Temporal changes in *Streptococcus pneumoniae* colonization in children following routine childhood immunization with pneumococcal conjugate vaccine in South Africa.

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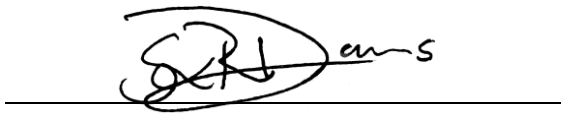
Declaration

‘Original published work 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, South Africa 2024

I, Sarah Leah Robina Downs, declare that this thesis and publications are my own, unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at any other university.



On the 28th Day of October , 2024 at Johannesburg

Dedication

This work is dedicated to my mom, Jo-Ann Mary Downs (1959-), and for her passion for Science, communities and public health.

Presentations emanating from this research:

1. SL Downs, CP Olwagen, L Van der Merwe, MC Nunes, and SA Madhi. Nanofluidic real-time PCR to discriminate vaccine associated pneumococcal serogroup 6 to individual serotypes *Molecular Biosciences Research Thrust (MBRT) Research Day*. **2019**. University of the Witwatersrand, Johannesburg, South Africa. Oral Presentation.

2. SL Downs, MC Nunes, L Van der Merwe, CP Olwagen, and SA Madhi. Temporal changes in pneumococcal colonization in under-5-year-old children eight years following routine infant immunization with pneumococcal conjugate vaccine (PCV) in rural South Africa. Faculty of Health Sciences Research Day. **2021**. University of the Witwatersrand, Online. **2020**. Oral Presentation

3. SL Downs, SA Madhi, L van der Merwe, MC Nunes and CP Olwagen. Nanofluidic Real-Time PCR to Discriminate Vaccine-Associated Pneumococcal Groups 6, 18 and 22 to Individual Serotypes. *International Meeting on Emerging Diseases and Surveillance (IMED)*. **2021**. Poster Presentation. Online. High-scoring abstract chosen for publication in the *International journal of infectious diseases*: IJID, March **2022**, 116: S118-S119 DOI: 10.1016/j.ijid.2021.12.280

4. SL Downs, MC Nunes, CP Olwagen, L Van der Merwe, and SA Madhi. Temporal changes in pneumococcal colonization in under-5-year-old children eight years following routine infant immunization with Pneumococcal Conjugate Vaccine (PCV) in rural South Africa. Vaccines. *International Society of Pneumonia and Pneumococcal Diseases Symposium (ISPPD-12)*; **2022**. Toronto Canada. Poster Presentation

5. SL Downs, MC Nunes, CP Olwagen, L Van der Merwe, and SA Madhi. Association of routine infant 2+1 13-Valent Pneumococcal Conjugate Vaccine (PCV) with *Streptococcus pneumoniae* carriage and serotype epidemiology in urban-dwelling South African children aged ≤5 years. *International Society of Pneumonia and Pneumococcal Diseases Symposium (ISPPD-12)*; **2022**. Toronto Canada. Poster Presentation

6. SL Downs, SA Madhi, L Van der Merwe, MC Nunes and CP Olwagen. High-throughput nanofluidic real-time PCR assay to evaluate serotype-specific *Streptococcus pneumoniae* colonization in the nasopharynx of healthy South African children. *International Society of Pneumonia and Pneumococcal Diseases Symposium (ISPPD-12)*; **2022**. Toronto Canada. Poster Presentation

7. SL Downs, SA Madhi, L Van der Merwe, MC Nunes and CP Olwagen. Nanofluidic real-time PCR to discriminate Pneumococcal Conjugate Vaccine (PCV) associated serogroup 6 to individual serotypes. *International Society of Pneumonia and Pneumococcal Diseases Symposium (ISPPD-12)*; **2022**. Toronto Canada. Poster Presentation.

8. SL Downs, MC Nunes, L Van der Merwe, SA Nzenze, CP Olwagen & SA Madhi. Temporal association of Pneumococcal Conjugate Vaccination on density and co-colonisation by *Streptococcus pneumoniae* serotypes and other common bacterial colonisers in rural South African children ≤60-months-old. *International Society of Pneumonia and Pneumococcal Diseases Symposium (ISPPD-13)*; **2024.** Cape Town, South Africa. E-poster with spotlight talk.

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1. SL Downs, SA Madhi, L Van der Merwe, MC Nunes & CP Olwagen. High-throughput nanofluidic real-time PCR to discriminate Pneumococcal Conjugate Vaccine (PCV)-associated serogroups 6, 18, and 22 to serotypes using modified oligonucleotides. *Nature Scientific Reports*. December **2021**. 11(1):23728 DOI: 10.1038/s41598-021-03127-9

2. SL Downs, SA Madhi, L Van der Merwe, MC Nunes & CP Olwagen. Optimization of a high-throughput nanofluidic real-time PCR assay to detect and quantify of 15 bacterial species and 92 *Streptococcus pneumoniae* serotypes. *Nature Scientific Reports*. March 2023. 13(1): 4588. DOI: 10.1038/41598-023-31820-4

3. SL Downs, CP Olwagen, L Van der Merwe, SA Nzenze, MC Nunes & SA Madhi. *Streptococcus pneumoniae* and other bacterial nasopharyngeal colonization nine years post-introduction of 13-valent pneumococcal conjugate vaccine in South African children. *International Journal of Infectious Diseases.* May **2023**. 134:45-52. DOI: 10.1016/j.ijid.2023.05.016.

Abstract

Background: *Streptococcus pneumoniae* remains a leading cause of morbidity and mortality in children <5 years old in sub-Saharan Africa. Pneumococcal conjugate vaccines (PCVs) reduce pneumococcal-associated disease by reducing vaccine-serotype (VT) circulation. Monitoring serotype-specific nasopharyngeal colonization can serve as a proxy to evaluate the impact of vaccination against VT disease. Sensitive tools for detecting pneumococcal serotypes and other bacterial species are required. Current real-time Polymerase Chain Reaction (PCR) methods do not fully distinguish 13-valent PCV (PCV13) serotypes from non-vaccine serotypes (NVT). The 7-valent-PCV (PCV7) was introduced in the South African immunization program in 2009 (replaced by PCV13 since 2011) using a 2+1 schedule (at 6, 14, and 40 weeks of age). In this study, the impact of routine PCV immunisation on pneumococcus, and other bacterial colonisation among rural (Agincourt, Mpumalanga) and urban (Soweto, Gauteng) dwelling South African children <5 years old, was evaluated 8-9 years after PCV introduction using a comprehensive, real-time PCR.

Methods: Designed and previously published assay-sets were combined into a 96-assay reaction set within a nanofluidic real-time PCR platform. The reaction-set was optimized using reference isolates and synthetic calibrators for analytical performance. To validate the reaction set, diagnostic performance was evaluated through blind analysis of 1 973 archived nasopharyngeal swab samples (NPS) previously serotyped with the referent standard culture-based Quellung method. The reaction set was applied to NPS collected from children across two study periods, during the early PCV introduction period and after 8-9 years of routine immunization. In Period-1, 630 NPS collected in May to October 2009 were available from Agincourt and 1135 NPS collected in May 2010 to February 2011 were available from Soweto. In Period-2, NPS were collected from 568 children in July 2017 to February 2018 in Agincourt, and 571 children in June to December 2018 in prospective colonization surveys.

Results: The real-time PCR reaction set was analytically sensitive (limit of detection $<10^2$ gene equivalents), efficient (90–110%), and had low variation between replicates (r^2 > 0.98) for relative quantification. The diagnostic sensitivity and specificity of the reaction set was >80% and >95% respectively for all assay-sets that targeted serotypes previously detected by Quellung at a prevalence >1%. Comparing Period-2 to Period-1, in Agincourt and Soweto, there was a lower overall (76.9% vs. 83.2%; adjusted Odds Ratio [aOR]: 0.65, 95% confidence interval [CI]: 0.48-0.87 and 49.4% vs. 68.1%; aOR: 0.66; 95% CI: 0.54-0.88, respectively) and PCV13-VT colonisation prevalence (14.3% vs. 51.0%; aOR: 0.16, 95%CI: 0.12-0.21 and 18.6% vs. 40.9%; aOR: 0.41; 95% CI: 0.3-0.56). In Period-2, residual colonization by 19F (5.3% and 8.1%) remained high in both settings compared with Period-1 (10.3%, aOR: 0.52, 95%CI: 0.33-0.82 and 6.6%; 75/1135; aOR: 2.0; 95% CI: 1.09-3.56, respectively). Non-vaccine-serotype (NVT) colonisation was higher in Period-2 in Agincourt (63.2%) than Period-1 (35.6%, aOR: 3.12, 95%CI: 2.45-3.97), while there was no difference in NVT in Soweto between the two periods (37.8% vs. 42.4%; aOR: 0.96, 95% CI: 0.72-1.26. In Agincourt, there was a higher prevalence in Period-2 compared with Period-1 of *Acinetobacter baumannii* (36.8% vs 1.1%, aOR: 50.11, 95%CI: 23.14-108.50) and *Klebsiella pneumoniae* (13.2% vs 0.6%, aOR: 22.16, 95%CI: 8.03-61.11).

Conclusions: The high-throughput nanofluidic real-time PCR method simultaneously detects 15 bacterial species and 92 pneumococcal serotypes, distinguishing to 57 single serotypes and 35 serotypes within 16 subgroups, using 73 serotyping, and 23 bacterial detection assay-sets in 96 samples (including controls), within a single qPCR run. There was an 80% and 59% reduction in colonization by PCV13-VT serotypes in Agincourt and Soweto, 8-9 years after introduction of routine immunisation with PCV, however, there was high residual prevalence PCV13-VT (14.3-18.6%) despite >90% of enrolled children >40 weeks being immunized with PCV in both settings, mainly due to serotype 19F.

Keywords

Pneumococcus, Pneumococcal conjugate vaccine, Children, Colonization, Real-Time PCR, Serotyping, Bacterial Colonization, Co-colonization

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In memory of my grandmother Edith Maud Whewell (1938 – 2020). I wish you could have been there for the end, so I could tell you it was worth it.

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Abbreviations

aliaA – gene, detoxification of hydrogen peroxide (H_2O_2)

AMR - Antimicrobial Resistance

AOM - Acute Otitis Media

aOR - Adjusted odds ratio

ART - Anti-Retroviral Therapy

ARTI - Acute respiratory tract infections

BgaA - β-galactosidase

BHQ - Blackhole Quencher

BLAST - Basic Local Alignment Search Tool

CAP – Community Acquired Pneumonia

CBPs - Choline-binding Proteins

cbpA - pneumococcal surface protein

CDC - Centers for Disease Control and Prevention

CDSCO - Central Drugs Standard Control Organization of India

CFR - Case Fatality Rate

CFU - Colony-Forming-Units

CHAMPS - Child Health and Mortality Prevention Surveillance

CI - Confidence Interval

CLWH - Children Living with HIV

COPD - Chronic Obstructive Pulmonary Disease

CPS - Capsular Polysaccharide Synthesis

Cq - Cycle of quantification

CRM197 - cross-reacting material of the nontoxic mutant of diphtheria toxin

CrI – Credible interval

CRP - C-Reactive Protein

dexB – pneumococcal gene, which encodes for the enzyme dextranase

DNA - Deoxyribonucleic Acid

DPO - Dual-Priming-Oligonucleotides

DRC - Democratic Republic of Congo

EPI - Expanded Program for Immunization

FDA - Food and Drug Administration, USA

GAVI – the Vaccine alliance, Global Alliance for Vaccines and Immunization

gBlocks - Synthetic double-stranded DNA template gene fragments

GCP - Good Clinical Practice

GE - Genome Equivalents

GMD - Geometric mean density

HDSS - Health and Demographics Surveillance Site

HIC - High-Income Countries

HIV - Human Immunodeficiency Virus

HR – Hazard Ratio

hRV - Human Rhinovirus

I^2 - measure of heterogeneity, statistical test

IAV - Influenza A Virus

IFC - Integrated Fluidic Circuit

IPD - Invasive Pneumococcal Disease

IQR – Interquartile range

LMIC - Low- and Middle-Income Countries

LNA - Locked Nucleic Acid

LOD - Limit of Detection

LRTI - Lower Respiratory Tract Infections

lyt*A* - the Autolysin gene

MDR - Multi-drug resistance

MGB - Minor Grove Binding

MIQE - Minimum Information for Publication of Quantitative Real-Time PCR Experiments

MLST/A - Multilocus Sequence Typing/Analysis

mPCR - Multiplex polymerase chain reaction

NanA - neuraminidase

NP - Nasopharynx

NPS - Nasopharyngeal swab samples

NTC - No-Template-Control

NTHi - Non-typeable Haemophilus influenzae

NVT - Non-Vaccine-Type

OM - Otitis Media

OR - Odds Ratio

PCR - Polymerase Chain Reaction

PCV - Pneumococcal Conjugate Vaccine

PCV7 - 7-valent Pneumococcal Conjugate Vaccine

PCV10 - 10-valent Pneumococcal Conjugate Vaccine

PCV13 - 13-valent Pneumococcal Conjugate Vaccine

PCV15 - 15-valent Pneumococcal Conjugate Vaccine

PCV20 - 20-valent Pneumococcal Conjugate Vaccine

PhiD-CV10 – polysaccharides conjugated to H. influenzae protein D, tetanus toxoid, and CRM197

pia*B* - permease gene of the pia ABC transporter

pIgR - Polymeric Immunoglobulin Receptor

PIV - Parainfluenza Virus

PLWH – People living with HIV

ply - Pneumolysin

PPV – Pneumococcal polysaccharide vaccine

psa*A* - pneumococcal surface adhesin A

qPCR - Quantitative polymerase chain reaction

r^2 - determination coefficient

RCT - Randomized Controlled Trial

ROC-AUC - Receiver Operating Characteristic - Area Under the Curve

RR – Risk Ratio

RSV - Respiratory Syncytial Virus

RTHC - Road to Health Card

SA - South Africa

SADC - Southern African Development Community

SARS-CoV-2 - Severe Acute Respiratory Syndrome Coronavirus 2

SD - Standard Deviation

SII-PCV10 - Serum Institute of India Pneumococcal Conjugate Vaccine 10

SNP - Single Nucleotide Polymorphism

SP2020 – gene, transcriptional regulation

SpxB – gene, pyruvate oxidase

SSI - Statens Serum Institut

ST - Sequence type

STA - Specific Target Amplification

STGG - Skim milk-tryptone-glucose-glycerin

StrH - β-N-acetylglucosaminidase

TAC - TaqMan Array Card

Tau^2 - Tau-squared, measure of the between-study variance or heterogeneity

TB - Tuberculosis

THB - Todd-Hewitt Broth

Tm - Melting Temperature

UI - Uncertainty Interval

UNICEF - The United Nations Children's Fund

USA - United States of America

VE - Vaccine Efficacy

VT - Vaccine Serotype

WHO - World Health Organization

WUENIC - WHO and UNICEF Joint Estimates of National Immunization Coverage

wzg – gene, regulation of capsule polysaccharide synthesis

wzh – gene, export of capsule polysaccharides.

wzd – gene, polymerization of capsule polysaccharides.

wze – gene, modification of capsule polysaccharides.

X^2 – Chi square, statistical test

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Chapter 1: Introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae is a ubiquitous commensal bacteria which colonizes humans at least at one point or another in their lives [1]. *S. pneumoniae* is a non-motile *Firmicutes*, gram-positive, α-haemolytic, lancet-shaped, and encapsulated diplococcus. Colloquially referred to as pneumococcus, *S. pneumoniae* are polysaccharide encapsulated bacteria that normally colonize the nasopharynx without causing disease but can shift to a virulent state as an opportunistic pathogen, progressing to life threatening disease [1-4]. The polysaccharide capsule, as the primary virulence factor, is highly variable and type specific [5]. There are 100 described antigenically distinct serotypes that vary in prevalence geographically, by age group and propensity to cause disease [6, 7]. When a new serotype of pneumococcus enters the nasopharynx, the persistence of that serotype and adherence to the epithelium of the nasopharynx is termed colonization [8]. While most serotypes are carried asymptomatically, approximately 23 serotypes cause the majority of invasive disease [9, 10]. In addition, some serotypes such as 1 and 5 have been associated with outbreaks of pneumococcal disease [11, 12]. Pneumococcus is a leading infectious cause of death in children under 5 years of age [13, 14] and of pneumonia-related mortality in all age groups, including adults >65 years old [15]. Due to the link between colonization and disease, along with the invasive potential of serotypes, colonization can be used as a marker of vaccine impact [8, 16].

1.2 Burden of Pneumococcal Disease

Although mostly carried asymptomatically, and with a low attack rate, pneumococcus is an important cause of serious invasive disease manifesting as meningitis, pneumonia, sepsis and bacteremia, and mucosal infections including non-invasive respiratory tract infections: otitis media, bronchitis, and sinusitis [10, 14]. Pneumococcus that is isolated in a normally sterile site such as cerebrospinal fluid, pleural fluid or blood is termed invasive pneumococcal disease (IPD) [17]. The risk of pneumococcal diseases is greatest in the very young (under two years of age), immunocompromised individuals and adults older than 65 years, due to the lack of a T cell–dependent immune response and/or immunosenescence [4]. In 2019 it was estimated that pneumococcus was responsible for 14% of under 5 year old mortality globally, with >90% deaths in lower income settings [18].

Lower respiratory tract infections (LRTI) are the fifth highest cause of mortality, and the highest infectious cause of death globally [19]. The Global Burden of Disease study estimated that 652 572 (95% uncertainty interval [UI] 586 475 - 720 612) children under 5 years of age and 1 080 958 (UI: 943 749 - 1 170 638) adults older than 70 years died from LRTI in 2016 [15]. In all age groups, pneumococcus caused more than half (55.4%; n=1 189 937 [UI: 690 445 - 1 770 660]) of all LRTI deaths (n=2 377 697 [UI: 2 145 584 - 2 512 809]) in 2016 [15]. Community acquired pneumonia (CAP) is the leading cause of mortality in children under 5 years of age in sub-Saharan Africa [13, 20, 21], with the highest proportion of these deaths attributed to pneumococcus[15, 22, 23]. In South Africa, the incidence if LRTI deaths in children under 5 years of age in 2016 was approximately 62.1 per 100 000 (49.7-77.7) [15].

Although pneumococcal meningitis is less common than pneumococcal LRTI, with an incidence rate in Africa of 21 per 100 000 (UI: 9-45) in children under 5 years of age, it

has a high case-fatality rate (CFR) of 61% (UI: 24–100) [14]. Pneumococcus is responsible for an estimated 41.1% of bacterial meningitis in African children under 18 years old [24]. Interventions to reduce meningitis mortality (that include conjugate vaccines) have resulted in a 54% reduction in mortality (93 deaths per 1000 live births vs. 43 per 1000) in children under 5 years of age globally between 1990 to 2015 [25].

In low- and middle-income countries (LMIC), otitis media (OM) and acute OM (AOM) and chronic post-AOM sequelae such as hearing loss, are preventable, by reducing the pathogens that cause them [26]. Around 80% of children aged under 4 years of age experience an OM episode (or multiple), resulting in 700 million annual cases in 2005 globally [27]. Pneumococcus is the most common cause of AOM [28], causing up to 50% of cases [22].

The risk of vaccine-serotype (VT) pneumococcal disease is reduced by immunization with the multivalent PCVs [10]. Prior to immunization with PCVs in the World Health Organization (WHO) Africa Region in 2008, pneumococcal pneumonia and meningitis was the leading causes of mortality in children under 5 years old, contributing to an estimated 447,300 deaths [19, 29]. The introduction of pneumococcal conjugate vaccines (PCV) is temporally associated with reductions in IPD [30]. A global modelling study estimated that in 2000, the year PCV was first licensed, pneumococcus was responsible for 735 000 deaths in Human Immunodeficiency Virus (HIV)-uninfected children under 5 years of age. By 2015, when 129 countries had introduced PCVs into immunization programs, deaths attributed to pneumococcus had declined to 294 000 (UI: 192 000–366 000) [14]. Nevertheless, in 2015, pneumococcus still accounted for 11% of all under 5 childhood deaths globally, [14] of which 52% (n=166 00) occurred in the Africa WHO region [14].

Data from 2019 indicates that the number of severe IPD cases in South Africa has remained stable for the period 2014 to 2019, with 44% (n=80/180) of the residual IPD in children under 5 years of age due to serotypes 8, 12F, 19F, 7 and 14. The overall incidence of IPD in South Africa in 2019 was 4 per 100 000 population, with the largest burden of disease in children under 5 years of age (20 per 100 000 population) [31].

While PCVs many prevent disease due to VT, the treatment of IPD and mucosal infections is further complicated by pneumococcal serotypes that are either partially susceptible to antimicrobials, or completely resistant [32]. Further to the reduction in overall IPD and mucosal infections afforded by PCVs, routine immunization decreases antimicrobial resistance (AMR), due to the inclusion of serotypes known to carry AMR genes, in PCV [33]. In South Africa, AMR-IPD was reduced by 80% in the under 2 year old age group in 2011-2012, compared with prior to routine 7-valent PCV (PCV7) immunization (2005-2008) in South Africa (from 2009) [34].

1.3 Risk factors for colonization and disease

Colonization by pneumococcus is affected by host and environmental risk factors. Pneumococcal colonization is higher in the winter or dry seasons when there is viral transmission and people gather indoors. Smoke exposure and air pollution, HIV-infection, malnutrition, creche attendance and high-density living settings are all risk factors for pneumococcal colonization and disease [4, 35-51]. The groups most at risk of developing IPD are infants, older adults (>65 years of age), immunocompromised and People Living with HIV (PLWH) [29, 34, 52-59] .

Across 16 African nations, there was a 2-times higher risk of mortality in children under 5 years of age from all cause LRTI in households utilizing solid fuels for heating and cooking indoors compared with households using electricity (adjusted hazard ratio: 2.35, 95% confidence interval [CI]: 1.22 to 4.52) [60]. In Soweto, South Africa, a 5-fold higher risk of presumed bacterial pneumonia was evident in children where the primary care giver was a smoker compared with healthy community controls (adjusted Odds Ratio [aOR]: 5.15, 95% CI: 2.94–9.03) [61].

Household crowding is also an important risk for pneumococcal colonization and disease [62-64]. In Soweto, Gauteng, South Africa, household crowding was associated with a 2-times higher risk of presumed bacterial pneumonia among HIV-uninfected children (aOR: 2.29, 95% CI: 1.89–2.78) compared with healthy community controls [61]. In Ethiopia, children living in households with one room had a 2.96 times higher risk of pneumococcal colonization compared with households with more than one room (95% CI: 1.46–5.99) [62].

Risk groups for death from pneumococcal disease also include children that are affected by poverty and malnutrition [65, 66]. In Soweto, there was a 6.7 times higher risk of presumed bacterial pneumonia in children under 5 years of age living with malnutrition (aOR: 6.68, 95% CI: 4.74–9.42) compared with community controls [61]. Malnutrition was also positively associated with pneumococcal colonization in Ethiopia (aOR: 2.07, 95% CI, 1.24-3.44) [67].

Collectively, children under 5 years of age are at the highest risk of colonization and residing with a child under 5 years of age increases the risk of acquisition of pneumococcus [62, 68, 69] particularly in LMIC. In Malawi, infants residing in close settings with other children under 5 years of age acquired pneumococcal colonization at a younger age (median time to first acquisition, 56.5 days) than infants without another

child under 5 years of age in the household (83.0 days; p=0.001). Generally mucosal immunity develops in infants from 6 months of age onward, hence young infants are also more susceptible to progression from colonization to pneumococcal disease [70]. In Ethiopia, a child under 5 years of age in the household carried a 2-3 times higher risk of colonization by pneumococcus compared with no children under 5 years of age living in the household in three separate studies (aOR: 1.80, 95% CI: 1.17–2.77; aOR: 2.84, 95% CI: 1.40–5.76; aOR: 2.9, 95% CI: 1.3-6.8) [62, 63, 67] and similarly so in Niger (aOR: 2.1, 95% CI: 1.5-2.9) [71], The Gambia (aOR: 4.06, 95% CI: 1.90–8.86) [72], and Kenya (1-2 siblings under-10 years old; HR: 1.69, 95% CI: 1.41-2.02) [73].

People of all ages, living with HIV, are highly susceptible to respiratory infections [74]. Prior to routine PCV immunization children living with HIV (CLWH) had 42 times greater risk of IPD compared with HIV-uninfected children [75, 76]. Two strategies in South Africa have reduced IPD in PLWH, and children in particular: The launch of the national Anti-Retroviral Therapy (ART) programme in April 2004 [75, 77] and childhood PCV vaccination introduced in April 2009 [78]. The prevention of mother to child transmission program was implemented in South Africa (2006) reducing vertical transmission of HIV, and thus CLWH from 9.6% in 2008 to 1.3% by 2016/17 [79, 80], compared with vertical transmission rate of 20-30% in 2001 [81], resulting in few children being born with HIV. While children that are HIV-exposed, but uninfected do not acquire pneumococcus at higher rates compared with HIV-unexposed and uninfected children [39, 82], HIV-exposed, but uninfected children in Soweto had a higher risk of presumed bacterial pneumonia (aOR: 2.33, 95% CI: 1.53–3.55) compared with HIV-unexposed and uninfected children [61]. This means that the use of ART does not necessarily reduce colonization by pneumococcus, but ART reduces susceptibility to IPD. Nzenze *et al*. found no difference in prevalence of pneumococcal colonization in PLWH on ART and those not on ART [59]. Nevertheless, Nunes *et al*. reported 41% reduction in IPD in CLWH under 2 years of age on ART compared with CLWH of the same age not on ART prior to the introduction of PCV [75].

Predicters of the early infant microbiome can indirectly influence risk of pneumococcal colonization. A high relative abundance of non-pathogenic species of *Dolosigranulum* and *Corynebacterium* in the nasopharynx, promoted through exclusive breastfeeding, have a protective role against pneumococcal colonization, [83] otitis media [84] and the development of wheezing and mild respiratory tract infections, compared with formula feeding [85].

1.4 Pneumococcal vaccines

An effective means of providing immunity and interrupting transmission of harmful serotypes is important to prevent morbidity, mortality, and health care costs associated with pneumococcal diseases. Furthermore, bacterial diseases often require antibiotic treatment, therefore prevention of pneumococcal disease is important for antibiotic stewardship [86]. Successful vaccine strategies must target the highest risk groups and involve vaccines that are efficacious and cost-effective. The main reservoir for most invasive serotypes is young children aged under 5 years, that transmit the bacteria to other age groups, including other children and adults [1, 59, 87, 88], and are the most effective age group to target with immunization. Since the development of the first whole cell vaccine in 1909, progress in vaccines to combat pneumococcal disease has included licenced vaccines that target serotype-specific regions of the polysaccharide capsule, including conjugation of polysaccharides to immunogenic proteins and serotype-independent protein or whole cell vaccines that are in development [89-91].

1.4.1 Pneumococcal Polysaccharide Vaccines.

The 23-valent polysaccharide vaccine (PPV23 known as Pneumovax®, Merck), was licenced in South Africa in 1992 [92], however PPV23 has not been in routine use in the country. PPV23 does not induce a robust anamnestic (memory) response, limiting its's effectiveness against pneumococcal disease beyond five years post-vaccination [93-95]. Furthermore, PPV23 does not prevent the acquisition of pneumococcal carriage and is not highly effective against non-invasive pneumococcal pneumonia [96, 97]. Finally, PPV23 is not immunogenic or effective in children under-2 years of age, due to them being unable to mount a T-cell independent response [97-101].

Vaccinating children with PCV and achieving high coverage in infancy, remains the key strategy to prevent pneumococcal colonization and infections from invasive or antibiotic resistant serotypes in both children under 5 years of age and at a population level, including other risk groups [10].

1.4.2 PCV to prevent disease in young children.

Through the conjugation of selected pneumococcal polysaccharides to carrier proteins, PCVs induce T-cell–dependent immunity to the serotypes included in the vaccine [97, 102, 103]. PCVs reduce the risk of nasopharyngeal (NP) acquisition of VT carriage through a serotype-specific adaptive immune response to capsular polysaccharides, thereby reducing transmission and disease [1, 8, 102-107]. The direct protection against pneumococcal NP colonization and disease in children targeted for immunization with PCVs, and in their unvaccinated contacts is well documented globally [17, 34, 59, 105, 107-116]. This includes reduction in IPD and all-cause pneumonia hospitalization [34, 107, 117, 118].

The introduction of PCVs have reduced the burden of disease and mortality by pneumococcus by more than 50% in South Africa and globally [14, 17]. Furthermore, immunization with PCV reduces pneumococcal disease incidence and severity, and provides herd immunity [34, 117, 119, 120]. The routine implementation of this vaccine into childhood immunization programs, has reduced the burden of pneumococcusin the general population through an indirect effect of reduced transmission of pneumococcus from the young vaccinated children to others in the community [91]. This includes reduction in IPD and all-cause pneumonia hospitalization in high- and middle-income countries [34, 107, 117, 118].

The first PCV licensed (PCV7, Prevnar®, Pfizer in 2000) targeted serotypes 4, 6B, 9V, 14, 18C, 19F and 23F (Table 1.1), through the conjugation of capsular specific polysaccharides to cross-reacting material of the nontoxic mutant of diphtheria toxin (CRM_{197}). The PCV7, however, excluded some serotypes that were epidemiologically important and prevalent in developing countries [34]. The PCV7 was licenced based on the vaccine efficacy (VE) in two randomised control trials (RCT) of a three primary dose schedule with a booster of 97.4% (95% CI: 82.7-99.9)[121] and 100% (95% CI: 75.4-100) [122] against VT-IPD; and 90% (95% CI: 58.3-98.9) [122] against overall IPD [122]. Furthermore, immunization with three primary doses and a booster resulted in a 89.1% (73.7-95.9) reduction in all IPD [121]. Thereafter, IPD by the non-PCV7 serotype 19A began to rise in the under 5 years of age group, from 2.6 cases per 100 000 in 1999 to 11.1 in 2007 [107]. Furthermore, serotype 1 that was not included in PCV7 frequently causes disease and outbreaks in Africa [123, 124]. Consequently, PCVs formulations were expanded to include additional serotypes.

Table 1.1: Serotype coverage of pneumococcal vaccines.

Serotype:	Year licenced	1	3	4	5	6A	6B	7F	8	9V	10A	11A	12F	14	15BC	18C	19A	19F	22F	23B	23F	24F	33F	38
PCV7*	2000																							
PCV9	-																							
PCV10	2008																							
SII-PCV10	2020																							
PCV12	-																							
PCV13	2009																							
PCV15	2022																							
PCV20	2021																							
PPV23	1992																							

*No longer in routine use

Countries that initially introduced PCV7 have since transitioned to higher valency PCVs. Current PCVs in use contain an additional three (10-valent PCV/PCV10/PHiD-CV10, Synflorix, GlaxoSmithKline, licensed in 2008: 1, 5 and 7F), six (13-valent PCV/PCV13, Prevnar-13®, Pfizer, licensed in 2009: 1, 3, 5, 6A, 7F and 19A), eight (15-valent PCV/PCV15, VAXNEUVANCE™, Merck, licensed in 2022: 1, 3, 5, 6A, 7F, 19A, 22F and 33F), and 13 (20-valent PCV/PCV20, Prevnar-20®, Pfizer, licensed in 2021 for use in older adults: 1, 3, 5, 6A, 7F, 19A, 8, 10A, 11A, 12F, 15B, 22F and 33F) serotypes in addition to those contained in PCV7 (Table 1.1, Figure 1.1). Furthermore, a 10-valent PCV (SII-PCV10; Pneumosil™, Serum Institute of India, Pvt. Ltd) contains PHiD-CV10 serotypes, except for 4 and 18C but with the addition of 19A. By 2023, PCVs have been introduced in most countries worldwide (n=163, Figure 1.1), with a further 13 countries planning an introduction, including with the assistance of the Global Alliance for Vaccines and Immunization (GAVI) in LMIC settings [125].

The licensure of PCV10 , also known as PHiD-CV10 (polysaccharides conjugated to *H. influenzae* protein D, tetanus toxoid and CRM_{197}) and PCV13, set to replace PCV7 in

immunization programs was based on immunogenic non-inferiority to the serotypes common to these two vaccines and PCV7 [126-129], in accordance with WHO recommendations for the licensure of new PCVs [130, 131], whereas few countries have yet introduced PCV15 and PCV20 (Figure 1.1). Both PCV15 and PCV20 have shown comparable immunogenicity and tolerability compared with PCV13 in infants [132, 133].

While the non-inferior immune responses were sufficient for approval and no large scale RCTs were conducted to discretely define protection against IPD for PCV10 and PCV13, a few smaller studies have demonstrated this protection. A systematic review of 19 studies, comprising RCTs, Case-control and cohort studies, consolidated findings on the efficacy and effectiveness of PCV10 and PCV13 in children under 5 years of age against VT-IPD and AOM. The systematic review delineated the VE of PCV13 against VT-IPD in the range of 86% to 96% in a 3+1 schedule and 67.2% to 86% in a 2+1 schedule, while the range of PCV10 VE against VT-IPD was 72.8% to 100% in a 3+1 schedule and 92% to 97% in a 2+1 schedule. Notably, both PCV formulations had limited effectiveness against serotype 3 IPD [134]. The VE was validated by a separate systematic review and meta-analysis that aimed to estimate the clinical effectiveness of all investigational and approved PCVs from PCV7, PCV9, PCV10, PCV11, and PCV13 in all age groups across multiple geographic regions and included immunocompetent and immunocompromised individuals for 21 RCTs using a random effects model. Despite the wide range of included participants, PCVs reduced the odds of IPD (odds ratio [OR]: 0.43; 95% CI: 0.36-0.51), the risk of pneumococcal pneumonia (RR:0.78; 95% CI: 0.62-0.97), all-cause pneumonia (RR: 0.93; 95% CI: 0.89-0.97) and pneumococcal AOM (RR: 0.57; 95% CI: 0.39-0.83) [135]. Discretely defined age and risk groups would have better described the effectiveness for targeted groups, however due to the smaller sample sizes this was not possible. For the most part, studies to investigate PCV effectiveness against disease outcomes have been heterogenous, making definitive measures in a meta-analysis untenable [136].

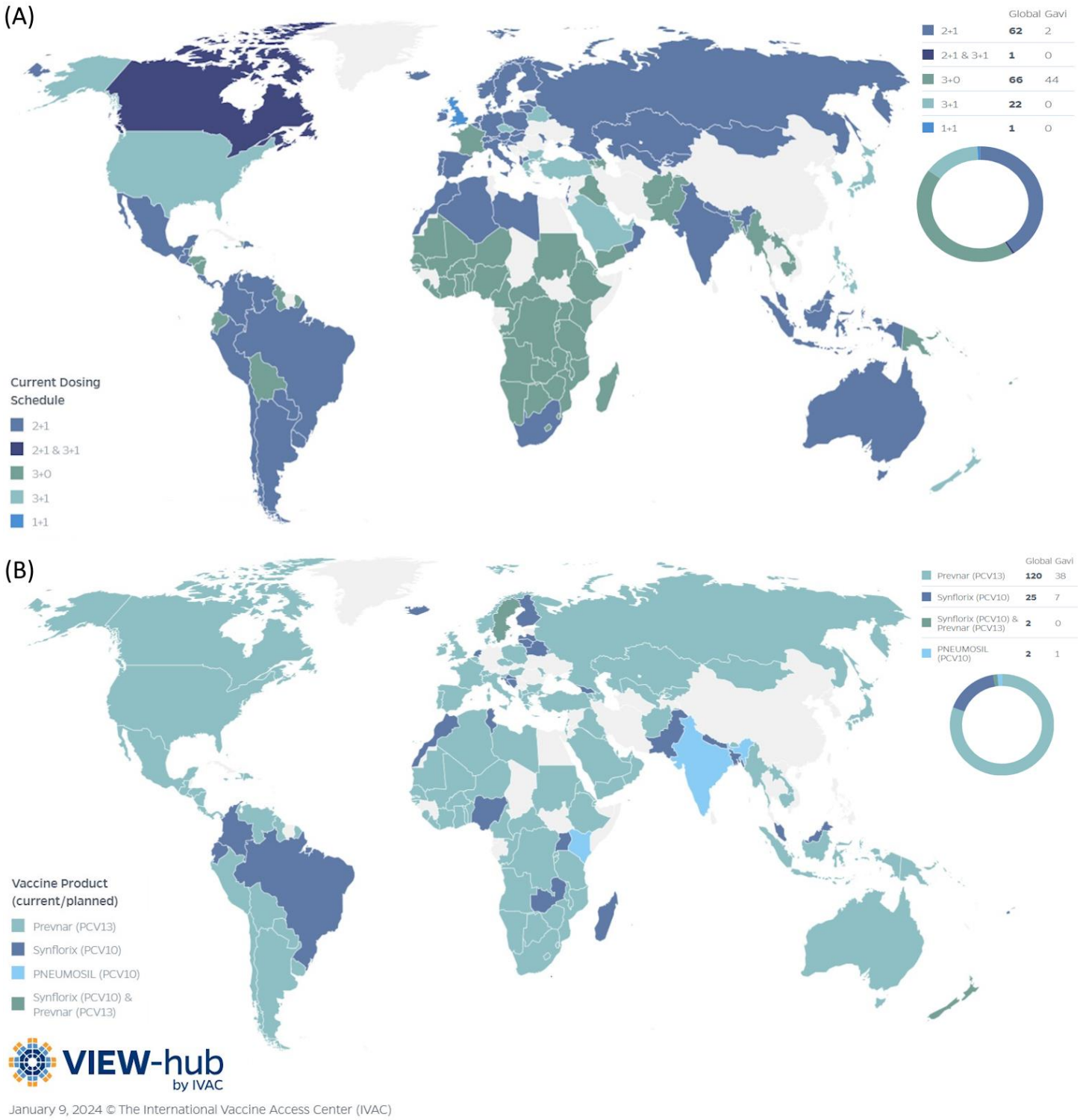

Figure 1.1: Current dosing schedules (A) and product use (B) for Pneumococcal Conjugate Vaccines (PCV) introduced into 163 countries worldwide.

Accessed at www.view-hub.org, January 2024.

Both PCV10 and PCV13 are WHO prequalified and currently in widest use, have comparable immunogenicity and impact on carriage and disease for the serotypes covered by both vaccines [10].

1.4.2.1 PCV dosing schedules

Immunity induced by PCV prevents the new acquisition of serotypes covered by the vaccine and thereby interrupts onward transmission; however, vaccination with PCVs does not clear serotypes already being carried [137, 138]. Additionally, colonization with VT prior to completing the primary immunization schedule, results in lower immunogenicity against the targeted serotypes [139]. For these reasons, the use of PCV earlier in infancy (6-14 weeks-of-age) is the most beneficial. For effective protection through childhood, a booster dose in older infants (around 9 months of age) elicits higher immunogenicity to VT which translates to a more persistent immune response, interruption of transmission and reduced susceptibility to disease [140-143].

Countries vary in both the valency of PCV, and the dose schedule used (Figure 1.1) [144]. Initially PCVs were recommended in a 3+1 schedule (2, 4, and 6 months of age and a booster at 12 to 15 months of age) [122], however in LMIC that have introduced PCVs, most administer PCV10 or PCV13 with two primary doses in early infancy (6 and 14 weeks of age) and a booster dose around 40 weeks of age (2+1 schedule) or three primary doses in infancy (6, 10 and 14 weeks of age), with no booster dose (3+0 schedule), shown in Figure 1.1A [10, 145]. The WHO currently advises the administration of PCV in a three-dose regimen, either 2+1 or 3+0 [10]. In South Africa the immunogenicity of a 2+1 PCV7 schedule demonstrated a superior immune response than when only three primary doses were administered [146]. The booster dose after 9 months of age is more important than the

number of primary doses in early infancy, and the booster dose results in a higher geometric mean concentration of IgG antibodies against VT post-booster, compared with the third primary dose in the absence of a booster [141, 147]. Furthermore, protection against VT after PCV immunization wanes over time, and a longer interval (two months) between two primary doses is more immunogenic than three primary doses spaced one month apart, implying that the 2+1 schedule is superior to the 3+0 schedule [146-148].

1.4.2.2 PCV in South Africa

South Africa was the first African country to include PCV7 in its Expanded Program for Immunization (EPI) in April 2009, implemented as a 2+1 schedule (6, 14 and 40 weeks) [34]. In South Africa, PCV7-VT were responsible for roughly 70% of IPD in children under 1 year of age in 2006 (prior to inclusion in the EPI) [24, 25]. According to a systematic review of pneumococcal colonization in sub-Saharan Africa, commonly carried serotypes prior to the introduction of PCVs, in order of prevalence included 19F, 6B, 14, 6A, and 23F [149], which were the same serotypes most prevalent in LMIC combined [46]. Except for 6A, these serotypes were all included in PCV7.

South Africa transitioned from PCV7 to PCV13 in May 2011, using the same 2+1 schedule and with an added catch-up campaign for children ≤6-years-old living with HIV or other high-risk conditions and any other children ≤3 years old [34, 59, 150]. The VT targeted by PCV13 were responsible for 86% of IPD in 2006 (Pre-PCV7) in South African in infants under 1 years of age [151]. In South Africa, a case-control study in children older than eight weeks old (median age 43 weeks; IQR: 17-112), showed that PCV7 in a 2+1 schedule in routine use, was 90% effective in reducing IPD from VT in HIV-uninfected children and 57% in CLWH [110]. Vaccine coverage in South Africa (up

to 3rd dose) has reportedly increased from 10% in 2009 [150, 152] when the vaccine was first introduced to 78.4 - 84.6% in 2017-18 according to official/administrative estimates (Figure 1.2) [153].

The routine use of PCV in South Africa since 2009 has reduced overall IPD. The incidence of IPD is highest in the age group targeted for immunization, children 0-12 months old in South Africa [154], with rates of just over 70 per 100 000 in 2009 during the introduction of the 7-valent PCV, compared with 21 cases per 100,000 in children aged 0-12 months in 2018 [154, 155]. Similarly, the incidence of IPD cases has been reduced in the 12-48 month age group following routine PCV immunization, where there were roughly 15 cases per 100 000 in 2009 compared with three per 100 000 in 2018 [154, 155]. Further to the overall reduction in IPD cases in South Africa, most IPD in 2018 was caused by 'replacement' non-vaccine serotype (NVT), meaning that VT were no longer causing IPD. Nine years after the introduction of PCV (2018), only 26% of IPD cases in children under 5 years of age were due to VT, compared with 85% in 2009 and the top five serotypes causing IPD in 2018 were serotypes 8 (NVT), 19F (VT), 16F (NVT), 19A (VT) and 12F (NVT) [154]. The routine use of PCVs in South Africa have averted an estimated 1116 (50% Credible Interval [CrI]: 559-1798) hospitalizations among HIV-uninfected urban-dwelling children of Soweto, Gauteng in 2014. In CLWH there was an estimated 33% (50% CrI: 6-52) reduction in the incidence of pneumonia hospitalizations, specifically, around 68 (50% CrI: 9 to 153) pneumonia hospitalizations were averted in Soweto children.

PCVs that aim to solve current shortfalls such as cost, or serotype coverage in LMIS are on various stages of development (Table 1.1). The more cost-effective 10-valent PCV (SII-PCV10; Pneumosil™, Serum Institute of India, Pvt. Ltd), which incorporates the most prevalent invasive serotypes (Table 1.1) before PCV introduction in LMICs was WHO prequalified in 2019 [156], approved by the Central Drugs Standard Control Organization (CDSCO) of India in 2020, and has been introduced in India [100, 157, 158]. A

12-valent PCV (PCV13 serotypes minus serotype 3) developed by SK Bioscience is in clinical trials and aims to reduce the cost of PCVs for LMICs, but has not yet been WHO prequalified or licensed for use [159]. Next-generation 15- and 20-valent PCVs [133, 160] have been approved for use in adults older than 18 years of age by the Food and Drug Administration (FDA) in the United States of America [161].

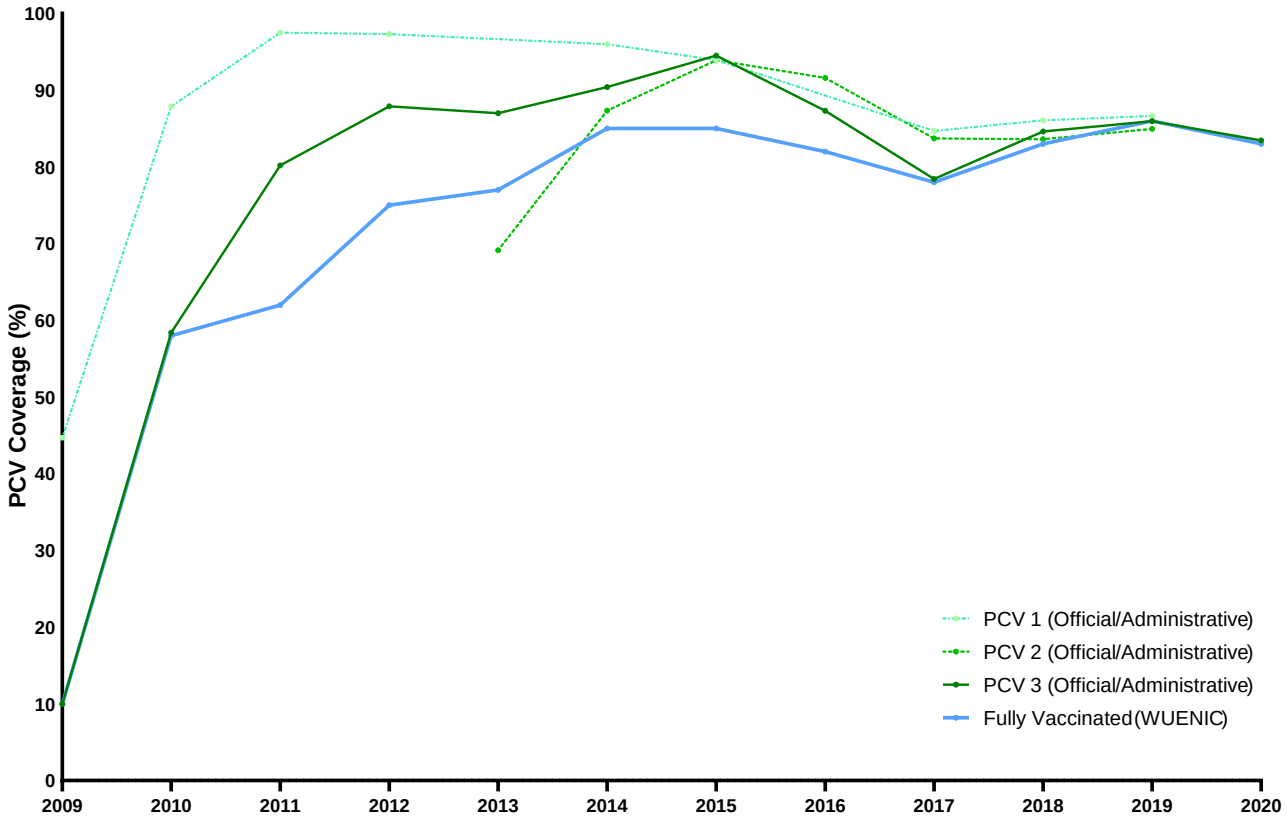

Figure 1.2: Annual administrative and Official pneumococcal vaccination coverage in South Africa reported through the World Health Organization (WHO)/United Nations Children's Fund (UNICEF) Joint Reporting Form on Immunization (JRF) and the WHO and UNICEF Joint Estimates of National Immunization Coverage (WUENIC) [153, 162].

WUENIC estimates include the percentage of infants who received three doses of pneumococcal conjugate vaccine (PCV) or two doses prior to their first birthday. Official

estimates are based on the administrative method i.e.: doses administered as part of the immunization schedule. PCV 1; PCV 2 and PCV 3 is the percentage in the target population that received one; two and three doses of PCV respectively.

1.5 Methods for pneumococcal serotyping

Pneumococcus employs diversity in the repeating saccharide units to evade host immune responses, while capsular protection and other virulence factors influence the colonization and disease potential of different serotypes [119, 163, 164]. Serotype level detection of pneumococcus is therefore important in colonization studies to assess vaccine effect (VT vs. NVT), disease potential, emerging or replacement serotypes and co-colonization.

Since around 1931, the gold standard for pneumococcal detection and serotype identification has been the culture based Quellung method (summarised in Table 1.2), while culture is not as analytically sensitive as molecular methods, and the reagents for Quellung can be costly [91, 114, 165, 166]. The premise of this method is the culture of pneumococcus from a clinical sample, which may be semi-quantitative. Pneumococcus is first identified based on colony morphology (alpha-haemolytic colonies) and sensitivity to optochin. From the cultured sample, one or two dominant pneumococcal colonies are selected and re-cultured for a pure isolate. Pure culture isolates are then mixed with serotype specific anti-sera and then the observation of serotype specific antibody binding that induces a characteristic opaque ring around the cell wall, is visualised through the microscope [91, 114, 165]. The method requires training and expertise, culture viable isolates, as well as the purchase of antisera. Organism viability may be affected by transport media, culture technique or antibiotic therapy prior to the clinical sample being taken, which may result in false negatives [165].

To avert the need for microscopy, the latex agglutination makes use of latex beads. Latex agglutination is performed on cultured isolates from clinical samples and follows a similar phenotypic serotyping premise as Quellung. Pneumococcus is identified based on colony morphology and optochin sensitivity, and serotype identification is based on antigenic reaction to antisera. Latex beads that are coated with serotype specific antibodies are mixed with the isolates, and the beads will agglutinate in the presence of the targeted serotype, which is macroscopically observed. Relative to the Quellung method, Latex agglutination is less expensive and faster to perform, however not all serotypes are distinguished, and similar to the Quellung method, latex agglutination relies on culture viability and the selection of dominant colonies [167].

Collectively, culture-based methods of serotyping detect and type the dominant (highest density) serotype. The Quellung and latex agglutination are WHO recommended pneumococcal serotyping methods, however culture-based methods are not effective at detecting multiple concurrent serotype colonization, low density colonization or for samples that are non-viable [168, 169]. This may then result in the masking of serotypes circulating in populations at lower density, by the favoured detection of higher density or dominant serotypes by culture based methods [170]. Bacterial density of pneumococcal serotypes in the nasopharynx is dynamic, and changes through the course of colonization [171]. In cross-sectional studies, low-density serotypes may indicate early stages of colonization before bacterial growth peaks or a late stage when bacterial density is declining, signaling clearance. [172]. Furthermore, low density serotypes may harbour antimicrobial resistance genes, that may be acquired by other serotypes through transformation. Although the clinical relevance of low-density serotypes is not well defined, their detection remains important for identifying residual VT serotype circulation in cross-sectional studies, and serotypes known to be invasive have been detected at low bacterial loads [173, 174].

