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Molecular investigation on the impact of the pneumococcal polysaccharide-protein conjugates vaccine (PCV) on bacterial nasopharyngeal colonization in children

Courtney Paige Olwagen

Student Number: 703434

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg 2017

DECLARATION

I, Courtney Paige Olwagen declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……

14th day of June 2017

DEDICATION

I dedicate this work to my husband Gareth, with love and gratitude for his unwavering support and belief in me. Without you this would not have been possible.

To my daughter Emily, you are an absolute delight, you have filled my life with love and laughter and for that I will be eternally grateful.

To my parents Mandy and Wayne, who have offered unconditional love and support and have always believed me.

PRESENTATIONS ARISING FROM THIS THESIS

1. *10th International Symposium on Pneumococci and Pneumococcal Diseases*. Olwagen CP, Adrian PV, Madhi SA. Comparison of traditional culture and qPCR for detection of pneumococcal colonisation by pneumococcal conjugate vaccine (PCV) status in African children. Glasgow, United Kingdom, Jun 26-30, 2016. (Poster presentation, P-354).

2. *10th International Symposium on Pneumococci and Pneumococcal Diseases*. Olwagen CP, Adrian PV, Madhi SA. Association of pneumococcal conjugate vaccine (PCV) immunisation and density of nasopharyngeal bacterial colonisation using a multiplex quantitative polymerase chain reaction assay. Glasgow, United Kingdom, Jun 26-30, 2016. (Poster presentation, P-422).

3. *Witwatersrand Faculty of Health Sciences Research Day.* Olwagen CP, Adrian PV, Madhi SA. Comparison of traditional culture and qPCR for detection of pneumococcal colonisation by pneumococcal conjugate vaccine (PCV) status in African children. Johannesburg, South Africa, Sep 19, 2016. (Poster presentation, ID-P-10).

4. *Witwatersrand Faculty of Health Sciences Research Day.* Olwagen CP, Adrian PV, Madhi SA. Association of pneumococcal conjugate vaccine (PCV) immunisation and density of nasopharyngeal bacterial colonisation using a multiplex quantitative polymerase chain reaction assay. Johannesburg, South Africa, Sep 19, 2016. (Poster presentation, ID-P-10).

5. *35th Annual meeting of the European Society for Paediatric Infectious Diseases.* Olwagen CP, Adrian PV, Madhi SA. Evaluation of the association of HIV-1 infection and density of common nasopharyngeal bacterial colonizers using the Biomark HD

nanofludic system in PCV7-immunized children living in South Africa. Madrid, Spain, May 23-27, 2017. (Poster presentation, ESP17-0583).

6. *35th Annual meeting of the European Society for Paediatric Infectious Diseases.* Olwagen CP, Adrian PV, Madhi SA. Performance of the Biomark HD real-time PCR System (Fluidigm) for the detection of common nasopharyngeal bacterial pathogens and serotyping *Streptococcus pneumoniae.* Madrid, Spain, May 23-27, 2017. (E poster oral, ESP17-0571).

ABSTRACT

Background: Nasopharyngeal colonisation is a pre-requisite for developing bacterial respiratory and invasive disease. Immunisation of children with the pneumococcal conjugate vaccine (PCV) impacts upon colonising pneumococcal serotypes, which in turn could also affect the biome of the nasopharynx in relation to colonisation by other bacteria. Due to limitations in standard culture methods, the association between PCV-immunisation and bacterial carriage density is still unclear, including among HIV-infected children. In this study we aimed to evaluate the effect of infant vaccination with the 7-valent PCV (PCV7) on vaccine-serogroup colonisation in order to determine whether the increase in non-vaccine serotype (NVT) colonisation was due to unmasking of previously low density colonising serotypes or increase in acquisition of NVT. Also, we evaluated the association between PCV7 immunisation and HIV-infection on the prevalence density of nasopharyngeal colonisation by other common potentially pathogenic bacteria.

Methods: A multiplex real-time qPCR assay was set up to detect 44 common pneumococcal serotypes and 5 bacterial pathogens including *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus* and *Streptococcus pyogenes*. All assays were optimised according to MIQE guidelines and their ability to detect multiple pneumococcal serotype/group and bacteria in archived nasopharyngeal swabs were evaluated. The multiplex qPCR assays were then used to evaluate vaccine-serotype, non-vaccine serotype and bacterial nasopharyngeal colonisation in achieved swabs of PCV7-vaccinated (at 6, 10 and 14 weeks of age) and PCV-unvaccinated African children at 9 and 15-16 months of age, prior to routine vaccination of children with PCV through the public immunisation program. In order to address the limitations of the qPCR assays, a nanofluidic real-time PCR assay was developed to simultaneously detect 53 pneumococcal serotypes, 6 serotypes of *H. influenzae* and 11 bacterial pathogens. Further, all assays were optimised and evaluated according to the MIQE guidelines and findings from Fluidigm and traditional qPCR assays were compared. Lastly, Fluidigm was used to evaluate the association of HIV-infection on the prevalence and density of nasopharyngeal colonisation at 9 and 16 months of age by common, potentially pathogenic bacteria including PCV7 pneumococcal serotypes, non-PCV7 serotypes, *Haemophilus influenzae*, non-typable *Haemophilus influenzae, Staphylococcus aureus, Moraxella catarrhalis, Streptococcus pyogenes, Neisseria*

meningitidis, Neisseria lactamica, Bordetella pertussis, Bordetella parapertusis, Bordetella bronchiseptica and *Bordetella holmesii* in achieved nasophartngeal swabs collected from PCV7-vacciniated HIV-infected and HIV-uninfected children.

Results: Molecular qPCR was more sensitive than culture in detecting multiple concurrent colonising pneumococcal serotypes as well as other common nasopharyngeal colonisers, with the majority of additional isolates detected by qPCR having a low carriage density ($<10^4$ CFU/ml). Further, qPCR identified a lower prevalence of PCV7-serotype colonisation among PCV7-vaccinated compared to PCV-unvaccinated children at 9 and 16 months of age [adjusted Odds Ratio (aOR): 0.37; 95% CI; 0.19-0.7 and 0.41; 95% CI; 0.26-0.63, respectively]; and an increase in NVT-serotype [aOR: 1.88; 95% CI; 1.02-3.48 and 2.2; 95% CI; 1.18-4.1] colonisation respectively. The increase in NVT carriage among PCV7-vaccinees was driven by serotype 19A, which increased by 53.4% (p=0.021) and 70.7% (p<0.001) at 9 and 16 months of age respectively. Further, 19A had a higher density of colonisation in PCV7-vaccinated groups compared to PCV-unvaccinated groups and was more likely to be identified as a primary than non-primary isolate in PCV7-vaccinated children alone. PCV immunisation was also associated with an increased prevalence of *H. influenzae* at 9 months (55.8% vs. 66.3%, p<0.001) and 16 months (72% vs. 62%, p=0.017) of age, while a temporary increase in the carriage prevalence of *S. aureus* was found in PCV7-vaccinated (18.9%) compared to PCV-unvaccinated children (11.1%, aOR 2.1; 95% CI 1.0-1.4; p=0.049) at 9 months of age only. The density of pneumococcus (4.68 vs. 4.28 CFU/ml; p=0.007), *H. influenzae* (3.86 vs. 4.34 CFU/ml; p=0.008), *M. catarrhalis* (2.98 vs. 3.52 CFU/ml; p<0.001) and *S. aureus* (3.06 vs. 4.02 CFU/ml; p=0.02) were also higher among PCV7-vaccinated compared to PCV-unvaccinated children at 9 months age, although this difference diminished with increasing age.

There was excellent concordance between the qPCR and Fluidigm for carriage prevalence and density of the majority of assays, with Fluidigm identifying an additional 7 pneumococcal serotypes and 11 bacterial species above those detected by qPCR. Further, discordant results between the two PCR methods were strongly associated with a low carriage density ($<10^2$ CFU/ml). Using molecular Fluidigm, a lower carriage prevalence of overall pneumococci (58.6% vs. 69.9%; p=0.02), non-vaccine serotypes (27.8% vs. 40%;

p=0.047) and *H. influenzae* (64.2% vs. 42.3%; *p*=0.01) was identified in HIV-infected children compared to HIV-uninfected children who were immunised with PCV7 at 9 months of age. No difference in the carriage prevalence of overall pneumococci was however found at 16 months of age (*p*=0.20), although the carriage prevalence of non-vaccine serotypes (50.9% vs. 60.4%; *p*=0.049) and *H. influenzae* (56% vs. 73.4%; *p*=0.02) was lower in HIV-infected children at 16 months of age. In addition, the density of overall pneumococcus was found to be higher in HIV-infected children (4.81 vs. 4.44 CFU/ml; *p*=0.014), despite the lower carriage prevalence at 9 months of age, which was driven by a higher density of vaccine serotypes/serogroups (4.21 vs. 3.72 CFU/ml; *p*=0.04). By 16 months of age, there was no difference in density of pneumococcal colonisation between the HIV-infected and HIV-uninfected children (*p*=0.89). No difference in the density of *H. influenzae* was found between HIV-infected and HIV-uninfected infants at 9 months of age (*p=0.08*); however, by 16 months of age, HIV-uninfected children had a higher density of overall *H. influenzae* colonisation (4.95 vs. 4.32 CFU/ml; *p*<0.001), which was largely due to the higher carriage density of NT*hinf* in HIV-uninfected children (5.0 vs. 4.23 CFU/ml; *p*<0.001).

Conclusion: Molecular qPCR assays were shown to be a promising alternative to WHO recommended culture in that multiple pneumococcal serotypes and other bacterial pathogens could be simultaneously detected as well as the bacterial load of each colonising bacteria quantified. The mechanism behind the vaccine effect was shown to be a combination of both serotype replacement and unmasking; however, the reduction in PCV7-serotype colonisation impacted on colonisation prevalence and density of other bacterial species of the nasopharynx and the clinical relevance of this needs further exploration in relation to mucosal and invasive disease outcomes, as well as for higher valence vaccines. While the higher carriage density of overall pneumococcus in HIV-infected children, despite the lower carriage prevalence might explain the higher invasive disease burden in HIV-infected compared to HIV-uninfected children even in the era of antiretroviral therapy treatment and PCV immunisation, future studies are required to provide clarity. Nevertheless, the findings from this thesis highlight the importance of continued surveillance of the circulation of pneumococcal serotypes as well as other bacterial pathogens especially in a population with a high burden of HIV-1 infection.

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ABBREVIATIONS

95% CI	95% confidence intervals
AOM	Acute otitis media
aOR	Adjusted odd ratio
ARI	Acute respiratory infection
BHI	Brain-heart infusion
cbpA	Choline binding protein A
CDC	Centre for Disease Control
CFU	Colony forming units
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CPS	Capsular polysaccharide
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
e	Amplification efficiency
EQA	External quality assessment
GlcNAc	N-acetyl-glycosamine
GMD	Geometric mean densities
HEU	HIV-exposed uninfected
HIC	High income countries
HIV	Human immunodeficiency virus
HREC	Human Research Ethics Committee
HUU	HIV-unexposed uninfected
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPD	Invasive pneumococcal disease
IVAC	International Vaccine Access Centre
LLD	Lower limits of detection
LMIC	Low-middle income countries
LTA	Lipoteichoic acid
LytA	Autolysin
MEE	Middle ear effusion
MGB	Minor grove binding
MILST	Multi-invasive-locus sequencing typing
NanA	Neuraminidase
NICD	National Institute for Communicable Diseases
NP	Nasopharyngeal
NPP	Negative predictive value
NT	Non-typable
NT*hinf*	Non-typable *H. influenzae*
NVT	Non-vaccine serotypes
^oC	Degree Celsius

OP	Oropharyngeal
OR	Odds ratio
PCR	Polymerase chain reaction
PCV	Pneumococcal polysaccharide-protein conjugate vaccines
PCV7	7-valent pneumococcal conjugate vaccine
PCV10	10-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
Ply	Pneumolysin
PPV	Positive predictive value
psaA	Pneumococcal surface antigen A
qPCR	Quantitative PCR
r^2	Correlation coefficient
RFLP	Restricted Fragment Length Polymorphism
RMPRU	Respiratory and Meningeal Pathogen Research Unit
SD	Standard deviation
STA	Specific Target Amplification
STGG	Skim milk-tryptone-glucose-glycerol
TA	Teichoic acid
VIMC	Vaccine Information Management system
VT	Vaccine targeted serotype
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1. *STREPTOCOCCUS PNEUMONIAE*

Streptococcus pneumoniae is an aetiological agent for both mucosal and invasive disease, and was responsible for approximately 411 000 deaths in children <5 years worldwide in 2013 [1], with children living in Sub-Saharan Africa being 14 times more likely to die, than children living in high income countries (HIC) according to the WHO.

1.1.1. Microbiology of *S. pneumoniae*

S. pneumoniae (pneumococcus) is a lancet-shaped gram-positive coccus that grows in pairs or short chains [2]. It was first isolated in 1881 by George Sternberg and Louis Pasteur and belongs to the mitis group within the genus streptococcus [3]. Further, *S. pneumoniae* is alpha-hemolytic, bile soluble and optochin-sensitive [2]. Cells are 0.5-1.25µM in diameter and are non-motile [4]. *S. pneumoniae* does not catalase or ferment glucose to lactic acid, and its optimum growth conditions include 5% CO_2 with an external catalase added to neutralise hydrogen peroxide produced [5].

1.1.2. Structure of *S. pneumoniae*

The surface of *S. pneumoniae* is made up of three main components, namely; the capsule polysaccharide, the plasma cell membrane and the cell wall (Figure 1.1). The cell wall consists of peptidoglycan, teichoic acid (TA) and Lipoteiochoic acid (LTA) [6]. The peptidoglycan backbone of the cell wall aids in anchoring the capsular polysaccharide and the cell wall polysaccharide [7]. In addition, there are a number of proteins and enzymes such as Pneumolysin (Ply), Autolysin (*LytA*), choline binding protein A (cbpA) and Pneumococcal surface antigen A (psaA) that are displayed on the surface of the pneumococcus. These proteins and enzymes contribute to the pathogenesis of the bacteria as they are either involved in direct interactions with the host or conceal the bacterium from host defence mechanisms [8].

The polysaccharide capsule is the outermost layer of the cell surface which encapsulates the pneumococcus [9]. It protects that bacteria from phagocytosis, inhibits complement activation and is involved in competition with other bacteria to colonise the pharynx [10-12]. The anti-phagocytic capsule forms the basis for pneumococcal classification, with 98 capsule serotypes

identified to date. Pneumococcal serotypes differ in their serological response, chemical structure and other related genetic mutations [13-15], with serotypes 1, 4, 5, 7F, 8, 12F, 14, 18C, 19A, 19F and 23F being responsible for majority of invasive pneumococcal disease (IPD) [15]. The capsule is thus an important virulence factor, with different serotypes having different invasive disease potentials, and some serotypes such as 1, 5, 14 and 19A are associated with a more severe outcome [14,15]. This is further demonstrated by the fact that un-capsulated pneumococci are generally not invasive [8].

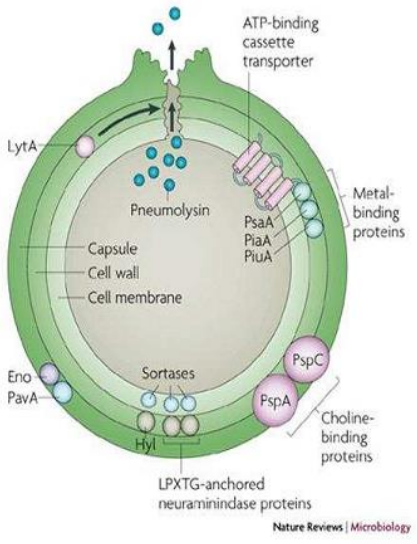


Figure 1.1: Structure of *Streptococcus pneumoniae* from Kadioglu *et al* [16].

1.1.3. Carriage of *S. pneumoniae*

S. pneumoniae is a common asymptomatic coloniser of the human nasopharynx in high income (HIC) and low-middle income (LMIC) countries [17-22], with carriage being a pre-requisite to pathogenesis of all pneumococcal disease as well as transmission between individuals [18,23-26]. The highest prevalence of colonisation has been observed in children <2 years [27], with most children being colonised within the first year of life [18,20,21,28], and some children in LMIC countries being colonised within days after birth [22]. Pneumococcal carriage prevalence varies widely, depending on age, ethnicity, socioeconomic living conditions and sample methods used to detect pneumococcus. However, carriage of pneumococcus increases gradually with age in healthy children. Syrjänen *et al*, reported an increase in the prevalence of colonisation from 9% to 43% in Finnish children [29], and Dagan *et al*, reported an increase from 26% to 62% in Israeli children at 2 and 24 months respectively [30]. Furthermore, colonisation prevalence may remain high in African settings in both children and adults [31],

with a high prevalence of nasopharyngeal (NP) carriage in LMIC countries also being associated with colonisation by multiple pneumococcal serotypes [22].

Children may be colonised with different pneumococcal serotypes repeatedly, with duration depending both on the child's age and the serotype carried [32-34]. Some serogroups such as 6, 14, 19 and 23, are acquired frequently and carried for longer periods, while serotypes 1 and 5 have a transient carriage [31,35]. In addition, the duration of colonisation is longer in children than adults, suggesting the acquisition of natural immunity against carriage [18,31].

1.1.4. Concurrent carriage of multiple pneumococcal serotypes

Concurrent colonisation of multiple pneumococcal serotypes was first observed in 1933 by Grundel *et al*; however, few studies since have documented concurrent colonisation with prevalence varying from 1.3% to 52% according to geographical distribution and method used (Table 1.1). Initial studies of concurrent pneumococcal colonisation relied on mouse inoculation assays. While these assays were sensitive in detecting multiple serotypes, they were expensive, labour intensive and not practical for use in large epidemiological studies [36]. Thus, the majority of subsequent studies have relied on serotyping individual dominant colonies isolated from culture with concurrent colonisation being reported in approximately 10% of such studies, which is likely an underestimation of the true prevalence of this phenomenon due to insufficient sensitivity of culture in detecting less-abundant colonising serotypes [36]. Further, Huebner *et al* reported that 299 colonies from each culture sample would need to be serotyped to have a 95% probability of detecting a second colonising pneumococcus which represents 1% of the colonising population, thus making culture impractical for detecting concurrent colonisation [37].

Other methods have also been used to investigate concurrent carriage of multiple pneumococcal serotypes such as conventional PCR [38,39], colony blot [40], Restricted Fragment Length Polymorphism (RFLP) [41] and microarray [42]. However, the majority of these methods rely on an initial culture step which could bias the results due to selection for better growing strains. Further, these tests are not-quantitative for the colonising serotypes and are thus unable to measure the density of colonisation and relative proportion of the individual colonising serotypes. Recently, Dhoubhadel *et al* and Messaudi *et al*, used quantitative real-

time PCR assays that were able to detect concurrent carriage of 50 and 40 pneumococcal serotypes respectively, with the added benefit of being able to quantify the relative proportion of concurrent colonising serotypes without relying on an initial culture step [43,44]. These methods were, however, limited in that they were not optimised to detect all known pneumococcal serotypes and thus concurrent carriage of multiple pneumococcal serotypes could still have been underestimated.

Improved methods that can detect multiple pneumococcal serotypes directly from a specimen so that the relative proportions of the colonising serotypes are not altered; can quantitatively measure the density of carriage of the colonising serotypes; are validated against the Quellung reaction to exclude any false-positive reactions; have the capacity to detect all known pneumococcal serotypes; and is practical and affordable are thus still needed [45].

From data available on the limited number of studies reporting concurrent carriage, prevalence differed by geographical regions with children in LMIC having a higher prevalence of concurrent colonisation than children from HIC, albeit with varying detection methods used (Table 1.1). Further, there is a paucity of data on concurrent colonisation in South Africa, with one study reporting a prevalence of 1.3% in children <5 years old [37]. As this study used conventional culture to investigate concurrent colonisation, it likely under-reported the true prevalence in a South African population, where a high prevalence of pneumococcal carriage has been reported [66,67]. This is further supported by the finding that populations with a higher prevalence of pneumococcal carriage tend to also have a higher prevalence of concurrent carriage of multiple pneumococcal serotypes.

Concurrent carriage of multiple pneumococcal serotypes has been shown to promote genetic recombination by horizontal gene transfer between serotypes through transformation, transduction or conjugate transfer [68]. Transformation of the CPS locus can result in capsule switching which could affect the performance of vaccines [69,70]. Improved methods to detect multiple pneumococcal serotypes with a high accuracy and efficiency can thus help us to gain a better understanding of the implications of concurrent colonisation, and to predict and monitor the emergence of new strains and thus the effectiveness of pneumococcal polysaccharide-protein conjugate vaccines (PCV).

Table 1.1: Summary of studies reporting concurrent colonisation of multiple pneumococcal serotypes *

Reference	Population	Method	Prevalence of concurrent colonisation	Specimen type	PCV status	Number of participants	Age of participants	Study period
Dowling *et al*, 1971[46]	USA	Culture	1.3%	OP swabs	Unvaccinated	-	-	June 1965 - June 1969
Gray *et al*, 1980 [18]	USA	Culture	8.0%	NP swabs	Unvaccinated	82	0 - 24 months	November 1974 - December 1975
Hansman *et al*, 1985 [47]	Australia	Culture	7.5%	NP swabs	Unvaccinated	174	<14 years	May 1981
Montgomery *et al*, 1990 [20]	Papua New Guinea	Culture	33.3%	NP swabs	Unvaccinated	158	-	January 1985 - March 1987
Gratten *et al*, 1994 [48]	Australia	Culture	20.6%	NP swabs	Unvaccinated	171	-	1990 - 1991
García-de-Lamas *et al*, 1997 [49]	Spain	Culture	64.0%	OP swabs	Unvaccinated	188	1 - 13 years	-
Kellner & Ford-Jones, 1999 [50]	Canada	Culture	2.2%	NP swabs	Unvaccinated	1322	Mean age 34 months	March - July 1995, and October 1995 - February 1996
Ussery *et al*, 1996 [51]	Alaska	Culture	3.2%	NP swabs	Unvaccinated	185	<5 years	1986 - 1990
Lloyd-Evans *et al*, 1996 [26]	Gambia	Culture	9.8%	NP swabs	Unvaccinated	1071	<5 years	June 1989 - May 1991
Lopez *et al*, 1999 [52]	Spain	Culture	6.7%	NP swabs	Unvaccinated	332	6 years	January - June 1997
Huebner *et al*, 2000 [37]	South Africa Israel	Culture	1.3% 2.4%	NP swab	Unvaccinated	-	1 - 60 months	-

St Sauver *et al*, 2000 [53]	USA	PFGE	5%	OP swabs	-	38	Children attending day care	February & June 1988
Syrjänen *et al*, 2001 [29]	Finland	Electrophoresis and latex agglutination	3.7% 3.9%	NP swabs & NP aspirates	Unvaccinated	329	2 - 24 months	April 1994 - July 1997
Sà-Leâo *et al*, 2002 [54]	Portugal	PFGE	10.8%	NP swabs	Unvaccinated	37	6 months - 6 years	January - March 1996
Bogaert *et al*, 2004 [40]	Netherlands	Colony dot blot	6.7% 15.4%	NP swab	Unvaccinated Vaccinated	26	1 - 7 years	April 1998 - January 2002
O' Brien *et al*, 2007 [55]	USA	Immunoblotting	8.1%	NP swabs	Unvaccinated and vaccinated	852	<2 years	April 1997 - October 2000
Hare *et al*, 2008 [56]	Aboginease	Culture	20%	NP swabs	Vaccinated	101	Children with otitis media	2004 - 2005
Kaltoft *et al*, 2008 [57]	Denmark	Culture	8% 12%	NP swabs	-	461 Kindergarten children 123 Nursery children	Mean age 52.4 months Mean age 23 months	-
Rivera-Olivero *et al*, 2009 [39]	Venezuela	Multiplex PCR	20%	NP swabs	Vaccinated	50	0 - 72 months	April 2004 - January 2005
Auranen *et al*, 2010 [58]	Denmark	Culture	20%	NP swabs	Unvaccinated	123	< 3 years	April 1999 - February 2001
Brugger *et al*, 2010 [41]	Switzerland	RFLP	6.7%	NP swabs	Vaccinated	-	-	2004 - 2009

Turner *et al*,2011[42]	Thailand	Culture Sweep serotyping Microarray	11.2% 43.2% 48.8%	NP swabs	Vaccinated	125	0 - 24 months	October 2007 - November 2008
Valente *et al*, 2012 [59]	Portugal	*ply*NCR-RFLP and microarray	20.1%	NP swabs	Vaccinated	492	18 - 71 months	Winter months (January - March) of 2001, 2006 & 2007
Lee *et al*, 2013 [60]	Korea	Culture and multiplex PCR	11.9%	NP swabs	Status not known	582	-	June 2006 - February 2007
Dhoubhadel *et al*, 2014 [43]	Vietnam	Nanofludic real time PCR	18.5% 7.1%	NP swab	vaccinated	595 with ARI 350 healthy children	<5 years	April 2008 - March 2009
Wyllie *et al*, 2014 [61]	Netherlands	Culture enriched qPCR	52%	Saliva	Vaccinated	50	5 - 10 years	June 2012
Kamng'ana *et al*, 2015 [62]	Malawi	Microarray	40 %	NP swabs	Unvaccinated	189	0 - 13 Years	2008 - 2012
Kandasamy *et al*, 2015 [63]	Nepal	Microarray	20.2%	NP swabs	Unvaccinated	600	6 weeks - 24 months	May - October 2012
Saha *et al*, 2015 [38]	Bangladesh	culture and PCR	22%	NP swabs	-	212	<5 years	-
Hjálmarsdóttir *et al*, 2016 [64]	Iceland	Culture Multiplex PCR	8.1% 23.5%	NP swabs	Unvaccinated	514	1.2 - 6.3 years	March - April 2009
Wyllie *et al*, 2016 [65]	Netherlands	Culture enriched qPCR	22%	NP swabs	Vaccinated	1169	11 - 24 months	Autumn/winter 2010-Autumn/winter 2013

*Pubmed and Scopus were used to search for studies reporting concurrent colonisation of multiple pneumococcal serotypes. ARI, acute respiratory infection. NP, nasopharyngeal. OP, oropharyngeal. PCV, pneumococcal conjugate vaccine. PCR, polymerase chain reaction. PFGE, Pulsed-field gel electrophoresis. qPCR, quantitative PCR. -, information not provided in publication.

1.2. INTERACTIONS BETWEEN PNEUMOCOCCUS AND OTHER NASOPHARYNGEAL COLONISING BACTERIA

Colonisation of the nasopharynx is a dynamic process where organisms are continuously acquired and eliminated, with clearance influenced by host immune responses [71]. Bacterial species, besides the pneumococcus that reside in the nasopharynx include *H. influenzae, M. catarrhalis, S. aureus,* and *N. meningitidis* [72]. Although there is emerging understanding of the interactions between these bacteria and the establishment of carriage and disease [73,74], the relevant mechanisms of such interactions remain unclear [74-77].

Association between colonising bacteria can be synergistic or competitive. Competition between residing nasopharyngeal flora can provide an opportunity for other species to occupy the vacated niche after antimicrobial treatment or vaccination targeted at specific bacterial species. Further, enzymes produced by one bacterial species might affect the growth or survival of other bacteria, and also horizontal gene transfer might occur between concurrently colonising organisms [78].

1.2.1. Association between *S. pneumoniae* and *S. aureus*

There is conflicting data with respect to the association between *S. pneumoniae* and *S. aureus* colonisation, with some studies showing a negative association, especially with respect to 7-valent pneumococcal serotypes (4, 6B, 9V, 14, 18C, and 23F) [77,79-81], whereas other studies have not established any such association or described the association to be independent of PCV7-serotypes [82,83]. The negative association between these bacteria is thought to be due to *S. pneumoniae* producing hydrogen peroxide which has a bactericidal effect on *S. aureus* [84].

1.2.2. Association between *S. pneumoniae* and *H. influenzae*

A synergistic relationship between *S. pneumoniae* and *H. influenzae* has been reported, with children colonised with pneumococcus being more likely to be colonised with *H. influenzae* [77,81]. Further, pneumococcus and *H. influenzae* have been shown to form biofilms which facilitates their survival, persistence and disease outcome after colonisation [85].

1.2.3. Biofilm formation

Biofilms are bacterial communities that aggregate on cell surfaces, and could be responsible for the conflicting data with respect to bacterial associations. Bacteria organised in biofilms, have different growth rates, metabolism, gene expression, and protein production than those of planktonic cultures [86]. Further, biofilms have been shown to result in weaker inflammatory responses and bacteria are more resistant to host defences and other environmental changes, thus having an increased capacity to evade the host immune system. Recently, pneumococci have been shown to form biofilms during carriage in animal studies, with colonisation associated with enhanced adhesive, increase resistance to antibiotic treatment, persistent carriage and decreased virulence [87-89]. In addition, carriage of *H. influenzae* has been shown to increase pneumococcal biofilm formations both *in vivo* and *in vitro* [90].

1.3. MECHANISM OF COLONISATION

S. pneumoniae encounters the mucosa of the host nasopharynx, which is covered with a mucus layer and epithelial cells. In healthy individuals, the mucosa inhibits the attachment of the bacteria by acting as a physical barrier and releasing antimicrobial substances such as lysozyme and lactoferrin which will either kill the bacteria or inhibit its growth [91]. If the pneumococcus is able to overcome these host defences, it attaches itself to the cell surface carbohydrate (N-acetyl glucosamine) receptors on non-inflamed epithelium of the respiratory tract [72], a process mediated by specific bacterial adhesion molecules such as PsaA and choline-binding proteins that form a bridge between the bacterial surface components and epithelial cell receptors [71,92-95] (Figure 1.2).

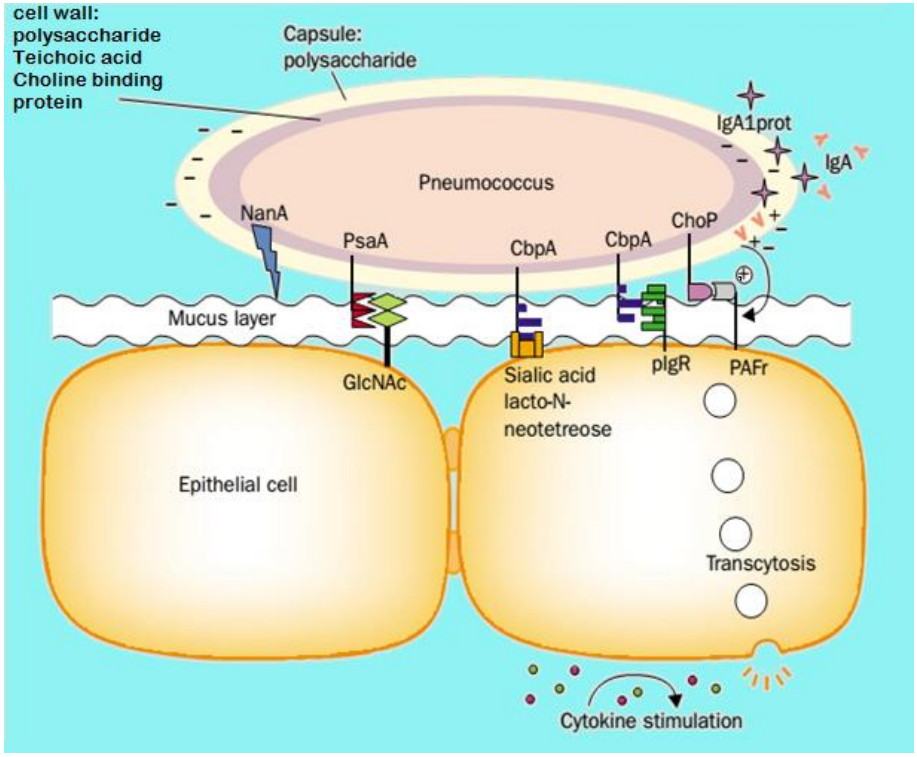


Figure 1.2: Interaction between *S. pneumoniae* and epithelial cells from Bogaert *et al* [72]

Pneumococcal enzyme Neuraminidase (NanA) decreases the viscosity of the mucus and exposes the N-acetyl-glycosamine (GlcNAc) receptors on the epithelial cells, thus facilitating its interaction with pneumococcal surface-associated proteins [72]. Other functions of pneumococcal enzymes include cell lysis, cell wall degradation, inhibiting ciliary function, providing nutrients to the bacteria and inhibiting opsonophagosytosis by human neutrophils [6,8,96]. Further, a thick capsule polysaccharide has an inhibitory effect on adherence and thus most pneumococcal isolates display phase variation between two forms distinguishable by their opaque or transparent colony morphologies. During the initial stages of colonisation, thinner capsules that are transparent and promote binding to host tissues dominate over thicker opaque capsules [16].

1.4. THE RELATIONSHIP BETWEEN COLONISATION AND MUCOSAL DISEASE

The human nasopharynx is constantly exposed to the outside environment, and as a result is colonised with multiple commensal bacterial pathogens that adhere to the mucosal epithelium such as *S. pneumoniae*, *S. aureus* and *H. influenzae* that are embedded in a complex

microbiota. Despite colonisation being asymptomatic, disruptions to the host immune defences, infection with respiratory viruses or underlying medical conditions such as chronic obstructive pulmonary disease (COPD), can result in a shift from nasopharyngeal commensal to causing mucosal or invasive disease [97] (Figure 1.3).

The nasopharyngeal niche is a major ecological reservoir of bacterial species and provides a source of potential opportunistic pathogens. Bacterial overgrowth and contiguous spread to the eustachian tube can result in negative middle ear pressure and capillary leakage of fluid into the middle ear resulting in otitis media [98]. Bacterial spread into sinus spaces result in sinusitis, while spread to the large airways could cause bronchitis and that of the lower airways present as pneumonia. High density colonisation of the nasopharynx can result in micro-aspiration of the organisms into the airways and into the lungs, resulting in pneumonia [72].

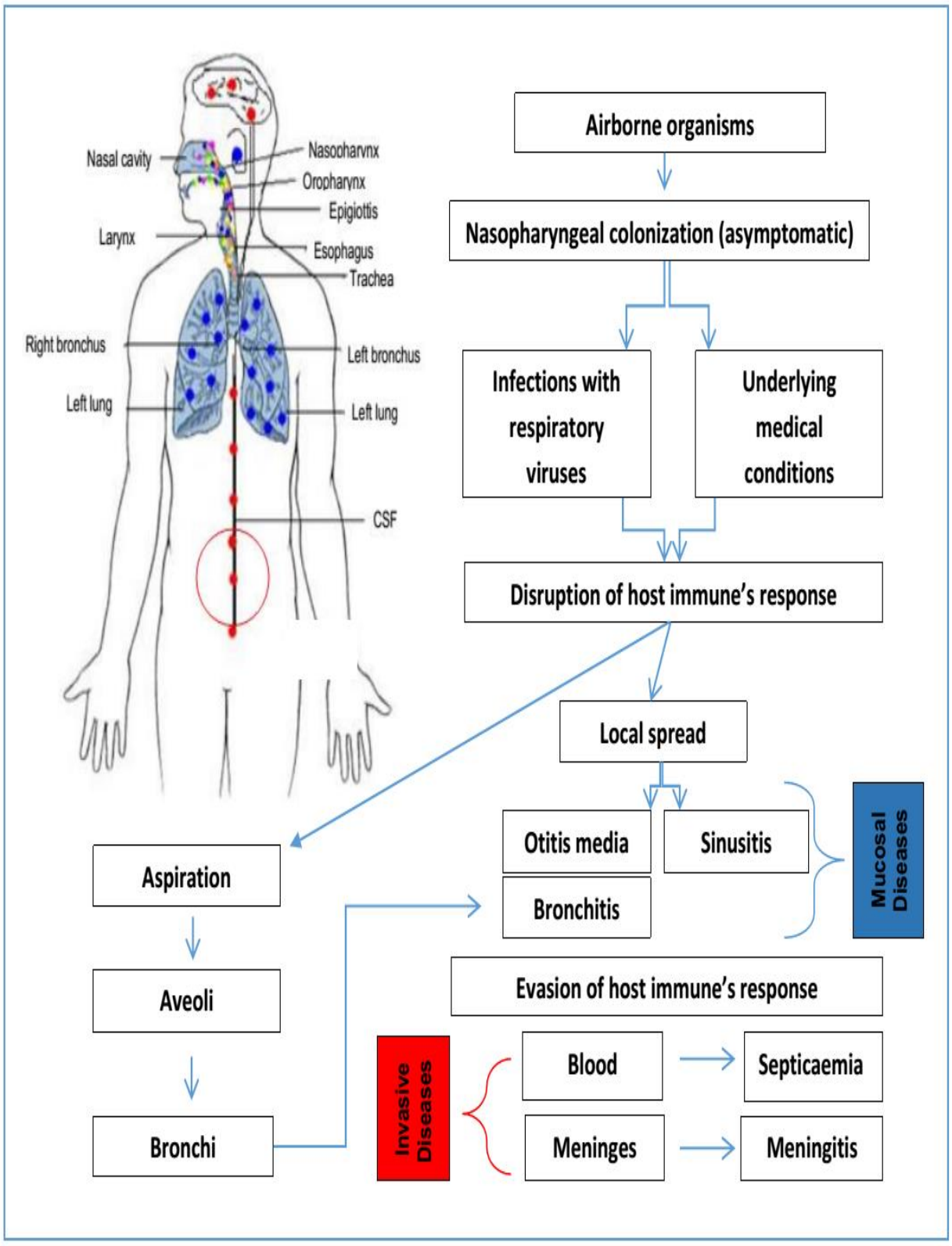


Figure 1.3: Pathogenic route for respiratory pathogens adapted from Bogaert *et al* [72]

Mucosal and invasive diseases are represented by blue and red dots respectively.

1.5. COMMENSAL BACTERIA AND INVASIVE DISEASE

Sub-optimal host immunity can result in high density colonisation of the nasopharynx, including due to host conditions such as young age and malnutrition that weaken the immune function; or disruption to the host mucosal barriers by infections such as influenza virus, HIV, and some cancers. Increase in nasopharyngeal density may result in the bacteria successfully breaching the mucosal barrier and causing invasion of sterile sites such as the blood and meninges, resulting in invasive diseases such as septicaemia, bacteraemia and meningitis.

The majority of invasive diseases are caused by immuno-evasive encapsulated organisms such as *S. pneumoniae* and *H. influenzae* type b; whereas un-encapsulated organisms such as non-typable *H. influenzae* and *M. catarrhalis* rarely cause invasive bacterial diseases. Further, isolates with some capsule structures are more efficient at causing invasive disease than others, as they are less susceptible to the hosts' immune response and thus pathogenesis of pneumococcal infections depends on the virulence of the bacteria [16].

1.6. INVASIVE DISEASE POTENTIAL OF PNEUMOCOCCAL SEROTYPES

Pneumococcal serotypes differ in their invasive disease potential with the majority of serotypes rarely causing serious disease [99]. A limited number of serotypes that differ according to temporal and geographical variations are responsible for the majority (~80%) of all invasive pneumococcal disease (IPD) [15], with serotype/groups 14, 16, 19, 18 and 23 causing the majority of IPD in Europe and the USA, and serotypes 1 and 5 being important in Africa and Asia [13,100].

Both the invasive disease potential and carriage prevalence of a serotype play a role in IPD. Although some serotypes have a low invasive disease potential, but a high prevalence of colonisation, they may contribute substantially to the burden of IPD, while other serotypes have high invasive potential but low carriage prevalence, with disease from these serotypes occurring in epidemics, in between which they are less common causes of IPD [101]. A limited number of studies which evaluated serotype invasiveness, by comparing prevalence of colonisation of specific serotypes in relation to their representation in IPD, identified serotypes 1, 4, 7F, 18C and 14 as having high invasive disease potential, and serotypes 3, 6A, 6B, 9V and 23F to have low invasive disease potential. Thus, as an example, even though

serotype 6B is responsible for a large percentage of IPD, in relation to carriage prevalence it is less likely to cause disease than serotype 14 [14,102,103]. This observation is further supported by Bruggerman *et al,* who found an inverse association between carriage prevalence and invasive disease potential, with the most commonly carried serotypes less likely to cause disease than the less commonly carried serotypes [101,104].

1.7. CHILDHOOD RISK FACTORS FOR IPD

Children <2 years are at the greatest risk for IPD , with risk factors including number of household contacts, lower socioeconomic status and host immunodeficiency [105]. Also, children attending day care, or who have siblings attending day care have a higher incidence of IPD, due to the close interactions between children that facilitate the transmission of the pneumococcus from one child to another [30,106-108], with the risk being highest in the first two months after day care is commenced [109]. Further, high rates of acute otitis media (AOM) in day care centre attendees mainly caused by the pneumococcus and *H. influenzae*, promotes an increase in antibiotic usage thus selecting for resistant pneumococci [110,111]. Several studies have shown an increasing number of household contacts to be associated with an increased incidence of pneumococcal carriage, transmission and disease [46,112,113], with young children being primarily responsible for the introduction of new serotypes into a household [112].

Low socioeconomic status, which is linked to low income, lower levels of education, and overcrowding has also been found to be a risk factor associated with an increased susceptibility of IPD. Further, asplenic children who are unable to clear opsonised bacteria from the host, as well as HIV-infected children who have defective antibody production and mucosal clearance, facilitates opportunistic infections with the pneumococcus and are thus at an increased risk of developing IPD [100,105,114]. Cases of IPD are more common in the winter months, which coincide with the cyclic pattern of respiratory viruses such as influenza and RSV, which act synergistically with the pneumococcus to disrupt the local immune defences and promote adherence to the respiratory epithelium [115-117].

1.8. PREVENTION AND TREATMENT OF UPPER AND LOWER RESPIRATORY TRACT INFECTIONS

Despite the effectiveness of antibiotics in treating mucosal and invasive infections, there remains a high morbidity and mortality rate. This is due to poor access to healthcare facilities, antibiotic resistance and the rapid onset of certain conditions such as meningitis and septicaemia in young infants, with insufficient time for treatment and intervention. This has led to an interest in preventative measures such as vaccination [118,119].

1.8.1. Pneumococcal polysaccharide vaccine (PPV)

To date, several vaccines have been developed and introduced. The pneumococcal polysaccharide vaccine (PPV; Pneumovax) was the first pneumococcal vaccine to be developed and was licensed for use in the United States in 1977. PPV is derived from 23 unique purified antigens directed against the most invasive capsular polysaccharides of *S. pneumoniae* (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F). Immunity is induced primarily through stimulation of B cells which releases IgM, without the assistance of T-cells, and thus does not induce memory responses and is poorly immunogenic in young children whose T-cell independent immune system is still immature [120]. Although this vaccine is effective in adults and older children, it is not effective in children under the age of two years [121,122].

1.8.2. Pneumococcal conjugate vaccines

Conjugate vaccines were then developed to improve the immunogenicity to the polysaccharide epitopes in children under the age of two years [123]. The conjugation of a protein molecule to the polysaccharides enhances the immunogenicity to the polysaccharide epitope by eliciting a T-cell dependent immune response, thus allowing for the development of an immunological memory and maturation of the immune response [124]. The first polysaccharide-protein conjugate vaccine to be licensed was HiB conjugate vaccine, which resulted in an elimination of HiB invasive disease in some countries [125].

This was followed by the introduction of a 7-valent pneumococcal conjugate vaccine (PCV7) which targeted seven of the most invasive serotypes namely; 4, 6B, 9V, 14, 18C, 19F and 23F [126,127]. PCV7 was licensed for use in the United States in 2000, but only introduced for routine

administration to young infants and children in South Africa in 2009 [126]. Since then, PCV7 has been effective at decreasing the incidence of vaccine-serotype IPD; however, some non-vaccine serotypes (NVT) still cause pneumococcal disease, and in some countries appear to be on the increase [128-132]. In order to prevent this shift, the repertoire of pneumococcal serotypes in the vaccine was expanded to include 13 serotypes [133]. By June 2016, 137 countries have introduced PCV into their national immunisation program, including 128 universal, 4 subnational and 5 high risk programs as shown in Figure 1.4 [International Vaccine Access *Centre (IVAC)*].

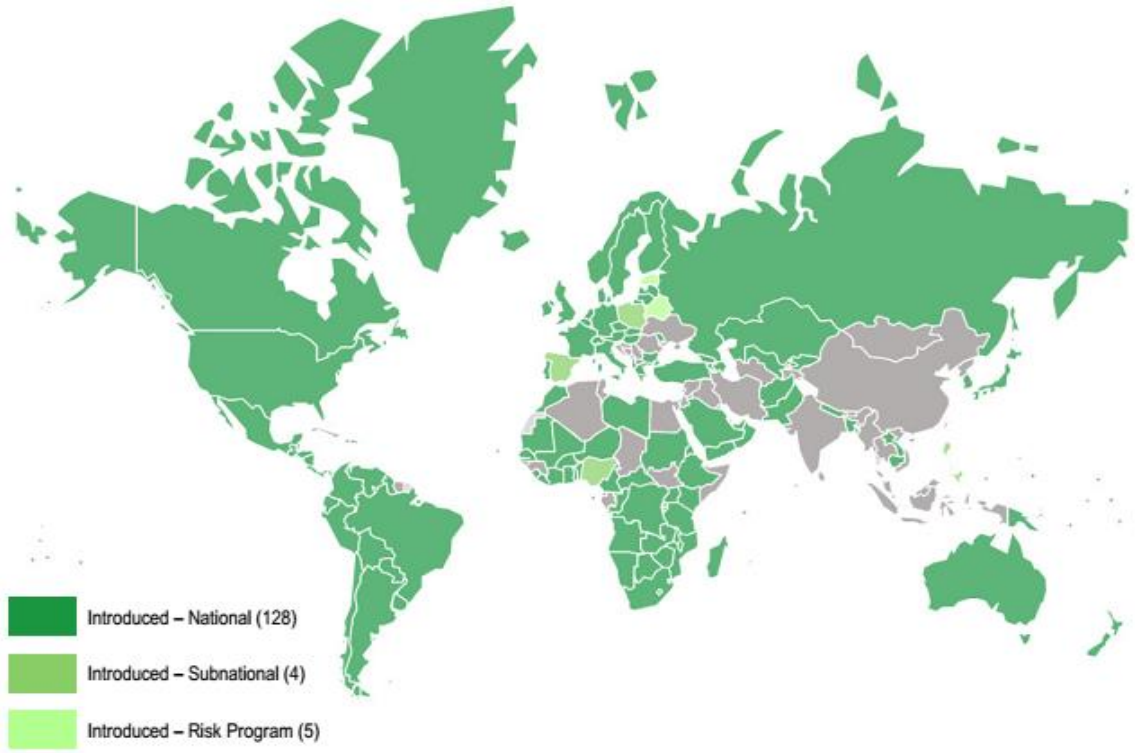


Figure 1.4: Geographical distribution of PCV

International Vaccine Access Centre (IVAC), Johns Hopkins Bloomberg School of Public Health, Vaccine Information Management system (VIMS). Global vaccine Introduction Report, June 2016.

1.9. EFFECT OF IMMUNISATION ON PNEUMOCOCCAL CARRIAGE AND SEROTYPE REPLACEMENT DISEASE

Pneumococcal vaccination has had an enormous impact on reducing nasopharyngeal carriage of vaccine targeted serotype (VTs) pneumococci and has resulted in an increase in the detection of carriage of NVTs [134]. This phenomenon has been referred to as the "serotype

replacement effect", whereby VTs appear to have disappeared from the host nasopharynx and been largely replaced by NVTs [135-137]. However, it remains unclear whether this apparent increase in NVT was due exclusively to serotype replacement of VT by NVT as a response to a vacant niche in the nasopharynx, or whether this process was due to the unmasking of underlying non-vaccine serotype pneumococci which previously colonised the NP at low levels and were undetectable by the standard methods recommended for carriage studies by WHO; Figure 1.5 [138,139].

