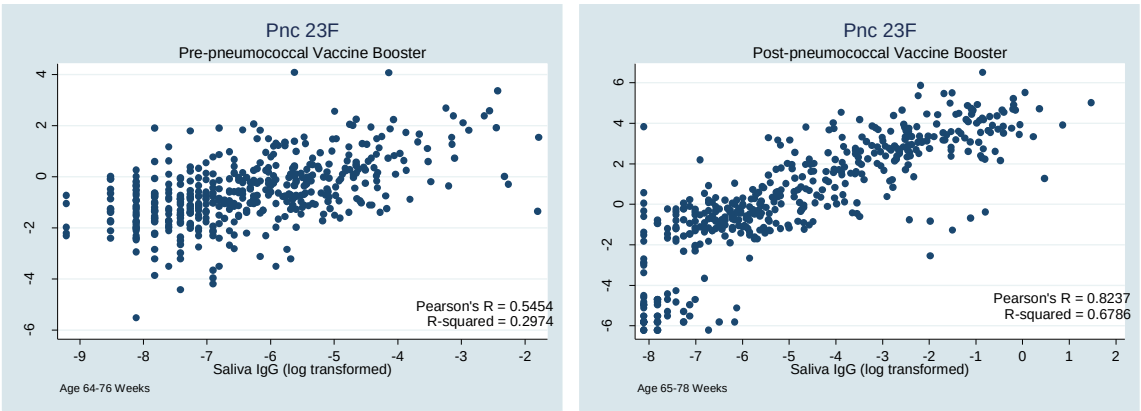

Figure 12: Serotype 23F pre and post booster scatter plots.

Serotype 23F had a moderate positive correlation coefficient of 0.5454 pre-booster vaccine at age 64-76 weeks, with a low linear association (r^2 = 0.2974). After the booster vaccine at age 65-78 weeks, the correlation coefficient and linear association increased to 0.8237 and 0.6786 (Figure 12) respectively.

4.6 SALIVA IGA AND IGG RESPONSE IN VACCINATED AND UNVACCINATED HIV NEGATIVE CHILDREN[7]

To measure the effectiveness of vaccination, the antibody immune response in children that are HIV negative unexposed born to HIV negative mothers and HIV negative exposed born to HIV positive mothers was evaluated. In these two groups, we compared the GMC's of the vaccinated and the unvaccinated, and examined the booster dose effect at age 65-78 weeks. Five sample-points were assessed to evaluate the difference in measured anti-pneumococcal polysaccharide antibody concentrations to eight serotypes, of which seven are representative of PCV7 and one serotype added to the experiments as control. Tables 22 to 26 describes the saliva IgA and IgG geometric concentrations in microgram per millilitre (µg/mL) unit with 95% confidence interval (CI) and p-value at all sample-points for HIV negative (unexposed and exposed), vaccinated and unvaccinated. Correlation was only observed at baseline (15-30 weeks) 3 to 6 weeks after three primary vaccine doses and at 65-78 weeks, 2 to 3 weeks after the booster vaccine dose.

Table 21: Vaccinated and unvaccinated saliva IgA & IgG GMC's at age 15-30 weeks

		Age 15-30 Weeks							
		Vaccinated		Unvaccinated					
Serotypes	N	Saliva IgA	Saliva IgG	Saliva IgA	Saliva IgG	P value[1]	P value[2]	R-value[3]	R-value[4]
Pnc 4	137-224	0.0028 (0.0022-0.0037)	0.0070 (0.0055-0.0090)	0.0016 (0.0013-0.0019)	0.0009 (0.0008-0.0011)	0.0002	<0.0001	0.7012	0.7835
Pnc 6B	135-190	0.0055 (0.0019-0.0033)	0.0045 (0.0034-0.0061)	0.0011 (0.0009-0.0013)	0.0012 (0.0010-0.0015)	<0.0001	<0.0001	0.6712	0.7700
Pnc 9V	140-219	0.0022 (0.0017-0.0029)	0.0101 (0.0079-0.0129)	0.0008 (0.0006-0.0009)	0.0011 (0.0009-0.0013)	<0.0001	<0.0001	0.5556	0.6977
Pnc 14	139-218	0.0197 (0.0140-0.0277)	0.0054 (0.0042-0.0069)	0.0017 (0.0014-0.0021)	0.0021 (0.0017-0.0025)	<0.0001	<0.0001	0.5319	0.6143
Pnc 18C	137-215	0.0012 (0.0009-0.0015)	0.0122 (0.0097-0.0155)	0.0006 (0.0005-0.0007)	0.0007 (0.0006-0.0009)	<0.0001	<0.0001	0.6029	0.6969
Pnc 19F	126-200	0.0042 (0.0032-0.0054)	0.0100 (0.0074-0.0134)	0.0030 (0.0025-0.0035)	0.0029 (0.0023-0.0037)	0.0326	<0.0001	0.6769	0.7528
Pnc 23F	136-219	0.0022 (0.0017-0.0029)	0.0123 (0.0096-0.0157)	0.0013 (0.0011-0.0016)	0.0010 (0.0008-0.0013)	0.0020	<0.0001	0.6606	0.7833
Pnc 7F	115-223	0.0014 (0.0011-0.0018)	0.0011 (0.0008-0.0015)	0.0014 (0.0012-0.0016)	0.0013 (0.0011-0.0016)	0.9358	0.4279	0.7384	0.7898

[1]P-Value: Saliva IgA (vaccinated children) vs. Saliva IgA (unvaccinated children), log transformed

[2]P-Value: Saliva IgG (vaccinated children) vs. Saliva IgG (unvaccinated children), log transformed

[3]R-Value: Saliva IgA vs. Saliva IgG (vaccinated children), log transformed

[4]R-Value: Saliva IgA vs. Saliva IgG (unvaccinated children), log transformed

At age 15-30 weeks (Table 22) post three primary vaccine doses, saliva IgA and IgG antibody concentrations against vaccine serotypes were high in children who received the vaccine. The highest observed concentration in the vaccine group was 0.0123 µg/mL for IgG and 0.0197 µg/mL for IgA against serotype 23F and 14 respectively. The antibody concentration in children who did not receive the vaccine was much lower in comparison to

[7] HIV negative exposed and unexposed data was combined to form a set that is used to compare unvaccinated and the vaccinated groups.

the vaccinated group for both IgG and IgA to vaccine serotypes ($P < 0.0001$ – $P = 0.0326$). The highest observed antibody concentration in the unvaccinated group was 0.0029 µg/mL and 0.0030 µg/mL against serotype 19F for saliva IgG and IgA respectively. There was no significant difference in antibody response against serotype 7F when IgG ($P = 0.4279$) and IgA ($P = 0.9358$) concentrations of vaccinated and unvaccinated groups were compared. Correlation coefficient between IgG and IgA concentrations was observed from moderate positives to high positives in both vaccinated and unvaccinated group. The high positive correlation coefficient in vaccinated group ($R = 0.7012$) and unvaccinated group ($R = 0.7835$) were observed against vaccine serotypes. Serotype 7F had high positive correlation coefficient ($R = 0.7384$) for vaccinated group and ($R = 0.7898$) for unvaccinated group.

Table 22: Vaccinated and unvaccinated saliva IgA & IgG GMC's at age 38-42 weeks

		Age 38-42 Weeks					
		Vaccinated		Unvaccinated			
Serotypes	N	Saliva IgA	Saliva IgG	Saliva IgA	Saliva IgG	P value[1]	P value[2]
Pnc 4	122-201	0.0021 (0.0017-0.0025)	0.0047 (0.0040-0.0055)	0.0014 (0.0011-0.0017)	0.0006 (0.0005-0.0008)	0.0057	<0.0001
Pnc 6B	117-201	0.0017 (0.0014-0.0021)	0.0062 (0.0051-0.0076)	0.0008 (0.0006-0.0010)	0.0009 (0.0007-0.0011)	<0.0001	<0.0001
Pnc 9V	122-201	0.0013 (0.0011-0.0015)	0.0060 (0.0052-0.0070)	0.0007 (0.0005-0.0008)	0.0009 (0.0008-0.0012)	<0.0001	<0.0001
Pnc 14	122-201	0.0106 (0.0086-0.0130)	0.0114 (0.0095-0.0137)	0.0015 (0.0012-0.0020)	0.0016 (0.0013-0.0019)	<0.0001	<0.0001
Pnc 18C	119-201	0.0006 (0.0005-0.0007)	0.0041 (0.0035-0.0048)	0.0003 (0.0003-0.0004)	0.0005 (0.0004-0.0006)	<0.0001	<0.0001
Pnc 19F	112-193	0.0037 (0.0030-0.0046)	0.0063 (0.0052-0.0076)	0.0030 (0.0023-0.0038)	0.0022 (0.0018-0.0027)	0.1558	<0.0001
Pnc 23F	122-201	0.0011 (0.0009-0.0013)	0.0040 (0.0034-0.0048)	0.0009 (0.0007-0.0012)	0.0008 (0.0006-0.0009)	0.2553	<0.0001
Pnc 7F	123-199	0.0016 (0.0013-0.0019)	0.0014 (0.0011-0.0017)	0.0012 (0.0010-0.0015)	0.0009 (0.0008-0.0011)	0.0550	0.0053

[1]P-Value: Saliva IgA (vaccinated children) vs. Saliva IgA (unvaccinated children), log transformed

[2]P-Value: Saliva IgG (vaccinated children) vs. Saliva IgG (unvaccinated children), log transformed

The saliva IgG and IgA antibody concentrations of the vaccinated group had decreased at age 38-42 weeks (Table 23) from age 15-30 weeks (Table 22), with the exception of serotypes 6B and 14 for IgG. Serotypes 6B and 14 IgG concentrations increased from 0.0045 µg/mL and 0.0054 µg/mL at age 15-30 weeks to 0.0062 µg/mL and 0.0114 µg/mL at age 38-42 weeks respectively. Children in the vaccinated group had significantly higher salivary IgG antibody concentration against vaccine serotypes ($P < 0.0001$) than children in the unvaccinated group. In the vaccinated group, IgA concentrations were significantly higher than IgA concentrations of the unvaccinated ($P < 0.0001$), the exception was serotype 19F ($P = 0.1558$) and 23F ($P = 0.2553$) (Table 23). The highest observed salivary IgG and IgA antibody concentrations in the vaccinated group was 0.0114 µg/mL and 0.0106 µg/mL against serotype 14 at age 38-42 weeks (Table 23). Saliva IgG and IgA antibody

concentrations at age 38-42 weeks (Table 23) for unvaccinated group remain unchanged from age 15-30 weeks (Table 22).