In 2006, the biosynthetic locus of the capsular polysaccharide synthesis (CPS) genes that confer serotype heterogeneity of 90 pneumococcal serotypes were published, enabling

the advancement of Polymerase Chain Reaction (PCR) and other molecular serotyping methods, that can circumvent the disadvantages of culture-based methods [5]. Pneumococcal serotypes are distinguished through antigenic diversity conferred by the glycosyltransferases in the polysaccharide capsule, which is external to the cell wall [175]. These are the result of mutation, duplication, insertion or deletion in the hypervariable region of the CPS locus (Figure 1.3). The basis of molecular serotyping is the detection of genetic diversity that distinguishes pneumococcal serotypes based on their capsule, by targeting the genes between 'dexB' and 'aliaA' (Figure 1.3) [5, 7, 176]. Molecular methods are more analytically sensitive for the detection of pneumococcus, especially at lower density, and can detect serotypes in multiple concurrent colonization and in culture negative, or non-viable samples that cannot be cultured [170, 173, 177-179]. A variety of conventional serotyping PCRs have been utilized including singleplex, multiplex and nested PCR formats, either directly or including broth enrichment, and have also been combined with other methods such as dot-blot detection, gel and capillary electrophoresis, fragment analysis, sequencing and pyrosequencing and electrospray ionization–mass spectrometry to further distinguish individual serotypes [180-189]. Notably, a conventional sequential multiplex PCR (mPCR) method was developed by the Centers for Disease Control and Prevention (CDC), United States of America (USA) to determine 29 broad or common serogroups/types, which were then sequentially narrowed down within pools to 40 singular serotypes [184, 190].

Real-time PCR is less time consuming than culture-based methods and conventional PCR and provides faster identification of pneumococcal serotypes [178]. Furthermore, real-time PCR is capable of quantifying bacterial load, that may be important in transmission potential and progression to disease [191-193]. Whilst real-time PCR has several advantages, genetic similarity of some serotypes can be an obstacle when many serotypes are included, as primers may bind to non-target serotypes, especially when multiplexing, which may compromise specificity [194]. To overcome this, molecular serotyping panels must be extensively validated against the gold standard Quellung method and cross

reactivity between serotypes investigated. Compliance with the published Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines provides methods of optimisation, validation, standardization and comparability between different world regions and specimens used [195], facilitates the use of real-time PCR for molecular serotyping of pneumococcus that is adaptable to different platforms and settings. A molecular method for serotyping should be comprehensive (include most serotypes), sensitive, specific, capable of detecting and quantifying serotypes at low density or in co-colonization, and reproducible through adaption to different platforms (e.g. real-time PCR instruments) and high- or low-throughput configurations in different settings [169].

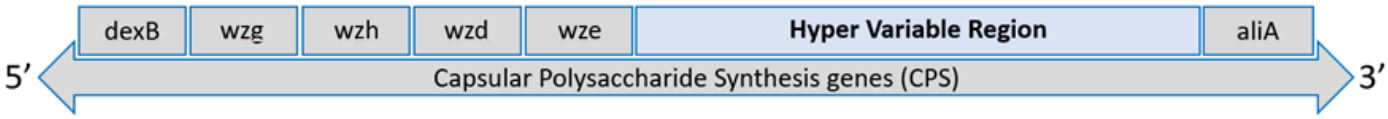

Figure 1.3: Simplified Genetic map of the Capsular Polysaccharide Synthesis Genes.

This region codes for the saccharide sequences that confer variance and virulence to the different serotypes. The region is flanked by the dexB gene in the 5' end, followed by the first 4 genes of the CPS (wzg, wzh, wzd and wze) which are highly conserved. The hypervariable region then codes the saccharides that differentiate the serotypes. The 3' end of the CPS locus is flanked by aliA. (Adapted from Bentley et al.(2006) [5] and Mavroidi et al.(2007) [7])

A disadvantage of molecular methods is that some non-pneumococcal nasopharyngeal or oral commensal species can acquire the capsular genes for pneumococcal serotypes that are the target of serotyping PCR, resulting in cross-reactivity with targeted molecular assays targeting serotype specific regions [196-201]. It is thus imperative to include validated and specific pan-pneumococcus genes to rule out other non-pneumococcal species in molecular serotyping schemes. Several ubiquitous pan-pneumococcus genes have been validated for use in molecular serotyping to confirm the presence of pneumococcus.

Autolysin (lyt*A*) has been the preferred gene for PCR detection of pneumococcus and quantification of bacterial load, whereas psa*A* (pneumococcal surface adhesin A); Xisco (a putative protein), pia*B* (permease gene of the pia ABC transporter) and recently SP2020 (putative transcriptional regulator gene) have also been described as sensitive and specific to pneumococcus [114, 166, 175, 202-208]. Some non-pneumococcal species may possess similar genes to lyt*A*, while some pneumococcus may have an atypical lyt*A* gene [209]. Therefore, it is recommended that multiple pan-pneumococcal genes are included in molecular serotyping to confirm pneumococcus, and the use of lyt*A* and pia*B* are regarded as sensitive and specific in combination [207, 210].

Since 2010, real-time PCR serotyping assays have been developed, to harness the advantages of rapid, cost-effective serotyping. Azzari *et al.* (2010) developed a real-time PCR to detect 21 serotypes, including VT and common NVT, however emerging and uncommon serotypes were not included, and some VT were detected in serogroups that included NVT (Table 1.3) [178]. Real-time PCR using TaqMan chemistry for pneumococcal serotyping has since been adapted to higher throughput methods, including the assay-sets designed by Azzari *et al.*, (2010) and additional novel serotyping assay-sets that have been designed (Table 1.3). Pholwat *et al.* (2016) optimized a PCR-based microfluidic TaqMan array card (TAC) made up of 53 assay-sets that detected 74 serotypes able to test up to seven clinical samples per card with 100% specificity and 97% (±9.7) efficiency [166]. Dhoubhadel *et al*. (2014) used nano-fluidic real-time PCR with SYBR Green chemistry to detect 50 serotypes, differentiating 16 individual serotypes and 13 sub-groups [175]. Olwagen *et al.* developed a reaction set to identify 55 serotypes and other nasopharyngeal colonizing pathogens across 96 samples at a time using nano-fluidic TaqMan chemistry on a Biomark HD System (Standard BioTools, formerly Fluidigm) with higher specificity, sensitivity and serotype resolution than standard quantitative PCR serotyping assays and culture methods [211]. To further improve sensitivity, specificity and serotype discrimination, assay sets utilising modified probes that include locked nucleic acids (LNA) and 'Blackhole Quencher (BHQ)-plus' were designed by Messaoudi *et al*. and Velusamy *et al*. (2020) respectively to improve

mismatch discrimination in difficult (A-T rich) or highly similar serotype target regions. Collectively, the assay-sets designed as part of these studies (Table 1.3) enable the development of comprehensive molecular serotyping schemes [193].

Expanding the array of serotypes detectable by higher throughput real-time PCR methods is essential. Serotypes that were previously detected as serogroups (e.g. Serogroups 6 and 18) and not individually distinguished may result in an underestimation of vaccine impact as they include both VT (6A, 6B, 18C) and NVT (6C, 6D, 18A, 18B, 18F), and some VT are targeted by PCV13 (6A), but not PCV10. Similarly, as higher valency PCVs (e.g. PCV15 and PCV20) have been developed, distinguishing previously grouped serotypes like 22F (usually detected as 22A/F) included in next-generation PCVs is important to predict the benefit, or evaluate the impact of these vaccines on serotype epidemiology. Furthermore, detecting, and distinguishing NVT is important for characterising replacement disease and unmasking low density circulating serotypes especially in high carriage settings, like LMIC [99]. In the past, the limited ability to fully serotype VT and NVT has been identified as a drawback of molecular serotyping for the purpose of assessing the impact of PCV, and due to the varying geographic prevalence of individual serotypes [194, 212]. In addition to real-time PCR, sensitive and specific deoxyribonucleic acid (DNA) microarray serotyping schemes that can also identify antimicrobial resistance genes have been developed, however, this method is more costly than PCR and requires equipment not ubiquitous to all labs [169, 213, 214].

Sequencing approaches to serotyping have included targeted sequencing of housekeeping genes, such as multilocus sequence typing/analysis (MLST/A) and ‘sequetyping’ using a primer pair on conserved regions flanking the hypervariable region of the CPS to detect differentiate serotypes using an automated pipeline [215, 216]. Recent advances in higher-throughput whole genome sequencing, automated pipelines and cost reductions in performing these methods have allowed the development of sequence-based serotyping, although sequencing is still more costly than real-time PCR based approaches [215, 217]. In

2016 the automated whole genome sequence analysis pipeline Pneumococcal Capsular Typing (PneumoCaT) was published, capable of detecting 89 serotypes individually and four serotypes to serogroup [218]. Similarly, seroBA was developed to identify 92 serotypes, and two subgroups [219] and more recently, Pneumococcal K-mer Integrated Typing (PneumoKITy) was optomized to rapidly distinguish 54 of the 93 serotypes in the Statens Serum Institut (SSI) Diagnostica serotyping panel (used for Quellung) to individual serotype and to detect 39 serotypes within serogroups or sub-serogroups [220]. The available equipment in the setting, technical expertise of laboratory personnel, reagent costs, and intended application should be considered when selecting a method to serotype pneumococcus.

1.6 Colonization and the pathway to disease

Colonization is determined as the presence of microorganisms without causing disease, symptoms or tissue damage [30]. Although innocuous in this state, asymptomatic colonization by pneumococcal serotypes in the nasopharynx is a source of transmission and a precursor to disease [8]. Progression to disease almost always includes initial asymptomatic pneumococcal nasopharyngeal colonization [8]. Due to the intrinsic link between colonization and disease, measuring serotype prevalence in the nasopharynx could be informative to determine the risk of disease [16], and to assess pneumococcal colonization is as important for surveillance of the impact of PCVs [221]. Further, the determination of bacterial density in the nasopharynx can bolster the usefulness of methods looking at colonization isolates to understand disease.

Table 1.2: Summary of pneumococcal serotyping methods.

Method	Type	Culture steps	Premise of Method
Quellung	Phenotypic	Culture dependant	Colony morphology (alpha-haemolytic; optochin sensitivity), microscopic observation of capsular swelling in the presence of antisera.
Latex agglutination	Phenotypic	Culture dependant	Colony morphology (alpha-haemolytic; optochin sensitivity), macroscopic observation of agglutination in the presence of antisera.
Sequential multiplex PCR	Molecular	Culture independent	Conventional PCR, Visualisation of bands of the correct size on electrophoresis
Real-time PCR	Molecular	Culture independent	PCR detection of fluorescence
Sequencing	Molecular	Culture independent	Characterization of genetic regions encoding the capsule

Estimates of colonization prevalence differ geographically, by sociodemographic status and by age group [6, 7]. Additionally, the estimated prevalence from different studies may not be directly comparable if different sample types are used (such as nasal, nasopharyngeal, or oropharyngeal samples; swabs or washes or a combination) [168, 210, 222]. Prevalence estimations may further vary depending on the sensitivity of the detection method, that is: culture-based or molecular-based techniques. Prevalence of pneumococcal colonization can be as high as 50-80% in children under 5 years of age depending on the setting [1, 113, 223-228].

Table 1.3: Real-time PCR methods using TaqMan chemistry developed for the detection and serotyping of pneumococcus.

Reference	Method	Assay-sets	Total serotypes	Sero/sub-groups	Individual serotypes
Azzari *et al*. (2010) [229] and Azzari *et al*. (2012) [230]	Singleplex, all novel	21	34	10	12
	Singleplex, expanded	29	43	11	19
	Novel assay-sets	8	9	1	7
Moore *et al*. (2010) [231]	Multiplex, all novel	12	18	4	8
Messaoudi *et al*. (2016) [193]	Multiplex, LNA* modified probes	40	67	17	23
Sakai *et al*. 2015 [232] and Pholwat *et al*. (2016) [166]	Singleplex quantitative and conventional PCR (group 6); adapted to TAC†	53	72	16	39
	Novel assay-sets	27	36	7	20
Sakai *et al*. (2017) [233]	Singleplex quantitative	64	89	20	45
	Novel assay-sets	11	16	5	6
Olwagen *et al*. (2017) [170] and Olwagen *et al*. (2019) [211]	Multiplex	24	55	11	14
	Nanofluidic quantitative	29	58	13	16
	Novel assay-sets	17	33	7	10
Pimenta *et al*. (2013) [234] and Velusamy *et al*. 2020) [235]	Triplex, all novel	21	37	10	11
	Updated quadriplex real-time PCR, BHQ-plus probes¤	48	64	13	34
	Novel assay-sets	27	27	3	23

Note: Assay set includes forward-, reverse-primer and probe; total serotypes detected includes all serotypes included in sero/sub-groups and individual serotypes. Sero/sub-groups were more than one serotype detected together and not distinguished to serotypes, but may have been typed further than the serogroup, eg: 6A/B/C/D would be a serogroup, but further distinguished to two sub-groups (6A/C and 6B/D) and counted as n=2; instead of n=1. Individual serotypes were distinguished from their serogroups with no cross reactivity with other serotypes, or an algorithm could be applied to assign a serotype, e.g. two assay-sets: 25A/F and 25A/F/38, would result in one serogroup (25A/F) and one individual serotype (38) but a total of 3 serotypes detected (25A/F/38). One method was excluded from the table as degenerate primers were used, and the number of serotypes/groups distinguished/detected was unclear [179]. *LNA is locked nucleic acid probe. †TAC is Taqman Array Card, a microfluidic real-time PCR. ¤BHQ-plus is a modified Blackhole Quencher

1.6.1 Pneumococcal adherence in the human nasopharynx.

The human nasopharynx is a respiratory surface composed of epithelial cells and mucosa [236] and along with pneumococcus, the nasopharynx is also commonly colonized by many other commensal bacteria [1]. Various intrinsic pneumococcal factors allow for nasopharyngeal colonization through adaption to low nutrient availability, adherence to the respiratory mucosa, and evasion of the host immune responses [1]. Pneumococcus adheres to epithelial cells through choline-binding proteins (CBPs), and colonization is enabled by sialidases such as Neuraminidases (e.g.: NanA) and other glycosidases such as BgaA (β-galactosidase) and StrH (β-N-acetylglucosaminidase) [1, 237]. These mediate partial interruption of the mucus layer of the epithelial cells through desialylation, thereby exposing host cell receptors, that are bound by pneumococcal surface adhesion protein A (PspA) within the cell wall [238, 239]. Desialylation of immunoglobulins and cell surface receptors also affects the immune response to pneumococci, preventing clearance [240]. Phosphocholine constitutes the pneumococcal membrane and cell wall associated "C-carbohydrate" teichoic and lipoteichoic acids. Usually, phosphocholine is bound by host C-Reactive Protein (CRP), activating complement and inflammatory pathways to protect the host [4, 65]. Importantly, the external pneumococcal capsule shields the cell wall from immune recognition as it interferes with opsonisation in phagocytosis, inhibits mucus binding, mucociliary clearance, and pneumococci are not killed in extracellular neutrophil traps [164, 237, 241-243]. Pneumococcus thereby evades the innate immune response, enabling colonization.

Colonization is mostly self-resolving, where pneumococcal serotypes are acquired and cleared without symptoms; however, serotypes with higher invasive potential that are not cleared through an effective mucosal immune response, may go on to cause disease [1]. In the progression from colonization to disease, pneumococcus generally migrate through the mucosal epithelium and endothelium by means of endocytosis, further disseminating

into other tissues and organ systems where they cause disease [70]. Bacterial pathogenic factors mainly relate to the polysaccharide capsule, which is the most important virulence factor [65]. Capsular protection (and other virulence factors) determines the colonization and disease potential of serotypes [163, 244, 245].

1.6.2 The benefit of commensal biofilms to bacterial colonization

Bacteria may form biofilms in the carrier state, that are an organised multicellular community of commensal bacteria [246-248]. Biofilm participation is beneficial to bacteria as it aids in evasion of the host's immune responses, the accumulation of pneumococcal biomass, provides an environment for genetic exchange through horizontal transfer and allows for the persistence of pneumococcal colonization even with antibiotic use [249, 250]. Usually participation in biofilms involves a reduced virulence phenotype [251]. In this way, biofilms are important in pneumococcal colonization, antibiotic resistance, transformation and immune evasion.

1.6.3 Bacterial Density in the Nasopharynx

Increasing bacterial load during carriage escalates the risk of transmission, dissemination into organ systems to cause disease [244, 252], and is a marker of invasive potential and severity of disease [191, 193, 252-260]. Usually, pneumococcal density is measured as a continuous value by quantitative real-time PCR (qPCR) or categorically by semi-quantitative culture methods. Density quantification using these techniques enables researchers to describe the role of accumulated bacterial mass in the escalation from the carrier to a disease state.

Pneumococcal density depends on serotype, indicating that the capsule is important in pneumococcal density [261]. In Vietnam the median density of typeable pneumococcus (2.5×10^6 Genome Equivalents [GE] /mL) from NP swab samples (NPS) was more than 200 times higher compared with pneumococci without the cps*A* gene, i.e. non-typeable isolates (1.0×10^4 GE/mL; $p<0.0001$) in radiologically confirmed pneumonia cases and healthy controls [191], underscoring the role of the capsule in accumulated pneumococcal density.

Despite the association of increased pneumococcal density with disease [191, 193, 253-260], NP density in the context of healthy colonization should be interpreted with caution. Density may be useful for the prediction of disease risk at a population level, but not as individual predictors of disease or diagnosis [191, 193, 253-260]. Pneumococcal density is influenced by epidemiological or ecological drivers of bacterial colonization, obscuring the characterization of disease progression. Factors involved in increased pneumococcal density include viral infection, antibiotic use, concurrent multiple serotypes or bacterial colonization, serotype naivety as a function of low prevalence or younger age, and carriage duration [171, 260, 262, 263]. Quantitative molecular methods are more useful in colonization studies when relative density is used to discern dominant and sub-dominant serotypes when multiple concurrent colonization is detected, than in prediction of disease [169].

While routine PCV immunization reduces VT prevalence [264], and reduced pneumococcal density during carriage is associated with reduced transmission [252], the direct effect of PCVs on NP density has not been well characterized for VT, NVT, or overall pneumococcus, summarized in Table 1.4. The carriage density of pneumococcus in the nasopharynx has been shown to change after PCV-related interventions, as summarised in Table 1.4, albeit with no conclusive trend. Vaccine impact on serotype prevalence has not been definitively linked to changes in serotype density during carriage.

In studies where pneumococcal NP density decreased following PCV immunization or routine implementation, the same temporal trends were generally observed for both VT and NVT, suggesting that this effect was not associated with PCV vaccination [263, 265, 266]. While some studies report a decrease in bacterial density, quantitative studies have also shown no alteration in pneumococcal NP density associated with vaccination, albeit due to study design [252, 267]. For example, a phase II PCV trial in Fiji showed overall pneumococcal NP density in PCV7-vaccinated children (17 months old) was similar to unvaccinated children [267], however, density was not analysed separately for VT and NVT. Further, cross-sectional surveys in children aged 6 weeks to 59 months in Mozambique showed no change in overall, VT or NVT colonization density pre- and post-PCV10 [252]. A limitation is that the suitability of the serotypes used as surrogate markers for PCV10-NVT (11A, 19A) and VT (19F) was not described. Some studies have indicated a shift to a higher density of NVT after vaccination, without changes in VT that could be a marker of serotype replacement [268, 269].

It is difficult to draw strong conclusions, as comparable studies have also reported overall increases in colonization density, for both NVT and VT temporally linked to PCV vaccination [270-272]. However, the uniform effect on VT and NVT implies these increases are not directly vaccine related.

In mice, experimental challenge with pneumococcus followed by live attenuated influenza vaccination reduced pneumococcal density upon infection with influenza virus compared with control mice (no influenza vaccination) and with PCV-vaccinated mice [273], suggesting that PCV does not reduce pneumococcal density and that factors such as viral infections and other pathogen interactions, may explain inconsistent trends and variability across studies in pneumococcal NP density following PCV introductions. The density of pneumococcal nasopharyngeal carriage is also variable within individuals and over time and density declines with time since acquisition [171].

Table 1.4: Temporal association of pneumococcal colonization density in the nasopharynx.

Site	Age (months)	Comparator	Group	Method	Summary Measure	Density (copies‡, CFU¥ or GE§ per mL)	Significance measure	Reference
Gambia	< 59	V1: Pre-PCV	Overall	Culture	Category	2.57		Roca *et al.* (2012) [263]
		V1: Post-PCV				2.11	p=0.067	
		V1: Pre-PCV	VT			2.87		
		V1: Post-PCV				2.48	p=0.071	
		V1: Pre-PCV	NVT			2.72		
		V1: Post-PCV				2.08	p=0.052	
		V2: Pre-PCV	Overall			2.76		
		V2: Post-PCV				1.99	p=0.002	
		V2: Pre-PCV	VT			2.70		
		V2: Post-PCV				1.94	p=0.042	
		V2: Pre-PCV	NVT			2.90		
		V2: Post-PCV				2.03	p=0.010	
	> 59	V1: Pre-PCV	Overall			2.44		
		V1: Post-PCV				1.88	p=0.001	
		V1: Pre-PCV	VT			2.14		
		V1: Post-PCV				1.78	p=0.021	
		V1: Pre-PCV	NVT			2.48		
		V1: Post-PCV				1.91	p=0.003	
		V2: Pre-PCV	Overall			2.44		
		V2: Post-PCV				1.75	p=0.001	
		V2: Pre-PCV	VT			2.42		
		V2: Post-PCV				1.78	p=0.003	
		V2: Pre-PCV	NVT			2.45		
		V2: Post-PCV				1.74	p=0.003	
Fiji	12-23	Pre-PCV	Overall	qPCR	Median	-		Dunne *et al.* (2018) [265]
		Post-PCV				-	p<0·0001	
		Pre-PCV	VT			4.98 $\log_{10}$ (IQR: 4.23-5.67)§		
		Post-PCV				4.29 $\log_{10}$ (IQR: 3.35-5.10)§	p=0.0238	
		Pre-PCV	NVT			-		
		Post-PCV				-	p=0.004	
		Vaccinated	Overall			4.85 $\log_{10}$§		
		Unvaccinated				5.16 $\log_{10}$§	p<0.001	

Site	Age (months)	Comparator	Group	Method	Summary Measure	Density (copies‡, CFU¥ or GE§ per mL)	Significance measure	Reference
		Vaccinated	VT			4.45 $\log_{10}$ §		
		Unvaccinated				5.16 $\log_{10}$ §	p=0.008	
		Vaccinated	NVT			4.86 $\log_{10}$ §		
		Unvaccinated				5.03 $\log_{10}$ §	p=0.033	
USA	7, 12, 18	PCV vs MnCC	VT	Culture	Category	-	OR: 0.61 (CI: 0.38–0.99)	O'Brien *et al.* (2007) [266]
			NVT			-	OR: 1.17 (CI: 0.79–1.72)	
	12-23							von Mollendorf *et al.* (2019) [270]
Mongolia		Unvaccinated	Overall	qPCR	Median	5.04 $\log_{10}$ (IQR: 4.10-5.76)		
		Vaccinated¤				5.87 $\log_{10}$ (IQR: 5.10-6.56)	p<0.001	
		Unvaccinated	VT			5.11 $\log_{10}$ (IQR: 4.23-5.76)		
		Vaccinated¤				5.80 $\log_{10}$ (IQR: 4.78-6.51)	p=0.002	
		Unvaccinated	NVT			4.79 $\log_{10}$ (IQR: 4.02-5.67)		
		Vaccinated¤				5.83 $\log_{10}$ (IQR: 5.08-6.51)	p<0.001	
Laos People's Democratic Republic	12-23	PCV vaccinated	Overall	qPCR	Median	6.00 $\log_{10}$ (IQR: 5.40–6.68)		Satzke *et al.* (2019) [271]
		Incomplete PCV				5.60 $\log_{10}$ (IQR: 4.82–6.37)	p<0.001	
South Africa	9	Vaccinated	Overall	qPCR	GMD	4.68 $\log_{10}$ ¥		Olwagen *et al.* (2017) [272]
		Unvaccinated				4.28 $\log_{10}$ ¥	p=0.007	
		Vaccinated	VT			3.80 $\log_{10}$ ¥		
		Unvaccinated				3.40 $\log_{10}$ ¥	p=0.048	
		Vaccinated	NVT			3.60 $\log_{10}$ ¥		
		Unvaccinated				3.10 $\log_{10}$ ¥	p= 0.018	
		Vaccinated	19A			3.76 $\log_{10}$ ¥		
		Unvaccinated				2.83 $\log_{10}$ ¥	p=0.046	
	16	Vaccinated	Overall			4.33 $\log_{10}$ ¥		
		Unvaccinated				4.44 $\log_{10}$ ¥	p=0.53	
		Vaccinated	VT			3.73 $\log_{10}$ ¥		
		Unvaccinated				3.59 $\log_{10}$ ¥	p=0.48	
		Vaccinated	NVT			3.74 $\log_{10}$ ¥		
		Unvaccinated				3.33 $\log_{10}$ ¥	p=0.08	
		Vaccinated	19A			4.15 $\log_{10}$ ¥		

Site	Age (months)	Comparator	Group	Method	Summary Measure	Density (copies‡, CFU¥ or GE§ per mL)	Significance measure	Reference
		Unvaccinated				3.04 $\log_{10}$ ¥	p=0.013	
Israel	7-24	PCV13-vaccinated	Overall	Culture	Mean cumulative density	2.96 (CI: 2.91-3.01)		Dagan *et al.* (2017) [269]
		PCV7-vaccinated				2.93 (CI: 2.88-2.98)	p=0.366	
		PCV13-vaccinated	PCV13VT			2.87 (CI: 2.79-2.96)		
		PCV7-vaccinated				2.92 (CI: 2.84-2.99)	p=0.470	
		PCV13-vaccinated	NVT			3.05 (CI: 2.99-3.11)		
		PCV7-vaccinated				2.96 (CI: 2.90-3.02)	p=0.041	
Peru	< 36	Pre-PCV	NVT	qPCR	Median	$1.9x10^5/5.28^*$ $\log_{10}$ ¥		Hanke *et al.* (2016) [268]
		Post-PCV				$5.5x10^5/5.74^*$ $\log_{10}$ ¥	p=0.046	
		Pre-PCV	VT			-		
		Post-PCV				-	p=0.210	
		Pre-PCV	Overall			-		
		Post-PCV				-	p=0.649	

Note: Dunne *et al.*, 2012 and Sigaúque *et al.*, 2018 [252, 267] referenced in text, not included; data was presented graphically in those article.
CFU=colony forming units, GE=gene equivalents, PCV is Pneumococcal Conjugate Vaccine, MnCC is the *Neisseria meningitidis* group C protein conjugate vaccine, V1 refers to universally vaccinated villages, V2 partially vaccinated (infants <30 months of age) villages, ¤1 dose of PCV or more, incomplete PCV refers to infants under vaccinated for their age-group, VT=vaccine serotypes, NVT=Non-vaccine serotypes, overall=all pneumococcus, qPCR is quantitative Polymerase Chain Reaction, culture was semi-quantitative culture, and the categories ranged from 1-4, GMD is geometric mean density, IQR is Inter-quartile range, CI is 95% Confidence Interval, all studies determined bacterial density from nasopharyngeal samples, *Converted from published value for comparison, and where two categories are listed the p-value is a comparison between the two values.

Nonetheless, qPCR is useful in pneumococcal colonization studies to describe the changing serotype epidemiology including multiple concurrent colonization with higher and lower density serotypes that are temporally associated with PCV introductions. Further, time since vaccination, age, season, carriage duration and other factors (such as receipt of other vaccines) may be more important in pneumococcal density than just a binary classification of vaccinated or not vaccinated in studies investigating temporal links between pneumococcal density and vaccination.

1.7 Pathogenic and Commensal Microbe interactions

Pneumococci exist within a diverse nasopharyngeal microbiome that is an important intermediary between colonization prevalence, pathogenicity [274, 275], and chronicity [276, 277]. The microbiome not only affects pneumococcal presence [278] but also serotype diversity [279]. There are differences in the human respiratory microbiome even within the upper respiratory tract [275] and this is important for the context of detection of pneumococcus within that environment. Within the nasopharynx, processes of ecological succession are influenced by environmental factors [47-50], microbe associations, disease, and host-immune responses in a serotype, and age-dependant manner [274, 280, 281]. Importantly, vaccination with PCVs may impact bacterial colonization dynamics within the nasopharynx. As the colonization prevalence of pneumococcal VT are reduced, this allows for replacement with other serotypes and bacterial species [282]. For this reason, continued monitoring of the temporal changes in pneumococcal colonization together with key bacterial species is important.

Along with pneumococcus, the most prevalent commensals with pathogenic potential in the nasopharynx include *H. influenzae*, *M. catarrhalis* and *S. aureus* [275, 283]. There is both a synergistic and a competitive relationship between co-carried pneumococcus and

H. influenzae but an antagonistic relationship between *S. pneumoniae* and *S. aureus* that is mostly age dependant [40, 284-288]. The relationship of pneumococcus to *M. catarrhalis* is less defined and changes with age. The mechanisms of interaction between these microbes are complex and often mediated through host immune responses [289]. By way of example, in pneumococcus, the production of hydrogen peroxide is mediated by the pyruvate oxidase (SpxB) gene, and results in bactericidal activity and targeted immune responses against some strains of *S. aureus*, [280, 290] other *Streptococcus* species, some strains of *H. influenzae*, *M. catarrhalis* and *Neisseria meningitidis* [291]. Further, pneumococcal neuraminidase (NanA) can desialylate the liposaccharides used by *H. influenzae* and *Neisseria meningitidis* to evade the immune system, increasing their susceptibility to immune-mediated clearance [292]. *H. influenzae* can in turn, modulate opsonophagocytic killing targeted toward *S. pneumoniae*, resulting in clearance [138, 293, 294].

1.7.1 Pneumococcal and bacterial interactions in South Africa

The relationship between pneumococcus, *S. aureus* and *H. influenzae* have been characterized in South Africa. A longitudinal study conducted pre-PCV (Jan 2007-May 2009) in Soweto (Gauteng, South Africa), assessed microbial colonization of the nasopharynx in children enrolled at 6-12 weeks-of-age and their mothers. In children ≤24 months of age, there was a negative association between *S. aureus* and *S. pneumoniae* colonization (aOR: 0.51, 95% CI: 0.39-0.67), whereas colonization with *H. influenzae* increased the odds of subsequent pneumococcal co-colonization (aOR: 1.75, 95% CI: 1.32-2.32), even where *H. influenzae* and *S. aureus* were carried together (aOR: 2.28, 95% CI: 1.31-3.97) [40]. Competitive interactions between pneumococcus and *S. aureus* are no longer evident in older children [295]. Further, there was no difference in *S. aureus* colonization by pneumococcal carrier status (26.3% in *S. pneumoniae* carriers vs. 36.0%

in non-carriers; p=0.11) in CLWH under 5 years of age hospitalized with severe pneumonia (Soweto). In contrast, colonization by *S. aureus* was lower in HIV-uninfected children that carried *S. pneumoniae* compared with non-carriers (5.5% vs. 21.3%; p=0.013). Although there was a difference in the mean age of CLWH and uninfected children (11.4 vs. 7.8 months; p=0.005) [(296)], age-related differences in *S. aureus* co-carriage have been described in children over four years old compared with under four years of age [(297)].

In Agincourt, (Mpumalanga, South Africa), a cross-sectional survey demonstrated that concurrent colonization by pneumococcus and *H. influenzae* decreased in children ≤12-years-of-age two years after the introduction of PCV (2011), compared with the year PCV was introduced (2009; aOR: 0.74; 95% CI: 0.63-0.87). Conversely, the odds of co-carriage of pneumococcus and *S. aureus* was similar between the two periods for children (aOR: 0.90; 95% CI: 0.55-1.45) in 2011 relative to 2009. The odds of carrying all three bacteria were reduced in the post-PCV era compared with pre-PCV in children (aOR: 0.64; 95% CI: 0.43-0.94) [(298)]. Co-colonization by *S. aureus* and pneumococcus may not have shown a difference between periods due to the broad age category analysed for children (under-12 years of age) as prevalence of colonization by both bacteria is age related in children [(297, 299)].

As PCVs reduce carriage of prevalent VT when introduced, overall pneumococcal carriage is reduced in the short term and the bacterial niche changes, allowing for increases in species like *S. aureus*, as demonstrated in the Netherlands [(300)]. Therefore, in addition to associative relationships between bacteria, serotype rearrangement of pneumococcus within the bacterial microbiome in response to PCV immunization may also influence the prevalence of non-pneumococcal colonizers in the nasopharynx, at least in the short term [(295)]. In the longer term, the bacterial landscape stabilizes as other non-VT serotypes occupy that niche, resulting in serotype replacement [(300)]. Incomplete serotype replacement in pneumococcal carriage was demonstrated in South Africa four

years following routine implementation of PCV7 (2009) and two years since transition to PCV13 (2011) [224]. The effect of PCV introduction on *H. influenzae* and *S. aureus* carriage described above, may also have changed in the longer term (after 8-9 years), as serotype replacement is expected to be complete in South Africa. It will therefore be important to assess the longer-term community structure of the nasopharyngeal microbiome in South Africa. Indeed, pneumococcal carriage surveys in the Netherlands, a country with a mature vaccination program, have shown that *H. influenzae*, non-typeable *H. influenzae* (NTHi) and *S. aureus* increased after the introduction of PCV, in children and their parents but stabilized in the longer term (seven years) when serotype replacement was complete [300, 301]. There was no difference between the periods for *M. catarrhalis*, except for increased prevalence in 24 month old children and their parents at 4.5 years after the introduction of PCV [301].

1.7.2 Pneumococcal density associations with other bacterial colonizers

Studies conducted in other world regions, including LMIS and high-income country (HIC) settings further elucidate the potential drivers and outcomes of inter-species relationships between pneumococcus, *H. influenzae* and *S. aureus* in the nasopharynx. A cohort study (Andes, Peru) showed that in young children, the synergistic association between *S. pneumoniae* and *H. influenzae* colonization in the NP (OR: 1.95–1.97, $p<0.01$) also included increased categorical bacterial density detected by qPCR (OR: 3.67, $p<0.001$; correlation coefficient: 0.32, $p<0.001$) whereas the antagonistic relationship between *S. pneumoniae* and *S. aureus* (OR: 0.61, $p<0.05$) had no density related associations [302]. Similarly, in children aged 12–24 months (Indonesia), there was a positive association in bacterial density of *S. pneumoniae* with *H. influenzae* (Spearman $\rho=0.430$, $p=0.002$) and *M. catarrhalis* (Spearman $\rho=0.400$, $p=0.0002$) in co-colonization [303]. The increased bacterial density in polymicrobial carriage of pneumococcus, *H.*

influenzae and *M. catarrhalis* were also shown in a mouse models [289, 304]. This infers that when *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* co-colonize the nasopharynx, together, each species accumulates to higher bacterial density.

1.7.3 The effect of polymicrobial carriage on serotypes

Co-carriage of bacterial species can select for pneumococcal serotypes in infants. Lysenko *et al*. (2010) showed in mice that *H. influenzae* mediated immunomodulation, and serotype specific virulence factors related to the pneumococcal capsule. More virulent pneumococcal serotypes may therefore arise during competition with co-colonizing *H. influenzae* [305]. When multiple commensal bacteria colonize concurrently, this may result in immune responses that clear carriage of pneumococcus [293]. Ultimately, pneumococcal serotypes able to resist innate immune responses are more likely to effectively colonize in the presence of other commensals and this may be a driver of serotype selection in a microbiome environment [294, 305]. To demonstrate this, Lewnard *et al*. (2016) showed that in children 2–30 months old pneumococcal and NTHi co-carriage resulted in higher density of both (increasing persistence) but lower serotype diversity that favoured fitter serotypes (greater degree of encapsulation; neutrophil resistance and metabolic efficiency) compared with pneumococcal carriage alone [279].

1.7.4 Viral interactions in colonization and disease

In addition to host, bacterial and pneumococcal factors described above, viral interactions are important in both pneumococcal colonization and disease. Viral infections can potentiate pneumococcal colonization [306] through alteration of the mucosal surface and

also facilitating bacterial adherence to epithelial cells [28, 307-309], including if the virus is detected in the absence of symptoms [310]. Furthermore, human adults with respiratory viral infections had a higher odds of pneumococcal colonization, and increased density when experimentally challenged with pneumococcus [311]. Viral infection, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) influenza, PIV, RSV, human metapneumovirus, human Rhinovirus, adenovirus, bocavirus and HIV induces changes to the metabolome of the host (dysbiosis), mucus production, exposure of host cell receptors, innate immunity and interferes in bacterial clearance, thereby promoting increased bacterial density [253, 260, 273, 307, 310-326]. Further, bacterial-viral synergism may also be the result of direct pathogen interactions that increase bacterial adherence and dissemination from the nasopharynx to other sites by enabling pneumococcal endocytosis into host cells across the endothelial layers enhancing progression from asymptomatic pneumococcal colonization to disease [65, 260, 316, 327, 328].

In summary, viral infections can precipitate higher rates of colonization, carriage density of pneumococcus and progression to disease. As these effects on colonization and density are usually transient [319], symptoms of respiratory illness should be noted during colonization studies that aim to assess temporal relationships with PCV introduction, and participants with respiratory symptoms excluded, as this may confound the conclusions.

1.8 Pneumococcal Carriage Dynamics in LMIC

Cross-sectional studies are cost-effective and provide a point estimate of prevalence and vaccine impact; however, they do not provide information on carriage dynamics and transmission [329, 330], which are important background considerations for interventions, including vaccination programs. Further, some invasive serotypes are less frequently detected in cross-sectional colonization studies compared with disease isolates [331] due to

transient carriage [65, 331-334], whereas these may be detected in multiple sampling points within the same participant. Specifically, invasive serotypes including 6B, 19F, and 23F are more frequently detected in colonization studies compared with serotypes 1, 5, 7F, 8, 33F, and 38 that are almost exclusively detected in invasive disease isolates [331, 334, 335]. The time from acquisition, and then to clearance of pneumococcal serotypes (carriage duration) is heterologous by serotype and varies with age, as time to clearance with subsequent carriage episodes is shorter [69, 171, 329, 333, 336-339].

The serotype specific rate of acquisition is dependent on local colonization prevalence which determines the exposure risk [69, 333]. As the prevalence of pneumococcus is higher in LMIC, the acquisition of initial carriage events occurs in early infancy, which carries a higher risk of respiratory disease [73, 336].

Longitudinal studies in LMIC have estimated time to first pneumococcal acquisition from 5-6.6 weeks (Kenya; Gambia; Thailand/Myanmar, all pre-PCV) [73, 329, 339], 8-9 weeks (Bangladesh, pre-PCV; Malawi, pre- and post-PCV; South Africa, post-PCV) [39, 68, 330, 340] and 18.5 weeks (Indonesia, no PCV) [171]. The age at first acquisition for Indonesian infants was older than in other LMIC. This may be related to the point prevalence reaching a peak (68%) at an older age of 12 months compared with other LMIC and enrolment from 8–12 weeks of age, which may have missed early carriage episodes. Nonetheless, infants in LMIC acquire their first colonization event early in infancy, and carriage prevalence peaks in the first few months of life compared with HIC where age at first colonization is six months and older, and carriage prevalence also peaks at an older age [341, 342].

Delaying the first carriage episode of invasive pneumococcal serotypes beyond the first few months of life, especially for more invasive serotypes, is important for reducing the risk of respiratory disease. Studies conducted in The Gambia elucidate the effect of PCV on the acquisition and transmission of colonization. Prior to the introduction of PCV,

77% of Gambian infants had been colonized by 2 months of age [338] and the carriage point prevalence was sustained at 80-90% in infants aged 3-12 months, with around half of carriage episodes involving PCV13-VT [339]. A cluster randomized control trial in The Gambia demonstrated that PCV immunization delayed the time to first acquisition of VT colonization in young infants compared with the pre-vaccination baseline. This benefit was increased in the intervention that included the vaccination of an entire village and not infants only, highlighting the role of community-level immunity and PCVs in increasing the time to first VT acquisition. At 8-weeks-of-life, up to 92% of infants had acquired pneumococcal carriage by any serotype. In villages where children were immunized with PCV7, fewer young infants not yet vaccinated (8 weeks-of-age) carried PCV7-VT (16.9%) and the acquisition rate of VT was lower compared with villages where PCV7 was not in use (PCV7-VT: 37.5%) [343]. Taken together, this implies that the impact of PCV on VT-carriage would accumulate as a routine immunization program matures, and that catch-up campaigns for older children during introduction are also beneficial, with further declines in VT-carriage and disease realized as transmission is reduced.

The benefits of routine immunization of children take time to accumulate in a population. Tigoi *et al*. (2012) estimated based on transmission within Kenyan households, that it would take more than 10 years for herd effects of PCV implementation within that population to be fully realized [73]. A microsimulation model based on Finnish data (a high-income setting) collected prior to the introduction of PCVs predicted a 5-10 year time frame (assuming 90% coverage for a vaccine with at least 50% efficacy) for elimination of vaccine serotypes in disease, and for serotype replacement to occur [344]. Due to the higher colonization prevalence in LMIC, it is likely that elimination of VT may be more protracted than this estimate. Considering this, consistent surveillance of circulating pneumococcal serotypes is especially important in LMIC, even with mature vaccine programs, where baseline prevalence of pneumococcal colonization was high.

Time from acquisition to clearance is variable by capsule type and partly explains differences in serotype prevalence [69, 333]. Serotypes that are carried for longer, are more prevalent, as there are increased opportunities for transmission [336, 345]. The LMIC studies vary in their estimates due to participant age, HIV-prevalence, and immunity, but usually carriage duration is no longer than three months in young children and a few days to weeks in adults [336, 338, 346]. In the Drakenstein community (semi-urban, low-income) in South Africa, the average duration of colonization in the first year of life was 30.3 days (95% CI: 26.0–34.5) and ranged between 13-65 days per serotype in 2012-2014 [340]. Also in South Africa, in rural and urban settings, carriage duration was almost doubled in CLWH under 5 years of age compared with age-matched HIV-uninfected children (107.9 days, 95% CI: 92.1–124.7 and 56.3 days, 95% CI: 51.1–62.1, respectively) [347], likely due to ineffective capsular-specific immune responses [348].

Pneumococcal carriage duration decreases with increasing age, with a shorter duration for each subsequent carriage episode after the first acquisition due to effective immune responses that accumulate with each carriage episode [39, 69, 171, 333, 337, 345]. The serotype level differences in duration of carriage and the density of carriage, especially in cases of concurrent colonization, could affect the probability of a serotype being sampled and detected in cross-sectional carriage surveys.

Estimates of disease measure severe symptomatic cases only, whereas transmission of pneumococcus is mostly from asymptomatic carriers, and therefore asymptomatic carriage is pertinent to risk of disease [22]. In addition to being a precursor to disease, carriage of pneumococcus plays an important role in the transmission of pneumococcal serotypes within families and communities [30, 349, 350]. Within household transmission usually originates from colonized children under 5 years of age [39]. As with most respiratory pathogens, horizontal transfer of carried pneumococcal serotypes to close contacts mostly occurs through respiratory droplets or contact with respiratory secretions, including through coughing, sneezing, breathing, talking or other close contact [1, 351].

Several bacterial and host factors contribute to serotype, age, and geographic heterogeneity in transmission rates [352]. Increased bacterial density and longer duration in carriage increase person-to-person transmission of serotypes. To demonstrate this, high bacterial density in a mouse model was shown to increase shedding of carried serotypes and therefore increase transmission [353].

Immune responses also play a role in pneumococcal transmission explaining the role of serotype-naïve young children and a mechanism for PCVs to reduce serotype-specific transmission. In mouse models, capsule-specific immunity decreased shedding independently of bacterial density, likely due to IgG mediated agglutination [354]. Reciprocally, non-specific immune responses in serotype-naïve infants play a part in increasing the likelihood of transmission. Host inflammatory responses to the pneumococcal carrier state, to ply, or infection with viral respiratory pathogens increase respiratory secretions resulting in increased shedding and transmission of carried pneumococcal serotypes [328, 355-358].

Bacterial factors and gene expression impact on a carried serotypes transmissibility. Expression of ply increases nutrient availability in the nasopharynx and promotes inflammation in the host, which in turn increases shedding of carried pneumococcal serotypes in nasal secretions and host-to-host transmission [359]. Mouse transmission models show that heterogeneity in capsular type, and expression of the capsule also affect transmission. Serotypes that are more effectively bound by mucin are shed less and have reduced transmissibility, whereas a greater negative charge afforded by a thicker polysaccharide capsule repels mucus binding [360]. Further, as carriage is a pre-requisite for transmission, it follows that routine immunization with PCVs reduces paediatric carriers in the population and the duration of carriage, and therefore reduces transmission [361]. Carriage duration is consistent through seasons, however other factors influence transmissibility, such as seasonal patterns in influenza like illnesses and therefore

transmission patterns are also seasonal [338].

As with colonization itself, pneumococcal transmission dynamics are variable with age. Young children (0-2 years old) that have not yet developed a spectrum of immune responses to pneumococcal serotypes are more frequently colonized and thereby sources of transmission [30]. Colonization with one serotype elicits serotype specific immunity that can prevent future homologous serotype re-acquisition but does not prevent acquisition of heterologous serotypes [362]. If brief colonization is established after re-acquisition of serotypes, these are likely to be less transmissible events, due to more effective clearance, shorter duration of carriage, and agglutination [39, 69, 333, 337, 345, 354]. Considering that colonization-acquired immunity accumulates with age and number of colonization events, reducing re-acquisition of serotypes therefore reduces transmission risk with age [328, 345].

Immunity acquired from PCV reduces the transmission of VT serotypes, shifting the proportion of circulating serotypes to dominance by NVT, that are generally less invasive. A longitudinal cohort study in the Navajo Nation and White Mountain Apache tribe assessed age-specific transmission and demonstrated that young infants (under 10 months of age) acquire serotypes from household members such as toddlers and parents, whereas toddlers and older children (up to five years old) are the main sources of introduction and transmission within households [363]. A dynamic model for serotype-specific transmission showed parameters that assumed carriage duration and onward transmission decreased with increasing age, and transmission mostly driven by the 0-5 year-old age group best predicted the observed data from rolling colonization surveys in Malawi [141]. Children under 5 years old are the main sources of transmission [39], and within-household accounts for the bulk of transmission compared with community contacts [364]. Contact between young children and with young children under 5 years of age accounts for most transmission of serotypes [350, 351]. Household level colonization is an important source of transmission [73] and can occur in all directions between adults and

children, however children under 5 years of age are more likely to introduce new serotypes from community settings like daycare into the household [39, 82, 333]. In South Africa, prior to the PCV-era, results of a stochastic transmission model fitted to longitudinal sampling of children under 2 years of age and their mothers demonstrated that the transmission direction of paediatric PCV7-VT was mostly from children to their mothers. Indeed, the force of infant-to-mother transmission ranged from 0.36-3.29 per 1000 days, whereas the reverse direction was 0.06–0.51 [82]. Interrupting acquisition of serotypes in young children with vaccination, therefore, interrupts transmission to other groups and results in population immunity.

1.9 Concurrent colonization with multiple serotypes

Standard culture-based pneumococcal serotyping schemes are inadequate for the detection of multiple concurrent serotypes as these methods often underestimate co-colonization [365, 366]. Additionally, investigating secondary and tertiary colonies on culture for characterising multiple colonization is not cost effective [366]. As molecular methods are increasingly employed for serotyping, the detection of co-colonization and low-abundance serotypes is facilitated, including with real time PCR and deep sequencing methods [169, 170, 174, 214]. A study conducted on the Thailand-Myanmar border demonstrated that microarray could detect 1.6-times as many serotypes as culture based methods in NPS, largely by detecting those at low abundance [214]. Similarly, in South Africa, qPCR could detect 6.4-times as many multiple colonization events compared with culture-based methods, with serotypes most commonly identified in multiple serotype colonization including 6A/B, 9B/F, 23F, 5, and 9A/L/N/V. [170]. The concurrent colonization of more than one serotype or co-colonization is common [65], especially in LMIC dwelling children [170, 367], however the frequency of multiple colonization is less than overall colonization itself [169, 248]. This is mostly due to serotype competition, where established serotypes can competitively inhibit the acquisition of a second serotype and

this competition is mediated through quorum sensing and bacteriocin expression from the resident serotype at higher bacterial density [248, 345, 368] . Where there is synergism in multiple serotype colonization, this is potentially facilitated through biofilm formation [369].

The impact of PCVs on VT serotype prevalence in colonization can allow for changes in other serotype composition, including in co-colonization. Moreover, as routine immunization with PCVs diminishes the prevalence or density of VT, serotypes initially believed to be carried at a lower rate may be unmasked rather as previously undetected (by culture-based methods) co-colonizing serotypes, present at a lower density in co-colonization, when employing molecular detection techniques [170, 272, 365]. As with other aspects of colonization, there are serotype level differences in co-colonization patterns. Some serotypes are often found in multiple colonization, whereas other serotypes like serotype 10A, 15B, 16F, 20 34, 35A, 35F, are usually found as solitary colonizers [370]. In LMIC with high colonization prevalence, the occurrence of multiple concurrent colonization in NP samples is reported in the range of 5.1%–40.0% of samples that were positive for pneumococcus, although this is dependent on the sensitivity of the employed serotyping method to detect co-colonization [169, 171, 367, 370]. Notably, a study that involved molecular serotyping of pneumococcus in saliva of Dutch school children demonstrated multiple concurrent colonization rate of 52% [371], albeit that some serotypes may have been non-pneumococcal species, or non-viable remnants of previous colonization.

Co-colonization becomes important in the context of ecological competition processes that drive genetic changes to pneumococcal serotypes. The acquisition of genetic elements between bacterial species or pneumococcal serotypes, is known as transformation, a process that necessitates physical proximity. This proximity is facilitated through co-colonization with various serotypes and other commensal bacteria. In instances where pneumococci acquire capsular genes from different serotypes, leading to a modification in their own capsule expression, this phenomenon is referred to as capsule switching. Pneumococcus incorporate external DNA resulting in changes in

capsular properties, as a result of selective pressure from vaccination, antibiotics, co-colonizers and other factors [372, 373]. Some examples of serotypes that have undergone capsular change include 19A, 22F, 33F, 15A and 35B [374-376]. Capsular changes also have implications for multi-drug resistance (MDR) [377]. Importantly, transformation opportunities afforded by multiple concurrent colonization and in the context of vaccination may also involve mechanisms to evade host immune clearance resulting from exposure or vaccine-induced responses, the transfer of antibiotic resistance and capsule switching genes between pneumococcal types [369, 373, 375, 378-384]. A Malawian study that enrolled children aged under 13 years revealed that up to 40% of children harboured multiple serotypes concurrently, and that the opportunities for transformation afforded by proximity led to genetic variations in serotypes [367]. Furthermore, co-colonization may increase the biomass of pneumococci [261, 365, 371], and this can trigger the pathway towards disease and increase the risk of transmission. In Vietnam, multiple serotype colonization was associated with acute respiratory tract infection (ARTI) (aOR: 2.92, 95% CI: 1.27–6.71) in children under 5 years of age, with 18.5% of cases carrying multiple pneumococcal serotypes compared with 7.1% of healthy controls (p=0.004). Here there was an association between specific serotype pairs (19F and 11, 19F and 15B/15C, 23F and 6A/6B) occurring in ARTI cases whereas serotypes 19A and 35B were isolated as single colonizers [385].