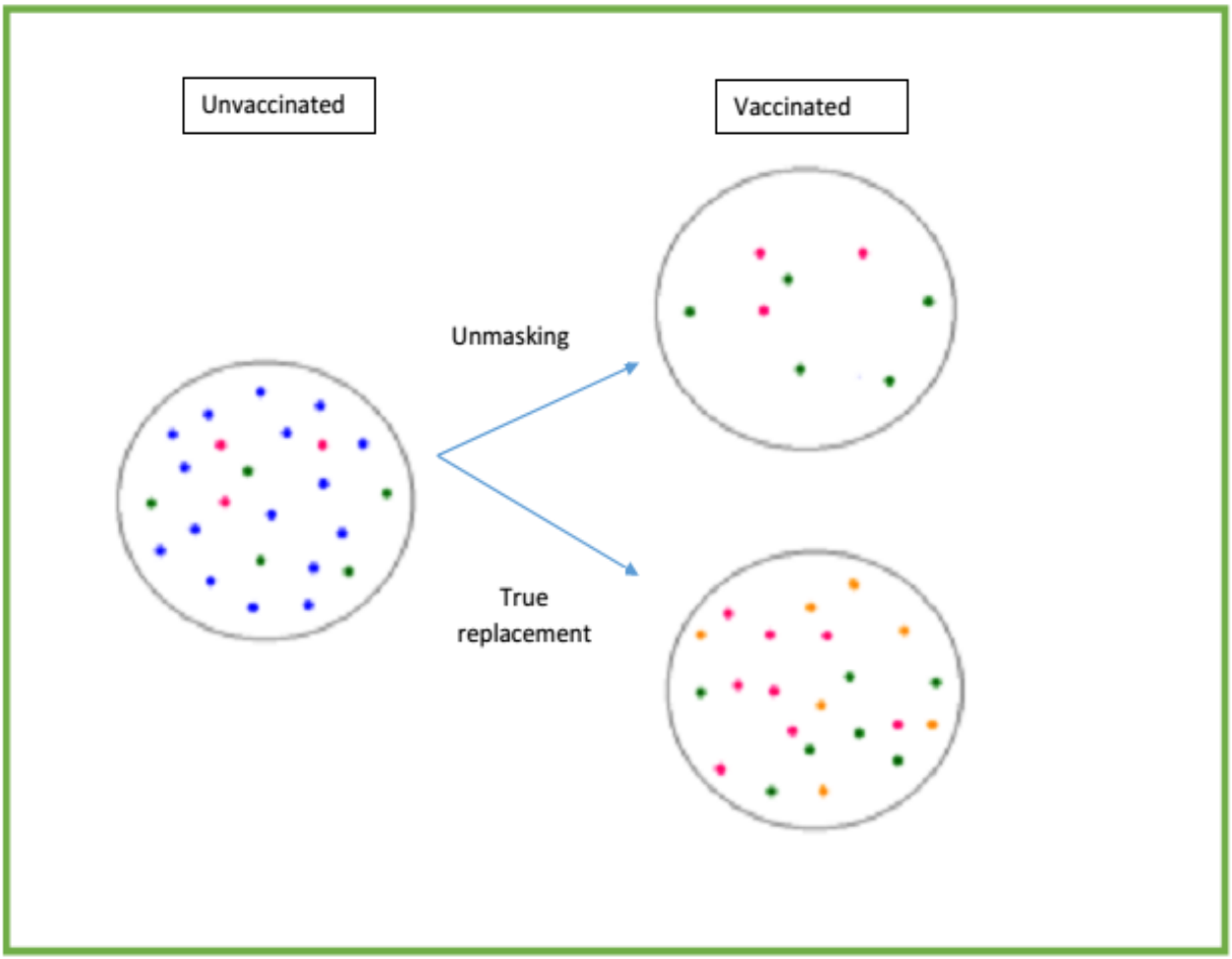


Figure 1.5: Simulation of the two possible mechanisms by which serotype replacement in the nasopharynx can occur following vaccination

Blue dots represent vaccine serotypes, while green, pink and yellow dots represent non-vaccine serotypes.

This vaccine effect has been observed in most studies investigating pneumococcal carriage post PCV-vaccination (Table 1.2), with serotypes 19A emerging as dominant NVT serotype following childhood immunisation with the 7-valent PCV [30,131,140]. In a study by Haung *et al* in USA, carriage of NVT was found to increase from 15% in 2000 to 29% in 2007, with serotypes 19A, 6A, 15B/C, 35B, and 11A being the most dominant NVTs isolated [141], while in a study by Nunes *et al* in South Africa, carriage of NVT was found to increase from 33.9% to 45%, with serotypes 6A and 19A being the most dominant NVTs isolated [142]. While these studies have been meaningful to some extent, the majority have relied on traditional culture methods that are non-quantitative for these colonising serotypes and thus the effect that PCV has on pneumococcal carriage density is not well understood, with available data from the limited number of studies being contradictory (Table 1.3); including O'Brien *et al,* reporting only a decrease in NVT carriage density in Navajo Indians [55]; Roca *et al*, reporting a decrease in both VT and NVT carriage density in The Gambia [143]; and Hanke *et al* reported an increase in NVT carriage density alone in Peru [144]. Further investigation is thus needed with more sensitive molecular methods to clarify these findings.

Table 1.2: Summary of studies evaluating the effect of infant vaccination with PCV on the prevalence of serotype-specific nasopharyngeal colonisation in children *.

Reference	Study vaccine	Study design	Country	Assay(s) used	Specimen type	Study period	Participants	Main Findings
Dagan *et al*, 1996 [30]	PCV7	Randomized control study	Israel	Culture	NP swabs	February - June 1994	12 - 18 months	Carriage of NVT was found to increase from 15% to 38% and from 22% to 32% in children who received 1 or 2 doses of PCV7 respectively, one year after PCV immunisation. Serotypes 6A and 19A were the most dominant NVTs isolated.
Mbelle *et al*, 1999 [145]	PCV9	Double-blind, randomized, placebo-controlled study	South Africa	Culture	Blood and NP swabs	-	Infants <6 weeks *n* = 500	Carriage of NVT was found to increase from 25% to 36%. Serotypes/groups 15, 6A and 19A were the most dominant NVTs isolated.
Dagan *et al*, 2002 [108]	PCV9	Double-blind, randomized study	Israel	Culture	NP swabs	August 1996 - February 1997	12-35 months *n* = 264	Carriage of NVT was found to increase from 34.1% to 43.7%. No data on carriage of serotypes/groups.
Lakshman *et al*, 2003 [146]	PCV7	Non-randomized, unblinded control study	UK	Culture	NP swabs	June 2000 - March 2001	2-5 years *n* = 607	Carriage of NVT increased in both summer and winter months. No data on carriage of serotypes/groups.
Ghaffar *et al*, 2004 [147]	PCV7	-	USA	Culture	NP swabs	September 2000-August 2001	2 - 18 months *n* = 278	Carriage of NVT was found to increase from 6% (2 month old children) to 18% (15 - 18 month old children). Serogroup 15 was the most dominant NVT isolated.

Pelton *et al*, 2004 [148]	PCV7	Surveillance study	USA	Culture	NP swabs	October 2000 - September 2003	2 - 24 months *n* = 1736	Carriage of NVT was found to increase from 7% to 16% in a 3 year period. Serogroups 15 (15A, 15B, 15C and 15F) and 11 (11A, 11C and 11D) were the most dominant NVTs isolated.
Huang *et al*, 2005 [149]	PCV7	-	USA	Culture	NP swabs	March 2001 - April 2004	<7 years *n* = 1736	Carriage of NVT was found to increase from 34% in 2001 to 55% in 2004. Serotypes/groups 11, 15 & 29 were the most dominant NVTs isolated.
O'Brien *et al*, 2007 [55]	PCV7	Group-randomized efficacy trial	USA	Culture and Immunoblot	NP swabs	April 1997 - October 2000	<2 years *n* = 8292	Increased risk of NVT colonisation among vaccinees.
Huang *et al*, 2009 [141]	PCV7	-	USA	Culture	NP swabs	winter season (2000 -2001; 2003 - 2004; 2006 - 2007)	3 months - 7 years *n* = 2638	Carriage of NVT increased from 15% in 2000 to 29% in 2007. Serotypes 19A, 6A, 15B/C, 35B, and 11A were the most dominant NVTs isolated.
Rodrigues *et al*, 2012 [150]	PCV7	Cross-sectional study	Portugal	Culture	NP swabs	February 2008 and 2009	3 months - <7 years *n* = 1142	No increase in overall NVT serotypes. Serotypes 3, 6C, 15B/C, 23A, 23B, 11A, 21, 16F, 35F and 24F were frequently isolated.
Roca *et al*, 2013 [151]	PCV7	Village-randomized study	Gambia	Culture, PCR(to differentiate 6A/C) and MILST (6A and 19A genotyping)	NP swabs	December 2003 -June 2008	<30 months *n* = 4792	Overall prevalence of NVT carriage was 31.2% and decreased with age. Serotype 19A being the most dominant NVT isolated, followed by 21 and 35B.

Grivea *et al*, 2014 [152]	PCV7	Cross-sectional study	Athens	Culture	NP swabs	December 2010 - June 2011	24 - 59 months *n* = 233	Serotypes 19A, 23B, 15B/C, 16F, 21, 11A, 15A, 6C, 10A, 22F, and 23A were the most dominant serotypes isolated.
Hammit *et al*, 2014 [153]	PCV10	Cross-sectional study	Kenya	Culture & qPCR	NP swabs	2009 - 2012	All ages *n* = 2031	Among children <5 years, carriage of NVT increased from 41% to 57% in 4 years. Serotypes 6A, 11A, 19A and 35B were the most dominant NVTs to be isolated in vaccinated groups.
Ueno *et al*, 2014 [154]	PCV7	Prospective study	Japan	Culture & PCR (*lytA*)	NP swabs	July 2010 - November 2012	<6 years *n* = 137	No difference in carriage of NVT (24.1% vs 23.4%) between unvaccinated and vaccinated groups respectively. Serotypes 6C was, however, the dominant NVT isolated.
Cohen *et al*, 2015 [155]	PCV7 & PCV13	Cross-sectional carriage study	France	Culture	NP swabs	October 2001 - June 2014	6 - 24 months *n* = 7991	Carriage of NVT increased from 17.2% to 24.3% from 2001 - 2012, but this effect greatly decreased after the implementation of PCV13 (21.4% - 3.8%). Serotype 19A was the most dominant NVT after PCV7, while serotypes 15B/C, 11A, 15A and 35B were the most dominant NVTs after PCV13 immunisation.
Daana *et al*, 2015 [156]	PCV7	Cross-sectional surveillance study	Palestine	Culture	NP swabs	May-July (2009 -2011)	<5 years *n* = 2755	Carriage of NVTs increased from 34% to 65%. Serotypes/groups 15B/C, 19B & 35B were the most dominant NVTs isolated.
Desai *et al*, 2015 [157]	PCV13	-	USA	Culture	NP swabs	July 2010 - June 2013	6 - 59 months *n* = 2048	Carriage of non-PCV13 serotypes increased from 68.4% to 97%. Serotypes 35B, 15B/C, 19A, 11A, 23B, 6C, 21 and 15A were the most dominant serotypes to be isolated during the entire study period.

Gladston *et al*, 2015 [158]	PCV7 and PCV13	-	UK	Culture and whole genome sequencing	NP swabs	October - march (2006/7 & 2010/11)	<4 years *n* = 1712	Carriage of NVT increased. Increase in the carriage prevalence of NVTs 21, 23B, 33F and 35F.
Gounder *et al*, 2015 [159]	PCV7	Cross-sectional study	Alaska	Culture	NP swabs	2000 - 2004 & 2008 - 2010	<5 years *n* = 3496	Carriage of NVTs increased from 43% to 95%. Serotype 19A was the most dominant NVT isolate.
Nunes *et al*, 2015 [66]	PCV7	Longitudinal prospective study	South Africa	Culture	NP swabs	December 2009 -April 2010	6-12 weeks *n* = 255	Carriage of NVT increased from 33.9 % to 45 %. Carriage of serotypes 6A and 19A was higher in vaccinated groups, but colonisation by other serotypes was not elaborated on.
Tòthpal *et al*, 2015 [160]	PCV7	-	Hungary	Culture	NP swabs	February 2010 - February 2012	3-6 years *n* = 1022	Carriage of NVT increased from 32.8 % to 41.7 %. Carriage of serotypes 11A, 35F, 19A, 6B, 15B, 3 and 28 were the most dominant NVTs isolated.
Bosch *et al*, 2016 [161]	PCV7 & PCV10	Surveillance study	Netherlands	Culture	NP swabs	October 2012 - March 2013	11 & 24 months old *n* = 660	Carriage of NVT increased from 29% to 50% (post-PCV7) and to 57% (post-PCV10) in 11 month old children. Carriage of NVT increased from 30% to 61% (post-PCV7) and to 55% (post-PCV10) in 24 month old children. Carriage of serotypes 19A, 6C, 23B, 23A and 11A were the most dominant serotypes to be isolated.
Chan *et al*, 2016 [162]	PCV13	Cross-sectional surveillance study	Hong-Kong	Culture & Multiplex PCR (40 serotypes/groups)	NP swabs	June 2013 - June 2014	2,12 & 18 month old children *n* = 1541	Pneumococcal carriage was low compared to other countries. Serotypes 15B/C, 6C, 23A and 15AF were the most dominant serotypes to be isolated.

Hamaluba *et al*, 2015 [163]	PCV7	Cross-sectional observational study	UK	Culture & Microarray	NP swabs	November 2010 - September 2011	22-55 months *n* = 575	Carriage of NVTs increased. Serotypes 6B, 3, 19A, 6C, 11A and 23B were dominant NVTs isolated.
Hanke *et al*, 2016 [144]	PCV7	Cross-sectional study	Peru	Culture and qPCR (Density based on *lytA*)	NP swabs	May - September (2009 & 2011)	<3 years *n* = 125	Carriage of NVT increased from 52 % to 71.2 %. Serotype 6C was the dominant NVT isolated.
Nunes *et al*, 2016 [164]	PCV7	Cross-sectional study	Portugal	Culture & Multiplex PCR (40 serotypes/groups)	NP swabs	January- March (1999 - 1999; 2001 - 2003; 2006 - 2007)	0-6 years *n* = 1092	Carriage of NVT increased from 35.7 % to 46.6 %. Serotypes 19A, 6C, 15A, 16F, 21, 23A and 23B were the most dominant isolates identified.
Soysal *et al*, 2016 [165]	PCV13	Retrospective surveillance study	Turkey	Culture & Multiplex PCR (40 serotypes/groups)	NP swabs	September 2011 - September 2013	0-18 years *n* = 2165	Decrease in PCV13 serotypes and a slight shift towards non-PCV13 serotypes. Serotypes/groups 6AB, 19F, 23F, 9AV, 12F, 15A/F and 22A/F most common isolates identified.

* Pubmed and Scopus were used to search for studies evaluating the effect of infant vaccination with PCV on the prevalence of serotype-specific nasopharyngeal colonisation. MILST, multi-invasive-locus sequencing typing. NP, nasopharyngeal. NVT, non-vaccine serotypes. PCV, pneumococcal conjugate vaccine. PCV7, 7-valent pneumococcal conjugate vaccine. PCV10, 10-valent pneumococcal conjugate vaccine. PCV13, 13-valent pneumococcal conjugate vaccine. PCR, polymerase chain reaction. qPCR, quantitative PCR. VT, vaccine serotypes. -, information not provided in publication.

Table 1.3: Summary of studies evaluating the effect of infant vaccination with PCV on the density of carriage of serotype-specific nasopharyngeal colonisation in children *.

Reference	Study vaccine	Study design	Country	Assay(s) used	Specimen type	Study period	Participants	Main Findings
O'Brien et al, 2007 (55)	PCV7	Group-randomized efficacy trial	USA	Culture and Immunoblot	NP swabs	April 1997 - October 2000	<2 years *n* = 8292	Density of VT decreased, no difference in the density of NVT (Note: Semi-quantitative culture used to determine carriage density).
Roca et al, 2012 (143)	PCV7	Clustered-randomized trial	Gambia	Culture	NP swabs	December 2003 -June 2008	<30 months *n* = 4792	Decrease in density of VT carriage following vaccination. Decrease in density of NVT carriage following vaccination. (Note: Semi-quantitative culture used to determine carriage density).
Hanke et al, 2016 (144)	PCV7	Cross-sectional study	Peru	Culture and qPCR (Density based on *lytA*)	NP swabs	May - September (2009 & 2011)	<3 years *n* = 125	No different in density of VT serotypes, carriage density of NVT increased.

*Pubmed and Scopus were used to search for studies evaluating the effect of infant vaccination with PCV on the density of carriage of serotype-specific nasopharyngeal colonisation. NP, nasopharyngeal. NVT, non-vaccine serotypes. PCV, pneumococcal conjugate vaccine. PCV7, 7-valent pneumococcal conjugate vaccine. qPCR, quantitative PCR. VT, vaccine serotypes.

1.10. EFFECT OF PCV IMMUNISATION ON CO-COLONISING BACTERIA

The human nasopharynx is colonised by a multitude of bacterial species embedded in a complex microbiota and shifts in colonising species may disrupt the natural balance. PCV induced effect on colonising bacterial species such as *M. catarrhalis, H. influenzae,* and *S. aureus* has not been fully investigated, especially with respect to changes in the microbiome, and its attribution to extended pressure or vaccine introduction [166] (Table 1.4). Wiertsema *et al,* found an increase in non-typable *H. influenzae* post-vaccination in Western Australia in children with otitis media [167], while another study by Xu *et al,* found that carriage of 19A was associated with a decrease in carriage of *H. influenzae* [74]. Further, there is contrasting data with respect to the effect that PCV has on *S. aureus* carriage, with most studies [168,169] reporting no change and others reporting a temporary increase in prevalence [170]. It is thus important not only to monitor changes in pneumococcal colonisation following PCV immunisation, but also monitor the epidemiology of colonisation by other bacteria in the nasopharynx, which could impact on changes in risk of disease by these bacteria and future strategies for vaccine development [166]. In addition, most of these NP studies on bacterial colonisation have used traditional culture methods and thus the effect that PCV has on the carriage density of common bacterial colonisers is still unknown, thus justifying further research with more sensitive molecular methods.

Table 1.4: Summary of studies investigating the effect of PCV immunisation on co-colonising bacteria*

Reference	Country	Study design	Study vaccine	Assay(s) used	Specimen type	Study period	Participants	Bacterial targets	Main findings
Block et al, 2004 [171]	USA	-	PCV7	Culture	Middle ear swabs	January 1992 - January 1998 & July 2000 - April2003	7 - 24 months *n* = 379	*S. pneumoniae, H. influenzae, M. catarrhalis, & S. pyogenes*.	Vaccination decreased pneumococcal carriage and increased non-typable *H. influenzae* carriage prevalence.
Revai *et al*, 2006 [172]	USA	Retrospective cross-sectional study	PCV7	Culture	NP swabs	October 1995 - December 2004	6 months - 4 years *n* = 580	*S. pneumoniae, H. influenzae & M. catarrhalis*	Vaccination increased the carriage prevalence of both *H. influenzae* and *M. catarrhalis.*
Cohen et al, 2007 [168]	France	Prospective study	PCV7	Culture	NP swabs	December 2003 -June 2006	6 - 24 months *n* = 1783	*S. pneumoniae & S. aureus.*	PCV had no effect on *S. aureus* carriage prevalence.
Madhi *et al*, 2007 [77]	South Africa	Randomized control trial	PCV9	Culture	NP swabs	March 2001 - April 2005	Mean age = 5.64 years *n* = 352	*S. pneumoniae, H. influenzae & S. aureus.*	No difference in the carriage prevalence of *S. aureus* and *H. influenzae* between vaccinated and unvaccinated groups 5.3 years after primary series and prior to general PCV immunisation.
Pymula *et al*, 2011 [173]	Czech Republic & Slovakia	Prospective randomized double-blind controlled study	PCV10	Culture, PCR & Immunoblot	NP swabs	-	6 - 24 months *n* = 381	*S. pneumoniae & H. influenzae*	Vaccination decreased the carriage prevalence of non-typable *H. influenzae.*
van Gils *et al, 2011* [170]	Netherlands	Randomized control trial	PCV7	Culture	NP swabs	July 2005 - February 2008	New-borns *n* = 1005	*S. pneumoniae* & *S. aureus.*	A trend towards higher colonisation of *S. aureus* after vaccination was observed.
Wiertsema *et al*, 2011 [167]	Australia	Carriage study	PCV7	Culture and PCR	NP swabs & MEE(middle ear effusion)	November 2007 -May 2009	<36 months *n* = 186	*S. pneumoniae, H. influenzae & M. catarrhalis*	Vaccination increased the carriage prevalence of non-typable *H. influenzae* in children with AOM.

Cohen *et al*, 2012 [174]	France	Prospective surveillance study	PCV7	Culture	NP swabs	1933 - 2000 & 2006 - 2009	6 - 30 months *n* = 4405	*S. pneumoniae, H. influenzae, M. catarrhalis,* & *S. aureus*	Vaccination decreased *S. pneumoniae, H. influenzae* and *S. aureus* carriage (Although not significant) and increased the carriage prevalence of *M. catarrhalis*.
Dunne *et al*, 2012 [175]	Fiji	Phase II pneumococcal vaccine trial	PCV7	Multiplex qPCR	NP swabs	2006 - 2007	17 months *n* = 161	*S. pneumoniae, H. influenzae, M. catarrhalis,* & *S. aureus*	Vaccination increased the carriage prevalence of NVT, while prevalence of other bacteria remained the same. The carriage densities of *S. pneumoniae* and *H. influenzae* remained the same, but density of *M. catarrhalis* was higher in indigenous children as a result of PCV immunisation.
Ho *et al*, 2012 [176]	Hong Kong	-	PCV7	Culture and PCR	NP swabs	September 2009 - April2010	2 - 5 years *n* = 2211	*S. pneumoniae* & *S. aureus.*	No difference in the carriage prevalence of *S. aureus* was found between vaccinated and control groups.
Spijkerman *et al*, 2012 [177]	Netherlands	Cross-sectional carriage study	PCV7	Culture	NP swabs	March 2006 - January 2011	11 & 24 months n ≈ 660	*S. pneumoniae, H. influenzae, M. catarrhalis,* & *S. aureus*	Vaccination decreased overall pneumococcal carriage at 11 months, but by 24 months the rates were similar between cohorts. A persistently higher rate of *S. aureus* and *H. influenzae* was observed after PCV immunisation.
Dukers - Muijrers *et al*, 2013 [178]	Netherlands	Cross-sectional study	PCV7	Culture	NP swabs	-	6 weeks-4 years *n* = 620	*S. pneumoniae* & *S. aureus.*	Vaccination increased the carriage prevalence of non-vaccine type pneumococci and *S. aureus.*

Hare *et al*, 2013 [179]	Australia and Alaska	Prospective cohort study	PCV7	culture	NP swabs	2004 - 2010	0.5 - 8.9 years *n* = 120	*S. pneumoniae, H. influenzae, M. catarrhalis,* & *S. aureus*	Vaccination increased the carriage prevalence of *S. aureus* and decreased the carriage prevalence of *H. influenza* in both cohorts. *M. catarrhalis* Carriage decline in Australian children only.
Biesbroek *et al*, 2014 [180]	Netherlands	Randomized control trial	PCV7	Culture and sequencing	NP swabs	-	12 and 24 months *n* = 200	*S. pneumoniae, H. influenzae, M. catarrhalis,* & *S. aureus*	Temporary shift in microbial community composition and increased bacterial diversity. Vaccination increased the relative abundance of non-pneumococcal streptococci and anaerobic bacteria. Vaccination increased the carriage prevalence of *H. influenzae* and *S. aureus.*
Hammit *et al*, 2014 [181]	Kenya	cross-sectional carriage study	PCV10	Culture and multiplex PCR	NP swabs	2009 - 2012	<5 years *n* = 2031	*S. pneumoniae, H. influenzae* & *S. aureus*	Vaccination decreased carriage prevalence of vaccine-type pneumococci & non-typable *H. influenzae.* No change in *S. aureus* carriage prevalence.
Nzenze *et al*, 2014 [182]	South Africa	Cross-sectional study	PCV7	Culture	NP swabs	May - October 2009 & 2011	0-2 years & 3-12 years *n = 5669*	*S. pneumoniae, H. influenzae* & *S. aureus*	Prevalence of *S. pneumoniae* and *H. influenzae* carriage prevalence decrease between the two study periods, while the prevalence of *S. aureus* remained unchanged.
Camilli *et al*, 2015 [183]	Italy	Cross-sectional study	PCV7 & PCV13	Culture and PCR	NP swabs	January - April 2012	<6 years *n* = 301	*S. pneumoniae & H. influenzae*	Vaccination increased *H. influenzae* nasopharyngeal carriage.

Bosch *et al*, 2016 [161]	Netherlands	Surveillance study	PCV10	Culture	NP swabs	October 2012 - March 2013	11& 24 months old *n* = 660	*S. pneumoniae, H. influenzae, M. catarrhalis,* & *S. aureus*	Vaccination decreased the overall pneumococcal carriage prevalence. While a temporary increase in the carriage prevalence of *S. aureus* was observed at 11 months but not at 24 months. The carriage prevalence of *H. influenzae* also increased while the prevalence of *M. catarrhalis* remained constant after vaccination.

* Pubmed and Scopus were used to search for studies evaluating the effect of PCV immunisation on co-colonising bacteria. AOM, acute otitis media. MEE, middle ear effusion. NP, nasopharyngeal. NVT, non-vaccine serotypes. OP, oropharyngeal swabs. PCV, pneumococcal conjugate vaccine. PCR, polymerase chain reaction. qPCR, quantitative PCR. VT, vaccine serotypes. -, information not provided in publication.

1.11. EFFECT OF IMMUNISATION ON INVASIVE PNEUMOCOCCAL DISEASE

PCV has greatly reduced the incidence of pneumococcal disease in both vaccinated and unvaccinated groups by reducing vaccine serotype transmission within the population, thus leading to heard immunity or indirect protection [134]. CDC has reported that the incidence of IPD caused by vaccine serotypes decreased by 94% within 7 years of PCV7 introduction, although the clinical impact of serotype replacement may not yet be fully established as it could lag behind the reduction in VT disease and will also depend on the invasiveness of the NVT [(30)].

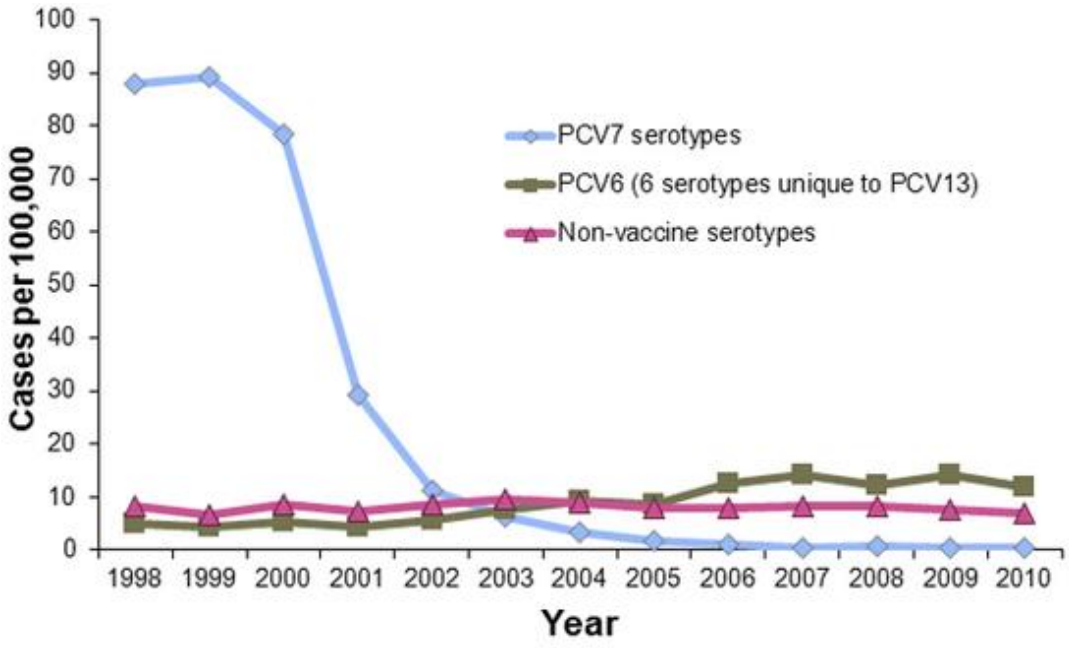


Figure 1.6: Rates of invasive pneumococcal disease amongst children aged <5years, 1988 - 2010 [(CDC)]

1.12. EPIDEMIOLOGY OF INVASIVE *STREPTOCOCCUS PNEUMONIAE* IN HIV-INFECTED INFANTS IN SOUTH AFRICA

In South Africa it is estimated that approximately 5% of children under the age of 5 years are infected with HIV. Among these children, 85% will develop a lower respiratory tract infection, with pneumonia being the most common cause of hospitalisation in HIV-infected children [119]. *S. pneumoniae* is the most important cause of invasive bacterial disease, with the burden of disease being 37 to 49 fold higher in HIV-infected compared to HIV-uninfected children [184,185]. This is most likely a result of decrease in the mucosal immunity (IgA and IgG) which allows for the persistent colonisation of pneumococcus and other bacteria, or decreased

immunological responsiveness to natural exposure and vaccination in the absence of antiretroviral treatment [71].

There are a limited number of comparative studies that have investigated the association between HIV-infection and pneumococcal colonisation, with results from these studies being contradictory with respect to the effect that HIV has on the overall carriage prevalence of pneumococcus (Table 1.5), with some studies showing no differences in the carriage prevalence between HIV-infected and HIV-uninfected children [186], and others studies from South Africa and The Gambia reporting that HIV-infected children have a higher prevalence of pneumococcal colonisation then their HIV-uninfected counterparts [77,187]. These studies were, however, all limited in that they relied on non-quantitative culture-based methods and thus the effect that HIV-infection has on pneumococcal carriage density is still unknown. Further, the majority of these studies involved populations that were not immunised with PCV and thus further investigation with more sensitive molecular methods on the association between HIV-infection and pneumococcal carriage prevalence and density in a PCV-vaccinated cohort is still needed.

Table 1.5: Summary of studies investigating the association between HIV-infection and bacterial colonisation in children *

Reference	Country	Study design	Assay(s) used	Study period	Bacterial targets	PCV-immunised	Main Findings
Rusen *et al*, 1997 [188]	Kenya	Cross-sectional	Culture	January 1990 -November 1990	*S. pneumoniae*	No	Pneumococcal carriage prevalence was higher in HIV-infected than HIV-uninfected children.
Leibovitz *et al*, 1999 [189]	Romania	Cross-sectional	Culture	May 1996	*S. pneumoniae*	No	Pneumococcal carriage prevalence was lower in HIV-infected than HIV-uninfected children.
Polack *et al*, 2000 [190]	USA	Cross-sectional	Culture	March - June 1997	*S. pneumoniae*	No	No difference in the carriage prevalence of pneumococcus between HIV-infected and HIV-uninfected children.
Zar *et al*, 2003 [191]	South Africa	Cross-sectional	Culture	1998	*S. pneumoniae, H. influenzae, S. aureus, K. pneumoniae, M. catarrhalis* & *M. tuberculosis*	No	No difference in the carriage prevalence of pneumococcus between HIV-infected and HIV-uninfected children. Carriage prevalence of *S. aureus* was higher in HIV-infected children.
Cardoso *et al*, 2006 [192]	Portugal	Observational & cross-sectional	Culture	November 2002 - May 2003	*S. pneumoniae*	No	No difference in the carriage prevalence of pneumococcus between HIV-infected and HIV-uninfected children.
McNally *et al*, 2006 [193]	South Africa	Cross-sectional	Culture	January 2001 -December 2002	*S. pneumoniae* & *S. aureus*	No	No difference in the carriage prevalence of pneumococcus between HIV-infected & HIV-uninfected children. Carriage prevalence of *S. aureus* was higher in HIV-infected children.

Madhi *et al*, 2007 [77]	South Africa	Cross-sectional	Culture	March 2001 - April 2005	*S. pneumoniae, H. influenzae* & *S. aureus*	Yes	HIV-infected children had a higher carriage prevalence of *S. pneumoniae* and *H. influenzae.* No difference in the carriage prevalence of *S. aureus* was found between HIV-infected and HIV-uninfected children.
Gill *et al*, 2008 [194]	Zambia	Longitudinal	Culture	December 2003 - September 2004	*S. pneumoniae*	No	Pneumococcal carriage prevalence was higher in HIV-exposed than HIV-unexposed infants.
Abdullahi *et al*, 2012 [195]	Kenya	Cross-sectional	Culture	October 2006 -December 2008	*S. pneumoniae*	Yes	Pneumococcal carriage prevalence was higher in HIV-infected than HIV-uninfected children.
Kinabo *et al*, 2013 [196]	Tanzania	Longitudinal	Culture & PCR	September 2005 - December 2009	*S. pneumoniae, H. influenzae* & *S. aureus*	No	Carriage with *S. aureus* was more common in HIV-infected children, while carriage of *S. pneumoniae* & *H. influenzae* was more common in HIV-uninfected children.
Nzenze *et al*, 2013 [197]	South Africa	Cross-sectional	culture	May - October 2009 & 2011	*S. pneumoniae*	Yes	No difference in the carriage prevalence of pneumococcus between HIV-infected and HIV-uninfected children.
Madhi *et al*, 2015 [198]	South Africa	Longitudinal	Culture	April 2005 - June 2006	*S. pneumoniae, H. influenzae* & *S. aureus*	Yes	Vaccine serotype colonisation was similar in PCV-immunised HIV-infected and HIV-uninfected children; however, a lower prevalence of overall pneumococcus, non-vaccine serotypes and *H. influenzae* in HIV-infected children was observed.

*Pubmed and Scopus were used to search for studies investigating the association between HIV-infection and bacterial colonisation in children.
HIV, human immunodeficiency virus. PCV, pneumococcal conjugate vaccine. PCR, polymerase chain reaction.

1.13. ASSOCIATION BETWEEN HIV-1 INFECTION AND COMMON BACTERIAL NASOPHARYNGEAL COLONISERS SUCH AS *S. AUREUS* AND *H. INFLUENZAE*

The effect of HIV-infection on other common nasopharyngeal colonisers such as *S. aureus* and *H. influenzae* is not clear, as a result of a limited number of studies that have focused only on non-quantitative culture based methods, predominantly in a PCV-unvaccinated population (Table 1.4). Further, results from these studies have been contradictory in that some studies have shown no difference in the carriage prevalence of *S. aureus* between HIV-infected and HIV-uninfected children [77], and others reported that HIV-infected children have a higher prevalence of *S. aureus* colonisation [191,193,196]. Also, while some studies have reported HIV-infected children to have a higher carriage prevalence of *H. influenzae* [77], others have rather reported a lower carriage prevalence of *H. influenzae* [196,198] or no difference between HIV-infected and HIV-uninfected groups [191].

1.14. INVESTIGATION OF SEROTYPE DISTRIBUTION

1.14.1. Quellung method for serotyping *Streptococcus pneumoniae*

Pneumococcal colonisation and serotype distribution is normally investigated by culture of the dominant colony on blood-agar media, followed by serotyping with specific antisera [199]. Despite the ubiquitous use of culture based methods for NP colonisation studies, there are several fundamental limitations with this method. These include that this traditional method generally detects only the dominant colonising serotype, and in the case of multiple serotype carriage, the minor serotypes will go undetected [200]. Further, these methods are not quantitative for the dominant serotype, and will not provide information of the relative quantitative distribution of the minor serotypes. Also, culture based methods tend to favour organisms which are more able to grow on laboratory media [127,133,134]. These limitations of culture-based methods, limit their ability to determine the true underlying mechanism of the increase in NVT colonisation following introduction of PCV [201].

1.14.2. Molecular methods: Real time qPCR

Real-time PCR has recently been developed not only to detect multiple *S. pneumoniae* serotypes that colonise the nasopharynx niche, but also to detect other respiratory pathogens such as *S. aureus* and *H. influenzae* directly from nasopharyngeal secretions. Quantitative real-time PCR has become an increasingly important platform in which multiple respiratory organisms can be detected from clinical specimens, with an increased sensitivity and specificity compared to standard culture methods. Several studies have illustrated the usefulness of qPCR. Stralin *et al*, developed a successful multiplex real-time PCR assay to simultaneously detect four respiratory pathogens, including *S. pneumoniae* and *H. influenzae* from a single sputum sample [202], while results from Corless *et al*, showed real-time PCR improved the diagnosis and detection of multiple causative agents of bacterial meningitis [203].

The ability to quantitatively detect multiple serotypes from a single nasopharyngeal specimen in both PCV-vaccinated and PCV-unvaccinated infants, enables better understanding of the dynamics of pneumococcal carriage and its relationship to disease. Serotyping pneumococci by qPCR, however, has two main shortcomings, including that a large sample volume is required to distribute across all the reactions, and that it is expensive. Further, processing multiple qPCR reactions is labour intensive and time consuming.

1.14.3. Molecular methods: Fluidigm

Fluidigm is a nanofluidic automated real-time PCR system that relies on microfluidic technology in which dynamic arrays of integrated fluidic circuits (IFCs) are used. These IFC's contain thousands of controlled valves and interconnected channels in which molecules of biological samples and reagents can be automatically mixed in a variety of grid like matrices. The instrument uses an array of non-fluidic chips called dynamic arrays for qPCR, in which a typical chip format allows for 9 216 PCR reactions (96.96 chip format; 96 samples x 96 assays) in a single qPCR run (www.fluidigm.co.za) [204].

Fluidigm has several advantages over standard qPCR in that it allows for a larger number of reactions per plate, thus making it more cost effective and less time consuming. Further, IFCs not only reduces the reaction volume from 10µL – 20µL down to 10nL scale, but the

technology also allows for validations as well as increased parallelism and throughput of qPCR reactions [204]. Fluidigm has a number of applications in biological automation including gene expression and genotyping, and has also recently been used to detect pneumococcal serotypes from nasopharyngeal samples. Dhoubhadel *et al*, showed this method to be specific with respect to detecting 50 different pneumococcal serotypes (17 individual serotypes and 33 serotypes in 12 groups), with good repeatability, accuracy and reproducibility [205].

Improved methods of detection of respiratory pathogens has provided a means to improve the health care of patients as optimal therapy can be initiated sooner, effectiveness of the treatment is improved and spread of disease can be reduced or even prevented [206]. A high bacterial load detected by real-time PCR in nasopharyngeal specimens can help predict the etiological role for *S. pneumoniae* in disease states [207].

1.15. RATIONAL FOR THE STUDY

The burden of HIV-infection in children in South Africa is high, with 1 in 8 children infected with HIV globally living in South Africa. Of these infants, 85% will develop a respiratory tract infection during the course of their HIV disease, with pneumonia being the most common cause of hospitalisation. *S. pneumoniae* is the most common cause of bacterial disease, with the burden of IPD being 37-49 fold higher in HIV-infected compared to HIV-uninfected children. While PCV has been effective in reducing serotype specific IPD by more than 80% in most countries, it is more effective in HIV-uninfected than HIV-infected infants [185,208]. Further, there is conflicting data with respect to the effect HIV-infection has on the overall carriage prevalence of pneumococcus, with some studies showing no difference in prevalence of pneumococcal carriage between HIV-infected and HIV-uninfected children, whilst other studies report that HIV-infected children have a higher prevalence of colonisation [209].

Concerns have also been raised about the long term effectiveness of PCV with limited serotypes, to sustain reduction in overall pneumococcal mucosal and invasive disease. There have already been reports of increase in disease from NVT pneumococcal serotypes and other bacteria such as *H. influenzae* [167,210]. Whilst these epidemiological studies provide some

insight as to what may be happening in the nasopharynx in response to PCV introduction, there are several shortcomings. The numbers of cases reported are often too few to make meaningful conclusions, especially when one stratifies into specific serotypes. In addition, the periods of analysis are often short, and could be influenced by factors such as fluctuations in the circulating respiratory viruses within a particular year.

Since NP colonisation is the precursor to diseases of the upper and lower respiratory tract, analysis of the NP tract flora can provide a simple means to signal changes, which could inform future trends in the long term effectiveness of these vaccines. The majority of studies of NP colonisation have focused on non-quantitative culture based methods, and compared changes in the dominant pneumococcal serotype. Whilst this has been meaningful to some extent, the lack of quantitative data has hindered our understanding of these dynamics. By applying quantitative real-time PCR and Fluidigm to quantitatively detect carriage of the most frequently isolated pathogens in the nasopharynx and in the case of the pneumococcus, down to serotype level, we can gain a better understanding of the impact of PCV on nasopharyngeal carriage and its potential relationship to pneumococcal disease.

1.16. STUDY OBJECTIVES

1. To develop a quantitative multiplex real-time PCR assay for the detection and density characterisation of multiple pneumococcal serotypes (44 serotypes/groups; 13 individual serotypes and 31 serotypes in 11 groups) on nasopharyngeal swabs, previously investigated by traditional culture methods.

2. To evaluate the effect of infant vaccination with 7-valent PCV (PCV7) on the density of serotype-specific nasopharyngeal colonisation, and also delineate the relative role of serotype replacement versus unmasking as the cause for the increase in non-vaccine serotype colonisation in PCV7-vaccinated children.

3. To evaluated the association of PCV7 immunisation of infants on the prevalence and density of nasopharyngeal colonisation by common, potentially pathogenic bacteria including PCV7 serotypes, non-vaccine serotypes, *Haemophilus influenzae*,

Staphylococcus aureus, Moraxella catarrhalis, Streptococcus pyogenes and *Neisseria meningitidis* using the qPCR assay.

4. To develop a novel Nanofludic real-time PCR assay that could simultaneously detect and quantify 11 bacterial pathogens, 53pneumococcal serotypes (16 individual serotypes and 37 serotypes in 13 groups) and 6 serotypes of *H. influenzae* (serotypes a-f).

5. To compare results from traditional qPCR and Fluidigm for the detection of bacterial colonisation and pneumococcal serotyping in PCV-vaccinated children.

6. To evaluate the association between HIV-infection and HIV-exposure on the prevalence and density of nasopharyngeal colonisation by common, potentially pathogenic bacteria including PCV7 serotypes, non-vaccine serotypes, *Haemophilus influenzae*, Non-typable *Haemophilus influenzae, Staphylococcus aureus, Moraxella catarrhalis, Streptococcus pyogenes, Neisseria meningitidis, Neisseria lactamica, Bordetella pertussis, Bordetella parapertusis, Bordetella bronchiseptica* and *Bordetella holmesii,* in PCV7-vaccinated children with the Biomark HD Nanofludic qPCR instrument.

CHAPTER 2: COMPARISON OF TRADITIONAL CULTURE AND MOLECULAR QPCR FOR DETECTION OF SIMULTANEOUS CARRIAGE OF MULTIPLE PNEUMOCOCCAL SEROTYPES IN AFRICAN CHILDREN

Streptococcus pneumoniae is a common coloniser of the human nasopharynx in both HIC and LMIC countries, with carriage being a pre-requisite to pathogenesis of pneumococcal disease as well as transmission between hosts [18,23-26]. To date almost 100 serotypes have been identified through their capsular polysaccharide and there is a paucity of data on concurrent carriage of multiple pneumococcal serotypes due to limitations of traditional recommended culture methods [139].

Pneumococcal colonisation and serotype characterisation is normally investigated by culture of the dominant organism followed by serotyping with specific antisera [199]. Despite the ubiquitous use of culture-based methods for nasopharyngeal colonisation studies, detection of concurrent colonisation by other serotypes of a lower density are often missed [200]. Further, traditional culture methods are not quantitative for the colonising serotypes. Molecular detection of pneumococci in the nasopharynx have several potential advantages over culture based methods, including detection of multiple serotypes from a single sample with high sensitivity, as well as quantitative PCR methods being able to measure the density of colonisation and relative proportion of colonising serotypes [211]. Interrogating beyond identification of only the dominant serotype colonising children, could also help inform the extent to which the increase in nasopharyngeal non-vaccine serotype colonisation following pneumococcal conjugate vaccine (PCV) immunisation is driven by unmasking of previously minor colonising serotypes or replacement acquisition by new serotypes.

In this chapter we aimed to evaluate the use of multiplex quantitative PCR (qPCR) for the detection and density characterisation of multiple pneumococcal serotypes on nasopharyngeal swabs, from PCV naive black-African children previously investigated by traditional culture methods.

2.1. MATERIAL AND METHODS

2.1.1. Study population

We retrospectively analysed archived nasopharyngeal swab samples collected from a cohort of PCV-unvaccinated, HIV-uninfected infants in Soweto, South Africa, who had previously been investigated by standard culture methods [142,185]. The parent study was a longitudinal, prospective study in which black-African HIV-infected and HIV-uninfected mothers and their infants were enrolled from January 2007 through to October 2007. The cohort included 250 PCV naïve infants and their mothers, including 125 infants born to HIV-infected women but who were HIV-uninfected (HEU); and 126 infants born to HIV-uninfected mothers. All infants born to HIV-infected mothers had a nonreactive HIV-polymerase chain reaction at enrolment and were not breastfed. Participants had five scheduled visits dictated by the child's age and a standardised questionnaire was administered at each study visit to gather demographic and clinical characteristics and to determine possible risk factors for *S. pneumoniae* carriage. The pneumococcal conjugate vaccine (PCV) immunisation of children was not available as standard-of-care during the study period.

One nasopharyngeal (NP) swabs were taken from each participant at each study visit, including at 9 and 15-16 months of age. Swabs were stored in skim milk-tryptone-glucose-glycerol (STGG) transport media at -70ºC at the Respiratory and Meningeal Pathogen Research Unit (RMPRU) in South Africa, as recommended by WHO [212]. The samples were previously cultured for *S. pneumoniae* using standard culture methods and pneumococcal serotyping undertaken using the Quellung method as described [36.] If pneumococcal colonies presented with different morphologies on the same plate, one colony representative of each was sub-cultured and serotyped.

2.1.2. Multiplex qPCR methods

Stored nasopharyngeal swab samples were thawed and processed. Briefly, NP swabs were vortexed for 30 seconds to release bacteria into the transport media and total nucleic acids were automatically extracted from STGG with the NucliSens ® easyMAG ® extraction system (BioMérieux, Marcy l'Etoile, France) according to manufactures instructions. Similarly, total nucleic acids were also extracted from pneumococcal reference strains

(positive control strains) grown in Todd-Hewitt broth supplemented with 5% yeast, with the NucliSens ® easyMAG ® extraction system according to manufactures instructions. Samples and pneumococcal reference strains were stored at – 20 °C.

Control strains for the pneumococcal serotypes 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 7C, 9A, 9L, 9N, 9V, 10A, 10B, 11A, 11B, 11C, 11D, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16F, 17A. 17F, 18A, 18B, 18C, 19A, 19B, 19F, 20, 21, 23A, 23B, 23F, 34 and 37 were obtained from the National Institute for Communicable Diseases (NICD). DNA from these strains were used to optimise PCR assays and as positive controls.

The sequences for oligonucleotide primers and dye-labelled MGB probes were taken from previously published sequences, or designed with the ABI primer express software package, version 3.0 (Applied Biosystems, ABI, Foster City, USA). All primers and probes were designed based on previously published CpsA (Capsular polysaccharide) genes; in which all sequences were obtained from the Gene Bank database http://www.ncbi.nlm.nih.gov/genbank/). Primer and Probe sequences are described in Table 2.1. Due to the high genotypic similarities between the capsule loci of certain serotypes, the qPCR method was unable to discriminate between some serotypes within a particular serogroup (i.e. 6A/B, 6C/D, 9A/L/N/V, 10A/B, 11A/B/C/F, 12A/B/F, 15A/B/C/F, 18A/B/C, 19B/F, 23A/B and 34/37/17A), but was able to identify serotypes 1, 3, 4, 5, 7C, 13, 14, 16F, 17F, 19A, 20, 21 and 23F individually.