Table 23: Vaccinated and unvaccinated saliva IgA & IgG GMC's at age 64-76 weeks

		Age 64-76 Weeks					
		Vaccinated		Unvaccinated			
Serotypes	N	Saliva IgA	Saliva IgG	Saliva IgA	Saliva IgG	P value[1]	P value[2]
Pnc 4	114-195	0.0017 (0.0014-0.0020)	0.0017 (0.0014-0.0020)	0.0015 (0.0011-0.0021)	0.0007 (0.0006-0.0009)	0.5330	<0.0001
Pnc 6B	111-197	0.0018 (0.0014-0.0024)	0.0046 (0.0037-0.0057)	0.0018 (0.0013-0.0026)	0.0014 (0.0012-0.0018)	0.9650	<0.0001
Pnc 9V	113-197	0.0016 (0.0013-0.0020)	0.0027 (0.0023-0.0032)	0.0017 (0.0012-0.0024)	0.0010 (0.0008-0.0013)	0.8247	<0.0001
Pnc 14	114-197	0.0084 (0.0067-0.0105)	0.0047 (0.0038-0.0058)	0.0048 (0.0032-0.0071)	0.0032 (0.0025-0.0042)	0.0158	0.0284
Pnc 18C	108-197	0.0006 (0.0004-0.0007)	0.0015 (0.0012-0.0018)	0.0005 (0.0004-0.0007)	0.0006 (0.0005-0.0008)	0.7161	<0.0001
Pnc 19F	113-197	0.0044 (0.0035-0.0057)	0.0036 (0.0029-0.0045)	0.0060 (0.0043-0.0082)	0.0037 (0.0030-0.0045)	0.1495	0.8601
Pnc 23F	111-197	0.0013 (0.0010-0.0017)	0.0022 (0.0018-0.0027)	0.0015 (0.0011-0.0021)	0.0010 (0.0008-0.0012)	0.5822	<0.0001
Pnc 7F	105-197	0.0013 (0.0011-0.0016)	0.0008 (0.0007-0.0009)	0.0015 (0.0012-0.0019)	0.0013 (0.0011-0.0015)	0.4108	0.0001

[1]P-Value: Saliva IgA (vaccinated children) vs. Saliva IgA (unvaccinated children), log transformed

[2]P-Value: Saliva IgG (vaccinated children) vs. Saliva IgG (unvaccinated children), log transformed

At a pre-booster vaccine baseline (age 64-76 weeks), a saliva sample was collected before the administration of pneumococcal booster vaccine (Table 24). As expected saliva IgG antibody concentrations continued to fall against all serotypes in the vaccinated group, but remained unchanged in the unvaccinated group. Saliva IgA antibody concentrations against some serotypes decreased when some increased in the vaccinated group and increased against all serotypes in the unvaccinated group. Children in the vaccinated group had significantly higher salivary IgG antibody concentrations against all ($P = 0.0284 – P < 0.0001$) but one serotype 19F ($P = 0.8601$) than the non-vaccine group concentrations (Table 24). Only serotype 14 had significant IgA antibody concentration ($P = 0.0158$) when the vaccinated group was compared to the unvaccinated group. In the vaccinated group the highest observed concentration was 0.0047 µg/mL for IgG and 0.0084 µg/mL for IgA both to serotype 14 respectively. However in the unvaccinated group only serotype 19F had high antibody concentrations of 0.0087 µg/mL and 0.0060 µg/mL for IgG and IgA respectively.

Saliva IgG and IgA antibody concentrations for all vaccine serotypes increased significantly 2 weeks after the booster dose at age 65-78 weeks (Table 25). Children in the vaccinated group had significantly higher salivary IgG and IgA antibody concentrations against all serotypes ($P < 0.0001$) than children in the unvaccinated group. The exception was serotype 19F ($P = 0.0606$) with comparable saliva IgA concentrations between vaccinated and unvaccinated groups. In the vaccinated group the highest observed concentration was 0.0208

µg/mL for IgG and 0.0191 µg/mL for IgA to both 6B and 14 serotypes respectively. However in the unvaccinated group both IgG and IgA concentrations continued to drop with high antibody concentration of 0.0021 µg/mL and 0.0035 µg/mL for IgG and IgA respectively.

Table 24: Vaccinated and unvaccinated saliva IgA & IgG GMC's at age 65-78 weeks

	Age 65-78 Weeks								
		Vaccinated		Unvaccinated					
Serotypes	N	Saliva IgA	Saliva IgG	Saliva IgA	Saliva IgG	P value[1]	P value[2]	R-value[3]	R-value[4]
Pnc 4	64-194	0.0052 (0.0040-0.0068)	0.0099 (0.0073-0.0135)	0.0006 (0.0005-0.0007)	0.0003 (0.0003-0.0004)	<0.0001	<0.0001	0.7150	0.5537
Pnc 6B	54-198	0.0062 (0.0440-0.0088)	0.0208 (0.0150-0.0288)	0.0005 (0.0004-0.0006)	0.0006 (0.0005-0.0008)	<0.0001	<0.0001	0.7607	0.4472
Pnc 9V	21-198	0.0039 (0.0029-0.0053)	0.0146 (0.0108-0.0197)	0.0006 (0.0005-0.0008)	0.0005 (0.0003-0.0008)	<0.0001	<0.0001	0.7929	0.2492
Pnc 14	75-199	0.0191 (0.0140-0.0259)	0.0180 (0.0139-0.0232)	0.0019 (0.0014-0.0026)	0.0015 (0.0012-0.0019)	<0.0001	<0.0001	0.7117	0.4286
Pnc 18C	47-198	0.0015 (0.0011-0.0020)	0.0088 (0.0064-0.0120)	0.0002 (0.0002-0.0002)	0.0003 (0.0002-0.0004)	<0.0001	<0.0001	0.7272	0.4596
Pnc 19F	74-199	0.0050 (0.0039-0.0064)	0.0086 (0.0064-0.0115)	0.0035 (0.0027-0.0046)	0.0021 (0.0017-0.0026)	0.0606	<0.0001	0.4247	0.4964
Pnc 23F	59-199	0.0043 (0.0032-0.0056)	0.0171 (0.0126-0.0233)	0.0007 (0.0005-0.0008)	0.0005 (0.0004-0.0006)	<0.0001	<0.0001	0.6798	0.3668
Pnc 7F	79-199	0.0010 (0.0008-0.0012)	0.0004 (0.0004-0.0005)	0.0010 (0.0008-0.0011)	0.0008 (0.0007-0.0010)	0.9107	<0.0001	0.4286	0.3092

[1]P-Value: Saliva IgA (vaccinated children) vs. Saliva IgA (unvaccinated children), log transformed

[2]P-Value: Saliva IgG (vaccinated children) vs. Saliva IgG (unvaccinated children), log transformed

[3]R-Value: Saliva IgA vs. Saliva IgG (vaccinated children), log transformed

[4]R-Value: Saliva IgA vs. Saliva IgG (unvaccinated children), log transformed

Correlation coefficient between IgG and IgA concentrations was observed from moderate positives to high positives in the vaccinated group (Table 25). The high positive correlation coefficient observed in vaccinated group was (R = 0.7929) against serotype 9V. Serotype 19F was the only exception of the vaccine serotypes with a low positive correlation coefficient (R = 0.4247) in the vaccinated group. Also serotype 7F had a low positive correlation coefficient (R = 0.4286) from the vaccinated group. IgG and IgA correlation in the unvaccinated group was observed from negligible to low positives, with the highest correlation coefficient of (R = 0.5537) (Table 25).

Table 25: Vaccinated and unvaccinated saliva IgA & IgG GMC's at age 102-116 weeks

	Age 102-116						
		Vaccinated		Unvaccinated			
Serotypes	N	Saliva IgA	Saliva IgG	Saliva IgA	Saliva IgG	P value[1]	P value[2]
Pnc 4	155-195	0.0018 (0.0015-0.0023)	0.0014 (0.0012-0.0018)	0.0013 (0.0010-0.0016)	0.0003 (0.0002-0.0003)	0.0163	<0.0001
Pnc 6B	179-194	0.0036 (0.0028-0.0046)	0.0068 (0.0054-0.0087)	0.0009 (0.0007-0.0011)	0.0004 (0.0004-0.0005)	<0.0001	<0.0001
Pnc 9V	162-195	0.0026 (0.0021-0.0033)	0.0030 (0.0024-0.0037)	0.0013 (0.0010-0.0016)	0.0005 (0.0004-0.0005)	<0.0001	<0.0001
Pnc 14	179-194	0.0057 (0.0044-0.0074)	0.0038 (0.0032-0.0045)	0.0028 (0.0021-0.0039)	0.0007 (0.0007-0.0008)	0.0007	<0.0001
Pnc 18C	160-185	0.0006 (0.0005-0.0008)	0.0016 (0.0013-0.0020)	0.0005 (0.0004-0.0006)	0.0003 (0.0003-0.0003)	0.1365	<0.0001
Pnc 19F	136-186	0.0051 (0.0040-0.0065)	0.0045 (0.0036-0.0057)	0.0059 (0.0045-0.0078)	0.0011 (0.0009-0.0012)	0.4164	<0.0001
Pnc 23F	60-193	0.0019 (0.0015-0.0024)	0.0040 (0.0031-0.0051)	0.0011 (0.0009-0.0013)	0.0005 (0.0004-0.0007)	0.0006	<0.0001
Pnc 7F	154-195	0.0009 (0.0008-0.0011)	0.0004 (0.0004-0.0005)	0.0015 (0.0013-0.0017)	0.0005 (0.0005-0.0006)	0.0002	0.0096

[1]P-Value: Saliva IgA (vaccinated children) vs. Saliva IgA (unvaccinated children), log transformed

[2]P-Value: Saliva IgG (vaccinated children) vs. Saliva IgG (unvaccinated children), log transformed

IgG and IgA antibody response post booster vaccine dose at age 102-116 weeks (Table 26) for vaccinated group dropped to low levels against vaccine serotypes. The highest observed concentration in the vaccine group was 0.0068 µg/mL for IgG and 0.0057 µg/mL for IgA against serotype 6B and 14 respectively. The vaccinated group had high saliva IgG and IgA concentrations compared to unvaccinated group ($P < 0.0001$), except for serotype 18C ($P = 0.1365$) and 19F ($P = 0.4164$). The unvaccinated group IgA antibody concentration was much higher at age 102-116 weeks (Table 26) when compared to concentrations of previous sampling at age 65-78 (Table 25). Although the unvaccinated group's antibody response was low, the highest concentration for IgG was 0.0011 µg/mL and 0.0059 µg/mL for IgA (Table 26) against serotype 19F.

4.7 DYNAMICS OF SALIVA ANTIBODY CONCENTRATIONS WITH RESPECT TO AGE IN THE FIRST TWO YEARS OF LIFE

The data outlined in this section was collected from children, who received a complete three primary doses of pneumococcal conjugate vaccine and is pooled from the groups: HIV uninfected and exposed and those HIV infected with or without early ART therapy. The aim of this section was to observe the overview of antibody response under two years of age in those who received the conjugate vaccine.

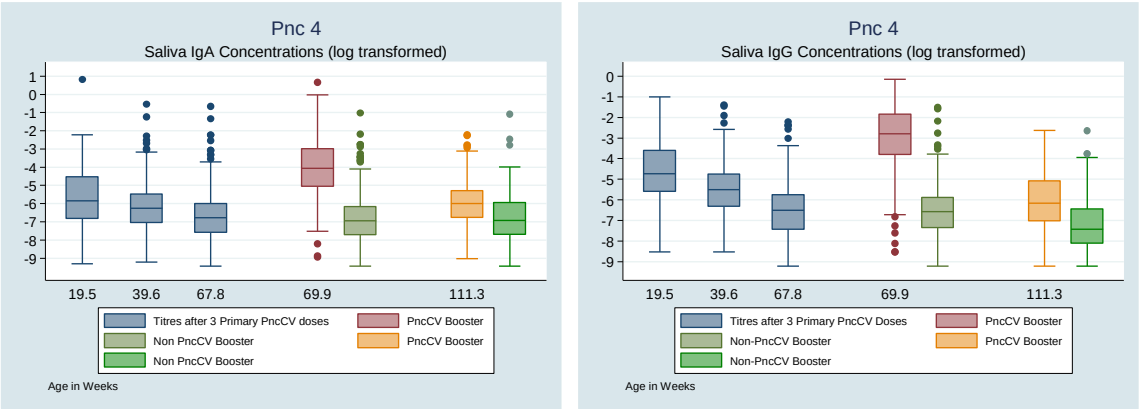

Figure 13: Saliva IgA and IgG concentration box plot for serotype 4.