1.10 Serotype replacement and unmasking

Once a serotype is acquired, and colonization is established, some serotypes may inhibit the acquisition of other serotypes through competitive interaction, until clearance [386]. In this way, prevalent serotypes occupy the nasopharyngeal niche. Pneumococcal vaccines have reduced colonization by vaccine-type (VT) pneumococci, leading to an increase in NVT colonization (i.e., replacement) as the ecological niche is vacated, allowing for the acquisition or enhanced detection (unmasking) of previously less common serotypes [33].

After PCV immunization the prevalence of serotypes not targeted by vaccines increase as these serotypes take advantage of reduced competition for occupation of the nasopharyngeal niche, termed serotype replacement [33, 386-388]. Studies conducted after the licencing and routine introduction of PCV have reported serotype replacement in colonization and less prominently in disease [33]. The serotypes involved in replacement colonization depends on the valency of the PCV in routine use, pre-PCV prevalence of colonization, co-colonizer relationships, antibiotic use, and intrinsic capsular factors [389].

Another mechanism whereby NVT appear to increase in prevalence post-vaccine is due to 'unmasking', where NVT were present as lower density colonizers prior to PCV introduction, but the reduction in circulating VT post-PCV enables their enhanced detection, especially using molecular methods [390]. A study that investigated serotype-specific colonization in PCV7-vaccinated children compared with PCV7-unvaccinated children in South Africa using qPCR at 9 and 15-16 months of age determined that the increase in NVT colonization after vaccination was due to both true serotype replacement, and increased detection of serotypes present at a lower density but not usually detected when co-carried with VT and using traditional culture-based methods, i.e. unmasking [272].

Post-PCV implementation, the overall colonization of pneumococci rebounds to pre-PCV levels over time, despite declines in VT colonization, due to replacement by NVT pneumococci. Nevertheless, this rebound in colonization does not translate to a proportional increase in IPD caused by NVT, likely due to most NVT having a less invasive phenotype [352, 390-392]. A data review and pooled analysis from 21 surveillance sites across four geographic regions (six in North America, 11 in Europe, three in Australasia, one in South America) included the incidence of IPD five years before and ten years after the introduction of PCV7, stratified by age [111]. When compared with the five-year pre-PCV introduction period, the incidence of VT-IPD in children under 5 years of age showed a significant annual reduction, persisting up to year 7 post-introduction

(relative risk [RR]: 0.03; 95% CI: 0.01–0.10). In contrast, the incidence of NVT-IPD increased over the same period (RR: 2.81, 95% CI: 2.12–3.71), while overall IPD decreased by year 1 (RR: 0.55, 95% CI: 0.46–0.65), with the reduction remaining relatively consistent through to year 7 (RR: 0.49, 95% CI: 0.35–0.68) [111]. This data highlights the positive impact of PCV immunization on IPD, despite serotype replacement.

This same phenomenon was also demonstrated in South Africa, whereby colonization by NVT increased by 35% (aOR: 1.35; 95% CI: 1.17–1.56) concomitant with a 50% (aOR: 0.50; 95% CI: 0.42–0.59) decrease in VT among under 2 year old children in a rural cohort, resulting in only a modest net decrease in pneumococcal colonization (from 83.0% to 73.4%) two years (2009 vs 2011) following the introduction of PCV [113]. Similarly, in an urban cohort in South Africa, overall colonization was reduced by 10.3% (from 61.4% to 55.1%) in children under 2 years of age, while the reduction in VT (-60.4%; from 36.1% to 14.3%) was offset by a 33.7% increase in NVT (from 27.3% to 41.2%) in the three years after PCV introduction (2012-2013) compared with the early PCV introduction period (2010-2011) [59]. In the same time frame, comparing a pre-PCV period (2005-2008) with a period three years after PCV was introduced in South Africa (2012), in children under 2 years of age the rate of IPD cases was reduced from 54.8 to 17.0 per 100,000 person-years. This involved an 85% (95% CI: -89 to -79) reduction in IPD cases from PCV7-VT and an associated 33% (95% CI: 15-48) increase in IPD cases from NVT [34]. Taken together, this means that as the prevalence of more invasive serotypes in VT colonization decreased, there was a corresponding increase in NVT colonization. Simultaneously, there was a significant decrease in overall disease incidence. Replacement of VT by NVT in asymptomatic colonization did not translate into substantial disease replacement. Still, it is crucial to comprehensively monitor shifts in colonization of serotypes not covered by vaccines, as NVT pneumococci with invasive potential and AMR contribute to residual pneumococcal disease [393]. Globally, serotypes involved in replacement disease are not consistent [393].

Serotype replacement in colonization is usually due to opportunism and competition. A meta-analysis conducted to identify serotypes in replacement IPD globally following the introduction of PCV established that the most prevalent replacement serotypes comprising 42.2% (95% CI: 36.1-49.5%) of IPD cases in children were 22F, 12F, 33F, 24F, 15C, 15B, 23B, 10A, and 38, varying in rank order and occurrence depending on region [389, 394]. In South Africa, 42.7% of IPD cases in children in 2012 (three years post-PCV) were due to NVT [34]. The NVT serotypes/groups predominantly identified in IPD in the post-PCV era (2018) were 8, 16F, and 12F in children ≤5 years-of-age [154]. The requirement for consistent serotype surveillance in the PCV-era is demonstrated by replacement disease from non-PCV7 serotypes 1, 5, 7F (included from PCV10), 3 and 19A (included from PCV13) [374, 375, 395] after the introduction of PCV7, but prior to transition to PCV10 or PCV13.

In many cases, replacement by NVT involves genotypes that were in low circulation prior to the introduction of PCVs, and subsequently expanded due to a vacated niche or were unmasked, however recombination events can lead to capsule switching [33, 375, 390, 396]. Exemplifying this, is serotype 19A where the increase in prevalence in the USA after the introduction of PCV7 involved stable, pre-existing genotypes, but also recombination events resulting in capsule switching driven by both immune pressure from vaccination and increased antibiotic use [375, 396]. This resulted in serotype 19A with sequence type (ST) 645 combining with ST247 usually associated with serotype 4 (targeted by PCV7) to produce a new vaccine escape and antibiotic resistant 19A ST2365 [375]. Nonetheless, most capsule switching events have been documented prior to PCVs, and therefore are not mainly driven by vaccines [389].

1.11 Colonization prevalence in African countries

Colonization by pneumococcus in early infancy is high in LMIC, especially in Africa. In Malawi, longitudinal sampling revealed that the overall colonization prevalence of pneumococcus increased from 30.6% at 6-weeks-of-age to 60.6% at 18-weeks-of-age [39]. Similarly, in South Africa, the overall colonization prevalence at 2 weeks-of-age was 3% (95% CI: 1–8%) and peaked at 24 weeks-of-age (64%, 95% CI: 56–69%) [340]. Furthermore, the highest rates of pneumococcus related morbidity and mortality have been reported in African children under 2 years of age, mainly due to more than 50% being colonized before 6 months of age, resulting in higher transmission and colonization, which is a precursor for disease [98, 119, 124, 397]. Furthermore, children living in LMIC also have a higher risk of progression to disease, as described in section 1.3.

1.11.1 Methods of Literature Review

To determine the prevalence of pneumococcal prevalence in African countries, and to delineate the prevalence of VT and NVT colonization at least two years following PCV introduction into the public immunization program, a literature search was conducted in MEDLINE/PubMed, to identify studies in English published between 01-Jan-2002 (PCV7 was first licenced in 2000) and 29-Oct-2023 that investigated the impact of PCV formulations on colonization of pneumococcus in African settings. The search terms included “PCV” or “pneumococcal conjugate vaccine”, “colonization” or “carriage” and “*Streptococcus pneumoniae*” or “pneumococcus” within the abstract. Reports were included if they were observational or cross-sectional studies assessing NP colonization; included healthy children aged under 60 months old and if any of the currently licensed PCV formulations in the region (7-, 10- and 13-valent) had been implemented in the public immunization program and universally available for all age-eligible children, with

or without a catch-up campaign for at least two years before the time the study was conducted. Studies were excluded for participants with symptoms of IPD, where participants were enrolled through health care facilities with current respiratory illness, where they were conducted among populations with chronic diseases not representative of the general population or if data was not available for VT and NVT. Study designs including systematic reviews, randomized controlled trials, case-control studies, case reports, longitudinal sampling, non-human subjects (animal or in-vitro), safety endpoints, immunogenicity endpoints, modelling, cost-effectiveness studies, commentaries, editorials, news reports and letters were also excluded.

Searches were imported into Rayaan.ai [398] to screen the titles and abstracts of all retrieved articles for eligibility and duplicate studies or studies utilizing the same data-sets were removed. If inclusion or exclusion criteria were not ascertained from the title and abstract, the study methods in the main article were used for screening, as detailed in the flow chart in Figure 1.4. Data extracted included the number of samples where pneumococcus (overall, VT and NVT) was detected, as well as the total number of children enrolled. The extracted data was assessed for heterogeneity and overall prevalence was stratified by net pneumococcal colonization, PCV13-VT and NVT. The data was further stratified by urban, rural and mixed rural/urban areas. The extracted data was expressed as a pooled point prevalence with 95% confidence intervals (CIs) using random-effects models in Stata 13, with the ‘metaprop’ module.

1.11.2 Results and discussion of Literature Review

A total of 789 publications were returned using the search criteria. After exclusion and inclusion criteria were applied, 12 publications from 10 African countries, with a total of

13 161 participants that were under 60 months old (Figure 1.4) were included in the final analysis. From the included publications, PCV had been in routine use for 2-7 years at the time the studies were conducted, utilizing a varying 3+0 dosing schedule [68, 225-228, 399-403] with the exception of South Africa, that utilized a 2+1 (6, 14 and 40 weeks) [59, 224] dosing schedule, detailed in Table 1.5 Furthermore, the coverage of three doses of PCV (WUNEIC) ranged from 75% to 96% (Table 1.5 and Figure 1.5) [153, 162]. Notably, Malawi, Kenya, and South Africa implemented a catch-up campaign for age groups older than the age eligible for vaccination at the timepoint when PCV was introduced (Malawi and Kenya) or transitioned to a higher valency (South Africa), detailed in Table 1.5 [68, 224, 227, 228]. In Malawi, children aged 0-12 received three doses as part of the catch-up campaign when PCV was introduced [68, 227]. In Kenya, all infants received three doses of PCV in the year after introduction of PCV10 while children living in Lwak, Kenya that were aged 1–4 years received up to 2 doses of PCV10 [228]. In South Africa, there was no catch-up campaign upon the introduction of PCV7, however children aged 0-36 months or 36–71 months with underlying conditions were included in a catch up campaign when South Africa transitioned to PCV13 [224].

For seven of the included studies, there was no information on the number of CLWH [225, 228, 399-403], whereas two studies excluded CLWH [68, 227], two studies compared CLWH and children without HIV-infection [59, 226], and one study described the prevalence of HIV in the study participants [224] (Table 1.5). In the case of studies that included CLWH and HIV-uninfected children, the prevalence of colonization was pooled. All studies utilized WHO recommended practices for the collection and storage of NPS from children [168]. Most studies (83%, 10/12) used an initial culture dependent step, with five (50%, N=10) of those studies subsequently using real-time mPCR or sequencing based ‘sequetyping’ for further serotyping (Table 1.6). Two studies used direct molecular detection and serotyping (mPCR) supplemented with Quellung or Sequetyping for further discrimination of individual serotypes.

There was a high amount of heterogeneity in the studies included, signaling a need for caution in interpreting the pooled estimates of overall, VT and NVT point prevalence

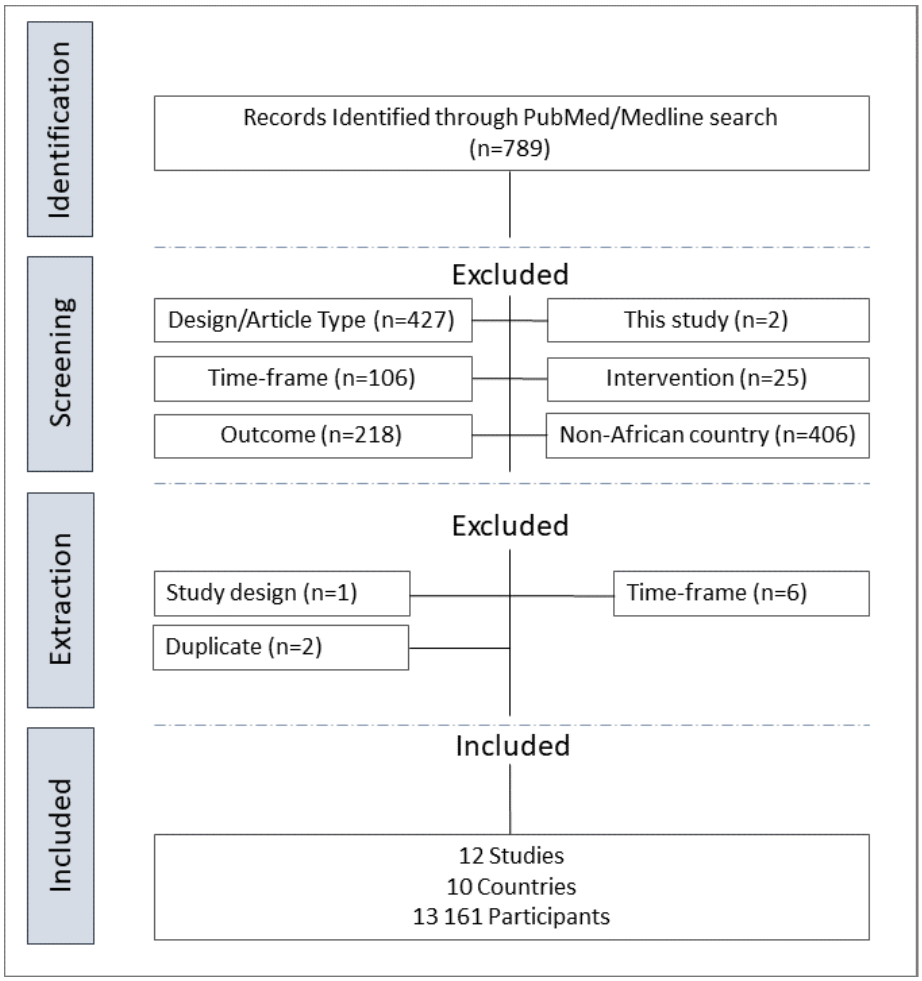

Figure 1.4: Flow diagram for literature review.

Individual studies could be listed under multiple exclusion reasons. "study design" for randomised control trials, case-control study; "time-frame" where PCV was not in routine use in the public immunization program for at least 2-years; "outcome" was not asymptomatic colonization*; "article type" was review or background articles; "Intervention" was not an approved PCV; "non-African"(population) where the study was not conducted in an African country; "Duplicate" studies where the same samples or data were used for multiple publications.*

between studies, including when stratified by urban, mixed urban/rural and rural settings (Figures 1.6-1.8). Here, the diverse detection and serotyping methods utilized (Table 1.6), the proportion of children immunized with PCV, the number of years of routine PCV-use (Table 1.5 and Figure 1.5) and methodological approaches to pneumococcal detection specific to each study may have contributed to the variation in pneumococcal colonization prevalence across studies. There were also differences in the ranges included in age categories among various studies, whereas colonization prevalence varies with age [69, 141]. This is demonstrated by comparing the colonization prevalence in the age categories in the Swarthout *et al*. (2020) paper (Malawi) where all other parameters of the study were consistent. Children aged 1-2 months old had a net colonization prevalence of 43% (95% CI: 30-58); 37% (95% CI: 3-43) and 58% (95% CI: 47-67) at 5, 6 and 7 years after the introduction of PCV respectively, compared with 80% (95% CI: 68-89); 85% (95% CI: 78-90); 81% (95% CI: 69-89) in children aged 4-12 months [227].

There was a potential risk of bias identified in three studies, due to the methods of assessing the outcome, i.e.: ascertaining pneumococcal colonization prevalence. In the study by Emgård *et al*. (2019), samples were collected, and bacterial culture conducted for the detection of pneumococcus in Tanzania. Positive isolates were subsequently transported in Skim milk-tryptone-glucose-glycerin media (STGG) to Sweden for further testing. Prior to transport, pneumococcus was cultured in only 32% (244/775) of samples, while there was no specific methodological bias identified from the culture methods described. After transport, the isolates were culture non-viable and therefore mPCR and sequetyping were used for detection and serotyping of pneumococcus. Further, 100uL of STGG was diluted in 900uL of phosphate buffered saline, prior to nucleic acid extraction [400]. The benefit of PCR-based methods is the detection of pneumococcus at a low copy number and in culture non-viable samples [170, 179]. Indeed, pneumococcus was detected in 85% (205/241) of the transported cultured pneumococcus isolates [400], despite the 1:10 dilution of the sample. Still, 15% of samples positive on culture prior to transport, were negative on PCR. Together, initial culture steps and long-distance transport may have

resulted in an underestimate of colonization in that study. Similarly, in the study by Mills *et al*. (2020), samples were initially cultured in Ghana and positive isolates later transported to Germany for further pneumococcal detection and serotyping (mPCR and Quellung) [(402)]. The proportion of pneumococcus detected from the initial culture steps before transport were not distinguished from those detected by PCR after transport, and thus could not be determined if the colonization prevalence detected by culture differed from that detected by PCR after long distance transport. Additionally, the study did not provide details about the volumes used for nucleic acid extraction, and there were no specific methodological aspects described that could be used to determine the plausibility of an underestimation of pneumococcal colonization in the study. In Birindwa *et al*. (2018) the authors describe that pneumococcus was detected in just 20.5% (163/794) of samples using culture for detection of pneumococcus, that was conducted in the Democratic Republic of Congo (DRC). Analogous to Emgård *et al*. (2019) described above, positive isolates were subsequently shipped to Sweden for further typing, and only 32 out of 151 shipped isolates were viable upon arrival, and thus the same molecular methods were employed for further serotyping. Nonetheless, pneumococcus was confirmed in 96.0% (145/151) of culture-positive isolates available for testing with PCR [(399)]. It is plausible that initial culture steps may have resulted in an underestimate of colonization. Here, the authors circumvented this by analyzing the proportions of VT and NVT out of the total number of isolates positive for pneumococcus, instead of prevalence out of all children. Here, data would not be comparable in this format, and was transformed into prevalence, which would introduce an underestimation that should be noted. The three studies highlight the importance of investing in cost effective and reproducible molecular methods in LMIC.

Table 1.5: Characteristics of reviewed colonization studies in Africa.

Author/Year	Locality	Enrolment Period	Vaccine (Years of use; introduction; transition)	Catch-up campaign; age in months (doses)	Study Coverage % (doses)	dosing schedule (weeks of age)	WUNEIC Coverage % (Year)	Age (months)	HIV status/ Prevalence	n
Birindwa *et al.* (2018) [399]	South-Kivu Congo	Jan 2014-Jun 2015	PCV13 (2; 2012; -)	-	-	3 + 0 (6, 10 & 14)	80 (2015)	1-60	-	794
Emgård *et al.* (2019) [400]	Moshi Tanzania	Feb-Apr 2015	PCV13 (3; 2012; -)	-	-	3 + 0 (4, 8, & 12)	92 (2015)	6-23	-	213
Heinsbroek *et al.* (2018) [68]	Karonga Malawi	Apr-Aug 2014	PCV13 (3; 2011; -)	0-12 (3)	-	3 + 0 (6, 10 & 14)	87 (2014)	1-2	HIV-	146
								4-5	HIV-	44
								12-48	HIV-	207
Kaboré *et al.* (2021) [401]	Bobo-Dioulasso Burkino Faso	Mar 2017	PCV13 (3; 2013; -)	-	71.0	3 + 0 (8, 12, & 16)	91 (2017)	1–11	-	201
					97.9			12–23	-	199
					74.0			24–59	-	204
Kobayashi *et al.* (2020) [228]	Kibera/Lwak Kenya	Oct-Dec 2013	PCV10 (2; 2011; -)	0-12 (3); Lwak only: 12-48 (2)	-	3 + 0 (6, 10 & 14)	75 (2013)	0-12	-	180
								12-59	-	485
Madhi *et al.* (2020) [224]	Agincourt South Africa	May-Oct 2011	PCV7/PCV13 (2; 2009; 2011)	0-36 & 36–71 months underlying conditions	45.1 (2)	2 + 1 (6, 14 & 40)	75 (2012)	0-9	3.0-3.2%	80
					37.8 (3)		75 (2012)	9-23	3.0-3.2%	828
					8.1 (3)		75 (2012)	24-59	3.0-3.2%	422
		May-Nov 2013	PCV7/PCV13 (4; 2009; 2011)	0-36 & 36–71 months underlying conditions	65.2 (2)		77 (2013)	0-9	3.0-3.2%	30
					82.2 (3)		77 (2013)	9-23	3.0-3.2%	404
					49.8 (3)		77 (2013)	24-59	3.0-3.2%	259

Author/Year	Locality	Enrolment Period	Vaccine (Years of use; introduction; transition)	Catch-up campaign; age in months (doses)	Study Coverage % (doses)	dosing schedule (weeks of age)	WUNEIC Coverage % (Year)	Age (months)	HIV status/ Prevalence	n
Mills *et al.* (2020) [402]	Cape Coast Ghana	Feb 2018	PCV13 (6; 2012; -)	-	-	3 + 0 (6, 10 & 14)	96 (2018)	0-59	-	513
Njuma-Libwea *et al.* (2020) [403]	Yaoundé Cameroon	Mar-Jul 2013	PCV13 (2; 2011; -)	-	-	3 + 0 (6, 10 & 14)	88 (2013)	24–36	-	198
		Mar-Jul 2015	PCV13 (4; 2011; -)				77 (2015)	24–36	-	689
Nzenze *et al.* (2015) [59]	Soweto South Africa	May 2012–Apr 2013	PCV7/PCV13 (3; 2009; 2011)	0-36 & 36–71 months underlying conditions	37.8-62.0 per age eligible dose	2 + 1 (6, 14 & 40)	75 (2012)	0-9	HIV-	344
									HIV+	52
								9–24	HIV-	447
									HIV+	92
								24–48	HIV-	166
									HIV+	182
Swarthout *et al.* (2020) [227]	Balantyre Malawi	Jun-Aug 2015	PCV13 (5; 2011; -)	0-12 (3)	98.7 (≥1)	3 + 0 (6, 10 & 14)	88 (2015)	24-59	HIV-	301
		Oct 2015-Apr 2016							HIV-	295
		May-Oct 2016					83 (2016)		HIV-	323
		May-Oct 2017	PCV13 (5; 2011; -)	0-12 (3)	98.7 (≥1)	3 + 0 (6, 10 & 14)	88 (2017)	1-2	HIV-	121
								24-59	HIV-	426
								12-24	HIV-	82
								4-12	HIV-	65
		Nov 2016-Apr 2017					88 (2017)	1-2	HIV-	46
									HIV-	121
							83 (2016)	12-24	HIV-	97

Author/Year	Locality	Enrolment Period	Vaccine (Years of use; introduction; transition)	Catch-up campaign; age in months (doses)	Study Coverage % (doses)	dosing schedule (weeks of age)	WUNEIC Coverage % (Year)	Age (months)	HIV status/ Prevalence	n
								4-12	HIV-	56
								24-59	HIV-	436
		Nov 2017-Jun 2018					88 (2017)	1-2	HIV-	133
								4-12	HIV-	56
								12-24	HIV-	83
								24-59	HIV-	444
		Jun-Dec 2018					92 (2018)	1-2	HIV-	92
								4-12	HIV-	57
								12-24	HIV-	69
								24-59	HIV-	387
Usuf *et al.* (2019) [225]	Serekunda/Sukuta Gambia	Mar-Jun 2011	PCV7/PCV13 (2; 2009; 2011)	-	-	3 + 0 (6, 10 & 14)	95 (2011)	6-12	-	339
		Mar-Jun 2012	PCV7/PCV13 (3; 2009; 2011)				98 (2012)	6-12	-	350
		Mar-Jun 2016	PCV7/PCV13 (7; 2009; 2011)				95 (2016)	6-12	-	351
Valenciano *et al.* (2021) [226]	Manhiça/Maputo/ Nampula Mozambique	Oct 2015–May 2016	PCV10 (2; 2013; -)	-	44	3 + 0 (6, 10 & 14)	80 (2015)	1–11	HIV+	88
								1–11	HIV-	78
								12–23	HIV+	127
								12–23	HIV-	184
								24–59	HIV+	394
								24–59	HIV-	302

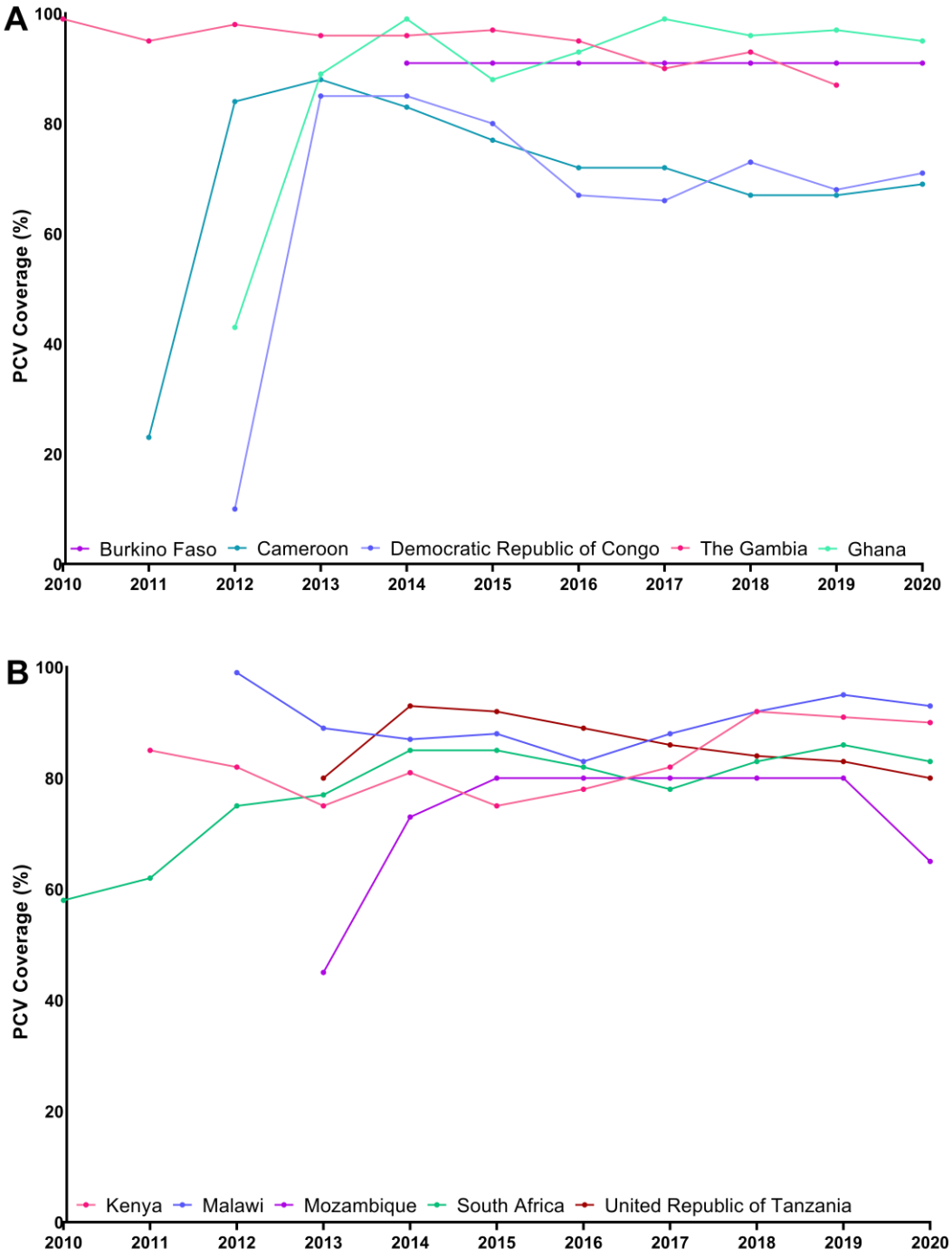

Figure 1.5: Annual pneumococcal vaccination coverage in African countries reported through the World Health Organization (WHO)/United Nations Children's Fund (UNICEF) Joint Reporting Form on Immunization (JRF) Estimates of National Immunization Coverage (WUENIC) [153, 162].

Separated into A: Country names beginning with A-K and B: Country names beginning with L-Z.

Table 1.6: Detection and serotyping method of reviewed colonization studies in South Africa.

Author/Year	Outcome measure (detection method)
Birindwa *et al.* (2018) [399]	Culture then mPCR for 40 serotypes supplemented with Sequetyping
Emgård *et al.* (2019) [400]	Culture then mPCR for 40 serotypes supplemented with Sequetyping
Heinsbroek *et al.* (2018) [68]	Culture, combination of Latex agglutination and Quellung serotyping
Kaboré *et al.* (2021) [401]	Culture then PCR, supplemented with Quelling serotyping
Kobayashi *et al.* (2020) [228]	mPCR serotyping
Madhi *et al.* (2020) [224]	Culture, then lyt*A* PCR to confirm pneumococcus and Quellung serotyping
Mills *et al.* (2020) [402]	Culture, then mPCR for 40 serotypes supplemented with Quellung serotyping
Njuma-Libwea *et al.* (2020) [403]	Sequential mPCR for 40 serotypes supplemented with Quellung serotyping
Nzenze *et al.* (2015) [59]	Culture, then lyt*A* PCR to confirm pneumococcus and Quellung serotyping
Swarthout *et al.* (2020) [227]	Culture, Latex agglutination serotyping
Usuf *et al.* (2019) [225]	Culture, Latex agglutination, molecular serotyping, sequencing
Valenciano *et al.* (2021) [226]	Culture, Quellung serotyping

1.11.2.1 Net pneumococcal colonization in African countries after routine PCV immunization

In African studies, the overall pooled estimate of prevalence of pneumococcal colonization in children under 60 months old was 67% (95% CI: 61-74%), although the actual prevalence is higher than this figure in many settings.

The pooled net pneumococcal colonization prevalence for the six studies that recruited children under 60 months old in urban areas was 67% (95% CI: 59-75; Figure 1.6). The prevalence was lower in two studies: Emgård *et al*. (2019) conducted in Tanzania and Mills *et al*. (2020) conducted in Ghana, where children aged 6-23 months and 0-59 months respectively were recruited after 3 and 6 years of routine PCV immunization,

respectively. The overall colonization prevalence was 26% (95% CI: 21-33) and 29% (95% CI: 26-34) respectively, which was lower than other included urban studies with similar aged children (Figure 1.6) [400, 402]. The prevalence in these two studies may be underestimated as discussed in section 1.10.2. The colonization prevalence was lower in the youngest age-group (1-2 months old) in Swarthout *et al*. (2020), conducted in Malawi and discussed above, and Nzenze *et al*. (2015), conducted in South Africa (under 9 months old: 49%, 95% CI: 44-54), whereas in older children in both studies, colonization prevalence was similar to the other studies that ranged between 60-91% (Figure 1.6), excluding Emgård *et al*. (2019) and Mills *et al*. (2020). The overall colonization prevalence was highest in Kenyan children in Kobayashi *et al*. (2020), in all age groups (0-12 months: 89%, 95% CI: 84-93; 13-60 months: 91%, 95% CI: 88-93) [228]. In that study, pneumococcal prevalence was assessed using direct mPCR with no intervening culture step, however in pre-PCV introduction time-points where colonization was assessed using culture-based methods, the prevalence was not different to when using PCR [228], meaning the higher colonization estimate were unlikely due to more sensitive molecular methods employed to detect pneumococcus in this study.

In three studies where recruitment was conducted in mixed urban and rural settings, the pooled net pneumococcal colonization prevalence was 75% (95% CI: 53-97), with similar estimates from The Gambia in Usuf *et al*. (2019) and from Mozambique in Valenciano *et al*. (2021) of 80-86% across all age groups (Figure 1.6) [225, 226]. In Birindwa *et al*. (2018), conducted in DRC, the prevalence was much lower, at 21% (95% CI: 18-23) [399], which is likely an underestimate due to sample viability, as detailed in 1.10.2.

The pooled pneumococcal colonization prevalence from the three studies conducted in rural settings was 64% (95% CI: 57-70) [68, 224, 403]. While the overall pneumococcal colonization in all children aged under 60 months was similar amongst the studies included in the analysis (range: 64-80%), the prevalence was lower in the youngest age

categories in the included studies. Specifically, from the Heinsbroek *et al*. (2018) data, pneumococcal colonization prevalence was estimated at 44% (95% CI: 36-52) in Malawian infants aged 1-2 months old. In a study by Madhi *et al*. (2020), the overall colonization prevalence in South Africa was 47% (95% CI: 30-64) in infants under 9 months old, after four years of routine PCV use (2013), while in the same age group after two years of routine use the overall pneumococcal prevalence was 73% (95% CI: 62-81) [68, 224]. In the latter case, the authors note that within the four years post PCV introduction, reductions in VT colonization were evident, however serotype replacement by NVT was not yet complete. Incomplete serotype replacement may have resulted in a lower net colonization prevalence in 2013, after the transition from PCV7 to PCV13 in South Africa (2011) [224]. In Njuma-Libwea *et al.* (2020), in Cameroon, colonization prevalence (62%; 95% CI: 58-65) in the four-year timepoint was not different to the two-year post-PCV timepoint (58%, 95% CI: 51-64). The authors described that samples were transported from Cameroon to Finland for pneumococcal detection and serotyping, and that samples were held at customs for the four- year post-PCV timepoint for over two months. The use of molecular detection methods should have circumvented any impact on bacterial viability, corroborated by the similar estimates in the four-year and two-year timepoints.

1.11.2.2 Colonization by VT in African countries after routine PCV immunization

The pooled prevalence of PCV13-VT colonization in African studies was 20% (95% CI: 17-23) after 2-7 years of PCV inclusion in immunization programs, including 17% (95% CI: 14-20) in urban settings, 24% (95% CI: 14-33) in mixed urban/rural settings, and 24% (95% CI: 16-31) in rural settings (Figure 1.7). The prevalence of PCV13-VT colonization was higher than the pooled prevalence in urban settings in Kobayashi *et al*.

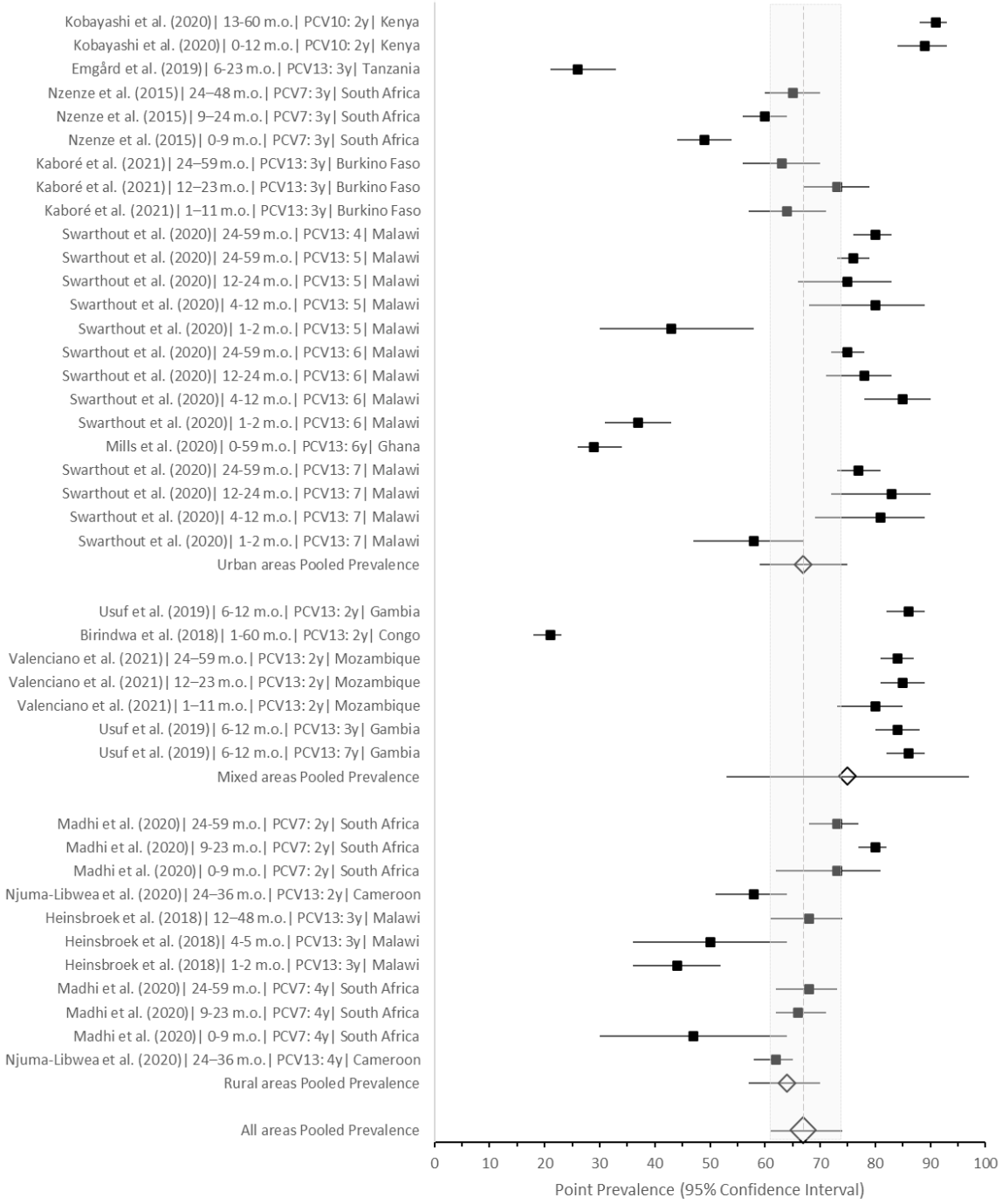

Figure 1.6: Pneumococcal colonization in African countries >2 years after pneumococcal conjugate vaccine (PCV) introduction.

The dotted vertical line and shaded area indicates the pooled prevalence estimate and 95% Confidence Interval. Studies arranged by years of PCV use, then by age category. Urban areas pooled prevalence: 67% (95% CI: 59-75); X^2=1201.8, p<0.01, I^2=98.4%, Tau^2=0.03; mixed areas pooled prevalence: 75% (95% CI: 53-97), X^2=1556.4, p<0.01,

I^2=99.6%, Tau^2=0.09; rural areas pooled prevalence: 67% (95% CI: 57-70), X^2=140.7, p<0.01, I^2=92.9%, Tau^2=0.01; all areas pooled prevalence: 67% (95% CI: 61-74%), X^2=2901.1, p<0.01, I^2=98.7%, Tau^2=0.04.

(2020) (Kenya, 2 years post-PCV, under 12 months old: 31%, 95% CI: 25-38; 13-60 months old: 33%, 95% CI: 29-37). While collective PCV13-VT were reported in Kobayashi *et al*. (2020) and analyzed here, Kenya utilizes PCV10 in the national immunization program. The prevalence of VT targeted by PCV10 in Kobayashi *et al*. (2020) was within the 95% CI for the pooled prevalence estimated here in urban settings for PCV13-VT, i.e.: 14% (25/180) for children under 12 months old, and 17% (82/485) in 13-60 months old. For the same reasons as discussed in 1.10.2; the prevalence of PCV13-VT in Emgård *et al*. (2019) (Tanzania, 3 years post-PCV, 6-23 months old: 6%, 95% CI: 4-10) and Mills *et al*. (2020) (Ghana, 6 years post-PCV, 0-59 months old: 11%, 95% CI: 9-14) were lower than the pooled estimates. The other studies conducted in urban settings were similar to the pooled estimates for urban settings and overall, with the exception of the 1–2 month old age group in the Malawian study by Swarthout *et al*. (2020), due to the young age.

In mixed urban/rural settings, Usuf *et al*. (2019) reported higher VT colonization in the two years post-PCV timepoint (Gambia, 6-12 months old: 33%, 95% CI: 29-39), but lower colonization in the seven years post-PV timepoint (Gambia, 6-12 months old: 11%, 95% CI: 8-15). In Valenciano *et al*. (2021) the reported colonization of PCV13-VT (Mozambique, 2 years post-PCV, 12-23 months old: 31%, 95% CI: 26-37; 24-59 months old: 39%, 95% CI: 35-42) was higher than the total pooled prevalence. In Mozambique, PCV10 is in routine use, and the prevalence of PCV10-VT reported in Valenciano *et al*. (2021) was lower than the pooled prevalence for children 12-23 months old (15.1%, 47/311) and the same as the pooled prevalence of PCV13-VT for children 24-59 months old (20.8%, 145/696). Furthermore, in the DRC, Birindwa *et al*. (2018) estimated lower PCV13-VT colonization (2 years post-PCV, 1-60 months old: 10%, 95% CI: 8-12)

compared with the pooled prevalence for mixed urban/rural settings and overall and is plausibly an underestimate, as discussed in section 1.10.2.

In rural settings, Madhi *et al*. (2020) reported higher prevalence of PCV13-VT two years post-PCV introduction than the pooled estimate for all studies (South Africa, under 9 months old: 38%, 95% CI: 28-48; 9-23 months old: 42%, 95% CI: 39-46; 24-59 months old: 44%, 95% CI: 39-49), whereas after four years of routine PCV immunization, the colonization prevalence of PCV13-VT was the same as the pooled PCV13-VT prevalence (under 9 months old: 20%, 95% CI: 10-37; 9-23 months old: 18%, 95% CI: 15-22; 24-59 months old: 20%, 95% CI: 15-25). All other estimates were within the confidence intervals of the pooled prevalence for rural settings, and all settings combined.

In summary, there were three studies with a risk of underestimating PCV13-VT prevalence, i.e.: Emgård *et al*. (2019) conducted in Tanzania, Mills *et al*. (2020) conducted in Ghana and Birindwa *et al*. (2018) conducted in the DRC, due to sample viability. In one study from Malawi, Swarthout *et al*. (2020), the delineation of an age category of young infants only 1-2 months old, resulted in a lower estimate of VT, due to age, whereas in other studies younger infants were included in broader age categories and would not have discerned this difference. In two studies conducted in Kenya, Kobayashi *et al*. (2020) and Mozambique, Valenciano *et al*. (2021), that utilize PCV10 in their immunization programs, the prevalence of PCV13-VT was higher than the other studies, however, PCV10-VT were within the range of the pooled estimates of PCV13-VT. Notably, two studies clearly demonstrated a higher impact of routine PCV use on VT over a seven-year period compared with just two years, i.e.: Usuf *et al*. (2019) in The Gambia and Madhi *et al*. (2020) in South Africa. Nevertheless, the pooled prevalence of PCV13-VT two to seven years after PCV introduction in African countries was high (20%, 95% CI: 17-23), including the lowest estimates in African settings (6-11%) [399, 400, 402], compared with that observed in higher income settings (0.7-4.0%), such as England,

USA, Norway and France [391, 404-408].

1.11.2.3 Colonization by NVT in African countries after routine PCV immunization

The pooled prevalence of NVT across the included African studies was 47% (95% CI: 41-54). When stratified by settings, the pooled prevalence was 51% (95% CI: 43-58) in urban settings, 51% (95% CI: 28-74) in mixed urban/rural settings, and 39% (95% CI: 35-44) in rural settings (Figure 1.8), respectively.

Consistent with the net pneumococcal prevalence, the prevalence of non-PCV types was lower than the pooled estimates in Emgård *et al*. (2019), Mills *et al*. (2020) and Birindwa *et al*. (2018), and lower than the respective estimates for urban and mixed settings (Tanzania, 6-23 months old: 20%, 95% CI: 15-26; Ghana, under 59 months old: 18%, 95% CI: 15-22 and DRC, 1-60 months old: 8%, 95% CI: 6-10).

In urban settings, the prevalence of NVT was lower than the pooled estimate in all settings and for urban settings in Swarthout *et al*. (2020) in the youngest age group (Malawi, 1-2 months, 28%, 95% CI: 23-34), and Nzenze *et al*. (2015) (South Africa, under 9 months old, 35%, 95% CI: 31-40) six and three years post-PCV respectively, which was accordant with the lower net pneumococcal prevalence in these age groups, as detailed in section 1.10.2. Within Swarthout *et al*. (2020), in Malawi, NVT colonization was higher than the pooled estimate for all settings seven years after PCV introduction in 12-24- and 24–59 month old children (68%, 95% CI: 56-78 and 61%, 95% CI: 56-66), after six years in children aged 4-12 months (67%, 95% CI: 58-75), and after four years in 24–59 month olds (60%, 95% CI: 56-63).

The prevalence of NVT in the mixed urban/rural setting in The Gambia (Usuf *et al*. 2019) in 6-12 month old children was higher than the pooled prevalence in all settings, three and seven years after PCV introduction (67%, 95% CI: 62-72 and 74%, 95% CI: 70-79) but there was no difference in the 2-year post-PCV timepoint (53%, 95% CI: 48-58), which is consistent with serotype replacement as VT prevalence was lower 3-7 years post-PCV in the same study.

In rural settings, the prevalence of NVT was lower than the pooled estimate in South Africa (Madhi *et al*., 2020), two-years post PCV for children 24–59 months old (32%, 95% CI: 28-37), as well as in Cameroon (Njuma-Libwea *et al.*, 2020) in children aged 24–36 months old, two- and four-years post PCV (31%, 95% CI: 25-38 and 37%, 95% CI: 33-40). Similarly, the prevalence of NVT was also lower in Malawi (Heinsbroek *et al.*, 2018) in 1-2 month old infants, three years post-PCV (31%, 95% CI: 24-39).

In summary, the prevalence of NVT was variable in African settings 2-7 years after the introduction of PCVs. Generally, there was an upward trend in NVT prevalence with a more extended duration since the integration of PCVs into routine immunization programs, which is in line with serotype replacement.

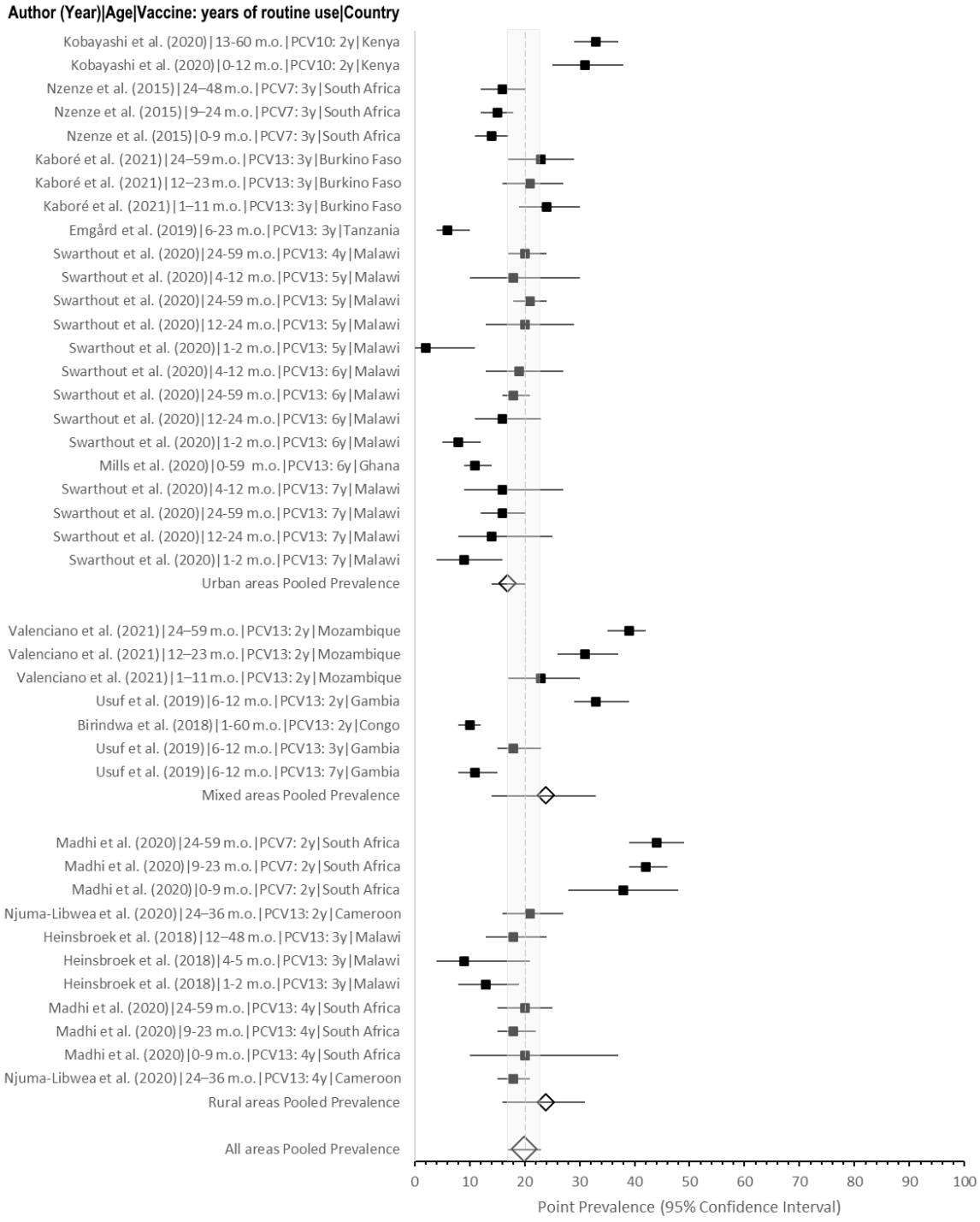

Figure 1.7: Pneumococcal 13-valent PCV serotype (PCV13-VT) colonization in African countries >2 years after Pneumococcal Conjugate Vaccine (PCV) introduction.

The dotted vertical line and shaded area indicates the pooled prevalence estimate and 95% Confidence Interval. Studies arranged by years of PCV use, then by age category. Urban areas pooled prevalence: 17% (95% CI: 14-20); X^2=233.0; p<0.01; I^2=90. 6%; Tau^2<0.01; mixed areas pooled prevalence: 24% (95% CI: 14-33); X^2=270.1; p<0.01;

I^2=97.8%; Tau^2=0.02; rural areas pooled prevalence: 24% (95% CI: 16-31); X^2=246.9; p<0.01; I^2=96.0%; Tau^2=0.01; All areas pooled prevalence: 20% (95% CI: 17-23); X^2=2901.1; p<0.01; I^2=98.7%; Tau^2=0.04.