Table 2.1: Primers and probe sequences for the qPCR detection of pneumococcal serotypes

Primers/Probe[a] name	Sequence (5′ - 3′)	serotype/serogroup	Source
lytA-Fwd	TCTTACGCAATCTAGCAGATGAAGC		
lytA-Rev	GTTGTTTGGTTGGTTATTCGTGC	*Streptococcus pneumoniae*	McAvin *et al*, 2001
lytA-Probe	(VIC)-TTTGCCGAAAACGCTTGATACAGGG-(MGB)		
1-Fwd	CGTGCGGTAATTGAAGCTATGA		
1-Rev	TGTGGCCCCAGCAACTCT	*Streptococcus pneumoniae*_serotype 1	Azzari *et al*, 2010
1-Probe	(FAM)-TGCTTGCCCTTGTATAGGGT-(MGB)		
3-Fwd	GGTCAGCAGAAAGTATGCATTGG		
3-Rev	TCGTTTATCCAGGGTCTGATGA	*Streptococcus pneumoniae*_serotype 3	Azzari *et al*, 2010
3-Probe	(VIC)-TATTGGATGTGGTTTATCGTGAAGA-(MGB)		
4-Fwd	TGGGATGACATTTCTACGCACTA		
4-Rev	CCGTCGCTGATGCTTTATCA	*Streptococcus pneumoniae*_serotype 4	Azzari *et al*, 2010
4-Probe	(FAM)-TCCTATTGGATGGTTAGTTGGTGA-(MGB)		
5-Fwd	TTACGGGAGTATCTTATGTCTTTAATGG		
5-Rev	CAGCATTCCAGTAGCCTAAAACTAGA	*Streptococcus pneumoniae*_serotype 5	Azzari *et al*, 2010
5-Probe	(VIC)-TTGTCTCAGCAACTCTATTTGGCTGTGGG-(MGB)		
6A/B/C/D-Fwd	AAGTTTGCACTAGAGTATGGGAAGGT		
6A/B/C/D-Rev	ACATTATGTCCRTGTCTTCGATACAAG	*Streptococcus pneumoniae*_serogroup 6A/B/C/D	Azzari *et al*, 2010
6A/B/C/D-Probe	(VIC)-TGTTCTGCCCTGAGCAACTGG-(MGB)		
6C/D-Fwd	TTGGGATGATTGGTCGTATTAG		
6C/D-Rev	CTCTTCAATTAGTTCTTCAGTTCG	*Streptococcus pneumoniae*_serogroup 6C/D	Azzari *et al*, 2010
6C/D-Probe	(FAM)-CCACGCAATTCGCCATC-(MGB)		
7C-Fwd	CGTCAGGAATAGGTGCAATCTCT		
7C-Rev	TGAAATTCCAAGCGAAGCAA	*Streptococcus pneumoniae*_serotype 7C	This Study
7C-Probe	(VIC)-TTCATCTATTGGTTCTTATGGTGTT-(MGB)		
9A/L/N/V-Fwd	TGGAATGGGCAAAGGGTAGTA		
9A/L/N/V-Rev	TCGGTTCCCCAAGATTTTCTC	*Streptococcus pneumoniae*_serogroup 9A/L/N/V	Azzari *et al*, 2010
9A/L/N/V-Probe	(FAM)-TTAATCATGCTAACGGCTCATCGA-(MGB)		

10A/B-Fwd	CCTCTCCTATCAACTATTACTCATTATACTACCT		
10A/B-Rev	AATAACCATAAGTCCCTAGATCATTCAAAG	*Streptococcus pneumoniae*_serotype 10A/B	Azzari *et al*, 2010
10A/B-Probe	(VIC)-TCATTACAACTCCCTATGTGACACGGGTCTTTT-(MGB)		
11A/B/C/D/F-Fwd	ACCGCATTTCTTATCGCACTATATT		
11A/B/C/D/F-Rev	TCTCCTTACCATCAAACATGTTAATCA	*Streptococcus pneumoniae*_serogroup 11A/B/C/D/F	This Study
11A/B/C/D/F-Probe	(FAM)-TGAATCAGTCTGACCGTTT-(MGB)		
12A/B/F-Fwd	GATTATTCGCTTGCCTCTTCATG		
12A/B/F-Rev	ATAGCCGAAATAAGCTTTCCAGAA	*Streptococcus pneumoniae*_serogroup 12A/B/F	Azzari *et al*, 2010*
12A/B/F-Probe	(FAM)-ATTTGTAAGCGGACGTGCGATT-(MGB)		
13-Fwd	TCGGATTTAGTAGTAACCCCATTGA		
13-Rev	TTCTTGATTGAGGATGCATTTCC	*Streptococcus pneumoniae*_serotype 13	This Study
13-Probe	(VIC)-AGTAGTAAGAGATCATATTCAAG-(MGB)		
14-Fwd	CGACTGAAATGTCACTAGGAGAAGAT		
14-Rev	AATACAGTCCATCAATTACTGCAATACTC	*Streptococcus pneumoniae*_serotype 14	Azzari *et al*, 2010
14-Probe	(VIC)-TGTCATTCGTTTGCCAATACTTGATGGTCTC-(MGB)		
15A/B/C/F-Fwd	TTGAATCAGGTAGATTGATTTCTGCTA		
15A/B/C/F-Rev	CTCTAGGAATCAAATACTGAGTCCTAATGA	*Streptococcus pneumoniae*_serogroup 15A/B/C/F	Azzari *et al*, 2010
15A/B/C/F-Probe	(FAM)-CTCCGGCTTTTGTCTTCTCTGT-(MGB)		
16F-Fwd	GCAACTGGTATTTTTGATATTGGAGAA		
16F-Rev	CAAAGGAATGCCATGCCATA	*Streptococcus pneumoniae*_serotype 16F	This Study
16F-Probe	(NED)-AAAATGCTAACTTCGTTGGAGG-(MGB)		
17F-Fwd	GTAAAGATTTCATGTCCTATAAGGGAGAA		
17F-Rev	AGGCGTCCCTGTTTATGAGAAG	*Streptococcus pneumoniae*_serotype 17F	This Study
17F-Probe	(FAM)-TTGTACATGGTCTGGATTT-(MGB)		
18A/B/C-Fwd	CCTGTTGTTATTCACGCCTTACG		
18A/B/C-Rev	TTGCACTTCTCGAATAGCCTTACTC	*Streptococcus pneumoniae*_serogroup 18A/B/C	Azzari *et al*, 2010
18A/B/C-Probe	(FAM)-AACCGTTGGCCCTTGTGGTGGA-(MGB)		
19A-Fwd	TTCGACGACGTATCAGCTTCA	*Streptococcus pneumoniae*_serotype 19A	Azzari *et al*, 2010

19A-Rev	TCATTGAGAGCCTTAACCTCTTCA		
19A-Probe	(VIC)-ACCCAAAACGGTTGACGCATTATACT-(MGB)		
19B/F-Fwd	GGTCATGCGAGATACGACAGAA		
19B/F-Rev	TCCTCATCAGTCCCAACCAATT	*Streptococcus pneumoniae*_serogroup 19B/F	Azzari *et al*, 2010
19B/F-Probe	(VIC)-ACCTGAAGGAGTAGCTGCTGGAACGTTG-(MGB)		
20-Fwd	AAAGATACTGGCTGAGGAGCTATCTATT		
20-Rev	AGTCAAAAGTACTCAACCATTCTGATATATTC	*Streptococcus pneumoniae*_serotype 20	Azzari *et al*, 2010
20-Probe	(VIC)-AGGATAAGGTCTACTTTGTGGGAGTTC-(MGB)		
21-Fwd	CCATTTGAAGGACCAGTTGTTG		
21-Rev	AAAAAGCCACTATCAGGAATACCAA	*Streptococcus pneumoniae*_serotype 21	This Study
21-Probe	(FAM)-AATGGCATTGCTTCGTAAA-(MGB)		
23A/B/F-Fwd	GGTGGACTTTCCGATGCAA		
23A/B/F-Rev	CACTGTCAACAAAAATGAGGTAATCTC	*Streptococcus pneumoniae*_serogroup 23A/B/F	This Study
23A/B/F-Probe	(VIC)-AAATGTCGGTATAGATAAAG-(MGB)		
23F-Fwd	TGCTATTTGCGATCCTGTTCAT		
23F-Rev	AGAGCCTCCGTTGTTTCGTAAA	*Streptococcus pneumoniae*_serotype 23F	Azzari *et al*, 2010
23F-Probe	(FAM)-TTTCTCCGGCATCAAACGTTAAG-(MGB)		
34/37/17A-Fwd	GGATACTATGTACGAACAGATGGACTTG		
34/37/17A-Rev	CTCACTAACTCGCCCGAATAAAC	*Streptococcus pneumoniae*_serogroup 34/37/17A	This Study
34/37/17A-Probe	(FAM)-CCGACTATACTCCATTTGA-(MGB)		

Probe[a]: Minor Grove Binding (MGB) dye labelled TaqMan probe.

2.1.3. Primer optimisation, standard curves and quantification of the real-time PCR assays

The qPCR's was optimised in 25µL reaction volumes containing 12.5µL 2x TaqMan gene expression master mix (ABI) and 0.25mM MGB dye labelled probe (ABI) in a primer concentration matrix. Reactions were amplified with the ABI 7500 Real Time PCR system (Applied Biosystems, ABI, Foster City, USA) as per standard manufactures instructions with standard cycling times. Standard concentration curves and lower limits of detection (LLD) for each target serotype was determined by amplifying serial dilutions of purified genomic DNA extracted from reference strains with the linear dynamic range being $10^1 – 10^7$ copies per standard curve. Target control DNA was harvested from early exponential phase cultures (OD_{600} =0.1), and the number of CFU/swab was estimated from Cq values relative to the standard curve, which is based on the quantitative culture of the control DNA.

The correlation coefficients (r^2) of each standard curve was determined and the amplification efficiency (e) of a given primer pair was calculated by $e = 10^{-1/m} -1$, where m is the slope of the standard curve. Lower limits of detection (LLD) or analytical sensitivity was determined by the minimum number of copies of DNA that could be measured repeatedly by the primers in the 10-fold serial dilutions. All primer pairs were tested with genomic DNA from all bacterial controls to determine the analytical specificity. Further, intra-assay variation (repeatability) was determined by running the same samples four times in the same assay run and presented as standard deviation (SD) for the copy number of the samples. A ratio of the difference between the observed copy number and the expected copy number was calculated to determine the accuracy of the assays, while the inter-assay variation (reproducibility) was determined by the SD of the copy number of the same sample tested in different runs.

Serotype targets were duplexed (Table 2.2) and paired reactions were tested to ensure that their Cq values did not shift by >1 Cq value, and that sensitivity and specificity remained the same as for singleplex reactions.

Table 2.2: Primer/probe duplex combinations

Primmer/probe combinations	Duplex reaction
5 & 21	A
20 & 23F	B
4 & 14	C
3 &17F	D
1 &23A/B/F	E
6A/B/C/D & 6C/D	F
10A/B & 15A/B/C/F	G
13 & 9A/L/N/V	H
19A & 18A/B/C	I
19B/F & 12A/B/F	J
11A/B/C/F & 16F	K
7C & 34/37/17A	L

2.1.3. Real-time PCR multiplex assay

Target DNA was pre-screened for pneumococcus by PCR for the *LytA* gene as described [213]. All *LytA* positive samples (Cq <35) were regarded as positive for pneumococci and tested for serotypes. Amplification data was analysed with the Applied Biosystems 7500 software, version 2.3 (Foster City, USA) with manually defined thresholds. Negative samples were defined as those with Cq values <35. Further, all *LytA* negative samples were tested for human GAPDH target to confirm the efficiency of the DNA extraction with all qPCR negative samples being positive for GAPDH.

2.1.4. Statistical analysis

Statistical analysis was performed with STATA Version 11.0 (Statacorp, Texas, USA). All *Lyt*A positive samples in which no serotype was identified were omitted in the multiple carriage analysis. The sensitivities of the qPCR assay and culture were tested with McNemar's test using culture as a reference, and when analysed for concordance, all serogroups were included as serotypes detected by qPCR vs. serotypes identified by culture. In the case of non-typable (NT) pneumococcus, we did not compare findings by culture and qPCR, as the qPCR method was not optimised to test for all pneumococcal serotypes and thus analysis could be misleading as a proportion of the NT detected by qPCR could belong to untested serotypes. For density of carriage, geometric mean densities (GMD) and 95%

confidence intervals (95%CI) of pneumococcal mean concentrations were calculated following $\log_{10}$ transformation on the data positive by qPCR. Further, the serotype specific propensity of whether a given serotype/serogroup was more likely to be found as a primary or non-primary isolate, was analysed using a two-tail Binomial test, in which the observed proportion of primary to non-primary isolates were compared. Results were considered significant when the *p*-value was < 0.05.

2.1.5. Ethical consent

Ethical consent for the initial enrolment was obtained from the Medical Human Research Ethics Committee (HREC) of the University of Witwatersrand (HREC: 050705). Approval for further testing of samples was obtained from the HREC (M120972). Written, informed consent had been obtained from the parents/guardians of all the study participants at the time of initial enrolment.

2.2. RESULTS

Molecular qPCR analysis involved 374 (83%) of the initial 450 nasopharyngeal swabs collected at either 9 or 15-16 months of age (Figure 2.1). Within the cohort, there were no differences in the prevalence of serotype colonisation detected by traditional culture methods between those in whom samples were and were not available (Appendix A). All children, in whom samples were available, were black-African, and 49.7% were male. Due to high similarities in findings at both 9 and 15-16 months of age, data was combined for the main analysis.

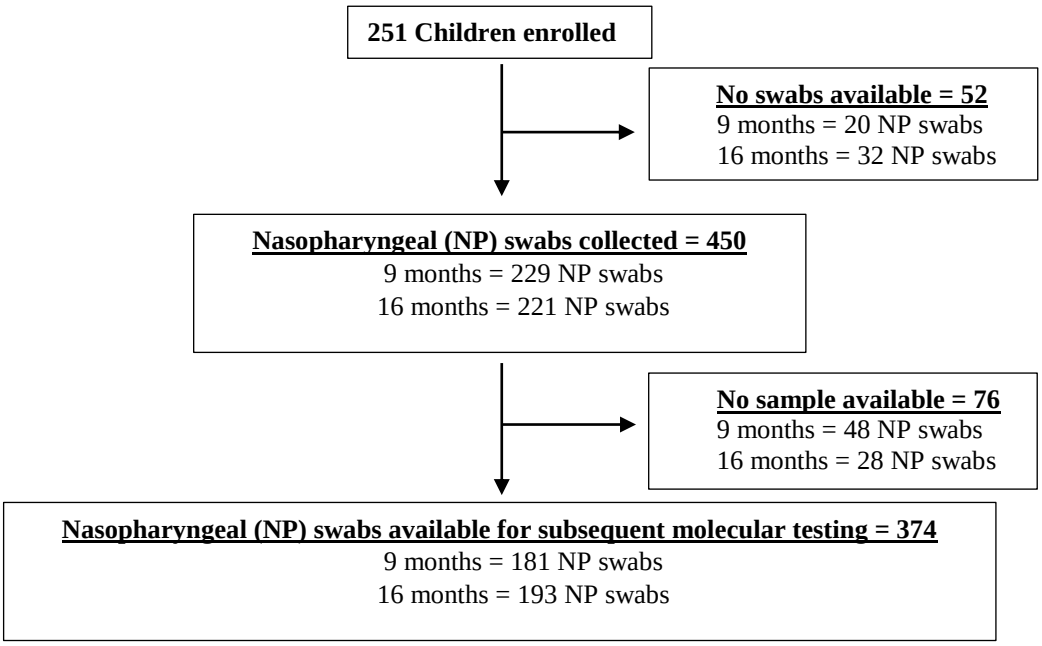


Figure 2.1: Schematic diagram of study population and sample availability

Diagram indicating the number of children initially enrolled in a PCV-unvaccinated cohort of HIV-uninfected children, as well as the number of nasopharyngeal (NP) swabs available for subsequent molecular qPCR analysis. No swabs were collected due to a missed study visit and the number of NP swabs available for molecular testing was defined by whether there was an adequate volume of sample remaining.

2.2.1. Performance of the real-time qPCR assay

All assays were effective in amplifying their respective targets with a high sensitivity, specificity and linearity with the exception of assay 10A/B in which only 10A was detected by the assay. The efficiency of all the assays ranged from 90% to 105% (Table 2.3). Within the linear dynamic range, the correlation coefficients (r^2) of all the assays were 0.99. Further, all assays showed high analytical sensitivities with their respective primer and probe pairs with the limit of detection being equivalent 10 copies per PCR for all respective assays, with exception to primers/probes that detected serotypes/serogroups 5, 23F, 14, 6A/B/C/D, 13 and 10A in which the LLD was 10 fold less sensitive (100 copies per PCR). All primer pairs and probes were tested with genomic DNA from all pneumococcal and bacterial controls, and no cross reactions occurred (Table 2.4). Both the inter-assay variation (repeatability) and inter-assay variation (reproducibility) for all respective assays was <0.1, while the accuracy for all respective assays was within ±0.1 (Table 2.3).

Table 2.3: Performance of the quantitative real-time PCR assay

Bacterial target	Efficiency (%)	Coefficient correlation (r^2)	Limit of detection (copies/PCR)	Intra-assay variation	Accuracy	Inter-assay variation
Streptococcus pneumoniae (lytA)	97	0.99	10	0.02	0.043	0.025
Streptococcus pneumoniae serotype 1	105	0.99	10	0.011	0.025	0.032
Streptococcus pneumoniae serotype 3	94	0.99	10	0.025	-0.013	0.025
Streptococcus pneumoniae serotype 4	91	0.99	10	0.074	-0.01	0.015
Streptococcus pneumoniae serotype 5	95	0.99	100	0.018	0.048	0.047
Streptococcus pneumoniae serogroup 6A/B/C/D	97	0.99	100	0.013	-0.26	0.023
Streptococcus pneumoniae serogroup 6C/D	92	0.99	10	0.011	-0.078	0.041
Streptococcus pneumoniae serotype 7C	102	0.99	10	0.008	-0.076	0.041
Streptococcus pneumoniae serogroup 9A/L/N/V	97	0.99	10	0.019	-0.059	0.023
Streptococcus pneumoniae serotype 10A/B	91	0.99	10	0.006	0.001	0.008
Streptococcus pneumoniae serogroup 11A/B/C/D/F	98	0.99	10	0.027	0.023	0.053
Streptococcus pneumoniae serogroup 12A/B/F	94	0.99	10	0.018	0.068	0.009
Streptococcus pneumoniae serotype 13	94	0.99	10	0.003	-0.023	0.003
Streptococcus pneumoniae serotype 14	91	0.99	100	0.078	-0.041	0.056
Streptococcus pneumoniae serogroup 15A/B/C/F	98	0.99	10	0.056	-0.059	0.038
Streptococcus pneumoniae serogroup 16F	92	0.99	10	0.03	-0.013	0.029
Streptococcus pneumoniae serotype 17F	99	0.99	10	0.054	-0.026	0.018
Streptococcus pneumoniae serogroup 18A/B/C	91	0.99	10	0.023	0.021	0.047
Streptococcus pneumoniae serotype 19A	92	0.99	10	0.001	0.054	0.005
Streptococcus pneumoniae serogroup 19B/F	93	0.99	10	0.006	0.001	0.003
Streptococcus pneumoniae serotype 20	102	0.99	10	0.015	0.054	0.05
Streptococcus pneumoniae serotype 21	101	0.99	10	0.098	0.075	0.11
Streptococcus pneumoniae serogroup 23A/B/F	96	0.99	10	0.013	-0.056	0.021
Streptococcus pneumoniae serotype 23F	90	0.99	100	0.002	-0.073	0.038
Streptococcus pneumoniae serogroup 34/37/17A	92	0.99	10	0.002	0.09	0.013

Table 2.4: Analytical specificity of the quantitative real-time PCR assay

| | | Bacterial targets | | |

2.2.2. Detection of pneumococcal carriage and serotype detection by culture and qPCR

There was a modest concordance between qPCR and culture for the detection of pneumococcus from infant NP swabs (*kappa* = 0.58), with the *LytA* qPCR assay being more sensitive than culture (82% vs. 71%; $p<0.001$); Table 2.5. The qPCR demonstrated high sensitivity (84%) and specificity (88%) compared to culture used as a referent standard for detection of pneumococcus, with the overall density of pneumococcal carriage being higher in culture-positive (4.4, 95% CI: 4.34 - 5.05 CFU/ml) than culture-negative samples (3.77; 95%CI: 3.59 - 3.95 CFU/ml); Figure 2.2.

Table 2.5: Yield of culture and molecular qPCR for the detection *Streptococcus pneumoniae* in nasopharyngeal swabs (n = 374)

	qPCR (-)	qPCR (+)	Total	*P*-value	Concordance (*Kappa*)	Sensitivity (%)	Specificity (%)
Culture (+)	8(2)	258(69)	266(71)				
Culture (-)	59(16)	49(13)	108(29)	<0.001	0.58	84	88
Total	67(18)	307(82)	374(100)				

Numbers are value (%), *p*-values of <0.05 were considered significant.

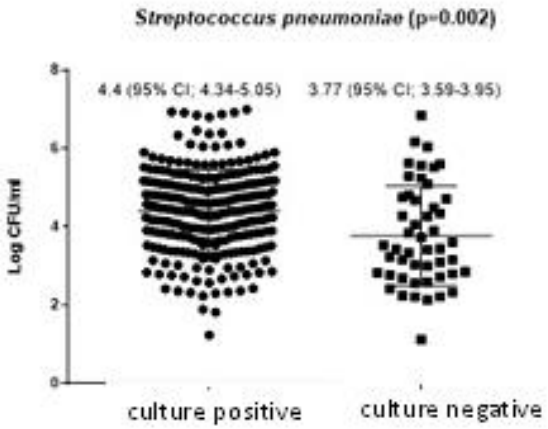


Figure 2.2: Density of pneumococcal carriage as determined by quantitative PCR (qPCR) grouped by culture positive and culture negative samples

Discordant results for identification of overall pneumococcus between qPCR and culture were strongly associated with density of pneumococcal carriage (Table 2.6). The majority (47/49; 96%) of qPCR positive but culture-negative samples had estimated copy numbers of $<10^4$ colony forming units (CFU) /ml per swab. Similarly, the majority (7/8; 87.5%) of qPCR negative but culture-positive samples were reported as "scant" (<5 colonies/plate) on culture. As pneumococcal density measured by qPCR increased, so did the detection by culture increase, with a perfect concordance (21/21; *Kappa*=1.0) between culture and qPCR at a carriage density of $>10^6$ CFU/ml.

Table 2.6: Detection of overall pneumococcal carriage by culture, stratified by colonisation density measured by quantitative PCR (qPCR)

Density (CFU/ml)	**Number culture positive/ number in density category by PCR (%)**
Undetected	8/67 (12)
0- 10^2	1/4 (25)
> 10^2-10^3	22/37 (59)
>10^3-10^4	64/81 (79)
>10^4-10^5	85/97 (88)
>10^5-10^6	65/67 (97)
>10^6 -10^7	18/18 (100)
>10^7-10^8	3/3 (100)
Total	266/374 (71)

CFU/ml, colony forming units per millimetre as measured by qPCR. Values are number culture positive/number in density category by qPCR (%).

There was a high concordance *(Kappa* = 0.73) between serotypes/groups identified by culture and those identified by qPCR, including for those specific serotypes which were individually identifiable on qPCR (*kappa* = 0.68); data not shown. The qPCR method, however, identified an additional 150 (57.5%) serotypes/serogroups above those identified by the culture method, including higher detection prevalence by qPCR of serotypes/serogroups 5 (6.61% vs. 0%; $p<0.001$), 9A/L/N/V (10.7% vs. 4%; $p<0.001$), 11A/B/C/D/F (3.5% vs. 1.1%, $p=0.002$), 13 (2.1% vs. 0.3%, $p=0.008$), 14 (7% vs. 4.8%, $p=0.002$), 18A/B/C (4.5% vs. 2.4%, $p=0.011$), 19B/F (18.7% vs. 15.5%, $p=0.033$) and 34/37/17A (2.1% vs. 1.1%, $p=0.05$) (Figure 2.3). Of

these additional serotypes detected by qPCR, 98.8% had densities <10^4 CFU/ml (Table 2.7). Further, 78% (117/150) of the additional serotypes detected by qPCR were co-carried with other pneumococcal serotypes, with only 12.8 % (15/117) of these, being the highest density colonising serotype (Table 2.8).

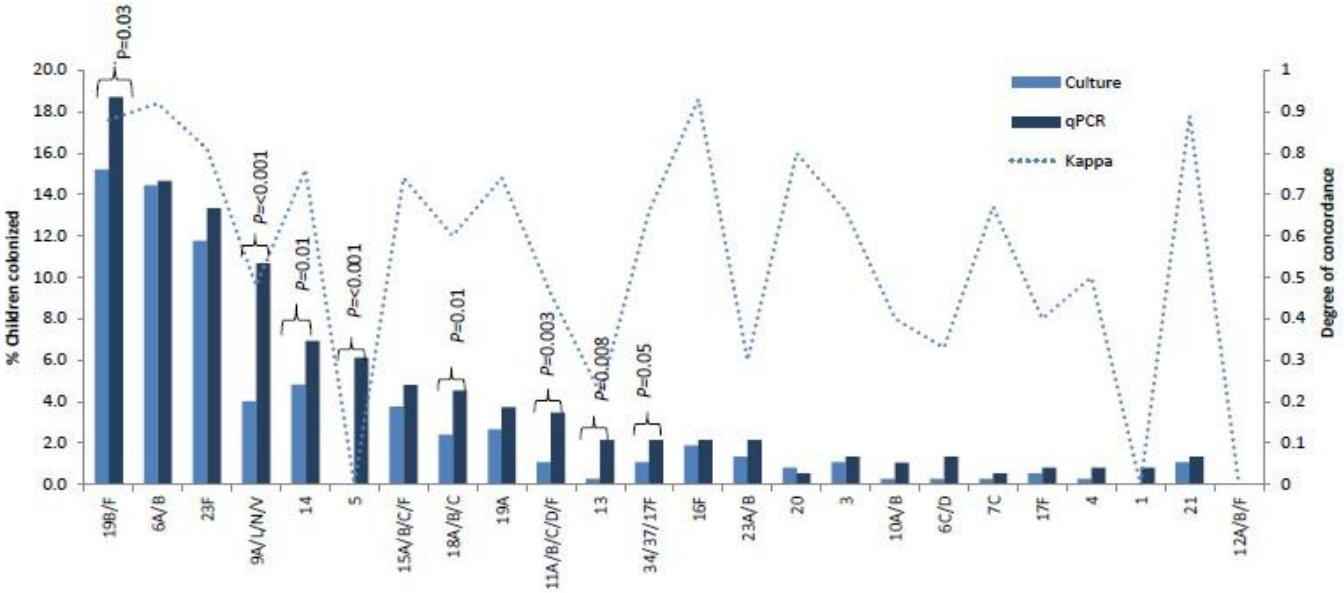


Figure 2.3: Overall prevalence of nasopharyngeal (NP) pneumococcal colonisation as detected by traditional culture and quantitative PCR (qPCR) in a PCV-unvaccinated, HIV-uninfected cohort.

Concordance between traditional culture and quantitative PCR (qPCR) were calculated using McNemar's test with serogroups detected by qPCR included as serotypes. *p*-values of <0.05 were considered significant. Serogroup 6A/B (54): 35.2% Serotype 6A and 64.8% Serotype 6B on culture method. Serogroup 6C/D (1): 100% Serotype 6C on culture method. Serogroup 11/A/B/C/D/F (4): 100% were serotype 11A on culture method. Serogroup 15/A/B/C/F (14): 21%, 64.3%, and 14.2% were serotypes 15A, 15B and 15C on culture method respectively. Serogroup 18A/B/C (9): 100% were serotype 18C on culture method. Serogroup 19B/F (58): 8.6% were serotype 19B and 91.4% were serotype 19F on culture method. Serogroup 23A/B (5): 60% were serotype 23A and 40% were serotype 23B on culture method. Serogroups 34/37/17A (4): 100% were serotype 34 on culture method.

Table 2.7: Detection of pneumococcal serotype carriage by culture, stratified by colonisation density measured by quantitative PCR (qPCR)

Pneumococcal density by qPCR (CFU/ml)	**Number of culture positive/number in density category by qPCR (%)**											
Serotype/group	**1**	**3**	**4**	**5**	**6A/B**	**6C//D**	**7C**	**9A/L/N/V**	**10A**	**11A/B/C/F**	**12A/B/F**	**13**
Undetected	0/371	1/369(0.3)	0/371	0/351	3/319(0.9)	0/369	0/372	1/333(0.3)	0/370	0/361	0/374	0/366
0- 10^2	0	0	0/1	0	0	0	0/1	0/19	0	0/7	0	0
> 10^2-10^3	0/2	0/1	0/1	0/23	7/7(100)	0/2	0	1/8(13)	0	0/2	0	0/5
>10^3-10^4	0	1/1(100)	1/1(100)	0	12/16(100)	0/2	1/1(100)	7/7(100)	0/1	2/2(100)	0	0/1
>10^4-10^5	0/1	1/2(50)	0	0	18/18(100)	0	0	5/6(83)	1/1(100)	2/2(100)	0	0/1
>10^5-10^6	0	1/1(100)	0	0	9/9(100)	0	0	1/1(100)	0/2	0	0	0
>10^6 -10^7	0	0	0	0	5/5(100)	1/1(100)	0	0	0	0	0	1/1(100)
>10^7-10^8	0	0	0	0	0	0	0	0	0	0	0	0
>10^8	0	0	0	0	0	0	0	0	0	0	0	0
Total	0/374	4/374(1.1)	1/374(0.3)	0/374	54/374(14.4)	1/374(0.3)	1/374(0.3)	15/374(0.3)	1/374(0.3)	4/374(1.1)	0/374	1/374(0.3)

Serotype/group	**14**	**15A/B/C/F**	**16F**	**17F**	**18A/B/C**	**19A**	**19B/F**	**20**	**21**	**23A/B**	**23F**	**34/37/17A**
Undetected	1/348(0.9)	2/356(0.6)	0/366	1/371(0.3)	1/357(0.3)	1/360(0.3)	0/304	1/372(0.3)	1/370(0.3)	3/365(0.8)	5/324(1.5)	0/366
0- 10^2	0	0	0	0/1	0/1	0/2	10/12(83.3)	0	0	0	0	1/2(50)
> 10^2-10^3	2/11(18)	1/3(33.3)	3/3(100)	0/1	1/7(14.3)	1/3(33.3)	8/12(66.7)	0	2/2(100)	2/8(25)	17/26(65.4)	1/4(25)
>10^3-10^4	10/10(100)	5/9(55.6)	3/3(100)	1/1(100)	4/5(80)	8/9(88.9)	21/23(91.3)	0	0	0/1	15/16(93.8)	1/1(100)
>10^4-10^5	4/4(100)	6/6(100)	0/1	0	3/4(75)	0	15/18(83.3)	2/2(100)	0	0	7/8(87.5)	1/1(100)
>10^5-10^6	1/1(100)	0	0	0	0	0	3/4(75)	0	2/2(100)	0	0	0
>10^6 -10^7	0	0	0	0	0	0	1/1(100)	0	0	0	0	0
>10^7-10^8	0	0	1/1(100)	0	0	0	0	0	0	0	0	0
>108	0	0	0	0	0	0	0	0	0	0	0	0
Total	18/374(4.8)	14/374(3.7)	7/374(1.9)	2/374(0.5)	9/374(2.4)	10/374(2.7)	58/374(15,5)	3/374(0.8)	5/374(1.3)	5/374(1.3)	44/374(13.4)	4/374(1.1)

Table 2.8: Yield of additional serotypes detected only by quantitative PCR (qPCR) in relation to being co-carriage with other pneumococcal serotypes

Serotype/group	Number of serotypes co-carried/additional serotypes detected by qPCR (%)	Number of dominating colonizing serotype/number of serotypes co-carried (%)*
1	2/3(75)	1/2(50)
3	2/2(100)	2/2(100)
4	2/2(100)	0/2
5	23/23(100)	1/23(4.3)
6A/B	3/4(75)	2/3(66.7)
6C/D	4/4(100)	0/4
7C	0/1	0
9A/L/N/V	19/26(73)	0/19
10A	2/3(75)	1/2(50)
11A/B/C/F	7/9(77.8)	2/7(28.6)
12A/B/F	0	0
13	6/7(85.7)	0/6
14	7/9(77.8)	1/7(14.3)
15A/B/C/F	5/6(83.3)	1/5(25)
16F	0/1	0
17F	1/2(50)	0/1
18A/B/C	7/9(77.8)	1/7(14.3)
19A	3/5(60)	0/3
19BF	12/13(92.3)	2/12(16.7)
20	0	0
21	0	0
23A/B	5/6(83.3)	1/5(20)
23F	6/11(54.5)	0/8
34/37/17A	1/4(25)	0/1
Total	117/150	15/117(12.8)

*Density of pneumococcal carriage was used to determine if the additional serotypes detected only by quantitative PCR (qPCR) were dominant colonising serotypes (Highest density coloniser).

2.2.3. Pneumococcal co-colonisation

Molecular qPCR was more sensitive than culture in detecting pneumococcal serotypes that were co-carried with two and three or more serotypes detected in 19.2% vs. 4.5% ($p<0.001$) and 9.4% vs. 0% ($p<0.001$) of swabs respectively (Table 2.9). A further 14% (43/307) of *Lyt*A positive NP swabs were negative for all the serogroups/serotypes we tested for, implying they were either non-typable, belonged to one of the untested serotypes, or were *Lyt*A positive non-pneumococcal streptococcal species. Due to the strong concordance between serotypes identified by qPCR also being identified by culture; however, we could assume that at least 11/43 (25.6%) of NT serotypes identified by qPCR belonged to one of the serotypes we did not test for (results not shown).

Table 2.9: Pneumococcal co-colonisation as measured by traditional culture and quantitative molecular PCR (*n* = 374)

	Culture	**qPCR**	***p*-value** [a]
Not colonised	108(29)	67(18)	<0.001
Colonised	266(71)	307(82)	
1 serotype	239(99.8)	176(57.3)	<0.001
2 serotypes	12(4.5)	59(19.2)	<0.001
3 + serotypes	0	29(9.4)	<0.001

Values are number (%). For multiple pneumococcal carriages % values are calculated based on total number of samples positive for *LytA*. PCV7, 7-valent pneumococcal conjugate vaccine. [a] as defined by McNemer's test. *p*-value <0.05 were considered significant.

Serotypes/serogroups 6A/B, 19BF, 23F, 5, and 9A/L/N/V were the most common serotypes to be simultaneously co-carried as detected by molecular qPCR (Figure 2.4), with serogroups 6A/B and 19B/F being the most common co-colonisers (prevalence 11/88, 12.5%), followed by serogroup 19B/F and serotype 5 (prevalence 8/88, 9.1%). Serotype/groups 6A/B (prevalence 52/264, 19.7%), 19B/F (prevalence 51/264, 19.3%) and 23F (prevalence 40/264, 15.2%) were the highest density colonisers from samples that tested positive by qPCR (dominant first colonising serotype), with serotype 5 (prevalence 21/88, 23.9%) and serogroup 9A/L/N/V (prevalence 7/29, 24%) being the most common second and third colonisers respectively, as determined by density of carriage from samples that tested positive by qPCR. Further, serogroup 6A/B was most frequently found as a primary isolate (prevalence 42/44; 95.5% occasions) with other co-colonising serotypes, serotypes/group 1, 3, 20, and 34/37/17A were identified as primary isolates only, and serotype 17F and 10A were respectively identified as second and third colonisers only. The serotype specific propensity of whether a given serotype/serogroup is more likely to be found as a primary or non-primary isolate is shown in Figure 2.5, with serotypes/serogroups 6A/B ($p<0.001$), 19B/F ($p<0.001$), 23F ($p<0.001$), 15A/B/C/F ($p<0.001$) and 34/37/17A ($p=0.008$) being more likely to be identified as a primary than a non-primary isolate and serotype 5 being more likely to be identified as a non-primary isolate ($p<0.001$).

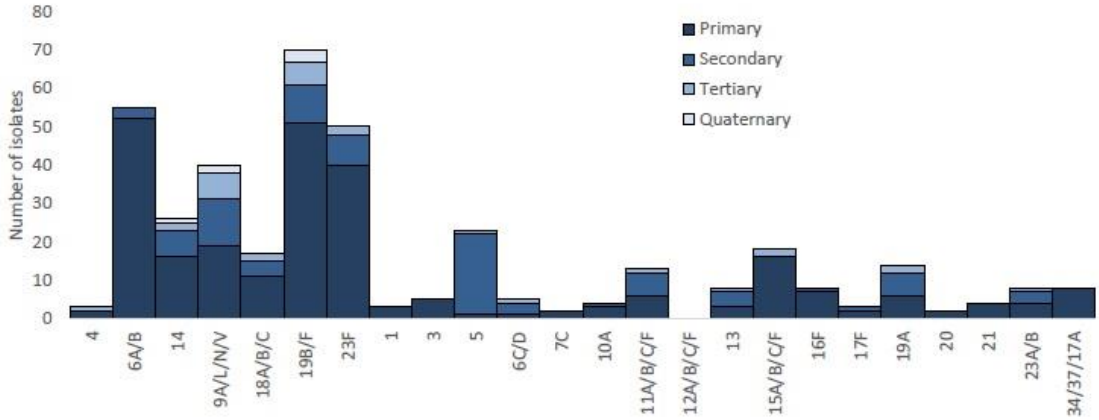


Figure 2.4: Serotype/group specific ranking of multiple pneumococcal carriage

Each isolate of *S. pneumoniae* was ranked according to its carriage density as determined by quantitative PCR (qPCR) to other isolates present in the same sample. Single colonisers were included in the analysis as primary colonising serotypes.

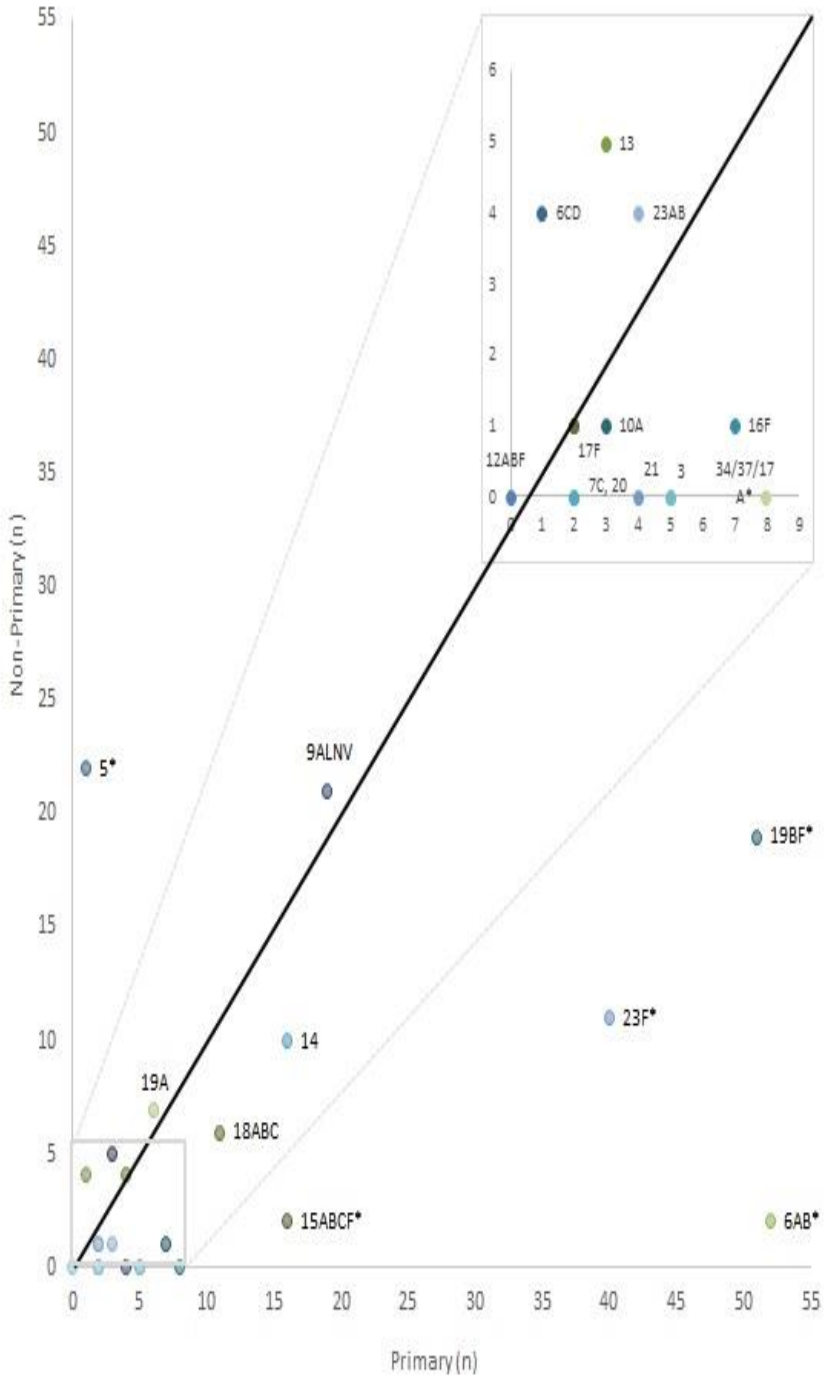


Figure 2.5: Serotype specific propensity of whether a given serotype/serogroup is more likely to be found as a primary or non-primary isolate

Pneumococcal serotypes identified from nasopharyngeal swabs of black South-African children were classified according to whether they occurred as a primary or non-primary isolate as determined by carriage density of qPCR positive samples. Single colonisers were included in the analysis as primary isolates. The box at the top of the graph is an inset of the blocked region at the bottom * Results were considered significant when the *p*-value was < 0.05.

2.3. DISCUSSION

The findings of our study showed that qPCR was more sensitive in detecting concurrent carriage with multiple pneumococcal serotypes, with at least 28.7% of all *LytA* positive nasopharyngeal samples having multiple serogroups/types identified, compared to 4.5% of culture positive cases. Further, we were able to quantify the relative proportion of these serotypes and found the majority of the additional serotypes detected by qPCR to have a lower bacterial load ($<10^4$ CFU/ml) than the dominant colonising serotype, indicating why these less dominant serotypes are likely missed by traditional culture methods that may be suboptimal for detecting low density carriage.

Serotypes/serogroups 6A/B, 19B/F, 23F, 5 and 9A/L/N/V were found as common co-colonisers of the nasopharynx. While serotypes/serogroups 6A/B, 19B/F, 23F and 9A/L/N/V are included among PCV-7 targeted serotypes, serotype 5 is a non-PCV7 serotype, hence immunisation with PCV7 could provide an opportunity for this serotype to occupy the vacant niche and contribute to replacement disease in our setting, as it has also been shown to have high invasive disease potential in South Africa [13,100] . Further, several models have demonstrated a competition between pneumococcal serotypes for both acquisition and persistence of colonisation [58,214,215], with more prevalent and higher colonising density serotypes being less metabolically demanding and thus better able to grow in a nutrient deficient environment. Our findings were in line with those from Dhoubhadel *et al*, in that concurrent colonising serotypes had different bacterial loads, with one serotype dominating over the other(s), which may explain the competition among concurrent colonising serotypes for growth due to limited nutrients and space [43]. Further, few studies have been powered to determine if certain pairs of serotypes occur more frequently together, with most finding no significant combinations above the 5% threshold error [59,215,216]. Our study, however, found serogroups 6A/B and 19B/F to be the most common concurrent colonisers, occurring in 12.5% of co-colonised children, followed by serogroup 19B/F and serotype 5, which were found in 9.1% of co-colonised children.

A limited number of studies have investigated for concurrent colonisation with pneumococcal serotypes in children, with rates varying from 1.5% to 40% according to geographical distribution and method used [33,37,62,139]. Findings from most of these studies, however, were limited in that the majority used non quantitative traditional culture based methods that were

insensitive in detecting multiple carriages, and therefore likely underestimated the true prevalence of this phenomenon [139]. Concurrent carriage of multiple pneumococcal serotypes in the present study was found in 28.7% of all colonised children. This finding was comparable to a recent study by Wyllie and collaborators that also used qPCR, where concurrent colonisation of multiple pneumococcal serotypes was found in 22% of colonised Nepalese children [65]. Other methods have also been used to investigate concurrent colonisation such as conventional PCR [38,39], colony blot [40], RFLP [41] and microarray [42], with the latter recently been shown to have the best performance overall for pneumococcal serotyping [217]. In studies done by Valente *et al* and Kandasamy *et al*, microarray found concurrent colonisation in 20% and 22% of colonised Portuguese and Nepalese children respectively [59,63]. While these results are comparable to our findings, Kamng'ona and colleagues found concurrent colonisation in 40% of Malawian children by microarray, a higher rate compared to our findings [62]. Our qPCR method, however, had the added benefit in that we were able to quantify the relative proportion of concurrent colonising serotypes, it did not rely on an initial culture step which might change the relative proportion of the various strains, and it is less labour intensive and less expensive.

Our study was limited in that the qPCR method did not detect all pneumococcal serotypes and thus co-carriage in samples colonised with these pathogens could have been missed and the rate of concurrent colonisation could have been underestimated. Secondly, our method was not able to discriminate between all serotypes within their respective serogroups; however, due to high concordance between culture and qPCR for the detection of serotypes, the relative proportion of these serotypes could be estimated as similar to the proportion detected by culture. We were also not able to compare non-typables detected by culture and qPCR, as the qPCR method was not optimised to test for all pneumococcal serotypes and thus analysis could be misleading as a proportion of the NT detected by qPCR would rather indicate that we did not have the assay to test for all serotypes, rather than it being true pneumococcal NT. In addition, the loss of sensitivity by qPCR when comparing results to culture was most probably a result of degradation of DNA as a result of long-term storage and/or repeated freezing and thawing of samples. As this was a retrospective study, there was no residual sample after testing available and therefore additional culture of the frozen samples to confirm sample failure was not possible. Further, we were not able to compare the densities of serotypes detected by qPCR in culture negative vs. culture positive swabs as our study was not adequately powered. Lastly a recent study by the PneuCarriage project group found qPCR

to have a large number of false positive results when testing pneumococcal spiked samples [217]. While this is concerning, the majority of false positives were for assays not included in our method. However, serotype 5 was the only serotype to be more commonly isolated as a non-primary than a primary isolate which raises a question of whether this serotype is unable to be cultured or if the qPCR method was giving off false positives for this assay, and thus further investigation is needed which was not possible in this study due to lack of residual sample remaining after molecular testing. However, a Cq cut off <35Cqs was implemented, and both positive and negative controls were used for all assays which should reduce this effect.

In conclusion, molecular detection of pneumococcal serotypes was more sensitive than culture and allowed us to detect multiple serotypes. The ability of qPCR to detect pneumococcus at a low carriage density will allow us to gain a better understanding of serotype carriage and its impact in disease prevalence.

CHAPTER 3: PNEUMOCOCCAL CONJUGATE VACCINE AND NASOPHARYNGEAL PNEUMOCOCCAL COLONISATION IN AFRICAN CHILDREN

The human nasopharynx is colonised with multiple commensal and some potentially pathogenic organisms, including *Streptococcus pneumoniae* [200.] Vaccination of infants with the pneumococcal conjugate vaccine (PCV) reduces their risk of acquisition of vaccine-serotype nasopharyngeal colonisation, whereas there is an increase in detection of non-vaccine serotype colonisation [134]. The basis for the increase in non-vaccine serotype colonisation could involve either replacement colonisation of the nasopharynx because of the vacant ecological niche resulting from PCV-vaccination reducing the nasopharyngeal colonisation acquisition rate of vaccine-serotype, or unmasking of prevailing non-vaccine serotype colonisers which were not previously recognised by traditional culture method that are biased in detecting only the dominant colonising serotype [136]. Molecular detection of pneumococci in the nasopharynx have several advantages over traditional culture based methods, including detection of multiple serotypes from a single sample with high sensitivity, as well as quantitative PCR methods being able to measure the density of colonisation and relative proportion of colonising serotypes as shown in chapter 2.

The aim of this chapter was to evaluate the effect of infant vaccination with 7-valent PCV (PCV7) on the density of serotype-specific nasopharyngeal colonisation to delaminate the relative role of serotypes replacement versus unmasking as the cause for the increase in non-vaccine serotype colonisation in PCV7-vaccinated children.

3.1 MATERIAL AND METHODS

3.1.1. Study population

We retrospectively analysed archived nasopharyngeal swab samples collected from a PCV-unvaccinated and PCV7-vaccinated cohort of HIV-uninfected infants in Soweto, South Africa. The parent study of the PCV7-vaccinated cohort initially enrolled 125 HIV-exposed-uninfected (HEU) and 125 HIV-unexposed infants (6 – 12 weeks old when enrolled) between April 2005 and June 2006 as part of a larger study investigating the effect of HIV-exposure and timing of ART initiation in HIV-infected children on immune response to PCV7 [142,185].

These infants were scheduled to receive three doses of PCV7 (i.e. Prevnar®; Wyeth Vaccines, NJ, USA) at 6, 10 and 14 weeks of age , as well as other scheduled childhood vaccines included in the public immunisation program at the time of study. In addition, a parallel cohort of of 251 PCV7 naïve infants and their mothers were enrolled between January 2007 through to October 2007, including 125 infants born to HIV-infected women but who were HIV-uninfected (HEU); and 126 infants born to HIV uninfected mothers (HIV-unexposed) which the population was fully described in chapter 2, section 2.1.1. [142]. During the course of both studies, PCV immunisation of children in Soweto (birth cohort 28,000 per annum) was limited mainly to study-participants (approximately 600) of the PCV7-vaccinated cohort, as the vaccine was only introduced into the pubic immunisation program in May 2009 [218].