Saliva IgG concentration at age 19.5 weeks for most vaccine serotypes was high which represents the baseline after 3 to 6 weeks of the last primary vaccine dose. The gradual drop

in antibody concentration thereafter was observed in some serotypes with exception to serotype 6B, 14 and control serotype 7F. Saliva IgA concentration at baseline was not that high, except serotype 4, and the drop in concentration was very slow.

Serotype 4 (Figure 13) IgG concentration at baseline (19.5 weeks) was higher than concentrations at both age 39.6 and 67.8 weeks and concentrations at 39.6 weeks are also higher than concentrations at 67.8 weeks. However, IgA concentration against serotype 4 (Figure 13) at 19.5 weeks was not that high as compared to age 39.6 and 67.8 weeks. Although the concentration at age 39.6 and 67.8 weeks compared to 19.5 weeks was low, both sample points were slightly comparable.

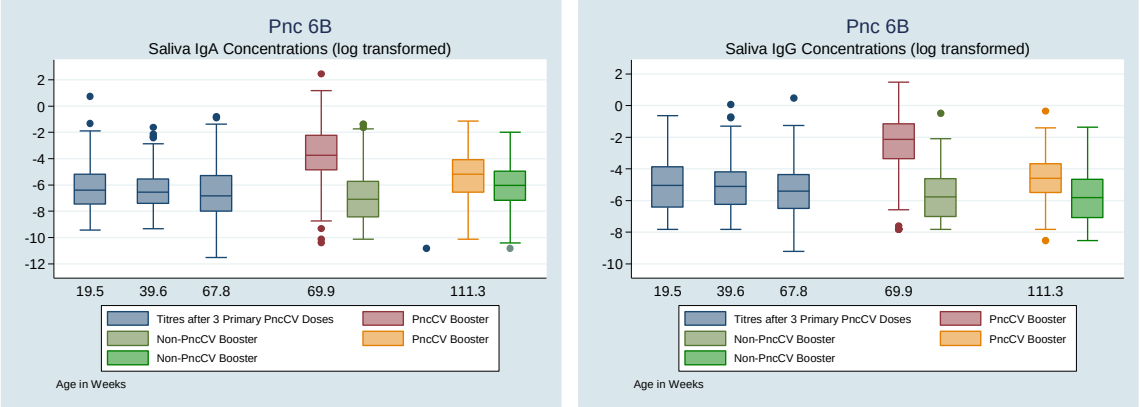

Figure 14: Saliva IgA and IgG concentration box plot for serotype 6B

Both saliva IgG and IgA concentrations against serotype 6B (Figure 14) at baseline (19.5 weeks), 39.6 and 67.8 weeks were comparable with no difference except for IgG at 19.5 weeks and IgA at 67.8 weeks with wide distribution antibody concentrations. The same trend was observed against serotype 9V (Figure 15), where IgA concentrations at age 19.5 weeks was high and widely distributed at 67.8 weeks and at 39.6 weeks very low. Although IgG concentration against serotype 9V followed the gradual drop from high at 19.5 weeks to low at 67.8 weeks (Figure 15).

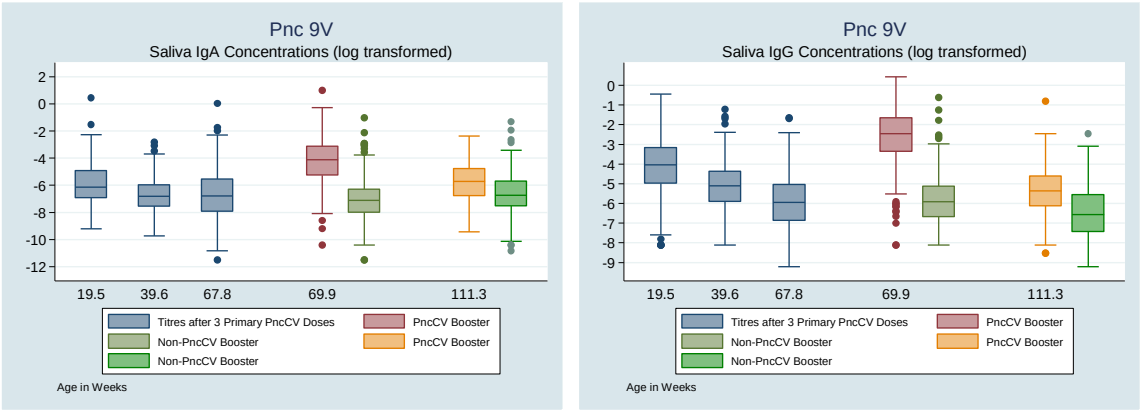

Figure 15: Saliva IgA and IgG concentration box plot for serotype 9V

Antibody concentration against serotype 14 (Figure 16) was different from other serotypes, in that IgG concentration at age 39.6 weeks were much higher than baseline concentration at 19.5 weeks and IgG concentration at age 67.8 weeks was wide and comparable to baseline (19.5 weeks). However, IgA concentration against serotype 14 was high at baseline, low and comparable at both 39.6 and 67.8 weeks.

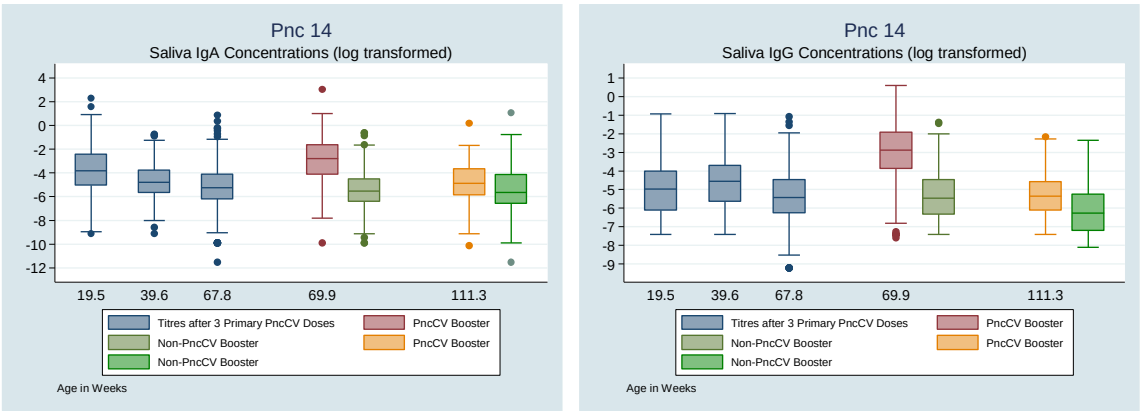

Figure 16: Saliva IgA and IgG concentration box plot for serotype 14

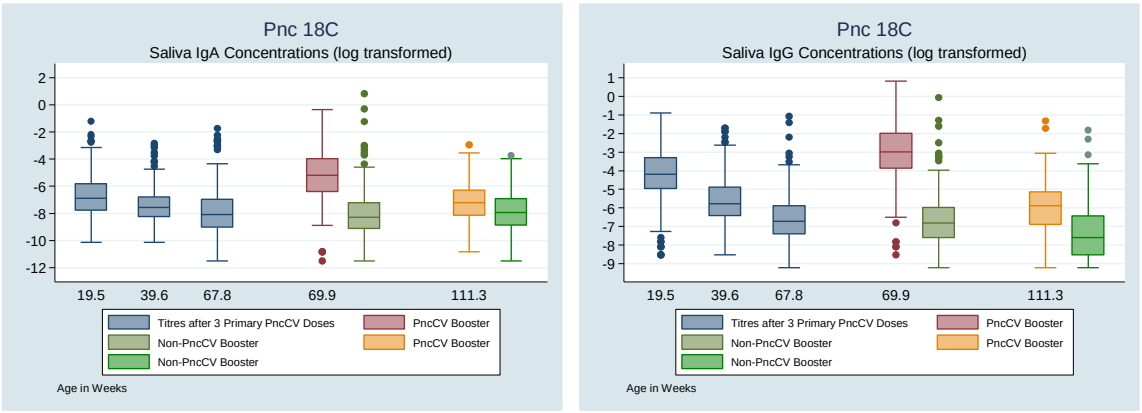

Figure 17: Saliva IgA and IgG concentration box plot for serotype 18C.

Both serotype 18C (Figure 17) and 19F (Figure 18) saliva IgG concentrations at baseline (19.5 weeks) were high and wide, and gradually dropped to low concentrations. Although at age 67.8 weeks for serotype 19F, IgG concentration was wider than IgG concentration at 39.6 weeks. Saliva IgA concentration for both serotype 18C and 19F were low, at age 19.5 weeks IgA concentration was higher (Figure 17) against serotype 18C than IgA at 36.9 and 67.8 weeks. However for serotype 19F, IgA concentrations at age 19.5, 36.9 and 67.8 weeks were much similar, with an exception at 67.8 week with a wider IgA concentration. The data show that the vaccine had no effect on saliva IgA against serotype 19F (Figure 18), because the antibody levels were all similar from baseline through to age 111.3 weeks.

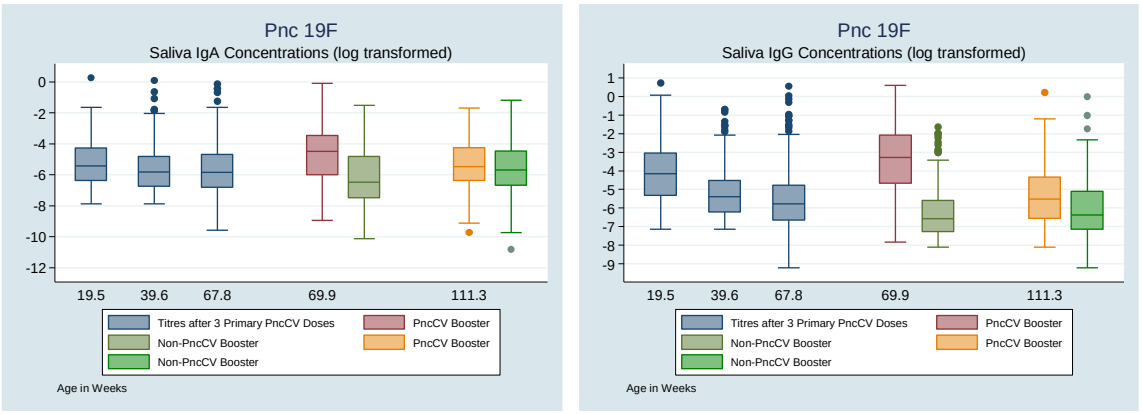

Figure 18: Saliva IgA and IgG concentration box plot for serotype 19F.