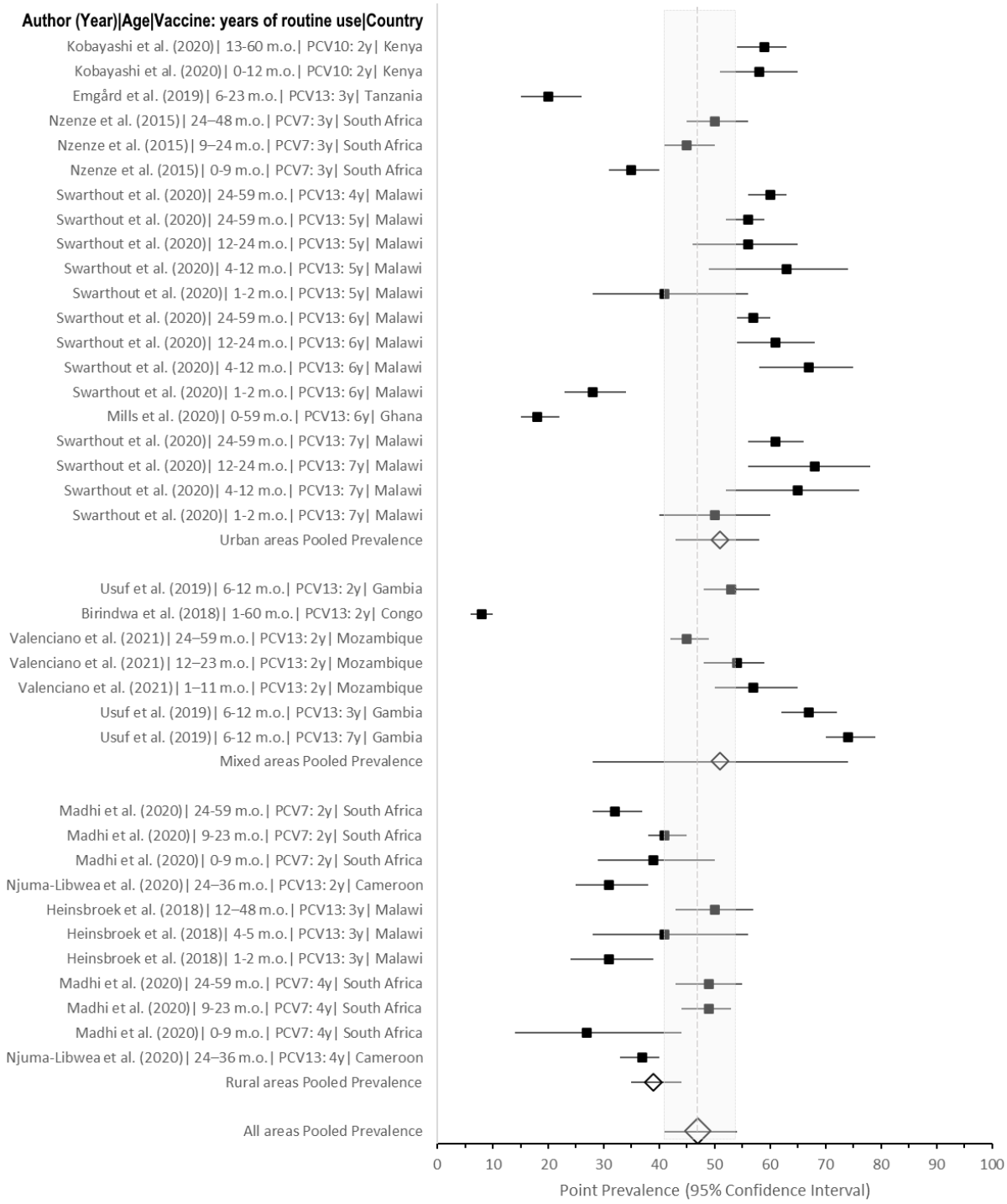

Figure 1.8: Pneumococcal non-13-valent PCV serotype (NVT) colonization in African countries >2 years after Pneumococcal Conjugate Vaccine (PCV) introduction.

The dotted vertical line and shaded area indicates the pooled prevalence estimate and 95% Confidence Interval. Studies arranged by years of PCV use, then by age category. Urban areas pooled prevalence: 51% (95% CI: 43-58), X^2=688.3; p<0.01; I^2=97.2%; Tau2=0.03; Mixed areas pooled prevalence: 51% (95% CI: 28-74), X^2=1334.8; p<0.01;

I^2=99.6%; Tau^2=0.10; Rural areas pooled prevalence: 39% (95% CI: 35-44, X^2=140.7; p<0.01; I^2=92.9%; Tau^2=<0.01; All areas pooled prevalence: 47% (95% CI: 41-54), X^2=2901.1; p<0.01; I^2=98.7%; Tau^2=0.04.

1.12 Justification, Aims and Objectives and sample size

Problem statement: Children are at higher risk of pneumococcal colonization and infections and account for most transmission. In South Africa, childhood PCV immunization has resulted in reductions in VT colonization and concomitantly in IPD among vaccinated and unvaccinated individuals. In a country with high rates of pneumococcal colonization and a high HIV burden, strict assessment of the pneumococcal serotype epidemiology and risk of disease are essential to assess and monitor the impact of PCV and inform any programmatic changes in product or dosing schedules. Long term impact is particularly important in LMIC with a background of high prevalence of VT colonization, where reduction in VT may be more protracted than in HIC. Furthermore, bacterial dynamics may have shifted after routine PCV immunization, and this must be investigated. In addition, vaccine coverage with three doses of PCV must be assessed in urban and rural settings. Replacement colonization by NVT also needs to be carefully monitored.

Aims: The broad aim of this study is to assess colonization after 8-9 years of routine PCV immunization using a high-throughput Real-Time PCR platform to provide up to date data on serotype distribution. This will provide vital information on the current prevalence of VT and risk of disease.

Primary objectives:

- To develop a high-throughput molecular method in a nanofluidic real-time PCR system for comprehensive pneumococcal serotyping to assess colonization using respiratory samples.
- To evaluate prevalence of colonization by the most frequent PCV13 individual serotypes in 2017 among children under 2 years old and 2-5 years old, eligible to have received at least one dose of PCV13 in Agincourt and compare this with baseline rates from 2009.
- To measure the individual colonization prevalence of the most frequent PCV13-serotypes in 2018 among children under 2 years old and 2-5 years old, eligible to have received at least one dose of PCV13 in Soweto and compare this with baseline rates from 2010-2011.

Secondary objectives:

- To assess other nasopharyngeal colonizers using a quantitative high-throughput molecular method to evaluate respiratory samples within a nanofluidic real-time PCR system.
- To assess colonization by VT and NVT in children ≤5 years old and evaluate the temporal association of routine childhood PCV immunization on colonization by non-vaccine serotypes and other bacterial species compared with at the onset of the immunization program.

Sample size calculation:

A minimum sample size of at least 396 was needed for 2017 and 2018 to detect 80% reduction in the most prevalent circulating PCV13-VT (4.2-13.9%: 19F; 6B; 6A; 23F; 19A and 14) in children under 5 years of age in 2017/18 compared with 2009/10 with an 80% power.

Chapter 2: High-throughput nanofluidic real-time PCR to discriminate Pneumococcal Conjugate Vaccine (PCV)-associated serogroups 6, 18, and 22 to serotypes using modified oligonucleotides. – Published Manuscript

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High-throughput nanofluidic real-time PCR to discriminate Pneumococcal Conjugate Vaccine (PCV)-associated serogroups 6, 18, and 22 to serotypes using modified oligonucleotides

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Current real-time high-throughput Polymerase Chain Reaction (qPCR) methods do not distinguish serotypes 6A from 6B, 18C from 18A/B and 22F from 22A. We established a nanofluidic real-time PCR (Fluidigm) for serotyping that included Dual-Priming-Oligonucleotides (DPO), a Locked-Nucleic-Acid (LNA) probe and TaqMan assay-sets for high-throughput serotyping. The designed assay-sets target capsular gene wci*P* in serogroup 6, wci*X* and wxc*M* in serogroup 18, and wcw*A* in serogroup 22. An algorithm combining results from published assay-sets (6A/B/C/D; 6C/D; 18A/B/C; 22A/F) and designed assay-sets for 6A/C; 18B/C/F; 18C/F, 18F and 22F was validated through blind analysis of 1973 archived clinical samples collected from South African children ≤ 5-years-old (2009–2011), previously serotyped with the culture-based Quellung method. All assay-sets were efficient (92–101%), had low variation between replicates ($R^2 > 0.98$), and were able to detect targets at a limit of detection (LOD) of < 100 Colony-Forming-Units (CFU)/mL of sample. There was high concordance (Kappa = 0.73–0.92); sensitivity (85–100%) and specificity (96–100%) for Fluidigm compared with Quellung for serotyping 6A; 6B; 6C; 18C and 22F. Fluidigm distinguishes vaccine-serotypes 6A, 6B, 18C, next-generation PCV-serotype 22F and non-vaccine-serotypes 6C, 6D, 18A, 18B, 18F and 22A. Discriminating single serotypes is important for assessing serotype replacement and the impact of PCVs on vaccine- and non-vaccine serotypes.

Streptococcus pneumoniae (pneumococcus) remains a leading cause of morbidity and mortality globally, causing an estimated 294 000 deaths (uncertainty range [UR] 192 000–366 000) in children 0–59 months without HIV in 2015[1]. Current PCV formulations include vaccine-serotypes (VT) 6A (PCV13), 6B and 18C (PCV13, PCV10). Serotype 22F is included in next-generation PCV15 and PCV20[2,3]. Before the introduction of PCVs, serogroup 6 was associated with 14–18% of invasive pneumococcal disease (IPD) episodes worldwide, whereas serotype 18C was the fifth most common serotype identified in IPD cases in developed nations[4,5]. Since the introduction of PCV, IPD due to serotypes 6A, 6B, and 18C have declined, however, 6A is still a common VT identified in carriage studies[6,7]. Serotype 22F has a high invasive disease potential and is increasing globally[8].

The referent standard for pneumococcus detection and serotype identification has been the culture-based Quellung method which is not very sensitive and is costly[9,10]. Real-time PCR provides faster diagnosis and higher sensitivity[11]; however, due to genetic similarity some serotypes have been difficult to distinguish with PCR. Serogroups 6A/B; 6C/D; 18A/B/C and 22A/F were detected using real-time PCR[11] and further distinguished with sequencing-based methods[12–14] or conventional PCR combined with gel electrophoresis[15]. Sequential multiplex PCR has now been expanded to differentiate serogroups 6 and 22 but not 18[16]. Distinguishing serogroups 6, 18,

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Assay	Culture strain	LOD (CFU/mL)†	Linear equation	R^2	Efficiency [$-1+10^{(-1/m)}$]
6A/B/C/D	6B	10^1	− 3.327x + 30.357	0.99	100%
6A/C	6A	10^2	− 3.4461x + 29.079	0.99	95%
6C/D	6C	10^2	− 3.303x + 25.549	0.99	101%
6C/D	6D	10^1	− 3.322x + 27.105	0.99	100%
18A/B/C	18C	10^2	− 3.3484x + 22.148	0.99	99%
18B/C/F	18C	10^1	− 3.3416x + 30.586	0.99	99%
18C/F	18C	10^2	− 3.3743x + 31.732	0.99	98%
16F/18F/28A/F	18F	10^1	− 3.5403x + 29.132	0.99	92%
22AF	22A	10^2	− 3.3701x + 26.527	0.99	98%
22F	22F	10^2	− 3.438x + 30.669	0.98	95%

Table 1. Performance of serogroup 6, 18 and 22 assay-sets in the Fluidigm. †All LOD calculations were based on triplicate dilutions of g-Blocks, except for assays 6A/C and 18C/F where relevant culture controls were included.

and 22 into single serotypes entirely in a high-throughput comprehensive molecular reaction-set has not been described.

Serotypes within serogroups 6, 18 and 22 are antigenically, biochemically, and genetically distinct[13,17–21]. The genetic basis of capsule variation in 6A and 6C relative to 6B and 6D involves a single nucleotide polymorphism (SNP) in the rhamnosyl-transferase gene (wci*P*α in 6A/C, wci*P*β in 6B/D)[18,22,23]. Serotype 6B 'sub-class II' (6E) is a genetic variant expressing a 6B capsule that is neither biochemically nor antigenically distinct from 6B[24]. Serotypes 6F, 6G, and 6H express hybrid capsules, serologically and biochemically distinct within serogroup 6[25,26]. Serotype 18A does not contain the acetyl-transferase gene (wci*X*) present in 18B/C/F. Relative to 18C/F, serotype 18B contains a SNP in wci*X*. Serotype 18F contains an additional acetyl-transferase gene (wcx*M*), also present in 16F and 28AF[21]. Within serogroup 22 the genetic locus from wcw*A* (glycosyl-transferase gene) to wcw*C* (acetyl-transferase gene) is heterogeneous[13].

In this study, nanofluidic real-time PCR (Fluidigm®) was used to distinguish individual serogroup 6 and serogroup 18 serotypes by including Dual Priming Oligonucleotide (DPO) forward primers to target the SNPs for 6A/C and 18C/F. Further, a thermodynamically modified Locked Nucleic Acid (LNA) probe was designed to detect 18C/F. Modified primer and probe strategies have not previously been utilised within the Fluidigm system.

Results

Performance of Fluidigm for identification of individual serotypes. When assessed using the standard culture control strains, all assays and applied algorithms amplified their respective targets effectively. The efficiency (90–110%) and slope ($-3.6 \geq \text{slope} \geq -3.3$) of each of the serogroup 6, 18 and 22 assay-sets were within the prescribed ranges in amplifying control strains of known CFU/mL, with low variation between replicates ($R^2 > 0.98$) (Table 1; Fig. 1).

The modified oligonucleotide strategies, including the DPO assays targeting 6A/C and 18C/F, combined with an LNA probe (18C/F), designed as part of this study were efficient (95% and 98% respectively), had low variation between replicates ($R^2 = 0.99$), and were able to detect 6A/C and 18C/F at a LOD of 10^2 CFU/mL (Table 1). Further, there was no cross-reactivity between serogroup 6 assay-sets (four serotypes) or serogroup 18 assay-sets (four serotypes) and the other 82 control strains for pneumococcal serotypes and eight nasopharyngeal colonisers included but not targeted by these assays.

Detection of individual serotypes by Fluidigm® and culture. In the 1973 archived clinical samples, the serogroup 6 assay-sets and the applied algorithms were able to distinguish serogroup 6 to individual serotypes with 96–99% specificity, 85–100% sensitivity and excellent concordance compared to Quellung for 6A,6B and 6C (Kappa = 0.86, 0.73 and 0.91 respectively). Serotype 18C was distinguished within serogroup 18 with 99.9% specificity, and 88.9% sensitivity and with excellent concordance compared to uellung (Kappa = 0.86). Finally, serotype 22F was detected with 100% specificity, 85.7% sensitivity and excellent concordance (Kappa = 0.92) compared with Quellung. There was no significant difference between Quellung and Fluidigm for detection of individual serotypes 6A, 6C, 18C and 22F (McNemar's test: $p > 0.05$). There was a significant difference in detection of serotype 6B (McNemar's test: $p < 0.01$), where 6B was detected in 73 additional samples with Fluidigm. Fluidigm detected the same serotypes as Quellung for most of these samples (83%; n = 43/52) in addition to serotype 6B. In the remaining samples no pneumococcus was detected by Quellung (n = 12) or were allocated as 6A (n = 9) due to the bacterial load for these samples approaching the limit of detection and not detected by the assay-set targeted at 6A/C.

Specificity, sensitivity, and concordance could not be assessed for serotypes 6D, 18A, 18B, 18F and 22A as these are minor serotypes and no positive samples were detected by Quellung for comparison with Fluidigm.

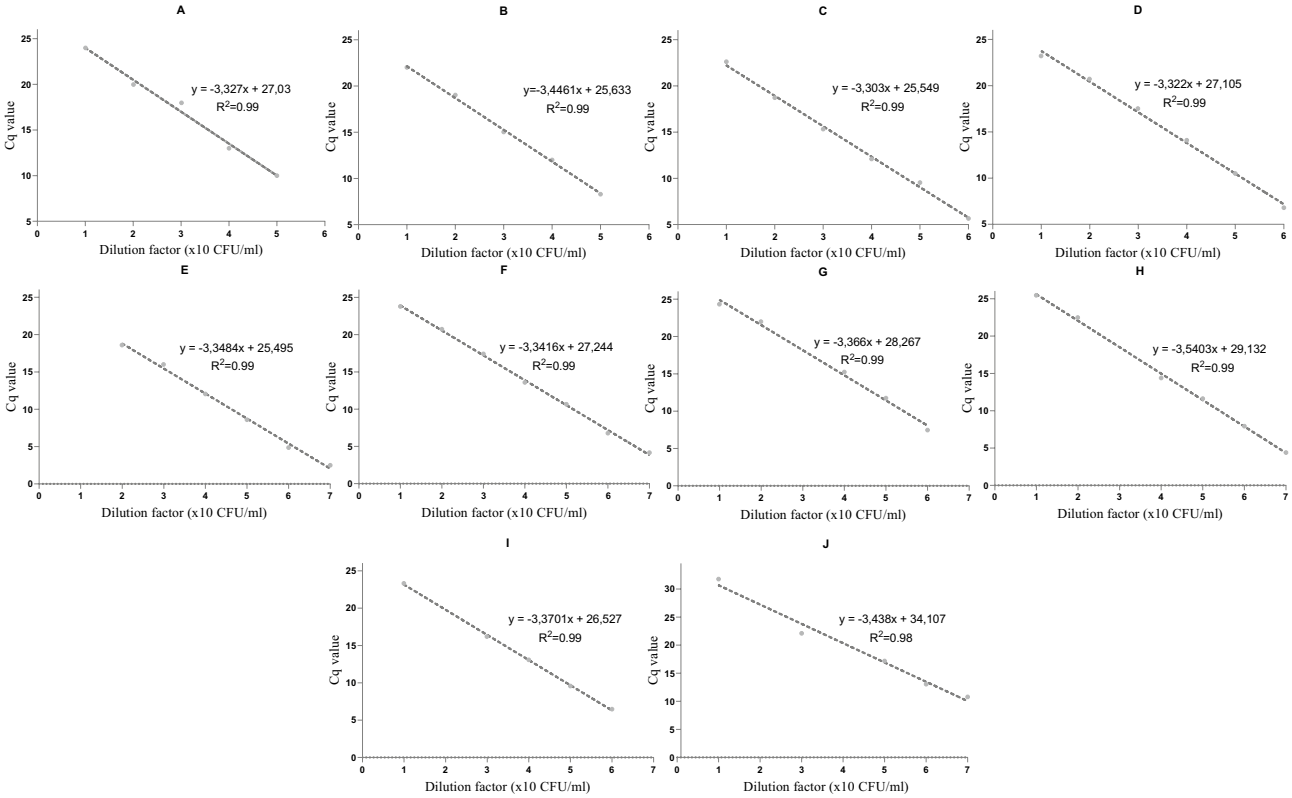

Figure 1. Standard curves constructed from duplicate serial dilutions to derive the linear equation, efficiency, and reproducibility for each serogroup 6, 18 and 22 assay-set. Panel A shows the culture standard for serotype 6B assessed with the assay-set to detect 6A/B/C/D; panel B is 6A assessed with the 6A/C assay-set; panel C is 6C assessed with the 6C/D assay-set; panel D is 6D with the 6C/D assay-set; panel E is 18C with the 18A/B/C/F assay-set; panel F is 18C with the 18B/C/F assay-set; panel G is 18C with the 18C/F assay-set; panel H is 18F with the 16F/18F/28A/F assay-set; panel I is 22A with the 22A/F assay-set and panel J is 22F with the 22F assay-set.

Discussion

The novel DPO, LNA, and other assay-sets designed here, combined with previously published serogroup 6, 18, and 22 assay-sets effectively discriminated between current PCV-formulation (PCV10, PCV13) VT (6A and 6B, 18C), next-generation PCV-formulation (PCV15, PCV20) VT 22F and NVT (6C, 6D, 18A, 18B, 18F and 22A) within a nanofluidic molecular serotyping reaction-set. Serogroups 6, 18, and 22 have previously been discriminated using conventional PCR[15,27,28], or sequencing-based methods[12,13,29]. Sequencing-based methods are expensive, not all laboratories have access to sequencing platforms, bioinformatics software and expertise. Further, some methods were unable to fully distinguish 6C/D or 18B/C[29]. Microarray assays have been developed, but these are also costly and require initial time-consuming culture steps[30]. Recently the CDC sequential multiplex real-time serotyping PCR was updated with two and three additional assay-sets respectively to detect and discriminate serogroup 6 (6B/D, 6A/B) and 22 (22A/F, 22A, 22F) respectively[16]. The CDC's unique 6B/D assay-sets utilized patented 'BHQ-plus' probes that include modified C and T nucleotides as duplex stabilizing chemistry to target the SNP that discriminates these serotypes. Our reaction-set was designed for comprehensive and high-throughput serotyping within a single nanofluidic real-time PCR, hence we used a compact strategy of assay-sets by designing just one additional assay-set each to further serotype serogroups 6 and 22. A benefit of our DPO primers is that they are not patented and may be purchased from any manufacturer globally to be incorporated with other probe chemistries if required.

No previous high-throughput real-time PCR assay has been reported to fully distinguish serogroup 6, 18, and 22[10,31–33] within a single reaction-set or made use of modified primer or probe strategies such as LNA-probes or DPO primers as utilised in this study. This is the first study to validate the use of thermodynamically modified assay-sets within the Fluidigm platform.

Comprehensive accurate serotyping is essential where VTs may still be circulating and to assess the relative benefit of next-generation PCVs that will include serotype 22F. Current published molecular assays, including the serogroup 6 and 18 described here, now enable detection of PCV13-VT 1, 3, 4, 5, 6A, 6B, 14, 18C, 19A, 19F, and 23F individually[10,11]. Our reaction-set is the first to distinguish all PCV13-serotypes except serogroup 9A/V and 7A/F to single serotypes. Relative to 9 V, 9A has a frameshift deletion in a G-polymer region surrounded by an AT-rich region[21]. Similarly, 7A lacks a side chain due to a frameshift mutation in the glycosyl transferase (wcw*D*) gene[21] located in a poly-T region within a GC region. These molecular features make the SNPs in 9 V

and 7F difficult to target and thermodynamically improbable to distinguish with molecular methods other than sequencing-based approaches.

The performance (sensitivity, specificity, and limit of detection) of the serogroup 6, 18, and 22 reaction-sets in the Fluidigm platform is comparable with other high-throughput strategies including the TaqMan Array Cards and the same Fluidigm platform including using different real-time PCR chemistry[10,33,34]. The performance of this reaction-set demonstrates that modified primers (DPO) or probes (LNA) are easily adapted to high-throughput real-time PCR, including where specific target pre-amplification is undertaken in multiplex (up to 34 assay-sets per tube). The successful combination of a DPO primer with a LNA probe to target 18C/F is a novel strategy. There is capacity for 96 samples and controls to be tested against 96 targets within a single run for the Fluidigm 96.96 Dynamic Array IFC (integrated fluidic circuit) that we utilized in the study, however the reaction-sets described here can be easily adapted to other real-time PCR platforms for lower through-put applications.

This study was limited in that non-pneumococcal *Streptococcus* species were not included in the validation of the assay-sets. Previous studies have validated the assay-sets for 6A/B/C/D, 6C/D, 18A/B/C, and 22AF against non-pneumococcal *Streptococcus*[10] and our algorithm included that samples must be detected with the serogroup 6 assay-set (6A/B/C/D), 18A/B/C, and 22AF to be assigned a serogroup 6, 18 or 22 type respectively, hence the designed assays are unlikely to detect other non-pneumococcal species. The methods described here are not currently aimed at detecting the hybrid serotypes 6F/G/H, as no reference specimens or clinical samples were available to validate the detection of these additional serotypes. Serotype 6B 'sub-class II' (6E) is a genetic variant of 6B, and the genetic regions targeted here are ubiquitous in 6B sub-classes so would be correctly assigned serotype 6B[24]. Prospective studies should include surveillance for hybrid serotypes and conduct confirmatory sequencing for isolates typed as serogroup 6 where PCVs are in use and residual serogroup 6 carriage is observed.

The Fluidigm could not be fully evaluated against the Quellung-method for detection of serotypes 6D, 18A/B/F or 22A in clinical isolates as these serotypes were not detected in the archived clinical samples by Quellung, most likely due to their low prevalence in Africa. Nevertheless, our algorithm correctly identified cultured control strains and would be able to correctly discern serotype 6D, 18A, 18F, 22A and 22F in clinical isolates. While the culture-based Quellung method is sensitive and specific, PCR is more sensitive and capable of detecting multiple co-colonising or culture non-typeable serotypes, even at a low density[35,36]. Genetic heterogeneity or the presence of a target of interest detected with molecular methods are not always accurate predictors of phenotypic capsule type and capsular expression in isolates[24]. Since the positive predictive value of Quellung is not perfect in cases of multiple serotype carriage or at low density, discrepant serotype positive designations by PCR are resolved as putative by confirming the presence of pneumococcal reference genes[37]. As Fluidigm PCR is compared to Quellung in this study, this may affect the measured concordance, for example serotype 6B where this serotype was detected in an additional 73 samples with Fluidigm. The benefit of real-time PCR is the enhanced detection of multiple serotypes carried concurrently compared with the Quellung method. Indeed, the serotypes assigned by the Quellung method were detected correctly in addition to serotype 6B in samples with multiple colonizing serotypes detected by our Fluidigm method. Further, the clinical samples that were re-analysed with Fluidigm as part of this study, had undergone multiple freeze–thaw cycles. This may have affected the sensitivity of Fluidigm compared with Quellung.

The DPO assay-sets targeting wci*P*α (6A/C) and wci*X* (18C/F) in conjunction with the designed wci*X* (18B/C/F), wcx*M* (18F), wcw*A* (22F), and published serogroup 6, 18 and 22 (6A/B/C/D; 18A/B/C; 22AF)[11] and 6C/D[38] assay-sets using the applied algorithms, can be used to correctly serotype circulating serogroup 6, 18 and 22 to individual serotypes using Fluidigm® real-time PCR in clinical samples.

Methods

DPO strategies overcome the limitations of primer design by combining the thermodynamic advantages of two isolated priming regions to enable sensitive and specific detection of SNPs in low GC regions[39,40]. LNA-based probes contain conformation-modified bases with a methylene bridge connecting the 2′-oxygen and 4′-carbon of the ribose ring in a nucleic acid base thereby increasing the melting temperature (Tm) for sensitive detection of low GC target regions[41].

Primer design. We included oligonucleotide primers and dye-labelled Minor Grove Binding (MGB) probes targeting serogroup 6 (6A/B/C/D), sub-group 6C/D, subgroup 18A/B/C, and serogroup 22 (22A/F) from published sequences[11,38] that have been optimized previously on the Biomark™ HD platform real-time PCR (Fluidigm)[33] in the reaction-set.

Genbank FASTA sequences of representative serogroup 6, 18, and 22F capsular genes (Table 2) were aligned separately for each serogroup in BioEdit using ClustalW Multiple alignment[42,43]. Standard primers and dye-labelled MGB probes were manually designed to detect the genes for wci*X* in 18B/C/F, wcx*M* in 18F, and wcw*A* unique to 22F, based on the Genbank accession numbers in Table 2. Sequences for serotypes 6B 'sub-class II' (6E), 6F, 6G, and 6H were included only for in silico analysis of their detection pattern. All oligonucleotide sequences that are listed in Table 2 were analysed to ensure specificity in silico using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/) prior to inclusion.

To further distinguish individual serogroup 6 and 18 serotypes, DPO primers and a LNA probe were newly designed (Table 3). The forward DPO for serogroup 6A/C and 18C/F were designed based on the Genbank sequence accession numbers in Table 2. The highly specific and shorter 3' 'foot' sequence to target the SNPs in 6A/C and 18C/F were designed based on the capsule gene locus for wci*P*α, and wciX_{CF} respectively with the 'interrogating nucleotide' (to detect the target SNP) located at the penultimate 3' position. The location of the longer and thermodynamically stable 5' 'anchor' sequence was predicated on the length of the non-complimentary 'bridge' sequence (that separates the two priming regions) and the location of the upstream SNP specific

Serotype	Genbank accession number:	References
6A	CR931638	Bentley et al.[44]
	JF911487–JF911497	Elberse et al.[45]
6B	CR931639	Bentley et al.[44]
	JF911498–JF911508	Elberse et al.[45]
	KT907353†; KU168827	Burton et al.[24]
6C	EF538714	Park et al.[18]
	HQ662201; HQ662202	Song et al.[20]
	JF911509; JF911510; JF911515	Elberse et al.[45]
6D	FJ899602	Jin et al.[46]
	HV580364*	Kapatai et al.[13]
	HQ662205; HQ662209; HQ662210; HQ662216; HQ662217	Song et al.[20]
6″E”	LT594599†	Kapatai et al.[13]
6F	KC832410	Oliver et al.[25]
6G	KC832411	Oliver et al.[25]
6H	KJ874439	Park et al.[26]
18A	CR931671	Bentley et al.[44]
18B	CR931672	Bentley et al.[44]
18C	CR931673	Bentley et al.[44]
18F	CR931674	Bentley et al.[44]
22A	CR931681	Bentley et al.[44]
22F	LT594600	Kapatai et al.[13]

Table 2. Serotype-specific capsular sequences used to design the Dual Priming Oligonucleotide primers (DPO); Locked Nucleic Acid (LNA) probes and standard assays. †Serotype 6B 'sub-class II' or 6E; *Sequenced by Park, I. and Nahm, M.H.

'foot' sequence (example for 6A/C in Fig. 2). A standard tagged Integrated DNA Technologies (IDT) PrimeTime® fluorescent probe (6A/C) and a PrimeTime LNA® real-time PCR probe (18C/F) and reverse primers were designed manually for both DPOs.

The final reaction-set included primer–probe sets which amplified target regions of serogroup 6 types with wci*P* (6A/B/C/D), wci*N*β (6C/D), wci*P*α (6A/C); serogroup 18 with wci*W* (18A/B/C), wci*X* (18B/C/F), wci*X* (18C/F), wcx*M* (18F) and serogroup 22 with wcw*V* (22A/F) and wcw*A* (22F) (Table 3).

Since each serotype was not targeted individually, an algorithm combining results from published primer–probe sets (6A/B/C/D, 6C/D, 18A/B/C, and 22A/F) and newly designed sets (6A/C, 18BCF, 18C/F, 18F and 22F) was applied to distinguish individual serogroup 6, 18 and 22 serotypes (Table 4 and further described in supplementary).

To confirm the presence of *S. pneumoniae*, pan-pneumococcal reference genes *LytA* (pneumococcal autolysin gene)[47] and PiaB (permease gene of the pia ABC transporter)[48] were also included in the reaction-set. Samples in which a serotype-specific target gene was detected, also needed to be positive for the two pneumococcal reference genes to be confirmed as *S. pneumoniae*.

Bacterial and pneumococcal reference isolates. Control strains for *S. pneumoniae* serotypes (1; 2; 3; 4; 5; 6A; 6B; 6C; 6D; 7A; 7B; 7F; 7C; 8; 9A; 9L; 9 N; 9 V; 10A; 10B; 10C; 11A; 11B; 11C; 11D; 11F; 12B; 12F; 13; 14; 15A; 15B; 15C; 15F; 16A; 16F; 17A; 17F; 18A; 18B; 18C; 18F; 19A; 19B; 19C; 19F; 20; 21; 22A; 22F; 23A; 23B; 23F; 24A; 24B; 24F; 25A; 25F; 27; 28A; 28F; 29; 31; 32A; 32F; 33A; 33B; 33C; 33D; 33F; 34; 35A; 35C; 35F; 35B; 36; 37; 38; 39; 40; 41A; 42; 43; 44; 45; 46; 47A; 47F; 48) and other nasopharyngeal species (*Acinetobacter baumannii*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Neisseria lactamica*, *Neisseria meningitidis*, *Staphylococcus aureus* and *Streptococcus pyogenes*) were obtained from the National Institute for Communicable Diseases, South Africa, Murdoch Children's Research Institute, Australia and Vaccines and Infectious Diseases Analytics Research Unit, Soweto, South Africa. DNA from these strains were used to optimize the PCR assay-sets.

Bacterial culture and DNA extraction. Control isolates were grown according to standard microtiter culture methods to quantify their relative density (colony forming units, CFU/mL) for use as reference standards and quantification calibrators. The density of viable cells (CFU/mL) was calculated using a serial dilution method (further detailed in Supplementary). Total DNA was extracted from 1 ml THB or BHI into 100 µL of elution buffer using the BioMérieux NucliSens easyMAG® automated benchtop nucleic acid extraction system (BioMérieux, Marcy l'Etoile, France) with standard reagents and protocols. Extracted DNA from reference strains were stored at – 20 °C until assayed. Where required, bacterial isolates of known CFU/mL were used as positive template controls, in addition to gBlocks™ synthetic external calibrators (Supplementary Tables S1 and S2).

Oligonucleotide	Sequence 5′–3′	Target	References
6A/B/C/D Forward	AAGTTTGCACTAGAGTATGGGAAGGT	wci*P*	Azzari et al.[11]
6A/B/C/D Reverse	CTTGTATCGAAGACAYGGACATAATGT		
6A/B/C/D Probe†	TGTTCTGCCCTGAGCAACTGG		
6A/C-DPO Forward	CATTGCTAGAGATGGTTCCTTCAGTTGATATTGATAAAGATTCGGGAG ACATGTCCAAACTGGC	wciP_{α}	This study
6A/C Reverse	CGATACAAGACCAGTTGC		
6A/C Probe‡	TTTGCACTAGAGTATGG		
6C/D Forward	TTGGGATGATTGGTCGTATTAG	wciN_{β}	Azzari et al.[11]
6C/D Reverse	CGAACTGAAGAACTAATTGAAGAG		
6C/D Probe†	CCACGCAATTCGCCATC		
18A/B/C Forward	CCTGTTGTTATTCACGCCTTACG	wci*W*	Azzari et al.[11]
18A/B/C Reverse	TTGCACTTCTCGAATAGCCTTACTC		
18A/B/C Probe†	AACCGTTGGCCCTTGTGGTGGA		
18B/C/F Forward	CAGGATTTCTAACTCTGATTGAA	wciX_{BCF}	This study
18B/C/F Reverse	AGCAAAATCTAACGTCCAGAG		
18B/C/F Probe†	CTTGTATGCTTATGGTCTTTTCGATTA		
18C/F-DPO Forward	CCAAATTGGAGTGTTTTACAAAGTATTAGCTCGATTTGCTGTACACGT CGACGCTTCAATTTCAGG	wciX_{CF}	This study
18C/F Reverse	TCTTTCAAATACAACTCTTAGATTTCCTTGTG		
18C/F LNA-Probe‡	TGagtTTATTGATAATttcC		
16F/18F/28AF Forward	TGGTTTCGGACTCTTTCGTGG	wcx*M*	This study
16F/18F/28AF Reverse	CTAAGATAGAAACTCCTTGTCCAATG		
16F/18F/28AF Probe†	GGTTGTACGTGGAATCGGATTTGGTC		
22AF Forward	TCTATTAAATAACCCATTGGAATTGAAACG	wcwV_{A}	Azzari et al.[11]
22AF Reverse	TCGCAATTGAAGACCACATAAACTG		
22AF Probe†	TCCGTAATTCGCTTATGGGCACATTCTCCA		
22F Forward	GAAGATTGTCCACCTTATATCC	wcwA_{F}	This study
22F Reverse	TCGGCACAATCAAAATATC		
22F Probe†	CGGTTATTTCACAAAAGACACGGTTGG		
LytA Forward	TCTTACGCAATCTAGCAGATGAAGC	LytA	Carvalho et al.[47]
LytA Reverse	GCACGAATAACCAACCAAACAAC		
LytA Probe†	CCGCAACTCATCAAGGATTTCTGTTACCA		
PiaB Forward	CATTGGTGGCTTAGTAAGTGCAA	PiaB	Brown et al.[48]
PiaB Reverse	TACTAACACAAGTTCCTGATAAGGCAAGT		
PiaB Probe†	TGTAAGCGGAAAAGCAGGCCTTACCC		

Table 3. Primer and probe assay-sets for serogroup 6, 18 and 22 detection and serotype discrimination. Nucleotides in lower case correspond to locked nucleic acid (LNA) modified bases. †5′-FAM- NFQ-3′ labelled dye (Thermofisher). ‡5′6-FAM- BHQ1-3′ labelled dye (Integrated DNA Technologies).

Clinical samples. Two cross-sectional surveys were undertaken in Agincourt (May–October 2009) and Soweto (May 2010–February 2011) to assess the prevalence of serotype-specific pneumococcal colonisation during the early introduction of PCV7 in South Africa[49,50]. Nasopharyngeal swabs (NPS) in STGG (Skim-milk-tryptone-glucose-glycerine transport media)[51] were collected from households (Agincourt) and children 0–12 years old (Soweto) and serotyped using the Quellung method, as previously described[49,50].

In this study, NPS in STGG from children aged 0 to 5-years-old were re-tested by Fluidigm to validate the assay-set for accurate serotyping in clinical samples compared with the Quellung method. Serotype data for 1973 samples were therefore available for comparison between Fluidigm PCR and Quellung method. Nucleic acids were extracted from 400 µL of STGG and eluted into 100 µL of elution buffer using the BioMérieux NucliSens easyMAG® according to standard manufacturer protocols. No-template-control (NTC) that was STGG alone was included in each batch of clinical samples (n = 23) that were extracted. Nucleic acids were stored at – 20 °C until assayed.

Fluidigm Real-time qPCR. The serogroup 6, serogroup 18 and serogroup 22 assay-sets designed in this study were included in a high-throughput nanofluidic real-time PCR serotyping panel on the Fluidigm platform that was optimised as described previously[33] and here was expanded to include other published assay-sets to increase the number of individual serotypes detected. The Fluidigm workflow is summarized in Fig. 3. Primers for all assay-sets were separated into three specific target amplification (STA) multiplex pools (Table S3) to prevent any cross-reactivity of primers.

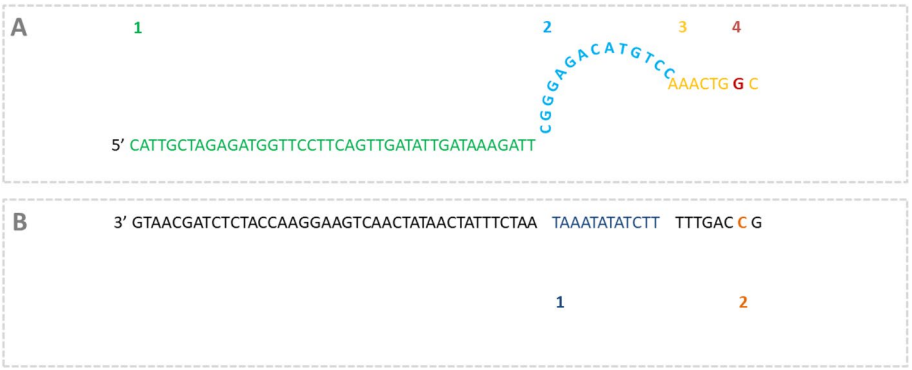

Figure 2. Schematic representation of the Dual Priming Oligonucleotide (DPO) forward primer (**A**) for the detection of wciPα and the target 6A/C sequence (**B**) as an example of the DPO designs used in this study. DPO primers combine the thermodynamic advantages of two priming regions, a short highly specific 'foot' sequence terminating at the 3' end, and a longer stable and sensitive 5' 'anchor' sequence with their action separated by a non-complimentary 'bridge' sequence that forms a bubble. The 'bridge' keeps the highly specific foot sequence close to the target sequence by connecting it to the anchor, increasing the frequency of target-primer hybrid formation to enable highly specific primer extension. Due to the dual priming nature of these oligonucleotides, the extension will not proceed if the 3' short foot sequence is mismatched. A1 shows the 'anchor' sequence which is the first priming region, A2 is the 'bridge' sequence which is non-complimentary to the intervening sequence (B1) which is not a priming region and the second priming region with the 'foot' sequence (A3) containing the interrogating nucleotide (A4) which targets the SNP on the wciPα target strand (B2).

Serotype	6A	6B	6C	6D	6F†	6G†	6H†	18A	18B	18C	18F	22A	22F
6A/B/C/D	+	+	+	+	+	+	+	–	–	–	–	–	–
6A/C	+	–	+	–	+	–	+	–	–	–	–	–	–
6C/D	–	–	+	+	–	–	–	–	–	–	–	–	–
18A/B/C	–	–	–	–	–	–	–	+	+	+	–	–	–
18BCF	–	–	–	–	–	–	–	–	+	+	+	–	–
18CF	–	–	–	–	–	–	–	–	–	+	+	–	–
16F/18F/28AF	–	–	–	–	–	–	–	–	–	–	+	–	–
22AF	–	–	–	–	–	–	–	–	–	–	–	+	+
22F	–	–	–	–	–	–	–	–	–	–	–	–	+

Table 4. Algorithm to discriminate serogroups 6, 18 and 22 to individual serotypes using the described reaction sets. †Theoretical detection pattern predicted in silico, no bacterial isolates or clinical isolates were available to confirm.

Specific target amplification/pre-amplification pools. The process of preparing each STA mix is detailed in Fig. 3 and further described in supplementary. For each pool, each included assay-set (n = 30–32 assay-sets per pool; Table S1) were combined to make up 200 µL of a multiplex STA pool. The final STA mix (5 µL) per sample contained 1.25 µL pooled assays at a final concentration of 45 nM for each primer, 1 µL Fluidigm Gene Expression (GE) Pre-Amp reagent, 1.50 µL ddH_2O and 1.25 µL of sample. Reactions were amplified in a T100 Thermal Cycler (Bio-Rad, Inc, CA, USA) and cycling conditions were: 95 °C for 2 min, 14 cycles of 95 °C for 15 s and 60 °C for 4 min, and a hold of 4 °C. STA products (5 µL) from the three PCR reactions were then combined and diluted with 10 µL H_2O to further dilute any remaining primers to less than 9 nM each in a final volume of 25 µL and tested in the Fluidigm.

The Fluidigm 96.96 GE Dynamic Array IFC™ was primed and loaded according to manufacturer specifications and placed in the Biomark™ HD for Fluidigm PCR, using the following thermal cycling conditions: 50 °C for 2 min, 70 °C for 30 min, 25 °C for 10 min, 50 °C for 2 min, 96.5 °C for 10 min followed by 40 cycles of 96 °C for 15 s, 60 °C for 60 s. Data were analysed with the supplied Real-Time PCR Analysis Software for the BioMark™ HD instrument (Fluidigm® Corporation, CA, USA) using manually defined thresholds and a Cycle of quantification (Cq) cut off value of 35.

Primer optimization. Secondary structures, self-annealing sites, and melting temperature (Tm) of each assay-set were predicted using the online tool OligoCalc[52]. Duplicate serial dilutions of total DNA from culture controls with CFU/mL of 10^0 to 10^6 were used to develop standard curves for all assay-sets to assess efficiency and reproducibility (R^2). Triplicate dilutions at 10^0 to 10^3 CFU/mL or copy number equivalents (for Synthetic gBlock external calibrators) were included to assess the limit of detection (LOD), that was defined as the low-

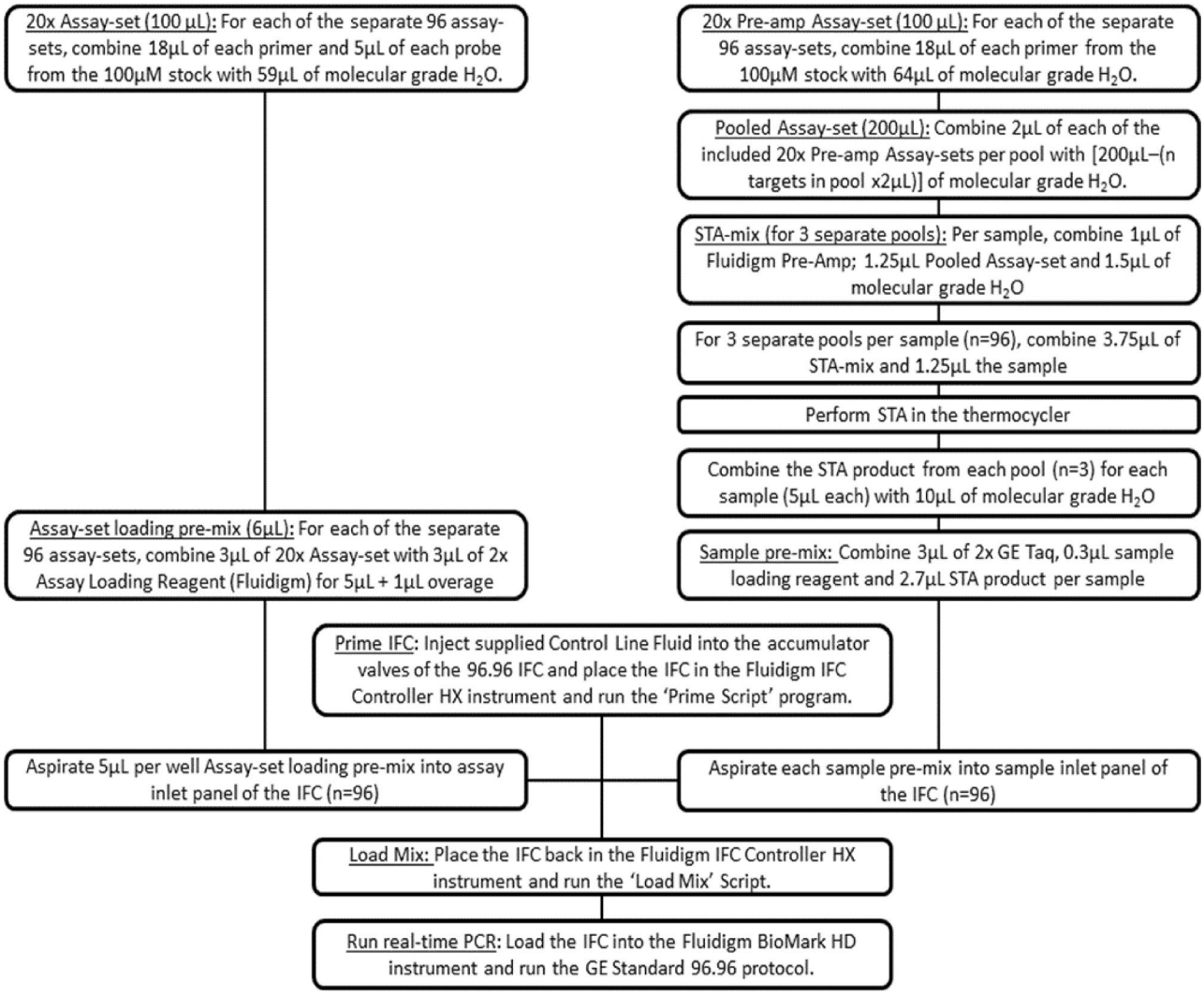

Figure 3. Flow diagram of the Specific Target Amplification (STA) within single 0.6 mL PCR tubes in the Bio-Rad T100 Thermal Cycler and the nano-fluidic high throughput real-time PCR within the 96.96 Gene Expression (GE) Dynamic Array Integrated Fluidic Circuit (IFC) carried out in the IFC Controller HX and the BioMark HD (Fluidigm).

est concentration that was detected in triplicate. Further, all assays were tested for cross-reactivity against 90 pneumococcal positive culture controls, including individual serotype 6,18, 22 serotypes and 8 other common nasopharyngeal bacterial colonisers.

Assay-set validation for use with clinical samples. To validate the serogroup 6,18 and 22 serotyping reaction-sets for use in clinical isolates, a blind analysis was conducted on 1973 clinical samples described above. Synthetic gBlock external calibrators (positive controls), and no-template controls (NTC) were included within each Fluidigm assay.

Statistical analysis. The slope (m) of the linear equation ($y = mx + c$) generated from the standard curves of tenfold serial dilutions of calibrators (bacterial culture controls or gBlock) were used to calculate the percent efficiency (E) for each assay-set ($E = [10^{(-1/m)-1}] \times 100$). Analyses were done in Stata, version 13.0, including applying relevant algorithms for serotype assignment. The McNemar's test was used to determine the sensitivity of the Fluidigm® assay-sets for serotyping serogroups 6, 18 and 22 to individual types compared to the gold standard culture-based Quellung. Cohen's kappa coefficient was used to concordance for each of the archived clinical samples serotyped using Fluidigm and the described algorithm compared with the Quellung results. Results were considered significant when p-values were ≤ 0.05.

Ethial approval. Ethical consent was obtained from the Medical Human Research Ethics Committee (HREC) of the University of Witwatersrand for the initial collection of the included samples (Soweto Cohort HREC: M090115; Agincourt Cohort HREC: M090114). Informed consent was obtained from all participants and their legal guardians as part of the original study in which the samples were obtained. All experimental

protocols were approved by Medical Human Research Ethics Committee (HREC) of the University of Witwatersrand (HREC: M170314). All methods were performed in accordance with the relevant local and international guidelines and regulations for Good Clinical Practice.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request.

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Author contributions

S.L.D., S.A.M., M.C.N and C.P.O. conceived and designed the study. S.L.D. was responsible for molecular assay design, data curation and analysis. C.P.O and S.L.D were responsible for gBlock design. S.L.D. and L.v.d.M. were responsible for lab analysis. Funding acquisition was undertaken by S.L.D., S.A.M., M.C.N and C.P.O. S.L.D drafted the manuscript and all authors reviewed and contributed to editing the manuscript.

Competing interests
The authors declare no competing interests.

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High-throughput nanofluidic real-time PCR to discriminate Pneumococcal Conjugate Vaccine (PCV)-associated serogroups 6, 18, and 22 to serotypes using modified oligonucleotides.

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Supplementary information:

Interpreting the Algorithm for serotyping serogroup 6, 18 and 22.

Samples/isolates detected with the ubiquitous serogroup 6 wci*P* (6A/B/C/D) assay-set and our newly designed 6A/C (wci*P*α) DPO assay-set, but not detected by the 6C/D (wci*N*β) assay-set were serotyped as 6A. Isolates that were detected with the same assay-sets (6A/B/C/D and 6A/C) but also detected with the 6C/D (wci*N*β) assay-set were serotyped as 6C. Isolates detected with 6A/B/C/D and 6C/D but not with the assay set for 6A/C were serotyped as 6D. Finally, isolates detected with 6A/B/C/D but not detected with the assay-sets for 6A/C or 6C/D were serotyped as 6B. Similarly, serogroup 18 was serotyped using the published primer-probe set targeting 18A/B/C and the designed sets targeting 18B/C/F, 16F/18F/28AF and the DPO targeting 18C/F. Isolates detected with the wci*W* targeted assay-set for 18A/B/C but not detected with other serogroup 18 assay-sets were designated as serotype 18A. Isolates detected with the 18A/B/C assay-set and the assay-set targeted at wci*X* (18B/C/F) but not detected with other assay-sets were serotyped as 18B. Isolates detected with the 18A/B/C assay-set, the 18B/C/F assay-set and the DPO assay-set targeted at wci*X* contained in 18C/F but not detected with the assay-sets for wcx*M* (16F/18F/28AF) were serotyped as 18C. Any isolates detected by all serogroup 18 assay-sets excluding 18A/B/C were serotyped as 18F. Isolates detected by 22A/F but not 22F were designated as 22A. Isolates that were detected by both serogroup 22 assay sets (22A/F and 22F) were designated 22F. The interpretation of the algorithm is summarised in the manuscript (Table 4).