Nasopharyngeal (NP) swabs were taken at several time intervals, including at 9 and 15-16 months of age in both cohorts. Swabs were stored in skim milk-tryptone-glucose-glycerol (STGG) transport media at the Respiratory and Meningeal Pathogen Research Unit (RMPRU) in South Africa, as recommended by WHO [212]. The samples were previously cultured for *S. pneumoniae* using standard culture methods and pneumococcal serotyping undertaken using the Quellung method as described [36.] Serotypes 4, 6B, 9V, 14, 18C, 19F or 23F were categorised as PCV7 vaccine-serotypes and the rest as non-vaccine serotypes.

3.1.2. Multiplex qPCR methods

The details for the multiplex qPCR have been described in chapter 2, section 2.1.2.

3.1.3. Statistical analysis

Statistical analysis was performed with STATA Version 11.0 (Statacorp, Texas, USA). Pearson X^2 test or student t-test was used to compare baseline characteristics between the cohorts. Comparisons and adjusted odd ratios of prevalence of bacterial colonisation between cohorts were analysed using logistic regression models adjusted for covariates, including race, passive smoke exposure, day care attendance, co-trimoxazole usage, and mean age in weeks at sample collection. For density of carriage, geometric mean densities (GMD) and 95% confidence intervals (95% CI) of pneumococcal mean concentrations were calculated following $\log_{10}$ transformation on the data, using analysis of covariance to adjust for possible covariates. Furthermore, the serotype specific propensity of whether a given serotype/serogroup was more likely to be found as a primary or non-primary isolate, was

analysed using a two-tail Binomial test, in which the observed proportion of primary to non-primary isolates were compared. Seorotype/serogroups were determined as being a primary or a non-primary isolate according to their respective carriage density, in which the primary isolate had a higher carriage density than the non-primary isolate(s). Results were considered significant when the *p*-values were <0.05.

3.1.4. Ethical consent

Ethical consent for the initial enrolment of the two cohorts was obtained from the Medical Human Research Ethics Committee (HREC) of the University of Witwatersrand [Vaccinated cohort: HREC: 040704, also registered under Clinical trials number NCT00099658, and the PCV-unvaccinated cohort: HREC: 050705]. Approval for further testing of samples was obtained from the HREC (M120972). Written, informed consent had been obtained from the parents/guardians of all the study participants at the time of initial enrolment.

3.2. RESULTS

Molecular qPCR analysis involved 713 (83%) of the initial 857 nasopharyngeal swabs collected from children, at either 9 or 15-16 months of age (Figure 3.1). Within each cohort, there were no differences in the colonisation patterns done by standard methodology, between those in whom samples were and were not available (Appendices A & B). Among the children in whom samples were available, 92% were African, and 52% were male (Table 3.1). Day care attendance was higher in PCV-vaccinated children at 9 (52% vs. 22.1%; $p<0.001$) and 16 months of age (53.6% vs.37.3%; $p=0.002$), while the mean age (in weeks) at time of sample collection was 40.7 (Standard deviation, SD 2.3) and 69.5 (SD 6) in PCV-unvaccinated children, and 39.3 (SD 3.1; $p<0.001$) and 66.7 (SD 1.8; $p<0.001$) in PCV-vaccinated children at 9 and 16 months of age respectively. Although the differences in age are small and thus unlikely to be epidemiologically or clinically significant in terms of the likelihood of affecting the results, we subsequently adjusted for this as well as the above mentioned differences using multivariate analysis.

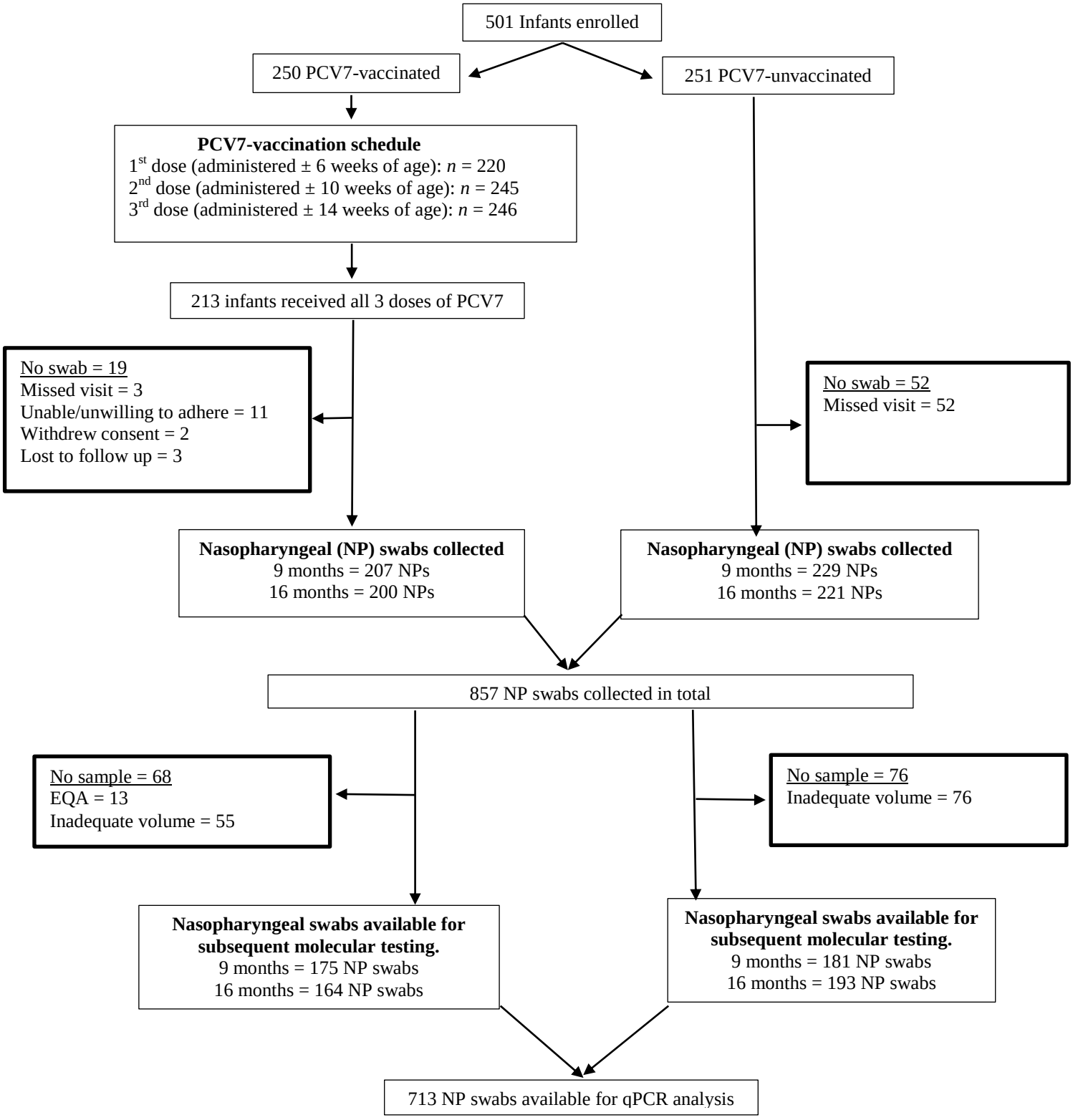


Figure 3.1: Schematic diagram of study population

Diagram indicating the number of children initially enrolled in a PCV-unvaccinated and PCV7-vaccinated cohort of HIV-uninfected children, as well as the number of nasopharyngeal (NP) swabs available for subsequent molecular qPCR analysis. PCV7-vaccinated participants were excluded from analysis if they did not receive all three doses of the pneumococcal conjugate vaccine (PCV) within protocol-defined window periods. The number of NP swabs available for molecular testing was defined by whether there was an adequate volume of sample remaining. EQA, samples were used for external quality assessment.

Table 3.1: Demographic features at two study visits when analysed for nasopharyngeal (NP) bacterial colonisation in PCV7-vaccinated and PCV-unvaccinated, HIV-uninfected children

	9 month old children			16 month old children		
	PCV-unvaccinated	**PCV7-vaccinated**	***p* -value**	**PCV-unvaccinated**	**PCV7-vaccinated**	***p* -value**
Number of children enrolled	250	251	-	250	251	-
Available NP swabs for molecular analysis	181	175	-	193	164	-
HIV status: Exposed (%)	111 (61.3)	104 (59.4)	0.40	99 (51.3)	97 (59.1)	0.24
: Unexposed (%)	70 (38.7)	71 (40.6)		94 (48.7)	67 (40.9)	
Mean birth weight (SD) grams	3061 (440)	3097 (482)	0.45	3079 (427)	3126 (476)	0.43
Male sex (%)	87 (48.1)	96 (54.9)	0.12	99 (51.3)	89 (54.3)	0.25
Race: Black African (%)	181 (100)	150 (85.7)	<0.001	193 (100)	132 (80.5)	<0.001
: Mixed ancestry (%)	-	25 (14.3)		-	32 (19.5)	
Smoking household contact (%)	66 (36.5)	81 (42.3)	0.003	65 (33.68)	64(39)	0.004
Co-trimoxazole prophylaxis (%)	95 (52.5)	81 (46.3)	0.2	71(36.8)	68(41.5)	0.15
Mean numbers of children (SD) < 5 years	1.6 (0.8)	1.6 (0.7)	0.6	1.6 (0.82)	1.5 (0.84)	0.21
Mean number of household contacts (SD)	5.3 (2.2)	5.2 (2.3)	0.81	5.2 (2.07)	5.1 (2.36)	0.17
Day care attendance (%)	40 (22.1)	91 (52)	<0.001	72 (37.31)	81 (53.64)	0.002
Breast feeding (%)	41 (22.7)	44 (25.1)	0.33	37 (19.2)	38 (23.3)	0.43
Mean age (SD) in weeks at visit	40.7 (2.3)	39.3 (3.1)	<0.001	69.5 (6.01)	66.7(1.6)	<0.001

Pearson X^2 test or student t-test was used to compare baseline characteristics between the cohorts and demographic features with a *p*-value <0.2 were included as possible cofounders in multivariate analysis. PCV7, Seven-valent pneumococcal conjugate vaccine. NP, nasopharyngeal. SD, standard deviation.

3.2.1. Pneumococcal co-carriage with respect to PCV7-vaccination status

Molecular qPCR detected one, two and three or more serotypes in 42.8%, 16.5% and 5.3% NP swabs respectively. The remaining 13.2% NP swabs were *LytA* positive, but negative for all the serotypes that were tested, implying they were either non-typable, belonged to untested serotypes, or were *LytA* positive non-pneumococcal streptococcal species. Failure to detect qPCR-typable pneumococci at 9 months of age was more common in PCV7-vaccinated (28.9%) than PCV-unvaccinated children (17.1%; p=0.01; Table 3.2); however, by 16 months of age, this difference was only of borderline significance (25% vs. 18.7 %; p=0.06). Further, PCV-unvaccinated children were more likely to have only a single serotype identified compared to PCV7-vaccinated children at both 9 (64.6% vs. 59.2%; p=0.025) and 16 months (50.3% vs. 44.7%; p=0.019) of age respectively.

Amongst co-carriers, concurrent colonisation by PCV7-serotypes and non-vaccine serotypes was lower among PCV7-vaccinated than PCV-unvaccinated children at both 9 (62.1% vs. 66.7%, p=0.006) and 16 (55.3% vs. 63.2%, p=0.004) months of age. Concurrent carriage of multiple PCV7-serotypes only was also lower in PCV7-vaccinated compared to PCV-unvaccinated children at 9 (13.8% vs. 30.7%; p=0.002) and 16 (2.6% vs. 22.4%; p=0.005) months of age. In contrast, concurrent carriage of multiple non-vaccine serotypes was higher in PCV7-vaccinated compared to PCV-unvaccinated children at 9 (24.1% vs. 2.6%, p=0.015) and 16 (42% vs. 14.3%, p=0.002) months of age.

Table 3.2: Co-colonisation by vaccine-types and non-vaccine type pneumococcus in HIV-uninfected children as measured by quantitative molecular PCR

	9 month old children			16 month old children		
	PCV7-unvaccinated (n=181)	PCV7-vaccinated (n=175)	p-value	PCV7-unvaccinated (n=193)	PCV7-vaccinated (n=164)	p-value
Not colonised	31(17.1)	50(28.9)	0.01	36(18.7)	41(25)	0.06
Colonised	150(82.9)	125(71.4)		157(81.3)	123(75)	
Not typable[a]	14(9.3)	22(17.6)	0.09	29(18.5)	29(23.4)	0.88
Single carriers	97(64.6)	74(59.2)	0.025	79(50.3)	55(44.7)	0.019
Multiple carriers	39(26)	29(23.2)	0.87	49(31.2)	39(31.7)	0.63
+ 1 VT	12(30.7)	4(13.8)	0.002	11(22.4)	1(2.6)	0.005
VT and NVT	26(66.7)	18(62.1)	0.006	31(63.2)	21(55.3)	0.004
+1 NVT	1(2.6)	7(24.1)	0.015	7(14.3)	16(42)	0.002

p -values were determined by multivariate logistic regression, controlling for race, smoking household contact, co-trimoxazole use, day care attendance and mean age at time of sample collection. Values are number (%).

PCV7, 7-valent pneumococcal conjugate vaccine. [a] Non-typables (NT), *LytA* positive, serotype negative samples. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7C, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 23A/B, and 34/37/17A). *p*-value <0.05 considered significant.

Among PCV-unvaccinated children, PCV7-serogroups 6A/B (prevalence: 52/264, 19.7%), 19B/F (prevalence: 51/264, 19.3%), and PCV7-serotype 23F (prevalence: 40/265, 15.2%) were the highest density colonising serotypes. Further, non-vaccine serotype 5was the most prevalent second colonising serotype ranked by having a lower density than the primary colonising serotype, but a higher density than the tertiary colonising serotype. Similarly, PCV7-serogroup 9A/L/N/V was the most prevalent tertiary colonising serotype/serogroup ranked by having a lower density than both the primary and secondary colonising serotypes (Figure 3.2).

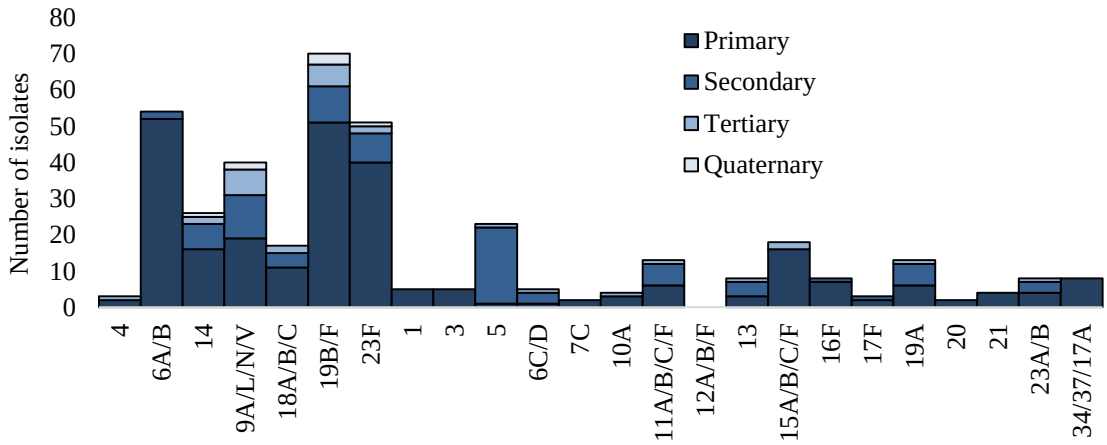


Figure 3.2: Serotype/group specific ranking of multiple pneumococcal carriage in PCV-unvaccinated children

Each isolate of *S. pneumoniae* was ranked according to its carriage density as determined by quantitative PCR (qPCR) to other isolates present in the same sample. Single colonisers were included in the analysis as primary colonising serotypes.

Among PCV7-vaccinated children, PCV7-serogroups 19B/F (prevalence: 43/197, 14.7%), 6A/B (prevalence: 29/197, 14.7%) and non-vaccine serotype 19A (prevalence: 29/197, 14.7%) were the highest density colonising serotypes and non-vaccine serotype 5 being the most common 2nd (prevalence: 11/68, 16.2%) and 3rd (prevalence: 4/9, 44.4%) colonising serotype based on its density of carriage (Figure 3.3).

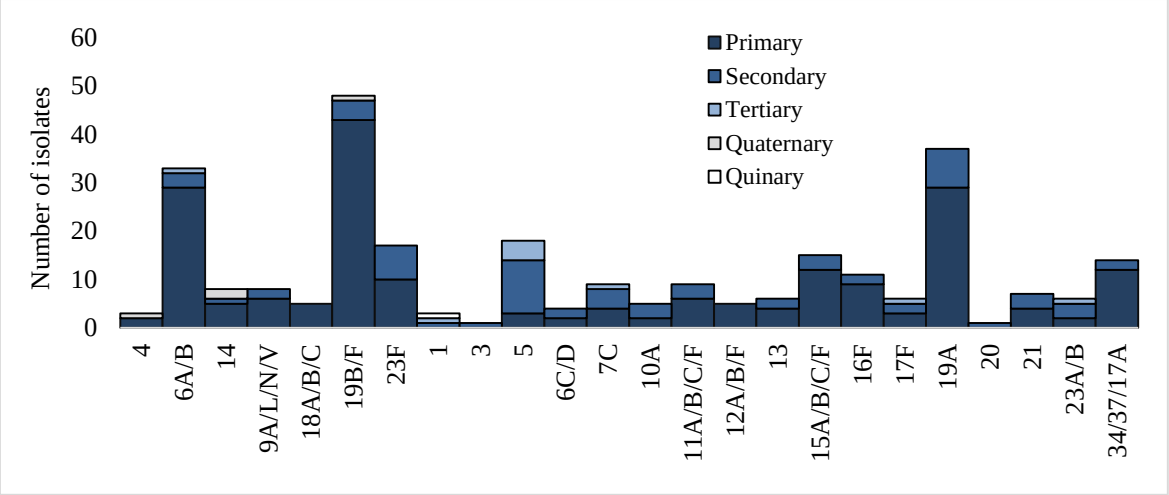


Figure 3.3: Serotype/group specific ranking of multiple pneumococcal carriage in PCV7-vaccinated children

Each isolate of *S. pneumoniae* was ranked according to its carriage density as determined by quantitative PCR (qPCR) to other isolates present in the same sample. Single colonisers were included in the analysis as primary colonising serotypes.

Further, the serotype specific propensity of whether a given serotype/serogroup is more likely to be found as a primary or non-primary isolate is shown in Figure 3.4, with vaccine serotypes/serogroups 6A/B ($p<0.001$ and $p<0.001$), 19B/F ($p<0.001$ and $p<0.001$) and 23F ($p<0.001$ and $p<0.001$) and non-vaccine serotypes/groups 15A/B/C/F ($p=0.035$ and $p<0.001$) and 34/37/17A ($p=0.035$ and $p=0.008$) being more likely to be identified as a primary than non-primary isolate in both PCV-vaccinated and PCV-unvaccinated groups. Non-vaccine serotype 19A was, however, more likely to be identified as a primary (78.4%) than non-primary isolate in PCV-vaccinated children alone (21.6%; $p<0.001$). Further, non-vaccine serotype 5 was more likely to be identified as a non-primary isolate and in both PCV-vaccinated ($p<0.008$) and PCV–unvaccinated groups ($p<0.001$). These results did not differ between time points.

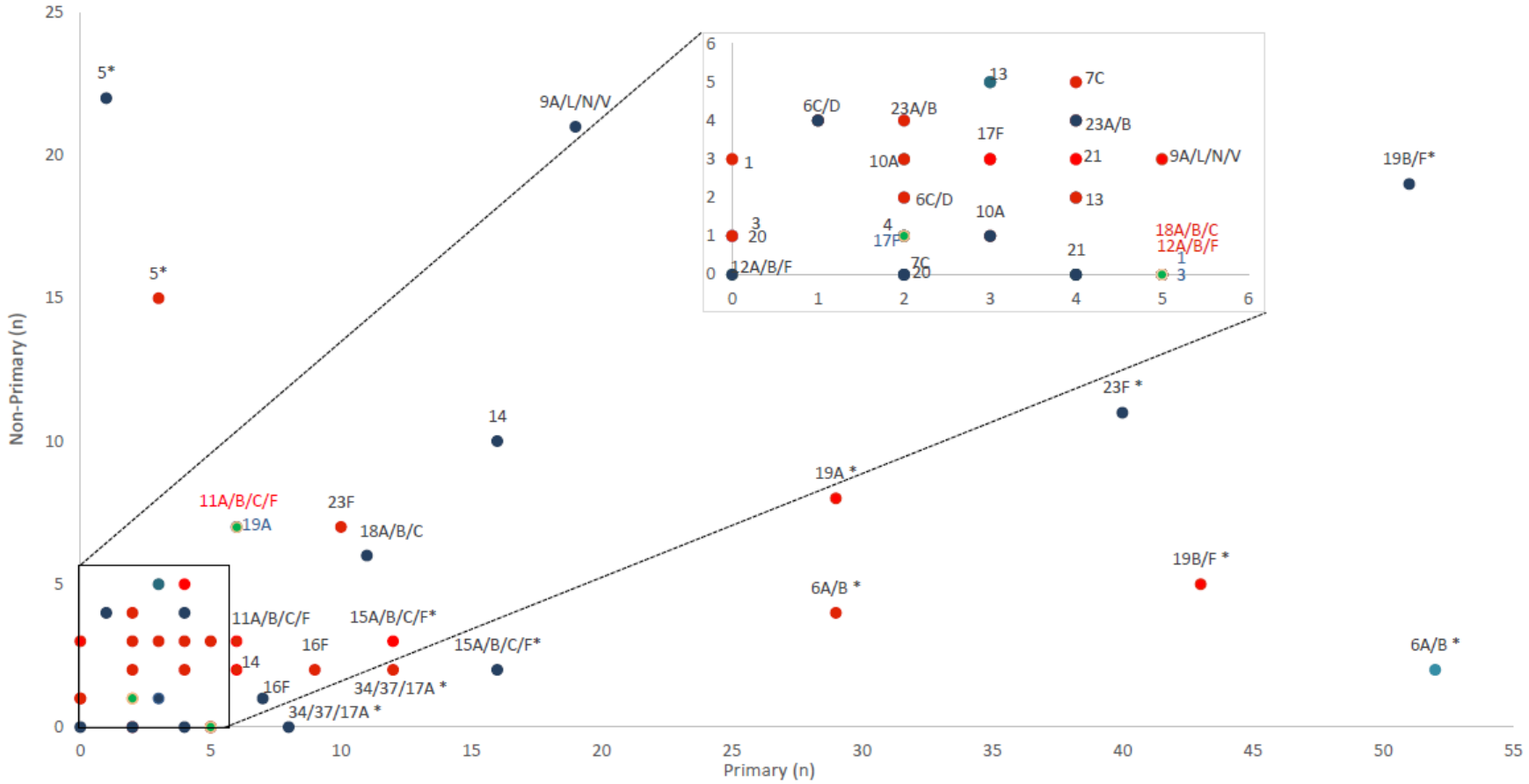


Figure 3.4:Serotype specific propensity of whether a given serotype/serogroup is more likely to be found as a primary or non-primary isolate in PCV7-vaccinated (red) and PCV-unvaccinated (blue) children

Pneumococcal serotypes identified from nasopharyngeal swabs were classified according to whether they occurred as a primary or non-primary isolate as determined by carriage density. Single colonisers were included in the analysis as primary isolates. The box at the top box at top of the graph is an inset of the blocked region at the bottom. Green dots represent two or more serotypes with the same colonising profile. * Results were considered significant when the *p*-value was <0.05.

The average densities of the 1st colonising serotype (3.59 vs. 3.99 CFU/ml, p=0.01), 2nd colonising serotype (3.9 vs. 4.38 CFU/ml, p=0.05) and 3rd colonising serotype (1.7 vs. 2.35 CFU/ml, p=0.005) were higher in PCV7-vaccinated compared to PCV-unvaccinated children at 9 months of age; however, no difference in densities were found by 16 months of age (Figure 3.5).

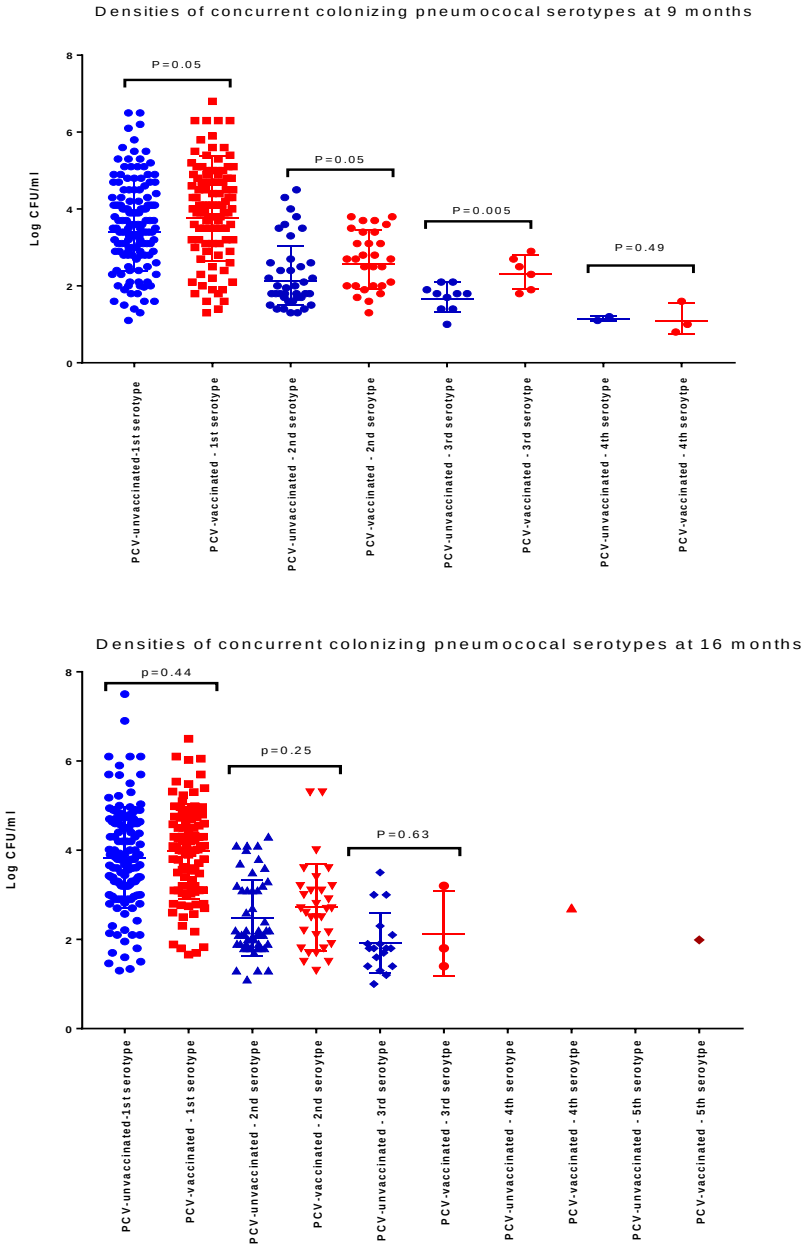


Figure 3.5: Density of concurrent colonising pneumococcal serotypes between PCV7-vaccinated and PCV-unvaccinated children, grouped by 1st, 2nd, 3rd and 4th colonising serotype as determined by density of carriage

3.2.2. Prevalence of NP bacteria as determined by qPCR with respect to PCV vaccination status

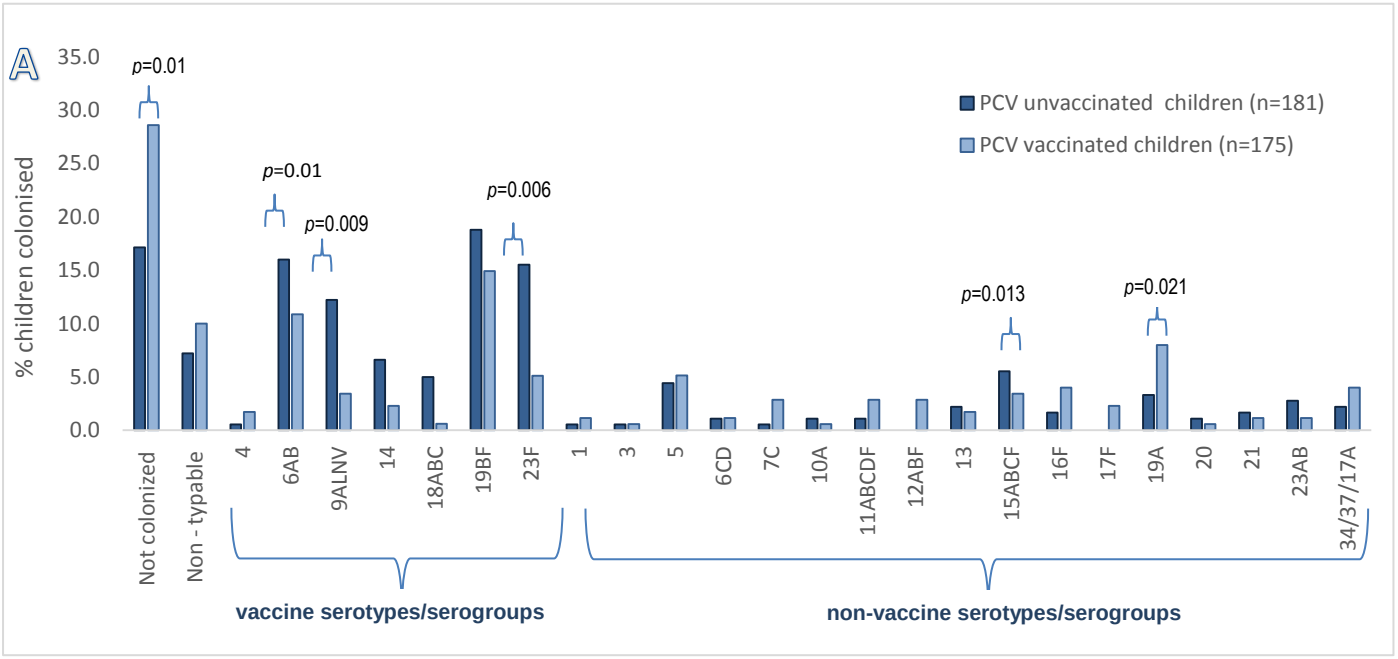
Overall prevalence of pneumococcal colonisation was lower in PCV7-vaccinated (71.4%) than PCV-unvaccinated children (82.9%; p=0.01; Table 3.3) at 9 months of age. The reduction in carriage was due to a lower prevalence of PCV7-serotype colonisation in PCV7-vaccinated (36%) than PCV-unvaccinated children (61.9% p=0.002), with PCV7-serogroup 9A/L/N/V (72.1% reduction, p=0.008) and 23F (67.1% reduction; p=0.006) showing the greatest decrease compared to prevalence in PCV-unvaccinated children; Figure 3.6a. A corresponding increase in prevalence of non-vaccine serotype colonisation in PCV7-vaccinated (40%) compared to PCV-unvaccinated children (33.7%; p=0.02) was evident at 9 months of age; which was driven largely by an increase in prevalence of a limited number of serotypes including serogroup 12A/B/F (100% increase; p=0.013) and 19A (53.4% increase; p=0.021).

By 16 months of age, there was no difference in overall pneumococcal colonisation between PCV7-vaccinated (75%) and PCV-unvaccinated children (81%; p= 0.06). The lower prevalence of PCV7-serotype colonisation in PCV7-vaccinated (32.9%) than PCV-unvaccinated children (51.8%; p=0.007), was largely offset by the increase in non-vaccine serotype colonisation among the PCV7-vaccinated children (62.2% vs. 37.8%; p=0.013); Figure 3.6b). PCV7-serogroup 9A/L/N/V (87.8% reduction, p<0.001) and vaccine-serotypes 14 (67.1% reduction, p=0.05) and 23F (57% reduction, p=0.02) showed the greatest decrease, while NVT serotypes 19A (70.7% increase p<0.001) and 21 (83.3% increase, p=0.04) showing the greatest increase.

Table 3.3: Prevalence of pneumococcal nasopharyngeal (NP) colonisation in PCV7-vaccinated and PCV-unvaccinated, HIV-uninfected children as measured by molecular qPCR

	9 month old children				16 month old children			
	PCV7-unvaccinated (n = 181)	PCV7-vaccinated (n = 175)	OR (95% CI), p -value*	aOR (95% CI), p -value*	PCV7-unvaccinated (n = 193)	PCV7-vaccinated (n = 164)	OR (95% CI), p -value*	aOR (95% CI), p -value*
LytA	150 (82.9)	125 (71.4)	0.52 (0.31-0.86), p = 0.01	0.45 (0.23 - 0.87), p = 0.01	157 (81)	123 (75)	0.68 (0.41-1.14), p = 0.147	0.55 (0.29-1.03), p =0.06
VT serotypes	112 (61.9)	63 (36)	0.35 (0.21- 0.58), p <0.001	0.37 (0.19 - 0.7), p = 0.002	100(51.8)	54(32.9)	0.44 (0.27-7.01), p <0.001	0.41(0.26-0.63), p = 0.007
NVT serotypes	54(33.7)	70(40)	1.74 (1.08 - 2.82), p = 0.002	1.88 (1.02 - 3.48), p = 0.02	73(37.8)	102 (62.2)	1.81 (1.12-2.93), p <0.001	2.2 (1.18-4.1), p = 0.01

Adjusted odd ratios (aOR) for pneumococcal colonisation were determined by multivariate logistic regression, controlling for race, smoking household contact, co-trimoxazole use, day care attendance and mean age at time of sample collection, by use of generalised estimating equations. PCV7, seven-valent pneumococcal conjugate vaccine. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 14, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7C, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 23AB, 34/37/17A). *p-values of <0.05 were considered significant.



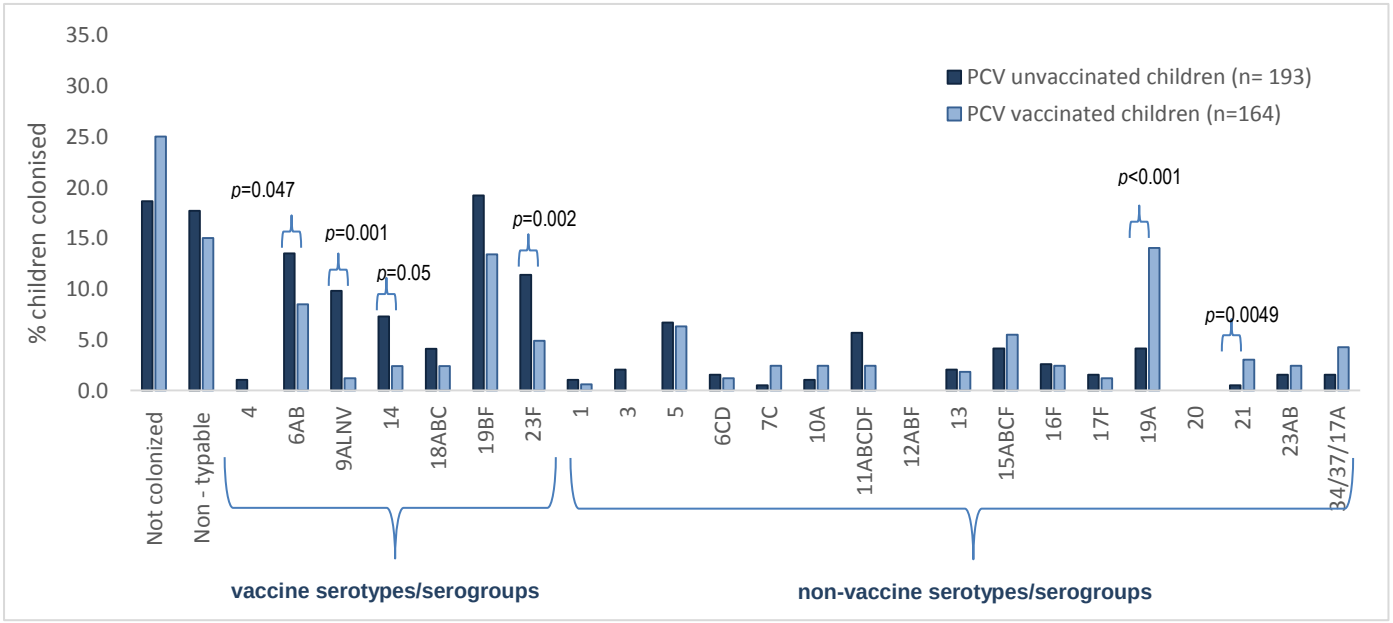


Figure 3.6: Prevalence of nasopharyngeal (NP) pneumococcal colonisation in PCV7-vaccinated and PCV-unvaccinated, HIV-uninfected children at a) 9 months and b) 16 months of age

p-values were determined by multivariate logistic regression, controlling for race, smoking household contact, co-trimoxazole use, day care attendance and mean age at time of sample collection, by use of generalised estimating equations. PCV7, seven-valent pneumococcal conjugate vaccine. *p*-values of <0.05 were considered significant.

3.2.3. Association of PCV vaccination and density of pneumococcal nasopharyngeal carriage

The density of pneumococcal carriage was higher in PCV7-vaccinated (4.68; 95% CI; 4.45-4.9 CFU/ml) than PCV-unvaccinated infants (4.28; 95% CI; 4.09-4.47 CFU/ml) at 9 months, of age (p=0.007); Table 3.4. This was associated with an increase in the density of overall vaccine serotypes/serogroup (3.8 vs. 3.4 CFU/ml; p=0.048), despite lower prevalence thereof; and an increase in overall density of non-vaccine serotypes/serogroups (3.6 vs. 3.1 CFU/ml; p=0.018). No difference in density of individual PCV7-serotypes/serogroups was found between PCV-unvaccinated and PCV7-vaccinated groups; however, the density of non-vaccine serotype 19A was higher in PCV7-vaccinated (3.76 vs. 2.83 CFU/ml; p=0.0463) compared to PCV-unvaccinated children, which was consistent with a higher carriage prevalence in PCV7-vaccinated children. Further, the density of non-vaccine serogroup 23A/B was also found to be higher in PCV7-vaccinated (4.48 vs. 2.07 CFU/ml; p=0.002) compared to PCV-unvaccinated children, despite no change in carriage prevalence. By 16 months of age, there was no overall difference in density of pneumococcal colonisation between the PCV7-vaccinated and PCV7-unvaccinated groups, although there was an increase in density of non-vaccine serotypes/groups in PCV7-vaccinated compared to PCV-unvaccianted children, including serotype/groups 5 (2.79 vs. 2.01 CFU/ml; p=0.015), 19A (4.15 vs. 3.04 CFU/ml; p=0.013), and 23A/B (3.59 vs. 1.58 CFU/ml, p=0.013); in addition to the higher prevalence of 19A alone.

Table 3.4: Density of nasopharyngeal carriage in PCV7-vaccinated and PCV7-unvaccinated, HIV-uninfected children as measured by quantitative PCR (qPCR)

	9 month old children			16 month old children		
	PCV7-unvaccinated (*n* = 181)	PCV7-vaccinated (*n* = 175)	*p*-value*	PCV7-unvaccinated (*n* = 193)	PCV7-vaccinated (*n* = 164)	*p*-value*
	GMD (95% CI)			GMD (95% CI)		
Pneumococcus (LytA)	4.28 (4.09-4.47)	4.68 (4.45-4.90)	0.007	4.33 (4.16-4.5)	4.44 (4.23-4.66)	0.53
Vaccine serotypes/groups	3.4 (3.2-3.6)	3.8 (3.5-4.1)	0.048	3.59 (3.36-3.83)	3.73 (3.4-4.0)	0.48
4	3.8[a]	2.18 (-2.1-6.47)		4.53[a]	-	
6A/B	4.41(4.0-4.82)	4.41 (3.9-4.92)	0.99	4.17 (3.66-4.68)	4.0 (3.44-4.58)	0.68
9A/L/N/V	2.52(1.96-3.08)	2.84 (1.48-4.19)	0.52	2.36 (1.77-2.96)	3.99 (0.81-6.04)	0.09
14	2.46(1.7-3.22)	3.66 (-0.68-6.65)	0.15	3.29 (2.68-3.91)	2.75 (1.29-4.20)	0.37
18A/B/C	2.80(2.26-3.49)	1.96[a]	0.39	3.58 (2.51-4.65)	4.30 (3.36-5.25)	0.32
19B/F	3.36(2.99-3.74)	3.79 (3.28-4.3)	0.16	3.52(3.12-3.96)	3.65 (3.12-4.17)	0.71
23F	2.9 (2.59-3.4)	3.76 (2.24-5.28)	0.84	3.04 (2.67-3.4)	3.44 (2.45-4.44)	0.71
Non - vaccine serotypes/groups	3.1 (2.7-3.4)	3.6 (3.0-3.4)	0.018	3.33 (3.0-3.66)	3.74 (3.42-4.06)	0.08
1	2.31[a]	2.29 (-1.03-5.61)		3.47 (-11.4- 18.31)	1.99[a]	
3	5.13[a]	3.55[a]		-	-	
5	2.0 (1.63-2.36)	2.29 (1.85-2.7)	0.23	2.01 (1.83-2.19)	2.79 (1.53-4.04)	0.015
6C/D	4.2 (-25.5-33.94)	2.76 (1.71-3.81)	0.60	2.99 (2.32-3.67)	3.99 (-12.12-20.1)	0.38
7C	3.25[a]	3.13 (0.5-5.76)		1.57 [a]	2.66 (1.51-3.79)	
10B	4.15 (-10.47-18.78)	3.09[a]		4.65 (4.0-5.3)	3.26 (2.15-4.36)	0.06
11A/B/C/D/F	1.79 (-2.66-6.24)	2.67 (1.32-4.0)	0.34	2.88 (1.9-3.87)	4.06 (0.82-7.3)	0.23
12A/B/F	2.44[a]	3.38(1.39-5.38)		-	-	
13	2.99 (0.22-6.01)	4.7 (2.86-6.54)	0.21	2.84 (1.08-4.61)	3.21 (1.38-5.04)	0.65
15A/B/C/F	3.66(3.11-4.2)	4.1 (3.4-4.79)	0.27	3.88 (3.23-4.53)	4.03 (3.27-4.79)	0.75
16F	3.26 (2.24-4.28)	3.65 (2.4-4.89)	0.65	4.84 (2.67-7.01)	5.07 (2.77-7.37)	0.84
17F	-	-		3.10 (-1.31-7.5)	2.48 (-12.1-17.05)	0.72
19A	2.83 (2.26-3.7)	3.76 (3.36-4.1)	0.046	3.04 (2.26-3.83)	4.15 (3.66-4.64)	0.013
20	4.20(2.33-6.07)	2.01[a]		-	-	
21	3.62 (-0.65-7.89)	3.32 (-13.7-20.36)	0.87	5.22[a]	2.82 (1.82-3.8)	
23A/B	2.07(1.48-2.67)	4.48 (0.82-8.14)	0.002	1.58 (1.28-1.87)	3.59 (0.88-6.32)	0.013
34/37/17A	2.83 (1.33-4.34)	3.76 (2.24-5.28)	0.84	4.34 (0.50-8.17)	4.67 (3.7-5.61)	0.71

For density of carriage geometric mean densities (GMD) and 95% confidence intervals (95% CI) of pneumococcal and bacterial mean concentrations were calculated following $\log_{10}$ transformations on data and compared with multivariate analysis controlling for race, smoking household contact, co-trimoxazole use, day care attendance and mean age at time of sample collection. PCV7, seven-valent pneumococcal conjugate vaccine. CI, confidence intervals.-, too few observations to calculate *p*-value.* *p*-values of <0.05 were considered significant.

3.3. DISCUSSION

In this study quantitative molecular qPCR was used to compare pneumococcal serotype/group specific colonisation in cohorts of PCV7-vaccinated and PCV-unvaccinated African children. Analysis clearly showed the vaccine effect, typified by a decrease in the prevalence of PCV7-serotypes and a corresponding increase in NVT serotypes [149,219,220]. This study, however, was also the first to show serotype replacement and unmasking as the cause for this increase in non-vaccine serotype colonisation in PCV7-vaccinated children.

Although serotype specific analysis was often limited by a small sample size for a given serotype, the carriage prevalence of the majority of NVT serotypes remained unchanged following PCV7-vaccination, with the exception of serotype 19A, for which prevalence of colonisation increased. Although this increase in serotype 19A has been previously documented in PCV7-vaccinated populations [141,219,221,222], the qPCR method also allowed quantification and showed an increase in density of this serotype, as well as this serotype being more commonly reported as a primary isolate in PCV7-vaccinated children, whilst being equally a primary (46.2%) or non-primary (53.8%) isolate in PCV-unvaccinated children. This would indicate that the increase in serotype 19A among PCV7-vaccinated children was due to a combination of replacement disease as well as unmasking of colonisation which would not have been identified using conventional culture methods. In addition, the increase in the carriage prevalence and density of PCV7-serotype 19A is of important clinical relevance as it could explain the emergence of serotype 19A as the major replacement serotype causing IPD following PCV7 introduction in several settings [223-225].

An increase in the colonisation density of non-vaccine serotype 5 and serogroup 23A/B was identified in PCV7-vaccinated compared to PCV-unvaccinated children, in the absence of an increase in overall colonisation prevalence. As serotype 5 is rarely seen in conventional carriage studies, these results suggest an unmasking of serotype 5 by the qPCR method. Nonetheless, serotype 5 has a high invasive disease potential in our setting [13,100] and an increase in the carriage density as a result of PCV-immunisation could enhance its ability to cause mucosal and invasive disease and potentially offset some of the effectiveness of PCV7 immunisation.

Further, a composition shift in co-colonised children from a mix of NVT's and VT's to almost pure NVTs as a result of PCV7 immunisation was observed, supporting an unmasking of serotype carriage; however, co-colonised children also had a lower density of colonisation with NVTs then singly colonised hosts, therefore further supporting that both an increase in detection probability (unmaking) and an increase in NVT acquisition (true replacement) played a role in the vaccine effect [134].

Of note among PCV7-vaccinated children, despite a lower prevalence of PCV7-serotytpe colonisation at 9 months of age, the density of colonisation of both PCV7-serotype and NVT pneumococci was higher in them compared to PCV-unvaccinated children. Although we might expect PCV vaccination to reduce the density of VT carriage, the opposite was found. One possible explanation for this observation is that pneumococcal carriage density may be influenced by the prevalence of antibodies to common pneumococcal surface antigens (CPAs). Ditse *et al*, found lower titers to CPAs in PCV7-vaccinated infants at 10 months, most likely a result of reduced pneumococcal exposure, although by 18 months these differences were not significant, which was consistent with colonisation density in our study [226]. Another explanation, however, could simply be that the vaccine is less effective in preventing colonisation with PCV7-serotypes among a subset of children who remained colonised, possibly due to a poorer immune response or other factors contributing to the colonisation by these PCV7-serotypes.

Our findings were similar to Ercibengoa *et al*, in that we detected multiple co–carriages in 21.9% of *LytA* positive swabs [227], much higher compared with other studies that relied mostly on culture based methods (1.3% - 12%) [37,50,57,60]. Despite the increase in NVT carriage as a result of the vaccine effect, the prevalence of concurrent pneumococcal carriage remained unchanged; however, the composition of multiple pneumococcal serotypes shifted from serotypes/groups 6A/B, 19B/F and 23F to serotypes/groups 6A/B, 19A and 19B/F being the dominant colonising serotype as a result of PCV vaccination. Serotype 5 remained the most common second coloniser in both groups.

Our study was limited in that a) the PCR method was not optimised to discriminate between all the VT serotypes within their respective serogroups. However, due to the high

concordance between serotypes identified by culture and qPCR, we can assume from colonisation data using traditional culture methods that 93.3%, 88.9% and 91.4% of all serogroups 9A/L/N/V, 18A/B/C and 19B/F identified by qPCR to be vaccine-serotypes 9V, 18C and 19F respectively. b) We had limited serotype coverage in the assay and thus the effect of other serotypes on the vaccine efficacy for protection against colonisation might be underestimated. c) Further, we were not able to identify non-typable pneumococcus which may have limited our understanding of the vaccine effect, as recent genome sequencing projects have shown an increase in non-typable isolates following PCV immunisation [228]. d) The study was not adequately powered to evaluate all serotype specific differences as the sample size of detected minor serotypes was too small. e) Our control group was enrolled after our vaccinated group. However, due to the limited coverage of PCV in the community it is thus unlikely that a broader indirect effect of the vaccine reducing transmission by young vaccinated children would have affected the nasopharyngeal colonisation patterns. f) Lastly, our study was not a randomised clinical trial and the cohorts were not matched and coincidental temporal fluctuations due to difference in year when the cohorts were enrolled and other unknown factors could have thus influenced the results, although this is unlikely.