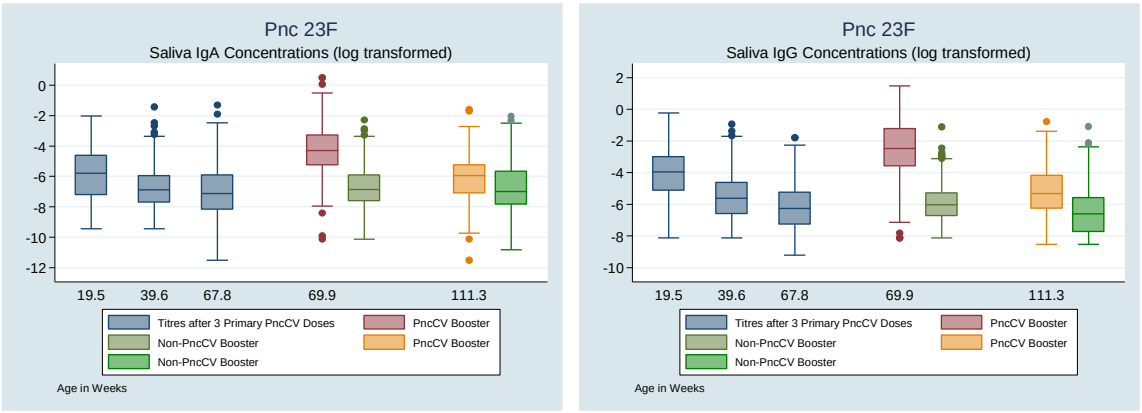

Figure 19: Saliva IgA and IgG concentration box plot for serotype 23F.

Saliva IgG and IgA concentrations were high at age 19.5 weeks against serotype 23F (Figure 19), and dropped to low concentration at 39.6 and 67.8 weeks. Only IgG concentration dropped gradually from baseline to 39.6 weeks and further dropped to low concentrations at 67.8 weeks. However, saliva IgA concentration was very high at baseline which dropped to low concentrations at 39.6 weeks, and increased from low to high and wide concentrations at 67.8 weeks (Figure 19). Serotype 7F antigen is not in the formulation of pneumococcal conjugate vaccine used in this study; the data shows that it had no effect in inducing both saliva IgG and IgA concentrations against serotype 7F antigen (Figure 20). Natural acquisition of serotype 7F pneumococcus increased IgG concentration at baseline only and the remaining sample points had similar concentrations. IgA concentration against serotype 7F was similar throughout the sample points and as expected, the booster vaccine had no effect on anti-7F antibody concentrations.

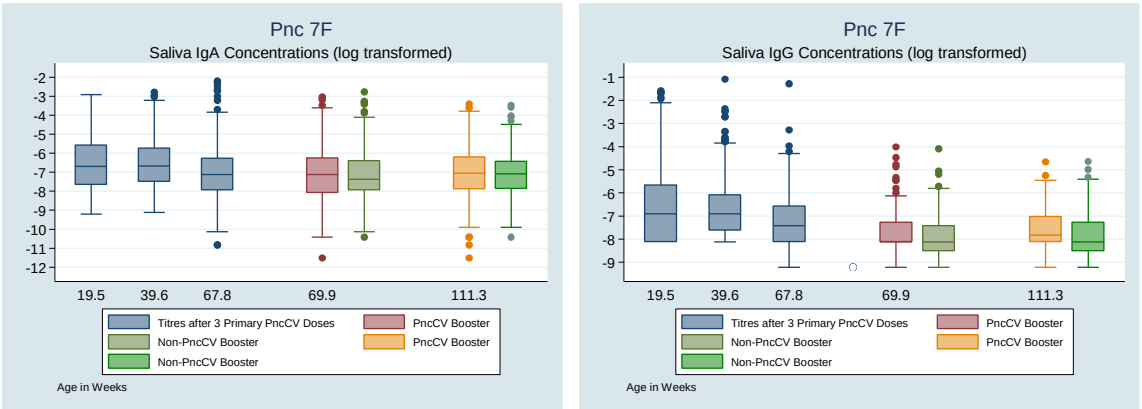

Figure 20: Saliva IgA and IgG concentration box plot for serotype 7F.

When pneumococcal booster dose was administered, there was a significant increase in antibody concentration and those that received placebo had no antibody concentration increase against all vaccine serotypes. The concentration of those that received placebo remained low compared to the concentration at age 111.3 weeks.

4.8 COMPARISON OF SALIVA IGA AND IGG IMMUNE RESPONSE TO VACCINATION WITH RESPECT TO HIV STATUS

When IgA and IgG from saliva were measured, the vaccine serotype antibody response was analysed in children that were born HIV negative, HIV positive and HIV exposed. The comparison was done with respect to HIV status with inclusion of HIV infected groups that received early and/or delayed ART therapy.

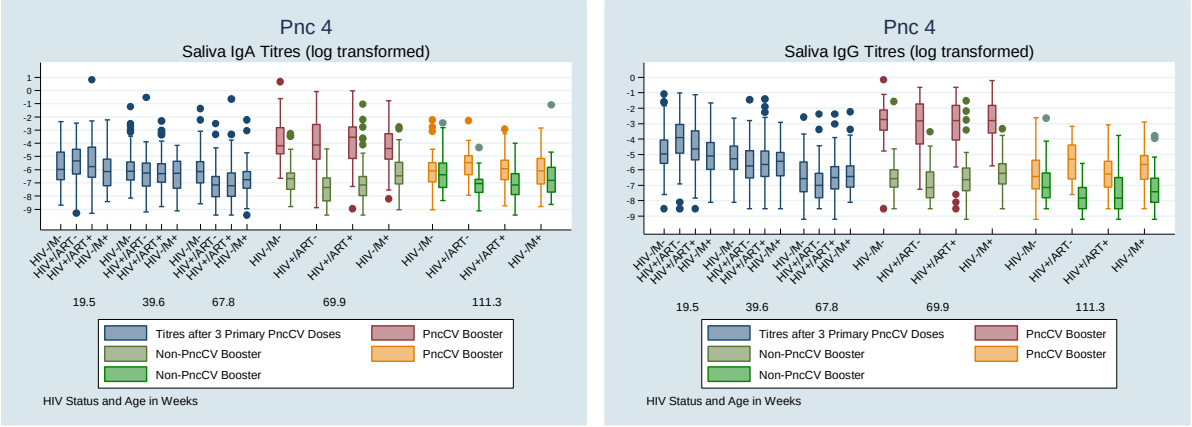

Figure 21: Serotype 4 saliva IgA and IgG concentration box plots for HIV status.

Saliva IgG and IgA antibody response against serotype 4 from four groups varied (Figure 21). At age 19.5 weeks, the HIV positive early ART therapy group had the widest distribution of saliva IgA concentrations, followed by HIV positive late ART therapy group. Both HIV negative groups yielded IgG and IgA concentrations that were lower than that of HIV positive groups, with HIV negative exposed concentration being the lowest. HIV positive group's IgA and IgG distribution dropped to low concentrations at 39.6 weeks and further dropped to lower levels at 67.8 weeks prior to booster vaccine. Although the HIV negative

group maintained a high IgA and IgG concentration observed at baseline (19.5 weeks), a wide distribution was observed at both 39.6 and 67.8 weeks.

IgA and IgG antibody distributions for HIV negative exposed groups at both 39.6 and 67.8 weeks was higher than the HIV positive and lower than the HIV negative group (Figure 21). After the pneumococcal booster vaccine at age 69.9 weeks, all the groups' IgA and IgG concentration levels increased significantly. HIV+/ART- booster recipients had the highest and widest antibody concentration distribution, followed by HIV+/ART+, then HIV-/M- and lastly HIV-/M+. At age 111.3 weeks the concentration for all groups' booster recipients dropped to low levels similar to that of non-booster recipients. Although HIV-/M- group IgA and IgG concentrations were the lowest of all groups, HIV+/ART- had the highest concentration of all groups.

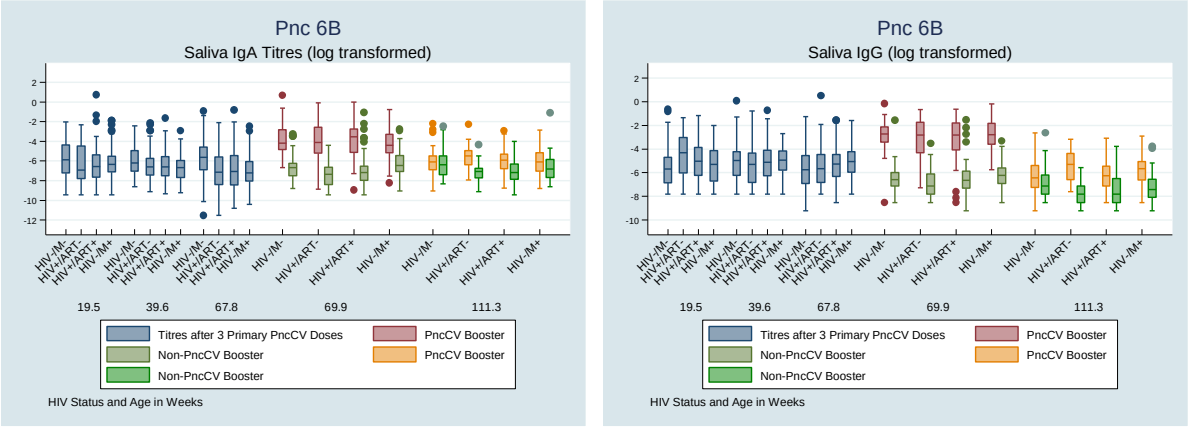

Figure 22: Serotype 6B saliva IgA and IgG concentration box plots for HIV status.

HIV-/M- group had high IgA concentration and least high IgG concentration at age 19.5 weeks against serotype 6B (Figure 22). HIV+/ART- group had the highest concentration levels for IgG and second highest for IgA, while age 67.8 weeks had the second highest for IgG and second last for IgA. However, HIV-/M+ group had the least high concentration levels at age 19.5, 39.6 and 67.8 weeks for IgA, but had the highest competing concentration levels at age 39.6 weeks and the highest levels at 67.8 weeks for IgG. At age 69.9 weeks HIV positive groups had the overall highest concentrations for both IgA and IgG that remained high at age 111.3 weeks for IgA with only HIV+/ART- for IgG.

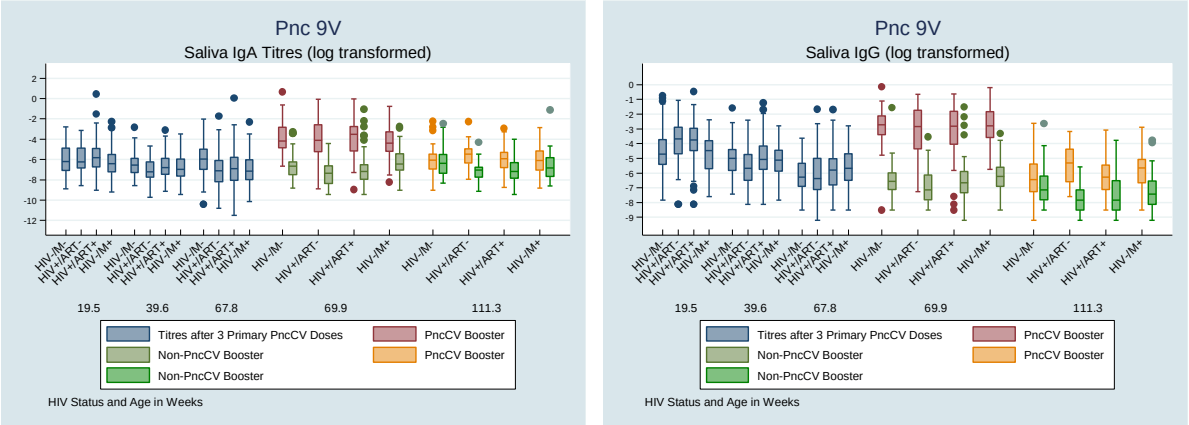

Figure 23: Serotype 9V saliva IgA and IgG concentration box plots for HIV status.