Bacterial culture and DNA extraction

Control isolates were grown according to standard microtiter culture methods to quantify their relative density (colony forming units, CFU/ml) for use as reference standards and quantification calibrators. Specifically, *S. pneumoniae* serotypes, *A. baumannii*, *K. pneumoniae*, *M. catarrhalis*, *N. lactamica*, *N. meningitidis*, *S. aureus* and *S pyogenes* control strains were streaked on 5% blood agar plates; *H. influenzae* strains were streaked onto chocolate agar plates for single colonies (using a four-way streak) and incubated in 5% CO_2 at 37°C for 12 hours. Single colonies (n=4-6) were picked and inoculated into Todd Hewitt Broth (THB) supplemented with 5% yeast (*S. pneumoniae*); THB (*A. baumannii*, *K. pneumoniae*, *M. catarrhalis*, *N. lactamica*, *N. meningitidis*, *S. aureus* and *S pyogenes*) and brain heart infusion (BHI) for *H. influenzae* strains and grown in CO_2 at 37°C and 5% CO_2 to an OD_{260} of 0.1 (*S. pneumoniae*) or 1.0 (all other bacterial strains) to obtain an optimum concentration of 1 x 10^7 CFUs/ml.

The density of viable cells (CFU/ml) was calculated using a serial dilution method. A total of 200µl of THB or BHI was aspirated into a microtiter plate and a 1:10 serial dilution was performed with sterile Phosphate-buffered saline (PBS). The lowest 6 dilutions were plated as triplicate 20µl drops on the relevant 5% blood agar plates or 'chocolate' agar plates and individual colonies in each triplicate drop were counted following 20h of growth at 37°C and 5% CO_2.

The lowest concentration containing 5-50 colonies was used to estimate the density using the formula:

$$Density\ (CFU/ml) = CFU\frac{Drop\ 1,\ \ Drop\ 2,\ \ Drop\ 3}{3} * 50 * dilution\ factor$$

Total DNA was extracted from 1ml THB or BHI into 100µl of elution buffer using the BioMérieux NucliSens easyMAG® automated benchtop nucleic acid extraction system (BioMérieux, Marcy l′Etoile, France) with standard reagents and protocols. Extracted DNA from reference strains were stored at -20ºC until assayed. Where required, bacterial isolates were used as calibrated positive template controls, in addition to synthetic external calibrators (gBlocks™).

Synthetic external calibrators

Synthetic double-stranded template gene fragments (gBlocks) were designed based on the sequences for each serotype or bacterial target according to the pool in which the respective assay-sets were included. For each pool, three gBlocks were designed including nine to thirteen target regions. Target sequences were aligned in BioEdit, and the primer and probe sequences were aligned to the target sequence for each target assay-set. The intervening base pairs were removed within the target sequence, leaving the primer and probe binding regions of the target for a shortened sequence. Buffer sequences of random base pairs non-complimentary to any of the assay-sets or their targets are inserted on the 5' and 3' end (around 20-50bp) of the oligonucleotide and in between each target (around 25bp and primer or probe sequence (around 5-10bp). The combined buffer and target sequences were analysed in the online IDT gBlocks Gene Fragments Design Tool to check for any secondary strugtures. Target sequences within gBlocks are easily quantified as there is an equimolar ratio of all templates. The gBlocks properties and sequences used in this study are included in Table S1 and S2.

Specific Target Amplification (STA) Pools

Primer stocks were made up to 100µM concentration. Working stock assay-sets (primers) were made up to 18µM primer concentration. For each pool, 2µL of working stock for each included assay-set (n=30-32 assay-sets per pool; Table S3) were combined with molecular grade double-distilled H_2O (200µL– [No. assay-sets x 2µL]) to make up a 200µL of a multiplex STA pool. The final STA mix (5µL) per sample contained 1.25µL pooled assays at a final concentration of 45nM for each primer, 1µL Fluidigm® Gene Expression (GE) Pre-Amp reagent, 1.50µL ddH_2O and 1.25µL of sample.

Fluidigm BioMark HD loading, sample, and assay preparation

Control line fluid (Fluidigm) was added into the accumulator and the Fluidigm IFC was primed in the Fluidigm IFC Controller HX prior to loading. Once primed, a total of 5µL assay premix (2.5µL assay; 2.5µL loading reagent) for each target was aspirated into 96 assay inlets (final concentration: 9µM primers and 2.5µM probe). Pooled STA product (2.25µL) for each of the 96 samples including relevant controls were combined with 2.75µL sample premixes (2.50µL GE Taq, 0.25µL sample loading reagent) in separate tubes and aspirated (5µL) into sample inlets. The Fluidigm IFC Controller HX was used to load the samples and assays into the chip. The loaded chip was then placed in the BiomarkHD for Fluidigm PCR, using the following thermal cycling conditions: 50°C for 2 min, 70°C for 30 min, 25°C for 10 min, 50°C for 2 min, 96.5°C for 10 min followed by 40 cycles of 96°C for 15 sec, 60°C for 60 sec.

Table S1: External synthetic calibrator (gBlock) properties.

gBlock™ name	Assay-Set Targets †	Length (bp)	Amount (ng/µl) ‡	Gene equivalents (Copy number)
A1: Pool A gBlock, no. 1	1, 4, 5, 6A/B/C/D, 33D, LytA, 19F (Atypical), 7A/F, 8, 9A/L/N/V	1088	8,09	$1,36 \times 10^{10}$
A2: Pool A gBlock, no. 2	10C/F, 11A/D, 14, 20, Hib, 16F, 22F, 15B/C, 18A/B/C, 19B/F	1147	6,73	$1,07 \times 10^{10}$
A3: Pool A gBlock, no. 3	11F, 24A, 28A/F, 17A, 35F/47F, 41F, 12A/B/F/44/46, 23A/B/F, Eco, Sag, hIS1001, Nme	1225	8,53	$1,27 \times 10^{10}$
B1: Pool B gBlock, no. 1	3, 6A/C, 7B/C/40, 9A/V, 10B, 11A/B/C/E/F, 12B, 13, 15A/B/C/F, 16A, 34/37/17A, 18B/C/F, 19C	1474	3,82	$4,73 \times 10^{9}$
B2: Pool B gBlock, no. 2	19F, 22A/F, 23F, 25A/F, 33C, 35B, 46, 47A/F, Xis	1023	7,71	$1,38 \times 10^{10}$
B3: Pool B gBlock, no. 3	BexA, Nla, Mca, Spy, Sau, 16S, PiaB, PtxS1, plS1001, IS481, Sor	1122	5,13	$8,34 \times 10^{9}$
C1: Pool C gBlock, no. 1	15A/F, Ply, 11B/C, 16F/18F/28A/F, 17F, 19A, 21, 23B, 24B/F, 25A/F/38, 27, 29	1284	7,04	$1,00 \times 10^{10}$
C2: Pool C gBlock no. 2	31, 32A/F, 33A/F/37, 33B, 34, 35A/C/42, 45, 48, 2, 10A, 9L/N	1252	5,28	$7,70 \times 10^{9}$
C3: Pool C gBlock no 3	Aba, Kpn, 23A, BexB, IgA1, Pji, 36, 39, 41A, 43, 6C/D.	1160	4,11	$6,47 \times 10^{9}$

† Listed in order of position on gBlock; ‡ Average value of 3 successive Q-bit measurements

Table S2: External synthetic calibrator (gBlock) sequences

gBlock name	gBlock sequence
A1	TACCAGACGATGCTTATCAATGACCCCTATATGTGTGATATCGTGCGGTAATTGAAGCTATGAGTTGTGCTTGCCCTTGTATAGGGTCTCCAGAGTTGCTGGGGCCACATCACCGCAGGTATCCCGTCGCCGGTTGGGATGACATTTCTACGCACTATCTTTCCTATTGGATGGTTAGTTGGTGATA GTTGATAAAGCATCAGCGACGGGGGTTGCTCTGGGTGCCATGAACTATTACGGGAGTATCTTATGTCTTTAATGGTTTGTCTCAGCAACTCTATTTGGCTGTGGAATTCTAGTTTTAGGCTACTGGAATGCTGGCAGCGACGACCACCACACACACATGCAAGTTTGCACTAGAGTATGGGAAG GTGTTGTTCTGCCCTGAGCAACTGGTGCTACTTGTATCGAAGACATGGACATAATGTTAACCTATGAACTCGATCAGAGAACGTATAGTCTTGCGACATTTCATCCACAACTAGTTTTTTATCAAAAAGACCTTGGCGAGGTGAGGTAACGCATGTGGAACATACCAAGCATCTCCATCTAAGTTC TTACGCAATCTAGCAGATGAAGCTATTTGCCGAAAACGCTTGATACAGGGTATGCACGAATAACCAACCAAACAACTATTAATATGGTATATTTATGTCGCGTCCTTAGTTCTGGTTATTCGGGAATCCAGTTATGAAGGTGAGCTAACAGTGCGACACTTCGATTCCGGTCCTCATCCGCCCTAC CGACAGTGGTACTAGAGGCGATGGCATGTGGCAAACCATAGTTGGCTATCGGCATGGTGGTGTTGTGAAATGATTAAGGAGGGCAAAAACGGTCTCCTAGCCACTCCAGCTATTCCCACTCATCAGTTTCCCATATGTTTCATTCTGATGGCAGATGGGTTGGGACGAGTAACGTTCGCTTCTTC AATTATTGATTGCCCACGCAATGGGGATGCTGGAATGGGCAAAGGGTAGTAAGTTAATCATGCTAACGGCTCATCGACGAGAAAATCTTGGGGAACCGATGGAGAGCCCGAGTTAAGGTGGAGCTCAAGTAAATGGTCTACCGACACTACTCTCAGAAG
A2	TTCCACGGCTCAAGTAAATGGTCTTGATTAGTAAGGCAGGATGCATAAACCAGGAGGTACCGAGTTATGGATGTTCTTATTGGCTTGTGGAACAATATTCACAGTCTTGCCCTGTTCACAATACAGAGTGGGGTTGGGAGGGGTAGATGTTATCATACACATGGCGGCCCAGCTACATTTATGGCA GTTATTGCTAAAGGAAAAATACCAATAGTTGTTCCGAGATTAAAGAAGTATTGGTGAGCATGTGAATGATCATCAGATGGATTGATACAACCCATGGCGACTGAAATGTCACTAGGAGAAGATATGTCATTCGTTTGCCAATACTTGATGGTCTCAGAGTATTGCAGTAATTGATGGACTGTATT ATACCATGTGCCATCCACAGTTCGTAAAAGATACTGGCTGAGGAGCTATCTATTCAGGATAAGGTCTACTTTGTGGGAGTTCAGTCGAATATATCAGAATGGTTGAGTACTTTTGACTCCGTCTGCTGATGATTGCGCATGGGTACATCTGTTCGCCATAACTTCATCTTAGCACCACAAAACTTC TCATTCTTCGAGCCTAATTATTAATCACCGAGATAGAAGCGTAAGGGGGTACTAGAGTAGATCGACAATGGACATTTTCCAAATGAAGATGTCCCACCTTATATCCAACAGAACGGTAGATATTTTGATTGTGCCGAAAGGTTTAGGAATAGCTGCATTAGAGCAACTGGTATTTTTGATATTGGA GAACAAAATGCTAACTTCGTTGGAGGTGTATGGCATGGCATTCCTTTGGGATTCTTGTTACCGTTAATTAGTTTGCTACAGGTTTTAGTATTGAGAAAGCGGATGATTGTAGCGATCCAACTATACCTCTTATATTGCTTTATGAATACTATTACTGGAACGCCTGTTGTTATTCACGCCTTACGTA ACCGTTGGCCCTTGTGGTGGATGGAGTAAGGCTATTCGAGAAGTGCAACTTGAAGATGAAGCCTGTGGTCATGCGAGATACGACAGAAAGACCTGAAGGAGTAGCTGCTGGAACGTTGAAATTGGTTGGGACTGATGAGGAGACTAAATCACTCACAAAGAAAATTTAACGCTTGATGTGATG ACCCCATAGTGTTCGAGGTCATTCCGAGTTATCCAG
A3	GATGACCTTCGTGGCGTGCTAGTATGCATAGTGTTCGAGATGACCTGCTTCATGCCATGTGGTCCAGCTACTTTTATGGCTACTCCAATAGTTGTTCCGAGGCAAAAGAATGGGGAGCATGTGAATGATCATCTTGGAGTTGCTAATTATGGGAAGTCTGCGGGTATTTTACGATATGCTGTGCAG AGTGTGCACGTGTAAGAGATGATGAGGCATGCATATTAGTCAATGCAACTACAGGTATTTTTGATATCGGAGTAGAAAATAGTAGGTTGATTGGCGGTGCTCAGGCGTTCACAAACGTAGTAAACTAGTGAGTACGGGGTCTAGGCATCTGATTATGTCATTCGATTGCTTGGAACCTACGGTAA CCCACGCCCATAATGAGCTTTTCAAACAGGAATTTTAGGATTTGCATATTGTTGGATATAGGTGGTCGTATATACTTGATGAATAAATCGGATTATTCGGACGACCAGTTGAACAGACCTATCTGTATGTTGATAATTTGTATGTCTCGCTCTAGGAATAATGCTCCTAGTTTTTGGGAGGAAGTG CTTTTACTTCTGTGCTAACAGTGGAGATATAGTTATGAATCATGAGAAAGCGGTTGCATTGATACGAAGAGAAGCATCGAAGATTATTCGCTTGCCTCTTCATGGATTTGTAAGCGGACGTGCGATTGTTCTGGAAAGCTTATTTCGGCTATTAAGGGTCTCCAGAGGTATTGTAGGTGGACTTTC CGATGCAAGAAATGTCGGTATAGATAAAGCGGAGATTACCTCATTTTTGTTGACAGTGTCATGAACGGTTGGTTATGTCCAAAGCGGCGATTTGGACGGCAGAGAAGGTACTGGAAAAAGAACTTCTGGCCTGGCAGGAGACTGCATCAGCTATCCTGGAACTCTAGTGGCTGGTGCATTGTAT TTTCACCAGCTGTATTAGGTACATGCTGATCAAGTGACAACTCCAGGGCCCTCGATGCTGGGAGCCGGTGGCGACAGCGAGACAGAATCCCGTGCAGATAGGCTTTTAGCTTGAGCGCGAAGTGAGCCAAGGCGGCCACATGGTTACCCATGGCGATGATGCACACTTAGGTGATTTACCTGCA TTCATGATGGCACAGCAACAAATCCTGTTTTCTCTATTATGATCCACAGGGTGGCACGATTATGCTAGTCATCACCGAATACGGTGTGGATACGTTAGCGTATCGGCGCCTGCA
B1	TTAACTTTAAGCTTGATGTGATGACCTTTTGCCATAGTGTTCGAGGTCATTGGTCAGCAGAAAGTATGCATTGGTTATTGGATGTGGTTTATCGTGAAGACCATCATCAGACCCTGGATAAACGAGCTGCACCTTAATCTTATCCGGTGCATTGCTAGAGATGGTTCCTTCAGTTGATATTGATAAA GATTATTTATATATAGAAAAACTGGCAGTTTGCACTAGAGTATGGGAAGGTGTTGTTGAGCAACTGGTCTTGTATCGAAGACATGGACATAATGTAACAACTAGTCATCATCCAGATATAGTCATTCCCAATCAGGGGTTGGTGGGTCAGTAATCGATAATGAGGGAAAAATTCATCATGGGATT TACCTTCTTTTAAGAGCGTTGTACTTGCAGAGGTTGAAGGTATCCTATATACTGCTTTAGGTATGTAGCGGTTGTCAATGTGTTTCTTTCAGATATTGGCAGATTCGGGAATAGGACCTGCAATAAAATATGAGATTGGTAAGGAATATTCTGGTCAACGGATTCCAATGCACTCGGTAACTTATG AATGGAATTCGTTTAAGTGAAAAGACATGTTGACATTCAGTAGTATGGAGATACCGCATTTCTTATCGCACTATATTTTGAATCAGTCTGACCGTTTGATGATTAACATGTTTGATGGTAAGGAGAAATTAGCTATATATAGTCTAGCTGGTTGCTGATCAAAAGGTCTATGTTTCACTTTGATGAT TTGGAAAGATTTTTATCTAATTGCTAAAAATCTTACTTTGAACCTAGGATATGCTGAACGATATGGATCGGATTTAGTAGTAACCCCATTGAGAGTAGTAAGAGATCATATTCAAGGTGGAAATGCATCCTCAATCAAGAATGCCATTATAGGGGAGAGATCTTGAATCAGGTAGATTGATTTCT GCTATGTCTCCGGCTTTTGTCTTCTCTGTATTAGGAAATATCATTAGGACTCAGTATTTGATTCCTAGAGGATGTTAGAACTATGTGGGATGCTAGCAGGAACTTTTCTAGGGGCTATACGCCATAATGGATTCATCCCGTGGGACGTTTCGGATTTGGACAGGGAGACAGATCGAGATGATTATC GTGGCTTCTGGATACTATGTACGAACAGATGGACTTGACCGACTATACTCCATTTGAGACGTTTATTCGGGCGAGTTAGTGAGTTGTTGTACGCCTTATGTACATGGCATTTGCAGGATTTCTAACTCTGATTGAATACTTGTATGCTTATGGTCTTTTCGATTAACAACTCTGGACGTTAGATTTTG CTGTCAGCGACGTATTTACTATGAGTAAATGGTTTTCAGATTACTTGATAGCTCTGAGTTCATTATGTGGGGGCAGGAACAAATCTTGACCACTCTCATAAGGAACGTAATGAGACATGGACTTACTATAAGACTTGACAGTAGAAGGAAAAGTCAAATCTGTCTCTTAG
B2	ATCTTCCTACAGGAAGGACGAAGCCAATATACAACCTTGTACGTCTTAGTAATGGATATACAGGTTCGGGAAATTGCAACAAGAAGTGGCAGATGATTTATCATTGCTTATGGCGCAGAGATGACGTATCTGGGGTGGATTGTATAGTCTATTAAATAACCCATTGGAATTGAAACGGCTTGGAG AATGTGCCCATAAGCGAATTACGGAACAGTTTATGTGGTCTTCAATTGCGAGGAGATTTGATTGAGGCAGACATTGCTATTTGCGATCCTGTTCATTCTTTCTCCGGCATCAAACGTTAAGGTTTACGAAACAACGGAGGCTCTATGTGAGATGTTTTATCACCGCTTATTTATGATACCAACTAG AATCAGCAGGACACCCGCTGGACTTACTGCAATACTGCGAGTAAATTATCAAAAGATATTCCATTTTTGAGGTGCGCTATTCCCTGTCACAGTCAGAGACAGTTTCAGCAAATCTTAGATGGAAGACAAGTTTGTGGATAGGACACGGTCCAACGTTTATAAGAGGTGTAGGCTATGCTCAGGGA GGATTATTTCTATTTCTAATCAAGGCATGGAGGTGGAGCATACAAGACAATTTAAACAATATTAGTAAAGCGCAGGTCAAGCAAATATTTTATATCGAGTTGTGCAGTCTTTACAAAGGTGCCTGGTTTGGCAAGTCGAAGTTTTTATATCTCTATTGGTTTGTAATCATTCTTTCTTCAATTCCTT TCTGAGCTTCGTCCAGTTCCTGGGATATTAGTAGCGATGCTGTGAATTATAGTAAAGAGGAATTGGTAGAGAGTTTGTGGATCCACTTGATGGAATGCCTGCTGCCCCGACGGATGATGGTTCAACTTTCTCAGTTTAATAGTTTAGTAAAGCAGGTGACTGGTAGGTAACACAGCAGTTGCTTGC GGTGCACTTGTAGGAGCAGCATTAGGAGCCACTGGTCTCTTAGGAACTGTAACTTTGACAAATATGGAGCAAATGATATTTTACCTGGTTTAAC
B3	ACTGTGAAGAAATACTCTTCGTATGATGTTGATCCAGACTATGTGACTCGACAGAATTTTCTGAATTAGGTGATTATCTTTATGAACAGGGATGAAAGCTCGGCTTGCATTTCTCTTTCTGTTGAATTTGATTGTCGCCAACAAACTATTGACAACTTCTGTTATTTTGCCCGAGAACCATTGTATC GTATTGGAGCGGACTAAAGCAAATATAGAACGTGATAAGAACCGCCAAGCCTCATGAGGCGCTGACGGCGGTGAGTGCCGCTTTACAACCACTGCTTTTGCAGCTGTTAGCCAGCCTGTTGTCTTGGCAGGCGATACAGTGGTCAGTGATCGCCGCACTCGCTACTATTTCTTACCTCAAATCC GCAACTCATCAAGGATTTCTGTTACCAACTATTACTGGTTTCCAAGACATTGTGACCGGGCAATACGCAGCTACTAGTAGCTCAGCAAATGCATCACAAACAGATAATGGCGTAAATAGGTGGTTCTGAAGATCCAACAGTATATAGTGCTGAGAGGATGGGACACGGTCCCTCCTACGGGAGG CAGCAGTTGTGCCAGCAGCCGCGGTAATACGGCAAACAGTAGATACCCTGGTAGTCCCATCAAGCATAAGCCATATAAGTTTTGCAATCATTGGTGGCTTAGTAAGTGCAAAGCTGTAAGCGGAAAAGCACTTGCCTTATCAGGAACTTGTGTTAGTATGTAAGGTACCTCATAACTGCCGCCAGC TCGTACTTCGACAATACGTCGACACTTATGGCGATAAATGCCGGCCGTATCGGATCTTTACCTTGCGATGCGTAACTGCCGTTTCGAACGCGTGGAATGGCGAAGACCCAGGGCGCACGCTGTCCGTTCTATTTGAAGCCAACGGCCTGCAATCATCCGATGTACGTGGCCAAGGCCGAACGCTT CATAGTTACGCTCACACCTACCAGAACTCAACAGTCGGCCTTGCGTGAGTGGGATGCTCAAGAGCCTGCTAAACCAGCAGATACGAAAGAAGCATCAAAGGCTGCTGTTGCTAGAAAGATCGCTTGGAACCTTTTAAGCACGCCGTAAACGACAATCAATACCATATTCCTTATTTAGAAGCCA GTATTGAGGCTC
C1	GATATGATAGTTGATGGAGACAGGTGACATATGACTACACTGTATCAAGTTGTTCATATATTCGTTATTTAGTGAATTGCTATACTCCGATGTTGGCAGCAGCAGTAATGTAGATTCTTATTCTGCAGGGAGAGCTCACATTAGTTTAATGCTCAGCGATAGCTTTCTCCAAGTGGAAGACCCCAG CAATTCAAGTGTTCGCGGAGCGGTAAACGATTTGTTGGCTAAGTGGCATCAAGAAATGGCCGCTATCAAATTTGGCGTATTGCTTATCACTTCCGTGGCAAGATTCTGGTGCTAAGATGGGGATCAACTATGCTCATAATCAGCATGCCAGATGTACCTTTGTCGTATTGGTTTCGGACTCTTTCG TGGATGGTTGTACGTGGAATCGGATTTGGTCCGCATTGGACAAGGAGTTTCTATCTTAGCAAAGCGTAAATTATTTGTTGGCAACTAGTAAAGATTTCATGTCCTATAAGGGAGAAGTTTGTACATGGTCTGGATTTATGATTGCTTCTCATAAACAGGGACGCCTAGTTAGTATCATCACCAGAT ATCTTTCGACGACGTATCAGCTTCACCACCCAAAACGGTTGACGCATTATACTTATGAAGAGGTTAAGGCTCTCAATGACAATTAGCAATAGATAATGGAGTTGATATGCCATTTGAAGGACCAGTTGTTGAAATGGCATTGCTTCGTAAAGTTGGTATTCCTGATAGTGGCTTTTTTATTGTAAT TGGTATCGTCTCTTTAGTGTTGAAGAAATTGCTCCAGAAACATATAGAGCTATTTATCTTTCGTGGTTTTTGTTTAGTGGTTGAGGCTAGTCTTTTGGTTTATATTAATTCCATTTCTGAAAGTAATTAGTAAGATTAACGGAAGATTGGTTGCTGACAATTTTGGAGCTGTTGGATGTTGGAGCTATT TTAAAAGTAGATGGAAGCAAGTCTTACGTAGAACCTCTCTGGATGAATTGCCACAGATTTGGAATATTTTGGTCGGTCACATGTCGCTTGTAGGACCAATAATAGGTAGAGGAAGTGATTCATTAGCGATTTAGCGACTGATATCCTATGTGGAAGGCGTTTGAAGGTGACTATCACGCGAGATC GATTTTGAGAGACTATTCGAGCAATTGGACGACTTCGAGTTGTGCCGTTTTTACAAACGCAGAGAAAAGACTAGGATTCAGAAAAATTTTAGAGGTGGGTACGCCTTGGAAGATTCGCATGAGAAATGACGTCCTCATCAGAGAGGGATAGCAAGTTATTGTCGGCG TGATTCAACTGTTCTAATTACCGCTCCAAGAGCATTTACAAACATAGGTCGGTCATTTTATTAGCAGAAGTTTTAAGTCACGGACATCCTTGCGGTTTTACGTGGGGGGACCCTTCTGGAGGATTGTGTACTTCCTGTTCTAGGCTTGGTGGAGCAGT GGTTGGGTTTGAACAAGAGAGACGCTATTTTCCTCTGGGAAGTGGTTTTGCTACATCTGGAACTGGTTCAGCAACTATACGCTGGCATGGCAGAAAAGTCCTAAGGTATTTCAGACGGTCTTAGAACCTGTCCAATGAAGAGCAAGACTTGACAGTCCTGTTAGTGCACCTGTATTTAACGATAA ATGGCGTGAACAACGAATAGGAGATGAAGGAGTTTTGAATGCTGTTCTGGAAGGACATGTCTTGATTGTCCGGTGGAGTAGGTCAAGATGGGAACTATTCAAGTACATTGGCGCTCCGTTACTCAGTATATTGGGAGGAACAGACACCCTAGCTATGGGATGAACCTGTGTTTCAAGCTTCCCCT TTAGACTTTCAAAATACCCAGGACACCCGTTCAACGATACGTGATACTTTGATTTCATTTTTTGTTATGGGTGGGGAGATGAGATATTGATCTAGCTACTTGACTAAAATATTTGAACTGCTAGGGAGCGAGGTCACTAAAAGTCATACAGCGAAATCGACTCGTCTACCAAGATAGGGAGAGCA GCATTAGGGGCAGGTTTTGCTTCATATGGGAGCAACGCTGCTTATGTGATTACTCTCCCCTGTAGCTAATGATAACTTTTGGCCGATTTTTGAGCAGTTGCTCCTCTATTTGTACCAACGGGGCGTGCGTTATGGACTGGCTGATGGTTCTCTAGGTCAACGTATTGGAACTCGTAGAAATTGGGA AAATAAAGGCTATTATTGGGTCAGGATTTATGCACAGTTTGGTGGTTCTATATCCTCTCCTATCAACTATTACTCATTATACTACCTATCATTACAACTCCCTATGTGACACGGGTCTTTTCTTTGAATGATCTAGGGACTTATGGTTATTCTGATGTTCATGGCTATTATGAAGCGTGGAATTTTCT ATACTGCAATAGGGCAATTCTTAGCCGGATTCTCTCACCTGTAGAATATGGTATTACTCTCCCCTGTAGCTAATGGTATCATTATAGTGGGGGTGTTATACGGCGAAACTATTTATGTTTGCCGC GAGCTCATGTCTAAGTGAAGTGGTCGATGGTGGGTCCTGTTCCGCTATTCCATCAAGATTTAGCTCGTCGTATTGGACTTTGGCAATGCAGATATCGGTACCCAGAAAATTACTCCTCAGCAAGAGGCACAGTTTGCTCCATTACAACCCAGGCCGAATATGACGAATTAACTACCGTCACCCGCC ACAGATTCATGAGCAGATCACCCGTCTGTTCCACGCGTTAGTGCGTTAAGTACTCCCCTCCATTACCCATTTGGTATGGGAGTAGGGAGTGTGGGAGAGGTTCACAAACAGCACTTTCTTCAGAGGAGGAATTGGAATGATAATTGCAACACTCAGTTTCGTTTTATTACCAAGCGCATTCTTCTT CGTCCATAACCTTCTCTAGCCTTGTTGTTACTGGCAAACTGATCATAATGCGAAATTTGCTCGCCATACTGCACAATTGGTTTAACCGCAGGCAAAGCATTTAATCTTGGGGTTACCATCACATCCTACGATTACATACTCACATCAAC GAGGCGGTGTAGCAATGGTTATTTCGTTCTTTGCTAATCAGGCAGACTGGCCTTGTCTATTCAGCCCTTCTGGTCTCATTGTAGCGGGCATTCTTGAGTTCCCAATTTACAATATAATCGCGAAGGAGAACATAACTATGGAGGACAAAAAAAATGAACTAACTCAAATAGTAACGTAAGTCAGGC GTATTCTTCACAAGGGAAATACCGCAAATAAACAAGAAATTACAGTATGTATCTCTTAAGGTTGAAAGCTAGACTATTCGGTCGTGGGACATTAAACCAAAGAGCTACCGAAGTTGAACTGCGAATATTTAAAG TTGAAAGAGGCTACATCAAATAGTTGGCGAGCTCGGTAGGGGTGAGTACTATTGGAGTGCTTTGGAAGTTACGGTGTGATTCAGTAGTAATTAGTATGGGACAGTTGGGATGATTGGTCGTATTAGTAGATGGCGAATTGCGTGGATCGAACTGAAGAACTAATTGAAGAGGTACATTACGCCA GACCGCCATGGCATACAGTGGTTACTTACGAAAGTATAGGCTTTTTGGT
C2	
C3	

Table S3: Assay-set pools for specific target amplification/Pre-Amplification (Pre-Amp)

Pool A (n=32)	**Pool B (n=32)**	**Pool C (n=34)**
1	3	2
4	6A/C	6C/D
5	7B/C, 40	9L/N
6A/B/C/D(F/G/H)	9A/V	10A
7A/F	10B	11B/C
8	11A/B/C/D/F/(E)	15A/F
9A/L/N/V	12B	16F, 18F, 28A/F
10C/F	13	17F
11A/D	15A/B/C/F	19A
11F	16A	21
12A/B/F, 44, 46	34, 37, 17A	23A
14	18B/C/F	23B
16F	19F	24B/F
15B/C	22A/F	25A/F, 38
18A/B/C	23F	27
19B/F	25A/F	29
20	33C	31
22F	35B	32A/F
23A/B/F	46	33A/F, 37
24A	47A/F	33B
28A/F	Pia*B*	34
33D	Bex*A*	35A/C, 42
17A	IS481	36
35F, 47F	Ptx*S1*	39
41F	pIS1001	41A
Eco	*Mca*	43
Lyt*A*	*Nla*	45
Sag	*Sau*	48
Hin-b	*Spy*	IgA1
hIS1001	Xisco	Ply
Nme	16S	Bex*B*
-	*Sor*	*Pji*
-	-	*Aba*
-	-	*Kpn*

Chapter 3: Optimization of a high-throughput nanofluidic real-time PCR to detect and quantify 15 bacterial species and 92 *Streptococcus pneumoniae* serotypes. – Published Manuscript

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Optimization of a high-throughput nanofluidic real-time PCR to detect and quantify of 15 bacterial species and 92 Streptococcus pneumoniae serotypes

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Sensitive tools for detecting concurrent colonizing pneumococcal serotypes are needed for detailed evaluation of the direct and indirect impact of routine pneumococcal conjugate vaccine (PCV) immunization. A high-throughput quantitative nanofluidic real-time PCR (Standard BioTools 'Fluidigm') reaction-set was developed to detect and quantify 92 pneumococcal serotypes in archived clinical samples. Nasopharyngeal swabs collected in 2009–2011 from South African children ≤ 5 years-old, previously serotyped with standard culture-based methods were used for comparison. The reaction-set within the 'Fluidigm' effectively amplified all targets with high efficiency (90–110%), reproducibility ($R^2 \geq 0.98$), and at low limit-of-detection (< 10^2 CFU/ml). A blind analysis of 1 973 nasopharyngeal swab samples showed diagnostic sensitivity > 80% and specificity > 95% compared with the referent standard, culture based Quellung method. The qPCR method was able to serotype pneumococcal types with good discrimination compared with Quellung (ROC-AUC: > 0.73). The high-throughput nanofluidic real-time PCR method simultaneously detects 57 individual serotypes, and 35 serotypes within 16 serogroups in 96 samples (including controls), within a single qPCR run. This method can be used to evaluate the impact of current PCV formulations on vaccine-serotype and non-vaccine-serotype colonization, including detection of multiple concurrently colonizing serotypes. Our qPCR method can allow for monitoring of serotype-specific bacterial load, as well as emergence or ongoing transmission of minor or co-colonizing serotypes that may have invasive disease potential.

The capsule of *Streptococcus pneumoniae* is composed of repeating polysaccharides genetically encoded by the Capsular Polysaccharide Synthesis (CPS) locus that confers serotype heterogeneity[1,2]. There are 100 biochemically and serologically distinct *S. pneumoniae* serotypes[3]. Capsular diversity is an important virulence factor in *S. pneumoniae*[4] and serotypes have different potential to cause disease in humans[5]. Pneumococcal Conjugate Vaccines (PCV) prevent acquisition of pneumococcal vaccine serotype (VT) carriage, which is a precursor to disease[6–10]. The first licensed PCV (PCV7) targeted seven serotypes, namely: 4, 6B, 9V, 14, 18C, 19F, and 23F. Current PCVs in use in our setting include an additional three (PCV10: 1, 5 and 7F) and six (PCV13: 1, 3, 5, 6A, 7F and 19A) serotypes. Next generation 15- and 20-valent PCVs are under clinical trials and include an additional two (22F and 33F)[11] and seven (8, 10A, 11A, 12F, 15B, 22F and 33F)[12] serotypes, respectively, compared with PCV13.

Surveillance of pneumococci colonization of the upper airway is useful to assess the impact of PCV immunization at a population level[13]. Pneumococcal colonization surveys are best performed using comprehensive, sensitive, and accurate serotyping methods able to detect multiple serotype co-carriage, including VT and non-vaccine serotypes (NVT). Consistent surveillance of circulating serotypes is needed to inform vaccination strategies and

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utilization of higher or alternate valency PCVs, including tailored to different geographic regions; and for the detection of emerging or replacement serotypes[13].

Culture-based Quellung methods remain the gold standard for *S. pneumoniae* serotyping; however, these methods are time-consuming, with low sensitivity, and are costly[14,15]. Compared with culture-based methods, Polymerase Chain Reaction (PCR)-based detection and serotyping do not rely on organism viability that can be negatively affected by antibiotic use, sample transport and storage conditions, are less time consuming, provide faster diagnosis and higher sensitivity[15]. Conventional and real-time PCR have been used, including single tube nested and multiplex PCRs with gel or capillary electrophoresis and combined with dot-blot detection, culture enrichment and sequencing-based analysis[16–28]. Real-time PCR methods have been developed with an increasing number of serotypes detected[21,29–31], including in high-throughput systems[15,32,33]. Further, quantitative PCR (qPCR) methods that are validated to determine bacterial load[29,31,32,34] are important for assessing the impact of vaccines in public health programs. Increased bacterial load in the nasopharynx may predict serotype-specific invasive disease potential[31].

Previously we developed a nanofluidic qPCR reaction-set that was able to simultaneously detect and quantify 55 pneumococcal serotypes[32], as well as a detection and typing method to distinguish serogroups 6A/B/C/D, 18A/B/C, and 22A/F into single serotypes[35] using the same platform. Here we expand on our method to detect and quantify an additional 38 serotypes—allowing for comprehensive pneumococcal serotyping (92 serotypes) and detection of 15 other bacterial species to assess colonization and bacterial load in respiratory samples.

Methods

Reaction-set development. To develop a comprehensive reaction-set for serotyping *S. pneumoniae* and detecting other species that may be present in the nasopharynx, within an in-house, nanofluidic, high-throughput, real-time PCR platform, a review of published assay-set (primers and probes) sequences was conducted. Representative GenBank FASTA sequences for serotype 19B (CR931676) and 19F (CR931678) capsular genes, the pneumococcal Xisco putative protein gene (NC_003028) and the *Haemophilus influenzae* Bex encapsulation gene (X54987) were aligned in BioEdit using ClustalW Multiple alignment[36,37]. Standard primers and dye-labelled MGB probes were manually designed to detect the 19F capsular polysaccharide gene *wzh* to differentiate 19F from 19B[1,2], to detect the pneumococcal Xisco gene and to detect the *H. influenzae* Bex*A* gene. All curated oligonucleotide sequences for each assay-set listed in Supplementary Table S1 were analyzed to ensure specificity *in-silico* using the Basic Local Alignment Search Tool (NCBI BLAST; http://www.ncbi.nlm.nih.gov/BLAST/) prior to inclusion in the reaction-set. The melting temperature (Tm) of individual oligonucleotides within each assay-set was determined using the online tools Oligocalc[38] and the Integrated DNA Technologies (IDT) OligoAnalyzer (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) to assess their suitability for inclusion within a uniform thermal profile and multiplexing. The IDT OligoAnalyzer tool was used to assess secondary structures and self-binding sites. The analytical efficacy (efficiency, sensitivity, and specificity) and diagnostic performance (sensitivity, specificity, and concordance) of the concatenated reaction-set within the nanofluidic high-throughput real-time qPCR ('Fluidigm', Standard BioTools) was established through calibrators and clinical samples respectively.

Bacterial strains. Control strains were grown according to standard microtiter culture methods to quantify their relative density as colony-forming units (CFU/ml) for use as quantification calibrators and to assess analytical efficacy. Total DNA was extracted and stored at − 30 °C until assayed, as described previously[35]. DNA from 89 isolates that were representative of the included pneumococcal serotypes and 15 other bacteria were used to optimize the PCR assay-sets and assess the analytical specificity of the assay-sets to their respective targets (pneumococcal serotypes: 1; 2; 3; 4; 5; 6A; 6B; 6C; 6D; 7A; 7B; 7F; 7C; 8; 9A; 9L; 9N; 9V; 10A; 10B; 10C; 11A; 11B; 11C; 11D; 11F; 12B; 12F; 13; 14; 15A; 15B; 15C; 15F; 16A; 16F; 17A; 17F; 18A; 18B; 18C; 18F; 19A; 19B; 19C; 19F; 20; 21; 22A; 22F; 23A; 23B; 23F; 24A; 24B; 24F; 25A; 25F; 27; 28A; 28F; 29; 31; 32A; 32F; 33A; 33B; 33C; 33D; 33F; 34; 35A; 35C; 35F; 35B; 36; 37; 38; 39; 40; 41A; 42; 43; 44; 45; 46; 47A; 47F; 48; *Acinetobacter baumannii*, *Bordetella holmesii, B. parapertussis, B. pertussis, Escherichia coli, Haemophilus influenzae-b*, *non-typeable Haemophilus influenzae*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Neisseria lactamica*, *Neisseria meningitidis*, *Staphylococcus aureus*, *Streptococcus algalactiae* and *Streptococcus pyogenes*). The control isolates were obtained from the National Institute for Communicable Diseases (NICD), South Africa, the Murdoch Children's Research Institute (MCRI), Australia and the South African Medical Research Council Vaccines and Infectious Diseases Analytics Research Unit (Wits-VIDA), Soweto, South Africa.

Synthetic external calibrators. We utilized synthetic double-stranded DNA (dsDNA) template gene fragments (gBlocks) as external calibrators (Supplementary Tables S2 and S3) for analytical efficacy and intra- and inter-assay variation. The gBlocks contained sequences for each pneumococcal serotype or other bacterial target according to the pool (Supplementary Table S4) in which the respective assay-sets were included. These were applied to assess the analytical sensitivity (Limit of Detection) and repeatability (intra- and inter-assay variation and Levy-Jennings plots) of the reaction-set. The design, properties and sequences of each gBlock have been detailed previously[35]. Briefly, for each of the three pools (A, B and C, Supplementary Table S4) three gBlocks were designed (nine in total) that each included nine to thirteen target regions. Target sequences within gBlocks are easily quantified as there is an equimolar ratio of all templates. The amount of gBlock synthetic DNA was measured using the Thermo Fisher Invitrogen Qubit 2.0 Fluorometer Qubit 2.0 with the Invitrogen Qubit dsDNA BR Assay Kit. The copy number, or the number of gene equivalents was calculated using the formula:

$$Number\ of\ Copies = \frac{amount(ng) * 6.022 \times 10^{23}\ molecules\ per\ mol}{length(bp) * 660 * 1 \times 10^{9}}$$

The amount was the average of the three Qubit measurements which was multiplied by the Avogadro's number (6.022×10^{23}). The length in base pairs was multiplied by the average weight of a dsDNA nucleotide (660 Daltons or g/mole) and converted back to ng (1×10^{9}).

Clinical samples (nasopharyngeal swabs) for validation. In May to October 2009 and May 2010 to February 2011, two carriage surveys were conducted in Agincourt, Mpumalanga, and Soweto, Gauteng respectively, to assess serotype-specific pneumococcal colonization during the early phase of introduction of PCV7 in South Africa which started in May 2009[39,40]. Carriage isolates from these cross-sectional surveys were previously cultured using standard microbiological methods and serotyped using the Quellung method. Here, we included all available archived nasopharyngeal swabs (NPS) from children aged 0 to 5-years-old to assess our method for pneumococcal serotyping in the age-groups where pneumococcal colonization was the highest (Fig. 1). Retrospectively, all archived samples, regardless of culture results were re-tested with 'Fluidigm', unless samples were depleted, or no Quellung results were available, to validate the reaction-set for accurate serotyping in clinical samples as outlined in Fig. 1. Out of the 1973 archived clinical samples available for the current analysis, 69.2% were positive for pneumococcus by culture.

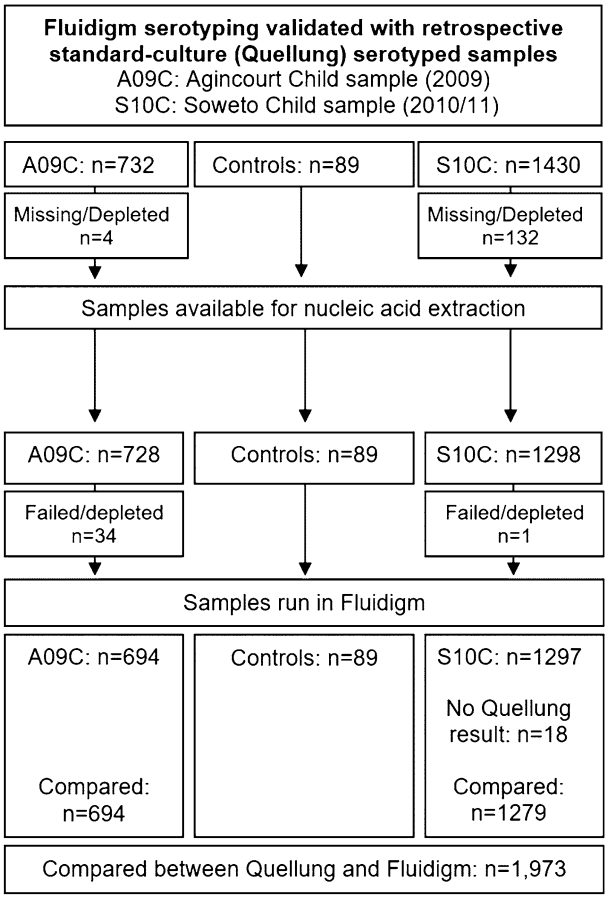

Figure 1. Validation flowchart describing the included archived clinical samples that were previously serotyped using the culture based Quellung method and that were compared with the Standard BioTools 'Fluidigm' real-time quantitative and serotyping PCR. A09C indicates samples collected from Agincourt (2009) children[39]. S10C indicates samples from Soweto (2010/11) children[40]. Samples were archived nasopharyngeal swabs in STGG as described in "Clinical samples (nasopharyngeal swabs) for validation" section, that were previously tested using the culture based Quellung method[39,40]. Archived clinical samples were used to assess the diagnostic performance of the 'Fluidigm' qPCR. Where samples were indicated as missing/depleted, these samples were not available for nucleic acid extraction as they were either exhausted through previous testing, or the sample vial was not located. Where samples are indicated as failed/depleted, the nucleic acid extraction failed and there was insufficient remaining sample to repeat this extraction. Control samples were culture strains used to assess the analytical performance of the 'Fluidigm' qPCR and are described in methods "Total nucleic acid extraction" section. All extracted samples and controls were run in the 'Fluidigm', however only samples with a Quellung result (positive or negative) were included in comparisons of diagnostic performance.

Total nucleic acid extraction. Samples (NPS in Skim-milk-tryptone-glucose-glycerin transport media [STGG]) that were stored at − 80 °C were thawed and vortexed at low speed for 30 s. Nucleic acids were extracted from 400 µl of STGG and eluted into 100 µl of elution buffer using the automated BioMérieux NucliSens easyMAG (BioMérieux, Marcy l'Etoile, France) nucleic acid extraction platform according to standard manufacturer protocols. One no-template-control (NTC) that was blank STGG was included in each batch of 23 clinical samples extracted. Nucleic acids were stored at − 30 °C until assayed.

BioMark HD Nanofluidic qPCR loading, sample, and assay preparation. Specific target amplification (STA) was done as per manufactures recommendations as the initial step (pre-amplification of DNA) for the Biomark HD system (Standard BioTools, formerly Fluidigm) as discussed previously[35]. In short, assay-sets (primers only) were separated into three STA multiplex pools (Supplementary Table S3) to prevent any primers' cross-reactivity. After STA, qPCR was carried out on either the Standard BioTools 96.96 Gene Expression (GE) Dynamic Array integrated fluidic circuit (IFC) (product number BMK-M-96.96) or the Flex Six 12.12 GE IFC (product number 100-6308) against a reaction-set that combines 96 samples with 96 assays-sets for 9 216 individual qPCR reactions per array or 12 samples with 12 assay-sets for 144 reactions per array, respectively. A flow diagram of the preparation and loading of the Standard BioTools ('Fluidigm') 96.96 IFC is detailed in Supplementary Fig. S1 and has been described in detail previously[35]. Briefly, for each sample, STA product from each of three pools (5 µL) and 10 µL of molecular grade H_2O was combined, to dilute any remaining primers. Subsequently, assay premix for each target was aspirated into the IFC assay inlets for a final concentration of 9 µM primers and 2.5 µM probe per reaction and samples (2.25 µL Pooled STA product, 2.50 µL Applied Biosystems TaqMan Gene Expression Master Mix, catalog number 4370074 and 0.25 µL Standard BioTools/Fluidigm sample loading reagent) were aspirated into sample inlets. The IFC was then run in the BiomarkHD thermocycler, using the manufacturer supplied thermal cycling conditions: 50 °C for 2 min, 70 °C for 30 min, 25 °C for 10 min, 50 °C for 2 min, 96.5 °C for 10 min followed by 40 cycles of 96 °C for 15 s, 60 °C for 60 s. Results were analyzed using the Fluidigm Real-time PCR Analysis software. Here, thresholds were manually defined; the baseline was automatically assigned, and a Cycle of quantification (Cq) cut off value of 38 was applied.

Analytical efficiency, sensitivity, specificity, and quantification of density. Regression calibration curves (standard curves) were prepared for each assay-set using duplicate tenfold serial dilutions of the targeted positive control strains or gBlocks. The standard curves included five data points; outliers at the end of the range that did not contain linear cycle of quantification (Cq) values were removed to define the linear dynamic range and determination coefficient (r^2) value. At high template concentration, PCR inhibitors are at a higher concentration, whereas in highly diluted samples, stochastic binding results in variability in amplification. Concentrated or diluted template, or dilution errors will therefore affect the amplification efficiency, resulting in unpredictable Cq values. Values that fall outside of the limit of linearity, are no longer predicted by the linear equation. For this reason, outliers are excluded. The efficiency (%) for each included assay-set was derived from the slope (m) of the linear equation ($y = mx + c$) generated from the standard curves using the equation:

$$E = \left[-1 + 10^{\left(-\frac{1}{m}\right)}\right] \times 100$$

A triplicate dilution-series of bacterial control strains or gBlocks at the lower end of the linear dynamic range (10^3–10^0 copies per µL) were used to determine the analytical sensitivity or Limit of Detection (LOD) defined as lowest CFU/mL or copies detected in triplicate of 'Fluidigm' qPCR. A DNA library was prepared of the targeted pneumococcal serotypes or other bacterial species at an average 10^3–10^4 CFU/mL. The analytical specificity was assessed by running the DNA library against each assay-set in the 'Fluidigm' qPCR.

For assay-sets that were within the prescribed ranges for efficiency (90–110%), relative quantification of bacterial density was extrapolated using the linear equation generated from the standard curves of the calibrators (control strains and gBlocks of known density) using the equation:

$$Density = 10^{\frac{Cq-c}{m}} \times \frac{extraction\ volume}{elution\ volume}$$

Where more than one assay-set detected or determined a serotype, the average density was calculated. For example, to calculate the density of serotype 6A:

$$Density\,6A = Density(6ABCD + 6AC)/2$$

The relative density between serotypes was used to assign hierarchy in multiple concurrent serotype carriage, with primary serotypes being those with the highest density, and sub-dominant or co-colonizing serotypes being those with lower relative density. Bland–Altman plots (difference vs. means) were constructed to compare the density of Lyt*A* to Pia*B*, and to compare the average pneumococcal density (Lyt*A* and Pia*B*) to the sum of serotype density per sample.

Diagnostic sensitivity. The diagnostic sensitivity was assessed through blinded re-analysis of the stored clinical samples (n = 1973; Fig. 1) that were previously cultured and serotyped using the Quellung method. The samples were re-tested with the qPCR reaction-set within the 96.96 IFC that included the extracted NTC and quantification calibrators (gBlocks listed in Tables 2 and 3, or control strains at 10^4 and 10^3 CFU/mL). McNemar's test was used to compare serotype detection in the qPCR reaction-set with that of the Quellung method. Samples needed to be positive for both pneumococcal reference genes Lyt*A* and Pia*B* to be assigned a positive

serotype. Where these pneumococcal genes were not detected, but a serotype-specific assay-set was positive, no serotype was assigned. Serotype designation by the 'Fluidigm' qPCR and Quellung were considered different where *p*-values were ≤ 0.05. Cohen's kappa coefficient was used to determine the serotype-specific concordance of 'Fluidigm' qPCR and Quellung per clinical sample. Kappa values of < 0.20, 0.21–0.40, 0.41–0.60, 0.61–0.80 and 0.81–1.00 were considered as poor, fair, moderate, good, and excellent agreement, respectively. Analyses were done in Stata version 13.0, including applying relevant algorithms for serotype assignment.

Repeatability and reproducibility. The repeatability (intra-assay variance) was determined using the standard deviation (SD) for the variance in Cq of gBlocks run in triplicate within the same IFC at 10^3 gene equivalents. The reproducibility (inter-assay variance) was defined as the standard deviation for Cq variance for the gBlocks between four different IFCs.