In conclusion, the ability of molecular qPCR to detect multiple concurrent colonising serotypes at a low carriage density allowed us to gain a better understanding of serotype carriage and indicates that the underlying mechanism for the increase in non-vaccine serotype colonisation in PCV vaccinated children is likely due to both serotype replacement as well as unmasking of underlying pre-existing colonising serotypes.

CHAPTER 4: EVALUATION OF THE ASSOCIATION OF PNEUMOCOCCAL CONJUGATE VACCINE IMMUNISATION AND DENSITY OF NASOPHARYNGEAL BACTERIAL COLONISATION USING A MULTIPLEX QUANTITATIVE POLYMERASE CHAIN REACTION ASSAY

Invasive and mucosal disease due to bacterial pathogens such as *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus, Streptococcus pyogenes* and *Neisseria meningitidis* follow on colonisation of the pharynx by these bacteria. Further, bacterial and host factors may contribute to interaction in the nasopharyngeal colonisation patterns by these bacteria [72,229]. Vaccination with pneumococcal conjugate vaccine (PCV) reduces the risk of nasopharyngeal colonisation acquisition by vaccine-serotypes; however, it increases colonisation by non-vaccine serotypes [134]. This increase in non-vaccine serotype colonisation could be due to increased risk of acquisition or "unmasking" of previous low-level nasopharyngeal colonisation by these serotypes [136]. Although the majority of these "replacing" non-vaccine serotypes have low invasive potential, they could still cause mucosal disease such as acute otitis media (AOM) [230] The PCV induced effect on pneumococcal nasopharyngeal colonisation could also affect colonisation patterns of other bacteria, including *S. aureus* which has an inverse association with *S. pneumoniae* colonisation and possibly particularly seven-valent PCV (PCV7) serotypes [27,80]; and *H. influenzae* which is positively associated with pneumococcal colonisation [83,175,231].

Since bacterial nasopharyngeal colonisation is the precursor to disease of the upper and lower respiratory tract, analysis of nasopharyngeal colonisation in PCV-vaccinated and PCV-unvaccinated children could provide a measure for predicting whether there might be changes in susceptibility to mucosal and systemic bacterial infections in these children as a result of PCV immunisation [229]. The majority of studies on nasopharyngeal colonisation have focused on non-quantitative culture based methods as recommended by a WHO working group [36]. This could underestimate the prevalence of pneumococcal colonisation and does not allow for quantitative evaluation of bacterial colonisation or the effect of PCV thereon, which may be associated with susceptibility for disease by these bacteria [232,233].

In this chapter, we evaluated the association of PCV7 immunisation of infants on the prevalence and density of nasopharyngeal colonisation at 9 and 16 months of age; by common, potentially pathogenic bacteria including PCV7 serotypes, non-vaccine serotypes, *Haemophilus influenzae, Staphylococcus aureus, Moraxella catarrhalis, Streptococcus pyogenes* and *Neisseria meningitidis.*

4.1. MATERIAL AND METHODS

4.1.1 Study population

As described in Chapter 3, Section 3.1.1.

Nasopharyngeal samples had been previously cultured for *S. pneumoniae*, *H. influenzae* and *S. aureus* using standard culture methods and the dynamics of colonisation over the first two years of life have been described [81,198]. In this chapter, we expand on the previous findings and explore the association of PCV7 immunisation on the density of colonisation by these bacteria, as well as colonisation by *M. catarrhalis, S. pyogenes* and *N. meningitidis* as measured by multiplex qPCR.

4.1.2. Multiplex qPCR methods

Control strains for *S. pneumoniae, H. influenzae, M. catarrhalis, S. aureus, N. meningitidis* and *S. pyogenes* were obtained from the National Institute for Communicable Diseases (NICD). DNA from these strains were used to optimise PCR assays and as positive controls. In addition, *Escherichia coli, Klebsiella pneumoniae,* and *Salmonella spp* strains were used as controls to test for non-specific PCR.

Stored nasopharyngeal swab samples were thawed and processed. Briefly, nasopharyngeal swabs were vortexed for 30 seconds to release bacterial DNA into the transport media and total nucleic acids were automatically extracted from STGG with the NucliSens ® easyMAG ® extraction system (BioMérieux, Marcy l'Etoile, France) according to manufacturer's instructions. Similarly, total nucleic acids were also extracted from pneumococcal reference strains grown in Todd-Hewitt broth supplemented with 5% yeast, S. *aureus, M. catarrhalis, S.*

pyogenes and *N. meningitidis* reference strains grown in Todd-Hewitt broth alone, and *H. influenzae* grown in Brain-Heart infusion (BHI) broth with the NucliSens ® easyMAG ® extraction system according to manufactures instructions. Samples and pneumococcal reference strains were stored at – 20 °C.

The sequences for oligonucleotide primers and dye-labelled MGB probes were taken from previously published sequences, or designed with the ABI primer express software package, version 3.0 (Applied Biosystems, ABI, Foster City, USA). Primer and Probe sequences are described in Table 4.1.

Table 4.1: Oligonucleotide primers and probe[a] sequences for quantitative molecular Real-time PCR detection of respiratory pathogens

Primers/Probe[a] name	Sequence (5′ - 3′)	Target gene	Gene Function	Bacteria	Source
lytA-Fwd	TCTTACGCAATCTAGCAGATGAAGC	*lytA*	Autolysin	*Streptococcus pneumoniae*	McAvin *et al.*, 2001
lytA-Rev	GTTGTTTGGTTGGTTATTCGTGC				
lytA-Probe	(VIC)-TTTGCCGAAAACGCTTGATACAGGG-(MGB)				
IgA1-Fwd	CAAAATTGCCAAGATTAAATGCTT	IgA1	Immunoglobin A1 protein	*Haemophilus influenzae*	Mc Gillivary *et al.*, 2005
IgA1-Rev	TGCTCGCCATACTGCACAA				
IgA1-Probe	(FAM)-CCTGCGGTTAAACC-(MGB)				
Nuc-Fwd	GCTCAGCAAATGCATCACAAA	Nuc	Thermonuclease	*Staphylococcus aureus*	This study
Nuc-Rev	CACTATATACTGTTGGATCTTCAGAACCA				
Nuc-probe	(FAM)-AGATAACGGCGTAAATA-(MGB)				
copB-Fwd	CCGCTTTTACAACCACTGCTT	copB	Outer-membrane protein	*Moraxella catarrhalis*	This study
copB-Rev	TGTATCGCCTGCCAAGACAA				
copB-probe	(NED)-CAGCTGTTAGCCAGCC-(MGB)				
spy-Fwd	GCACTCGCTACTATTTCTTACCTCAA	Spy1258	Transcriptional regulator	*Streptococcus pyogenes*	CDC 2008
spy-Rev	GTCACAATGTCTTGGAAACCAGTAAT				
spy-Probe	(VIC)-CCGCAACTCATCAAGGATTTCTGTTACCA-(MGB)				

SodC-Fwd	GCACACTTAGGTGATTTACCTGCAT	SodC	Cu-Zn superoxide dimutase	*Neisseria meningitidis*	Thomas *et al.*, 2011
SodC-Rev	CCACCCGTGTGGATCATAATAGA				
SodC-Probe	(FAM)-CATGATGGCACAGCAACAAATCCTGTTT-(MGB)				

Probe[a]: Minor Grove Binding (MGB) dye labelled TaqMan probe.

4.1.3. Primer optimisation, standard curves and quantification of the real-time PCR assays

Optimisation of primers and probes for overall pneumococcus and its respective serotypes/serogroups has been explained in detail in chapter 2, section 2.1.3. In terms of the other bacterial targets, standard concentration curves and lower limits of detection (LLD) for each target bacteria were determined as described previously, with the exception of the bacterial control DNA being harvested at an OD_{600} = 1.0 (early exponential phase) instead of OD_{600} = 0.1. Similarly, correlation coefficients (r^2), analytical sensitivity, intra-assay variation, accuracy and inter-assay variation were determined as described previously in chapter 2.

All additional bacterial targets not described in chapter 2 showed good sensitivity and specificity with their respective primer and probe pairs with the limit of detection equivalent to 10 copies per PCR. Furthermore, the efficiency of these assays ranged from 91-96 %. Within the linear dynamic range, the correlation coefficients (r^2) of all the assays were 0.99. All primer pairs and probes were tested with genomic DNA from all pneumococcal and bacterial controls, and no cross reactions occurred. In addition, both the inter-assay variation (repeatability) and inter-assay variation (reproducibility) for all respective assays was <0.1, while the accuracy for all respective assays was within ± 0.1.

Bacterial targets were duplexed (Table 4.2) and paired reactions were tested to ensure that the Cq values did not shift by >1 Cq value, and that sensitivity and specificity remained the same as for single-plex reactions.

Table 4.2: Primer/probe duplex combinations

Primmer/probe combinations	Duplex reaction
S. pneumoniae & N. meningitidis	A
S. aureus & M. catarrhalis	B
H. influenzae & S. pyogenes	C

4.1.4. Real-time PCR multiplex assay

The density of *S. pneumoniae, H. influenzae¸ M. catarrhalis, S. aureus, S. pyogenes and N. meningitidis* were measured in colony forming units (CFU)/ml. Amplification data was analysed with the Applied Biosystems 7500 software, version 2.3 (Foster City, USA) with a manually defined threshold. Negative samples were defined as those with Cq values <35.

All samples positive for pneumococcus were further serotyped as described previously in chapter 2. Briefly; Serotypes/serogroups 4, 6A/B, 9A/L/N/V, 14, 18A/B/C, 19B/F or 23F were categorised as PCV7 vaccine-serotypes and serotypes/serogroups 1, 6C/D, 7C, 10A, 11A/B/C/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 23A/B, and 34/37/17A as non-vaccine serotypes. Samples negative for all tested bacteria were re-tested for human GAPDH to confirm the efficiency of the DNA extraction and the absence of PCR inhibitors, with all qPCR negative samples being positive for GAPDH

4.1.5. Statistical analysis

Statistical analysis was performed with STATA Version 11.0 (Statacorp, Texas, USA). Pearson X^2 test or student t-test was used to compare baseline characteristics between the cohorts. Comparisons and adjusted odd ratios of prevalence of bacterial colonisation between cohorts were analysed using logistic regression models adjusted for covariates, including race, passive smoke exposure, day care attendance, co-trimoxazole usage, and mean age in weeks at sample collection. For density of carriage, geometric mean densities (GMD) and 95% confidence intervals (95% CI) of bacterial mean concentrations were calculated following $\log_{10}$ transformation on the data, using analysis of covariance to adjust for possible covariates. To assess whether colonisation by one bacterial species was associated with colonisation by another, logistic regression models with generalised estimating equations (GEE) were used. Spearman correlations were used to compare the degree of correlation between densities of each pair of bacteria. Results were considered significant at a *p*-value of < 0.05.

4.1.6. Ethics approval

As described in chapter 3, section 3.1.4.

4.2. RESULTS

Molecular qPCR analysis involved 713 (83%) of the initial 857 nasopharyngeal swabs collected from children, at either 9 or 15-16 months of age (Figure 4.1). Within each cohort, there were no differences in the colonisation patterns done by standard methods between those in whom samples were and were not available (Appendices A and B). Among the children in whom samples were available, 92% were black African, and 52% were male (Table 4.3). Day care attendance was higher in PCV-vaccinated children, while the mean age in weeks at the time of sample collection being 40.7 (Standard deviation, SD 2.3) and 69.5 (SD 6) in PCV-unvaccinated children, and 39.3 (SD 3.1) and 66.7 (SD 1.8) in PCV-vaccinated children at the 1st and 2nd visits, respectively. Although the differences in age are small and thus unlikely to be epidemiologically or clinically significant in terms of the likelihood of affecting the results, we subsequently adjusted for these differences as well as the above mentioned using multivariate analysis.

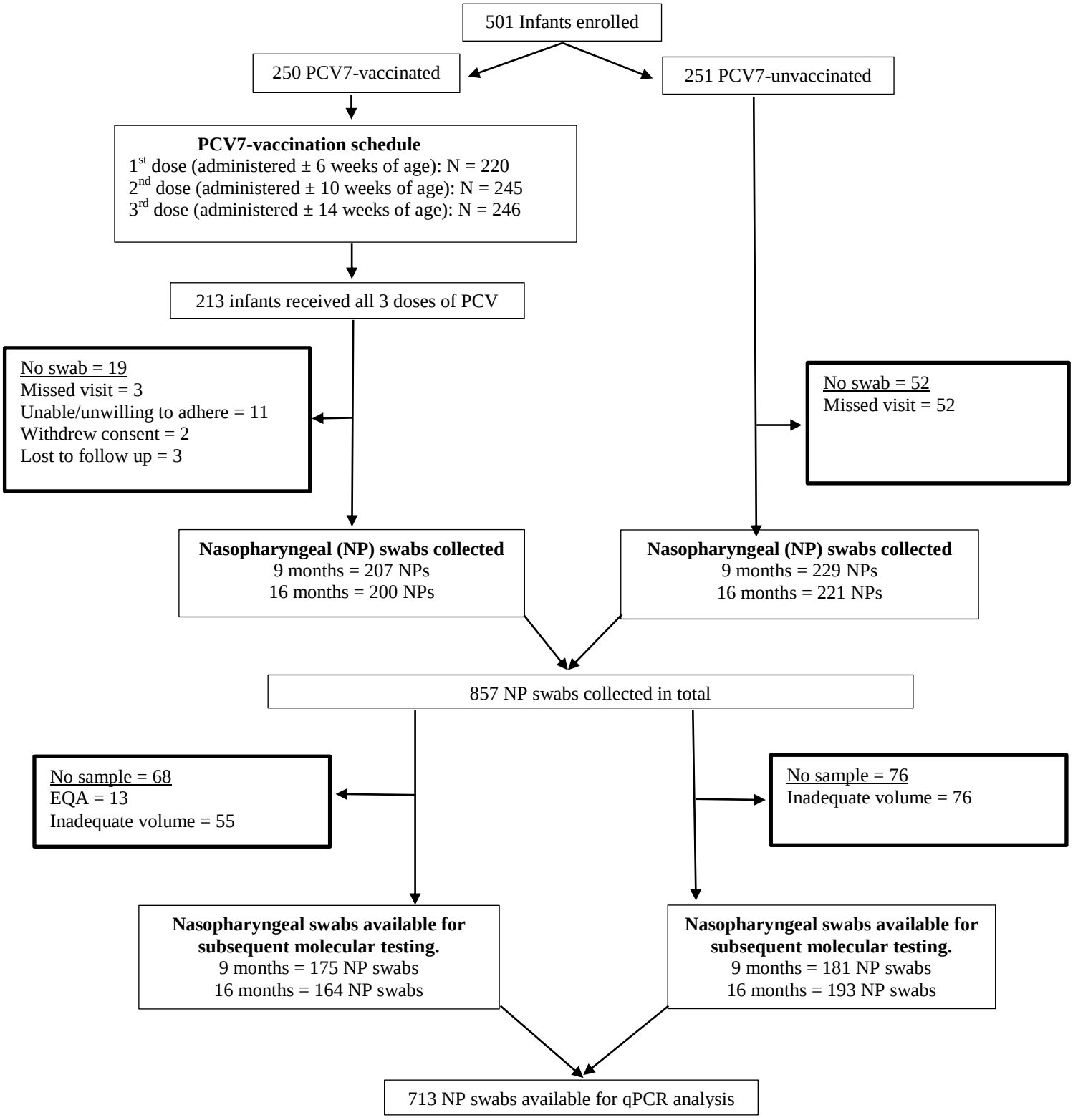


Figure 4.1: Schematic diagram of study population

Diagram indicating the number of children initially enrolled in a PCV-unvaccinated and PCV7-vaccinated cohort of HIV-uninfected children, as well as the number of nasopharyngeal (NP) swabs available for subsequent molecular qPCR analysis. PCV7-vaccinated participants were excluded from analysis if they did not receive all three doses of the pneumococcal conjugate vaccine (PCV) within protocol-defined window periods. The number of NP swabs available for molecular testing was defined by whether there was an adequate volume of sample remaining. EQA, samples were used for external quality assessment.

Table4.3: Demographic features at two study visits when analysed for nasopharyngeal (NP) bacterial colonisation in PCV7-vaccinated and PCV-unvaccinated, HIV-uninfected children

	9 month old children			16 month old children		
	PCV-unvaccinated	PCV7-vaccinated	*p*-value	PCV-unvaccinated	PCV7-vaccinated	*p*-value
Number of children enrolled	250	251	-	250	251	-
Available NP swabs for molecular analysis	181	175	-	193	164	-
HIV status: Exposed (%)	111 (61.3)	104 (59.4)	0.40	99 (51.3)	97 (59.1)	0.24
: Unexposed (%)	70 (38.7)	71 (40.6)		94 (48.7)	67 (40.9)	
Mean birth weight (SD) grams	3061 (440)	3097 (482)	0.45	3079 (427)	3126 (476)	0.43
Male sex (%)	87 (48.1)	96 (54.9)	0.12	99 (51.3)	89 (54.3)	0.25
Race: Black African (%)	181 (100)	150 (85.7)	<0.001	193 (100)	132 (80.5)	<0.001
: Mixed ancestry (%)	-	25 (14.3)		-	32 (19.5)	
Smoking household contact (%)	66 (36.5)	81 (42.3)	0.003	65 (33.68)	64(39)	0.004
Co-trimoxazole administered (%)	95 (52.5)	81 (46.3)	0.2	71(36.8)	68(41.5)	0.15
Mean numbers of children (SD) < 5 years	1.63 (0.8)	1.6 (0.7)	0.6	1.6 (0.82)	1.5 (0.84)	0.21
Mean number of household contacts (SD)	5.3 (2.2)	5.2 (2.3)	0.81	5.2 (2.07)	5.1 (2.36)	0.17
Day care attendance (%)	40 (22.1)	91 (52)	<0.001	72 (37.31)	81 (53.64)	0.002
Breast feeding (%)	41 (22.7)	44 (25.1)	0.33	37 (19.2)	38 (23.3)	0.43
Mean age (SD) in weeks at visit	40.7 (2.3)	39.3 (3.1)	<0.001	69.5 (6.01)	66.7(1.6)	<0.001

Pearson X^2 test or student t-test was used to compare baseline characteristics between the cohorts and demographic features with a *p*-value <0.2 were included as possible cofounders in multivariate analysis. PCV: Seven-valent pneumococcal conjugate vaccine. NP, nasopharyngeal. SD, standard deviation.

4.2.1. Detection of bacterial carriage by culture and qPCR

There was modest concordance between qPCR and culture for the detection of pneumococcus from infant NP swabs at both 9 months (*kappa* = 0.59) and 16 months of age (*kappa* = 0.59). *LytA* qPCR assay was more sensitive than culture in detecting pneumococcus in both PCV-unvaccinated (83% vs. 74%; p=<0.001) at 9 months; and 81% vs. 68% (p<0.001) at 16 months age; Table 4.4); and among PCV7-vaccinated children (71% vs. 57%; p<0.001) at 9 months of age; and 77% vs. 61% (p<0.001) at 16 months of age.

Similarly, there was modest concordance between qPCR and culture for the detection of *H. influenzae* from infant NP swabs at 9 months (*kappa* = 0.50) and 16 months of age (*kappa* = 0.61). The qPCR assay was more sensitive than culture in detecting *H. influenzae* in both PCV-unvaccinated children at 9 months (56% vs. 31%; p=<0.001) and 16 months of age (62% vs. 58%; p=0.04); and PCV7-vaccinated children (66% vs. 48% (p<0.001) at 9 months and 72% vs. 54% (p<0.001) at 16 months of age.

There was also a moderate concordance between qPCR and culture for the detection of *S. aureus* from infant NP swabs at 9 months (*kappa*=0.068) and 16 months (*kappa*=0.065) of age. While there was no significant difference found between the methods in PCV-unvaccinated children at 9 months of age (p=0.53*)*, the qPCR method was more sensitive than culture for the detection of *S. aureus* in PCV-unvaccinated children at 16 months of age (19% vs. 13%; p=0.004). Amongst PCV7-vaccinated children, the qPCR method was more sensitive than culture in detecting *S. aureus* at both 9 (19% vs. 13%; p=0.004) and 16 (17% vs. 11%; p=0.03) months of age; Table 4.4.

M. catarrhalis, *S. pyogenes* and *N. meningitidis* were not tested by culture and thus could not be compared to molecular qPCR. The overall detection prevalence, by qPCR at 9 months of age was 60.1% (214/356), 3.7% (13/356) and 2% (7/356) respectively; and 60.2% (215/357) 5.3% (19/357) and 1.1% (4/357) at 16 months of age.

Table 4.4: Number (%) of bacterial culture and molecular qPCR for the detection of *S. pneumoniae, H. influenzae* and *S. aureus* in nasopharyngeal swabs

	9 month old children						16 month old children					
	PCV-unvaccinated			PCV7-vaccinated			PCV-unvaccinated			PCV7-vaccinated		
	Culture (-)	**Culture (+)**	**Total**	**Culture (-)**	**Culture (+)**	**Total**	**Culture (-)**	**Culture (+)**	**Total**	**Culture (-)**	**Culture (+)**	**Total**
S. pneumoniae												
PCR (-)	27 (15)	4 (2)	31 (17)	45 (26)	5 (3)	50 (29)	32 (17)	4 (2)	36 (19)	39 (24)	2 (1)	41 (25)
PCR (+)	20 (11)	130 (72)	150 (83)	31 (18)	94 (54)	125 (71)	29 (15)	128 (66)	157 (81)	25 (15)	98 (60)	123 (75)
Total	47 (26)	134 (74)	181 (100)	76 (43)	99 (57)	175 (100)	61 (32)	132 (68)	193 (100)	64 (39)	100 (61)	164 (100)
H. influenzae												
PCR (-)	78 (43.1)	2 (1)	80 (44)	59 (34)	4 (2)	63 (36)	68 (35)	5 (3)	73 (37)	42 (26)	4(2)	46 (28)
PCR (+)	47 (26)	54 (30)	101 (56)	32 (18)	80(48)	112(66)	14 (7)	106 (55)	120 (62)	33 (20)	85 (52)	118 (72)
Total	125 (69.1)	56 (31)	181 (100)	91 (52)	84 (48)	175 (100)	92(48)	111 (58)	193 (100)	75 (46)	89 (54)	164 (100)
S. aureus												
PCR (-)	157 (87)	4 (2)	161 (89)	141 (81)	1 (1)	142 (81)	156 (81)	1 (1)	157 (81)	131 (80)	5 (4)	137 (84)
PCR (+)	6 (3)	15 (8)	20 (11)	11 (6)	22 (13)	33 (19)	11 (6)	25 (13)	36 (19)	15 (9)	13 (8)	28 (17)
Total	163 (90)	18 (10)	181 (100)	152 (87)	23 (13)	175 (100)	167 (87)	26 (13)	193 (100)	146 (89)	18 (11)	164 (100)

The qPCR method demonstrated high sensitivity and specificity for all bacteria for which culture was undertaken and used as a referent standard, with 96.8%, 95.6% and 82.3% of culture-positive swabs being positive by qPCR and 90.5%, 94.3% and 97.5% of qPCR negative swabs being negative by culture for *S. pneumoniae, H. influenzae* and *S. aureus,* respectively (Table 4.5). Discordant results between culture and qPCR results were strongly associated with the density of carriage, with 88% (242/275) of culture negative, PCR positive samples having a bacterial load of $<10^5$ CFU/ml. Similarly, 93% (42/45) of PCR negative, culture positive samples were scored as "scant" (<5 colonies/plate). This trend was observed across all bacterial pathogens, with the overall concordance between culture and PCR being 98% (50/51), 94% (77/82) and 100% (5/5) for *S. pneumoniae, H. influenzae* and *S. aureus* respectively when estimated PCR loads were $> 10^6$ CFU/ml.

Table 4.5: Detection of bacterial carriage by culture, stratified by colonisation density measured by quantitative PCR (qPCR)

Pneumococcal density by qPCR (CFU/ml)	Number culture positive/ number in density category by PCR (%)				
	9 month old children		16 month old children		
	PCV-unvaccinated	PCV7-vaccinated	PCV-unvaccinated	PCV7-vaccinated	Total
S. pneumoniae					
Undetected	4/31 (13)	5/50 (10)	4/36 (11)	2/41 (5)	15/159 (9)
0-10^2	1/2 (50)	0/1	0/2	0/3	1/8 (13)
>10^2-10^3	14/20 (70)	5/16 (31)	8/17 (47)	6/14 (43)	33/67 (49)
>10^3-10^4	31/38 (82)	11/21 (52)	33/43 (77)	17/21 (81)	92/123 (75)
>10^4 -10^5	40/46 (87)	26/30 (87)	45/51 (88)	31/37 (84)	142/164 (87)
>10^5-10^6	34/34 (100)	33/37 (89)	31/33 (94)	34/38 (89)	132/142 (93)
>10^6 -10^7	9/9 (100)	16/17 (94)	9/9 (100)	6/6 (100)	40/41 (98)
>10^7-10^8	1/1 (100)	3/3 (100)	2/2 (100)	4/4 (100)	10/10 (100)
Total	134/181 (74)	99/175 (57)	132/193 (68)	100/164(61)	465/713 (65)
H. influenzae					
Undetected	2/80 (3)	4/63 (6)	5/73 (7%)	4/46 (9)	15/262 (6)
0-10^2	0/10	0/5	-	0/1	0/16
>10^2-10^3	5/22 (23)	5/14 (36)	5/9 (56)	2/6 (33)	17/51 (33)
>10^3-10^4	12/22 (55)	13/23 (57)	12/16 (75)	9/17 (53)	46/78 (59)
>10^4 -10^5	15/20 (75)	28/34 (82)	23/27 (85)	17/25 (68)	83/106 (78)
>10^5-10^6	19/24 (79)	27/29 (93)	36/38 (95)	20/27 (74)	102/118 (86)
>10^6 -10^7	3/3 (100)	7/7 (100)	25/25 (100)	28/33 (85)	63/68 (93)
>10^7-10^8	-	-	5/5 (100)	9/9 (100)	14/14 (100)
Total	56/181 (31)	84/175 (48)	111/193 (58)	89/164 (54)	340/713 (48)
S. aureus					
Undetected	4/161 (2)	4/145 (3)	1/157 (1)	6/137 (4)	15/598 (3)
0-10^2	-	-	-	0/1	0/3
>10^2-10^3	5/9 (56)	6/13(50)	9/16 (56)	4/13 (31)	24/51 (54)
>10^3-10^4	4/6 (67)	3/5 (60)	4/6 (67)	7/12 (58)	18/29 (71)
>10^4 -10^5	2/2 (100)	3/4 (75)	4/5 (80)	-	9/11 (82)
>10^5-10^6	3/3 (100)	4/5 (80)	6/7 (86)	1/1 (100)	15/16 (94)
>10^6 -10^7	-	2/2 (100)	2/2 (100)	-	4/4 (100)
>10^7-10^8	-	1/1 (100)	-	-	-
Total	18/181 (10)	23/175 (13)	26/193 (13)	18/164 (11)	85/713 (12)

CFU/ml, colony forming units per millimetre as measured by qPCR. PCV7, seven-valent pneumococcal conjugate vaccine. Values are number culture positive/number in density category by qPCR (%).

4.2.2. Prevalence of NP bacteria as determined by qPCR with respect to PCV vaccination status

At least one bacteria was detected in 93.8 % (669/713) of all infant NP swabs, with 76.2% (543/713) of individuals being co-colonised with two or more species. At 9 months of age, overall pneumococcal colonisation was lower in PCV7-vaccinated (71.4%) than PCV-unvaccinated children (82.9%) (Adjusted odd ratio [aOR] 0.45; 95% confidence intervals [CI] 0.23-0.87; p= 0.018; Figure 4.2). The net reduction in carriage was due to a lower prevalence of vaccine-serotype colonisation in PCV7-vaccinated (36%) than PCV–unvaccinated children (62%, aOR 0.37; 95% CI 0.19-0.7; p=0.002). However, there was a corresponding increase in prevalence of non-vaccine serotype colonisation among the PCV7-vaccinated children (40% vs. 33.7%; aOR 1.88; 95% CI 1.02-3.48; p=0.023, Figure 4.2). By 16 months of age, there was no difference in overall pneumococcal colonisation between PCV7-vaccinated (75%) and PCV-unvaccinated children (81%; aOR 0.55; 95% CI 0.29-1.03; p= 0.062). This was due to the magnitude of lower prevalence of PCV7-serotype colonisation in PCV7-vaccinated (32.9%) than PCV-unvaccinated children (51.8%; aOR 0.41; 95% CI 0.26-0.63; p=0.007), being largely offset by the increase in non-vaccine serotype colonisation among the PCV7-vaccinated children (62.2% vs. 37.8%; aOR 2.2; 95% CI 1.18-4.1; p=0.013); Figure 4.2).

In contrast, the prevalence of colonisation by *H. influenzae* was higher in PCV7-vaccinated than PCV-unvaccinated children, including 66.3% vs. 55.8% (aOR 1.56, 95% CI 1.01-2.39; p=0.023), respectively, at 9 months of age; and 72% vs. 62% (aOR 1.88; 95% CI 1.07-3.3; p=0.017; Figure 4.2), respectively, at 16 months of age. Further, the prevalence of colonisation by *S. aureus* was higher in PCV7-vaccinated (18.9%) than PCV-unvaccinated children (11.1%, aOR 2.1; 95% CI 1.0-1.4; p=0.049) at 9 months of age; however, no difference between PCV7-unvaccinated (18.7%) and PCV-vaccinated (16.5%; aOR 0.81; 95% CI 0.41-1.14; p=0.14) children was found at 16 months of age.

No significant differences in the prevalence of colonisation at either 9 or 16 months of age was evident for, *M. catarrhalis, S. pyogenes* or *N. meningitidis* in respect to PCV7 vaccination status.

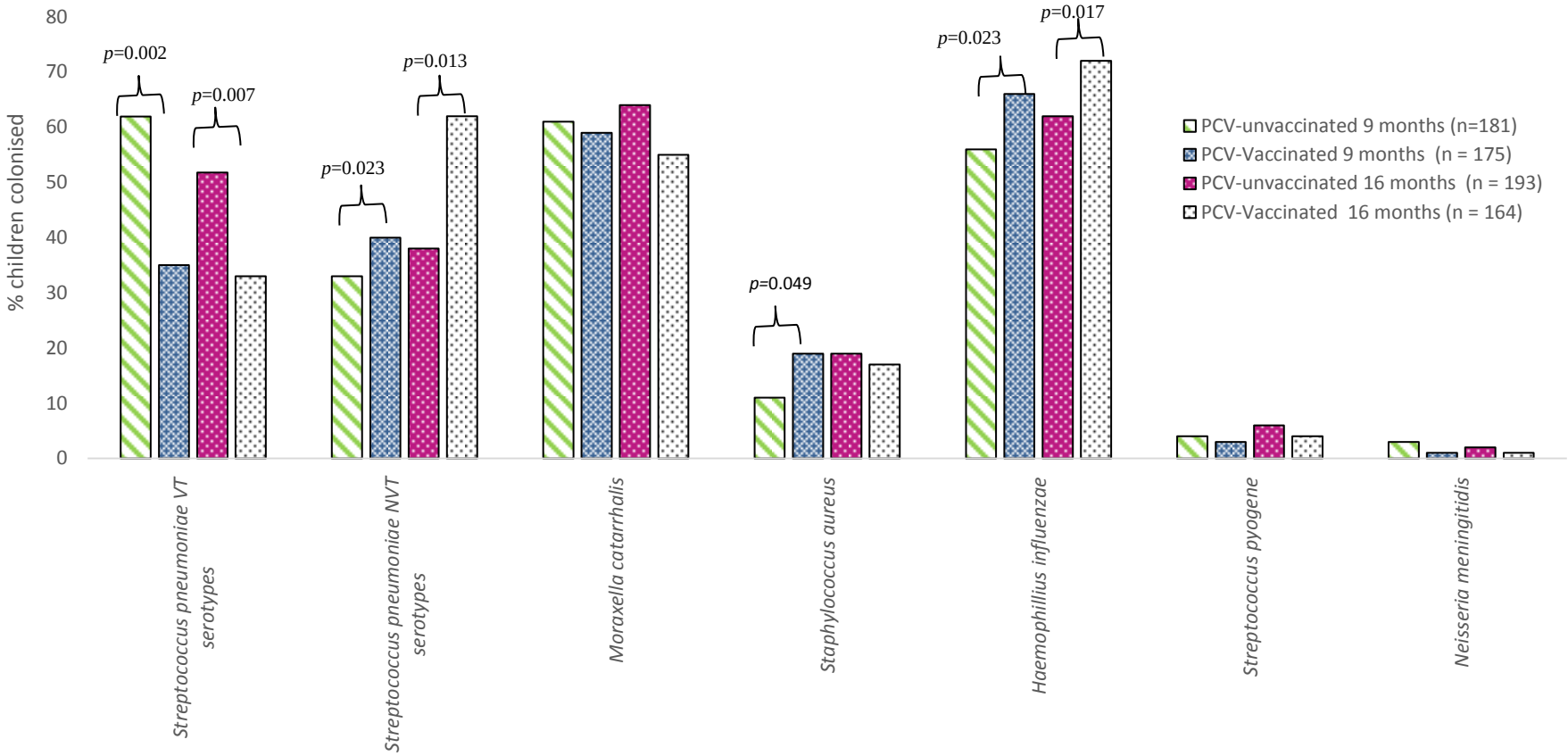


Figure 4.2: Prevalence of nasopharyngeal (NP) bacterial colonisation in PCV7-vaccinated and PCV-unvaccinated, HIV-uninfected children as measured by molecular qPCR

p-values were determined by multivariate logistic regression, controlling for race, smoking household contact, co-trimoxazole use, day care attendance and mean age at time of sample collection, by use of generalised estimating equations. PCV7, seven-valent pneumococcal conjugate vaccine. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 14, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7C, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 23A/B, 34/37/17A). * *p*-Values of <0.05 were considered statistically significant.

Logistic regression models demonstrated *S. pneumoniae* and *H. influenzae* to be positively associated with each other in PCV7-vaccinated children ($p<0.001$ and $p=0.015$) and PCV-unvaccinated children ($p=0.016$ and $p=0.023$) at 9 and 16 months of age respectively (Table 4.6), with associations being independent of PCV7-serotype group. Similarly, *S. pneumoniae* and *M. catarrhalis* colonisation were found to be positively associated with each other in both PCV7-vaccinated children ($p=0.002$ and $p=0.021$) and PCV-unvaccinated children ($p=0.008$ and $p=0.008$) at 9 and 16 months of age respectively. These associations were independent of PCV7-serotype group. *H. influenzae* and *M. catarrhalis* were found to be positively associated with each other in PCV7-vaccinated ($p=0.023$ and $p=0.019$) and PCV-unvaccinated children ($p<0.001$ and $p=0.003$) at 9 and 16 months of age respectively. The low prevalence of *S. pyogenes* and *N. meningitidis* colonisation limited comparisons from being made in relation to association of colonisation by other bacteria.

Table 4.6: Association of nasopharyngeal bacterial colonisation as determined by quantitative molecular PCR among PCV7-vaccinated and PCV-unvaccinated children

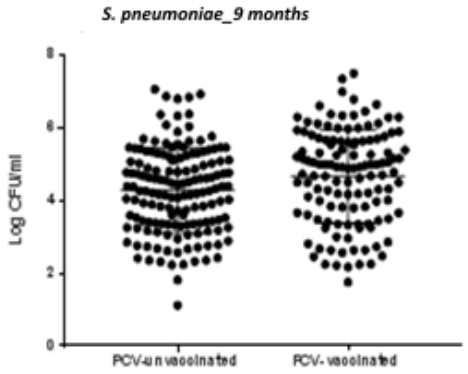
Bacterial association	9 month old children				16 month old children			
	PCV7-vaccinated (*n* = 175)		**PCV-unvaccinated (*n = 181*)**		**PCV7-vaccinated (*n = 164*)**		**PCV-unvaccinated (*n = 193*)**	
	aOR (95% CI)	*p*-value *	aOR (95% CI)	*p*-value *	aOR (95% CI)	*p*-value *	aOR (95% CI)	*p*-value *
S. pneumoniae & H. influenzae	4.8 (2.22-10.4)	<0.001	4.38 (2.6-5.17)	0.016	2.56 (1.2-5.47)	0.015	1.89(0.91-3.93)	0.023
VT *& H. influenzae*	1.19(0.52-2.71)	0.68	1.24(0.47-2.89)	0.87	1.82(0.81-4.06)	0.14	1.25(0.69-2.25)	0.46
NVT *& H. influenzae*	2.64(1.05-6.61)	0.058	0.94(0.49-1.81)	0.86	2.7(1.13-6.52	0.09	1.81(0.96-3.4)	0.07
S. pneumoniae & M. catarrhalis	2.96 (1.51-5.83)	0.002	2.33 (1.05-5.18)	0.011	2.11 (1.08-5.3)	0.021	2.72 (1.26-5.85)	0.008
VT *& M. catarrhalis*	0.48(0.23-1.02)	0.06	0.77(0.37-1.59)	0.47	2.75(1.22-6.18)	0.06	1.75(0.96-3.21)	0.07
NVT *& M. catarrhalis*	1.48(0.7-3.14)	0.30	0.85(0.43-1.67)	0.63	1.43 (0.76-2.67)	0.26	1.82(0.96-3.45)	0.07
S. pneumoniae & S. aureus	0.76 (0.34-1.71)	0.50	0.58 (0.19-1.73)	0.33	2.13 (0.69-6.56)	0.19	0.52 (0.22-1.2)	0.12
VT & S. aureus	0.81(0.32-2.06)	0.66	0.68(0.23-2.03)	0.49	1.2(0.51-2.83)	0.67	0.74(0.36-1.54)	0.43
NVT & *S. aureus*	1.49(0.58-3.87)	0.41	2.32(0.78-6.9)	0.13	1.34(0.59-3.07)	0.48	0.52(0.23-1.19)	0.12
S. pneumoniae & S. pyogenes	0.59 9(0.1-3.6)	0.57	ND		ND		ND	
VT & *S. pyogenes*	0.6(0.05-6.79)	0.68	0.26(0.6-1.14)	0.07	ND		ND	
NVT & *S. pyogenes*	1.61(0.14-18.25)	0.7	4.61(0.9-23.6)	0.07	ND		ND	
S. pneumoniae & N. meningitidis	ND		ND		ND		ND	
H. influenzae & M. catarrhalis	2.64 (1.87-4.09)	0.023	5.38 (2.81-10.32)	<0.001	4.84 (3.1-6.78)	0.019	2.59 (1.35-4.63)	0.003
H. influenzae & S. aureus	1.02 (0.46-2.28)	0.96	0.96 (0.38-2.45)	0.94	0.87 (0.33-2.26)	0.77	0.67 (0.31-1.44)	0.31
H. influenzae & S. pyogenes	2.07 (0.23-18.96)	0.52	1.34 (0.31-5.77)	0.70	ND		ND	
H. influenzae & N. meningitidis	0.5(0.03-8.07)	0.62	2.41(0.25-23.7)	0.45	ND		ND	
M. catarrhalis & S. aureus	0.69(0.32-1.48)	0.34	0.47 (0.19-1.21)	0.12	1.75 (0.7-4.37)	0.23	0.62 (0.29-1.32)	0.214
M. catarrhalis & S. pyogenes	1.05 (0.17-6.45)	0.96	0.36 (0.8-1.46)	0.17	ND		ND	
M. catarrhalis & N. meningitidis	ND		0.62 (0.08-4.53)	0.64	ND		ND	
S. aureus & S. pyogenes	2.99 (0.48-18.65)	0.24	1.15 (0.14-9.93)	0.89	2.4 (0.44-13.14)	0.22	1.63 (0.42-6.39)	0.481
S. aureus and N. meningitidis	ND		ND		ND		ND	

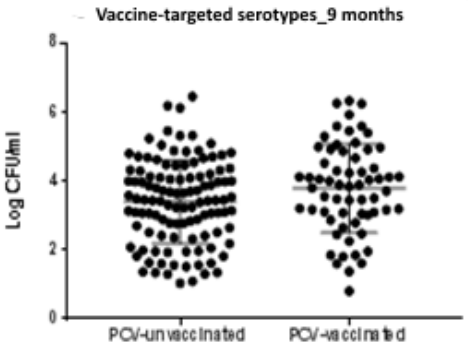
Adjusted odds ratios (aORs) for bacterial associations were determined by multivariate logistic regression, controlling for race, smoking house hold contact, co-trimoxazole use, day care attendance and mean age at time of sample collection, by use of generalised estimating equations. * *p*-values of <0.05 were considered statistically significant. ND, not done as too few observations available for analysis. CI, confidence intervals. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 14, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7C, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 23A/B, 34/37/17A).

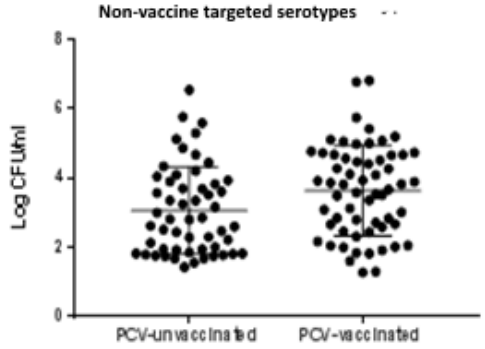
4.2.3. Association of PCV vaccination and density of bacterial nasopharyngeal carriage

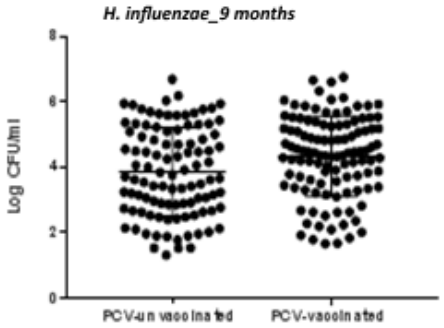
The overall density of pneumococcal carriage was higher in PCV7-vaccinated than PCV-unvaccinated children at 9 months of age, with titers of 4.68 (95%CI: 4.45-4.90) CFU/ml compared to 4.28 (95%CI: 4.1-4.44) CFU/ml respectively (p=0.007). This was driven by an increase in carriage density, albeit lower prevalence of colonisation, of VT serotypes, with titers of 3.8(3.5-4.1) CFU/ml compared to 3.4(3.2-3.6) CFU/ml respectively (p=0.048) and by an increase in NVT serotypes, with titers of 3.6 (95%CI: 3.3-4) CFU/ml compared to 3.1 (95%CI: 2.7-3.4) CFU/ml, respectively (p=0.018). There was, however, no difference in density of colonisation between the PCV7-vaccinated and -unvaccinated groups at 16 months of age (figure 4.3).

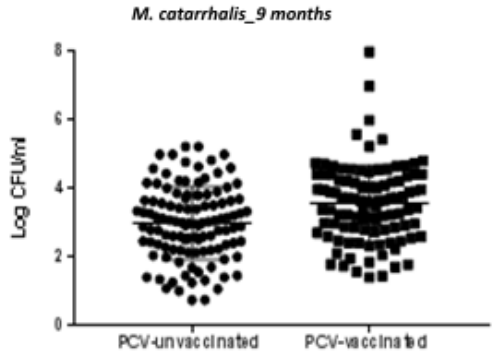
A similar trend was noted for the density of the other bacteria at 9 months of age, where the density of colonisation was higher in PCV7-vaccinated than PCV-unvaccinated children for *H. influenzae* (4.34 vs. 3.86 CFU/ml; p=0.008), *M. catarrhalis* (3.52 vs. 2.98 CFU/ml; p<0.001) and *S. aureus* (4.02 vs. 3.06 CFU/ml; p=0.02). There was however, no difference in density of colonisation between PCV7-vaccinated and PCV-unvaccinated groups at 16 months of age. No significant differences in density of colonisation were observed for *S. pyogenes* and *N. meningitidis* between PCV7-vaccinated and PCV-unvaccinated children.

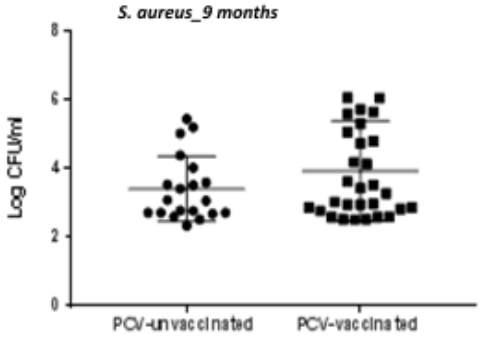

S. pneumoniae_9 months
Log CFU/ml
PCV-unvaccinated
PCV-vaccinated

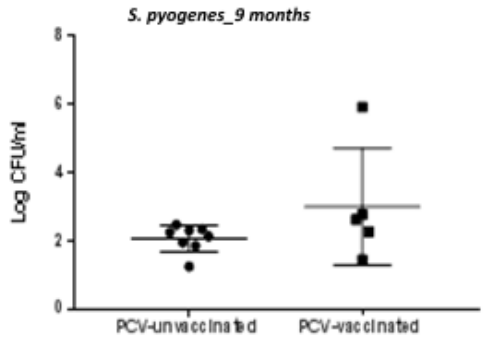

Vaccine-targeted serotypes_9 months
Log CFU/ml
PCV-unvaccinated
PCV-vaccinated

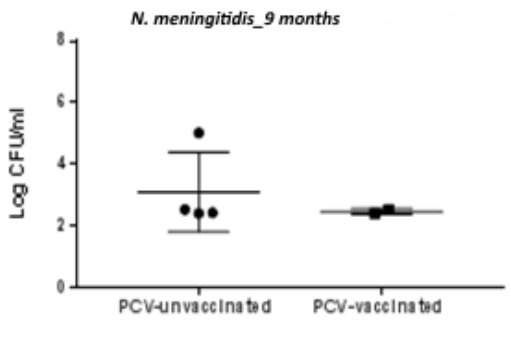

Non-vaccine targeted serotypes
Log CFU/ml
PCV-unvaccinated
PCV-vaccinated


H. influenzae_9 months
Log CFU/ml
PCV-unvaccinated
PCV-vaccinated


M. catarrhalis_9 months
Log CFU/ml
PCV-unvaccinated
PCV-vaccinated


S. aureus_9 months
Log CFU/ml
PCV-unvaccinated
PCV-vaccinated


S. pyogenes_9 months
Log CFU/ml
PCV-unvaccinated
PCV-vaccinated


N. meningitidis_9 months
Log CFU/ml
PCV-unvaccinated
PCV-vaccinated

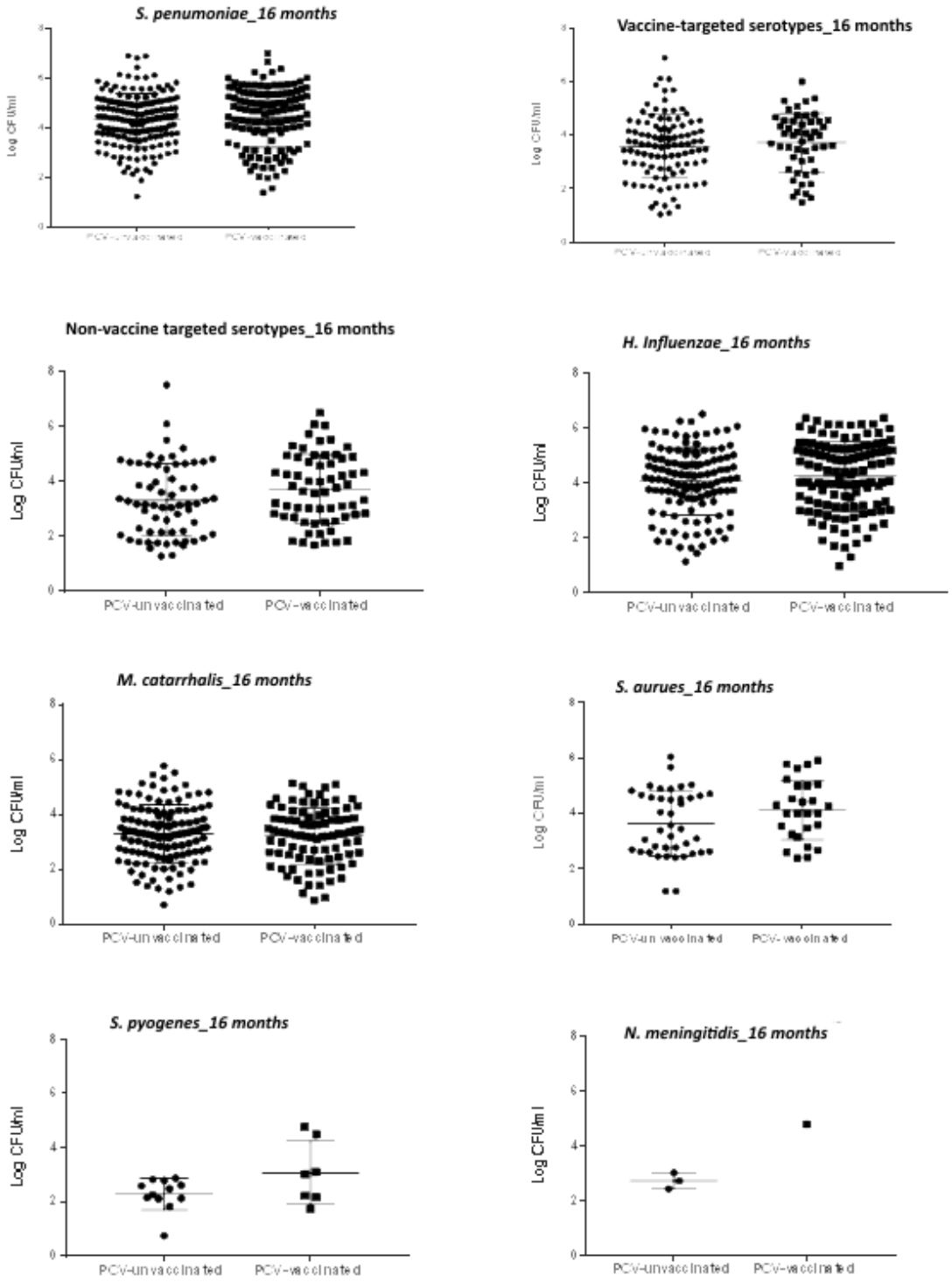


Figure 4.3: Density of nasopharyngeal bacterial carriage in PCV7-vaccinated and PCV-unvaccinated, HIV-unifected children as measured by quantitative molecular qPCR

For density of carriage geometric mean densities (GMD) and 95% CI intervals (95% CI) of pneumococcal and bacterial mean concentrations were calculated following $\log_{10}$ transformations on data and compared with multivariate analysis controlling for race, smoking house hold contact, co-trimoxazole use, day care attendance and mean age at time of sample collection. PCV7, seven-valent pneumococcal conjugate vaccine. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7C, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 23A/B, 34/37/17A). *p*-values of <0.05 were considered significant.