For serotype 9V (Figure 23), group HIV-/M- and both HIV positive groups had comparable concentrations at age 19.5 weeks for IgA, and the lowest concentration was in the HIV-/M+ group. For IgG, HIV positive groups had the highest concentrations and HIV negative groups low concentrations. However, at age 39.6 weeks for IgA, only group HIV+/ART- had low concentration and the rest of the groups had comparable and high concentration levels that was the same trend for IgG. At age 67.8 weeks, HIV-/M- group had high concentration levels, while other groups had low concentration levels that were comparable for IgA.

For IgG, HIV-/M+ group had high antibody concentrations, with HIV+/ART- having high and wide antibody concentrations followed by the HIV+/ART+ and HIV-/M- groups respectively. Saliva IgG had the highest booster concentration at age 69.9 weeks, higher than IgA concentration to all groups. All groups' antibody concentrations were comparable to each other for IgA without significant differences and the same was observed for IgG. HIV negative group's concentrations fell quickly to comparable levels to non-booster recipients at age 111.3 weeks, while the HIV positive group's concentration remained higher than that of the non-booster recipients for IgA. For IgG, the groups had a higher concentration than non-booster recipients.

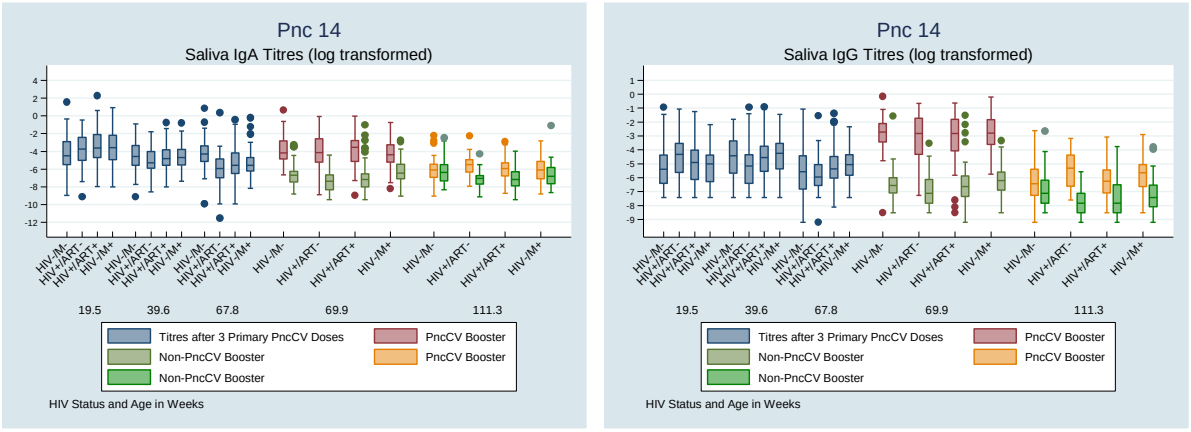

Figure 24: Serotype 14 saliva IgA and IgG concentration box plots for HIV status.

Saliva IgA response to serotype 14 at age 19.5 weeks (Figure 24), HIV+/ART+ had the highest concentration, followed by HIV-/M+, then HIV+/ART- and lastly the HIV-/M- group. For IgG, both HIV positive groups had the highest concentration and HIV negative groups had the lowest concentration. At age 39.6 and 67.8 weeks, IgA and IgG had the same trends where the HIV negative group had the highest concentration levels followed by HIV positive groups. High significant IgG concentrations were observed in all groups, compared to IgA at 69.5 weeks after the booster vaccine. IgA concentration dropped significantly to low concentrations observed in non-booster groups at age 111.3 weeks and IgG concentration also dropped to low concentrations.

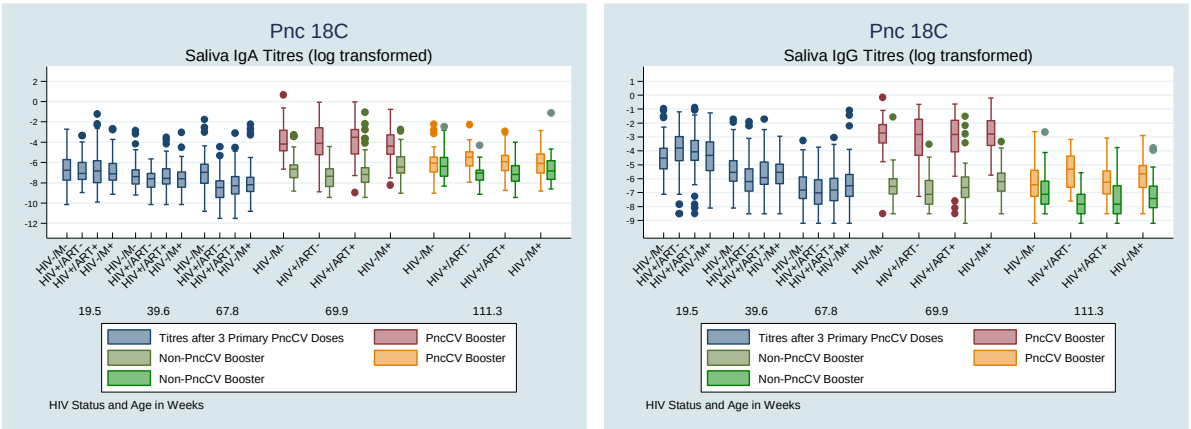

Figure 25: Serotype 18C saliva IgA and IgG concentration box plots for HIV status.

Serotype 18C (Figure 25) IgA concentration at age 19.5 to 67.8 weeks were significantly low and comparable. However, IgG concentrations were high in all groups at age 19.5 weeks and decreased at age 39.6 weeks and decreased further at age 67.8 weeks. HIV-/M- group had the lowest concentration at age 19.5 week compared to other groups, but had the highest concentrations at age 39.6 and 67.8 weeks. Similarly, the HIV+/ART- group had high concentration at age 19.5 weeks and low concentration at age 39.6 and 67.8 weeks. When HIV+ART+ and HIV-/M+ were compared to each other, HIV+/ART+ had higher IgA and IgG concentrations than HIV-/M+ at age 19.5 weeks. At age 39.6 weeks, both groups had slightly comparable concentrations and HIV-/M- had high IgA and IgG concentration at age 67.8 weeks. Both IgA and IgG concentration in all groups increased after booster vaccination, and IgG was most significant. IgA concentration dropped significantly to concentration levels of non-booster groups and IgG concentration dropped to high concentration levels above that of non-booster groups at age 111.3 weeks.

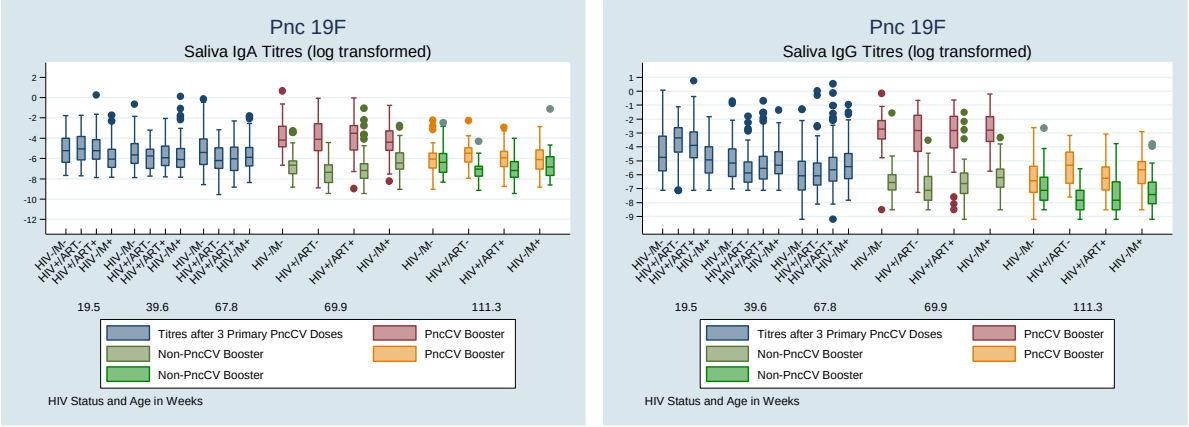

Figure 26: Serotype 19F saliva IgA and IgG concentration box plots for HIV status.

Saliva IgA concentration levels to serotype 19F (Figure 26) at age 19.5, 39.6 and 67.8 weeks were comparable, with the exception of HIV-/M+ that had the lowest concentration at age 19.5 weeks as compared to other groups. Also the exception was the HIV-/M- group with the highest concentrations at both ages 39.6 and 67.8 weeks. On the other hand, saliva IgG concentrations varied from one age group at a sampling point. HIV+/ART+ had the highest concentration at 19.5 age weeks, while HIV-/M- had the highest concentration at age 39.6 weeks and lastly HIV-/M+ had the highest concentrations at age 67.8 weeks. HIV+/ART- had

the lowest concentration at both age 39.6 and 67.8 weeks, also HIV-/M- had the lowest and the widest concentration coupled with outliers.

After the booster dose, vaccine recipients had the highest concentration for both IgA and IgG compared to non-booster recipients. The IgA concentration of all groups was highest at age 69.9 weeks, and at age 111.3 weeks the concentration levels were comparable to those of the non-vaccine recipients. Furthermore, vaccine recipients' IgG concentration levels at 69.9 were significantly higher and concentration levels at age 111.3 weeks were decreased but still higher than the concentration of the non-vaccine recipients.

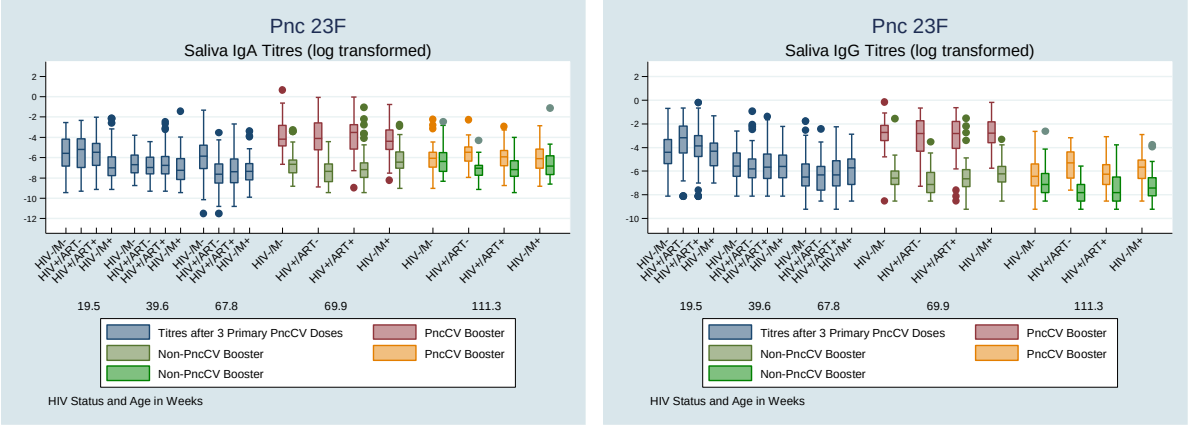

Figure 27: Serotype 23F saliva IgA and IgG concentration box plots for HIV status.