Ethical approval. Ethical approval was obtained from the Medical Human Research Ethics Committee (HREC) of the University of Witwatersrand for the initial sample collection (Soweto Cohort HREC: M090115; Agincourt Cohort HREC: M090114). Written informed consent was obtained from all legal guardians of the participants as part of the original study in which samples were obtained. Molecular methods and the use of samples in this analysis was approved by the HREC of the University of Witwatersrand (HREC: M170314). All methods were performed in accordance with the relevant local and international guidelines and regulations for Good Clinical Practice.

Results

Comprehensive serotyping reaction-set. A total of 96 assay-sets were included in the final 'Fluidigm' qPCR reaction-set. The qPCR could detect 92 pneumococcal serotypes and differentiate these into 35 serotypes in 16 groups and 57 individual types and identify 15 other bacterial species (Fig. 2, Supplementary Table S4).

Analytical performance of the 'Fluidigm' qPCR. Amplification efficiency across the reaction-set was excellent for 91 assay-sets that were within the prescribed range of 90–110% (Table 1). For these assay-sets, the data points were accurately predicted by the linear equation of the regression curves ($r^2 > 0.98$, Table 1), allowing for relative quantification of bacterial load using the slope of the linear equation. The linear dynamic range for these assay-sets was at least 5-log fold. Further, Levy-Jennings plots constructed using assay-set specific reference calibrators were within ± 2.0 SD of the mean Cq value across all plates. This validated the use of the designed calibrators (gBlocks). The assay-sets for the detection of *E. coli*, *Pneumocystis jiroveci*, *S. algalactiae*, the *Xisco* gene of *S. pneumoniae* and the Bacterial 16S ribosomal RNA gene (listed in Supplementary Table S1) did not perform well within this reaction-set, as the efficiency was < 90% or > 110% or $r^2 < 0.98$ (Supplementary Table S5).

Analytical sensitivity, specificity, and quantification of density. The analytical sensitivity was high for 91 included assay-sets (LOD: 10–100 gene equivalents, Table 1). The assay-sets performed consistently, with Cq values of replicates (n = 3) amplified concurrently within a reaction-plate (intra-assay) or in consecutive reaction-plates (inter-assay) within ± 0.1 SD of the mean. Of the 96 assay-sets within the 'Fluidigm' qPCR, 94 assay-sets were specific to their targeted bacterial control calibrators, and there was no observed cross-reactivity between assay-sets. Two reaction-sets, namely 10A and 33B detected other serotypes within the same group—i.e., 10B and 33C respectively. These assay-sets were thus renamed accordingly—10A was renamed 10A/B and 33B was renamed 33B/C. A serotyping algorithm based on the detection pattern of all assay-sets against all targeted bacterial strains was applied to dissect some individual serotypes, namely: 6A, 6B, 6C, 6D, 10A, 11E, 12A/F/44, 18A, 18B, 18C, 18F, 19B, 22A, 23A, 38, 37, 33AF, 35F, 47A, 47F, non-typeable *S. pneumoniae*, non-type-b *H. influenzae*, non-typeable *H. influenzae*, *B. pertussis*, *B. holmesii* and *B. parapertussis* (Table 2). Assay-sets for serotypes 10B and 33C had been included within the reaction-set and therefore the specificity of serotype allocation by the algorithm was not affected by cross reacting serotypes, as 10A and 33B were not detected by these respective assay-sets. For example, serotype 10A would be detected by assay-set 10A and not assay-set 10B and would therefore be assigned serotype 10A; whereas serotype 10B would be detected by assay-set 10A and assay-set 10B and therefore be assigned serotype 10B.

Diagnostic sensitivity, specificity, and agreement of the 'Fluidigm' qPCR. As the diagnostic performance was compared with the Quellung method, this was only applied to pneumococcal serotypes. Further, the diagnostic performance of the 'Fluidigm' could not be assessed for assay-sets that targeted bacterial species or pneumococcal serotypes that were not previously detected in archived clinical samples using culture-based methods (listed in footnote of Table 3). The 'Fluidigm' qPCR method was capable of correctly classifying pneumococcal serotypes compared with the referent standard (Quellung) and the receiver operator curve area under the curve (ROC-AUC) was greater than 0.73 for all compared assay-sets (Table 3). 'Fluidigm' qPCR distinguished targets with > 80% sensitivity (Table 3) in the 1 973 archived clinical samples for 36 assay-sets. The diagnostic sensitivity for six assay-sets (1; 7B/C/40; 23A; 29; 33B and 35A/C/42) was lower (50–74%, Table 3) as the prevalence of the targeted serotypes detected by Quellung was ≤ 1% whereas additional targeted serotypes were detected using 'Fluidigm' qPCR (Fig. 3). The remaining assay-sets could not be assessed as explained above. The diagnostic specificity was high (> 95%) for all assay-sets where targeted serotypes were previously detected by Quellung (Table 3).

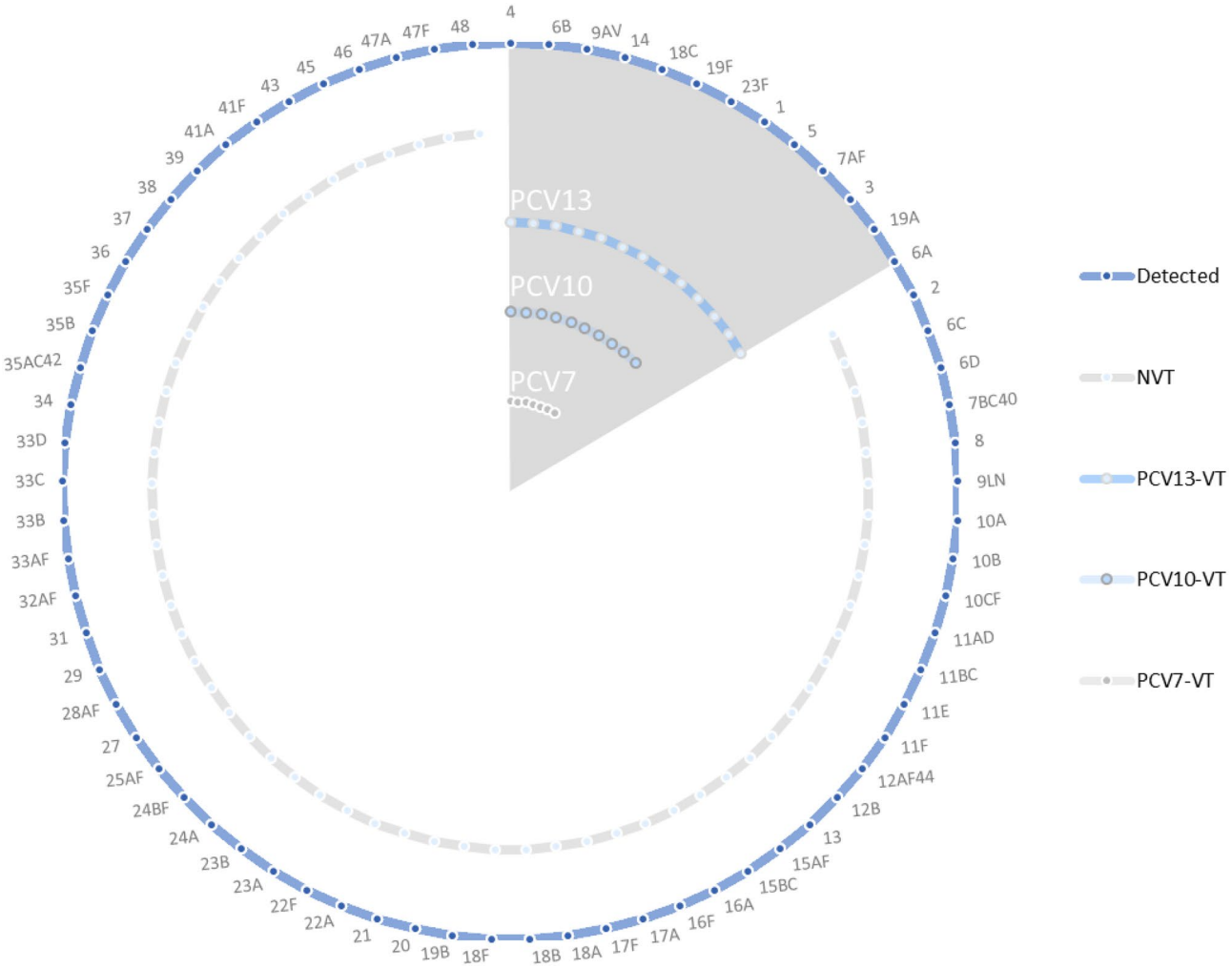

Figure 2. Summary web of the 92 pneumococcal serotypes (57 individual serotypes, and 35 serotypes within 16 serogroups) detected by the 'Fluidigm' reaction-set. Highlighted in grey are PCV7 serotypes: 4, 6B, 9A/V, 14, 18C, 19F and 23F; PCV10 serotypes: PCV7 serotypes and 1, 5, 7A/F; PCV13 serotypes: PCV10 serotypes and 3,6A, & 19A. NVT: serotypes not included in PCV13.

There was good concordance between culture and 'Fluidigm' qPCR, with the majority of the assay-sets detecting the same serotype by both methods (kappa 0.61–0.8) with the exception of serotype/groups 1, 5 and 23A that had a fair concordance (kappa 0.41–0.60) and serotypes 7A/F, 11B/C, 12A/F/44, 33A/F, 35A/C/42, 35F, and 38 that had a moderate concordance (kappa 0.21–0.40). Where concordance was rated as fair or moderate, this was due to the detection of these serotypes in additional samples by 'Fluidigm' qPCR compared with Quellung (serotypes 5; 23A; 12A/F/44; 35A/C/42 and 38), and further, where there were less than five total samples detected by the Quellung method (serotypes 1; 7A/F; 11B/C; 33A/F and 35F) (Fig. 3). For one assay-set (serotype 29) the concordance was rated as poor ($\kappa < 0.20$). For this target, 'Fluidigm' qPCR detected one out of two samples serotyped as 29 using the culture based Quellung method, however, a further eight samples that were negative using Quellung, were serotyped as 29 using 'Fluidigm' qPCR. 'Fluidigm' qPCR was more sensitive in detecting all respective serotypes/groups compared to culture (McNemar's test: $p < 0.05$; Table 3) except for serotypes 6C, 7A/F, 7B/C/40, 9L/N, 10A, 11B/C, 15B/C, 18C, 20, 21, 22F, 23B, 25A/F and 33B, where there was no difference in classification detected between the two methods (McNemar's test: $p > 0.05$; Table 3). The 'Fluidigm' reaction-set was able to identify an additional 39.1% (826/2113) serotypes above those detected by culture for the compared assay-sets in Table 3, and conversely culture detected 132 (9.3% of 1419) serotypes not identified by 'Fluidigm' qPCR (Table 3, Fig. 3).

Quantification of serotypes and bacterial density. The GMD (Geometric Mean Density) for all serotypes and bacterial targets are presented in Supplementary Table S6, and summarised in Supplementary Fig. S2, and the hierarchy of co-colonising pneumococcal serotypes is presented in Supplementary Table S7. Out of the additional serotypes detected by 'Fluidigm' qPCR, 72.6% (831/1 144) were co-colonising, or lower density serotypes relative to the primary, or highest density serotype. The GMD of the additional serotypes detected by

Assay-set	Calibrator	Efficiency (%) $[-1+10^{(-1/m)}]$	LOD (g-Block)	Linear equation	r^2
1	St 1	98	10^1	y = −3.3761x + 24.236	1.00
2	St 2	95	10	y = −3.4361x + 26.784	1.00
3	St 3	108	10^1	y = −3.1449x + 22.45	1.00
4	St 4	100	10^1	y = −3.32x + 28.393	1.00
5	St 5	99	10^1	y = −3.3421x + 25.415	1.00
6A/B/C/D(F/G/H)	St 6B	100	10^1	y = −3.327x + 30.357	0.99
6A/C	St 6C	97	10^2	y = −3.4039x + 30.277	0.97
6C/D	St 6C	97	10	y = −3.384x + 27.769	0.99
7A/F	St 7F	95	10^1	y = −3.4605x + 26.292	1.00
7B/C/40	St 7C	102	10^1	y = −3.2729x + 27.595	0.99
8	St 8	110	10^2	y = −3.1071x + 24.264	1.00
9A/L/N/V	St 9A	102	10^1	y = −3.268x + 25.114	1.00
9A/V	St 9 V	101	10^1	y = −3.3014x + 24.495	0.99
9L/N	St 9N	99	10	y = −3.3583x + 28.397	1.00
10A	St 10A	99	10	y = −3.3555x + 24.534	1.00
10A/B	St 10B	100	10^1	y = −3.3263x + 23.409	0.99
10C/F	St 10C	96	10^2	y = −3.4327x + 28.19	1.00
11A/B/C/D/E/F	St 11A	102	10^1	y = −3.2846x + 26.706	0.99
11A/D	St 11D	91	10^2	y = −3.5656x + 29.722	1.00
11B/C	St 11B	100	10^2	y = −3.3137x + 25.745	1.00
11F	St 11F	103	10^2	y = −3.2412x + 30.921	0.99
12A/B/F/44/46	St 12B	100	10^2	y = −3.3217x + 22.458	1.00
12B	g-Block	97	10^1	y = −3.4084x + 26.027	1.00
13	St 13	105	10	y = −3.2135x + 24.699	1.00
14	St 14	100	10^2	y = −3.3232x + 26.189	0.99
15A/B/C/F	St 15A	97	10	y = −3.3908x + 26.255	1.00
15A/F	St 15A	109	10^2	y = −3.119x + 23.396	0.99
15B/C	St 15B	98	10^2	y = −3.3707x + 25.31	0.99
16A	g-Block	102	10	y = −3.2674x + 24.3	0.99
16F	St 16F	104	10^2	y = −3.2321x + 25.116	1.00
16F/18F/28AF	St 18F	108	10^1	y = −3.1525x + 24.213	0.99
17A	St 17A	91	10^1	y = −3.5508x + 23.693	1.00
17A/F	St 17F	99	10^1	y = −3.3389x + 26.05	0.98
18A/B/C/F	St 18C	99	10^2	y = −3.3484x + 22.148	1.00
18B/C/F	St 18C	98	10	y = −3.363x + 23.995	1.00
18C/F	St 18C	98	10^2	y = −3.3743x + 31.732	0.99
19A	St 19A	94	10^2	y = −3.483x + 23.801	1.00
19B/F	St 19B	108	10^2	y = −3.1345x + 20.812	1.00
19F	g-Block	110	10^1	y = −3.1075x + 26.863	1.00
20	St 20	99	10^2	y = −3.3405x + 25.595	0.99
21	St 21	103	10^2	y = −3.2409x + 27.273	0.99
22A/F	St 22F	98	10^2	y = −3.3701x + 26.527	1.00
22F	St 22F	95	10^2	y = −3.438x + 30.669	0.98
23A/B/F	St 23B	94	10^2	y = −3.4648x + 29.288	0.98
23B	St 23B	97	10^2	y = −3.3888x + 28.063	1.00
23F	St 23F	102	10^1	y = −3.2754x + 22.908	0.99
24A	St 24A	94	10^1	y = −3.4846x + 25.594	0.99
24B/F	St 24F	103	10^2	y = −3.2571x + 25.548	1.00
25A/F	St 25F	90	10^1	y = −3.5977x + 23.92	0.99
25A/F/38	St 25A	93	10^2	y = −3.5069x + 24.259	1.00
27	St 27	95	10^2	y = −3.4509x + 24.242	0.99
28AF	g-Block	92	10^2	y = −3.517x + 24.565	1.00
29	g-Block	105	10^2	y = −3.2145x + 26.526	1.00
31	g-Block	104	10	y = −3.2831x + 22.833	1.00
32A/F	St 32A	98	10	y = −3.3804x + 22.641	0.99
33A/F/37	St 33A	100	10	y = −3.3257x + 23.074	0.99
Continued					

Assay-set	Calibrator	Efficiency (%) [$-1+10^{(-1/m)}$]	LOD (g-Block)	Linear equation	r^2
33B/C	St 33C	97	10	y = −3.406x + 25.401	1.00
33C	St 33C	96	10^1	y = −3.4155x + 25.436	1.00
33D	g-Block	109	10	y = −3.1097x + 25.098	1.00
34/17A	St 34	97	10	y = −3.4042x + 24.341	1.00
34/37/17A	St 37	100	10	y = −3.3282x + 27.087	1.00
35A/C/42	St 35C	100	10	y = −3.3161x + 25.966	0.99
35B	St 35B	103	10^1	y = −3.251x + 27.268	0.98
35F/47F	St 35F	90	10^2	y = −3.5757x + 24.976	0.98
36	St 36	102	10^1	y = −3.2814x + 26.331	1.00
39	g-Block	106	10	y = −3.462x + 21.292	1.00
41A	St 41A	101	10	y = −3.3039x + 24.147	1.00
41F	g-Block	94	10^2	y = −3.4843x + 22.181	0.98
43	g-Block	105	10	y = −3.1997x + 27.245	0.99
45	St 45	90	10	y = −3.6009x + 26.052	1.00
46	g-Block	98	10^1	y = −3.3655x + 21.273	1.00
47AF	St 47F	95	10^1	y = −3.442x + 24.074	0.99
48	St 48	91	10	y = −3.5707x + 24.586	0.99
Acinetobacter baumanii	*A. baumanii*	94	10^1	y = −3.4785x + 26.193	0.99
Bordetella holmesii (h*IS*)	g-Block	91	10^2	y = −3.5491x + 29.271	1.00
B. pertussis/holmesii (*IS481*)	*B. pertussis*	90	10^2	y = −3.5933x + 25.235	0.99
B. parapertussis (p*IS*)	*B. parapertussis*	95	10	y = −3.4358x + 21.956	0.99
B. pertussis/bronchiseptica/parapertussis (Ptx*S*)	*B. parapertussis*	97	10	y = −3.3903x + 22.329	0.99
Haemophilus influenzae (Bex*A*)	*H. influenzae*, type b	93	10^1	y = −3.513x + 25.169	1.00
H. influenzae (Bex*B*)	*H. influenzae*, type b	102	10^1	y = −3.2784x + 26.106	1.00
H. influenzae, type b	*H. influenzae*, type b	97	10^2	y = −3.3953x + 22.967	0.99
H. influenzae (Ig*A1*)	*H. influenzae*, type b	97	10	y = −3.3905x + 21.628	1.00
Klebsiella pneumoniae	g-Block	99	10	y = −3.3499x + 26.09	0.98
Morexella catarrhalis	*M. catarrhalis*	97	10^1	y = −3.4079x + 26.561	0.99
Neiserria lactamica	*N. lactamica*	102	10^1	y = −3.2662x + 25.872	0.98
Neiserria meningitidis	*N. meningitidis*	99	10^2	y = −3.3496x + 24.255	0.98
Staphylooccus aureus	*S. aureus*	91	10^1	y = −3.5547x + 29.696	1.00
Streptococcus oralis	g-Block	108	10^1	y = −3.1503x + 26.386	1.00
Streptococcus pneumoniae (Lyt*A*)	St 4	99	10^1	y = −3.3582x + 26.927	1.00
S. pneumoniae (Pia*B*)	St 4	97	10^1	y = −3.394x + 26.863	0.99
S. pneumoniae (Ply)	St 4	96	10^1	y = −3.4107x + 26.927	1.00
S. pyogenes	g-Block	99	10	y = −3.3488x + 25.05	1.00

Table 1. Analytical performance of 93 optimized assay-sets in the high-throughput BioMark HD nano-fluidic real-time PCR ('Fluidigm').

'Fluidigm' qPCR was lower than where the serotype designation was concordant between 'Fluidigm' qPCR and Quellung [2.3 (95% Confidence Interval/CI: 2.2–2.4) and 4.1 (95% CI: 4.0–4.2); Fig. 4].

There was excellent agreement between the pneumococcal density calculated from the Lyt*A* and Pia*B* assay-sets (line of bias: −0.2) when compared using Bland–Altman plots (Supplementary Fig. S3A). Further, only 6.9% (58/836) of the data points were outside of the narrow 95% CI (−1.7 to 1.3). The agreement between average pneumococcal density (Lyt*A* and Pia*B*) and the sum of all serotype density (all serotype-specific assay-sets) was acceptable (line of bias: −1.2, Supplementary Fig. S3B) with 5.8% (45/776) of the data outside of the 95% CI (−6.7 to 4.0). In 7.8% (65/836) of samples positive for pneumococcus, no serotypes were detected, and in a further 3.6% (30/836) the difference between the pneumococcal density and the total serotype density was above the upper CI of the Bland–Altman plot.

Discussion

We have developed and validated a nanofluidic qPCR reaction-set that is sensitive, specific, and reproducible for the accurate detection and quantification of 92 pneumococcal serotypes and other colonizing bacteria within 96 specimens (including calibrators) per reaction-plate. The performance of the assay-sets described here is comparable with other high-throughput reaction-reaction sets to detect pneumococcal carriage, including in the Standard BioTools 'Fluidigm' platform[32,33] and in TaqMan Array Cards[15] for LOD (< 10^3 CFU/ml); efficiency (90–110%); linear dynamic range (fivefold) and linearity ($R^2 > 0.98$). Notably, the detection of additional serotypes

	6A	6B	6C	6D	10A	10B	11E	12A/F/44	18A	18B	18C	18F	19B	19F	22A	22F	23A
6A/B/C/D	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
6A/C	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6C/D	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
10A/B	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
10B	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–
11A/B/C/D/E/F	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
11A/D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11B/C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12A/B/F/44/46	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
12B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16F/18F/28AF	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
17A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
17A/34/37	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18A/B/C	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
18BCF	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–
18CF	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–
19B/F	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–
19F	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
22AF	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
22F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
23A/B/F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
23B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
23F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
25A/F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
25A/F/38	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
28A/F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
33A/F/37	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
33B/C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
33B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
34	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
35F/47F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
46	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
47A/F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IS481	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PtxS1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
hIS1001	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
pIS1001	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IgA1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BexA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BexB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Hin–b	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

	25A/F	33A/F	33B	33C	35F	37	38	47A	47F	*B. pertussis*	*B. holmesii*	*B. parapertussis*	*B. bronchiseptica*	*H. influenzae*	*H. influenzae–b*	NTHI
6A/B/C/D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6A/C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6C/D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10A/B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11A/B/C/D/E/F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11A/D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11B/C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12A/B/F/44/46	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Continued

	25A/F	33A/F	33B	33C	35F	37	38	47A	47F	*B. pertussis*	*B. holmesii*	*B. parapertussis*	*B. bronchiseptica*	*H. influenzae*	*H. influenzae–b*	NTHI
12B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16F/18F/28AF	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
17A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
17A/34/37	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
18A/B/C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18BCF	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18CF	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
19B/F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
19F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
22AF	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
22F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
23A/B/F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
23B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
23F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
25A/F	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
25A/F/38	+		–	–	–	–	+	–	–	–	–	–	–	–	–	–
28A/F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
33A/F/37	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–
33B/C	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
33B	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
34	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
35F/47F	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–
46	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
47A/F	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–
IS481	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–
PtxS1	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
hIS1001	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
pIS1001	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–
IgA1	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
BexA	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
BexB	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
Hin–b	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–

Table 2. The algorithm for designating bacterial strains based on analytical specificity of assay-sets to their targets.

(39.1%) by the 'Fluidigm' qPCR panel described here with 96 assay sets, is comparable to the 'Fluidigm' panel described previously (also 39.1%), that included 48 assay sets[32]. Compared with the gold-standard culture based-Quellung method, our reaction-set was 98.8% accurate for the classification of pneumococcal serotypes. Further, the average sensitivity across the qPCR reaction-set was 89.1% compared with Quellung.

Previous molecular serotyping techniques have detected common serotypes; however, other emerging or uncommon NVT were not detected. Expanding the array of serotypes detectable by higher throughput methods has become increasingly important as NVT replace VT in carriage[41–43]. It is also important to detect and assign bacterial load or density to multiple serotype carriage (co-colonization) directly from clinical specimens without an intervening culture step, which is an advantage of molecular techniques over standard culture-based methods[44]. Previously, incomplete serotyping has been described as a disadvantage of molecular serotyping in our setting (South Africa) where serotype prevalence differs from other geographic regions[45,46]. To the best of our knowledge, the 'Fluidigm' qPCR method described here is the most comprehensive high-throughput quantitative molecular serotyping reaction-set developed to date.

Notably, Pai et al.[25,26] developed a conventional multiplex PCR method to determine 29 common serogroups/types, sequentially narrowed down to 40 singular serotypes. Azzari et al.[21] used real-time PCR to detect 21 serotypes. Sakai et al.[29] then designed 27 new assay-sets to detect 72 serotypes/groups. Pholwat et al.[15] optimized a PCR-based Microfluidic TaqMan array card (TAC) covering 74 serotypes in up to seven clinical samples per card. Sakai et al.[30] further developed a qPCR reaction-set to detect 46 individual serotypes and 33 serotypes within 20 serogroups. In the 'Fluidigm', Dhoubhadel et al.[33] used SYBR Green chemistry to detect 50 serotypes (16 individual serotypes and 13 sub-groups). Messoudi et al.[31] adapted Pai et al.[26] assay-sets into a sequential real-time multiplex PCR to detect 40 serotypes or groups using Locked Nucleic Acid (LNA) modified probes. Together, these studies have furthered development into molecular serotyping and provided useful assay-sets for

Serogroup/type	Sensitivity (%)	Specificity (%)	Correctly classified (%)	ROC AUC*	Cohen's Kappa	Concordant samples	Discordant Fluidigm	Discordant Quellung	p-value†
1	66.7	99.4	99.4	0.83	Fair (0.40)	4	10	2	0.039
3	88.5	98.4	98.3	0.93	Moderate (0.57)	23	30	3	<0.001
4	100	99.0	99.0	1.00	Moderate (0.48)	9	19	0	<0.001
5	100	97.5	97.5	0.99	Fair (0.24)	8	49	0	<0.001
6A	92.9	98.8	98.4	0.96	Excellent (0.88)	131	22	10	0.050
6B	91.7	95.9	95.7	0.94	Good (0.72)	122	74	11	<0.001
6C	100	99.9	100	1.00	Excellent (0.91)	5	1	0	1.00
7A/F	100	99.9	99.9	1.00	Moderate (0.50)	1	2	0	0.500
7B/C/40	70.0	99.5	99.2	0.85	Good (0.65)	14	9	6	0.607
8	100	99.6	99.7	1.00	Good (0.67)	6	6	0	0.031
9A/V	93.1	99.3	99.2	0.96	Good (0.78)	27	13	2	0.007
9L/N	90.0	99.7	99.7	0.95	Good (0.75)	9	5	1	0.219
10A	87.5	99.5	99.5	0.94	Good (0.73)	14	8	2	0.109
11A/D	85.4	99.0	98.7	0.92	Good (0.73)	35	19	6	0.015
11B/C	100	99.9	99.9	1.00	Moderate (0.50)	1	2	0	0.500
12A/F/44	100	99.3	99.3	1.00	Moderate (0.60)	10	13	0	<0.001
13	96.8	99.1	99.1	0.98	Good (0.78)	30	16	1	<0.001
14	86.6	97.6	97.3	0.92	Good (0.67)	58	45	9	<0.001
15A/F	94.1	99.3	99.3	0.97	Good (0.71)	16	12	1	0.003
15B/C	88.9	99.0	98.6	0.94	Excellent (0.84)	80	18	10	0.185
16F	96.2	98.9	98.9	0.98	Excellent (0.82)	51	20	2	<0.001
17F	96.6	99.1	99.1	0.98	Good (0.76)	28	16	1	<0.001
18C	94.4	99.9	99.9	0.97	Excellent (0.92)	17	2	1	1.00
19A	94.4	97.2	97.2	0.96	Good (0.74)	85	51	5	<0.001
19B	88.9	97.7	97.7	0.93	Fair (0.26)	8	44	1	<0.001
19F	96.5	95.7	95.8	0.96	Good (0.80)	195	75	7	<0.001
20	85.2	99.4	99.2	0.92	Good (0.75)	23	11	4	0.119
21	100	99.7	99.8	1.00	Excellent (0.81)	11	5	0	0.063
22F	85.7	100	100	0.93	Excellent (0.92)	6	0	1	1.00
23A	58.8	97.8	97.5	0.78	Fair (0.28)	10	42	7	<0.001
23B	81.3	99.3	98.9	0.90	Good (0.77)	39	13	9	0.524
23F	84.2	96.6	95.8	0.90	Good (0.71)	112	62	21	<0.001
25A/F	100	99.8	99.9	1.00	Fair (0.40)	1	3	0	0.250
29	50.0	99.5	99.5	0.75	Poor (0.18)	1	8	1	0.039
31	100	99.4	99.5	1.00	Good (0.64)	9	10	0	0.002
33A/F	100	99.4	99.4	1.00	Moderate (0.42)	4	11	0	0.001
33B	50.0	99.7	99.7	0.75	Fair (0.22)	1	6	1	0.125
34	97.7	99.5	99.5	0.99	Excellent (0.89)	43	9	1	0.022
35A/C/42	73.3	98.9	98.7	0.86	Moderate (0.46)	11	21	4	<0.001
35B	100	99.2	99.3	1.00	Good (0.65)	13	14	0	<0.001
35F	83.3	99.3	99.3	0.91	Moderate (0.41)	5	13	1	0.002
38	91.7	99.1	99.1	0.95	Moderate (0.55)	11	17	1	<0.001

Table 3. Diagnostic performance of the 'Fluidigm' high-throughput BioMark HD nano-fluidic real-time qPCR Fluidigm. There were no samples for which serotypes 2; 10B; 10C/F; 17A; 18A; 18B; 18F; 22A; 24A/B/F; 28A/F; 33D; 36; 37; 41; 43; 45; 47A/F and 48 were detected with the referent standard Quellung method for comparison of diagnostic performance. *ROC AUC: Receiver operator curve, area under curve, †McNemar's Test.

use in our 'Fluidigm' qPCR that we have further optimized within multiplex STA pools to detect and quantify 92 pneumococcal serotypes, distinguishing to 16 serogroups and 57 individual serotypes. Prior to our study, modified probes have not been utilized in a high throughput system such as micro- or nano-fluidic PCR systems or recommended in the Standard BioTools 'Fluidigm' platform. Our study has shown that these modified probe strategies work well in high-throughput 'Fluidigm' nano-fluidic qPCR.

Due to the aetiological link between high bacterial load, transmission risk and the invasive potential of serotypes, quantification of bacterial load in carriage studies is important to assess vaccine impact. Apart from serotypes 12F, 8, 24F, 33F, 38 and 10A, NVT involved in carriage serotype replacement have been shown to have low invasive potential according to case-to-carrier ratios analyses[47,48]; however, molecular studies have shown

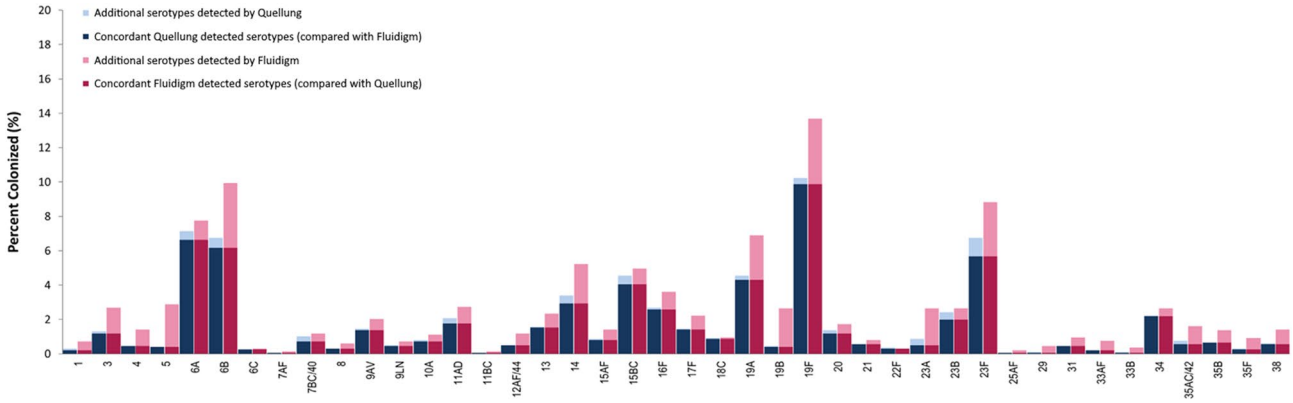

Figure 3. Prevalence of individual *Streptococcus pneumoniae* serotypes detected by the 'Fluidigm' qPCR reaction-set compared with the culture based Quellung method. Serotypes detected in samples by both 'Fluidigm' and Quellung are classified as concordant, whereas samples detected by only one method are classified as additional serotypes.

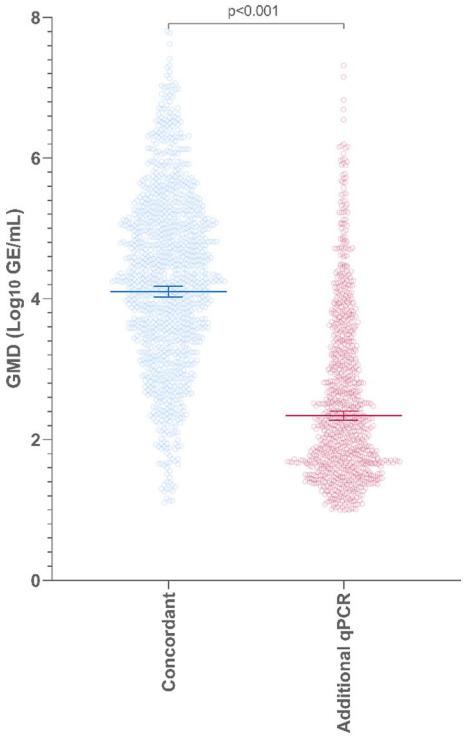

Figure 4. The Geometric Mean Density (GMD) expressed as Log10 Gene Equivalents (GE)/mL determined by 'Fluidigm' qPCR. Concordant serotypes (blue) were detected by both the referent standard culture based Quellung method and 'Fluidigm'. Additional serotypes are those that were detected by 'Fluidigm' qPCR only (red).

that high bacterial density may predict invasive potential of these colonizing serotypes. Out of the additional serotypes contained in PCV15 (22F, 33F) and PCV20 (8, 10A, 11A, 12F and 15BC), 'Fluidigm' qPCR identified an additional 0.3% of serotype 8; 0.4% of 10A; 1.0% of 11A/D; 0.7% of 12A/F/44; 0.9% of 15B/C and 0.6% of 33A/F compared with culture. A molecular study (multi-site: South Africa, Brazil, Mali, Cambodia and France) comparing the nasopharyngeal bacterial load of invasive isolates with asymptomatic carriage isolates for synonymous serotypes, found a fivefold higher mean bacterial load in invasive isolates (263.4×10^5 CFU/ml; $\pm 57.07 \times 10^5$ vs. 49.9×10^5 CFU/ml $\pm 67.4 \times 10^5$)[31].

Bacterial prevalence may also be intrinsically linked to bacterial load that increases the risk of onward transmission. For example, serotype specific bacterial load and prevalence were compared in a Vietnamese study that included children under 5 years-of-age admitted to hospital with acute respiratory infection (ARI) and healthy

age-matched children. Here, high bacterial load per serotype was associated with higher prevalence in both ARI cases (Spearman's $\rho = 0.44$, n = 186; $p < 0.0001$) and healthy children (Spearman's $\rho = 0.41$, n = 115; $p < 0.0001$).

Our reaction-set did not include the assay-sets previously published for 19F-Atypical, 19C and 23A; however, we did not find any indications in the in silico analysis that these assay-sets do not detect their respective targets, and these assay-sets have worked well in other studies[15,29,30]. Serotype 23A was still detected in our reaction-set using an algorithm that combines the results of the serogroup 23 (23A/B/F) assay-set and the serotype 23B- and 23F-targeted assay-sets whereby a sample positive for 23A/B/F but negative for 23B and 23F would be ascribed 23A.

While *LytA* and *PiaB* in combination are described as sensitive and specific for pneumococcal detection, and the density calculated using these assay sets is concordant, Tavares et al.[49] have since recommend using the putative transcriptional regulator gene SP2020 combined with *LytA* for increased specificity. These four assay-sets (19F-Atypical, 19C, 23A and SP2020) should be further validated and included in the reaction-set to increase the detection to 94 serotypes, 16 serogroups and 59 individual serotypes. The assay-sets for the detection of *E. coli*, *P. jiroveci*, *S. agalactiae*, the *Xisco* gene of *S. pneumoniae* and the Bacterial 16S ribosomal RNA gene were not well optimized within this reaction-set and may therefore only be used for qualitative detection of targets.

Challenges exist for distinguishing within some serogroups to singular serotypes by our reaction-set. These include that a high degree of cross-reactivity between genetically similar serotypes limits discrimination at serotype level. For example, serotypes 7A/F and 9A/V differ genetically by only a single nucleotide in a difficult to target region (low GC content) with runs of homogonous base pairs that would result in mis-priming for all high-throughput strategies described here[2]. These will require more specialist strategies that do not fit within our uniform thermal profile. Further, some serotypes interconvert in situ and may not be clinically characterized easily into separate serotypes, such as serotypes 11A and 11E, 15B and 15C, or 35B and 35D, which exist on a 'spectrum' due to incomplete loss or gain of acetylation or transferases[50,51]. The latter two serogroups, although they contain molecularly distinct serotypes, have problematic clinical distinction, and therefore should be interpreted with caution.

Other limitations to this study include that our method requires experienced personal and specialized equipment (Standard BioTools, 'Fluidigm') that is not affordable in many settings; however, this method can be easily adapted to other real-time qPCR platforms. Where serotypes are not distinguished to individual serotypes, the bacterial load for serotypes detected within groups could be over-estimated if there is co-carriage of more than one serotype within a serogroup, for example serogroup 12A, 12F and serotype 44, or serotypes 7B, 7C and serotype 40. As colonization by most of these serotypes is not high in our setting, this is not expected to have a very relevant impact. The discrimination of these serotypes individually will be important in settings where these serotypes are in circulation or to assess emergence of non-vaccine types in the post-vaccine era.

The referent standard Quellung method is limited to the detection of higher density and dominant colonizers, whereas our 'Fluidigm' qPCR can detect multiple concurrent colonization, including at lower density. This is due to the inclusion of a comprehensive serotyping method (assay-sets covering most serotypes) and a high analytical sensitivity to detect low density colonizers. These benefits of qPCR translate to diagnostic comparisons of these methods under-estimating the diagnostic sensitivity and the concordance of 'Fluidigm' qPCR compared with the culture based Quellung methods, especially where the prevalence of serotypes detected by the referent standard is low. This was indeed the case for serotypes 1; 7B/C/40; 23A; 29; 33B and 35A/C/42.

A further limitation for diagnostic comparison in our study includes the use of retrospective samples that have undergone multiple freeze–thaw cycles and have been stored for around 10 years, which can affect the ability of any diagnostic method to accurately detect pathogens. The Quellung method was undertaken in 2010 (soon after sample collection) and detected an additional 9.3% of serotypes that were not detected by 'Fluidigm' qPCR. In contrast, the analytical sensitivity and specificity of the 'Fluidigm' qPCR was demonstrated across the reaction-set when newly prepared culture or gBlock calibrators were used. Additionally, the 'Fluidigm' qPCR reaction-set could detect 39.1% more serotypes that were missed by Quellung. Here, the density (GMD) of the additional serotypes detected by 'Fluidigm' qPCR was around two times lower than for serotypes concordantly detected by 'Fluidigm' qPCR and Quellung and most (72.6%) were sub-dominant serotypes that would be less likely to be detected by the Quellung method. Nonetheless, our method was able to serotype the 1 973 selected samples with good concordance and sensitivity compared with Quellung.

The use of qPCR-based serotyping relies on detection of short target sequences within a larger capsular polysaccharide synthesis locus, to assign serotypes. The recent description of serotype 14-like pneumococci highlights a limitation of short genetic identifiers. In Papua New Guinea, four NPS isolates from hospitalized children with acute lower respiratory tract infection were assigned serotype 14 (an invasive VT) by qPCR. These isolates were 'non-typeable' (non-encapsulated) using the Quellung, latex agglutination and PneumoCaT methods. The authors describe a divergent serological profile and the ability to escape vaccine-elicited immunity to capsular serotype 14, despite partial genetic similarity. Genomic sequencing of the 14-like isolates revealed mutations that interrupt the capsular biosynthetic loci, whereas the short PCR targeted serotype 14 sequence was intact[52]. Additionally, small changes within target sequences, such as single nucleotide polymorphisms (SNPs) are not easily discerned using standard qPCR methods, whereas these single point genetic changes can alter the capsular structure, as exemplified by serotypes 6A and 6B[53]. The implication from these two examples is that in some cases, molecular serotyping may not identify antigenic diversity or new serotypes, and alternate serotyping methods should be used to resolve discrepancies.

In 7.8% of samples where both Lyt*A* and Pia*B* were detected, no serotype was detected. Within the context of our method, the differential detection of pneumococcus and serotypes may indicate the presence of either non-typeable (NT) pneumococci or, less likely, serotypes not included in our reaction-set. In a further 3.8% of pneumococcal positive samples the difference in the pneumococcal density and the total serotype density was above the upper bound confidence interval of the Bland–Altman plot, which may be a useful strategy to identify

NT pneumococci present in co-colonization with other serotypes. This method of detecting NT pneumococci in co-colonisation using the confidence intervals of the Bland–Altman plots would however underestimate NT present at lower density.

A limitation of our method involves the detection of capsular serotype target regions that have been acquired by non-pneumococcal species present in the nasopharynx. While we included that samples need to be positive for both Lyt*A* and Pia*B*, where non-pneumococcal serotype homologues are present in co-colonization with true pneumococcal serotypes, these would be assigned as pneumococcal serotypes. An example of this phenomenon is *Streptococcus mitis* that express a serotype 1 capsule[54]. In target assay-sets where qPCR concordance with Quellung was rated as poor, fair or moderate using Cohen's kappa (serotypes 1; 5; 7A/F; 11B/C; 12A/F/44; 23A; 11B/C; 29; 33A/F; 35A/C/42; 35F and 38), this was due to the detection of these serotypes in additional samples by qPCR. When detected in co-colonisation, we cannot exclude that these additional serotypes detected are always true pneumococci. In the Bland–Altman plot for the agreement between average pneumococcal density and serotype density, where the total density of multiple concurrent colonising serotypes was higher than the total pneumococcal density and outside of the lower bound 95% CI, this may indicate the presence of non-pneumococcal serotype homologues. Here we propose additional sequencing analysis be undertaken where necessary for the confirmation of serotypes in co-colonization, where other non-pneumococcal *Streptococci* are detected.

One of the large benefits of our study is the description of performance per assay-set according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines[55] and the large sample size of Quellung-serotyped isolates available for validation of our method.

Although to set-up the reaction-set within the Standard BioTools 'Fluidigm' qPCR platform is initially expensive, advantages include a large reduction of labor costs and waiting time due to the high number of data points, as many samples are tested against many different serotypes run within one plate. Our method is relatively fast to perform and can yield results of 92 serotypes and 15 bacterial targets for 180 samples (two IFC runs) within one day. The cost per nasopharyngeal sample is around USD $36 which is highly favorable, considering the comprehensive serotyping and quantitative information generated per sample. The concatenated reaction-set within the Standard BioTools 'Fluidigm' qPCR will be a benefit in large carriage studies. The method should be further validated for other clinical specimens, such as blood, sputum, cerebrospinal fluid, and other sites where pneumococcus may be found.

Data availability

The datasets generated and/or analysed during the current study and the Stata codes are available from the corresponding authors on reasonable request.

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Author contributions

S.L.D., S.A.M., M.C.N. and C.P.O. conceptualized and designed the study, and acquired funding. S.L.D. was responsible for the reaction-set design, S.L.D. and C.P.O. were responsible for gBlock design. S.L.D. and L.v.d.M. performed the experiments and the qPCR analysis. S.L.D. performed the statistical analysis. S.L.D. wrote the initial manuscript and all authors reviewed, contributed to, and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Supplementary Material

Optimization of a high-throughput nanofluidic real-time PCR to detect and quantify 15 bacterial species and 92 Streptococcus pneumoniae serotypes

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Keywords: Pneumococcus (*Streptococcus pneumoniae*)$_1$, PCV (Pneumococcal Conjugate Vaccine)$_2$, Molecular Diagnostics$_3$, Infectious Disease$_4$, quantitative PCR$_5$, carriage$_6$.