Spearman correlation coefficients showed the overall densities of *S. pneumoniae* and *H. influenzae* to be weakly correlated in PCV7-vaccinated (*rho*=0.338; $p<0.001$ and *rho*=0.309, $p=0.0025$) and PCV-unvaccinated children (*rho*=0.4 and 0.464; $p<0.001$) at 9 and 16 months of age respectively. These correlations were independent of PCV7–serotype group.

A weak positive correlation was also found between densities of *S. pneumoniae* and *M. catarrhalis* in PCV7-vaccinated (*rho*=0.333, $p=0.002$) children at 9 months of age, while a moderate correlation was found between the densities of *S. pneumoniae* and *M. catarrhalis* in PCV-unvaccinated (*rho*=0.45, $p=0.005$) children at 9 months of age. These correlations were evident for vaccine serotypes (*rho*=0.423, $p=0.016$ and *rho*=0.3186, $p=0.006$) in both cohorts respectively. A similar trend was noted at 16 months of age, with a weak positive correlation being found between the densities at both PCV7-vaccinated (*rho*=0.25, $p=0.0035$) and PCV-unvaccinated (*rho*=0.343, $p=0.0013$). These correlations were, however, independent of PCV7-serotype group. A weak correlation was also identified between densities of *H. influenzae* and *M. catarrhalis* in PCV-vaccinated children (*rho*=0.36, $p=0.048$ and *rho*=0.2523, $p=0.04$) and PCV-unvaccinated (*rho*=0.19, $p=0.03$ and *rho*=0.343, $p=0.0013$) at 9 and 16 months of age respectively. These associations were independent of PCV7–serotype group. No significant correlations with density of colonisation were found between other bacteria (Table 4.7).

Table 4.7: Correlation of density of nasopharyngeal bacterial colonisers as determined by quantitative molecular PCR among PCV7-vaccinated and PCV-unvaccinated children

Bacterial correlation	9 month old children				16 month old children			
	PCV7-vaccinated (*n* = 175)		**PCV-unvaccinated (*n* = 181)**		**PCV7-vaccinated (*n* = 164)**		**PCV-unvaccinated (*n* = 193)**	
	Rho	*p*-value *	*Rho*	*p*-value *	*Rho*	*p*-value *	*Rho*	*p*-value *
S. pneumoniae & H. influenzae	0.338	<0.001	0.4	<0.001	0.309	0.0025	0.46	<0.001
VT *& H. influenzae*	0.085	0.59	0.287	0.78	0.1472	0.24	0.2321	0.16
NVT *& H. influenzae*	0.152	0.27	0.09	0.61	-0.0858	0.58	0.3095	0.026
S. pneumoniae & M. catarrhalis	0.333	0.002	0.4488	0.005	0.252	0.0035	0.343	0.0013
VT *& M. catarrhalis*	0.423	0.016	0.3186	0.006	0.0857	0.48	0.2106	0.24
NVT *& M. catarrhalis*	0.251	0.15	0.0791	0.61	0.1239	0.4	0.1619	0.34
S. pneumoniae & S. aureus	-0,489	0.84	0.28	0.31	0.192	0.38	-0.063	0.74
VT & S. aureus	0.7364	0.07	-0.3143	0.54	0.432	0.21	0.151	0.54
NVT & *S. aureus*	0.086	0.78	0.033	0.93	-0.373	0.19	0.611	0.34
S. pneumoniae & S. pyogenes	ND		ND		0.143	0.76	0.360	0.15
VT & *S. pyogenes*	ND		ND		0.867	0.33	0.1	0.83
NVT & *S. pyogenes*	ND		ND		ND		0.31	0.46
S. pneumoniae & N. meningitidis	ND		ND		ND		ND	
H. influenzae & M. catarrhalis	0.360	0.048	0.19	0.03	0.2523	0.04	0.343	0.0013
H. influenzae & S. aureus	-0.139	0.56	-0.351	0.29	-0.159	0.54	-0.165	0.48
H. influenzae & S. pyogenes	-0.4	0.60	0.1	0.87	0.679	0.093	-0.077	0.81
H. influenzae & N. meningitidis	ND		ND		ND		ND	
M. catarrhalis & S. aureus	0.286	0.30	0.519	0.15	0.412	0.11	0.24	0.32
M. catarrhalis & S. pyogenes	ND		0.5	0.67	ND		0.436	0.18
M. catarrhalis & N. meningitidis	ND		ND		ND		ND	
S. aureus & S. pyogenes	ND		ND		ND		ND	
S. aureus and N. meningitidis	ND		ND		ND		ND	

Bacterial correlations were determined by spearman's coefficients. ND, not done as too few observations available for analysis. PCV7, seven-valent pneumococcal conjugate vaccine. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7C, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 23A/B, 34/37/17A). *p*-values of <0.05 were considered significant.

4.3. DISCUSSION

This study successfully established a highly sensitive molecular qPCR method to examine the effect that PCV has on common bacterial nasopharyngeal colonisers in PCV7-vaccinated and PCV-unvaccinated children in South Africa. Our analysis was in agreement with culture based methods, and showed a decrease in the carriage prevalence of pneumococcus and a corresponding increase in the carriage prevalence of *H. influenzae* associated with PCV vaccination [234]. Molecular qPCR allowed us to also investigate the density of carriage and showed that the overall density of *H. influenzae* was also higher in PCV7-vaccinated infants, which could possibly explain the moderate effects that PCV has on otitis media (OM), even with the vaccine having up to 80% efficacy in reducing vaccine-type pneumococcal colonisation[235-237]. This is further supported by studies done in USA which showed the incidence of *H. influenzae* acute otitis media to increase after PCV immunisation [238].

There is contradicting data with respect to the effect that PCV has on *S. aureus* carriage, with most studies reporting a temporary increase in the prevalence of colonisation and others reporting no change. Our findings are in line with studies done in the Netherlands were a temporary increase in the colonisation prevalence of *S. aureus* was found after PCV7 immunisation suggesting the effect there on to be around PCV7 vaccinations in infancy and subsequently diminishing after non-vaccine serotypes or other bacterial species colonise the vacant niche or by an age-dependent maturation of the immune system[170,177,239]. The qPCR method, however, also allowed quantification and showed a temporary increase in the density of *S. aureus*, raising concerns of the indirect effect PCV-vaccination may have on both *S. aureus* carriage and infection, and may possibly explain the rise in otitis media cases following immunisation with PCV [240]. Two cross sectional studies, one in primary care visiting children from Massachusetts and the other in children from France with otitis media reported no change the prevalence of colonisation by *S. aureus* following PCV immunisation [168,169]. These studies, however, were limited in that they included children of varying ages and therefore the temporary effect of PCV there on *S. aureus* carriage could easily have been missed. Further, the studies involved PCV-vaccinated populations with established heard effects and the effect of infant vaccination with PCV may have thus been less pronounced due to a pre-established low circulation and carriage of vaccine-type pneumococcal serotypes.

Nevertheless, the vaccine effect, typified as a decrease in vaccine-type serotypes and a corresponding increase in NVT serotypes as a result of PCV vaccination has been well documented [149,219,220]; however, the findings from this study suggest that after the eradication of common PCV colonisers such as vaccine-type pneumococci, momentum is directed by both non-vaccine type serotypes and other bacterial species to colonise the vacant niche and thus direct bacteria-bacteria interactions may impact clinically important nasopharyngeal dynamics.

Our findings are in line with other studies, in that positive associations with respect to prevalence were reported between pneumococcus, *H. influenzae* and *M. catarrhalis* [83,175,231]. Whilst these observations may well be associated with non-bacterial factors such the presence of respiratory viruses which may cause an ubiquitous increase in bacterial outgrowth in mucosal secretions, these common observations could also be associated with the quality of nasopharyngeal swab that was collected, and as such, justifies further research into these interactions.

The majority of studies of NP colonisation have focused on non-quantitative culture based methods, which have compared changes in the prevalence of bacterial carriage. Whilst this has been meaningful to some extent, the lack of quantitative data has hindered our understanding of these dynamics. Quantitative real-time PCR has become an increasingly important diagnostic tool in which multiple respiratory organisms can be detected from clinical specimens, with an increased sensitivity and specificity compared to standard culture methods. Furthermore, the ability to quantitatively detect multiple bacterial species within nasopharyngeal specimens provided a means for a better understanding of carriage and its relationship to disease. Improved methods of detection of respiratory pathogens will provide a means to improve the health care of patients as optimal therapy can be initiated sooner, effective treatment is improved and spread of disease can be reduced or prevented. Furthermore, a high bacterial load detected by real-time PCR in nasopharyngeal specimens can help predict the aetiological role for these bacterial species in invasive bacterial disease. However, the real-time qPCR has some disadvantages in that it requires expensive reagents and equipment, and a large sample volume is required to distribute across all reactions.

Our study was limited, as only a selection of NP organisms were analysed and thus analysis at a microbiome level may be advantageous. Furthermore, nasopharyngeal swabs do not favour the isolation of *S. aureus* and *S .pyogenes* in which anterior nares and throat swabs are better for detection, respectively [241,242]. The prevalence of these bacteria could have thus been underestimated. Lastly, the qPCR method was not able to discriminate between all serotypes within their respective serogroup; however, due to the high concordance between serotypes identified by culture and qPCR, we can assume from colonisation data using traditional culture methods, that 71.4%, 80% and 92.6% of all serogroups 9A/L/N/V, 18A/B/C and 19B/F identified by qPCR to be vaccine-serotypes 9V, 18C and 19F, respectively (Chapter 2).

In conclusion, there is a natural balance between pneumococci and co-colonising bacterial species. Interfering with this may lead to disequilibrium within the host, which could affect susceptibility to disease from other serotypes and bacteria species. On-going surveillance, especially on the implication of such changes in the nasopharyngeal biome on particularly mucosal infections are warranted.

CHAPTER 5: PERFORMANCE OF THE BIOMARK HD REAL-TIME PCR SYSTEM (FLUIDIGM) FOR THE DETECTION OF COMMON NASOPHARYNGEAL BACTERIAL PATHOGENS AND SEROTYPING *STREPTOCOCCUS PNEUMONIAE*

The human nasopharynx is a common ecological niche for several respiratory pathogens including *S. pneumoniae, H. influenzae, M. catarrhalis,* and *S. aureus* [71]. Interactions between these bacteria and the establishment of carriage and disease is not well known [73,74], with findings being contradictory and relevant mechanisms to explain concurrent colonisation being unclear [74-77]. The majority of studies of nasopharyngeal colonisation have focused on non-quantitative culture based methods and thus the lack of quantitative data has hindered our understanding of these dynamics [199]. Recently molecular quantitative PCR-based methods have been developed and allowed for quantitative detection of multiple serotypes from a single nasopharyngeal specimen (Chapter 2). Serotyping pneumococci by qPCR, however, still has two main shortcomings, including that large sample volume is required to distribute across all the reactions, and that it is expensive (approximately R26 per sample per assay). Further, processing multiple qPCR reactions is labour intensive and time consuming.

Fluidigm is a nanofluidic automated real-time PCR system that relies on microfluidic technology in which dynamic arrays of integrated fluidic circuits (IFCs) are used. These IFC's contain thousands of controlled valves and interconnected channels in which molecules of biological samples and reagents can be automatically mixed in a variety of patterns. The instrument uses an array of non-fluidic chips called dynamic arrays for qPCR, in which a typical chip format allow for 9 216 PCR reactions (96.96 chip format; 96 samples x 96 assays) in a single qPCR run (www.fluidigm.co.za) [204]. Further, Fluidigm has several advantages over standard qPCR in that it allows for a larger number of reactions per plate, thus making it more cost effective (approximately R7 per sample per assay) and less time consuming. Further, IFCs not only reduces the reaction volume from 10µL – 20µL down to 10nL scale, but the technology also allows for validations as well as increased parallelism and throughput of qPCR reactions [204].

This chapter aimed to develop a novel Nanofludic real-time PCR assay that could simultaneously detect and quantify 11 bacterial pathogens, 53 pneumococcal serotypes (16 individual serotypes and 37 serotypes in 13 groups) and 6 serotypes of *H. influenzae*

(serotypes a-f) in 96 different samples (92 samples and 4 controls) in a single PCR run. In addition we compared results from traditional quantitative PCR and Fluidigm for the detection of bacterial colonisation and pneumococcal serotyping in a PCV-vaccinated cohort of black-African children.

5.1. MATERIAL AND METHODS

5.1.1. Study population

We retrospectively analysed archived nasopharyngeal swab samples collected from a PCV7-vaccinated cohort of HIV-uninfected infants in Soweto, South Africa. Detailed information of the study cohort has been described in Chapter 3. Samples were previously screened for *S. pneumoniae, H. influenzae, S. aureus, M. catarrhalis, S. pyogenes* and *N. meningitidis* by conventional real-time polymerase chain reaction (qPCR) (Chapter 4), and pneumococcal serotyping was done on all samples that tested positive for *S. pneumoniae* (Chapter 3). In this Chapter, we compare previous findings by conventional real-time PCR to those of the Biomark HD system (Fluidigm) and in addition screened for an extra 9 serotypes/serogroups (7A/F, 8, 22A/F, 25A/25F/38 and 35B), and 5 bacteria (*B. bronchiseptica, B. parapertusis, B. pertussis, B. holmesii* and *N. lactamica*) as well as serotyped *H. influenzae.*

5.1.2. Bacterial and pneumococcal reference isolates

Control strains for the pneumococcal serotypes (1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 7A, 7F, 7C, 8, 9A, 9L, 9N, 9V, 10A, 10B, 11A, 11B, 11C, 11D, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A. 17F,18A, 18B, 18C, 19A, 19B, 19F, 20, 21, 22A, 22F,23A, 23B, 23F,25A, 25F, 34, 35B and 37), *H. influenzae* serotypes (serotypes a-f), *M. catarrhalis, S. aureus, N. meningitidis* and *S. pyogenes* were obtained from the National Institute for Communicable Diseases (NICD). Additional isolates for *B. bronchiseptica* (ATCC ® 4617), *B. parapertusis* (ATCC ® 15311), *B. pertussis* (ATCC ® 2397) and *N. lactamica* (ATCC ® 23970) were purchased from Davies Diagnostics (South Africa), while an isolate for *B. holmesii* (ATCC ® 51541) was purchased from LGC standards South Africa. DNA from these strains were used to optimise PCR assays and as positive controls.

5.1.3. DNA extraction

Stored nasopharyngeal swab samples were thawed and processed. Briefly, NP swabs were vortexed for 30 seconds to release bacteria into the transport media and total nucleic acids were automatically extracted from STGG with the NucliSens ® easyMAG ® extraction system (BioMérieux, Marcy l'Etoile, France) according to manufactures instructions. Similarly, total nucleic acids were also extracted from pneumococcal reference strains (positive control strains) grown in Todd-Hewitt broth supplemented with 5% yeast, *S. aureus, M. catarrhalis, S. pyogenes, N. meningitidis* and *N. lactamica* reference strains grown in Todd-Hewitt broth alone, *H. influenzae* serotypes a-f grown in Brain-Heart infusion (BHI) broth and Bordetella species grown in potato broth with the NucliSens ® easyMAG ® extraction system according to manufacturer's instructions. Samples and reference strains were stored at - 20 °C.

5.1.4. Nanofluidic real-time PCR

The sequences for oligonucleotide primers and FAM dye-labelled MGB probes were taken from previously published sequences, or designed with the ABI primer express software package, version 3.0 (Applied Biosystems, ABI, Foster City, USA). Primer and Probe sequences are described in Table 5.1. GAPDH was included to confirm the efficiency of the DNA extraction and BexA to confirm the presence of non-typable *H. influenzae.* Due to the high genotypic similarities between the capsule loci of certain pneumococcal serotypes, the qPCR method was unable to discriminate between some serotypes within a particular serogroup (i.e. 6A/B, 6C/D, 7A/F, 9A/L/N/V, 11A/B/C/D/F, 12A/B/F, 15A/B/C/F, 18A/B/C, 19B/F, 22A/F, 23A/B, 25A/25F/38 and 34/37/17A), although able to identify serotypes 1, 3, 4, 5, 7C, 8, 10A, 13, 14, 16F, 17F, 19A, 20, 21, 23F and 35B individually.

Table 5.1: Oligonucleotide primers and probe[a] sequences for quantitative molecular Nanofludic real-time PCR detection of respiratory pathogen

Target Name	Primer/Probe name	Forward primer	Reverse primer	Probe	Reference
Streptococcus pneumoniae	lytA	TCTTACGCAATCTAGCAGATGAAGC	GTTGTTTGGTTGGTTATTCGTGC	TTTGCCGAAAACGCTTGATACAGGG	McAvin *et al*, 2001
Haemophilus influenzae	IGA	CAAAATTGCCAAGATTAAATGCTT	TGCTCGCCATACTGCACA A	CCTGCGGTTAAACC	McGillivary *et al*, 2005
Haemophilus influenzae type A	HiA	GCAACCATCTTACAACTTAGCGAATAC	GGTCTGCGGTGTCCTGTGTT	AAGTGAAGCATGTCGCCATTCGTCCA	Maaroufi *et al*, 2007
Haemophilus influenzae type B	HiB	TGTTCGCCATAACTTCATCTTAGC	CTTACGCTTCTATCTCGGTGATTAATAA	CACAAAACTTCTCATTCTTCGAGCCTA	Maaroufi *et al*, 2007
Haemophilus influenzae type C	HiC	TCTGTGTAGATGATGGTTCAGTAG	TTAGGATATTTACGCTGCCATT	TGCAGCTAAGATTATT	Maaroufi *et al*, 2007
Haemophilus influenzae type D	HiD	TATTGATGACCGATACAACCTGTTTAAA	CCAGAAATTATTTCTCCGTTATGTTGA	AATGGTTGTAAAACTCTTCT	Maaroufi *et al*, 2007
Haemophilus influenzae type E	HiE	GTTGAAAACAAACCGCACTTT	ATCTTTAATTACCAGATCCCTTTCAT	AACGAATGTAGTGGTAGTTAGA	Maaroufi *et al*, 2007
Haemophilus influenzae type F	HiF	GGATAATCAAATACCACATTGGCTTA	GTAGATTAGCCTCAATAACATGTGAATTAA	TCATCGTGAGATCATTGATCACGAT	Maaroufi *et al*, 2007
BexA	BexA	CTGAATTRGGYGATTATCTTTATGA	ACAATCAAAYTCAACHGAAAGHGA	AGGGATGAAAGCYCGRCTTGCAT	Maaroufi *et al*, 2007
Staphylococcus aureus	SA	GCTCAGCAAATGCATCACAAA	CACTATATACTGTTGGATCTTCAGAACCA	AGATAACGGCGTAAATA	This Study
Moraxella catarrhalis	MCAT	CCGCTTTTACAACCACTGCTT	TGTATCGCCTGCCAAGACAA	CAGCTGTTAGCCAGCC	This Study
Streptococcus pyogenes	SPY	GCACTCGCTACTATTTCTTACCTCAA	GTCACAATGTCTTGGAAACCAGTAAT	CCGCAACTCATCAAGGATTTCTGTTACCA	CDC 2008
Neisseria meningitidis	SodC	GCACACTTAGGTGATTTACCTGCAT	CCACCCGTGTGGATCATAATAGA	CATGATGGCACAGCAACAAATCCTGTTT	Thomas *et al.*, 2011
Neisseria lactamica	LacZ	TTGCCCGAGAACCATTGTATC	GCGGTTCTTATCACGTTCTATATTTG	TATTGGAGCGGACTAAA	This Study
Bordetella pertussis and *Bordetella holmesii*	IS481	CAAGGCCGAACGCTTCAT	GAGTTCTGGTAGGTGTGAGCGTAA	CAGTCGGCCTTGCGTGAGTGGG	Tatti et al, 2011
Bordetella holmesii	hIS1001	GGCGACAGCGAGACAGAATC	GCCGCCTTGGCTCACTT	CGTGCAGATAGGCTTTTAGCTTGAGCG C	Tatti et al, 2011
Bordetella parapertusis	pIS1001	TCGAACGCGTGGAATGG	GGCCGTTGGCTTCAAATAGA	AGACCCAGGGCGCACGCTGTC	Tatti et al, 2011
Bordetella pertussis, *Bordetella parapertusis* and *Bordetella bronchiseptica*	PtxS	CGCCAGCTCGTACTTC	GATACGGCCGGCATT	AATACGTCGACACTTATGGCGA	Tatti et al, 2011
Streptococcus pneumoniae serotype 1	1	CGTGCGGTAATTGAAGCTATGA	TGTGGCCCCAGCAACTCT	TGCTTGCCCTTGTATAGGGT	Azzari *et al*, 2010
Streptococcus pneumoniae serotype 3	3	GGTCAGCAGAAAGTATGCATTGG	TCGTTTATCCAGGGTCTGATGA	TATTGGATGTGGTTTATCGTGAAGA	Azzari *et al*, 2010
Streptococcus pneumoniae serotype 4	4	TGGGATGACATTTCTACGCACTA	CCGTCGCTGATGCTTTATCA	TCCTATTGGATGGTTAGTTGGTGA	Azzari *et al*, 2010
Streptococcus pneumoniae serotype 5	5	TTACGGGAGTATCTTATGTCTTTAATGG	CAGCATTCCAGTAGCCTAAAACTAGA	TTGTCTCAGCAACTCTATTTGGCTGTGGG	Azzari *et al*, 2010
Streptococcus pneumoniae serogroup 6A/B/C/D	6ABCD	AAGTTTGCACTAGAGTATGGGAAGGT	ACATTATGTCCRTGTCTTCGATACAAG	TGTTCTGCCCTGAGCAACTGG	Azzari *et al*, 2010
Streptococcus pneumoniae serogroup 6C/D	6CD	TTGGGATGATTGGTCGTATTAG	CTCTTCAATTAGTTCTTCAGTTCG	CCACGCAATTCGCCATC	Azzari *et al*, 2010
Streptococcus pneumoniae serogroup 7A/F	7A/F	GATGGCATGTGGCAAACCA	TTTGCCCTCCTTAATCATTTCAC	TTGGCTATCGGCATGGTGGT	Azzari *et al*, 2010
Streptococcus pneumoniae serotype 7C	7C	CGTCAGGAATAGGTGCAATCTCT	TGAAATTCCAAGCGAAGCAA	TTCATCTATTGGTTCTTATGGTGTT	This Study
Streptococcus pneumoniae serotype 8	8	CCACTCATCAGTTTCCCATATGTTT	TCAATAATTGAAGAAGCGAACGTT	TGATGGCAGATGGGTTGGGACGAG	Azzari *et al*, 2010
Streptococcus pneumoniae serogroup 9A/L/N/V	9A/L/N/V	TGGAATGGGCAAAGGGTAGTA	TCGGTTCCCCAAGATTTTCTC	TTAATCATGCTAACGGCTCATCGA	Azzari *et al*, 2010
Streptococcus pneumoniae serotype 10A	10A	CCTCTCCTATCAACTATTACTCATTATACTACCT	AATAACCATAAGTCCCTAGATCATTCAAAG	TCATTACAACTCCCTATGTGACACGGGTCTTTT	Azzari *et al*, 2010
Streptococcus pneumoniae serogroup 11A/B/C/D/F	11ABCDF	ACCGCATTTCTTATCGCACTATATT	TCTCCTTACCATCAAACATGTTAATCA	TGAATCAGTCTGACCGTTT	This Study
Streptococcus pneumoniae serogroup 12A/B/F	12ABF	GATTATTCGCTTGCCTCTTCATG	ATAGCCGAAATAAGCTTTCCAGAA	ATTTGTAAGCGGACGTGCGATT	Azzari *et al*, 2010

Streptococcus pneumoniae serotype 13	13	TCGCATTTAGTAGTAACCCCTTGA	TTCTTGATTGAGGATGCATTTCC	AGTAGTAAGAGATCATATTCAAG	This Study
Streptococcus pneumoniae serotype 14	14	CGACTGAAATGTCACTAGGAGAAGAT	AATACAGTCCATCAATTACTGCAATACTC	TGTCATTCGTTTGCCAATACTTGATGGTCTC	Azzari *et al*, 2010
Streptococcus pneumoniae serogroup 15A/B/C/F	15ABCF	TTGAATCAGGTAGATTGATTTCTGCTA	CTCTAGGAATCAAATACTGAGTCCTAATGA	CTCCGGCTTTTGTCTTCTCTGT	Azzari *et al*, 2010
Streptococcus pneumoniae serogroup 16F	16F	GCAACTGGTATTTTTGATATTGGAGAA	CAAAGGAATGCCATGCCATA	AAAATGCTAACTTCGTTGGAGG	This Study
Streptococcus pneumoniae serotype 17F	17F	GTAAAGATTTCATGTCCTATAAGGGAGAA	AGGCGTCCCTGTTTATGAGAAG	TTGTACATGGTCTGGATTT	This Study
Streptococcus pneumoniae serogroup 18A/B/C	18ABC	CCTGTTGTTATTCACGCCTTACG	TTGCACTTCTCGAATAGCCTTACTC	AACCGTTGGCCCTTGTGGTGGA	Azzari *et al*, 2010
Streptococcus pneumoniae serotype 19A	19A	TTCGACGACGTATCAGCTTCA	TCATTGAGAGCCTTAACCTCTTCA	ACCCAAAACGGTTGACGCATTATACT	Azzari *et al*, 2010
Streptococcus pneumoniae serogroup 19B/F	19BF	GGTCATGCGAGATACGACAGAA	TCCTCATCAGTCCCAACCAATT	ACCTGAAGGAGTAGCTGCTGGAACGTTG	Azzari *et al*, 2010
Streptococcus pneumoniae serotype 20	20	AAAGATACTGGCTGAGGAGCTATCTATT	AGTCAAAAGTACTCAACCATTCTGATATATTC	AGGATAAGGTCTACTTTGTGGGAGTTC	Azzari *et al*, 2010
Streptococcus pneumoniae serotype 21	21	CCATTTGAAGGACCAGTTGTTG	AAAAAGCCACTATCAGGAATACCAA	AATGGCATTGCTTCGTAAA	This Study
Streptococcus pneumoniae serotype 22A/F	22AF	TCTATTAAATAACCCATTGGAATTGAAACG	TCGCAATTGAAGACCACATAAACTG	TCCGTAAT”T”CGCTTATGGGCACATTCTCCA	Azzari *et al*, 2010
Streptococcus pneumoniae serogroup 23A/B/F	23ABF	GGTGGACTTTCCGATGCAA	CACTGTCAACAAAAATGAGGTAATCTC	AAATGTCGGTATAGATAAAG	This Study
Streptococcus pneumoniae serotype 23F	23F	TGCTATTTGCGATCCTGTTCAT	AGAGCCTCCGTTGTTTCGTAAA	TTTCTCCGGCATCAAACGTTAAG	Azzari *et al*, 2010
Streptococcus pneumoniae serogroup 25A/25F/38	25AF/38	GTCTTACGTAGAACCTCTCTGGATGA	TGGTCCTACAAGCGACATGTG	TTGCCACAGATTTGGAATATT TTGGTCGG	This Study
Streptococcus pneumoniae serogroup 34/37/17A	34/37/17A	GGATACTATGTACGAACAGATGGACTTG	CTCACTAACTCGCCCGAATAAAC	CCGACTATACTCCATTTGA	This Study
Streptococcus pneumoniae serotype 35B	35B	GCATGGAGGTGGAGCATACA	TGTAAAGACTGCACAACTCGATATAAAA	CAATTTAAACAATATTAGTAAAGCGCAGGTCAAGCAAA	Azzari *et al*, 2010

Probe[a]: Minor Grove Binding (MGB) FAM labelled TaqMan probe.

5.1.4.1. Pre-amplification of DNA

Specific target amplification (STA) was done per manufactures recommendations as the initial step (pre-amplification of DNA) for the Biomark HD system (Fluidigm). Briefly two separate pools of primers were prepared by mixing 24X TaqMan assays in each, to a final concentration of 0.2X per assay (Table 5.2). PCR reactions were carried out in 5µL volumes each containing 2.5µL TaqMan PreAmp Master Mix (Fluidigm, CA, USA), 1.25µL of pooled assay and 1.25µL of DNA. Reactions were amplified with the T100 Thermal Cycler (Bio-Rad Laboratories, CA, USA) and cycling conditions included an initial activation at 95ºC for 10 minutes followed by 14 two-step cycles (denaturation at 95ºC for 15 seconds and annealing/extension at 60ºC for 4 minutes). STA products from the two assays were combined and diluted 1:5 with dH_2O and stored at -80 ºC.

Table 5.2: Primer pools for Specific Target Amplification (STA) to be used in an initial step (pre-amplification of DNA) for the Biomark HD system (Fluidigm)

Pool A	**Pool B**
Streptococcus pneumoniae (*lytA*)	GAPDH
Haemophilus influenzae	*Haemophilus influenzae* type D
Haemophilus influenzae type A	*Haemophilus influenzae* type F
Haemophilus influenzae type B	BexA
Haemophilus influenzae type C	*Staphylococcus aureus*
Haemophilus influenzae type E	*Moraxella catarrhalis*
Neisseria meningitidis	*Streptococcus pyogenes*
Bordetella holmesii	*Neisseria lactamica*
Streptococcus pneumoniae serotype 1	*Bordetella pertussis* and *Bordetella holmesii*
Streptococcus pneumoniae serotype 4	*Bordetella parapertusis*
Streptococcus pneumoniae serotype 5	*Bordetella pertussis*, *Bordetella parapertusis* and *Bordetella bronchiseptica*
Streptococcus pneumoniae serogroup 6A/B/C/D	*Streptococcus pneumoniae* serotype 3
Streptococcus pneumoniae serotype 7C	*Streptococcus pneumoniae* serogroup 6C/D
Streptococcus pneumoniae serotype 8	*Streptococcus pneumoniae* serogroup 7A/F
Streptococcus pneumoniae serogroup 9A/L/N/V	*Streptococcus pneumoniae* serogroup 11A/B/C/D/F
Streptococcus pneumoniae serotype 10A	*Streptococcus pneumoniae* serogroup 12A/B/F
Streptococcus pneumoniae serotype 14	*Streptococcus pneumoniae* serotype 13
Streptococcus pneumoniae serotype 19A	*Streptococcus pneumoniae* serogroup 15A/B/C/F
Streptococcus pneumoniae serogroup 19B/F	*Streptococcus pneumoniae* serogroup 16F
Streptococcus pneumoniae serotype 21	*Streptococcus pneumoniae* serotype 17F
Streptococcus pneumoniae serogroup 23A/B/F	*Streptococcus pneumoniae* serogroup 18A/B/C
Streptococcus pneumoniae serotype 23F	*Streptococcus pneumoniae* serotype 20
Streptococcus pneumoniae serogroup 25A/25F/38	*Streptococcus pneumoniae* serotype 22A/F
Streptococcus pneumoniae serogroup 34/37/17A	*Streptococcus pneumoniae* serotype 35B

5.1.4.2. Real-time qPCR using the Biomark HD System (Fluidigm)

The qPCR assays were carried out with 96.96 dynamic arrays (Fluidigm Corporation, CA, USA) according to manufactures instructions. Briefly 10X assay mixture and sample pre-mix were prepared, with each assay being prepared in duplicate. An IFC controller (Fluidigm) was used to prime the 96.96 dynamic arrays IFC Chip (Fluidigm) with control line fluid. 5µL of assay and sample mix was then transferred into the appropriate inlets of the primed chip and loaded with the IFC controller. After loading, the chip was placed in the Biomark instrument for quantitative PCR at 95ºC for 10 minutes followed by 40 cycles at 95ºC for 15 seconds and 60 ºC for 1 minute. The data was analysed with real-time PCR Analysis Software in the BIOMARK instrument (Fluidigm Corporation, CA, USA).

5.1.4.3. Primer optimisation, Standard curves and quantification in the nanofludic real-time PCR system.

Standard concentration curves and lower limits of detection (LLD) for each target bacteria were determined by amplifying four independent 10X serial dilutions of purified genomic DNA extracted from reference strains with the linear dynamic range being $10^1 – 10^7$ copies per standard curve. Target control DNA was harvested from early exponential phase cultures (OD_{600} = 0.1 for pneumococcus and OD_{600} = 1.0 for other bacteria), and the number of CFU/swab was estimated from Cq values relative to the standard curve, which is based on the quantitative culture of the control DNA.

Correlation coefficients (r^2) of each standard curve were determined and the amplification efficiency (e) of a given primer pair was calculated by $e = 10^{-1/m} -1$, where m is the slope of the standard curve. Lower limits of detection (LLD) or analytical sensitivity was determined by the minimum number of copies of DNA that could be measured repeatedly by the primers in the 10-fold serial dilutions. All primer pairs were tested with genomic DNA from all bacterial controls in a single Fluidigm run to determine the analytical specificity. Further, intra-assay variation (repeatability) was determined by running the same samples four times in the same assay run and presented as standard deviation (SD) for the copy number of the samples. A ratio of the difference between the observed copy number and the expected copy number was calculated to determine the accuracy of the assays, while the inter-assay variation (reproducibility) was determined by the SD of the copy number of the same sample tested in different runs. A summary of the criteria for performance acceptance by the Fluidigm assays are summarised in Table 5.3.

Table 5.3: Minimum acceptance criterion for the qPCR assays as defined by the MIQE* guidelines[243].

	Definitions/acceptance criterion according to MIQE guidelines.
Correlation coefficients	Correlation coefficients indicate the width of the spreading of individual data around the linear regression line. The higher the r^2 value the more robust the assay is. Acceptable range: $r^2 \geq 0.98$.
Efficiency	The PCR efficiency is dependent on the assay. Acceptable range: 90% -110%
Lower limit of detection	The limit of detection is the lowest amount of DNA in a sample that can be detected with reasonable certainty (95% probability) by the assay. The most sensitive LOD theoretically possible is 3 copies per PCR.
Specificity	Analytical specificity refers to the qPCR assay detecting the appropriate target sequence rather than other nonspecific targets also present in the sample. Acceptable assay: An assay that will only generate amplification signals for its intended target sequences and should not produce an amplification signal when the target specific assays are applied to individual PCRs of pure genomic DNA from other bacterial species.
Accuracy	The difference between experimentally measured and actual concentrations, presented as fold changes or copy number estimates. Acceptable range: The accuracy for all respective

	assays should be within ±0.1fold change
Repeatability	The precision and robustness of the assay with the same sample repeatedly analysed in the same assay and is expressed as a standard deviation for the Ct variance Acceptable range: SD<0.167
Reproducibility	The variations in the results of the same sample run in different runs/assays. Acceptable range: SD<0.167

*MIQE, Minimum Information for Publication of Quantitative Real-Time PCR experiments.

5.1.5. Statistical analysis

Statistical analysis was performed with STATA Version 11.0 (Statacorp, Texas, USA). The sensitivities of the standard qPCR assay and the nanofluidic qPCR were determined for all bacterial targets and pneumococcal serotypes that were tested by both methods with McNemar's test using qPCR as a reference. For density of carriage, geometric mean densities (GMD) and 95% confidence intervals (95% CI) of bacterial mean concentrations were calculated following $\log_{10}$ transformation on the data and the densities for bacterial targets and pneumococcal serotypes detected by qPCR and Fluidigm were compared with a paired student t-test. Results were considered significant when the *p*-value was < 0.05.

5.1.6. Ethics approval

Ethical consent for the initial enrolment of the cohort was obtained from the Medical Human Research Ethics Committee (HREC) of the University of Witwatersrand [Vaccinated cohort: HREC: 040704, also registered under Clinical trials number NCT00099658]. Approval for further testing of samples was obtained from the HREC (M140907). Written, informed consent had been obtained from the parents/guardians of all the study participants at the time of initial enrolment.

5.2. RESULTS

Nanofluidic qPCR analysis involved 335 (82.3%) of the initial 407 nasopharyngeal swabs collected at either 9 or 15-16 months of age (Figure 5.1). 83.4% children in whom samples were available, were black-African, and 49.1% were male. Due to similarities in findings at both 9 and 15-16 months of age, data was combined for main analysis.

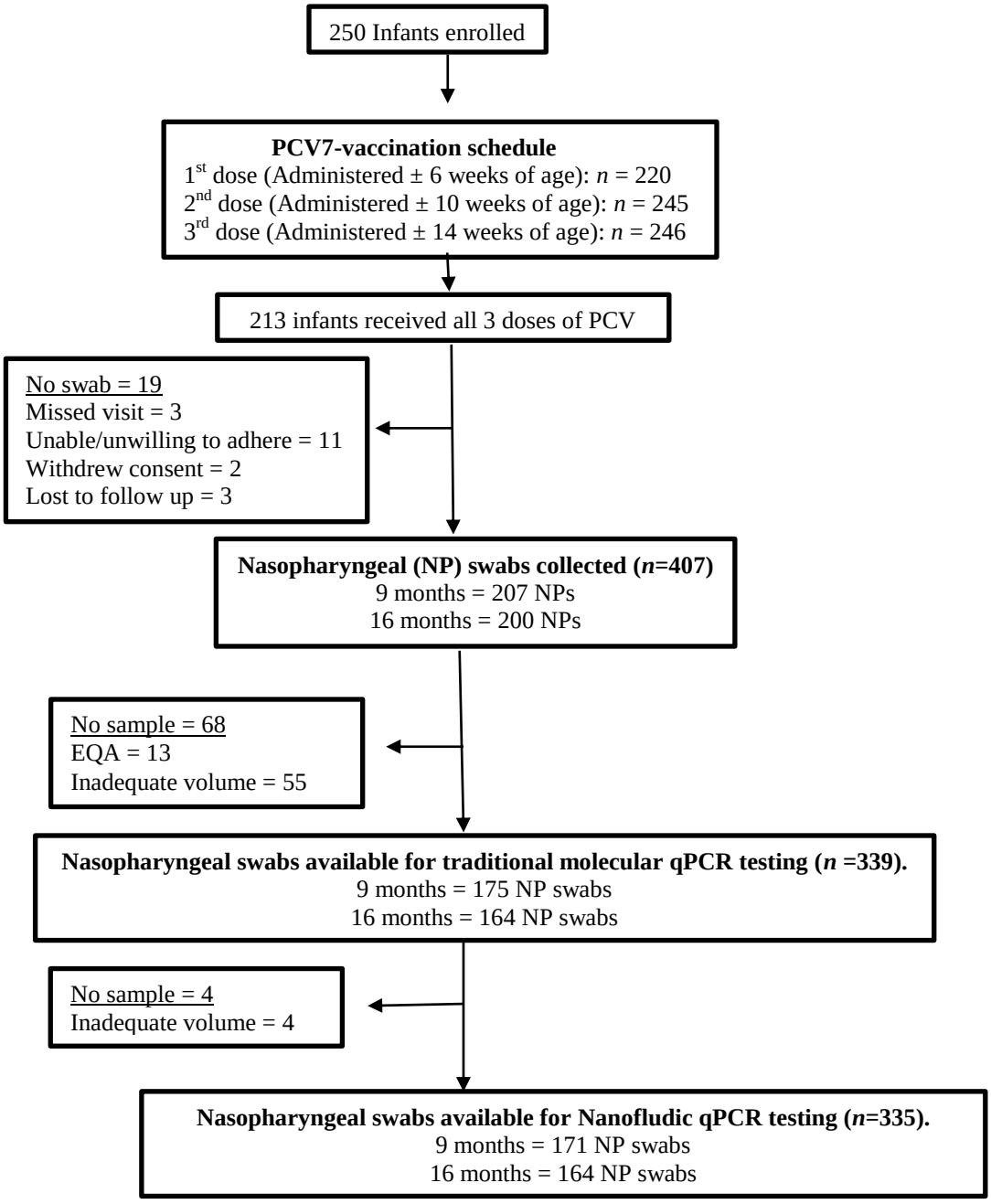


Figure 5.1: Schematic diagram of study population

Diagram indicating the number of children initially enrolled in the PCV7-vaccinated cohort of HIV-uninfected children, as well as the number of nasopharyngeal (NP) swabs available for subsequent traditional molecular qPCR analysis and Biomark HD system (Fluidigm). PCV7-vaccinated participants were excluded from analysis

if they did not receive all three doses of the pneumococcal conjugate vaccine (PCV) within protocol-defined window periods. The number of NP swabs available for molecular testing was defined by weather there was an adequate volume of sample remaining. EQA, samples were used for external quality assessment.

5.2.1. Performance of the nanofluidic real-time PCR system

All assays were effective in amplifying their respective targets with a high sensitivity, specificity and linearity in the Biomark HD system, with the efficiency of the assays ranging from 89% to 105% (Table 5.3). Within the linear dynamic range, the correlation coefficients (r^2) of the assays were 0.99, except for assays detecting *H. influenzae* E, IS481 (*B. pertussis* and *B. holmesii*) and *S. pneumoniae* serotype 19A, where r^2 was 0.98. All assays showed high analytical sensitivities with their respective primer and probe pairs with the limit of detection equivalent 10 copies per PCR for all respective assays, with exception to primers/probes that detected *H. influenzae* serotype E, IS481 (*B. pertussis* and *B. holmesii*) and pneumococcal serotypes/serogroups 5, 6A/B/C/D, 6C/D, 13 and 21 in which the lower limit of detection was 10 fold less (100 copies per PCR).

All primer pairs and probes were tested with genomic DNA from all bacterial controls in a single PCR run, and no cross reactions occurred (Table 5.4). Both the inter-assay variation (repeatability) and inter-assay variation (reproducibility) for all respective assays was <0.1, while the accuracy for all respective assays was within ±0.1 (Table 5.4).

Table 5.4: Performance of the nanofluidic real-time PCR system

Bacterial target	Efficiency (%)	Coefficient correlation (r2)	Limit of detection (copies/PCR)	Intra-assay variation (SD)	Accuracy (fold change)	Inter-assay variation (SD)
Streptococcus pneumoniae	104	0.99	10	0.011	0.028	0.045
Haemophilus influenzae	96	0.99	10	0.05	-0.062	0.09
Haemophilus influenzae type A	97	0.99	10	0.031	-0.025	0.038
Haemophilus influenzae type B	91	0.99	10	0.008	-0.078	0.041
Haemophilus influenzae type C	104	0.99	10	0.013	0.065	0.032
Haemophilus influenzae type D	101	0.99	10	0.002	0.083	0.03
Haemophilus influenzae type E	105	0.98	100	0.014	0.039	0.009
Haemophilus influenzae type F	93	0.99	10	0.03	0.05	0.068
Moraxella catarrhalis	97	0.99	10	0.02	-0.057	0.015
Staphylococcus aureus	96	0.99	10	0.007	0.004	0.049
Neisseria lactamica	100	0.99	10	0.063	0.023	0.026
Neisseria meningitidis	91	0.99	10	0.029	-0.049	0.13
ptxs1	99	0.99	10	0.025	-0.081	0.018
HIS1001	104	0.99	10	0.045	-0.071	0.131
PIS1001	91	0.99	10	0.009	0.068	0.021
IS481	99	0.98	100	0.042	0.013	0.12
Streptococcus pyogenes	105	0.99	10	0.018	-0.069	0.09
Streptococcus pneumoniae serotype 1	93	0.99	10	0.011	0.034	0.013
Streptococcus pneumoniae serotype 3	93	0.99	10	0.002	-0.049	0.05
Streptococcus pneumoniae serotype 4	96	0.99	10	0.002	0.06	0.023
Streptococcus pneumoniae serotype 5	105	0.99	100	0.012	0.055	0.041
Streptococcus pneumoniae serogroup 6A/B/C/D	94	0.99	100	0.011	-0.059	0.029
Streptococcus pneumoniae serogroup 6C/D	91	0.99	100	0.013	-0.026	0.047
Streptococcus pneumoniae serogroup 7A/F	98	0.99	10	0.008	-0.054	0.034
Streptococcus pneumoniae serotype 7C	90	0.99	10	0.027	-0.068	0.015
Streptococcus pneumoniae serotype 8	101	0.99	10	0.002	0.104	0.019
Streptococcus pneumoniae serogroup 9A/L/N/V	96	0.99	10	0.006	-0.059	0.016
Streptococcus pneumoniae serotype 10A	90	0.99	10	0.083	-0.001	0.005
Streptococcus pneumoniae serogroup 11A/B/C/D/F	93	0.99	10	0.027	0.011	0.021
Streptococcus pneumoniae serogroup 12A/B/F	89	0.99	10	0.003	0.045	0.029
Streptococcus pneumoniae serotype 13	96	0.99	100	0.011	-0.067	0.039
Streptococcus pneumoniae serotype 14	91	0.99	10	0.009	-0.027	0.027
Streptococcus pneumoniae serogroup 15A/B/C/F	102	0.99	10	0.078	-0.052	0.083
Streptococcus pneumoniae serogroup 16F	105	0.99	10	0.011	-0.033	0.025
Streptococcus pneumoniae serotype 17F	92	0.99	10	0.01	-0.041	0.01
Streptococcus pneumoniae serogroup 18A/B/C	99	0.99	10	0.014	-0.016	0.09
Streptococcus pneumoniae serotype 19A	90	0.98	10	0.018	0.077	0.025
Streptococcus pneumoniae serogroup 19B/F	98	0.99	10	0.004	0.009	0.021
Streptococcus pneumoniae serotype 20	103	0.99	10	0.014	0.069	0.003
Streptococcus pneumoniae serotype 21	96	0.99	100	0.111	0.083	0.122
Streptococcus pneumoniae serotype 22AF	99	0.99	10	0.019	-0.067	0.046
Streptococcus pneumoniae serogroup 23A/B/F	103	0.99	10	0.074	-0.081	0.022
Streptococcus pneumoniae serotype 23F	97	0.99	10	0.006	0.02	0.038
Streptococcus pneumoniae serogroup 25A/25F/38	101	0.99	10	0.015	0.012	0.087
Streptococcus pneumoniae serogroup 34/37/17A	101	0.99	10	0.001	-0.079	0.008
Streptococcus pneumoniae serotype 35B	100	0.99	10	0.013	0.005	0.029

Table 5.5: Analytical specificity of the nanofluidic real-time PCR system

| | Bacterial target | | |

5.2.2. Detection of bacterial nasopharyngeal carriage by conventional real-time PCR and nanofluidic real-time PCR (Fluidigm)

There was excellent concordance between conventional qPCR and Fluidigm for the detection of *S. pneumoniae* (*Kappa* = 0.98), *H. influenzae* (*kappa* = 0.97), *M. catarrhalis* (*Kappa* = 0.98), *S. aureus* (*kappa* = 0.98) and *S. pyogenes* (*kappa* = 0.96) and a high concordance between conventional qPCR and Fluidigm for the detection of *N. meningitidis* (*Kappa*=0.75; Figure 5.2, Table 5.5). Discordant results between the methods were strongly associated with density of carriage, with all additional bacteria detected either by qPCR or Fluidigm having an estimated copy numbers of $<10^2$ colony forming units (CFU)/ml per swab (Figure 5.3). The sensitivity and specificity of the Fluidigm method compared to conventional qPCR were high with all assays having a sensitivity >92% and a specificity of >99% (Table 5.5).