Serotype 23F saliva IgA concentration to HIV-/M- and HIV+/ART- were highest, followed by HIV+ART+ then lastly HIV-/M+ at age 19.5 weeks (Figure 27). HIV+/ART- group had the highest concentrations for IgG, which were followed by HIV+/ART+, HIV-/M- and lastly HIV-/M+. All the groups had the same comparable concentration at age 39.6 weeks for both IgA and IgG. Saliva IgA and IgG concentrations of all the groups were higher at age 69.9 weeks, which decreased at age 111.3 weeks.

Serotype 7F was a control serotype to determine natural antibody response (Figure 28). IgA antibody response against serotype 7F was similar to most vaccine serotypes. HIV-/M-, HIV+/ART- and HIV+/ART+ groups had high IgA and IgG antibody concentrations, only the HIV-/M+ group had low IgA and IgG concentrations at age 19.5 weeks. However, the

IgA antibody response at age 39.6 and 67.8 weeks was high in the HIV-/M- group, and the rest of the groups sharing similar concentration levels.

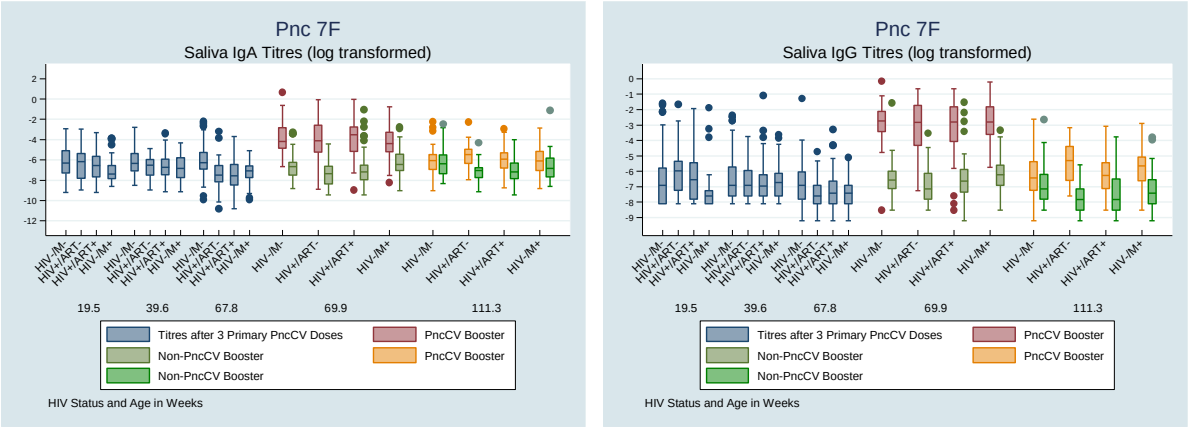

Figure 28: Serotype 7F saliva IgA and IgG concentration box plots for HIV status.

Similarly, the HIV-/M- group had high and wide IgG antibody response at age 39.6 and 67.8 weeks and the rest of the groups sharing similar concentration levels. After the booster vaccine dose, high IgA and IgG response was observed in vaccine recipients and low IgA and IgG response observed in non-vaccine recipients against serotype 7F.

4.9 CONCLUSION

With the optimisation of the assay conditions, the validation was proved successful by meeting the targets of each parameter. Serum IgG concentrations of the vaccine candidates were 5 fold higher compared to non-vaccine candidates to all serotypes although saliva IgG concentrations of the vaccinated candidates were 2 fold higher compared to unvaccinated candidates. The p-value of <0.0001 was significant when comparing IgG for the vaccinated and unvaccinated candidates for both serum and saliva. The correlation between serum and saliva IgG yielded a high coefficient of correlation of above or over 0.8 to all serotypes at age 69.9 weeks. There was no difference in antibody concentrations between HIV negative and HIV exposed. Vaccine boosters elevated concentrations that lasted a longer period at age 69.9 weeks.

CHAPTER 5

DISCUSSION

5.1 INTRODUCTION

This dissertation is part of a research study carried out from 2005 to 2012 in Johannesburg and Cape Town. The main objective of the parent study was to conduct an investigation on the immunogenicity and safety of pneumococcal conjugate vaccine administered to HIV infected and HIV uninfected children. The second objective of the parent study was to evaluate the qualitative and quantitative antibody response to polysaccharide antigens in PCV7. Since little is known about the effects of PCV7 vaccination on salivary antibodies, this was added to the parent study and forms the subject of this thesis. Induced immunoglobulin A (IgA) and immunoglobulin G (IgG) antibody concentrations were measured in saliva and the IgG concentration level was measured in serum. Natural acquired antibody response to pneumococcal polysaccharide was compared to vaccine induced antibody response. As stipulated in chapter 1: Section 1.5.1, the aim of this study was to evaluate the relationship and/or difference in antibody induction by naturally occurring polysaccharide and purified polysaccharide. Mucosal immunity which is made up of abundant IgA antibodies in saliva, is the first line of defence against microbial attack by bacteria such as pneumococcus. However, IgG in saliva is activated once the epithelial barrier has been bridged and infection beyond epithelial is imminent (Cerutti et al., 2011:273-274).

5.2 THE OPTIMISATION AND VALIDATION OF LUMINEX ASSAY FOR THE MEASUREMENT OF SALIVA IGA AND IGG ANTIBODIES

The validation of pneumococcal capsular polysaccharide-specific antibody concentration by Luminex multiplex assay to monitor the IgA and IgG immune response to PCV7 and through pneumococcus natural exposure in saliva was reported in the results section. In the developmental stage, we optimised the conjugation reaction by binding each of eight pneumococcal capsular polysaccharides to a microsphere.

Specificity assays demonstrated that the multiplex assay detected pneumococcal capsular polysaccharide specific IgA and IgG in saliva and this suggests that the antigenic determinant epitopes on the capsular polysaccharides were not affected during conjugation to the microsphere. Together with high reference serum percentage recovery for IgA (99.62-106.33%) and IgG (99.00-102.88%) proved the assay's specificity and reproducibility. Addition of pneumococcal purified polysaccharide antigen resulted in over 99.80% inhibition in signal for 89-SF standard for both IgA and IgG (Table 12). This boosted the performance of the assay specificity of vaccine serotypes, as well as the control serotype 7F.

Precision is the second important parameter that assessed the inter- and intra-precision assay for control serum CV's and saliva duplicate sample CV's respectively. The inter-precision assay CV variability for control serum of both IgA and IgG was consistently below 30% (Table 13 & 14). Similarly, Intra-assay IgA and IgG variation was also shown to be very low, with a CV of 10% and below for all serotypes (Table 15 & 16). Low and consistent intra-assay variability of reference serum SF89, with CV of less than 8.95 to pneumococcal serotypes was reported with a Luminex assay (Klein et al., 2012:1280).

The serum and saliva protective concentration of vaccine serotype-specific pneumococcal antibody concentrations by Luminex has not been clearly defined. A quantified protective concentration of 0.35 µg/mL against vaccine serotypes is globally accepted, which ELISA was used to measure (Ekström et al., 2007:1794). It is however difficult to measure the saliva protective concentrations to vaccine serotype capsular polysaccharide with ELISA methods. The saliva ELISA assays produce low concentration readings due to low dynamic ranges (Nieminen et al., 1999:769) and it is less sensitive (Martín-Martín et al., 2013) compared to the Luminex assay and correlation between the two methods is low. If the correlate of protection for salivary Igs can be established, then it would provide a less invasive way of collecting samples. The serum/saliva IgG ratio is about 20:1, then the cut off for protection of saliva IgG is probably around 0.35/20 (0.175 µg/mL).

5.3 RESPONSE, COMPARISON AND CORRELATION OF SALIVA AND SERUM IGG IN VACCINATED AND UNVACCINATED CHILDREN

After 3 primary doses of PCV7, anti-pneumococcal IgG concentration against vaccine serotypes was very high at age 15-30 weeks and gradually decreased thereafter. IgG in serum reached the highest concentration of 7.37 µg/mL against serotype 19F at age 15-30 weeks. Serotype 19F was found to have the highest IgG concentration against it, one month after three doses of pneumococcal conjugate vaccine in serum (Nurkka et al., 2001:28). IgG continued to drop to high concentrations of 1.93 µg/mL and 2.63 µg/mL at age 38-42 weeks and 64-76 weeks respectively, except for serotype 14 (7.17 µg/mL) at age 38-42 weeks.

Similarly, IgG concentrations in saliva was high at age 15-30 weeks, reaching a concentration of 0.0173 µg/mL, and further dropped to high concentrations of 0.0098 µg/mL and 0.0048 µg/mL at age 38-42 weeks and 64-76 weeks respectively. The antibodies in both saliva and serum dropped to low concentrations in a similar manner throughout the sample points. There have been suggestions that the origin of most saliva IgG is the same as that of serum IgG, and it transudates through the capillaries from serum (Nieminen et al., 1998:635; Brandtzaeg, 2013:19).

Two weeks after the pneumococcal booster vaccine (age 65-78 weeks), IgG concentrations in both serum and saliva increased to 8.84 µg/mL and 0.0169 µg/mL respectively. Rodenburg et al. (2012:e46920) also reported an increase in saliva IgG concentrations after pneumococcal vaccine booster dose, supporting the idea that IgG in saliva originates from serum.

In this study the anti-pneumococcal polysaccharide specific saliva and serum IgG correlated strongly and positively post booster vaccination. Although correlation varied from one serotype to another, at least a minimum correlation coefficient of 0.6236 was observed. It was not only in this study that serum and saliva IgG correlated, but also in a PCV7 study where four vaccine serotypes 4, 6B, 19F and 23F were correlated (Korkeila et al., 2000:1218-1226). However, it is important to report that saliva IgG absolute concentrations differ extensively from serum IgG absolute concentrations from serotype to serotype; although one study reported higher mucosal anti-pneumococcal IgG concentrations in the lungs of healthy and HIV-infected subjects than in plasma (Twigg, 2005:418).

The overall fold difference of saliva IgG concentrations between the vaccinated group and unvaccinated group ranged between 0.54 to 28.33 fold in all sample points (Data not shown). The ratio for serum/saliva was also comparable when correlated, similar to the data from a study done by Rodenburg and colleagues where serum IgG levels were 20 fold higher to saliva Ig levels and also highly correlated (Rodenburg et al., 2012:e46916-e46923). The highest fold difference was observed at age 15-30 weeks (5.08 – 21.71), 6 weeks after vaccination and 65-78 weeks (3.71 – 28.33), 2 weeks after booster vaccination. Saliva IgG concentration's difference against serotype 7F between vaccinated and unvaccinated was well below 1.5 folds, observed in all sample points. This however could mean that some IgG in the unvaccinated group could be locally produced through stimulation by natural colonisation, as suggested by Brandtzaeg in both his works (2007:303; 2013:19).