Supplementary Table 1: Oligonucleotide sequences for the 'Fluidigm' qPCR reaction-set (96 assay-sets) for detection of 92 pneumococcal serotypes, 15 other bacterial and one fungal species including the use of Dual Priming Oligonucleotides (DPO; n=2), Locked Nucleic Acid probes (LNA; n=3) and internally quenched probes (n=8)

Assay-set name	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3')	Gene Target	Reference
1 †	CGTGCGGTAATTGAAGCTATGA	TGTGGCCCCAGCAACTCT	FAM-TGCTTGCCCTTGTATAGGGT-NFQ	wch*D*	Azzari *et al.* 2010 [1]
2 ‡	TTATGGACTGGCTGATGGTTCTC	AAATCCTGACCCAATAATAGCCTTT	FAM-AGGTCAACG/ZEN/TATTGGAAC TCTTAGAAATTGGGAAA-IABkFQ	wzy	Pholwat *et al.* 2016 [2]
3 †	GGTCAGCAGAAAGTATGCATTGG	TCGTTTATCCAGGGTCTGATGA	VIC-TATTGGATGTGGT TTATCGTGAAGA-NFQ	tnp	Azzari *et al.* 2010 [1]
4 †	TGGGATGACATTTCTACGCACTA	CCGTCGCTGATGCTTTATCA	FAM-TCCTATTGGATG GTTAGTTGGTGA-NFQ	wzy	Azzari *et al.* 2010 [1]
5 †	TTACGGGAGTATCTTATGTCTTTAATGG	CAGCATTCCAGTAGCCTAAAACTAGA	VIC-TTGTCTCAGCAACT CTATTTGGCTGTGGG-NFQ	wzy	Azzari *et al.* 2010 [1]
6A/B/C/D (F/G/H) †	AAGTTTGCACTAGAGTATGGGAAGGT	ACATTATGTCCRTGTCTTCGATACAAG	VIC-GTTCTGCCCTGAGCAACTGG-NFQ	wci*P*	Azzari *et al.* 2010 [1]
6A/C ‡	CATTGCTAGAGATGGTTCCTTC AGTTGATATTGATAAAGATTCG GGAGACATGTCCAAACTGGC	CGATACAAGACCAGTTGC	FAM-GTTTGCACT/ZEN/AGAGTA TGGGAAGGTGTTGT-IABkFQ	wci*P*α	Downs *et al.* 2021 [3]
6C/D †	TTGGGATGATTGGTCGTATTAG	CTCTTCAATTAGTTCTTCAGTTCG	FAM-CCACGCAATTCGCCATC-NFQ	wciN_{β}	Azzari *et al.* 2010 [1]
7A/F †	GATGGCATGTGGCAAACCA	TTTGCCCTCCTTAATCATTTCAC	FAM-TTGGCTATCGGCATGGTGGT-NFQ	wcw*H*	Azzari *et al.* 2010 [1]
7B/C/40 ‡	TCCAGATATAGTCATTCCCAATCAG	AAAGAAGGTAAATCCCATGATGAATT	FAM-TCCCTCATTATCGATTA CTGACCCACCA-BHQ1	wcx*U*	Pholwat *et al.* 2016 [2]
8 †	CCACTCATCAGTTTCCCATATGTTT	TCAATAATTGAAGAAGCGAACGTT	FAM-TGATGGCAGAT GGGTTGGGACGAG-NFQ	wzx	Azzari *et al.* 2010 [1]
9A/L/N/V ‡	TGGAATGGGCAAAGGGTAGTA	TCGGTTCCCCAAGATTTTCTC	FAM-TTAATCATGCTAACGG CTCATCGA-BHQ1	mna*A*	Olwagen *et al.* 2017 [4]
9A/V‡	AGGTATCCTATATACTGCTTTAGG	CGAATCTGCCAATATCTGAAAG	FAM-ACACATTGA CAACCGCTACA-BHQ1	wzx	Pholwat *et al.* 2016 [2]

Assay-set name	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3')	Gene Target	Reference
9L/N ‡	CGTGGAATTTTCTATACTGCAATAGG	CTACTGCTACGATACCATATTCTACAG	FAM-CAATTCTTAG CCGGATTCTCTC-BHQ1	wzx	Pholwat *et al.* 2016 [2]
10A/B †	CCTCTCCTATCAACTAT TACTCATTATACTACCT	AATAACCATAAGTCC CTAGATCATTCAAAG	VIC-TCATTACAACTCCCTA TGTGACACGGGTCTTTT-NFQ	wcr*D*	Azzari *et al.* 2010 [1]
10B †	AAATATGAGATTGGT AAGGAATATTCTGG	GTCTTTTCACTTAAACGAATTCCATTC	FAM-AACGGATTCCAATGC ACTCGGTAACT-NFQ	wcr*D*	Pholwat *et al.* 2016 [2]
10C/F †	CGAGTTATGGATGTTCTTATTGGC	CCCAACCCCACTCTGTATTG	FAM-ACAGGGCAAGACTGT GAATATTGTTCCA-NFQ	wcj*G*	Sakai *et al.* 2017 [5]
11A/B/C/ D/F/(E) †	ACCGCATTTCTTATCGCACTATATT	TCTCCTTACCATCAAACATGTTAATCA	FAM-TGAATCAGTCTGACCGTTT-NFQ	wzx	Olwagen *et al.* 2017 [4]
11A/D †	CGGCCCAGCTACATTTATGG	TGATCATTCACATGCTCACCAA	FAM-AAATACCAATAGTTGT TCCGAGATTAAAGAAGT-NFQ	wch*K*	Pholwat *et al.* 2016 [2]
11B/C †	TCAAATTTGGCGTATTGCTTATCA	TGATTATGAGCATAGTTGATCCCC	FAM-TCCGTGGCAAGATTCT GGTGCTAAG-NFQ	wzy	Sakai *et al.* 2017 [5]
11F †	TGGTCCAGCTACTTTTATGGC	TGATCATTCACATGCTCCCC	VIC-ACTCCAATAGTTGTTC CGAGGCAAAAGA-NFQ	wch*K*	Pholwat *et al.* 2016 [2]
12A/B/F/44/ 46 †	GATTATTCGCTTGCCTCTTCATG	ATAGCCGAAATAAGCTTTCCAGAA	FAM-ATTTGTAAGCG GACGTGCGATT-NFQ	mna*B*	Azzari *et al.* 2010 [1]
12B †	GGTTGCTGATCAAAAGGTCTATG	AGGTTCAAAGTAAGATTTTTAGCAA	FAM-AGATAAAAATCTTTCC AAATCATCAAAGTGA-NFQ	wzx	Pholwat *et al.* 2016 [2]
13 †	TCGGATTTAGTAGTAACCCCATTGA	TTCTTGATTGAGGATGCATTTCC	VIC-AGTAGTAAGAG ATCATATTCAAG-NFQ	wzy	Olwagen *et al.* 2017 [4]
14 †	CGACTGAAATGTCACTAGGAGAAGAT	AATACAGTCCATCAA TTACTGCAATACTC	VIC-TGTCATTCGTTTGCCA ATACTTGATGGTCTC-NFQ	wch*L*	Azzari *et al.* 2010 [1]
15A/B/C/F ‡	TTGAATCAGGTAGATTGATTTCTGCTA	CTCTAGGAATCAAAT ACTGAGTCCTAATGA	FAM-CTCCGGCTTT TGTCTTCTCTGT-BHQ1	wzx	Azzari *et al.* 2010 [1]
15A/F ‡	CGTTATTTAGTGAATTGCTATACTC	TCCCTGCAGAATAAGAATCTAC	FAM-TACtGCtGCtGCcAACA-BHQ1	wzy	Messaoudi *et al.* 2016 [6]
15B/C ‡	TTTGCTACAGGTTTTAGTATTGAG	AAAGCAATATAAGAGGTATAGTTGG	FAM-CGCtACaATcATcCGCT-BHQ1	wzy	Messaoudi *et al.* 2016 [6]

Assay-set name	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3')	Gene Target	Reference
16A †	GCTAGCAGGAACTTTTCTAGGG	TCCCTGTCCAAATCCGAAAC	FAM-CCCACGGGATGAATC CATTATGGCG-NFQ	wcx*R*	Sakai *et al.* 2017 [5]
16F †	GCAACTGGTATTTTTGATATTGGAGAA	CAAAGGAATGCCATGCCATA	FAM-AAAATGCTAAC TTCGTTGGAGG-NFQ	wzy	Olwagen *et al.* 2017 [4]
17A †	TGATTATGTCATTCGATTGCTTGG	AAATCCTAAAATTCCTGTTTGAAAAGC	FAM-ATTATGGGCGTG GGTTACCGTAGG-NFQ	wzy	Sakai *et al.* 2017 [5]
17F †	GTAAAGATTTCATGT CCTATAAGGGAGAA	AGGCGTCCCTGTTTATGAGAAG	FAM-TTGTACATGGTCTGGATTT-NFQ	Wzx	Olwagen *et al.* 2017 [4]
18A/B/C/F ‡	CCTGTTGTTATTCACGCCTTACG	TTGCACTTCTCGAATAGCCTTACTC	FAM-AACCGTTGGC CCTTGTGGTGGA-BHQ1	wzy	Azzari *et al.* 2010 [1]
18B/C/F ‡	CAGGATTTCTAACTCTGATTGAA	AGCAAAATCTAACGTCCAGAG	FAM-CTTGTATGCTTATGG TCTTTTCGATTA-BHQ1	wciX_{BCF}	Downs *et al.* 2021 [3]
18C/F ‡	CCAAATTGGAGTGTTTTACAA AGTATTAGCTCGATTTGCTGT ACACGTCGACGCTTCAATTTCAGG	TCTTTCAAATACAACT CTTAGATTTCCTTGTG	FAM-TGagtTTATTGATAATttcC-BHQ1	wciX_{CF}	Downs *et al.* 2021 [3]
18F: 16F/18F/28AF ‡	TGGTTTCGGACTCTTTCGTGG	CTAAGATAGAAACTCCTTGTCCAATG	FAM-GGTTGTACGTGGAAT CGGATTTGGTC-BHQ1	wcx*M*	Downs *et al.* 2021 [3]
19A †	TTCGACGACGTATCAGCTTCA	TCATTGAGAGCCTTAACCTCTTCA	VIC-ACCCAAAACGGTTG ACGCATTATACT-NFQ	wzy	Azzari *et al.* 2010 [1]
19B/F †	GGTCATGCGAGATACGACAGAA	TCCTCATCAGTCCCAACCAATT	VIC-ACCTGAAGGAGTAG CTGCTGGAACGTTG-NFQ	wzy	Azzari *et al.* 2010 [1]
19F †	CAGGTTCGGGAAATTGCAA	ATCTCTGCGCCATAAGCAATG	VIC-AGAAGTGGCAGATGATT-NFQ	wzh	This study
20 †	AAAGATACTGGCTGAGGAGCTATCTATT	AGTCAAAAGTACTCAA CCATTCTGATATATTC	VIC-AGGATAAGGTCTACT TTGTGGGAGTTC-NFQ	wci*L*	Azzari *et al.* 2010 [1]
21 †	CCATTTGAAGGACCAGTTGTTG	AAAAAGCCACTATCAGGAATACCAA	FAM-AATGGCATTGCTTCGTAAA-NFQ	wzy	Olwagen *et al.* 2017 [4]
22A/F †	TCTATTAAATAACCC ATTGGAATTGAAACG	TCGCAATTGAAGACCACATAAACTG	FAM-TCCGTAATTCGCTTA TGGGCACATTCTCCA-NFQ	wcw*V*	Azzari *et al.* 2010 [1]
22F ‡	GAAGATTGTCCACCTTATATCC	TCGGCACAATCAAAATATC	FAM-CGGTTATTT/ZEN/ CACAAAAGACACGGTTGG-IABkFQ	wcwA_F	Downs *et al.* 2021 [3]

Assay-set name	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3')	Gene Target	Reference
23A/B/F †	GGTGGACTTTCCGATGCAA	CACTGTCAACAAAAATGAGGTAATCTC	FAM-AAATGTCGGTATAGATAAAG-NFQ	wchV	Olwagen *et al.* 2017 [4]
23B †	TTGAAGAAATTGCTCCAGAAACAT	CCAAAAGACTAGCCTCAACCACTAA	FAM-TAGAGCTATTTATCTTT CGTGGTTTT-NFQ	wzx	Pholwat *et al.* 2016 [2]
23F †	TGCTATTTGCGATCCTGTTCAT	AGAGCCTCCGTTGTTTCGTAAA	FAM-TTTCTCCGGCA TCAAACGTTAAG-NFQ	wzy	Azzari *et al.* 2010 [1]
24A †	CTTGGAGTTGCTAATTATGGGAAG	ATCTCTTACACGTGCACACTC	FAM-CACAGCATATCGTAA AATACCCGCA-NFQ	wzx	Pholwat *et al.* 2016 [2]
24B/F ‡	TCTGAAAGTAATTAG TAAGATTAACGGAAG	TCCATCTACTTTTAAAATAGCTCCAAC	FAM-CCACAGTCCCAAAAT TGTCAGCAACC-BHQ1	wzy	Sakai *et al.* 2017 [5]
25A/F †	ATACCAACTAGAATCAGCAGGAC	AAATGGAATATCTTT TGATAATTTACTCGC	VIC-CCGCTGGACTTACTGCAATA-NFQ	wcy*E*	Pholwat *et al.* 2016 [2]
25A/F/38 †	GTCTTACGTAGAACCTCTCTGGATGA	TGGTCCTACAAGCGACATGTG	FAM-TTGCCACAGATTTGG AATATTTTGGTCGG-NFQ	wciI	Olwagen *et al.* 2017 [4]
27 †	AGCGATTTAGCGACTGATATCC	TCTCAAAATCGATCTCGCGTG	FAM-TGTGGAAGGCGT TTGAAGGTGACT-NFQ	wha*K*	Pholwat *et al.* 2016 [2]
28A/F †	CAACTACAGGTATTTTTGATATCGGAG	GTTTACTACGTTTGTGAAGCGC	FAM-AGAAAATAGTAGGTT GATTGGCGGTGCT-NFQ	wcx*P*	Sakai *et al.* 2017 [5]
29 †	TTCGAGTTGTGCCGTTTTTACA	GGCGTACCCACCTCTAAAATTTT	VIC-TGAATCCTAGTCTTTTCTCTGCG-NFQ	wcr*J*	Pholwat *et al.* 2016 [2]
31 †	GCAGAAGTTTTAAGTCACGGAC	AGCATTACAGATGTCACTAAGGG	FAM-CCCCCACGTAAAACCGCAAGG-NFQ	wzy	Pholwat *et al.* 2016 [2]
32A/F †	GTACTTCCTGTTCTAGGCTTGG	CCCAGAGGAAAATAGCGTCTC	FAM-TTGTTCAAACC CAACCACTGCTCC-NFQ	wzy	Sakai *et al.* 2017 [5]
33A/F/37 †	GGAACTGGTTCAGCAACTATACG	GGTTCTAAGACCGTCTGAAATACC	FAM-TAGGACTTTTCTGCCATGCC-NFQ	wzy	Pholwat *et al.* 2016 [2]
33B/C †	CCTGTTAGTGCACCTGTATTTAAC	GCATTCAAAACTCCTTCATCTCC	FAM-TTCGTTGTTCACGCCATTTA-NFQ	wci*N*	Pholwat *et al.* 2016 [2]
33C †	CAGAGACAGTTTCAGCAAATCTTAG	AGCCTACACCTCTTATAAACGTTG	FAM-CCGTGTCCTATCCAC AAACTTGTCTTCC-NFQ	Wzy	Sakai *et al.* 2017 [5]
33D ‡	CGTATAGTCTTGCGACATTTCA	TTCCACATGCGTTACCTCAC	FAM-CACAACTAG/ZEN/TTTTTTA TCAAAAAGACCTTGGC-IABkFQ	wci*N*	Pholwat *et al.* 2016 [2]
34/37/17A †	GGATACTATGTACGAACAGATGGACTTG	CTCACTAACTCGCCCGAATAAAC	FAM-CCGACTATACTCCATTTGA-NFQ	wci*B*	Olwagen *et al.* 2017 [4]

Assay-set name	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3')	Gene Target	Reference
34 †	CGGTGGAGTAGGTCAAGATG	GTCTGTTCTCCCCAATATACTGAG	FAM-ACGGAGCGCCAATG TACTTGAATAGTT-NFQ	Wzy	Pholwat *et al.* 2016 [2]
35A/C/42 †	TGTTTCAAGCTTCCCCTTTAGA	AAATGAAATCAAAGTATCACGTATCG	FAM-TTCAAAATACCCAG GACACCCGTTCA-NFQ	wcr*K*	Pholwat *et al.* 2016 [2]
35B †	GCATGGAGGTGGAGCATACA	TGTAAAGACTGCACAACTCGATATAAAA	FAM-CAATTTAAACAATATTAG TAAAGCGCAGGTCAAGCAAA-NFQ	wcr*J*	Azzari *et al.* 2010 [1]
35F/47F †	GTGGTCGTATATACT TGATGAATAAATCG	ACATACAAATTATCA ACATACAGATAGGTC	FAM-TTCAACTGGTCGTCCGAATA-NFQ	Wzy	Pholwat *et al.* 2016 [2]
36 †	CTTGTCTATTCAGCCCTTCTGG	CGCGATTATATTGTAAATTGGGAACT	VIC-AGAATGCCCGCTACAATGAG-NFQ	Wzy	Pholwat *et al.* 2016 [2]
39 †	CAAAAAAATGAACTA ACTCAAATAGTAACG	ATACTGTAATTTTCTTGTTTATTTGCGG	FAM-AAGTCAGGCGTATTC TTCACAAGGGAAA-NFQ	wcr*G*	Pholwat *et al.* 2016 [2]
41A †	GCAAATAGATGTATCCCAGTTAACAC	GGTAGCTCTTTTGGTTTAATGTCC	FAM-CGACCGAATAGTCT AGCTTCAAAGG-NFQ	wci*B*	Pholwat *et al.* 2016 [2]
41F †	TTTTTGGGAGGAAGTGCTTTT	AACCGCTTTCTCATGATTCATAACT	FAM-CTTCTGTGCTA ACAGTGGAGAT-NFQ	Wzx	Pholwat *et al.* 2016 [2]
43 †	AGAGGCTACATCAAATAGTTGGC	GAATCACACCGTAACTTCCAAAG	FAM-TCCAATAGTACTCA CCCCTACCGAGC-NFQ	Wzx	Pholwat *et al.* 2016 [2]
45 †	TCTAGCTACTTGACTA AAATATTTGAACTG	GACGAGTCGATTTCGCTGTAT	VIC-CTTTTAGTGACCTCGCTCCC-NFQ	Wzy	Pholwat *et al.* 2016 [2]
46 †	CGAAGTTTTTATATCTCTATTGGTTTG	TATCCCAGGAACTGGACGAA	FAM-TCATTCTTTCTTCAAT TCCTTTCTGA-NFQ	Wzy	Sakai *et al.*2015 [7]
47A/F †	AGGAATTGGTAGAGAGTTTGTGG	GAAAGTTGAACCATCATCCGTC	FAM-CACTTGATGGA ATGCCTGCTGCC-NFQ	whaI	Pholwat *et al.* 2016 [2]
48 †	CAGGTTTTGCTTCATATGGGAG	ATCGGCCAAAAGTTATCATTAGC	FAM-CGCTGCTTATGTGTA TTACTCTCCCCTG-NFQ	Wzy	Sakai *et al.* 2017 [5]
Acintobacter baumanii †	TTTAGCTCGTCGTATTGGACT	CCTCTTGCTGAGGAGTAATTTT	FAM-TGGCAATGCAG ATATCGGTACCCA-NFQ	blaOXA -51-like	Gadsby *et al.*, 2015 [8]
Bordetella holmesii †	GGCGACAGCGAGACAGAATC	GCCGCCTTGGCTCACTT	FAM-CGTGCAGATAGGCTT TTAGCTTGAGCGC-NFQ	hIS*1001*	Tatti *et al.*, 2011 [9]

Assay-set name	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3')	Gene Target	Reference
B. parapertussis †	TCGAACGCGTGGAATGG	GGCCGTTGGCTTCAAATAGA	FAM-AGACCCAGGGCGCACGCTGTC-NFQ	pIS*1001*	Tatti *et al.*, 2011 [9]
B. pertussis/ Holmesii †	CAAGGCCGAACGCTTCAT	GAGTTCTGGTAGGTGTGAGCGTAA	FAM-CAGTCGGCCTT GCGTGAGTGGG-NFQ	IS*481*	Tatti *et al.*, 2011 [9]
B. pertussis/ bronchiseptica/ parapertussis †	CGCCAGCTCGTACTTC	GATACGGCCGGCATT	FAM-AATACGTCGAC ACTTATGGCGA-NFQ	ptx*S1*	Tatti *et al.*, 2011 [9]
Escherichia coli †	GTCCAAAGCGGCGATTTG	CAGGCCAGAAGTTCTTTTTCCA	FAM-ACGGCAGAGAAGGTA-NFQ	uid*A*	Lee *et al.*, 2006 [10]
Haemophilus influenzae †	CTCAGTTTCGTTTTATTACCA	CCAGTAACAACAAGGCTA	FAM-CGCATTCTTCTTCGTC CATAACCTTC-NFQ	BexB	This Study
H. influenzae †	CTGAATTRGGYGATTATCTTTATGA	ACAATCAAAYTCAACHGAAAGHGA	FAM-AGGGATGAAA GCYCGRCTTGCAT-NFQ	BexA	Maaroufi *et al*,. 2007 [11]
H. influenzae †	CAAAATTGCCAAGATTAAATGCTT	TGCTCGCCATACTGCACAA	FAM-CCTGCGGTTAAACC-NFQ	IgA1	Olwagen *et al.* 2017 [4]
H. influenzae, type b †	TGTTCGCCATAACTTCATCTTAGC	CTTACGCTTCTATCTCGGTGATTAATAA	FAM-CACAAAACTTCTCAT TCTTCGAGCCTA-NFQ	Bcs3`	Maaroufi *et al.*, 2007 [11]
Klebsiella pneumoniae ‡	AGGCCGAATATGACGAAT	GGTGATCTGCTCATGAA	FAM-ACTACCGT CACCCGCCACA-BHQ1	glt*A*	Gadsby *et al.*, 2015 [8]
Morexella catarrhalis †	CCGCTTTTACAACCACTGCTT	TGTATCGCCTGCCAAGACAA	FAM-CAGCTGTTAGCCAGCC-NFQ	tonB	Olwagen *et al.* 2017 [4]
Neiserria Lactamica †	TTGCCCGAGAACCATTGTATC	GCGGTTCTTATCACGTTCTATATTTG	FAM-TATTGGAGCGGACTAAA-NFQ	lacZ	Olwagen *et al.* 2017 [4]
Neiserria meningitidis †	GCACACTTAGGTGATTTACCTGCAT	CCACCCGTGTGGATCATAATAGA	FAM-CATGATGGCACAGCA ACAAATCCTGTTT-NFQ	SodC	Dolan Thomas *et al.*, 2011 [12]
Pneumocystis jiroveci ‡	CCATCACATCTACGATTAC	GAACGAAATAACCATTGC	FAM-ACTCACATC AACGAGGCGGT BHQ1	Msg-A1	Jensen, 2002 (Modified) [13]
Staphylooccus aureus †	GCTCAGCAAATGCATCACAAA	CACTATATACTGTTGGATCTTCAGAACCA	FAM-AGATAACGGCGTAAATA-NFQ	Tfp	Olwagen *et al.* 2017 [4]
Streptococcus pneumoniae ‡	TCTTACGCAATCTAGCAGATGAAGC	GTTGTTTGGTTGGTTATTCGTGC	FAM-TTTGCCGAAAACG CTTGATACAGGG- BHQ1	LytA	McAvin *et al.*, 2001 [14]
S. pneumoniae ‡	AGCAGGTGACTGGTAGGTAAC	CTCCTAATGCTGCTCC	FAM-CAGTTGCTT/ZEN/GCG GTGCACTTG-IABkFQ	Xisco	This Study

Assay-set name	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3')	Gene Target	Reference
S. pneumoniae †	AGCGATAGCTTTCTCCAAGTGG	CTTAGCCAACAAATCGTTTACCG	FAM-ACCCCAGCAAT TCAAGTGTTCGCG-NFQ	Ply	Greiner *et al.*2001 [15]
S. pneumoniae ‡	CATTGGTGGCTTAGTAAGTGCAA	TACTAACACAAGTTC CTGATAAGGCAAGT	FAM-TGTAAGCGG/ZEN/AAAAG CAGGCCTTACCC-IABkFQ	PiaB	Trzciński *et al.*2013 [16]
S. pyogenes †	GCACTCGCTACTATTTCTTACCTCAA	GTCACAATGTCTTGGAAACCAGTAAT	FAM-CCGCAACTCATCAAG GATTTCTGTTACCA-NFQ	Spy	CDC 2008; Kodani, 2011 [17]
S. algalactidae †	GAACTCTAGTGGCTGGTGCATTG	GGAGTTGTCACTTGATCAGCATGT	FAM-ATTTTCACCAGCTGTATTAG-NFQ	Cfb	CDC [18]
S. oralis ‡	ACCAGCAGATACGAAAGAAGCAT	AGGTTCGGGCAAGCGATCTTTCT	FAM-AAGGCTGCT/ZEN/GTT GCTGAAGAAGT-IABkFQ	gtf*R*	Alvarez, 2013 [19]
Bacterial 16S ribosomal RNA gene ‡	TCCTACGGGAGGCAGCAGT	GGACTACCAGGGTATCTAATCCTGTT	FAM-CGTATTACC/ZEN/GCG GCTGCTGGCAC-IABkFQ	16S	Nadkarni *et al.*, 2002 [20]

Note: † Ordered from Thermofisher; ‡ ordered from Integrated DNA Technologies; NFQ is Nonfluorescent quencher; IABkFQ is an Iowa Black Fluorescent Quencher; BHQ1 is Black Hole Quencher 1.

Supplementary Table 2: External synthetic calibrator (gBlock) properties.

gBlock™ name	Assay-Set Targets †	Length (bp)	Amount (ng/µl) ‡	Gene equivalents (Copy number)
A1: Pool A gBlock, no. 1	1, 4, 5, 6A/B/C/D, 33D, LytA, 19F (Atypical), 7A/F, 8, 9A/L/N/V	1088	8,09	$1,36 \times 10^{10}$
A2: Pool A gBlock, no. 2	10C/F, 11A/D, 14, 20, Hib, 16F, 22F, 15B/C, 18A/B/C, 19B/F	1147	6,73	$1,07 \times 10^{10}$
A3: Pool A gBlock, no. 3	11F, 24A, 28A/F, 17A, 35F/47F, 41F, 12A/B/F/44/46, 23A/B/F, Eco, Sag, hIS1001, Nme	1225	8,53	$1,27 \times 10^{10}$
B1: Pool B gBlock, no. 1	3, 6A/C, 7B/C/40, 9A/V, 10B, 11A/B/C/E/F, 12B, 13, 15A/B/C/F, 16A, 34/37/17A, 18B/C/F, 19C	1474	3,82	$4,73 \times 10^{9}$
B2: Pool B gBlock, no. 2	19F, 22A/F, 23F, 25A/F, 33C, 35B, 46, 47A/F, Xis	1023	7,71	$1,38 \times 10^{10}$
B3: Pool B gBlock, no. 3	BexA, Nla, Mca, Spy, Sau, 16S, PiaB, PtxS1, plS1001, IS481, Sor	1122	5,13	$8,34 \times 10^{9}$
C1: Pool C gBlock, no. 1	15A/F, Ply, 11B/C, 16F/18F/28A/F, 17F, 19A, 21, 23B, 24B/F, 25A/F/38, 27, 29	1284	7,04	$1,00 \times 10^{10}$
C2: Pool C gBlock no. 2	31, 32A/F, 33A/F/37, 33B, 34, 35A/C/42, 45, 48, 2, 10A, 9L/N	1252	5,28	$7,70 \times 10^{9}$
C3: Pool C gBlock no 3	Aba, Kpn, 23A, BexB, IgA1, Pji, 36, 39, 41A, 43, 6C/D.	1160	4,11	$6,47 \times 10^{9}$

† Listed in order of position on gBlock; ‡ Average value of 3 successive Q-bit measurements

Supplementary Table 3: External synthetic calibrator (gBlock) sequences

gBlock name	gBlock sequence
A1	TACCAGACGATGCTTATCAATGACCCCTATATGTGTGATATCGTGCGGTAATTGAAGCTATGAGTTGTGCTTGCCCTTGTATAGGGTCTCCAGAGTTGCTGGGGCCACATCACCGCAGGTATCCCGTCGCCGGTTGGGATGACATTTCTACGCACTATCTTTCCTATTGGATGGTTAGTTGGTGATA GTTGATAAAGCATCAGCGACGGGGGTTGCTCTGGGTGCCATGAACTATTACGGGAGTATCTTATGTCTTTAATGGTTTGTCTCAGCAACTCTATTTGGCTGTGGGAATTCTAGTTTTAGGCTACTGGAATGCTGGCAGCGACGACCACCACACACACATGCAAGTTTGCACTAGAGTATGGGAAG GTGTTGTTCTGCCCTGAGCAACTGGTGCTACTTGTATCGAAGACATGGACATAATGTTAACCTATGAACTCGATCAGAGAACGTATAGTCTTGCGACATTTCATCCACAACTAGTTTTTTATCAAAAAGACCTTGGCGAGGTGAGGTAACGCATGTGGAACATACCAAGCATCTCCATCTAAGTTC TTACGCAATCTAGCAGATGAAGCTATTTGCCGAAAACGCTTGATACAGGGTATGCACGAATAACCAACCAAACAACTATTAATATGGTATATTTATGTCGCGTCCTTAGTTCTGGTTATTCGGGAATCCAGTTATGAAGGTGAGCTAACAGTGCGACACTTCGATTCGGTTCCTCATCCGCCCTAC CGACAGTGGTACTAGAGGCGATGGCATGTGGCAAACCATAGTTGGCTATCGGCATGGTGGTGTTGTGAAATGATTAAGGAGGGCAAAAACGGTCTCCTAGCCACTCCAGCTATTCCCACTCATCAGTTTCCCATATGTTTCATTCTGATGGCAGATGGGTTGGGACGAGTAACGTTCGCTTCTTC AATTATTGATTGCCCACGCAATGGGGATGCTGGAATGGGCAAAGGGTAGTAAGTTAATCATGCTAACGGCTCATCGACGAGAAAATCTTGGGGAACCGATGGAGAGCCCGAGTTAAGGTGGAGCTCAAGTAAATGGTCTACCGACACTACTCTCAGAAG
A2	TTCCACGGCTCAAGTAAATGGTCTTGATTAGTAAGGCAGGATGCATAAACCAGGAGGTACCGAGTTATGGATGTTCTTATTGGCTTGTGGAACAATATTCACAGTCTTCGCCCTGTTCACAATACAGAGTGGGGTTGGGAGGGTAGATGTTATCATACACATGGCGGCCCAGCTACATTTATGGCA GTTATTGCTAAAGGAAAAATACCAATAGTTGTTCCGAGATTAAAGAAGTATTGGTGAGCATGTGAATGATCATCAGATGGATTGATACAACCCATGGCGACTGAAATGTCACTAGGAGAAGATATGTCATTCGTTTGCCAATACTTGATGGTCTCAGAGTATTGCAGTAATTGATGGACTGTATT ATACCATGTGCCATCCACAGTTCGTAAAAGATACTGGCTGAGGAGCTATCTATTCAGGATAAGGTCTACTTTGTGGGAGTTCAGTCGAATATATCAGAATGGTTGAGTACTTTTGACTCCGTCTGCTGATGATTGCGCATGGGTACATCTGTTCGCCATAACTTCATCTTAGCACCACAAAACTTC TCATTCTTCGAGCCTAATTATTAATCACCGAGATAGAAGCGTAAGGGGGTACTAGAGTAGATCGACAATGGACATTTTCCAAATGAAGATGTCCACCTTATATCCAACAGAACGGTAGATATTTTGATTGTGCCGAAAGGTTTAGGAATAGCTGCATTAGAGCAACTGGTATTTTTGATATTGGA GAACAAAATGCTAACTTCGTTGGAGGTGTATGGCATGGCATTCCTTTGGGATTCTTGTTACCGTTAATTAGTTTGCTACAGGTTTTAGTATTGAGAAAGCGGATGATTGTAGCGATCCAACTATACCTCTTATATTGCTTTATGAATACTATTACTGGAACGCCTGTTGTTATTCACGCCTTACGTA ACCGTTGGCCCTTGTGGTGGATGGAGTAAGGCTATTCGAGAAGTGCAACTTGAAGATGAAGCCTGTGGTCATGCGAGATACGACAGAAAGACCTGAAGGAGTAGCTGCTGGAACGTTGAAATTGGTTGGGACTGATGAGGAGACTAAATCACTCACAAAGAAAATTTAACGCTTGATGTGATG ACCCCATAGTGTTCGAGGTCATTCCGAGTTATCCAG
A3	GATGACCTTCGTGGCGTGCTAGTATGCATAGTGTTCGAGATGACCTGCTTCATGCCATGTGGTCCAGCTACTTTTATGGCTACTCCAATAGTTGTTCCGAGGCAAAAGAATGGGGAGCATGTGAATGATCATCTTGGAGTTGCTAATTATGGGAAGTCTGCGGGTATTTTACGATATGCTGTGCAG AGTGTGCACGTGTAAGAGATGATGAGGCATGCATATTAGTCAATGCAACTACAGGTATTTTTGATATCGGAGTAGAAAATAGTAGGTTGATTGGCGGTGCTCAGCGCTTCACAAACGTAGTAAACTAGTGAGTACGGGGTCTAGGCATCTGATTATGTCATTCGATTGCTTGGAACCTACGGTAA CCCACGCCCATAATGAGCTTTTCAAACAGGAATTTTAGGATTTGCATATTGTTGGATATAGGTGGTCGTATATACTTGATGAATAAATCGGATTATTCGGACGACCAGTTGAACAGACCTATCTGTATGTTGATAATTTGTATGTCTCGGCTCTAGGAATAATGCTCCTAGTTTTTGGGAGGAAGTG CTTTTACTTCTGTGCTAACAGTGGAGATATAGTTATGAATCATGAGAAAGCGGTTGCATTGATACGAAGAGAAGCATCGAAGATTATTCGCTTGCCTCTTCATGGATTTGTAAGCGGACGTGCGATTGTTCTGGAAAGCTTATTTCGGCTATTAAGGGTCTCCAGAGGTATTGTAGGTGGACTTTC CGATGCAAGAAATGTCGGTATAGATAAAGCGGATTACCTCATTTTTGTTGACAGTGTCATGAACGGTTGGTTATGTCCAAAGCGGCGATTTGGACGGCAGAGAAGGTACTGGAAAAAGAACTTCTGGCCTGGCAGGAGACTGCATCAGCTATCCTGGAACTCTAGTGGCTGGTGCATTGTAT TTTCACCAGCTGTATTAGGTACATGCTGATCAAGTGACAACTCCAGGGCCCTCGATGCTGGGAGCCGGTGGCGACAGCGAGACAGAATCCCGTGCAGATAGGCTTTTAGCTTGAGCGCGAAGTGAGCCAAGGCGGCCACATGGTTACCCATGGCGATGATGCACACTTAGGTGATTTACCTGCA TTCATGATGGCACAGCAACAAATCCTGTTTTCTCTATTATGATCCACACGGGTGGCACGATTATGCTAGTCATCACCGAATACGGTGTGGATACGTTAGCGTATGCGGCCTGCA
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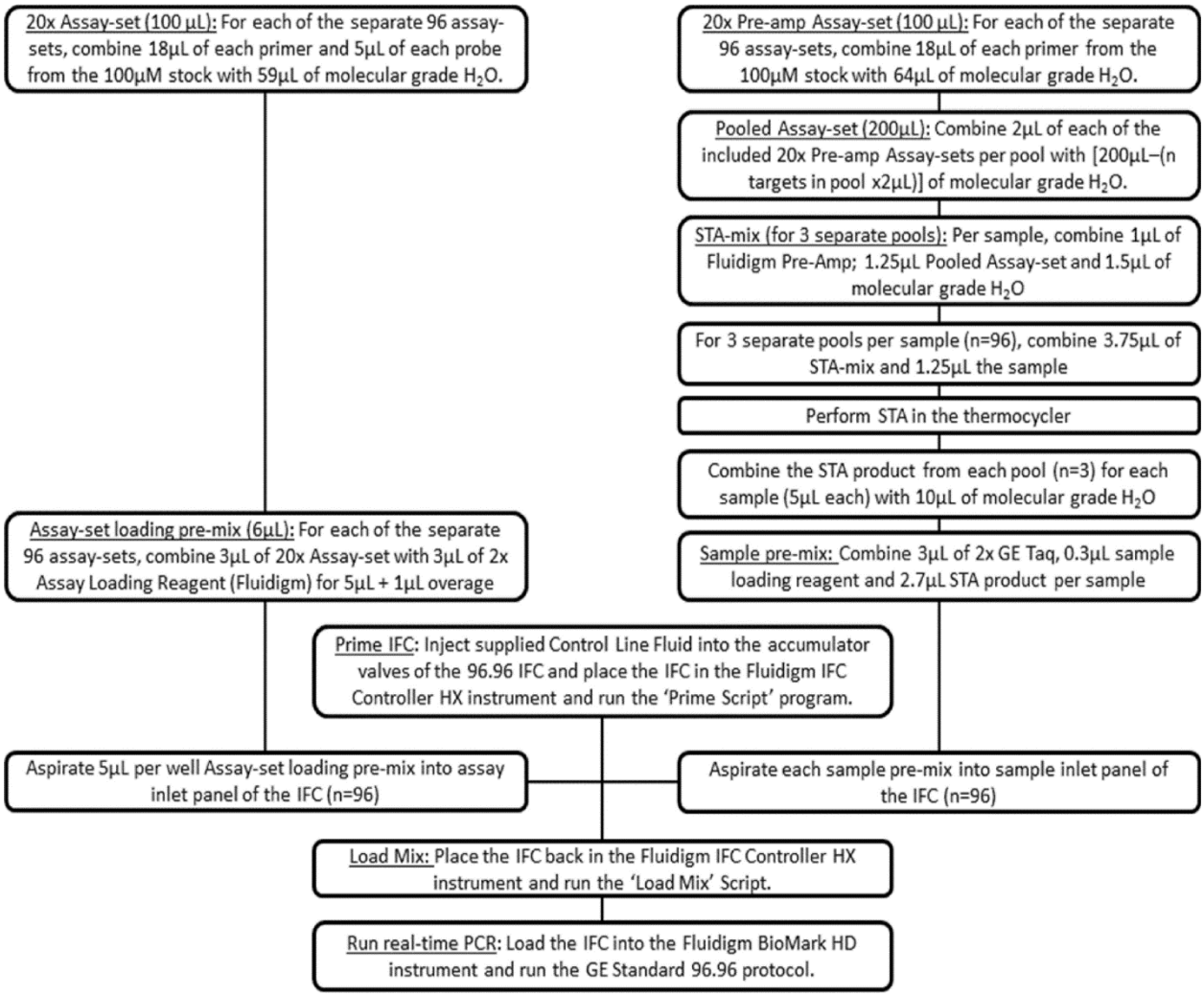
C1	TGATTCAACTGTTCTAATTACCGCTCCAAGAGCATTTACAAACATAGGTCGGTCATTTTATTAGCAGAAGTTTTAAGTCACGGACATCCTTGCGGTTTTACGTGGGGGGACCCTTAGTGACATCTGTAATGCTGAGAAGGCTCTCGGAGGATTGTGTACTTCCTGTTCTAGGCTTGGTGGAGCAGT GGTTGGGTTTGAACAAGAGAGACGCTATTTTCCTCTGGGAAGTGGTTTTGCTACATCTGGAACTGGTTCAGCAACTATACGCTGGCATGGCAGAAAAGTCTAAGGTATTTCAGACGGTCTTAGAACCTGTCCAATGAAGAGCAAGACTTGACAGTCCTGTTAGTGCACCTGTATTTAACGATAA ATGGCGTGAACAACGAATAGGAGATGAAGGAGTTTTGAATGCTGTTCTGGAAGGACATGTCTTGATTGTCCGGTGGAGTAGGTCAAGATGGGAAACTATTCAAGTACATTGGCGCTCCGTTACTCAGTATATTGGGGAGAACAGACACCCTAGCTATGGGATGAACCTGTGTTTCAAGCTTCCCCT TTAGACTTTCAAAATACCCAGGACACCCGTTCAACGATACGTGATACTTTGATTTCATTTTTTGTTATGGGTGGGGAGATGAGATATTGATCTAGCTACTTGACTAAAATATTTGAACTGCTAGGGAGCGAGGTCACTAAAAGTCATACAGCGAAATCGACTCGTCTACCAAGATAGGGAGAGCA GCATTAGGGGCAGGTTTTGCTTCATATGGGAGCAACGCTGCTTATGTGTATTACTCTCCCCTGTAGCTAATGATAACTTTTGGCCGATTTTGACAACGGGGCGTGCGTTATCGGTAACGATTTGGACTCGTCTATTGTACCAAGATAGGGAGAGCA AAATAAAGGCTATTATTGGGTCAGGATTTATGCACAGTTTGGTGGTTCTATATCCTCTCCTATCAACTATTACTCATTATACTACCTATCATTACAACTCCCTATGTGACACGGGTCTTTTCTTTGAATGATCTAGGGACTTATGGTTATTCTGATGTTCATGGCTATTATGAAGCGTGGAATTTTCT ATACTGCAATAGGGCAATTCTTAGCCGGATTCTCTCACCTGTAGAATATGGTATCGTAGCAGTAGGATTCAATGTACAAGTGGTATCATTATAGTGGGGGTGTTATACGGCGAAACTATTTATGTTAGTTTTCCGC
C3	GAGCTCATGTCTAAGTGAAGTGGTCGATGGTGGGTCCTGTTCCGCTATTCCATCAAGATTTAGCTCGTCGTATTGGACTTTGGCAATGCAGATATCGGTACCCAGAAAATTACTCCTCAGCAAGAGGCACAGTTTGCTCCATTACAACCCAGGCCGAATATGACGAATTAACTACCGTCACCCGCC ACAGATTCATGAGCAGATCACCCGTCTGTTCCACGCGTTAGTGCGTTAAGTACTCCCCTCCATTACCCATTTGGTATGGGAGTAGGGAGTGTGGGAGAGGTTCACAAACAGCACTTTCTTCAGAGGAGGAATTGGAATGATAATTGCAACACTCAGTTTCGTTTTATTACCAAGCGCATTCTTCTT CGTCCATAACCTTCTCTAGCCTTGTTGTTACTGGAAACTGATCATAATGCGAAATTTGCTCGCCATACTGCACAATTGGTTTAACCGCAGGCAAAGCATTTAATCTTGGCAATTTTGGGGTTACACAGACATCGACACACACGAGTACATCTACCATCACATCTACGATTACATACTCACATCAAC GAGGCGGTGTAGCAATGGTTATTTCGTTCTTTGCTAATCAGGCAGACTGGCCTTGTCTATTCAGCCCTTCTGGTCTCATTGTAGCGGGCATTCTTGAGTTCCCAATTTACAATATAATCGCGAAGGAGAACATAACTATGGAGGACAAAAAAATGAACTAACTCAAATAGTAACGTAAGTCAGGC GTATTCTTCACAAGGAAATACCGCAAATAAACAAGAAAATTACAGTATGTATCTCTTAAGGTTGAATAGAAAGTTTTGAGCAAATAGATGTATCCCAGTTAACACTACCTTTGAAGCTAGACTATTCGGTCGTGGGACATTAAACCAAAAGAGCTACCGAAGTTGAACTGCGAATATTTAAAG TTGAAAGAGGCTACATCAAATAGTTGGCGAGCTCGGTAGGGGTGAGTACTATTGGAGTGCTTTGGAAGTTACGGTGTGATTCAGTAGTAATTAGTATGGGACAGTTGGGATGATTGGTCGTATTAGTAGATGGCGAATTGCGTGGATCGAACTGAAGAACTAATTGAAGAGGTACATTACGCCA GACCGCCATGGCATACAGTGGTTACTTACGAAAGTATAGGCTTTTTGGT

Supplementary Table 4: Assay-set pools for specific target amplification/Pre-Amplification (Pre-Amp)

Pool A (n=32)	Pool B (n=32)	Pool C (n=34)
1	3	2
4	6A/C	6C/D
5	7B/C, 40	9L/N
6A/B/C/D(F/G/H)	9A/V	10A
7A/F	10B	11B/C
8	11A/B/C/D/F/(E)	15A/F
9A/L/N/V	12B	16F, 18F, 28A/F
10C/F	13	17F
11A/D	15A/B/C/F	19A
11F	16A	21
12A/B/F, 44, 46	34, 37, 17A	23A
14	18B/C/F	23B
16F	19F	24B/F
15B/C	22A/F	25A/F, 38
18A/B/C	23F	27
19B/F	25A/F	29
20	33C	31
22F	35B	32A/F
23A/B/F	46	33A/F, 37
24A	47A/F	33B
28A/F	Pia*B*	34
33D	Bex*A*	35A/C, 42
17A	IS481	36
35F, 47F	Ptx*S1*	39
41F	pIS1001	41A
Eco	*Mca*	43
Lyt*A*	*Nla*	45
Saq	*Sau*	48
Hin-b	*Spy*	IgA1
hIS1001	Xisco	Ply
Nme	16S	Bex*B*
-	*Sor*	*Pji*
-	-	*Aba*
-	-	*Kpn*



Supplementary Figure 1: Flow diagram of the Specific Target Amplification (STA) within single 0.6ml PCR tubes in the Bio-Rad T100 Thermal Cycler and the nano-fluidic high throughput real-time PCR within the 96.96 Gene Expression (GE) Dynamic Array integrated fluidic circuit (IFC) carried out in the IFC Controller HX and the BioMark HD (Standard BioTools 'Fluidigm').

Supplementary Table 5: Analytical performance of 5 assay-sets not well optimized in the high-throughput BioMark HD nano-fluidic real-time PCR ('Fluidigm')

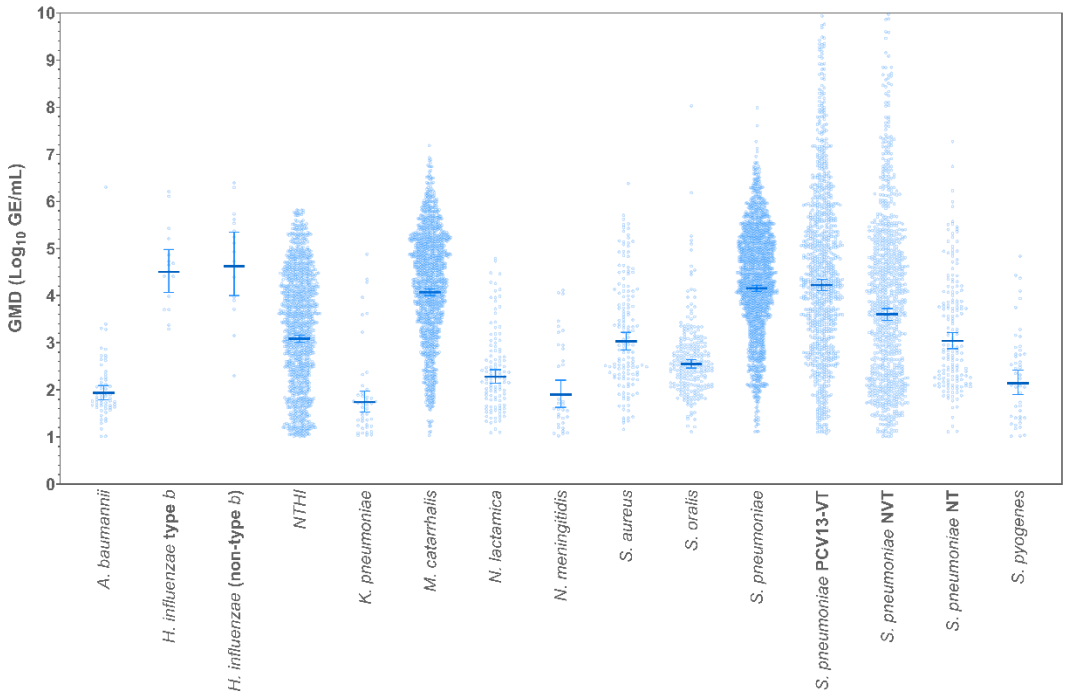
Assay-set	Calibrator	Efficiency (%) [-1+10$^{(-1/m)}$]	LOD (g-Block)	Linear equation	R^2
Bacterial 16*S*	g-Block	205	10^1	y=-2,0649x+23,561	0.91
Streptococcus pneumoniae (Xisco)	St 4	89	10^2	y=-3.6201x+27.495	0.99
Pneumocystis jiroveci	g-Block	91	10^2	y=-3.5599x+31.266	0.94
Streptococcus algalactiae (GBS)	GBS	113	10^2	y=-3.0364x+24.213	0.99
Escherichia coli	*E.coli*	131	10^2	y=-2.7535x+21.717	0.99

Supplementary Table 6: Geometric Mean Density (GMD) of bacterial and pneumococcal serotype targets quantified using the high-throughput BioMark HD nano-fluidic real-time PCR ('Fluidigm' qPCR)

Target	GMD Log_{10} Gene Equivalents (GE)/mL (95%CI)
Overall *S. pneumoniae*	4.2 (4.1-4.2)
Sum of serotype density	4.9 (4.7-5.0)
Lyt*A*	4.4 (4.4-4.5)
Pia*B*	4.2 (4.1-4.2)
PCV7-VT	4.1 (4.0-4.2)
PCV13-VT	4.2 (4.0-4.3)
NVT	3.2 (3.1-3.4)
NT	3.0 (2.9-3.2)
1	1.8 (1.3-2.7)
3	1.7 (1.4-2.0)
4	3.2 (2.9-3.6)
5	1.8 (1.7-2.0)
6A	5.2 (5.0-5.4)
6B	4.2 (4.1-4.4)
7AF	2.1 (1.6-2.7)
9AV	2.9 (2.5-3.4)
14	3.3 (3.1-3.6)
18C	3.1 (2.5-3.8)
19A	2.6 (2.3-2.8)
19F	4.0 (3.8-4.2)
23F	3.8 (3.6-3.9)
2	1.7 (0.2-13.0)
6C	4.7 (3.3-6.7)
6D	6.3
7BC40	3.9 (3.3-4.5)
8	2.5 (1.8-3.5)
9LN	4.3 (3.8-4.9)
9like	1.7 (1.5-2.0)
10A	2.6 (2.0-3.5)
10B	0.9 (0.7-1.1)

Target	GMD Log_{10} Gene Equivalents (GE)/mL (95%CI)
10CF	2.6 (2.3-3.0)
11AD	3.9 (3.6-4.2)
11BC	3.1 (1.2-7.8)
11F	-
12AF44	0.9 (0.6-1.3)
12B	-
13	2.9 (2.5-3.4)
15AF	3.5 (3.1-4.0)
15BC	4.5 (4.3-4.8)
15like	2.0 (1.9-2.2)
16A	1.4 (1.0-2.1)
16F	3.7 (3.4-4.0)
17A	-
17F	3.3 (2.9-3.8)
18F	-
18A	0.7 (0.5-0.9)
18B	2.1 (1.5-2.9)
19B	0.4 (0.3-0.7)
20	2.7 (2.3-3.3)
21	4.2 (3.4-5.0)
22A	1.8 (1.7-1.9)
22F	5.8 (4.7-7.1)
23A	3.3 (3.0-3.7)
23B	4.1 (3.9-4.4)
24A	2.5 (1.7-3.7)
24BF	1.4
25AF	-
27	1.8 (1.0-3.2)
28AF	-
29	2.1 (1.8-2.4)
31	1.9 (1.3-2.8)
32AF	1.1 (0.6-2.2)
33AF	1.2 (0.8-1.7)
33B	1.9 (1.6-2.2)
33C	1.9 (1.2-3.1)
33D	-
34	3.4 (3.1-3.7)
35AC42	2.9 (2.4-3.4)
35B	3.1 (2.6-3.7)
35F	2.0 (1.5-2.5)
36	1.9 (1.3-2.9)
37	-
38	-
39	0.5 (0.4-0.7)

Target	GMD Log_{10} Gene Equivalents (GE)/mL (95%CI)
41A	1.6 (0.6-4.7)
41F	0.6 (0.4-0.9)
43	2.8 (2.3-3.4)
45	1.7 (1.5-2.0)
46	3.1 (2.6-3.7)
47F	1.6 (0.3-8.0)
47A	1.2 (1.0-1.3)
48	1.4 (0.9-2.1)
A. baumannii	1.9 (1.7-2.0)
K. pneumoniae	1.1 (0.9-1.4)
M. catarrhalis	4.1 (4.0-4.1)
N. lactamica	2.2 (2.1-2.4)
N. meningitidis	1.2 (0.9-1.5)
S. aureus	3.0 (2.9-3.2)
S. oralis	2.5 (2.5-2.6)
S. pyogenes	1.6 (1.4-1.9)
H. influenzae type b	4.5 (4.1-5.0)
H. influenzae (non-type b)	4.6 (4.0-5.3)
NTHI	2.5 (2.4-2.6)
B. pertussis	-
B. holmesii	5.2 (4.1-6.4)
B. parapertussis/bronchiseptica	1.5 (0.5-4.6)



Supplementary Figure 2: Geometric Mean Density (GMD) measured in Log10 Gene Equivalents (GE)/mL.

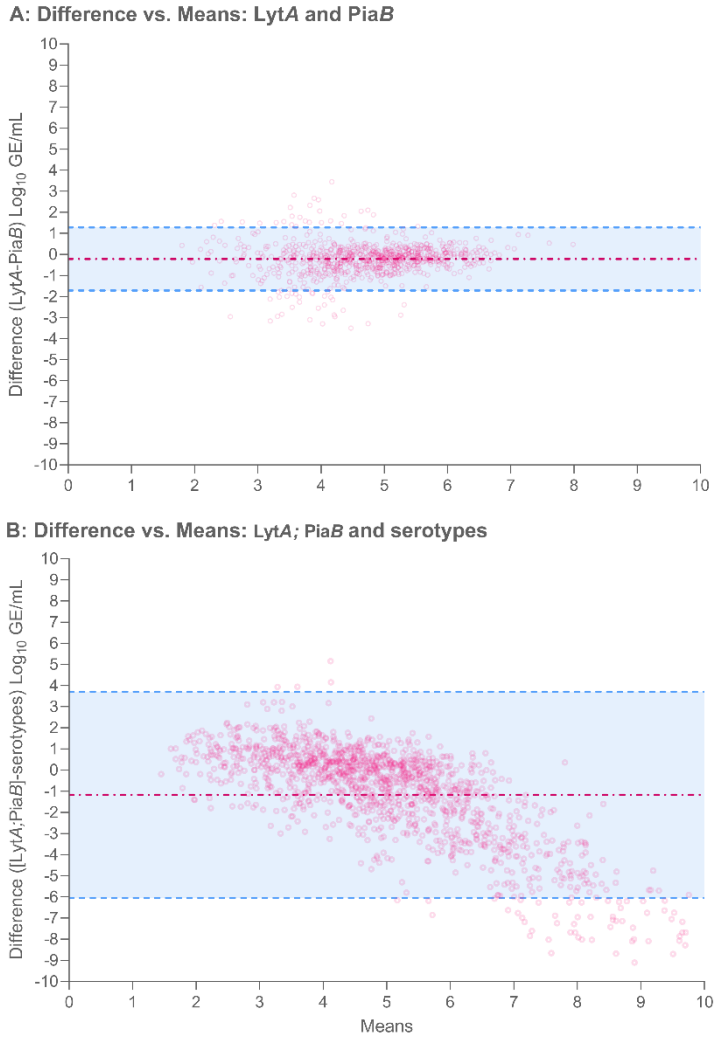
GMD was determined using 'Fluidigm' qPCR. Error Bars represent 95% Confidence intervals. PCv13VT include vaccine serotypes (1, 3, 4, 5,6A, 6B, 7A/F, 9A/V, 14, 18C, 19A, 19F and 23F); NVT, all serotypes/serogroups not included in PCV13. NT, non-typeable S. pneumoniae.

Supplementary Table 7: Hierarchy of co-colonising pneumococcal serotypes detected in Nasopharyngeal Swab samples collected from Rural (Agincourt, 2009) and Urban (Soweto, 2010) children 0-60 months-of-age.