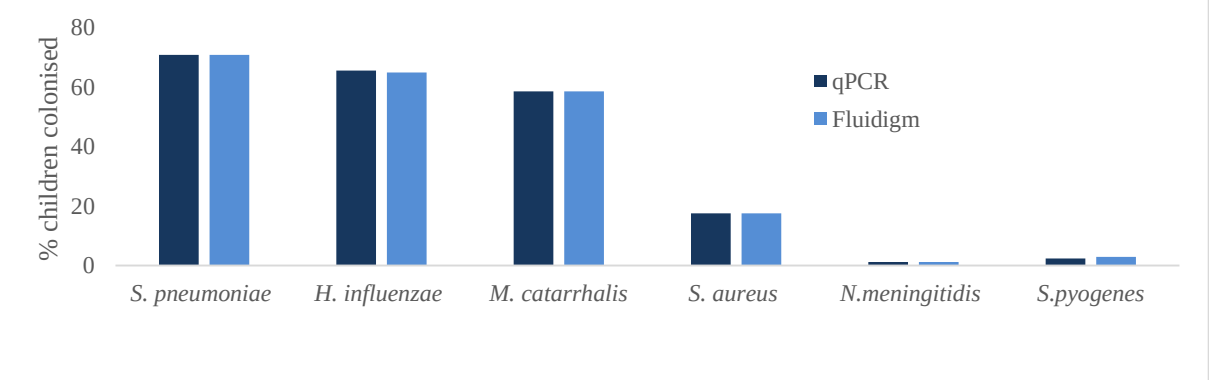


Figure 5.2: Prevalence of nasopharyngeal (NP) bacterial colonisation in PCV7-vaccinated, HIV-uninfected children as measured by traditional molecular real-time PCR (qPCR) and the Biomark HD System (Fluidigm)

p-values of <0.05 was considered significant as determined by McNemar's test.

Table 5.6: Concordance between traditional real-time PCR (qPCR) and the Biomark HD System (Fluidigm) for the detection of common of nasopharyngeal pathogens in PCV7-vaccinated, HIV-uninfected children

Bacterial Target	qPCR*	Fluidigm*	*p*-value	Concordance (*kappa*)	Sensitivity (%)	Specificity (%)
S. pneumoniae	244(72.8)	246(73.4)	0.16	0.98	98.8	100
H. influenzae	230(68.7)	232(69.3)	0.32	0.97	98.7	99
M. catarrhalis	189(56.4)	192(57.3)	0.08	0.98	98.4	100
S. aureus	57(17)	55(16.4)	0.16	0.98	100	99.3
S. pyogenes	11(3.3)	12(3.6)	0.32	0.96	91.7	100
N. meningitidis	3(0.9)	5(1.5)	0.16	0.75	91.7	100

* Values are number (%).

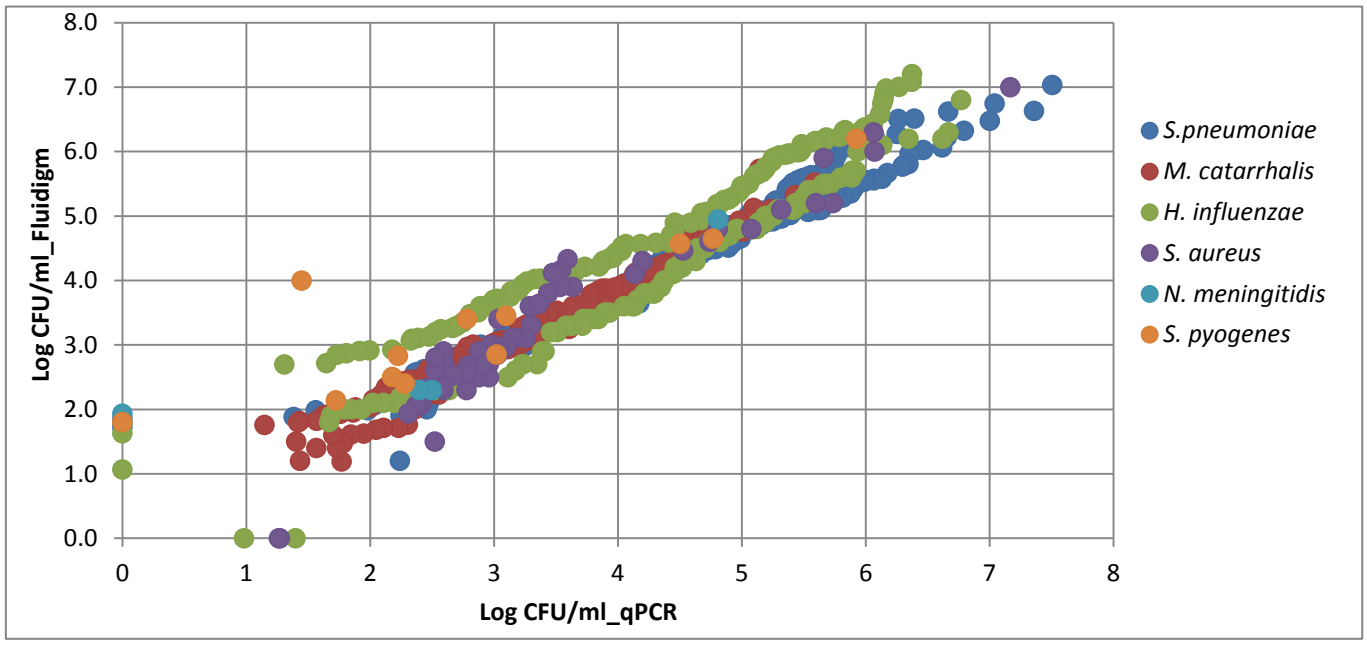


Figure 5.3: Density of colonisation by common nasopharyngeal bacterial pathogens as determined by traditional real-time PCR (qPCR) and the Biomark HD System (Fluidigm)

B. bronchiseptica, B. parapertusis, B. pertussis, B. holmesii and *N. lactamica* were not tested by conventional qPCR and thus could not be compared to Nanofluidic real-time PCR; however, the overall detection prevalence by Fluidigm was 0.3%, 0%, 0%, 0%, and 4.2 % respectively. Further, *H. influenzae* was not serotyped by conventional qPCR and thus could not be compared to Fluidigm; however, of the *H. influenzae* positive samples, 3.9% were

serotype A, 1.3% were serotype B, 2.6% were serotype C, 1.3% were serotype D, 1.7% were serotype E, 4.3% were serotype F and 83.6% were non-typable.

5.2.3. Detection of pneumococcal serotype carriage by conventional real-time PCR and nanofluidic real-time PCR (Fluidigm)

There was excellent concordance between conventional qPCR and Fluidigm for all the assays detecting *S. pneumoniae* serotypes/serogroups (*kappa* >0.8), with exception of primers/probes detecting serotypes/serogroups 1, although concordance was still high (*kappa* =0.75); Figure 5.4, Table 5.7. The Fludigm method, however, detected an additional 7 serotypes above those identified by qPCR while the qPCR method detected an additional 2 serotypes above those detected by Fluidigm.

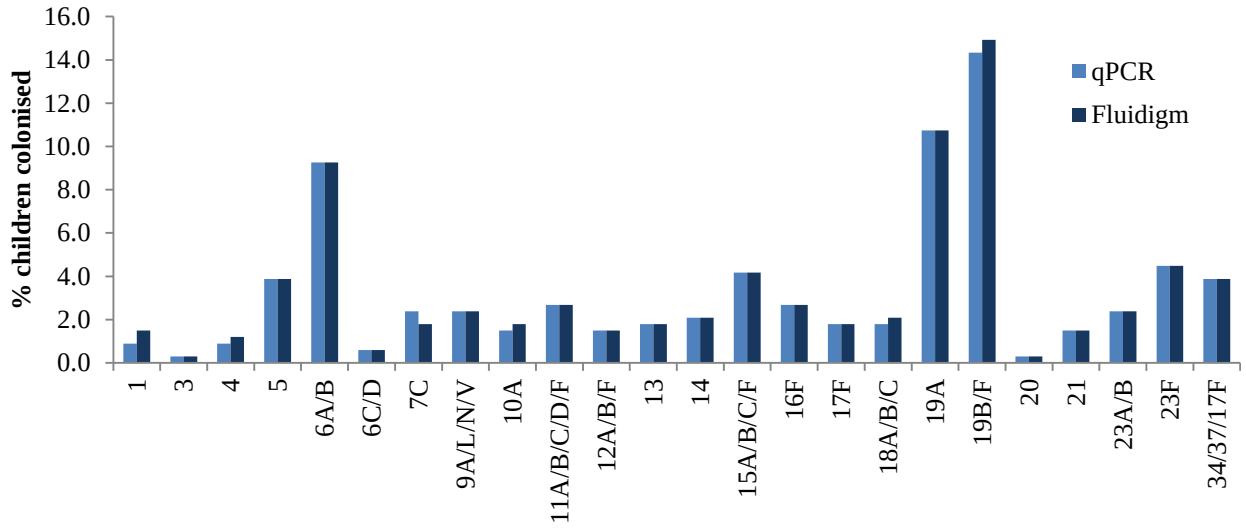


Figure 5.4: Prevalence of pneumococcal serotype colonisation in PCV7-vaccinated, HIV-uninfected children as measured by traditional molecular real-time PCR (qPCR) and the Biomark HD System (Fluidigm)

p-values of <0.05 was considered significant.

Table 5.7: Concordance between traditional real-time PCR (qPCR) and the Biomark HD System (Fluidigm) for the detection of pneumococcal serotypes in PCV-vaccinated, HIV-uninfected children

Serotype	qPCR *	Fluidigm*	*p*-value	Concordance (*kappa*)	Sensitivity (%)	Specificity (%)
LytA	244(72.8)	247(73.7)	0.08	0.98	98.8	100
1	3(0.9)	5(1.5)	0.16	0.75	60	100
3	1(0.3)	1(0.3)	-	1	100	100
4	3(0.9)	4(1.2)	0.32	0.86	75	100
5	13(3.9)	13(3.9)	-	1	100	100
6A/B	31 (9.3)	31 (9.3)	-	1	100	100
6C/D	2(0.6)	2(0.6)	-	1	100	100
7C	8(2.4)	6(1.8)	0.16	0.85	100	99.3
9A/L/N/V	8(2.4)	8(2.4)	-	1	100	100
10A	5(1.5)	6 (1.8)	0.32	0.91	83.3	100
11A/B/C/D/F	9(2.7)	9(2.7)	-	1	100	100
12A/B/F	5(1.5)	5(1.5)	-	1	100	100
13	6(1.8)	6(1.8)	-	1	100	100
14	7(2.1)	7(2.1)	-	1	100	100
15A/B/C/F	14(4.2)	14(4.2)	-	1	100	100
16F	9(2.7)	9(2.7)	-	1	100	100
17F	6(1.8)	6(1.8)	-	1	100	100
18A/B/C	6(1.8)	7(2.1)	0.32	0.93	85.7	100
19A	36(10.7)	36(10.7)	-	1	100	100
19B/F	48(14.3)	50(14.9)	0.08	0.96	94	100
20	1(0.3)	1(0.3)	-	1	100	100
21	5(1.5)	5(1.5)	-	1	100	100
23A/B	8(2.4)	8(2.4)	-	1	100	100
23F	15(4.5)	15(4.5)	-	1	100	100
34/37/17F	13(3.9)	13(3.9)	-	1	100	100

* Numbers are value (%)

Discordant results between the methods were strongly associated with density of carriage, with all additional serotypes/groups detected either by qPCR or Fluidigm having estimated copy numbers of $<10^2$ colony forming units (CFU)/ml per swab (Figure 5.5). The sensitivity and specificity of the Fluidigm method compared to conventional qPCR were high with the majority of the assays having a sensitivity > 80%, with exception of serotypes 1 and 3 which had sensitivities of 60% and 75% respectively. All Fluidigm assays, however, had a high specificity (≥99.3%), Table 5.6.

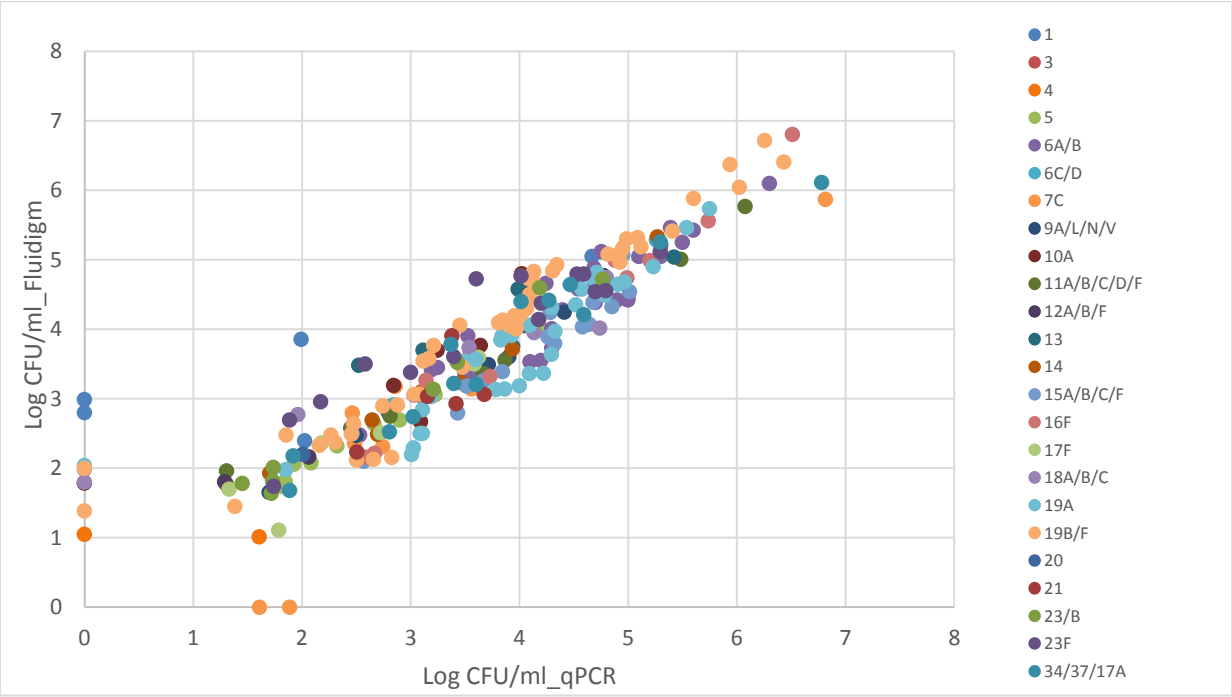


Figure 5.5: Density of colonisation of pneumococcal serotypes as determined by traditional real-time PCR (qPCR) and the Biomark HD System (Fluidigm)

Serotypes/serogroups 7A/F, 8, 22A/F, 25A/25F/38 and 35B were not tested by conventional qPCR and thus could not be compared to nanofluidic real-time PCR; however, the overall detection prevalence by Fluidigm was 0.3%, 0.9%, 0.6%, 2.7% and 0.3% respectively for these serotypes

5.2.4. Density of bacterial nasopharyngeal carriage as determined by conventional qPCR and Fluidigm

There was excellent correlation between the two methods (*Rho* >0.95) for measuring the density of carriage with no significant differences found for all bacterial pathogens and pneumococcal serotypes measured by conventional qPCR and Fluidigm (Table 5.8).

Table 5.8: Density of bacterial nasopharyngeal carriage as determined by conventional molecular real-time Polymerase chain reaction and the Biomark HD System (Fluidigm)

Bacteria	Density detected by qPCR	Density detected by Fluidigm	*p*-value
S. pneumoniae	4.54(4.37-4.70)	4.39(4.24-4.54)	0.49
H. influenzae	4.38(4.21-4.55)	4.56(4.39-4.73)	0.18
M. catarrhalis	3.38(3.23-3.52)	3.29(3.15-3.44)	0.37
S. aureus	3.48(3.17-3.79)	3.41(3.08-3.75)	0.13
S. pyogenes	3.09(2.14-4.03)	3.68(3.04-4.32)	0.11
N. meningitidis	3.24(-0.13-6.61)	2.96(0.20-5.73)	0.18
Pneumococcal serotypes/serogroups			
1	2.90(-0.92-6.71)	3.76(0.46-7.07)	0.22
3	ND	ND	-
4	2.85(-0.27-5.97)	2.30(-1.20-7.20)	0.79
5	2.52(2.01-3.02)	2.46(2.0-2.91)	0.09
6AB	4.25(3.88-4.61)	4.07(3.77-4.36)	0.06
6CD	4.05(-1131-19.42)	3.60(-5.11-12.30)	0.54
7C	3.49(1.73-5.25)	3.27(1.89-4.67)	0.33
9ALVN	3.31(2.52-4.10)	3.23(2.52-3.95)	0.16
10A	3.23(2.47-3.98)	3.62(2.64-4.1)	0.17
11ABCDF	3.29(2.03-4.56)	3.21(2.16-4.27)	0.51
12ABF	3.38(1.39-5.38)	3.58(1.76-5.40)	0.19
13	3.96(2.85-5.06)	4.03(3.13-4.94)	0.82
14	3.40(2.06-4.74)	2.93(2.37-3.48)	0.29
15ACF	3.81(2.93-4.68)	3.54(3.01-4.07)	0.08
16F	4.50(3.43-5.56)	4.23(3.01-5.44)	0.14
17F	2.54(1.55-3.53)	2.55(1.67-3.44)	0.88
18ABC	3.84(2.39-5.28)	3.84(2.96-4.73)	0.98
19A	3.82(3.48-4.17)	3.75(3.4-4.10)	0.26
19BF	3.87(3.49-4.24)	4.14(3.78-4.51)	0.66
20	ND	ND	-
21	3.38(2.23-4.53)	3.13(2.58-3.68)	0.55
23AB	2.92(-0.76-4.92)	2.58(0.31-4.86)	0.16
23F	3.64(3.12-4.16)	3.98(3.44-4.51)	0.18
34/37/17A	3.90(3.08-4.72)	3.41(2.88-3.94)	0.06

Numbers are geometric mean densities (95% Confidence intervals). ND, not done as too few observations to measure 95% confidence intervals. *p*-values of <0.05 were considered significant as determined by a paired student t-test.

5.3. DISCUSSION

A reliable nanofluidic real-time PCR system that was able to simultaneously detect 11 bacterial pathogens, 53 pneumococcal serotypes (16 individual serotypes and 37 serotypes in 13 groups) and 6 serotypes of *H. influenzae* (serotypes a-f) in 96 different samples (92 samples and 4 controls) in a single PCR run was successfully established according to MIQE guidelines[243]. Further, findings from traditional quantitative PCR and Fluidigm in a PCV7-vaccinated cohort of black-African children were compared and excellent concordance between the methods was found for both the carriage prevalence and density for bacterial species and pneumococcal serotypes.

There was however some discordance between the methods with the qPCR method detecting an additional 3 bacterial species and 2 pneumococcal serotypes above those detected by Fluidigm, and the Fluidigm method detecting an additional 11 bacterial species and 7 pneumococcal serotypes above those detected by qPCR. Nevertheless, these small differences (<5%) in the detection by the methods are expected as according to Poisson's distribution the assays had a 95% probability of detecting their respective targets with reasonable certainty [243], which was further supported by the discordance between the methods being associated with a lower carriage density ($<10^2$CFU/ml). Further, the Fluidigm method involved a pre-amplification step which pre-selected for its' respective targets and may explain why the method was able to detect additional bacteria and pneumococcal serotypes/serogroups of a lower carriage density above those detected by the qPCR method.

While culture remains the gold standard for bacterial carriage and pneumococcal serotype characterisation, these assays lack quantitative data which limits our understanding of nasopharyngeal carriage and its relation to disease severity [18,23-26]. Recently several molecular methods have been developed to overcome the shortcomings of these traditional culture

assays; however, they are still limited in that a large sample volume is required to distribute across all reactions, it is expensive and processing multiple qPCR reactions is labour intensive and time consuming (Chapter 2). Further, there are a limited number of quantitative studies that have described colonisation of a diverse number of bacterial pathogens and pneumococcal serotypes together. The Fluidigm assay was, however, able to overcome some of the shortcomings of traditional qPCR assays as it allows for a larger number of reactions per plate, thus making it more cost effective and less time consuming.

The Biomark HD system is a new instrument that has only been used to serotype pneumococcus by one other group; however, their assay differed in that they used Evagreen (a dsDNA-binding dye) chemistry instead of TaqMan probes to quantify bacterial carriage, it was only able to detect 50 pneumococcal serotypes (17 individual serotypes and 33 serotypes in 12 groups) and did not include any additional bacterial pathogens [43,205]. However, in terms of performance the assays were comparable.

The study was limited in that the PCR method was not able to discriminate between all the serotypes within their respective serogroups. Further, as an initial step for gene expression on the Biomark HD Fluidigm system, manufacturers recommend STA for each sample to be done by combining all primers in a pool. However, due to the high similarity between some pneumococcal primers, non-specific binding of some primers to each other resulted which was prevented by separating primers into two separate pools and then combining the STA products from the two assays. Careful monitoring and optimisation is thus needed in future studies especially if more pneumococcal targets are to be added. Lastly when the sensitivity of some of the assays was lower in generally involved serotypes/groups with a lower prevalence of colonisation and thus our study was limited in power to have certainty. As this was a retrospective study, there was no residual sample after testing available to retest the samples

and to confirm the discordant results between the methods which would have been beneficial in providing clarity.

In conclusion we developed a highly sensitive and specific assay that could simultaneously detect common pneumococcal serotypes, *H. influenzae* serotypes and other common nasopharyngeal bacterial pathogens and provide quantitative data in a single PCR run. Improved methods of detection of respiratory pathogens can provide a means to improve the health care of patients as optimal therapy can be initiated sooner, effectiveness of the treatment is improved and spread of disease can be reduced or even prevented [206]. Further, a high bacterial load detected by real-time PCR in nasopharyngeal specimens might help predict the aetiological role for *S. pneumoniae* and other bacterial pathogens in bacterial disease [207].

CHAPTER 6: EVALUATION OF THE ASSOCIATION OF HIV-1 INFECTION AND DENSITY OF COMMON NASOPHARYNGEAL BACTERIAL COLONISERS USING THE BIOMARK HD NANOFLUDIC SYSTEM IN PCV7-IMMUNISED CHILDREN LIVING IN SOUTH AFRICA

Approximately 5% of children <5 years of age in South Africa are HIV-infected, with *S. pneumoniae* being the most important cause of invasive bacterial disease and the burden of disease being 40-fold greater in HIV-infected compared to HIV-uninfected children, even in the era of PCV [244]. A limited number of comparative studies have investigated the association between HIV-infection and pneumococcal colonisation, with conflicting results on whether HIV-infection is associated with differences in prevalence of pneumococcal nasopharyngeal colonisation compared to HIV-uninfected children [186]. While some studies have reported no difference in the carriage prevalence between HIV-infected and HIV-uninfected children, others from South Africa and The Gambia reported a higher prevalence of pneumococcal colonisation in HIV-infected children [77,187]. Further, the effect that HIV-infection has on the prevalence of nasopharyngeal colonisation by other bacteria such as *H. influenzae* and *S. aureus* is also unclear. Whereas Madhi *et al* reported no difference in the carriage prevalence of *S. aureus* between HIV-infected and HIV-uninfected children in South Africa [77], others have reported higher prevalence of colonisation from South Africa and Tanzania in HIV-infected children [191,193,196]. Also, some studies have reported HIV-infected children to have a higher prevalence of *H. influenzae* colonisation [77], while others have reported a lower carriage prevalence of *H. influenzae* [196,198] or no difference between HIV-infected and -uninfected children [191].

These studies were, however, limited in having only used routine culture as recommended by WHO, [36] which might be less sensitive than molecular detection methods and also does not provide insight into the density of colonisation. Also, the majority of these studies involved a population that were not immunised for PCV; and thus further investigation with more sensitive molecular methods on the association between HIV-infection and pneumococcal carriage prevalence and density in a PCV immunised cohort is needed.

In this chapter, we applied a Nanofludic real-time PCR assay to evaluate the effect of HIV-infection and HIV-exposure on prevalence and density of nasopharyngeal colonisation at 9 and 16 months of age by common, potentially pathogenic bacteria including PCV7 pneumococcal serotypes, non-PCV7 serotypes, *Haemophilus influenzae*, non-typable

Haemophilus influenzae, Staphylococcus aureus, Moraxella catarrhalis, Streptococcus pyogenes, Neisseria meningitidis, Neisseria lactamica, Bordetella pertussis, Bordetella parapertusis, Bordetella bronchiseptica and *Bordetella holmesii.*

6.1. MATERIAL AND METHODS

6.1.1. Study population

Archived nasopharyngeal swabs collected from a PCV7-vaccinated cohort of HIV-infected and HIV-uninfected infants in South Africa were retrospectively analysed. Detailed information of the cohort has been described [142,185]. Briefly, participants included HIV-infected infants with a $CD4^+$ T-lymphocytes cells ≥25% randomised to initiate ART immediately (ART-Immed); or when clinically and or immunologically indicated as per then WHO recommendations for ART initiation in children (ART-Def), HIV-exposed-uninfected (HEU) and HIV-unexposed-uninfected (HUU) infants who were 6 – 12 weeks old when enrolled between April 2005 and June 2006. Infants were scheduled to receive three doses of PCV7 (i.e. Prevnar®; Wyeth Vaccines, NJ, USA) at 6, 10 and 14 weeks of age, as well as other recommended childhood vaccines in South Africa, including *H. influenzae* type-b conjugate vaccine (HibCV)[185,245]. During the course of the study, PCV immunisation of children in Soweto (birth cohort 28,000 per annum) was limited mainly to study-participants (approximately 600), as the vaccine was only introduced into the pubic immunisation program in May 2009 [218].

Nasopharyngeal (NP) swabs were taken at several time intervals, including at 9 and 15-16 months of age. Swabs were stored in skim milk-tryptone-glucose-glycerol (STGG) transport media at the Respiratory and Meningeal Pathogen Research Unit (RMPRU) in South Africa, as recommended by WHO [212].

6.1.2. Real-time qPCR using the Biomark HD System (Fluidigm)

Stored nasopharyngeal swab samples were thawed and processed. Briefly, NP swabs were vortexed for 30 seconds to release bacteria into the transport media and total nucleic acids were automatically extracted from STGG with the NucliSens ® easyMAG ® extraction system (BioMérieux, Marcy l'Etoile, France) according to manufacturer's instructions. Samples were stored at – 20 °C. DNA extracts were quantified for 11 bacterial pathogens, 53 pneumococcal serotypes (16 individual serotypes and 37 serotypes in 13 groups) and 6

serotypes of *H. influenzae* (serotypes a-f) with the Nanofludic real-time qPCR system as described in Chapter 5. Briefly, Specific target amplification (STA) was done per manufactures recommendations as the initial step (pre-amplification of DNA) for the Biomark HD system (Fluidigm). The qPCR assays were then carried out on the STA products with 96.96 dynamic arrays (Fluidigm Corporation, CA, USA) according to manufactures instructions and bacterial loads were quantified using standard curves. Data was analysed with Real-Time PCR Analysis Software in the BIOMARK instrument (Fluidigm Corporation, USA).

6.1.3. Statistical analysis

Statistical analysis was performed with STATA Version 11.0 (Statacorp, Texas, USA). Pearson X^2 test or student t-test was used to compare baseline characteristics between the cohorts. Comparisons and adjusted odd ratios of prevalence of pneumococcal and bacterial colonisation between cohorts were analysed using logistic regression models adjusted for covariates, including race, gender, study centre, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. Since we did not identify any difference in the prevalence and density of colonisation for all bacteria between the ART-immed and ART-Def groups, as well as between the HIV-exposed uninfected and HIV-unexposed uninfected groups in our analysis, we subsequently analysed these children as a composite group of HIV-infected and HIV-uninfected children respectively (Appendices D & E). The associations of nasopharyngeal bacterial colonisation between groups were determined by multivariate logistic regression models controlling for the above mentioned cofounders. For density of carriage, geometric mean densities (GMD) and 95% CI intervals (95%) of pneumococcal and bacterial mean concentrations were calculated following $\log_{10}$ transformation on the data, using analysis of covariance to adjust for possible covariates. Spearman correlations were used to compare the degree of correlation between densities of each pair of bacteria. Results were considered significant when the *p*-value was <0.05.

6.1.4. Ethics approval

As described in chapter 5, section 5.16.

6.2. RESULTS

Nanofluidic qPCR analysis was undertaken on 763 (85.1%) of the initial 897 nasopharyngeal swabs collected at either 9 or 15-16 months of age (Figure 6.1). Within each cohort, there were no differences in the colonisation patterns done by standard methodology, between those in whom samples were available and not available (Appendices B and C). Ninety percent of the children for whom samples were available, were black-African, and 45.8% were male (Table 6.1). The mean age in weeks at time of sample collection did not differ between groups, being 39.6 (standard deviation; SD 1.5) and 39.3 (SD 3.2) weeks at 9 months of age (p=0.13) and 67.9 (SD 1.9) and 67.7 (1.6) weeks at 15-16 months of age (p=0.15) in HIV-infected and HIV-uninfected children, respectively. The mean birth weight was lower in HIV-infected (2967grams) than their HIV-uninfected counterparts (3092 grams; p=0.02) at 9 months and at 15-16 months of age (2958 vs. 3126 grams; p=0.001). Co-trimoxazole prophylaxis was more frequently administered in HIV-infected children at both 9 months (99% vs. 46.2%; p<0.001) and 15-16 months (98.6% vs. 41.4%; p<0.001) of age. Furthermore, breastfeeding was higher in HIV-uninfected children than HIV-infected children at 9 months (25.4% vs. 1.4%; p<0.001) and 15-16 months of age (23.2% vs. 1.4%%; p<0.001).

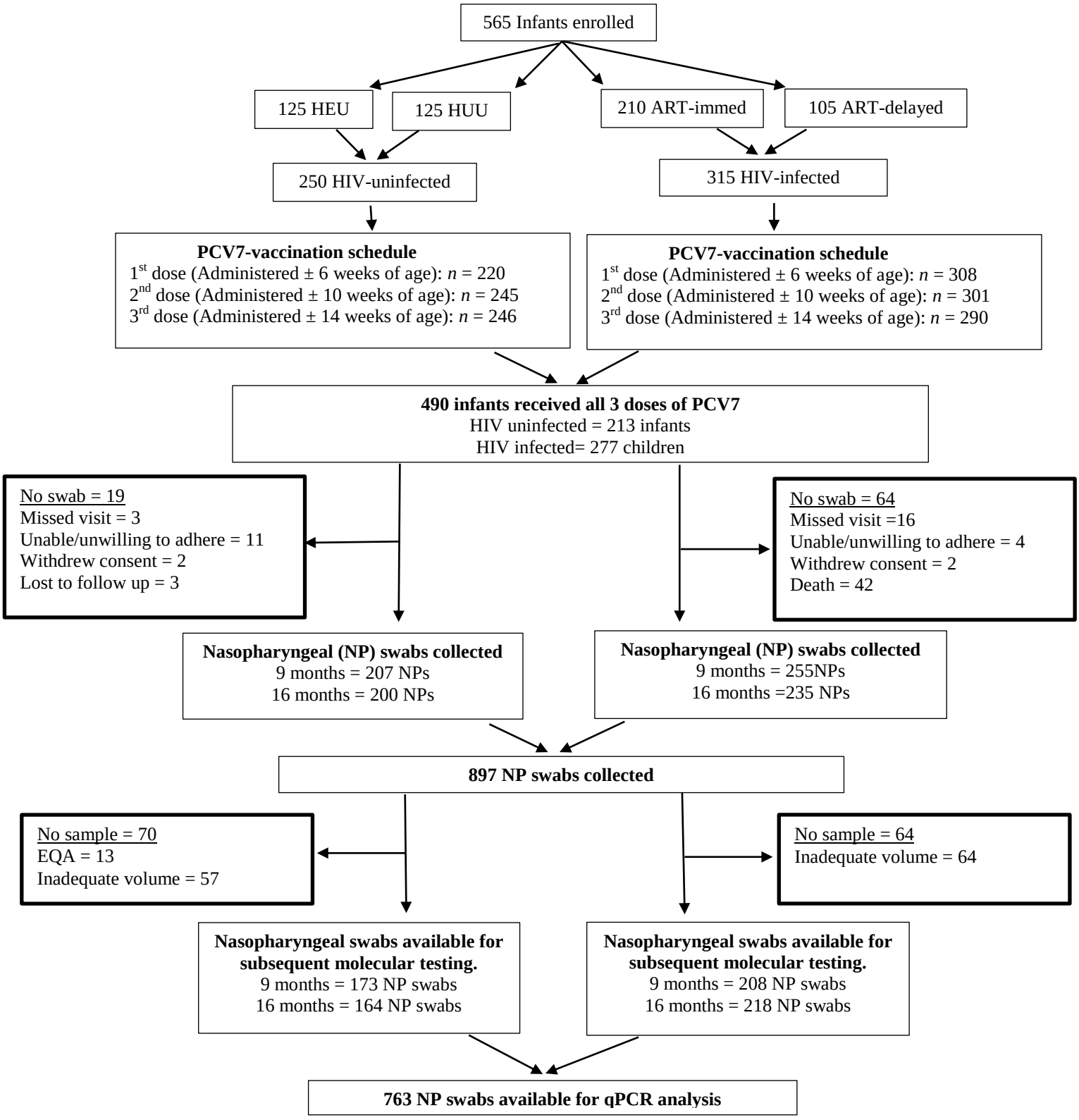


Figure 6.1: Schematic diagram of study population

Diagram indicating the number of children initially enrolled in a PCV7-vaccinated cohort of HIV-uninfected and HIV-infected children, as well as the number of nasopharyngeal (NP) swabs available for subsequent nanofluidic real-time PCR analysis. Participants were excluded from analysis if they did not receive all three doses of the pneumococcal conjugate vaccine (PCV) within protocol-defined window periods. The number of NP swabs available for molecular testing was defined by whether there was an adequate volume of sample remaining. HUU, HIV-unexposed uninfected. HEU, HIV-exposed uninfected. EQA, samples were used for external quality assessment.

Table 6.1: Demographic features at two study visits when analysed for nasopharyngeal (NP) bacterial colonisation in HIV-infected and HIV-uninfected, PCV7-vaccinated children

	9 month old children			16 month old children		
	HIV-infected	HIV-uninfected	*p*-value	HIV-infected	HIV-uninfected	*p*-value
Available samples for molecular analysis	208	173		218	164	
Mean birth weight (SD) grams	2967(489)	3092(448)	0.02	2958(451)	3126(476)	0.001
Male sex (%)	80(45.5)	96 (55.5)	0.001	85(39)	89(54.3)	0.003
Race: Black African (%)	199 (95.7)	148(85.5)	0.001	211(96.8)	132(80.5)	<0.001
: Mixed ancestry (%)	9(4.3)	25(14.5)		7(3.2)	32(19.5)	
Smoking household contact (%)	90(43.3)	79(45.7)	0.49	92(44.4)	64(39)	0.57
Co-trimoxazole prophylaxis (%)	206(99)	80(46.2)	<0.001	215(98.6)	68(41.4)	<0.001
Mean number of household contacts (SD)	5.1(2.4)	5.3(2.3)	0.53	5.0(1.9)	5.1(2.4)	0.67
Breast feeding (%)	3(1.4)	44(25.4)	<0.001	3(1.4)	38(23.2)	<0.001
Mean age (SD) in weeks at visit	39.6(1.5)	39.3(3.2)	0.23	67.9(1.9)	67.7(1.6)	0.25

Pearson X^2 test or student t-test was used to compare baseline characteristics between the cohorts and demographic features with a *p*-value <0.2 were included as possible cofounders in multivariate analysis.

6.2.1. Prevalence of bacterial colonisation with respect to HIV-status

6.2.1.1. *Streptococcus pneumoniae* colonisation

In the multivariate adjusted analysis, prevalence of overall pneumococcal colonisation was lower in the composite groups of HIV-infected (58.6%) than HIV-uninfected children (69.9%, p=0.02; Table 6.2) at 9 months of age, largely due to the lower prevalence of non-vaccine-serotype colonisation (27.8% vs. 40%, respectively; p=0.047), whilst there was no difference in prevalence of vaccine-serotype colonisation (37.5% vs. 36%; p=0.48). The serotype-specific analysis was limited by the low number of serotypes identified in each group; however, the prevalence of non-vaccine serogroup 15A/B/C/F was 3.3 (8.2% vs. 3.5%; p=0.029) fold higher in HIV-infected than HIV-uninfected children at 9 months of age (Figure 6.2). By 16 months of age, there was no difference in the overall pneumococcal colonisation between the composite groups of HIV-infected and HIV-uninfected children (p=0.26), although the prevalence of non-vaccine serotype colonisation remained 1.7 fold higher in HIV-uninfected (60.4%) than HIV-infected children (50.9%, p=0.049; Table 6.2). Other differences included that the prevalence of vaccine serogroup 6A/B was 3.7 fold higher in HIV-uninfected (7.9%) than HIV–infected children (2.3%; p=0.03; Figure 6.3).

6.2.1.2. H. influenzae

Prevalence of overall *H. influenzae* colonisation was lower in the composite groups of HIV-infected (42.3%) than HIV-uninfected children at 9 months of age (64.2%, p=0.003; Table 6.2), which was largely due to the lower prevalence of non-typable *H. influenzae* colonisation (37.5% vs. 54.8%, p=0.006). Similarly, the prevalence of overall *H. influenzae* colonisation was also lower in the composite groups of HIV-infected (56%) than HIV-uninfected children at 16 months of age (73.4%, p=0.018), which was also largely due to lower prevalence of non-typable *H. influenzae* colonisation (50% vs. 59.2%, p=0.04).

6.2.1.3. Other bacterial nasopharyngeal colonisers

No difference in the carriage prevalence of *S. aureus, S. pyogenes, N. meningitidis, N. lactamica, B. pertussis, B. parapertusis, B. holmesii* and *B. bronchiseptica* were found between HIV-infected and HIV-uninfected groups at 9 and 16 months of age respectively (Table 6.2); however, the carriage prevalence of *M. catarrhalis* was lower in the in HIV-infected (43.3% than -uninfected children (57.8%, p=0.03) at 9 months of age only.

Table 6.2: Prevalence of nasopharyngeal (NP) colonisers in HIV-infected and HIV-uninfected, PCV7- vaccinated children

	9 month old children				16 month old children			
	HIV-infected *n* = 208	HIV-uninfected *n* = 173	OR (95% CI); *p*-value	aOR (95% CI); *p*-value	HIV-infected *n = 218*	HIV-uninfected *n= 164*	OR (95% CI); *p*-value	aOR (95% CI); *p*-value
S. pneumoniae	122(58.6)	121(69.9)	0.61(0.40-0.93); *p*=0.02	0.58(0.35-0.96); *p*=0.04	149(68.4)	125(76.2)	0.67(0.43-1.07); *p*=0.09	0.74(0.45-1.24); *p*=0.26
VT	78(37.5)	63(36)	1.20(0.75-1.91): *p*=0.47	1.24(0.71-2.18); *p*=0.48	74(33.9)	55(33.5)	1.27(0.80-2.03); *p*=0.31	0.85(0.50-1.44); *p*=0.55
NVT	58(27.8)	70(40)	0.57(0.36-0.89); *p*=0.01	0.59(0.35-1.01);*p*=0.047	111(50.9)	105(64)	0.59(0.38-.90); *p*=0.015	0.65(0.41-1.04); *p=0.049*
H. influenzae	88(42.3)	111(64.2)	0.44(0.29-0.67); *p<0.001*	0.48(0.29-0.79); *p=0.003*	122(56)	121(73.4)	0.45(0.29-0.70); *p*<0.001	0.50(0.31-0.81); *p*=0.005
Hinf A	0	1(0.6)	-	-	4(1.8)	8(4.9)	0.36(0.11-1.23); *p*=0.1	0.45(0.12-1.73); *p*=0.25
Hinf B	3(3.4)	2(1.2)	1.25(0.21-7.57):*p*=0.81	1.56(0.15-15.8); p=0.7	1(0.5)	1(0.6)	0.75(0.05-12.1); *p*=0.84	0.49(0.23-8.98); *p*=0.63
Hinf C	1(1)	0	-	-	0	6(3.7)	-	-
Hinf D	2(2.3)	1(0.9)	1.67(0.15-18.57):*p*=0.68	2.57(0.22-30.67); *p*=0.46	2(0.9)	2(1.2)	0.75(0.10-5.38); *p*=0.78	0.49(0.06-3.98); *p*=0.51
Hinf E	1(1.1)	2(1.2)	0.41(0.04-4.59):*p*=0.47	0.33(0.02-5.97); *p=0.45*	1(0.5)	2(1.2)	0.37(0.03-4.15); *p*-0.42	0.26(0.02-2.99); *p*=0.28
Hinf F	4(4.8)	4(2.3)	0.82(0.20-3.36):*p*=0.79	3.41(0.43-26.78); *p=0.24*	5(2.3)	6(3.7)	0.62(0.19-2.06); *p*=0.43	0.46(0.11-1.61); *p*=0.21
NT*Hinf*	78(37.5)	97(54.8)	0.47(0.31-0.71):*P*<0.001	0.54(0.32-0.93); *p*=0.03	109(50)	97(59.2)	0.69(0.46-1.04); *p*=0.07	0.56(0.53-1.37); *p*=0.04
M. catarrhalis	90(43.3)	100(57.8)	0.55(0.37-0.84): *p*=0.005	0.55(0.32-0.94); *p*=0.03	125(57.3)	92(56.1)	1.05(0.70-1.58); *p*=0.81	1.04(0.66-1.64); *p*=0.86
S. aureus	34(16.3)	30(17.2)	0.99(0.53-1.88); *p=0.99*	0.77(0.36-1.67); *p*=0.51	31(14.2)	25(15.2)	1.07(0.59-1.93); *p*=0.82	0.99(0.52-1.94); *p*=0.99
S. pyogenes	1(0.5)	5(2.9)	0.16(0.02-1.40); *p*=0.1	0.31(0.02-4.71); *p*=0.4	2(0.9)	7(4.3)	0.21(0.04-1.01); *p*=0.05	0.20(0.03-1.20); *p*=0.08
N. meningitidis	3(1.44)	2(1.2)	1.25(0.27-7.58); *p*=0.81	1.54(0.15-15.7); *p*=0.72	1(0.5)	3(1.8)	0.24(0.03-2.34); *p*=0.23	0.23(0.02-2.52); *p*=0.23
N. lactamica	7(3.4)	5(2.9)	1.17(0.36-3.75); *p*=0.79	1.35(0.30-6.02); *p*=0.7	11(5.1)	9(5.5)	0.92(0.37-2.26); *p*=0.85	0.54(0.20-1.46); *p*=0.23
B. pertussis	0	0	-	-	0	0	-	-
B. parapertusis	1(0.5)	0	-	-	0	0	-	-
B. Holmesii	0	0	-	-	0	0	-	-
B. bronchiseptica	0	1(0.6)	-	-	0	0	-	-

Adjusted odd ratios (aOR) for bacterial colonisation were determined by multivariate logistic regression, controlling for race, gender, study centre, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. 95% CI, 95% confidence levels. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7A/F, 7C, 8, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 22A/F, 23A/B, 25A/25F/38, 34/37/17A and 35B). -, too few observations to calculate *p*-value. A *p*-value of <0.05 was considered significant.

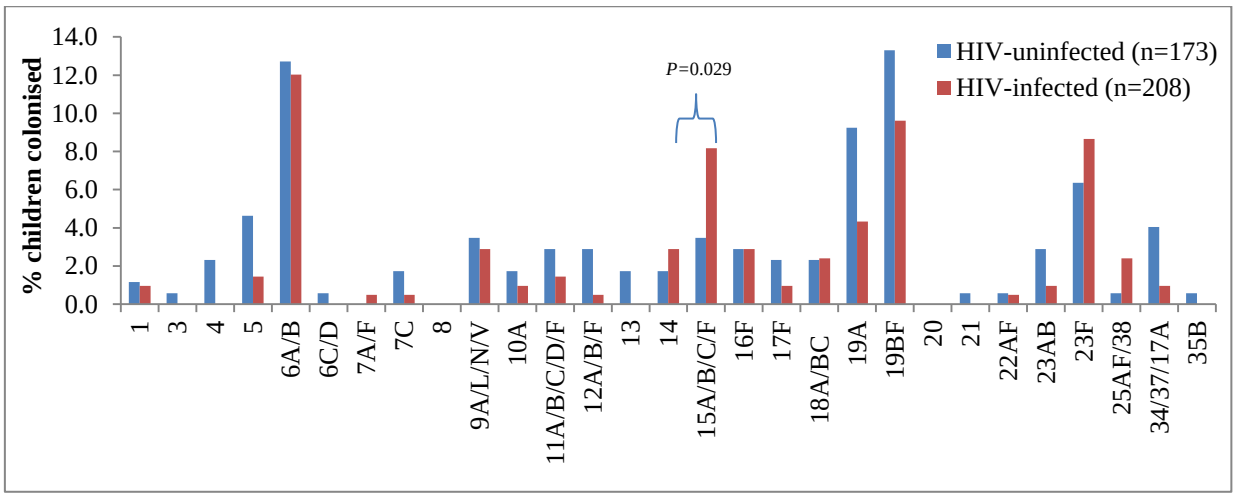


Figure 6.2: Prevalence of nasopharyngeal (NP) pneumococcal colonisation among the HIV-infected and HIV-uninfected children at 9 months

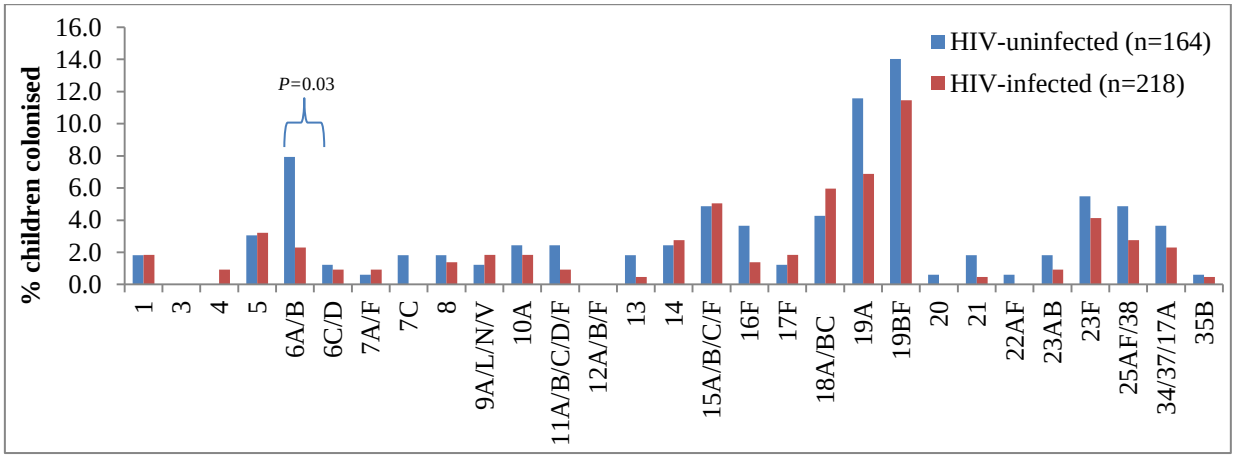


Figure 6.3: Prevalence of nasopharyngeal (NP) pneumococcal colonisation among the HIV-infected and HIV-uninfected children at 16 months

p-values were determined by multivariate logistic regression, controlling for race, gender, study centre, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. * *p*-values of <0.05 were considered significant.