5.4 SALIVA IGA AND IGG RESPONSE IN VACCINATED AND UNVACCINATED HIV NEGATIVE CHILDREN

Mucosal immune response has been viewed as the primary defence against colonising microorganisms by preventing adhesion to the mucosal epithelium (Mantis et al., 2011:604). The effective mucosal antibodies consist of predominantly IgA specific antibodies that are produced locally (Mazanec et al., 1993:430) and IgG transported across mucosal epithelium (Kelly et al., 2004:168). However, natural exposure through colonisation to bacterial polysaccharide results in a poor immune response in children less than two years of age while vaccines induced higher antibody responses (Sigurdardottir et al., 2014:417-424).

Post primary PCV7 vaccination, vaccinated group IgG and IgA antibody concentrations against vaccine serotypes were high at baseline (15-30 weeks) when compared to the unvaccinated group ($P < 0.05$). The concentrations began to fall at age 38-42 weeks, which continued to fall further at age 64-76 weeks only against vaccine serotypes, while serotype 7F IgA and IgG concentration was unchanged. A point was reached where IgA concentrations in the vaccinated group was no different from unvaccinated group ($P > 0.05$) at pre-booster baseline (64-76 weeks), and only serotype 19F for IgG ($P = 0.8601$) (Table 23). Post booster vaccination (65-78 weeks), both IgG and IgA concentrations in vaccinated group increased significantly compared to the unvaccinated group ($P < 0.0001$), except for serotype 19F ($P = 0.0606$) and serotype 7F ($P = 0.9107$) for IgA (Table 24). The findings of Nurkka et al.

(2004:44) were similar to that of this study were GMCs ranges rose significantly from low to high concentrations for salivary IgA following booster vaccination.

The conjugate vaccine is capable of inducing antibody response to only vaccine serotype and ineffective to induce antibody concentration to unvaccinated serotype such as serotype 7F. These findings also show that the conjugate vaccine can elicit saliva antibody responses even though it is administered parenterally. Kauppi et al. (1995:292) reported an increase in saliva antibody concentration in response to parenterally administered Hib conjugate.

We also observed a moderate positive correlation (R = 0.5319 – R = 0.7384) between saliva IgA and IgG concentrations at baseline (15-30 weeks) following the last of 3 primary vaccinations in the vaccinated group. High positive correlation (R = 0.6143 – R = 0.7898) in the unvaccinated group was observed also at baseline. At pre-booster baseline (64-76 weeks), a negligible correlation was observed in the vaccinated group (Data not shown) that increased to high positive correlation (R = 0.6798 – R = 0.7929) after booster vaccination at age 65-78 weeks (Table 24). The only exception was serotype 19F with low positive correlation (R = 0.4247). There is a link between the two antibody production systems because IgA and IgG in saliva are stimulated in a similar manner that needs more investigation.

5.5 DYNAMICS OF SALIVA ANTIBODY CONCENTRATIONS WITH RESPECT TO AGE IN THE FIRST TWO YEARS OF LIFE

It has been reported that infants below the age of 2 years have an immature immune response to capsular polysaccharides (Bonhoeffer et al., 2006:929; Hussein et al., 2015:3). However, children in this study managed to produce measurable saliva IgG and IgA antibody concentrations 3 to 6 weeks after primary PCV7 vaccination (19.5 weeks) against all vaccine serotypes. Saliva IgG concentration against serotype 7F was low when compared to other serotypes, and IgA concentration against 7F was higher than IgG at age 19.5 weeks. Saliva IgA antibody concentration against most vaccine serotypes dropped slower than saliva IgG antibody concentration at age 39.6 and 67.8 weeks. There was no significant drop of saliva IgA antibody concentration against serotype 7F at baseline, at age 39.6 weeks and age 67.8 weeks (Figure 20).

Two weeks after booster PCV (69.9 weeks), saliva IgG and IgA concentration against all vaccines increased to high levels, except serotype 7F. In this study antibody concentrations to vaccine serotypes increased during three primary vaccine doses and depleted post vaccination, furthermore increasing after vaccine booster dose and the same was observed by Black et al. (2000:193).

5.6 COMPARISON OF SALIVA IGA AND IGG IMMUNE RESPONSE TO VACCINATION WITH RESPECT TO HIV STATUS

S. pneumoniae is a leading cause of childhood pneumonia, IPD and meningitis, with high burden of disease in developing countries. Together with human immunodeficiency virus (HIV), they lead to disease associated mortality in Africa, especially in sub-Saharan children that increases the risk of pneumococcal infection (Iwajomo et al., 2011:534). Infants and young children with human immunodeficiency virus (HIV) infection have been found to have higher incidence and burden of systemic infections caused by *S. pneumoniae* (Jones et al., 1998:2183). Similarly, HIV-exposed uninfected infants have been reported to be at high risk of pneumococcal infection when compared to HIV-unexposed uninfected infants (Gray & Zar, 2010:209).

Post primary PCV7 vaccination, saliva IgG and IgA concentrations against all vaccine serotypes were high at baseline (19.5 weeks) for all the groups. However, the HIV positive children on delayed AR therapy (HIV+/ART-) managed to produce high saliva IgG concentrations against all vaccine serotypes, including control serotype 7F at baseline. The high IgG concentration was not maintained as it dropped quite more rapidly than other groups at age 39.6 and 67.8 weeks against all serotypes. Also saliva IgA concentration of HIV+/ART- group dropped more rapidly than most groups. A similar decrease in antibody concentrations was also observed after the third-dose of pneumococcal conjugate vaccine in 5 vaccine serotypes 6B, 23F, 14, 18C and 19F by King et al. (1998:363).

The HIV positive children on early ART (HIV+/ART+) had the second highest saliva IgA and IgG concentrations at baseline (19.5 weeks) against all serotypes. The group IgG concentration did no fall as rapidly as it was observed in the HIV+/ART- group; it rather dropped in a constant manner at age 39.6 and 67.8 weeks. HIV negative children (both HIV-

/M- and HIV-/M+ groups) saliva IgA concentration did not drop rapidly, it was rather maintained from age 19.5 weeks right through to age 67.8 weeks.

After the booster vaccination at age 69.9 weeks, high saliva IgG and IgA antibody concentrations were observed against all vaccine serotypes and also against control serotype 7F. Vaccinated children in all groups had high and similar IgG and IgA antibody concentrations while children who received placebo had low IgG and IgA antibody concentrations. However, both IgG and IgA concentrations of HIV+/ART group were high and wide compared to other groups. Even when IgG and IgA of all groups dropped at age 111.3 weeks, HIV+/ART- IgG and IgA antibody concentrations were higher and wider than other groups.

5.7 CONCLUSION

The contribution added to the main programme by this study was the information explaining the impact of the pneumococcal conjugate vaccine on mucosal immunity. The validation data showed that the Luminex assay is the most suitable platform that is currently available, to measure, quantify and identify antibody concentrations with high sensitivity and low cross-reactivity from non-target antigens. The correlation between the serum and saliva IgG antibody concentration response after immunisation with pneumococcal conjugate vaccine was significant. Vaccinated cohorts produced higher antibody concentration than unvaccinated cohorts. Saliva IgG production correlated with saliva IgA.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 INTRODUCTION

In this study it is indicated that the PCV7 can induce an immune response that results in serotype-specific antibody production. It is also indicated that pneumococcal exposure can induce an effective natural immunity to vaccine and non-vaccine serotypes beyond the age of 18 months. Even though the vaccine is administered intramuscularly, it can promote the production of a saliva IgA and IgG antibody response.

6.2 RESEARCHERS CONTRIBUTION

As part of the sub-study that evaluated the antibody concentration in saliva and serum samples, this researcher was responsible for the following:

- The initial responsibilities were to receive and process the samples prior to assaying and testing.
- The overall set up for the optimization and validation of the Luminex assay.
- Quantification of pneumococcal antibody concentrations in both saliva and serum using Luminex and ELISA assays respectively.
- Capturing and cleaning of data in accordance with GCLP (Good Clinical and Laboratory Practice) guidelines prior to statistical analysis.
- Statistical tests such as student's t-test, Pearson's correlation including Wilcoxon signed ranks and Mann-Whitney tests were used in data analysis.
- Data analysis and reporting.

6.3 LIMITATION TO THE STUDY

The major limitation of the study was methodological, there was too much random variation caused by the inconsistency of the sample (saliva) due to different flow rates and viscosity, (pilot studies demonstrated a 10-fold variance in Ig concentration on samples collected the

same day on adults) whereas serum is a more consistent body fluid to work with. There was also a problem with the sensitivity of the assay. It was clearly better than the ELISA used by Nurkka et al. (2004:44), that has only a small proportion of samples above the detection threshold, but there were still too many samples close to the LOD, where the assay measurements are less accurate. If you combine these two factors, it creates a lot of noise in the measurement system that prohibits the establishment of good correlations between the Ig types.

The lack of similar research studies made it difficult to gather enough information to support the overall outcome of this study. Research studies form the basis of literature review and help to lay a foundation for understanding the research problem investigated. The reason for this lack of data is due to the nature of these studies that require huge sample population size and the financial resources to support the cost of the vaccines, participant allowances, assays, and medical care needed for such a study. The impact of this limitation is significant enough, in that few studies supported similar objectives while other objectives had less data support.

Assessment of antibody concentration using two methods of quantification indicated problems when comparisons were calculated. Serum IgG antibodies were quantified with an ELISA method while saliva IgG and IgA antibodies were quantified with a Luminex bead-based method. Though the statistical comparisons were significant during serum and saliva IgG correlation, the fold difference was 2 to 10 depending on the sample-point. It was obvious from the absolute values that IgG concentrations levels were different, since one of the methods was sensitive enough to limit the background while the other was less sensitive. Though the aim was to calculate the correlation, it would have been more informative and ideal if one methodology was used to compare the same IgG levels from these two distinct sites.

6.4 MAIN FINDINGS

6.4.1 Literature

S. pneumoniae is a gram-positive bacterium that normally inhabits the human nasopharynx as normal flora. It usually colonises the upper respiratory tract and spreads to other parts of the body to cause disease especially in the lungs, the blood and the meninges. There are about 90

distinct serotypes that are differentiated by the makeup of the capsular polysaccharide, a very virulent substance of the pneumococcus. The capsular polysaccharide is capable of invoking a host immune response together with bacterium pathogenic proteins. However, a matured immune system of a host can defend against the disease initiation by responding with specific antibodies to the capsule and the proteins. This mechanism of defence is only effective in adults and children over the age of 2 years. An infant's antibody response immunity is incapable of responding to capsular polysaccharide infection, without help from a conjugate vaccine. Pneumococcal conjugate vaccine is manufactured to assist with priming the infant's immune system to elicit a T-cell dependent immune response to capsular polysaccharide. This also assists in the generation of memory B-cells' immune response for the continuous encounter of the same capsular polysaccharide. The current study was established to evaluate the mucosal and humoral antibody response to capsular polysaccharide of the 7-valent vaccine serotypes.

6.4.2 Prevalence

Burden of disease is underestimated in developed countries, but in developing countries the burden estimates are close to non-existent. The reason for this underestimation and lack of information is due to health facilities that are out of reach to communities that need them and undocumented disease cases. The source of information is obtained from academically funded projects with a limited number of participants, yielding limited information to draw enough conclusions. As it has been shown in recent studies, the risk of IPD is greatest among young children in the first two years of life. The rate of IPD was defined as the number of episodes or cases per 100 000 child-years. In a study conducted in South Africa, the incidence of vaccine specific-serotype invasive disease was estimated to be at 112 cases per 100 000 child-years.

6.4.3 Validation

The appropriateness of the optimisation and validation of the assay depends mainly on the objectives and the goals of the study. The validation assessed in this study included the required and necessary parameters and procedures that demonstrated the ability of this optimised assay to quantitatively measure IgA and IgG antibody concentrations in saliva. Limit of detection (LOD) and limit of quantification (LOQ) were the first parameters to be

assessed and all serotype ranges were determined. The second parameters to be assessed include specificity and sensitivity that ascertained the limit of acceptability of the assay. Precision was also looked at to address the reproducibility of an assay with the ability to measure linearity and parallelism of samples serially diluted against a reference curve. The findings were favourable for the usage of the method (See chapter 4: Results, section 4.3). The Luminex bead-based assay was a favourable method and more suitable for the detection of small quantities and concentrations of Igs in saliva because the assay was more sensitive.

6.4.4 Antibody Response in Vaccinated and Unvaccinated Population

The observation of the immune response in saliva and serum for IgG concentration has shown that after PCV booster administration, the serotype–specific antibody levels increase and correlate. It was observed that there was a concentration difference in the children who received the vaccine and children who did not receive the vaccine in both the HIV negative and HIV exposed groups. The vaccine played a major role in eliciting an effective immune response to vaccine serotypes and also a natural exposure to pneumococcus elicited some response to vaccine serotypes. It has been widely reported that infants have an immature immune response to unconjugated capsular polysaccharide, especially before they reach the age of two. Anti-pneumococcal concentration levels to four groups, the HIV uninfected, HIV exposed and HIV infected between those who are receiving early and delayed ART therapy were also different. However, the difference may be due to the administered ART therapy at different ages.

6.5 SIGNIFICANCE OF THE STUDY

The most significant thing about the study is the uniqueness of the parent study cohort obtained, i.e. a large HIV infected cohort and the fact that there is an early and delayed ART arm.

The most significant thing about the study objectives is that it is one of the few studies to look at salivary antibodies with a sensitive Luminex bead-based method, and to explore the correlation between serum and saliva IgG, and the relationship between saliva IgG and IgA.

6.6 EXPECTED OUTCOMES

6.6.1 Impact on Health Policies

The findings of this study would add value to a lack of information on salivary antibodies as a correlate of protection to infections that are initiated in the mucosal area. However, saliva as a first line of defence is suitable for immune induction by oral vaccination that is being researched currently. The information generated in this study would be freely available to researchers exploring the potential of mucosal vaccines.

Mucosal data supports the use of early administration of HAART as is evident by the fact that those who received early ART therapy had stronger saliva IgA and IgG responses than those that had delayed HAART. This supports the government policy of perinatal screening of mothers for HIV so that the neonates can start on HAART as soon as possible as there are now proven benefits with respect to impact on mucosal immunity.

6.6.2 Scientific Knowledge and Contribution

The design of the study provided data to highlight the differences between mucosal and humoral immune response to PCV vaccination. The two immune systems respond with different dynamics following vaccine exposure. The IgG in saliva matched the concentration and response of the IgG in serum, providing proof that IgG in saliva originates from the blood. The production of IgA in saliva could also be stimulated through intramuscular (IM) vaccination, however the response was shorter lived than salivary and serum IgG. There are few to no studies that compared how well anti-pneumococcal capsular polysaccharide antibodies, particularly the IgA and IgG in saliva correlate, until our study.

6.6.3 Capacity Building

This project resulted in pioneering the use of Luminex technology to improve the sensitivity of immune assays, and thus allow the detection of antibodies in low concentration samples such as saliva. Several junior technologists and in-service trainees were also mentored in these methods. Through this study, a Master’s degree will be obtained and an article will be published in a peer reviewed journal.

6.6.4 Contribution to the community

The community leadership where the study was conducted will get feedback regarding the outcome of the study and its benefits. This would be done by an article published in a peer reviewed journal and the community health forum that aims at encouraging parents to participate in vaccination programs for their infants, children above two years and the elderly.

6.7 CONCLUSION

The following conclusions can be drawn from this study:

- The Luminex assay was optimised and validated to produce reliable data from low concentration samples such as saliva.
- Pneumococcal conjugate vaccines induced pneumococcal capsular polysaccharide specific antibodies in saliva. However, there are differences between the vaccines' ability to induce mucosal immune response and there are also serotype specific differences in the antibody concentrations and in the proportion of positive samples after a series of vaccinations.
- Salivary IgG appeared to be derived mainly from serum, but some local IgG production may took place.
- The pneumococcal conjugate vaccine in this study was able to induce mucosal immune memory: the anti-pneumococcal IgA concentrations also increased with age in the saliva of unvaccinated children.
- A booster immunisation with a pneumococcal conjugate vaccine induced stronger salivary antibody responses than a natural booster.

6.8 RECOMMENDATION

Pneumococcal conjugate vaccine and natural contacts with bacteria have all been found to induce specific antibodies in saliva. According to literature on respiratory infections, local antibodies have a role in pneumococcal colonisation defence. However, the actual importance of salivary anti-pneumococcal antibodies in humans is not completely clear. It is unclear

which is more important, IgA or IgG or how much of mucosal antibody concentrations are needed to prevent an acquisition of a disease. In serum, surrogates of protection against pneumococcal disease have been sought for many years. Serum anti-pneumococcal capsular polysaccharide IgG concentration 0.35 μg/mL has been suggested as a surrogate of protection against invasive disease at population level.

Salivary antibody concentrations reflect antibody levels in the nasopharynx where bacteria colonise the human body. It is unclear e.g., whether there is a difference between vaccine-induced and natural antibodies with respect to the avidity and functional activity. Neither is it known whether there are differences between the qualities of salivary antibodies after a polysaccharide booster compared with a conjugate booster.

The researcher recommends the following:

- Future studies should investigate the role of mucosal immunity against pneumococcal disease in humans, and try to establish correlates of protection for these potential protective classes of immunoglobulins against:
  - IPD
  - Acquisition of pneumococcal colonisation
  - clearance of colonisation
- Protein vaccines against pneumococcal disease are being developed currently. The ability of these vaccines to induce mucosal immunity should be studied and the possible role of mucosal immunity as a surrogate of protection should be considered.
- A combination of nasal and parenteral administration could be considered. That would offer protection against different serotypes and further induce both systemic and mucosal immunities.

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University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee: (Medical)
FWA Registered No IRB 00001223

SECRETARIAT: Suite 189, Private Bag x2600, Houghton 2041, South Africa • Tel: +27-11-274-9200 • Fax: +27-11-274-9281

FAXED AND MAIL

06 January 2005

Dr SA Madhi,
Department Director
Respiratory and Meningeal Pathogens Rese
Chris Hani Baragwanath Hospital
First Floor, West Wing, Nurses Residence
Old Potch Rd, Diepkloof, Soweto
2013

Fax: 011 989 9886

Dear Dr Madhi,

PROTOCOL: CIPRA - SA PROJECT 4 - EVALUATION OF QUANTITATIVE AND QUALITATIVE ANTIBODY RESPONSES TO STREPTOCOCCUS PNEUMONIAE AND HAEMOPHILUS INFLUENZAE TYPE B CONJUGATE VACCINES AMONGST HIV-1 EXPOSED-INFECTED CHILDREN THAT ARE RECEIVING VS. THOSE NOT RECEIVING ANTIRETROVIRAL THERAPY, AS WELL AS AMONG HIV-1 EXPOSED-UNINFECTED CHILDREN AND HIV-1 UNEXPOSED-UNINFECTED CHILDREN - A MULTICENTER TRIAL OF THE COMPREHENSIVE INTERNATIONAL PROGRAM FOR RESEARCH ON AIDS

ETHICS REFERENCE NO: 040704

RE : APPROVAL FOR PROTOCOL VERSION 1.0 DATED 24 NOVEMBER 2004 AND PATIENT INFORMATION LEAFLET AND INFORMED CONSENT FORM VERSION 1.0 DATED 24 NOVEMBER 2004

We acknowledge receipt of your letter dated 10 December 2004 with the following documentation pertaining to the above-captioned trial.

Amendment Date:	24-Nov-04	Amendment Version:	Protocol Version 1.0
Amendment Number:	1.0	Received Date:	10-Dec-04

The following has been approved by the Wits Human Research Ethics Committee: (Medical)

* Protocol Version 1.0 dated 24 November 2004
* Patient Information Leaflet and Informed Consent Form, Version 1.0 dated 24 November 2004

1. THIS APPROVAL IS SUBJECT TO THE FOLLOWING PROVISOS:

* A copy of the MCC Approval and/or MCC Notification letter must be submitted to the Ethics Regulatory Office Secretariat before the study commences.

* The study is conducted according to the protocol submitted to the University of the Witwatersrand, Human Research Ethics Committee. Any amendments to the protocol must first be submitted to the Human Research Ethics Committee for approval.

* During the study, the University of the Witwatersrand, Human Research Ethics Committee is informed immediately of :

- Any Unexpected Serious Adverse Events or Unexpected Adverse Drug Reactions, which, in the Investigator and/or the Sponsor's opinion are suspected to be related to the study drug. (International and Local Reports).
- Any data received during the trial which, may cast doubt on the validity of the continuation of the study .

* The University of the Witwatersrand, Human Research Ethics Committee is notified of any decision to discontinue the study and the reason stated.

* The Investigators authorised by this approval participate in this study. Additional Investigators shall be submitted to the University of the Witwatersrand, Human Research Ethics Committee for approval prior to their participation in the study.

* In the event of an authorised Investigator ceasing to participate in the study, the University of the Witwatersrand, Human Research Ethics Committee must be informed and the reason for such cessation given.

2. PRINCIPLES OF INFORMED CONSENT:

* The University of the Witwatersrand, Human Research Ethics Committee requires that in all studies, the Principles of Informed Consent are adhered to. This applies to volunteers as well as patients.

3. PROGRESS REPORTS:

* The University of the Witwatersrand, Human Research Ethics Committee requests that the MCC Progress Reports be submitted twice a year (March and September) to the Office for Pharmaceutical Trials and a report of the final results, at the conclusion of the study.

4. TRANSPORT AND STORAGE OF BLOOD AND TISSUE SAMPLES IN SOUTH AFRICA:

* If blood specimens are to be stored for future analysis and is planned that such analysis will be done outside Wits then the blood must be stored at Wits with release of sub-samplesonly once projects have been approved by the local Research Ethics Committee applicable to where the research will be done as well as by the Wits Human Research Ethics Committee: (Medical).

5. REIMBURSEMENT TO PATIENTS FOR TRANSPORT:

* The Human Research Ethics Committee: (Medical) does not agree with the views as stipulated by the Medicines Control Council of SA and that reimbursement will be appropriate according to the situation and to the discretion of the Principal Investigator.

6. GENETIC TESTING

* Please note that in the future the Human Research Ethics Committee: Medical; will unlikely approve open-ended genetic testing as this does not fit the Human Research Ethics Committee criteria.

7. WE AWAIT YOUR RESPONSES AS REQUESTED:

* MCC Approval and/or Notification before the above study may commence.

* Kindly forward the above to the undersigned at fax: 011 274 9281 at your earliest convenience.

The above has been noted for the Ethics Committee information and records.

KINDLY FORWARD TO THE RELEVANT INVESTIGATORS / CRA / STUDY CO-ORDINATORS

Regards,

PROF MERRYLL VORSTER

For and on behalf of the Human Research Ethics Committee: (Medical)