6.2.2. Association of bacterial colonisation between common nasopharyngeal (NP) pathogens stratified by HIV-status

Logistic regression models showed *S. pneumoniae* to be positively associated with *H. influenzae* in HIV-uninfected ($p<0.001$) and HIV-infected ($p<0.001$) children at 9 months of age, with associations being driven by PCV7-serotypes, non-vaccine serotypes and NT*hinf* (Table 6.3). Similarly, a positive association between pneumococcus and *H. influenzae* was found at 16 months in both HIV-infected ($p=0.04$) and HIV-uninfected ($p=0.036$) children; however, a positive association between non-vaccine serotypes and NT*hinf* were only found in HIV-infected children ($p=0.004$); although positive trends between vaccine-serotypes, non-vaccine serotypes and NT*hinf* were still observed in both groups.

Positive associations between *M. catarrhalis* and pneumococcus were found in HIV-infected ($p<0.001$) and HIV-uninfected ($p<0.001$) children at 9 months of age, with associations being driven by both PCV7-serotypes and non-vaccine serotypes. By 16 months of age, however, this association was only found in HIV-infected children ($p<0.001$), with associations being driven by non-vaccine serotypes alone ($p=0.001$); although, positive trends between *M. catarrhalis*, overall pneumococcus, PCV7-serotypes and non-vaccines serotypes were still observed in both HIV-infected and HIV-uninfected children. Furthermore, *M. catarrhalis* and *H. influenzae* were found to be positively associated with each other in both HIV-uninfected ($p=0.03$) and HIV-infected ($p=0.001$) children at 9 months of age; however, by 16 months of age, this association was no longer significant. In Addition, a positive association was found between *M. catarrhalis* and NT*hinf* in HIV-infected children at both 9 ($p=0.005$) and 16 ($p=0.03$) months of age respectively.

Table 6.3: Association of nasopharyngeal bacterial colonisation as determined by quantitative molecular PCR among PCV7-vaccinated and PCV-unvaccinated children

	9 month old children		16 month old children	
	HIV-infected	HIV-uninfected	HIV-infected	HIV-uninfected
	aOR (95% CI); *p*-value	aOR (95% CI); *p*-value	aOR (95% CI); *p*-value	aOR (95% CI); *p*-value
S. pneumoniae & M. catarrhalis	4.14(2.22-7.75);*p*<0.001	4.59(2.24-9.43);*p*<0.001	3.65(1.98-6.73);*p*<0.001	1.69(0.79-3.60);*p*=0.17
VT & *M. catarrhalis*	2.06(1.08-3.90);*p*=0.028	3.20(1.41-7.23);*p*=0.005	1.77(0.93-3.37);*p*=0.08	1.94(0.91-4.11);*p*=0.082
NVT & *M. catarrhalis*	2.40(1.21-4.74);*p*=0.012	2.03(1.04-3.95);*p*=0.037	2.98(1.53-5.80);*p*=0.001	1.34(0.70-2.59);*p*=0.37
S. pneumoniae & S. aureus	0.65(0.39-1,13);*p*=0.056	0.48(0.37-1.23);*p*=0,18	0.55(0.25-1.22);*p*=0.14	0.94(0.05-5.30);*p*=0.08
VT & S. aureus	0.45(0.22-1.95);*P*=0.73	0.16(0,03-0.52);*P*=0.07	0.38(0.13-1.16);*p*=0.09	1.10(0.39-3.08);*p*=0.86
NVT & *S. aureus*	0.99(0.28-3.84);*P*=0.84	0.54(0.09-3.56);*p*=0.65	0.76(0.31-1.85);*p*=0.54	1.94(0.77-4.89);*p*=0.16
S. pneumoniae & H. influenzae	4.41(2.33-8.33);*p*<0.001	4.36(2.10-9.07);*p*<0.001	1.83(1.01-3.30);*p*=0.04	2.37(1.06-5.28);*p*=0.036
VT & *H. influenzae*	3.37(1.75-6.49);*p*<0.001	2.98(1.27-6.99);*p*=0.012	1.39(0.75-2.59);*p*=0.29	1.50(0.65-3.47);*p*=0.34
NVT & *H. influenzae*	4.2(2.10-8.39);*p*<0.001	2.75(1.34-5.62);*p*=0.006	2.95(1.54-5.66);*p*=0.001	1.89(0.89-4.04);*p*=0.1
S. pneumoniae & NThinf	2.99(1.62-5.52);*p*<0.001	3.11(1.52-6.35);*p*=0.002	1.56(0.86-2.81);*p*=0.14	1.09(0.50-2.38);*p*=0.82
VT *& NThinf*	2.36(1.24-4.48);*p*=0.009	3.90(1.68-9.07);*p*=0.002	1.13(0.61-2.09);*p*=0.69	1.08(0.42-1.82);*p*=0.72
NVT *& NThinf*	3.0(1.52-5.91);*p*=0.001	2.49(1.26-4.90);*p*=0.008	2.47(1.32-4.61);*p*=0.004	1.34(0.69-2.61);*p*=0.39
VT and NVT	0.53(0.24-1.21);*p*=0.13	1.23(0.58-2.50);*P*=0.59	0.73(0.36-1.47);*p*=0.38	0.75(0.36-1.56=5);*p*=0.43
M. catarrhalis & S. aureus	0.48(0.18-1.22);*p*=0.12	1.09(0.41-2.89);*p*=0.87	0.45(0.20-1.0);*p*=0.052	1.79(0.67-4.78);*p*=0.25
M. catarrhalis & H. influenzae	2.82(1.57-5.08);*p*=0.001	2.01(1.06-3.81);*p*=0.03	1.74(1.0-3.03);*p*=0.051	0.97(0.47-2.0);*p*=0.94
M. catarrhalis & NThinf	2.36(1.30-4.26);*p*=0.005	1.65(0.88-3.09);*p*=0.11	1.87(1.06-3.27);*p*=0.03	1.03(0.53-2.0);*p*=0.92
S. aureus & H. influenzae	1.35(0.57-3.22);*p*=0.49	0.99(0.37-2.64);*p*=0.98	1.18(0.53-2.61);*p*=0.69	0.73(0.27-1.97);*p*=0.54
S. aureus & NThinf	1.71(0.72-4.09);*p*=0.22	1.07(0.41-2.82);*p*=0.89	1.29(0.59-2.84);*p*=0.52	1.06(0.41-2.75);*p*=0.9

Adjusted odd ratios (AOR) for bacterial colonisation were determined by multivariate logistic regression, controlling for race, gender, study centre, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. HUU, HIV-unexposed-uninfected. HEU, HIV-exposed-uninfected. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7A/F, 7C, 8, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 22A/F, 23A/B, 25A/25F/38, 34/37/17A and 35B). NT, non-typable *H. influenzae.* 95% CI, 95% confidence levels. A *p*-value of <0.05 was considered significant.

6.2.3. Association of HIV-status and density of bacterial nasopharyngeal carriage

The density of overall pneumococcal colonisation was higher in HIV-infected than HIV-uninfected infants at 9 months of age, with titers of 4.81 (95% CI; 4.59-5.03) CFU/ml compared to 4.44 (95% CI; 44.24-4.63) CFU/ml respectively (p=0.014; Table 6.4, despite HIV-infected children having a lower carriage prevalence than HIV-uninfected counterparts. Other differences include a higher density of VT in HIV-infected compared to HIV-uninfected children (4.21 vs. 3.72p=0.04). By 16 months of age, however, there was no difference in density (p=0.89) and prevalence of pneumococcal colonisation between the HIV-infected and HIV-uninfected children. No difference in the density of *H. influenzae* was found between HIV-infected and HIV-uninfected infants at 9 months of age (*p=0.18;* Table 6.4); however, by 16 months of age HIV-uninfected children had a higher density of overall *H. influenzae* colonisation, with titers of 4.95 (95% CI; 4.73-5.17) CFU/ml compared to 4.32 (4.07-4.57) CFU/ml respectively, which was largely due to the higher carriage density of NT*hinf* in HIV-uninfected children (5.0 vs. 4.23; p<0.001). No difference in density of *S. aureus, S. pyogenes, N. meningitidis, N. lactamica, B. pertussis, B. parapertusis, B. holmesii* and *B. bronchiseptica* was found between the groups.

Table 6.4: Density of nasopharyngeal bacterial carriage stratified by HIV status

	9 month old children			16 month old children		
	HIV-infected *n* = 208	HIV-uninfected *n* = 173	*p*-value	HIV-infected *n = 218*	HIV-uninfected *n= 164*	*p*-value
S. pneumoniae	4.81(4.59-5.03)	4.44(4.24-4.63)	0.04	4.49(4.26-4.72)	4.39(1.18-4.61)	0.89
VT	4.21(3.12-5.11)	3.72(3.45-3.99)	0.04	3.60(3.30-3.89)	3.61(3.30-3.93)	0.93
NVT	3.48(3.16-3.79)	3.29(3.03-3.54)	0.35	3.45(3.09-3.81)	3.34(3.09-3.59)	0.42
M. catarrhalis	3.55(3.33-3.77)	3.27(3.06-3.48)	0.42	3.49(3.29-3.69)	3.24(3.04-3.44)	0.24
H. influenzae	5.0(4.72-5.29)	4.73(4.49-4.97)	0.08	4.32(4.07-4.57)	4.95(4.73-5.17)	<0.001
NT*hinf*	4.92(4.62-5.23)	4.71(4.45-4.97)	0.07	4.23(3.97-4.50)	5.0(4.75-5.24)	<0.001
S. aureus	3.93(3.44-4.42)	4.03(3.43-4.63)	0.39	2.21(1.44-3.0)	3.03(2.71-3.34)	0.82
S. pyogenes	3.44	4.27(3.89-4.64)	-	-	-	-
N. meningitidis	4.87(-1.67-11.40)	2.04(1.81-2.27)	0.2	8.83	2.93(0.07-5.79)	-
N. lactamica	2.68(1.64-3.72)	2.24(1.92-2.57)	0.15	2.57(2.14-3.0)	2.21(1.85-2.56)	0.3

For density of carriage geometric mean densities (GMD) and 95% CI intervals (95% CI) of pneumococcal and bacterial mean concentrations were calculated following $\log_{10}$ transformations on data and compared with multivariate analysis controlling for race, gender, study centre, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7A/F, 7C, 8, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 22A/F, 23A/B, 25A/25F/38, 34/37/17A and 35B).m NT*hinf*, non-typable *H. influenzae.* -, too few observations to calculate a *p*-value. A *p*-value <0.05 was considered significant.

Spearman correlation coefficients showed the overall densities of *S. pneumoniae, H. influenzae* and *M. catarrhalis* to be positively associated with each other in both HIV-infected and HIV-uninfected children at both study visits, with associations by vaccine-serotype, non-vaccine serotype and NT*hinf* (Table 6.5). Furthermore, a negative association between densities of *S. pneumoniae* and *S. aureus* was found in HIV-infected children at 9 months of age only.

Table 6.5: Correlation of density of nasopharyngeal bacterial colonisers as determined by Nanofludic real-time qPCR Stratified by HIV-status

	9 month old children		16 month old children	
	HIV-infected *n* = 208 *Rho*; *p*-value	HIV-uninfected *n* = 173	HIV-infected *n = 218*	HIV-uninfected *n= 164*
S. pneumoniae & M. catarrhalis	0.336; *p*=0.005	0.271;*P*=0.013	0.308;*p*=0.002	0.321;*p*=0.005
VT *& M.catarrhalis*	0.255; *p*=0.05	0.30; *p*=0.03	0.372;*p*=0.018	0.389;*p*=0.03
NVT *& M. catarrhalis*	0.462;*p*=0.01	0.255; *p*=0.11	0.239;*p*=0.05	0.391;*p*=0.012
S. pneumoniae & S. aureus	-0.659:*p*=0.01	0.0964; *p*=.0.73	0.007;*p*=0.97	0.139;*p*=0.36
VT & S. aureus	0.143; *p*=0.76	0.2; *p*=0.75	0.371;*p*=0.46	NP
NVT & *S. aureus*	-0.714; *p*=0.11	0.143;*p*=0.76	0.201;p*p*=0.52	NP
S. pneumoniae & H. influenzae	0.329;*p*=0.01	0.422; *p*<0.001	0.441;*p*<0.001	0.368; *p*<0.001
VT *& H. influenzae*	0.22; *p*=0.02	0.262; *p*=0.01	0.419;*p*=0.01	0.552; *p*<0.001
NVT *& H. influenzae*	0.328;*p*=0.03	0.272; *p*=0.03	0.287;*p*=0.05	0.381;*p*=0.005
S. pneumoniae & NThinf	0.27;*p*=0.04	0.363;*p*=0.002	0.421;*p*= *p*<0.001	0.262;*P*=0.024
VT & NThinf	0.112;*p*=0.56	0.221;*p*=0.24	0.408;*p*=0.025	0.429;*p*=0.03
NVT & NThinf	0.244;*p*=0.21	0.313; *p*=0.052	0.259;*p*=0.1	0.359;*p*=0.21
M. catarrhalis & S. aureus	-0.428;*p*=0.34	-0.518;*p*=0.08	0.007;*p*=0.97	0.259;*p*=0.1
M. catarrhalis & H. influenzae	0.266;*p*=0.04	0.344;*p*=0.004	0.237;*p*=0.04	0.502;*p*<0.001
M. catarrhalis & NThinf	0.296;*p*=0.05	0.342;*p*=0.01	0.288;*p*=0.02	0.514;*p*=0.001
S. aureus & H. influenzae	-0.084;*p*=0.8	0.147;*p*=0.65	NP	0.036;*p*=0.9
S. aureus & NThinf	-0.084;*p*=0.79	0.054;*p*=0.87	NP	0.016;*p*=0.96

Bacterial correlations were determined by spearman's coefficients. HUU, HIV-unexposed-uninfected. HEU, HIV-exposed-uninfected. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7A/F, 7C, 8, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 22A/F, 23A/B, 25A/25F/38, 34/37/17A and 35B). NT*hinf*, non-typable *H. influenzae.* NP, study not powered to calculate AOR and *p*-value due to too few observations. A *p*-value <0.05 was considered significant.

6.3. DISCUSSION

To our knowledge this is the first study to investigate the association of HIV-1 infection and the density of bacterial carriage of common nasopharyngeal colonisers such as *S. pneumoniae, H. influenzae* and *S. aureus* in a PCV7-immunised cohort. Our findings were in line with those from a previous study undertaken by us, which used WHO recommended culture to find a lower carriage prevalence of overall pneumococci, non-vaccine serotypes and *H. influenzae* in HIV-infected children [198]. The current study, however, expanded on the previous analysis in that the Nanofludic real-time PCR method allowed for quantification and showed the density of overall pneumococcal carriage to be higher in HIV-infected children despite a lower carriage prevalence, which could possibly explain the higher disease burden in HIV-infected compared to HIV-uninfected children [245].

While there were fundamental differences in the baseline characteristics of HIV-infected and HIV-uninfected children including a higher administration rate of co-trimoxazole prophylaxis in HIV-infected children and a higher rate of breastfeeding in HIV-uninfected children, the effect on pneumococcal carriage prevalence and density remained unchanged even after adjusting for these possible cofounders. Nevertheless, antibiotic usage including co-trimoxazole prophylaxis in general is higher in HIV-infected children and thus should be considered when interpretation the findings of our study especially since recent studies in Zambia showed co-trimoxazole to increase the risk of colonisation with co-trimoxazole resistant pneumococci within 6 weeks of starting prophylaxis and other studies reporting resistance as high as 50% – 80% in HIV-infected children which is predominantly found in vaccine-type pneumococci [246-249]. Antimicrobial exposure in HIV-infected children can thus increase their risk to shed non-vaccine pneumococci due to their sensitivity to antimicrobials and further retain their vaccine serotypes which were consistent to our findings.

Further, the HIV-infected children included in this study were initiated on antiretroviral treatment immediately upon diagnosis of their HIV-infection status or within the first year of life which might also explain the lower carriage prevalence of overall pneumococcus in the HIV-infected cohort as PCV have been shown to induce a more durable and functional antibody response in individuals on ART at the time of vaccination compared to ART-naïve individuals, independently of the baseline CD4+ cell count [250]. The possible effects of ARTs on pneumococcal carriage density; however, are

unknown and the clinical relevance of this warrants further studies as the temporary increase in pneumococcal carriage density observed in this study might explain why invasive pneumococcal disease remains high in HIV-infected individuals despite ART usage [251]. Further, it is possible that the PCV induced vaccine effect as shown in Chapter 3, were PCV7 immunisation resulted in a temporary increase in the density of pneumococcal carriage, was more pronounced in HIV-infected children who have underlying immunosuppressive mediators and might explain why an increase in the prevalence density of overall pneumococcus was observed in these children. In addition, the increase in the prevalence density of overall pneumococcus appeared to be driven by vaccine serotypes suggesting that failure of the immune response as a result of HIV-infection allowed for invasive vaccine serotypes to be carried at a higher carriage density, although further investigation is needed to provide clarity.

In addition, we also identified that carriage prevalence and density of *H. influenzae* to be higher in HIV-uninfected children, which was consistent with our previous findings by culture [198] and to another study done in Tanzania [196], albeit differing from another South African study which reported that the prevalence of *H. influenzae* was higher in HIV-infected children [77]. The increase in the carriage prevalence and density was driven by NT*hinf* most likely due to the low detection rate of the serotypes a-f. Also while we found no change in the carriage prevalence or density of *S. aureus*, other studies from South Africa [191,193] and Tanzania [196] found the carriage prevalence of *S. aureus* to be higher in HIV-infected children. Differences in the findings from these studies could possibly be attributed to the different PCV-vaccination status of the children; however, additional studies in PCV7-vaccinated cohorts with more sensitive molecular methods are still needed to clarify these associations.

Positive associations with respect to prevalence were found between pneumococcus, *H. influenzae* and *M. catarrhalis* in both HIV-infected and HIV-uninfected children, with associations being driven by vaccine serotypes, non-vaccine serotypes and NT*hinf*. This finding is contradictory to other studies that found these associations in HIV-negative children alone [72,77]. The HIV-infected children in our study, however, differed from previous studies on bacterial colonisation interactions in that children received antiretroviral treatment and were thus relatively immunocompetent.

Furthermore, no association between pneumococcus and *S. aureus* was found in all groups which are consistent with findings from another study in South Africa [193].

Our study was limited in that the Fluidigm method was not optimised to discriminate between all serotypes within their respective serogroups. Furthermore, the study was not adequately powered to evaluate the difference in carriage density of serotypes/serogroups as the sample size of detected minor serotypes was too small. Only a selection of NP organisms were analysed and thus analysis at a microbiome level may be advantageous. Lastly, nasopharyngeal swabs do not favour the isolation of *S. aureus* and *S. pyogenes* in which anterior nares and throat swabs are better for detection, respectively [241,242]. The prevalence of these bacteria could have thus been underestimated and the analysis on *S. aureus* prevalence needs to be interpreted with caution.

Since NP colonisation is the precursor to disease of the upper and lower respiratory tract, analysis of the NP tract flora can provide a simple means to signal changes, which could inform future trends in the long term effectiveness of these vaccines, especially in children with HIV-1 infection whom have a higher disease burden. The ability of the Biomark HD system to simultaneously detect multiple pneumococcal serotypes and bacterial pathogens at a low carriage density allowed us to gain a better understanding of the association of HIV-infection and nasopharyngeal bacterial carriage. In our study we showed the carriage density of overall pneumococcus to be higher in HIV-infected children, despite the lower carriage prevalence which might explain the higher invasive disease burden in HIV-infected compared to HIV-uninfected children as a high nasopharyngeal density of *streptococcus pneumoniae* has previously been shown to be associated with invasive pneumococcal pneumonia [252].

CHAPTER 7: INTEGRATED DISCUSSION AND CONCLUSIONS

While culture remains the WHO recommended referent standard for evaluating pneumococcal nasopharyngeal colonisation and serotype characterisation, these assays lack quantitative data which has hindered our understanding of the association between PCV-immunisation on pneumococcal and bacterial nasopharyngeal carriage, including in HIV-infected children. The finding presented in this thesis, however, supports the use of molecular qPCR methods as alternatives to standard culture and future bacterial/pneumococcal colonisation studies should strongly considered using these methods, especially if investigating a population with a high HIV disease burden.

While the qPCR methods were limited for pneumococcal serotyping in that they did not test for all known pneumococcal serotypes and that they were unable to differentiate between some serotypes that had high genetic similarities, the knowledge gained by being able to quantify the density of each colonising serotype outweighed these limitations. Future studies using these methods; however, could focus on expanding the number of assays so that all known pneumococcal serotypes are tested for and additional testing such as RFLP and sequencing could be done to further discriminate some serotypes with high genetic similarities. Nevertheless, by using sensitive molecular qPCR methods to compare the circulation of pneumococcal serotypes as well as other bacterial pathogens in a PCV7-vaccinated cohort of HIV-infected and HIV-uninfected children, as well as a PCV-unvaccinated cohort, we were able to make several important findings.

When comparing the circulation of pneumococcal serotypes in a PCV7-vaccinated and PCV-unvaccinated cohort of children, the PCV7 vaccine effect was clearly shown and for the first time the mechanism behind this effect was shown to be both serotype replacement colonisation of the nasopharynx because of the vacant ecological niche and unmasking of prevailing non-vaccine serotype colonisers which were not previously recognised by traditional culture method that are biased towards detecting the dominant colonising serotype. Although serotype specific analysis was often limited by a small sample size for a given serotype, findings from two non-PCV7 serotypes namely 19A and 5 were of importance. The increase in the prevalence and carriage density of

serotype 19A was shown to be a combination of both serotype replacement disease as well as unmasking, while the increase in the colonisation density of serotype 5 which is rarely seen in conventional culture studies was unmasked by the qPCR method. The findings are of important clinical relevance as serotype 5 has a high invasive disease potential in our setting [18, 19] and an increase in the carriage density as a result of PCV-immunisation could enhance its ability to cause mucosal and invasive disease and potentially offset some of the effectiveness of PCV7 immunisation. Further, the increase in the carriage prevalence and density of PCV7-serotype 19A could explain the emergence of serotype 19A as the major replacement serotype causing IPD following PCV7 introduction in several settings which is probably a result of 19A being commonly characterized by clones that carry a high prevalence of antimicrobial resistance, thus facilitating its survival where other NVT's perish [15-17] .

Although there are now 10 and 13 valent PCV vaccines available that have been expanded to include serotypes 5 and 19A, PCV7 was only introduced into the public immunisation program in May 2009 in South Africa and has only recently being replaced by PCV10 and PCV13. Serotype 19A and 5 are thus largely considered to be non-vaccine serotypes that have a high invasive disease potential in our settings and monitoring trends in the circulation of these serotypes post PCV7 immunisation is of importance. Further, the increase in the detection of serotype 5 and 19A by molecular qPCR methods highlights the importance of continued surveillance of the circulating pneumococcal serotypes after the introduction of PCV10 and PCV13 with sensitive molecular methods that are able to detect serotypes at a low carriage density and thus accurately determine the effectiveness of these vaccines in reducing/eliminating carriage by these serotypes.

While PCV7 immunisation has been effective in reducing the morbidity and mortality in children < 5 years of age, there are concerns about the downstream effects of this vaccine as well as other to provide a vacant niche for bacteria that are resistant to antibiotics to colonise the host resulting in an increase in invasive disease caused by these bacteria in which treatment will be challenging. Findings from this thesis support the notion that after the eradication of common PCV7 colonisers such as vaccine-type pneumococci, momentum is directed by both non-vaccine type serotypes and other bacterial species to colonise the vacant niche and thus direct bacteria-bacteria interactions may impact clinically important nasopharyngeal dynamics. The prevalence and carriage density of

H. influenzae and *S. aureus* were shown to be higher in PCV-vaccinated children and could explain the moderate effects that PCV has on otitis media (OM), even with the vaccine having up to 80% efficacy in reducing vaccine-type pneumococcal colonisation[17-19] although additional studies are still needed for clarity. Further, the temporary increase in the colonisation prevalence and density of *S. aureus* highlights the effect to be around PCV7 vaccinations in infancy and subsequently diminishing after non-vaccine serotypes or other bacterial species colonise the vacant niche or by an age-dependent maturation of the immune system[21-23]. These results highlighted the natural balance found between pneumococci and co-colonising bacterial species and interfering with this may lead to disequilibrium within the host, which could affect susceptibility to disease from other serotypes and bacteria species. On-going surveillance, especially on the implication of such changes in the nasopharyngeal biome on particularly mucosal infections are thus warranted.

One of the most interesting findings of this study was the higher carriage density of overall pneumococci observed in HIV-infected PCV7 immunised children, despite them having lower carriage prevalence. While this might explain why invasive pneumococcal disease remains high in HIV-infected individuals despite ART usage, one cannot ignore other possible contributors such as the high rate of antibiotic usage in HIV-infected children as well as the HIV-infected children being initiated on antiretroviral treatment immediately upon diagnosis of their HIV-infection status or within the first year of life, especially since the possible effects of ARTs on pneumococcal carriage density is unknown. Nevertheless these findings are of important clinical relevance and warrants further study. It is, however, possible that the PCV induced vaccine effect was also more pronounced in HIV-infected children who have underlying immunosuppressive mediators and might explain why an increase in the prevalence density of overall pneumococcus was observed in these children. In addition, the increase in the prevalence density of overall pneumococcus appeared to be driven by vaccine serotypes suggesting that failure of the immune response as a result of HIV-infection allowed for invasive vaccine serotypes to be carried at a higher carriage density, although further investigation is needed to provide clarity. Nonetheless these findings highlight the importance of continued surveillance of circulation of pneumococcal serotypes especially in a population with a high burden of HIV-1 infection.

The findings from this thesis were limited in that the qPCR assays were unable to discriminate between all vaccine serotypes within their respective serogroups due to the high genotypic

similarities between the capsule loci of certain pneumococcal serotypes. Furthermore, not all pneumococcal serotypes and bacterial pathogens were tested for and thus carriage with samples colonised with these pathogens could have been missed and the rate of concurrent colonisation with multiple pneumococcal serotypes underestimated. The effect of other serotypes on the vaccine efficacy for protection against colonisation (VEcol) might have thus also been underestimated. In addition, the study was not adequately powered to evaluate all serotype specific differences as the sample size to detect minor serotypes was too small, particularity with respect to evaluating the difference in the carriage density of specific serotypes/serogroups. The loss of sensitivity when comparing the qPCR assays to each other, as well as when comparing traditional qPCR to culture was most probably a result of degradation of DNA as a result of long-term storage and/or freezing and thawing of samples. Further, nasopharyngeal swabs do not favour the isolation of *S. aureus* and *S .pyogenes* in which anterior nares and throat swabs are better for detection, respectively [238, 239]. The prevalence of these bacteria could have thus been underestimated and may explain why no change in *S. aureus* prevalence due to PCV-vaccination was reported. In addition, the control group was enrolled after our vaccinated group. However, due to the limited coverage of PCV in the community it is thus unlikely that, a broader indirect effect of the vaccine reducing transmission by young vaccinated children, would have affected the nasopharyngeal colonisation patters. Lastly, our study was not a randomised clinical trial and the cohorts were not matched. Coincidental temporal fluctuations and other unknown factors could have thus influenced the results.

While this study was meaningful in improving our understanding of the association of PCV7 immunisation and HIV-1 infection on bacterial carriage prevalence and density, analysis was done in a PCV-7 immunised cohort and has now been replaced by PCV-13 in South Africa. The extent of serotype replacement may thus differ after the reductions of serotypes 19A and 5 colonisation which are included in PCV-13, and the ability of other bacterial species to colonise the vacant niche might be changed. Further investigation of pneumococcal and bacterial carriage in a PCV-13 immunised cohort with sensitive molecular methods is thus still needed.

In conclusion, improved molecular methods that were able to quantitatively detect multiple pneumococcal serotypes and other common nasopharyngeal bacterial pathogens at a low carriage density allowed us to gain a better understanding of the association of PCV immunisation and HIV-

1 infection on the carriage density of vaccine-serotypes, non-vaccine serotypes and other common bacterial pathogens, in which other studies have been limited. There is a natural balance between pneumococci and co-colonising bacterial species, and interfering with this may lead to disequilibrium within the host, which could affect susceptibility to disease from other serotypes and bacteria species. Since NP colonisation is the precursor to diseases of the upper and lower respiratory tract, analysis of the NP tract flora can provide a simple means to signal changes, which could inform future trends in the long term effectiveness of these vaccines, especially in children with HIV-1 infection who have a higher disease burden. On-going surveillance, especially on the implication of such changes in the nasopharyngeal biome on particularly mucosal infections are warranted. Improved methods of detection of respiratory pathogens can provided a means to improve the health care of patients as optimal therapy can be initiated sooner, effectiveness of the treatment is improved and spread of disease can be reduced or even prevented. Further, a high bacterial load detected by real-time PCR in nasopharyngeal specimens might help predict the etiological role for *S. pneumoniae* and other bacterial pathogens in bacterial disease.

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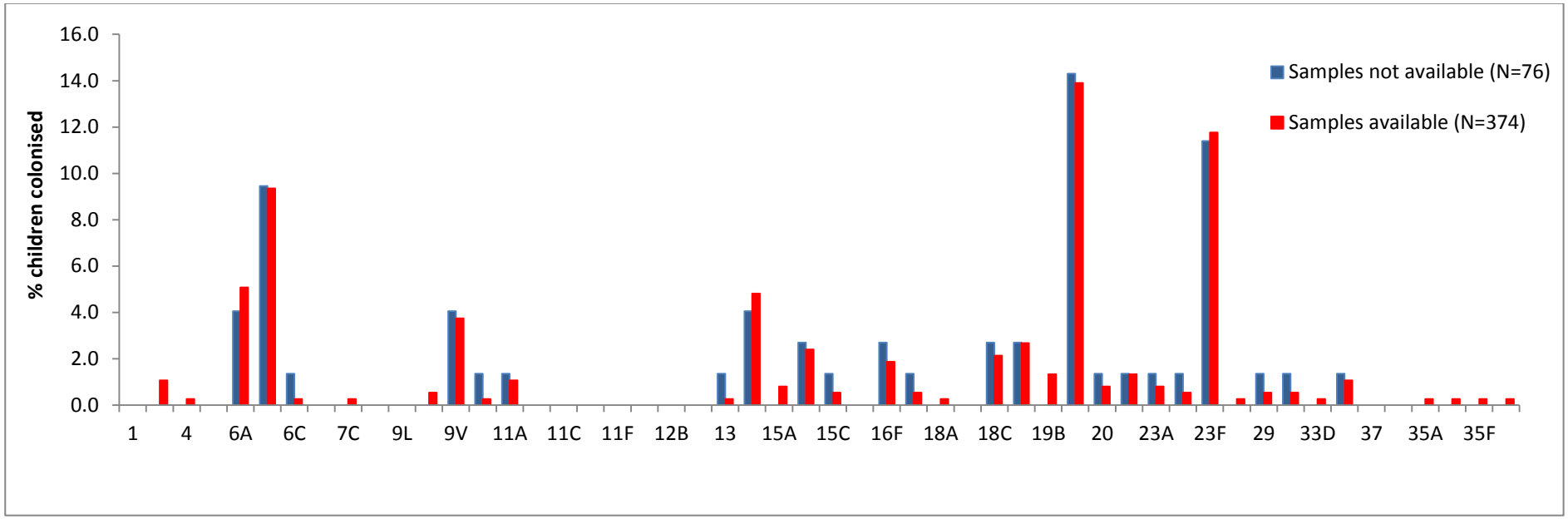
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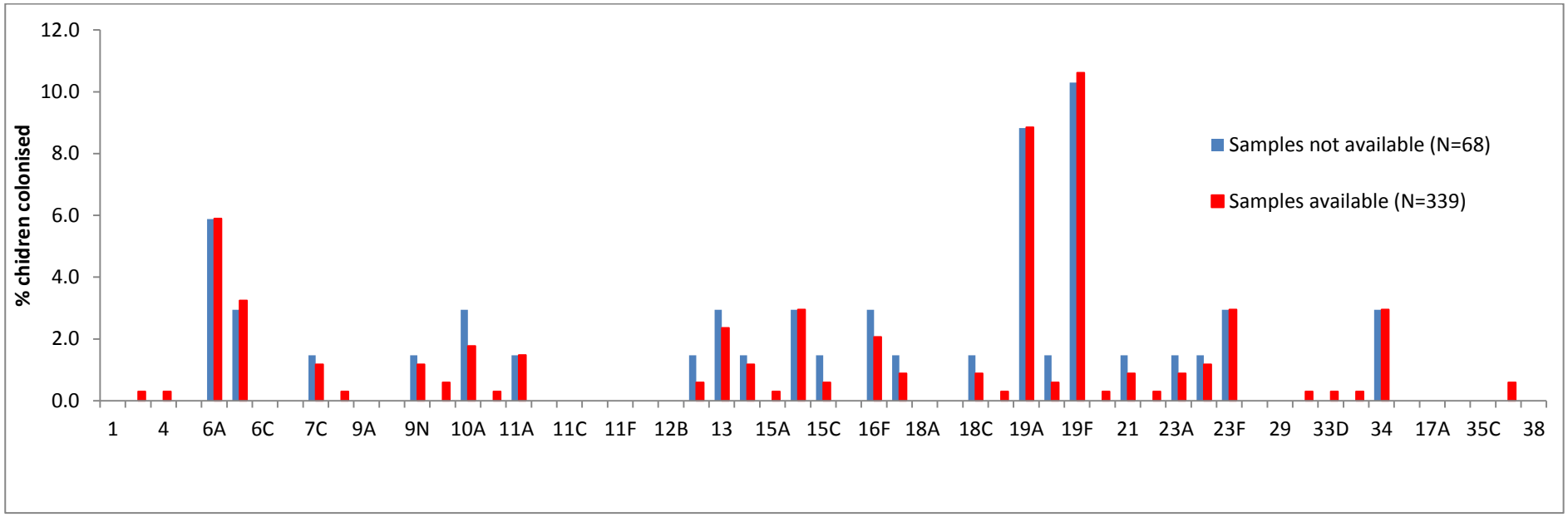
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APPENDICES

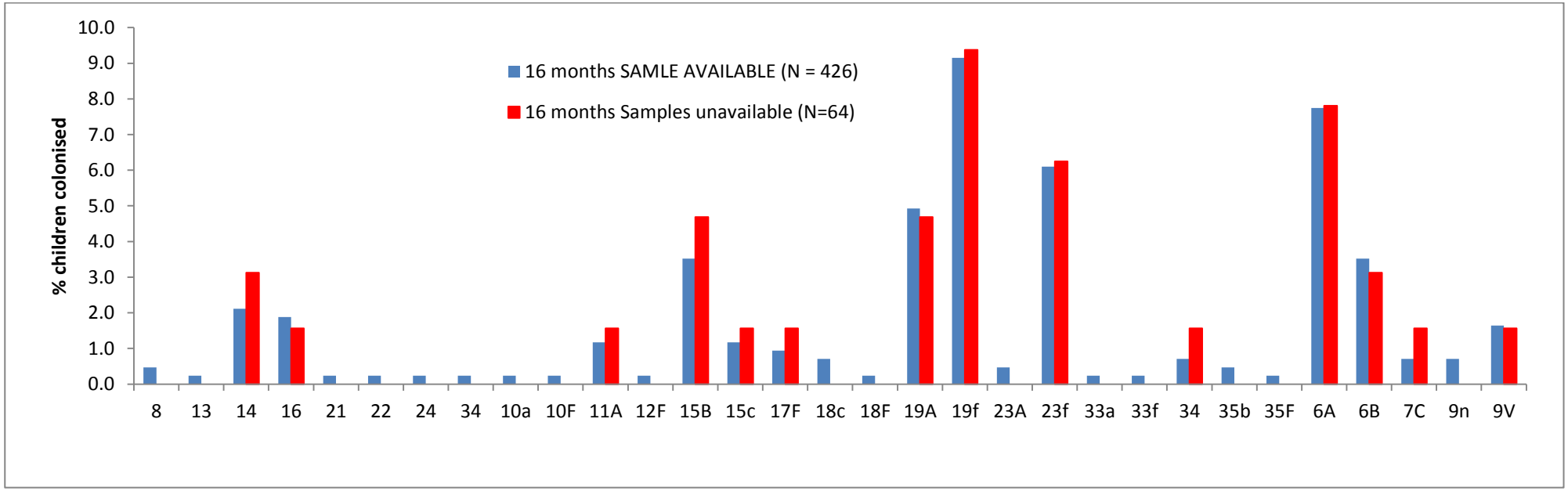
Appendix A: Prevalence of pneumococcal serotypes in PCV-unvaccinated HIV-uninfected children between samples available and not-available for subsequent molecular qPCR testing



Appendix B: Prevalence of pneumococcal serotypes in PCV7-vaccinated HIV-uninfected children between samples available and not-available for subsequent molecular qPCR testing



Appendix C: Prevalence of pneumococcal serotypes in PCV7-vaccinated HIV-infected children between samples available and not-available for subsequent molecular qPCR testing.



Appendix D: Prevalence of nasopharyngeal (NP) colonisers between HIV-exposed uninfected children as well as between HIV-infected children who received immediate or delayed ART therapy

	HEU	HUU	aOR (95% CI)	*p*-value	ART-def.	ART-immed.	aOR (95% CI)	*p*-value
9 month old children	*n=102*	*n=71*			*n=156*	*n=52*		
S. pneumoniae	68(66.7)	53(74.6)	0.46(0.16-1.32)	0.059	94(60.3)	28(53.9)	1.3(0.69-2.44)	0.42
VT	19(18.6)	21(29.6)	0.40(0.14-1.15)	0.09	41(26.3)	14(26.9)	0.96(0.48-1.97)	0.93
NVT	31(30.4)	29(40.8)	0.41(0.16-1.03)	0.06	38(24.4)	10(19.2)	1.35(0.62-2.95)	0.45
H. influenzae	67(65.7)	44(61.9)	1.17(0.46-3.01)	0.73	69(44.2)	19(36.5)	1.38(0.72-2.63)	0.33
Hinf A	-	1(1.4)	-		0	0	-	-
Hinf B	1(1)	1(1.4)	0.23(0.001-45.57)	0.58	2(1.3)	1(1.9)	0.66(0.06-7.46)	0.74
Hinf C	1(1)	0	-		0	0	-	-
Hinf D	1(1)	0	-		2(1.3)	0	-	-
Hinf E	2(2)	0	-		1(0.6)	0	-	-
Hinf F	1(1)	3(4.2)	-		4(2.56)	0	-	0.24
NThinf	60(58.8)	37(52.1)	1.48(0.59-3.71)	0.4	60(38.5)	18(34.6)	1.18(0.61-2.78)	0.62
M. catarrhalis	55(53.9)	45(63.4)	0.80(0.32-2.0)	0.63	73(46.8)	17(36.7)	1.81(0.94-3.5)	0.08
S. aureus	18(17.6)	12(16.9)	1.0 (0.37-2.7)	0.99	16(10.3)	8(15.4)	0.63(0.25-1.57)	0.32
S. pyogenes	1(1)	4(5.6)	0.13(0.01-1.71)	0.12	0	1(1.9)	-	-
N. meningitidis	1(1)	1(1.4)	-		3(1.9)	0	-	-
N. lactamica	0	5(7)	-		7(4.5)	0	-	-
B. pertussis	0	0	-		0	1(2.3)	-	-
B. parapertusis	0	0	-		0	0	-	-
B. Holmesii	0	0	-		0	0	-	-
B. bronchiseptica	0	1(1.4)	-		0	0	-	-
16month old children	*n*=97	*n*=67			*n*=154	*n*=56		
S. pneumoniae	74(76.2)	51(76.1)	1.03(0.44-1.78)	0.23	109(70.8)	40(71.4)	0.80(0.42-1.54)	0.51
VT	26(26.8)	20(30)	0.66(0.29-1.49)	0.32	43(27.9)	16(28.6)	0.86(0.43-1.73)	0.67
NVT	42(43.3)	25(37.3)	1.62(1.06-2.50)	0.067	47(30.5)	16(28.6)	1.20(0.61-2.38)	0.6
H. influenzae	67(69.1)	54(80.6)	0.57(0.25-1.33)	20	88(57.1)	34(60.7)	0.93(0.50-1.72)	0.82
Hinf A	4(4.1)	4(6)	0.59(0.12-3.02)	0.53	4(2.6)	0	-	-
Hinf B	0	1(1.5)	-	-	1(0.1)	0	-	-

Hinf C	4(4.1)	2(3)	0.94(0.13-7.0)	0.95	0	0	-	-
Hinf D	1(1)	1(1.5)	0.36(0.02-6.56)	0.49	2(1.3)	0	-	-
Hinf E	1(1)	1(1.5)	-	-	1(0.1)	0	-	-
Hinf F	5(5.2)	1(1.5)	3.69(0.28-47.43)	0.33	3(2)	2(3.6)	0.54(0.09-3.30)	0.5
NThinf	53(54.6)	44(56.7)	0.82(0.39-1.73)	0.6	79(51)	30(53.6)	0.93(0.50-1.72)	0.82
M. catarrhalis	53(54.6)	37(55.2)	1.06(0.57-1.99)	0.85	86(55.8)	39(69.9)	0.53(0.28-1.03)	0.06
S. aureus	15(15.5)	10(14.9)	1.0(0.40-2.49)	0.99	22(14.3)	9(16.1)	0.84(0.35-2.05)	0.71
S. pyogenes	3(3.1)	4(6)	0.60(0.87-4.23)	0.61	2(1.3)	0	-	-
N. meningitidis	1(1)	2(3)	0.19(0.01-2.12)	0.2	1(0.1)	0	-	-
N. lactamica	4(4.1)	5(7.5)	0.27(0.06-1.18)	0.08	7(4.6)	4(7.1)	0.62(0.17-2.20)	0.56
B. pertussis	0	0	-	-	0	0		
B. parapertusis	0	0	-	-	0	0		
B. Holmesii	0	0	-	-	0	0		
B. bronchiseptica	0	0	-	-	0	0		

Adjusted odd ratios (aOR) for bacterial colonisation were determined by multivariate logistic regression, controlling for race, gender, study centre, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. 95% CI, 95% confidence levels. ART-immed, HIV-infected children with CD4+ cell count ≥25% started on ART at the time of first dose of PCV-7. ART-def., HIV-infected children with CD4+ cell count ≥25% at time of first PCV-7 dose that were started on antiretroviral treatment (ART) based on clinical or immunological indications. HUU, HIV-unexposed uninfected. HEU, HIV-exposed uninfected. VT, vaccine serotypes/serogroups (4, 6AB, 9ALNV, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7A/F, 7C, 8, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 22A/F, 23A/B, 25A/25F/38, 34/37/17A and 35B). -, too few observations to calculate p-value. A *p*-values of <0.05 were considered significant.

Appendix E: Density of nasopharyngeal bacterial carriage stratified by HIV-exposure as well as weather HIV-infected children received immediate or delayed ART therapy

	HUU	HEU	*p*-value	ART-def.	*ART-immed*	*p-value*
9 month old children						
S. pneumoniae	4.48(4.20-4.75)	4.43(4.15-4.72)	0.34	4.83(4.56-5.09)	4.76(4.34-5.17)	0.8
VT	4.49(4.11-4.87)	4.80(4.29-5.31)	0.42	5.29(4.89-5.69)	5.04(4.36-5.73)	0.53
NVT	4.67(4.35-5.0)	4.64(4.24-5.05)	0.91	4.85(4.46-5.24)	4.67(4.11-5.24)	0.66
M. catarrhalis	3.22(2.34-3.51)	3.35(3.04-3.66)	0.75	3.55(3.29-3.81)	3.56(3.16-3.96)	0.96
H. influenzae	4.86(4.49-5.24)	4.68(4.35-5.0)	0.85	5.0(4.67-5.33)	4.99(4.37-5.61)	0.97
NT*hinf*	4.82(4.41-5.22)	4.64(4.30-4.99)	0.79	4.87(4.36-5.14)	4.91(4.26-5.34)	0.67
S. aureus	3.512.83-4.19)	4.37(3.45-5.29)	0.82	3.98(3.31-4.66)	3.82(2.99-4.64)	0.74
S. pyogenes	4.20(3.73-4.67)	4.56	-	-	-	-
N. meningitidis	2.06	2.02	-	-	-	-
N. lactamica	-	-	-	-	-	-
16 moth old children						
S. pneumoniae	4.39(4.08-4.70)	4.39(4.07-4.71)	0.85	4.46(4.19-4.73)	4.59(4.09-5.08)	0.65
VT	4.54(3.87-5.20)	4.75(4.37-5.13)	0.98	4.61(4.23-4.99)	5.29(5.58-6.0)	0.08
NVT	4.64(4.29-4.99)	4.69(4.22-5.17)	0.8	4.92(4.58-5.26)	4.62(4.04-5.19)	0.36
M. catarrhalis	3.27(2.90-3.63)	3.23(2.99-3.47)	0.51	3.52(3.29-3.75)	3.43(3.01-3.84)	0.66
H. influenzae	5.10(4.79-5.41)	4.83(4.51-5.14)	0.054	4.31(4.01-4.61)	4.45(3.98-4.93)	0.63
NT*hinf*	5.08(4.73-5.43)	4.93(5.58-5.28)	0.15	4.31(4.00-4.62)	4.45(3.97-4.87)	0.56
S. aureus	3.17(259-3.76)	2.93(2.51-3.35)	0.29	2.16(1.98-3.05)	2.28(2.00-2.97)	0.78
S. pyogenes	3.25(1.61-4.89)	3.33(0.47-6.19)	-	-	-	-
N. meningitidis	3.19(-10.26-16.64)	2.41	-	-	-	-
N. lactamica	2.15(1.71-2.59)	2.28(1.27-3.29)	0.67	2.74(2.04-3.44)	2.28(1.96-2.61)	0.28

For density of carriage. Geometric mean densities (GMD) and 95% CI intervals (95%) of pneumococcal and bacterial mean concentrations were calculated following log 10 transformations on data and compared with multivariate analysis controlling for race, gender, study centre, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. ART-immed, HIV-infected children with CD4+ cell count ≥25% started on ART at the time of first dose of PCV-7. ART-def., HIV-infected children with CD4+ cell count ≥25% at time of first PCV-7 dose that were started on antiretroviral treatment (ART) based on clinical or immunological indications. HUU, HIV-unexposed uninfected. HEU, HIV-exposed uninfected. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7A/F, 7C, 8, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 22AF, 23AB, 25A/25F/38, 34/37/17A and 35B).m NT*hinf*, non-typable *H. influenzae*. -, too few observations to calculate *p*-value. A *p*-value of <0.05 was considered significant.

Appendix F: Ethics certificates

R14/49 Mrs Courtney Olwagen

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140907

NAME: (Principal Investigator)	Mrs Courtney Olwagen
DEPARTMENT:	School of Pathology Respiratory and Meningeal Pathogens Research Unit Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	Molecular Investigations on Impact of the Pneumococcal Polysaccharide-Protein Conjugate Vaccine (PCV) on Bacterial Nasopharyngeal Colonization in Children
DATE CONSIDERED:	03/10/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof SA Madhi and Dr PV Adrian
APPROVED BY:	Professor A Woodiwiss, Co-Chairperson, HREC (Medical)
DATE OF APPROVAL:	24/10/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Ms Courtney P Olwagen

CLEARANCE CERTIFICATE	M120972
PROJECT	Molecular Investigations on Impact of the Pneumococcal Polysaccharide-Protein Conjugate Vaccine (PCV) on Bacterial Naso-pharyngeal Colonization in Children
INVESTIGATORS	Ms Courtney P Olwagen.
DEPARTMENT	Clinical Microbiology and Infectious Diseases
DATE CONSIDERED	28/09/2012
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 28/09/2012 CHAIRPERSON .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr Peter Adrian

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the

Appendix G: Turnitin report

PhDthesis16November2016.docx

ORIGINALITY REPORT

%8	%6	%5	%1
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Madhi, Shabir A., Alane Izu, Marta C. Nunes, Avye Violari, Mark F. Cotton, Patrick Jean-Philippe, Keith P. Klugman, Anne von Gottberg, Nadia van Niekerk, and Peter V. Adrian. "Longitudinal study on Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus nasopharyngeal colonization in HIV-infected and -uninfected infants vaccinated with pneumococcal conjugate vaccine", Vaccine, 2015. Publication	%2
2	www.planetxmap.com Internet Source	<%1
3	www.ncbi.nlm.nih.gov Internet Source	<%1
4	International Journal of Housing Markets and Analysis, Volume 6, Issue 1 (2013-05-27) Publication	<%1
5	tampub.uta.fi Internet Source	<%1
6	Fernanda Rodrigues. "Paediatric nasopharyngeal ecology in the era of	<%1