Serogroup/type	**Primary Coloniser % (n/N)** †	**Co-Colonisation % (n/N)** †	**Second Serotype % (n/N)** †	**Third Serotype % (n/N)** †	**≥ Four Serotypes % (n/N)** †
PCV7 VT	42.6 (302/709)	**57.4 (407/709)**	47.4 (336/709)	8.5 (60/709)	1.6 (11/709)
Additional PCV13 VT	26.7 (96/359)	**73.3 (263/359)**	42.9 (154/359)	23.1 (83/359)	7.2 (26/359)
PCV13 VT	43.8 (425/971)	**56.2 (546/971)**	48.6 (472/971)	7.6 (74/971)	
NVT	**67.1 (621/926)**	32.9 (305/926)	10.0 (93/926)	20.8 (193/926)	2.1 (19/926)
NT	**50.6 (85/168)**	49.4 (83/168)	49.4 (83/168)		
1	23.1 (3/13)	**76.9 (10/13)**	30.8 (4/13)	46.2 (6/13)	
3	33.3 (17/51)	**66.7 (34/51)**	47.1 (24/51)	15.7 (8/51)	3.9 (2/51)
4	51.7 (15/29)	48.3 (14/29)	37.9 (11/29)	10.3 (3/29)	
5	23.2 (13/56)	**76.8 (43/56)**	42.9 (24/56)	17.9 (10/56)	16.1 (9/56)
6A	**93.7 (133/142)**	6.3 (9/142)	6.3 (9/142)		
6B	**80.6 (158/196)**	19.4 (38/196)	16.3 (32/196)	3.1 (6/196)	
7A/F	33.3 (1/3)	**66.7 (2/3)**	66.7 (2/3)	0.0 (/3)	
9A/V	56.3 (18/32)	43.8 (14/32)	34.4 (11/32)	9.4 (3/32)	
14	62.1 (59/95)	37.9 (36/95)	32.6 (31/95)	3.2 (3/95)	2.1 (2/95)
18C	88.2 (15/17)	11.8 (2/17)	11.8 (2/17)		
19A	60.2 (71/118)	39.8 (47/118)	23.7 (28/118)	8.5 (10/118)	7.6 (9/118)
19F	78.3 (191/244)	21.7 (53/244)	17.2 (42/244)	3.7 (9/244)	0.8 (2/244)
23F	81.9 (136/166)	18.1 (30/166)	13.3 (22/166)	4.2 (7/166)	0.6 (1/166)
2		100.0 (2/2)	50.0 (1/2)		50.0 (1/2)
6C	100.0 (5/5)				
6D	100.0 (1/1)				
7B/C/40	70.0 (14/20)	30.0 (6/20)	25.0 (5/20)	5.0 (1/20)	
8	46.2 (6/13)	53.8 (7/13)	46.2 (6/13)	7.7 (1/13)	
9-Like	26.1 (6/23)	73.9 (17/23)	30.4 (7/23)	21.7 (5/23)	21.7 (5/23)
9L/N	71.4 (10/14)	28.6 (4/14)	21.4 (3/14)	7.1 (1/14)	
10A	75.0 (15/20)	25.0 (5/20)	10.0 (2/20)	10.0 (2/20)	5.0 (1/20)
10B	5.3 (1/19)	94.7 (18/19)	26.3 (5/19)	31.6 (6/19)	36.8 (7/19)
10C/F	10.0 (1/10)	90.0 (9/10)	70.0 (7/10)	20.0 (2/10)	
11A/D	70.0 (35/50)	30.0 (15/50)	28.0 (14/50)	2.0 (1/50)	

Serogroup/type	Primary Coloniser % (n/N) †	Co-Colonisation % (n/N) †	Second Serotype % (n/N) †	Third Serotype % (n/N) †	≥ Four Serotypes % (n/N) †
11B/C	66.7 (2/3)	33.3 (1/3)	33.3 (1/3)		
12A/F/44	23.5 (4/17)	76.5 (13/17)	17.6 (3/17)	52.9 (9/17)	5.9 (1/17)
13	65.9 (27/41)	34.1 (14/41)	29.3 (12/41)	4.9 (2/41)	
15A/F	55.0 (11/20)	45.0 (9/20)	45.0 (9/20)		
15B/C	94.3 (66/70)	5.7 (4/70)	5.7 (4/70)		
15-Like	33.1 (40/121)	66.9 (81/121)	37.2 (45/121)	19.8 (24/121)	9.9 (12/121)
16A		100.0 (5/5)	20.0 (1/5)	20.0 (1/5)	60.0 (3/5)
16F	79.7 (47/59)	20.3 (12/59)	18.6 (11/59)	1.7 (1/59)	
17F	61.4 (27/44)	38.6 (17/44)	29.5 (13/44)	6.8 (3/44)	2.3 (1/44)
18A	15.4 (6/39)	84.6 (33/39)	46.2 (18/39)	30.8 (12/39)	7.7 (3/39)
18B	37.5 (6/16)	62.5 (10/16)	43.8 (7/16)	12.5 (2/16)	6.3 (1/16)
19B	14.6 (7/48)	85.4 (41/48)	45.8 (22/48)	16.7 (8/48)	22.9 (11/48)
20	79.4 (27/34)	20.6 (7/34)	11.8 (4/34)	8.8 (3/34)	
21	80.0 (12/15)	20.0 (3/15)	20.0 (3/15)		
22A	19.0 (16/84)	81.0 (68/84)	42.9 (36/84)	31.0 (26/84)	7.1 (6/84)
22F	100.0 (6/6)				
23A	52.9 (27/51)	47.1 (24/51)	45.1 (23/51)	2.0 (1/51)	
23B	84.0 (42/50)	16.0 (8/50)	16.0 (8/50)		
24A	33.3 (1/3)	66.7 (2/3)		66.7 (2/3)	
24B/F		100.0 (1/1)			100.0 (1/1)
27	28.6 (2/7)	71.4 (5/7)	14.3 (1/7)	28.6 (2/7)	28.6 (2/7)
29		100.0 (8/8)	75.0 (6/8)	12.5 (1/8)	12.5 (1/8)
31	44.4 (8/18)	55.6 (10/18)	16.7 (3/18)	27.8 (5/18)	11.1 (2/18)
32A/F	16.7 (1/6)	83.3 (5/6)	0.0 (/6)	50.0 (3/6)	33.3 (2/6)
33A/F	28.6 (4/14)	71.4 (10/14)	21.4 (3/14)	14.3 (2/14)	35.7 (5/14)
33B		100.0 (5/5)	60.0 (3/5)	20.0 (1/5)	20.0 (1/5)
33C	12.5 (1/8)	87.5 (7/8)	25.0 (2/8)	12.5 (1/8)	50.0 (4/8)
34	75.6 (34/45)	24.4 (11/45)	20.0 (9/45)	4.4 (2/45)	
35A/C/42	62.5 (20/32)	37.5 (12/32)	18.8 (6/32)	6.3 (2/32)	12.5 (4/32)
35B	60.0 (15/25)	40.0 (10/25)	24.0 (6/25)	12.0 (3/25)	4.0 (1/25)
35F	25.0 (4/16)	75.0 (12/16)	37.5 (6/16)	25.0 (4/16)	12.5 (2/16)
36		100.0 (3/3)	33.3 (1/3)	66.7 (2/3)	
39	11.1 (1/9)	88.9 (8/9)	22.2 (2/9)	44.4 (4/9)	22.2 (2/9)
41A	20.0 (1/5)	80.0 (4/5)	20.0 (1/5)	20.0 (1/5)	40.0 (2/5)
41F	8.3 (1/12)	91.7 (11/12)	16.7 (2/12)	33.3 (4/12)	41.7 (5/12)
43	27.3 (3/11)	72.7 (8/11)	54.5 (6/11)	9.1 (1/11)	9.1 (1/11)
45	10.0 (2/20)	90.0 (18/20)	65.0 (13/20)	15.0 (3/20)	10.0 (2/20)
46	66.7 (4/6)	33.3 (2/6)	16.7 (1/6)		16.7 (1/6)
47A	10.3 (3/29)	89.7 (26/29)	48.3 (14/29)	24.1 (7/29)	17.2 (5/29)
47F		100.0 (2/2)	50.0 (1/2)		50.0 (1/2)
48	20.0 (1/5)	80.0 (4/5)	20.0 (1/5)		60.0 (3/5)
Total	**59.7 (1403/2352)**	40.3 (949/2352)	25.5 (599/2352)	9.6 (226/2352)	5.3 (124/2352)

† n is the number of isolates in each category for each period and N is the total isolates identified as each serotype/group. The rank was determined according to colonization density.



Supplementary Figure 3: Concordance of pneumococcal quantification by LytA, PiaB and the sum of all serotype-specific assay-sets (Bland-Altman plots depicting the difference vs means).

Panel A compares pneumococcal density determined by the Lyt*A* and Pia*B* assay-sets in 'Fluidigm' qPCR. Panel B compares average pneumococcal density (Lyt*A* and Pia*B*) to the total density of all serotype-specific assay-sets in 'Fluidigm' qPCR. The light blue shaded region indicates the 95% limits of agreement and the dark pink dotted line represents the line of bias (mean difference). The y-axis on both graphs is limited to the range of -10 to 10 Log10 Gene Equivalents (GE)/mL.

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Chapter 4: Nasopharyngeal colonization by *Streptococcus pneumoniae* and other common bacteria eight years following introduction of pneumococcal conjugate vaccine in rural South African children ≤5 years old. – Prepared Manuscript

Nasopharyngeal colonization by Streptococcus pneumoniae and other common bacteria eight years following introduction of pneumococcal conjugate vaccine in rural South African children ≤5 years old.

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Keywords: Streptococcus pneumoniae, asymptomatic colonisation, PCV, serotyping, 19F, co-colonisation, density

Abstract:

Background: *Streptococcus pneumoniae* remains a leading cause of morbidity and mortality in children <5 years old in sub-Saharan Africa. Monitoring serotype-specific nasopharyngeal colonisation can serve as a proxy to evaluate the effect of vaccination against vaccine-serotype (VT) disease. We evaluated the impact of 13-valent pneumococcal conjugate vaccine (PCV13, 2+1 schedule) immunisation on pneumococcus, and other bacterial colonisation among rural South African children <60 months old, eight years after PCV introduction.

Methods: Nasopharyngeal swabs collected during two cross-sectional surveys in Agincourt, Mpumalanga from May to October 2009 (Period-1, 630) and July 2017 to February 2018 (Period-2, 568) in healthy children 0-60 months old were tested for 92 pneumococcal serotypes and 15 other bacteria using a high-throughput nano-fluidic real-time PCR assay.

Results: Comparing Period-2 to Period-1, there was a lower net pneumococcal prevalence (76.9% vs. 83.2%, adjusted Odds Ratio [aOR]: 0.65, 95%CI: 0.48-0.87) and PCV13 VT colonisation prevalence (14.3% vs. 51.0%; aOR: 0.16, 95%CI: 0.12-0.21). In Period-2 VT 19F (5.3%) and 6B (4.8%) dominated, albeit at a lower prevalence compared with Period-1

(10.3%, aOR: 0.52, 95%CI: 0.33-0.82 and 15.2%, aOR: 0.26, 95%CI: 0.16-0.41, respectively). Non-vaccine-serotype (NVT) colonisation was higher in Period-2 (63.2%) than Period-1 (35.6%, aOR: 3.12, 95%CI: 2.45-3.97), driven by 16F (8.1% vs. 0.3%) and 23B (5.1% vs. 3.5%). Non-typeable pneumococci were higher in Period-2 (21.8%) compared with Period-1 (12.5%, aOR: 1.94, 95%CI: 1.42-2.67). Other differences included a higher prevalence in Period-2 of *Acinetobacter baumannii* (36.8% vs 1.1%, aOR: 50.11, 95%CI: 23.14-108.50), *Klebsiella pneumoniae* (13.2% vs 0.6%, aOR: 22.16, 95%CI: 8.03-61.11), *Streptococcus pyogenes* (2.5% vs 0.2%, aOR: 14.49, 95%CI: 1.89-111.09) and *Neisseria lactamica* (8.1% vs 4%, aOR: 2.14, 95%CI: 1.28-3.57), whereas *Streptococcus oralis* (0.5% vs 2.2%, aOR: 0.21, 95%CI: 0.06-0.77) and *Moraxella catarrhalis* (60.4% vs 67.8%, aOR: 0.72, 95%CI: 0.56-0.91) were lower.

Conclusions: There was an 80% lower prevalence of PCV13-VT serotype colonisation eight years after introduction of routine immunisation with PCV, however, there was high residual prevalence of 19F and 6B colonisation. Furthermore, the clinical relevance of temporal changes in colonization by other bacteria warrant further investigation.

1. Background

Streptococcus pneumoniae is a polysaccharide-encapsulated bacteria that colonises the nasopharynx [(1)]. There are more than 100 described capsular types that vary in prevalence according to geographical location and the age of the host [(2, 3)]. Despite most colonisation events usually being asymptomatic, pneumococcus remains the leading infectious cause of under 5 mortality and pneumonia-related deaths [(4, 5)].

Children <5 years old are the age-group most susceptible to pneumococcal disease and the main reservoir of carriage and ongoing transmission [(6, 7)]. Forty-eight percent of under-5 childhood deaths from lower respiratory tract infections occur in sub-Saharan Africa, yielding an incidence of 215.1 deaths per 100 000, many of which are associated with pneumococcus [(4)]. The formulation of the initial 7-, 10- and 13-valent pneumococcal conjugate vaccines (PCV), targeted inclusion of the most common invasive serotypes which caused invasive pneumococcal disease (IPD) in the pre-PCV era [(8)]. In South Africa, routine childhood PCV immunisation was associated with a reduction in PCV7-VT colonisation among children <5 years old by 40% two years after implementation (2009 vs 2011) [(9)], and there was a 70% and 66% reduction in PCV7-VT and additional six VT in PCV13, respectively, in children 9-59

months old by 2013 [10] . Further, PCV immunisation in South Africa reduced net IPD by an estimated 62% by 2013 [11], and there was a 23-33% reduction in all-cause pneumonia mortality by 2016 in children aged one month to four years which was attributed to PCV immunization [12]. In a rural area in the Mpumalanga province, the incidence (per 100,000) of IPD declined from 8.37 in 2009 to 2.23 in 2017, across all age-groups [13, 14]. Nevertheless, there was residual PCV13-VT colonization (18.4%) in children from the rural Mpumalanga setting up to four years (2013) after introduction of PCV in the public immunisation program [10].

Recent surveys on pneumococcal carriage have been undertaken in urban South African settings between 2018-21 [15, 16]. Nevertheless, it is important to also evaluate carriage separately in rural settings within the same country, as carriage prevalence differs between urban and rural locales, due to sociodemographic risk factors, such as crowding [17]. Following routine PCV immunization in African countries, the residual carriage of PCV13-VT ranges between 9-32% in rural settings (2-4 years later) [10, 18-21] and 9-19% (2-9 years later) in urban regions [16]. In contrast, VT colonization in children in United Kingdom was <4% and <1% at two and six years, respectively, after routine childhood PCV immunization [22, 23]. Nevertheless, there is a paucity of data on the long-term effect of routine childhood PCV immunisation on VT carriage in children from rural African settings.

We aimed to evaluate the temporal association of routine childhood PCV immunization on nasopharyngeal serotype-specific pneumococcal and other common bacteria in children aged less than five years of age in a rural South African setting, eight years after introduction.

2. METHODS

2.1 Study design

The 7-valent PCV (PCV-7; vaccine serotypes [VT]: 4, 6B, 9V, 14, 18C, 19F and 23F) was introduced into the South African public immunization program in April 2009, using a 2+1 (6, 14 and 40 weeks of age) dosing schedule [24]. Subsequently, the South African immunization program transitioned to the vaccinating with the 13-valent PCV (PCV-13, which included PCV7-VT plus 1, 3, 5, 6A, 7F and 19A serotypes) in May 2011.

Two cross-sectional surveys using random household sampling were undertaken in a rural setting in the Agincourt Health and Demographics Surveillance Site (HDSS), Mpumalanga, South Africa. The Agincourt HDSS is a miscellany of rural villages surrounding the Agincourt Primary Health Care Clinic, located in north-eastern Mpumalanga (Supp. Figure 1). The region is low resourced with high unemployment and has a large migrant population. The site covers an area of 420km^2 with around 110,000 individuals of all ages participating in annual census surveys [25].

Nasopharyngeal swab samples (NPS) were collected from children ≤60 months old enrolled between May-October 2009 (Period-1) during the early introduction period of PCV7. The details of the Period-1 cohort and culture-based Quellung estimates of pneumococcal and serotype prevalence have been reported previously and served as a baseline for the current study [9]. Briefly, households with a child ≤24 months old were selected from 27 villages within the Agincourt HDSS. If at least one person older than 12 years of age was willing to participate, then all consenting household members and their healthy children older than 9 months were enrolled. All children ≤60 months old were included in the current analysis (Figure 1). For Period-2, NPS were collected from children ≤60 months old enrolled within the same community between July 2017 and February 2018. Households with a child ≤60 months old were randomly selected across 32 villages. Healthy children ≤60 months old were enrolled, irrespective of their PCV immunisation-status. Trained field workers explained the study and the informed consent information sheet to parents/guardians in their language. Participants were excluded if a parent/guardian was not available/willing to provide written informed consent, the child had any febrile illness (fever >37.5℃) on the day of screening, received antibiotic treatment (not including co-trimoxazole prophylaxis) in the 21 days before screening, the primary caregiver was unwilling to discuss theirs or their child's HIV-status or treatment. If eligible, and consent was provided, children were enrolled and sampled on the same day with no follow-up visits.

The study team moved systematically through villages, recruiting all eligible children per household, with only the index child contributing to the required sample size. If a selected household was excluded, the study team moved to the next household without attempting to revisit, for example when illness resolved, or antibiotics had been discontinued. If no

parent/legal guardian was at home during traditional hours (weekdays, 08:00-16:00), households were revisited on Saturday. Swabs from both study periods were tested for 92 pneumococcal serotypes and 15 other bacterial species as described [26].

For the current study, all available samples, and data for children ≤60 months old (Figure 1) collected during Period-1 were retrospectively analysed. In Period-1 and Period-2 data gathered included information on health and risk factors for pneumococcal colonisation (symptoms of allergies or respiratory infections, HIV-status, and treatment, cotrimoxazole prophylaxis, tuberculosis (TB) exposure and/or treatment, hospitalisation within three months of the sampling date, medical conditions, daytime social contact, and breastfeeding status) and HIV-status. In Period-1, HIV-status was self-reported by the guardian, and in Period-2 HIV-infection status (if available) and vaccination status were transcribed from the child's road to health card (RTHC). If RTHC was unavailable, the primary caregiver self-reported if the child received vaccinations, and the ages at which they were vaccinated. Children were regarded as fully vaccinated for PCV if they received all scheduled routine immunisations for their age. In Period-1, NPS (aluminium-shafted, Dacron swab, Medical Wire and Equipment Co. Ltd., Corsham, Wiltshire, England) were archived in skim milk-tryptone-glucose-glycerol (STGG) transport media at -80°C, detailed elsewhere [27]. In Period-2 a single NPS (FLOQSwabs, COPAN Diagnostics, Murrieta, California, United States of America) was collected from each child and immediately inoculated into 1.5mL STGG medium and placed on ice in a cold box, and transferred to −80°C storage at the Agincourt Laboratory within 6 hours of sample collection in accordance with World Health Organization (WHO) recommendations [28]. At the end of the study period, samples were transported to the Vaccines, and Infectious Diseases Analytics Research Unit (Wits-VIDA) on dry ice and stored at −80°C until processed.

2.2 Sample size calculation

Six-hundred and thirty archived NPS collected and previously analysed with culture-based serotyping during Period-1 were available for retrospective testing on the nanofluidic quantitative PCR (qPCR) method for the current study (Figure 1). All samples were tested regardless of previous culture results. A minimum sample size of 408 was needed for Period-2 to detect 80% reduction in the most prevalent circulating PCV13-VT (19F; 6B; 6A; 23F;

19A and 14) in children <5 years of age in 2017 compared with 2009 (Period-1) with an 80% power.

2.3 Quantitative PCR

Total nucleic acids were extracted from 400uL of thawed and vortexed (30 seconds) STGG into 100uL elute using the automated NucliSens® easyMAG® extraction system and reagents (BioMérieux, Marcy l'Etoile, France) according to manufacturer protocols and stored at –20°C until tested. The total nucleic acid extracts were then tested using quantitative PCR (qPCR) to detect 15 bacterial species (*Acintobacter baumannii*; *Bordetella holmesii*; *B. parapertussis*; B. pertussis; *B. bronchiseptica*; *Haemophilus influenzae*; *H. influenzae-b*; non-typeable *H. influenzae* (NTHI); *Klebsiella pneumoniae*; *Morexella catarrhalis*; *Neisseria lactamica*; *Neisseria meningitidis*; *Staphylooccus aureus*; *Streptococcus pneumoniae*; *Streptococcus pyogenes* and *S. oralis*) and 92 pneumococcal serotypes. The pneumococcal serotype-specific assay-sets could differentiate 35 serotypes into 16 serogroups and 57 serotypes individually. The design, optimization, and validation of the high-throughput Standard BioTools (formerly Fluidigm) nanofluidic real-time qPCR method has been detailed previously [26, 29]. Standard BioTools 96.96 Gene Expression (GE) Dynamic Array integrated fluidic circuit (IFC) (product number BMK-M-96.96) and associated instruments were used according to manufacturer protocols.

2.4 Statistical analyses

The qPCR results were analysed using Standard BioTools Real-time PCR Analysis software. Statistical analyses were conducted using STATA Version 13.1 (StataCorp, Texas, USA), and graphical representations were generated using GraphPad Prism and Excel. The cycle of quantification (Cq) values were transformed into density (Log_{10} genomic equivalents [GE]/mL), with cut-off values applied based on assay-set limits of detection. Algorithms were then employed for bacterial species and pneumococcal serotype determination, following previously described protocols [26, 30]. Samples were deemed positive for pneumococcus only if the two pneumococcal reference genes included (LytA, PiaB) were detected. The allocation of a serotype was contingent upon the presence of pneumococcus, confirmed by the pneumococcal reference genes. Bland-Altman plots were generated to compare the agreement between the average pneumococcal density (LytA, PiaB) and the sum of the densities of all detected serotypes within a sample. In cases where pneumococcal

density exceeded that of the cumulative serotype density, the upper 95% limit of agreement between pneumococcal density and cumulative density of detected serotypes, was used to quantify non-typeable *S. pneumoniae*, and this would include samples positive for pneumococcus but where no serotype was detected.

Pneumococcal serotypes were classified into overall pneumococcus (any sample testing positive for pneumococcus), PCV13-VT (1, 3, 4, 5, 6A, 6B, 7AF, 9AV, 14, 18C, 19A, 19F and 23F); or non-vaccine-type (NVT, any serotype not included in PCV13); and were also assessed by individual serotype. For NPS where more than one serotype was detected within a category (NVT or VT) this was counted once per individual sample.

Children were grouped into age categories according to the SA-EPI PCV schedule to describe PCV coverage in Period-2. Demographic characteristics and factors that impact the risk for pneumococcal colonisation were compared between Period-1 and Period-2 using the X^2 test or the Student t-test where appropriate. Risk factors with different frequency between the periods (p-values of <0.1) were included in an adjusted multivariate analysis.

Logistic regression models were used to evaluate the temporal relationship between the two time periods and the prevalence of pneumococcus, VT, NVT, individual serotypes, and other bacteria, adjusting for breastfeeding status, HIV infection, antibiotic usage, co-trimoxazole prophylaxis, and tuberculosis treatment. The adjusted odds ratios (aOR) and 95% confidence intervals (CI) are reported. In instances where aOR could not be computed, Fisher's exact test was utilized to assess statistical significance.

For density comparison, the mean $\log_{10}$ GE per ml were calculated for overall pneumococcus, VT, NVT, individual serotypes, and each bacterial species. Where more than one serotype or bacterial species were found concurrently in the same sample, the relative density was used to assign hierarchy in analyses of co-colonisation for each participant. Solitary colonisation or the highest ranked coloniser (highest density) were designated as the dominant colonisers, and all other colonisers as co-colonisers. Co-colonisation events in Period-2 were compared

with Period-1. To describe the relationship between bacterial co-colonizers, a colonization hierarchy matrix was constructed based on a relative target density. The highest density colonizer was the primary colonizer and where lower-density colonisers were present, these were classified as co-colonisers. The matrix was translated into paired relationships with each dominant and co-coloniser. Where more than two bacteria were detected in co-colonization, these were paired separately with the dominant bacterium, e.g., Bacterium 1 > Bacterium 2 > Bacterium 3 would be Bacterium 1 > Bacterium 2; Bacterium 1 > Bacterium 3, and therefore would not directly represent the prevalence of the dominant bacterium, but rather the number of times each multiple concurrent colonisation relationship occurred. Alluvial Sankey diagrams were constructed using the paired relationships in flourish.studio (available at https://flourish.studio).

2.5 Ethics approval

The study was approved by the Human Research Ethics Committee (HREC) at the University of Witwatersrand (M090114 and M170314). Additionally, approval from the Department of Health for Mpumalanga Province was granted (MP2017RP35-643) before the study commenced. All sample collection and laboratory procedures adhered to applicable guidelines and regulations. For minors, written informed consent was obtained from the primary guardian during the initial enrolment process.

3.0 Results

The final analysis included 630 archived NPS from Period-1 and 568 from Period-2 (Figure 1). The mean age of children in Period-2 was higher compared with Period-1 (31.6 vs. 25.8 months, $p<0.001$). Within the ≤24-months age category, children enrolled in Period-2 were younger compared with Period-1 (12.7 vs. 17.4 months; $p<0.001$), whereas in the 25-60-months age category, children were older (41.4 vs. 37.9 months; $p<0.001$). In Period-2, a lower proportion of children were breastfeeding (18%, 102/568) or were ever breastfed (85%, 483/568) compared with Period-1 (30.3%, 191/630 and 92.7%, 584/630; respectively; both $p<0.001$). In Period-1, 4.8% (30/630) of children were on antibiotics at enrolment, which was an exclusion criterion in Period-2. There were no other significant differences in the demographic characteristics between the study periods; Table 1.

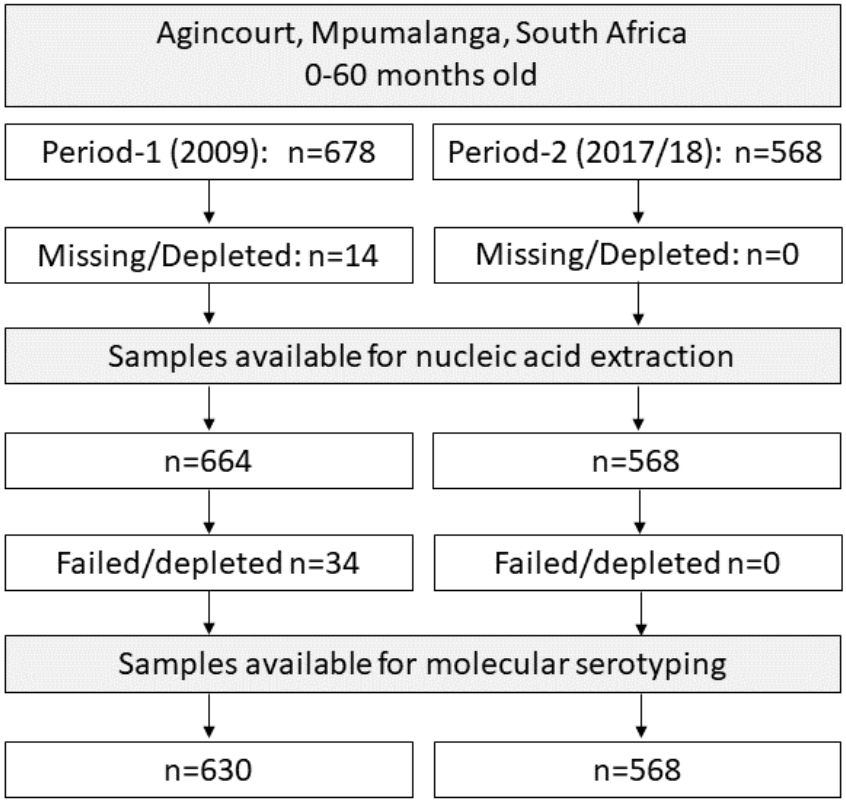

Figure 1: Flow diagram of samples from children ≤60 months old in Agincourt in 2009 (Period-1) and 2017/18 (Period-2).

In Period-1, data on PCV immunisation was not collected per dose, and only fully immunized children (3 doses) could be compared with Period-2. During Period-2, 94.4% (475/503) of children older than 40 weeks, were fully (three doses) vaccinated with PCV (Supp. Table 1) compared with 61.1% (385/630) in Period-1 ($p<0.001$). All children aged 14-40 weeks were up to date for their age in Period-2, having received both primary doses of PCV (100%, 34/34), and 95.5% (21/22) of children aged 6-14 weeks had received their initial PCV dose.

3.1 Pneumococcal carriage prevalence

Among children ≤60 months old, the overall pneumococcal colonisation prevalence was 35% lower in Period-2 (76.9%, 437/568) compared with Period-1 (83.2%, 524/630; aOR: 0.65, 95%CI: 0.48-0.87), which was driven by a 84% relative reduction in prevalence of PCV13-VT (14.3%, 81/568 vs. 51%, 321/630; aOR: 0.16, 95%CI: 0.12-0.21; Figure 2A, Supp. Table

2). There was no difference in the overall pneumococcus prevalence between the study periods when stratified by age-groups of ≤24 and 25-60 months (Figure 2C and 2E, Supp. Table 3-4). There was, however, a reduction in PCV13-VT prevalence in the ≤24 (88% reduction: 12.3%, 24/195 vs. 52.6%, 195/371; aOR: 0.12, 95%CI: 0.07-0.19; Supp. Table 3) and 25-60 months (82%: 15.3%, 57/373 vs. 48.7%, 126/259; aOR: 0.18, 95%CI: 0.12-0.27; Supp. Table 4) age-groups in Period-2 (Figure 2D and 2F).

Vaccine serotypes 1, 4, 6A, and 18C were not detected in any child during Period-2, compared with prevalence of 0.6% (4/630), 1.3% (8/630), 7.6% (48/630), 0.8% (5/630), respectively in Period-1 (Figure 2B and Supp. Table 2). Further, there was lower prevalence of colonization in Period-2 compared with Period-1 for serotypes 23F (-92%; 0.7% [4/568] vs. 7.9% [50/630]; aOR: 0.08, 95%CI: 0.03-0.23), 14 (-86%; 0.9% [5/568] vs. 6.2% [39/630]; aOR: 0.14, 95%CI: 0.05-0.35), 6B (-74%; 4.8% [7/568] vs. 15.2% [96/630]; aOR: 0.26, 95%CI: 0.16-0.41), and 19A (-63%; 1.4% [8/568] vs. 3.7%; [23/630]; aOR: 0.37, 95%CI: 0.16-0.86%); Figure 2B, Figure 3, Supp. Table 2. Vaccine serotypes with a residual prevalence of more than 1% in Period-2 were 19F (5.3% [30/568] vs 10.3% [65/630] in Period-1; aOR: 0.52, 95%CI: 0.33-0.82), 6B (4.8% [7/568] vs. 15.2% [96/630]; aOR: 0.26, 95%CI: 0.16-0.41), 19A (1.4% [8/568] vs. 3.7% [23/630]; aOR: 0.37, 95%CI: 0.16-0.86%), 3 (1.4% [8/568] vs 1.6% [10/630]; aOR: 0.88, 95%CI: 0.34-2.25), and 5 (1.2% [7/568] vs 1.0% [6/630]; aOR: 1.20, 95%CI: 0.40-3.66).

Table 1: Characteristics of children ≤60 months old enrolled in two cross-sectional surveys undertaken in Agincourt Health and Demographic Surveillance System, South Africa.

	Period 1 (2009; N=630)	**Period 2 (2017; N=568)**	**p-value***
0-60 months: mean age in months (SD)	25.8 (±12.9)	31.6 (±16.3)	<0.001
≤ 24 months: mean age in months (SD)	17.4 (±3.9); 371	12.7 (±7.4); 195	<0.001
> 24 months: mean age in months (SD)	37.9 (±11.8); 259	41.4 (±9.8); 373	<0.001
Currently breastfeeding, % (n)	30.3% (191)	18.0% (102)	<0.001
Ever breastfed, % (n)	92.7% (584)	85.0% (483)	<0.001
Duration of breastfeeding in months, mean (SD)	15.5 (±6.8)	14.4 (±6.9)	0.004
Weight, Kg, mean (SD)	11.3 (±2.6), n=623	13.2 (±3.8), n=566	<0.001
Height, cm, mean (SD)	80.8 (±10.4), n=597	91.1 (±48.4), n=567	<0.001
Daytime social contact: Stays home, % (n)	29.8% (188)	35.4% (201)	0.041
Daytime social contact: Stays home with other children <5, % (n)	35.1% (221)	25.5% (145)	<0.001
Daytime social contact: Attends daycare (≥4 hrs/day; ≥3 times/week), % (n)	34.8% (219)	37.7% (214)	0.294
Daytime social contact: Other daytime social contact or unknown, % (n)	0.3% (2)	1.4% (8)	0.038
Children living with HIV (CLWH), % (n/N)	2.5% (3/120)	0.5% (2/409)	0.739
Cotrimoxazole prophylaxis, % (n)	0.2% (1)	0.5% (3)	0.268
Treated for Tuberculosis in the past year, % (n)	0.8% (5)	0.7% (4)	0.868
Current antibiotic treatment. n (%)	4.8 (30)	0 (0)	<0.001
Hospitalised in last 3 months, % (n)	1.4% (9)	1.1% (6)	0.563

*****Student t-test or Pearson X^2 test was used to compare characteristics between the cohorts and those with P-values <0.01 were considered different between the periods; only variables known to affect colonisation by pneumococcus were adjusted for in the regression analyses, n: number of individuals with investigated outcomes.
N: total number of individuals with available information on the characteristic. N was only included in rows where different from that indicted in the column header.

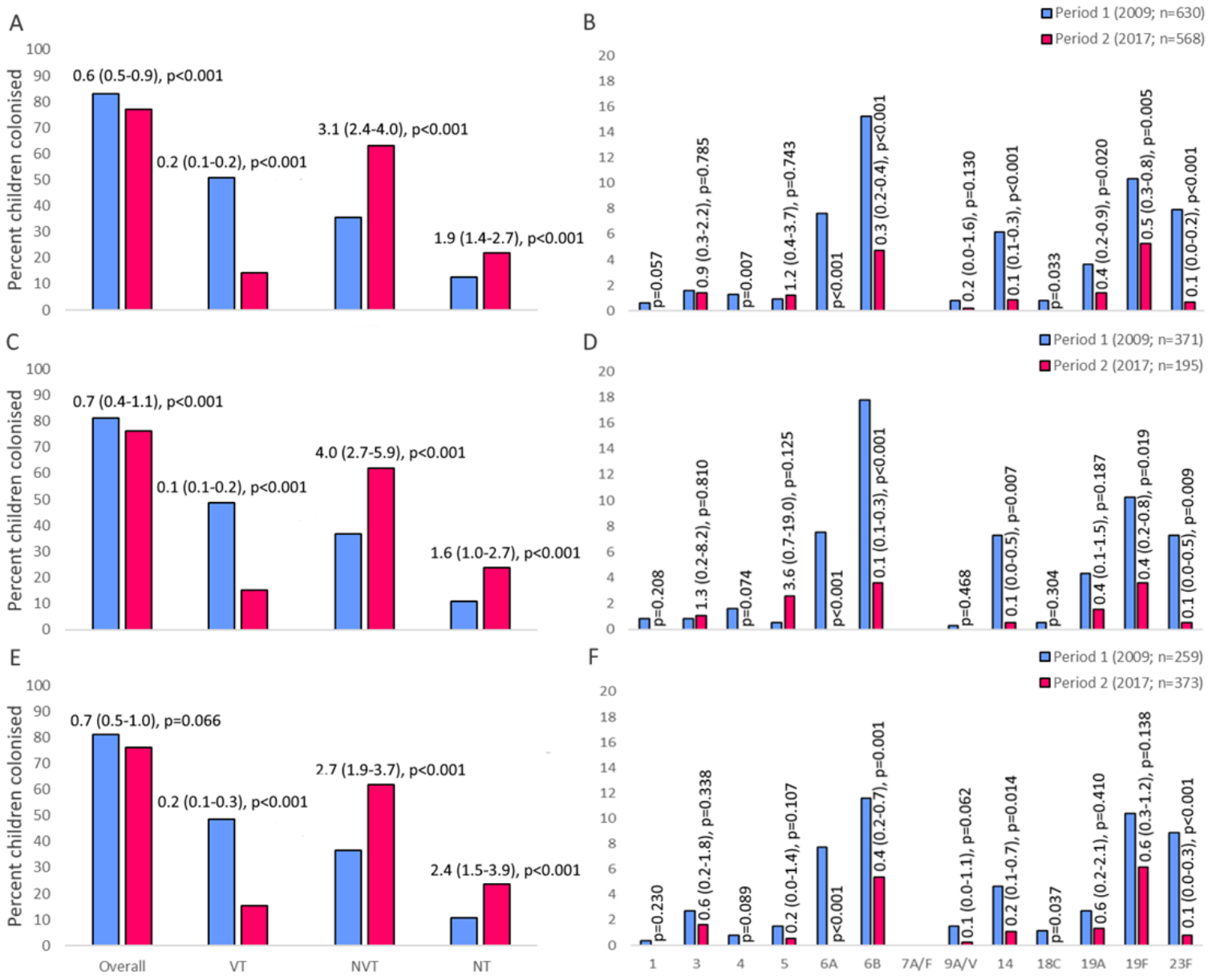

Figure 2: Grouped colonisation prevalence of *Streptococcus pneumoniae* and individual vaccine serotypes in children aged ≤60 months, stratified by age (0-24 and 25-60 months).

Panels A and B include all children ≤60 months, Panels C and D include children 0-24 months, and Panels E and F include children 25-60 months. Panels A, C, and E are grouped colonisation prevalence and Panels B, D, and F are individual vaccine serotypes. Overall includes all colonisation by pneumococcus.

VT: PCV13 vaccine serotypes (1, 3, 4, 5,6A, 6B, 7A/F, 9A/V, 14, 18C, 19A, 19F and 23F); VT (excl 3; 19F): are all PCV13 serotypes excluding serotypes 3 and 19F. NVT: non-PCV13-vaccine serotypes; NT: non-typeable S. pneumoniae; adjusted Odds Ratios (aOR) with 95% Confidence Intervals (CI) and p-values are included. For reference, all OR, p-values and proportions, see supplementary Table 2.

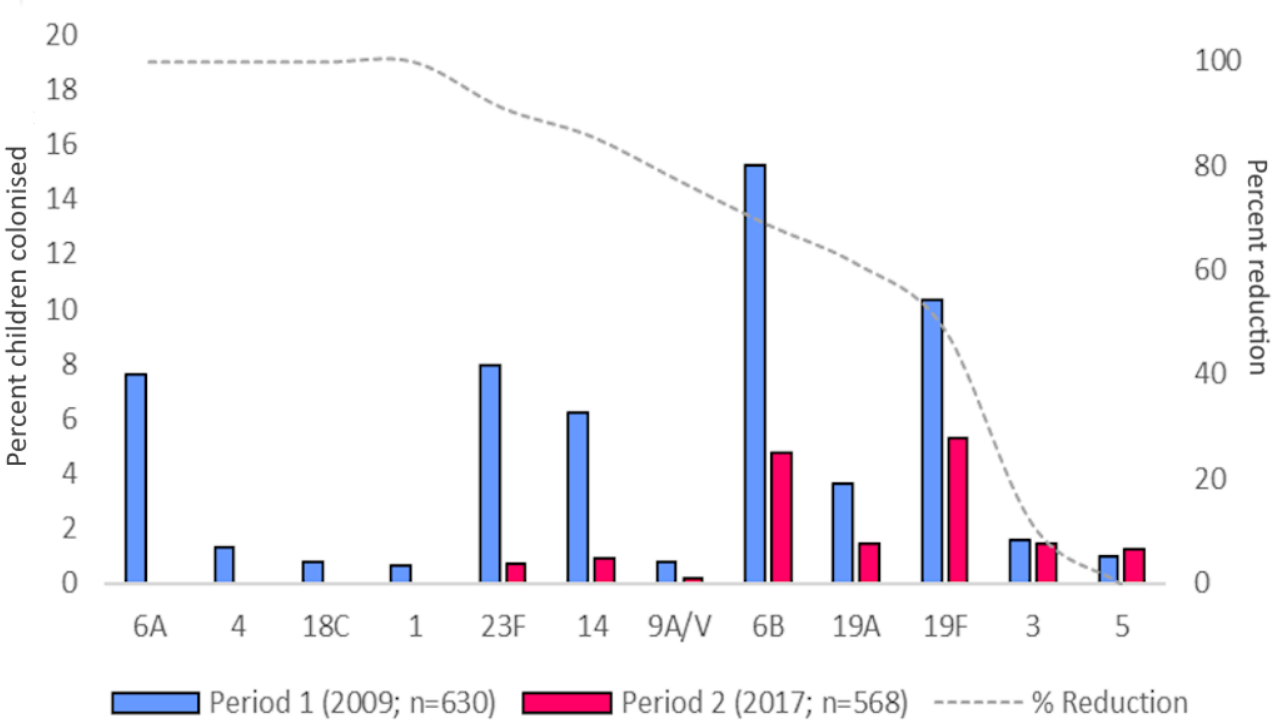

Figure 3: Percent reduction in vaccine serotypes prevalence in children aged ≤60 months.

The percent reduction is calculated based on the relative reduction in the prevalence of each serotype in Period-2 compared with Period-1, arranged in the order of highest to lowest reduction in individual serotypes in Period-2 compared with Period-1. For reference, all aOR, p-values and proportions, see supplementary Table 2.

In contrast, the prevalence of NVT was 3.1 times (95%CI: 2.45-3.97) higher in Period-2 (63.2% [359/568]) compared with Period-1 (35.6% [224/630]; being similarly so in children ≤24 (65.6% [128/195] vs. 34.8% [129/371]; aOR: 3.98, 95%CI: 2.70-5.87) and 25-60 (61.9% [231/373] vs. 36.7% [95/259]; aOR: 2.67, 95%CI: 1.90-3.74); Figure 4 and Supp. Table 2. The increase in NVT in Period-2 compared with Period-1 was driven by serotypes 16F (8.1% [46/568] vs. 0.3% [2/630]; aOR: 27.33, 95%CI: 6.59-113.33), 21 (3.7% [21/568] vs. 0.2% [1/630]; aOR: 22.42, 95%CI: 3.00-167.71), and 15AF (2.8% [16/568] vs. 0.3% [2/630]; aOR: 8.86, 95%CI: 2.02-38.86). Furthermore, there was a 1.9 times higher prevalence of non-typeable pneumococcus in Period-2 in children ≤60 months (21.8% [124/568] vs. 12.5% [79/630]; aOR: 1.94, 95%CI: 1.42-2.67); including in age groups ≤24 (18.5% [36/195] vs.

13.8% [51/371]; aOR: 1.64, 95%CI: 0.99-2.71; Supp. Table 3) and 25-60 months old (23.6% [88/373] vs. 10.8% [28/259]; aOR: 2.43, 95%CI: 1.52-3.88; Supp. Table 4).

3.2 Pneumococcal carriage density

Pneumococcal density in Period-2 was lower than in Period-1 for overall (3.8, 95%CI: 3.7-4.0 vs. 4.1 $\log_{10}$ GE/ml, 95%CI: 4.0-4.2) and PCV13-VT (3.2, 95%CI: 2.7-3. 8 vs. 4.2 $\log_{10}$ GE/ml, 95%CI: 4.0-4.5); (Supp. Figure 2 and Supp. Table 5). However, there were no differences between the density of NVT (3.5, 95%CI: 3.4-3.7 vs. 3.6 $\log_{10}$ GE/ml, 95%CI: 3.4-3.8) or non-typeable pneumococcus (3.6, 95%CI: 3.4-3.8 vs. 3.6 $\log_{10}$ GE/ml, 95%CI: 3.3-3.8), between the study periods (Supp. Figure 2, Supp. Table 5). While there were no significant differences in the mean densities for the majority of the individual serotypes, the density of serotype 19F was lower in Period-2 (4.0 $\log_{10}$ GE/ml, 95%CI: 3.7-4.4) compared with Period-1 (4.6 $\log_{10}$ GE/ml, 95%CI: 4.4-4.9); Supp. Figure 3-4 and Supp. Table 5.

3.3 Multiple concurrent colonisation by pneumococcal serotypes

Of the children with pneumococcal colonization in Period-1, 19.7% (128/651) were colonised by multiple serotypes, including 16.4% (n=107), 2.0% (n=13) and 1.2% (n=8) with two, three and four or more serotypes, respectively. Similarly, 18.2% (97/533) of children with pneumococcal colonization had multiple serotypes in Period-2; including 13.9% (n=74), 3.8% (n=20) and 0.6% (n=3) with two, three and four or more serotypes, respectively; Supp. Table 6, Supp. Figure 5.

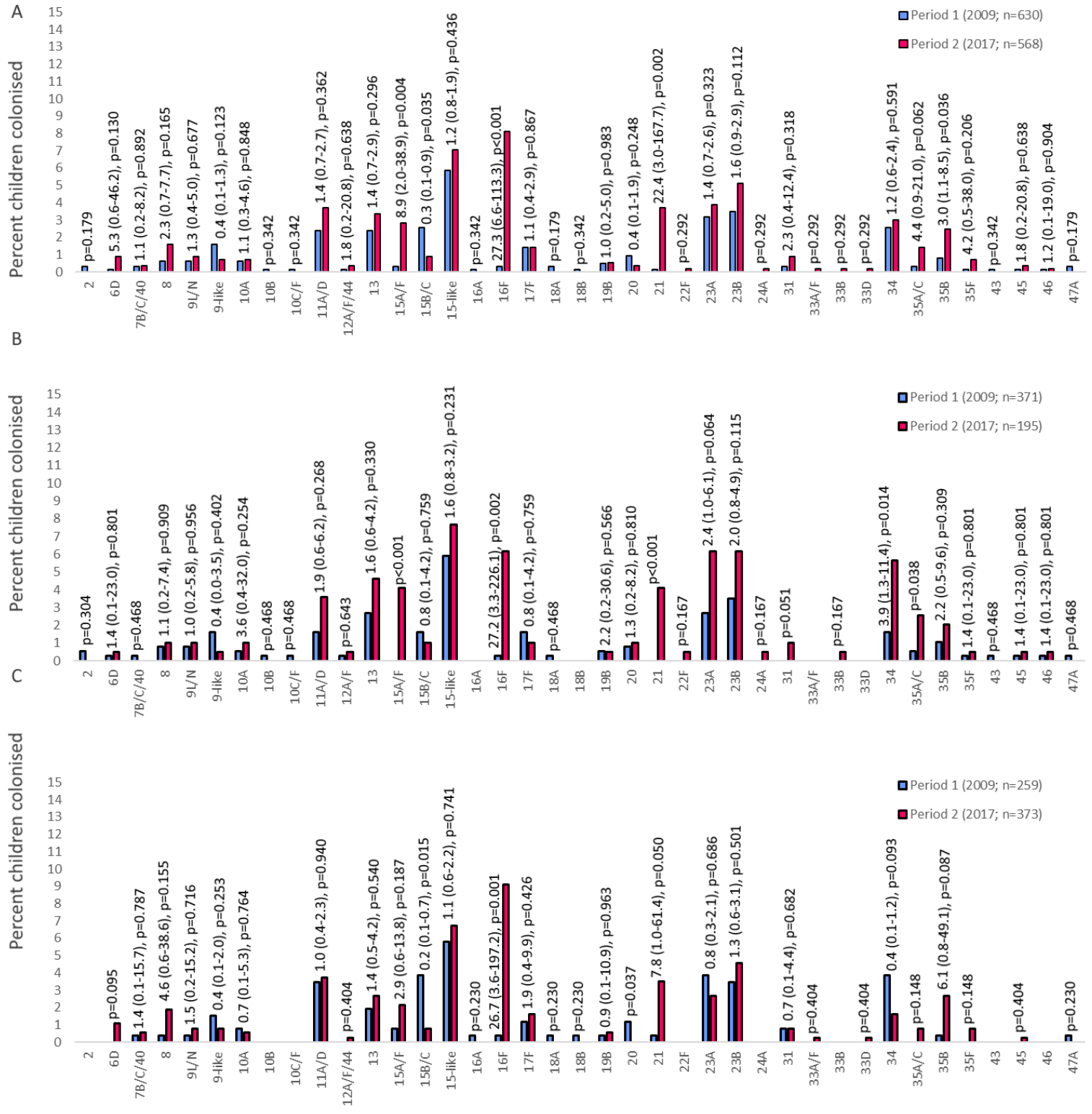

Figure 4: Colonisation prevalence of individual non-vaccine serotypes (NVT) in children aged ≤60 months, stratified by age (0-24 months and 25-60 months).

NVT: non-PCV13-vaccine serotypes. Panel A includes all children ≤60 months, Panel B includes children 0-24 months and Panel C includes children 25-60 months; Adjusted Odds Ratios (aOR) with 95% Confidence Intervals (CI) calculated using logistic regression and p-values are included. For reference, all aOR, p-values and proportions, see supplementary Table 2.

3.4 Colonisation prevalence and density of other bacterial colonisers

There was a 50- and 22-times higher prevalence of colonisation in Period-2 compared with Period-1 of *Acinetobacter baumannii* (36.8% [209/568] vs. 1.1% [7/630]; aOR: 50.11, 95%CI: 23.14-108.50) and *Klebsiella pneumoniae* (13.2% [75/568] vs 0.6% [4/630]; aOR: 22.16, 95%CI: 8.03-61.11), respectively. Also, there was a higher prevalence of colonization in Period-2 compared with Period-1 of *Streptococcus pyogenes* (2.5% [14/568] vs 0.2% [1/630]; aOR: 14.49, 95%CI: 1.89-111.09), *Neisseria lactamica* (8.1% [46/568] vs 4% [26/630]; aOR: 2.14, 95%CI: 1.28-3.57); Figure 5, Supp. Table 7.

In contrast, there was a lower prevalence of colonisation in Period-2 compared with Period-1 of *Streptococcus oralis* (0.5% [3/568] vs. 2.2% [14/630]; aOR: 0.21, 95%CI: 0.06-0.77) and *Moraxella catarrhalis (*60.4% [343/568] vs. 67.8% [427/630]; aOR: 0.72, 95%CI: 0.56-0.91); Figure 5, Supp. Table 7.

The density of colonizing bacteria were similar over the two periods, except for a higher density of *K. pneumoniae* in Period-2 (2.5 $\log_{10}$ GE/ml, 95%CI: 2.4-2.7) compared with Period-1 (1.6 $\log_{10}$ GE/ml, 95%CI: 1.1-2.4); Supp. Figure 6 and Supp. Table 8.

3.5 Multiple concurrent bacterial carriage

Children colonized with *S. pneumoniae* had a 2.32 (95%CI: 1.78-3.04) higher odds of being co-colonised with other investigated bacteria in Period-2 (52.4%, 229/437), compared with Period-1 (33.4%; 175/524). In both periods, pneumococcus was a co-coloniser to *M. catarrhalis*, non-typeable and *Haemophilus influenzae* type b, and *S. aureus*, whereas in Period-2, pneumococcus also co-colonised children carrying *K. pneumoniae, N. lactamica* and *S. oralis* as dominant bacteria (Figure 6, Supp. Table 8 and 9). Also, *A. baumannii* (91.9%, 192/209 vs. 85.7%, 6/7; aOR: 1.88, 95%CI: 0.21-17.10) had higher odds of being detected as co-colonisers to other bacterial species in Period-2 (*M. catarrhalis, S. pneumoniae*, non-typeable *Haemophilus influenzae, K. pneumoniae, N. lactamica, Haemophilus influenzae*, *S. oralis* and *S. aureus*) compared with Period-1 (*M. catarrhalis* and *S. pneumoniae*). *M. catarrhalis* was associated with a 0.40 (95%CI: 0.30-0.55) lower odds of

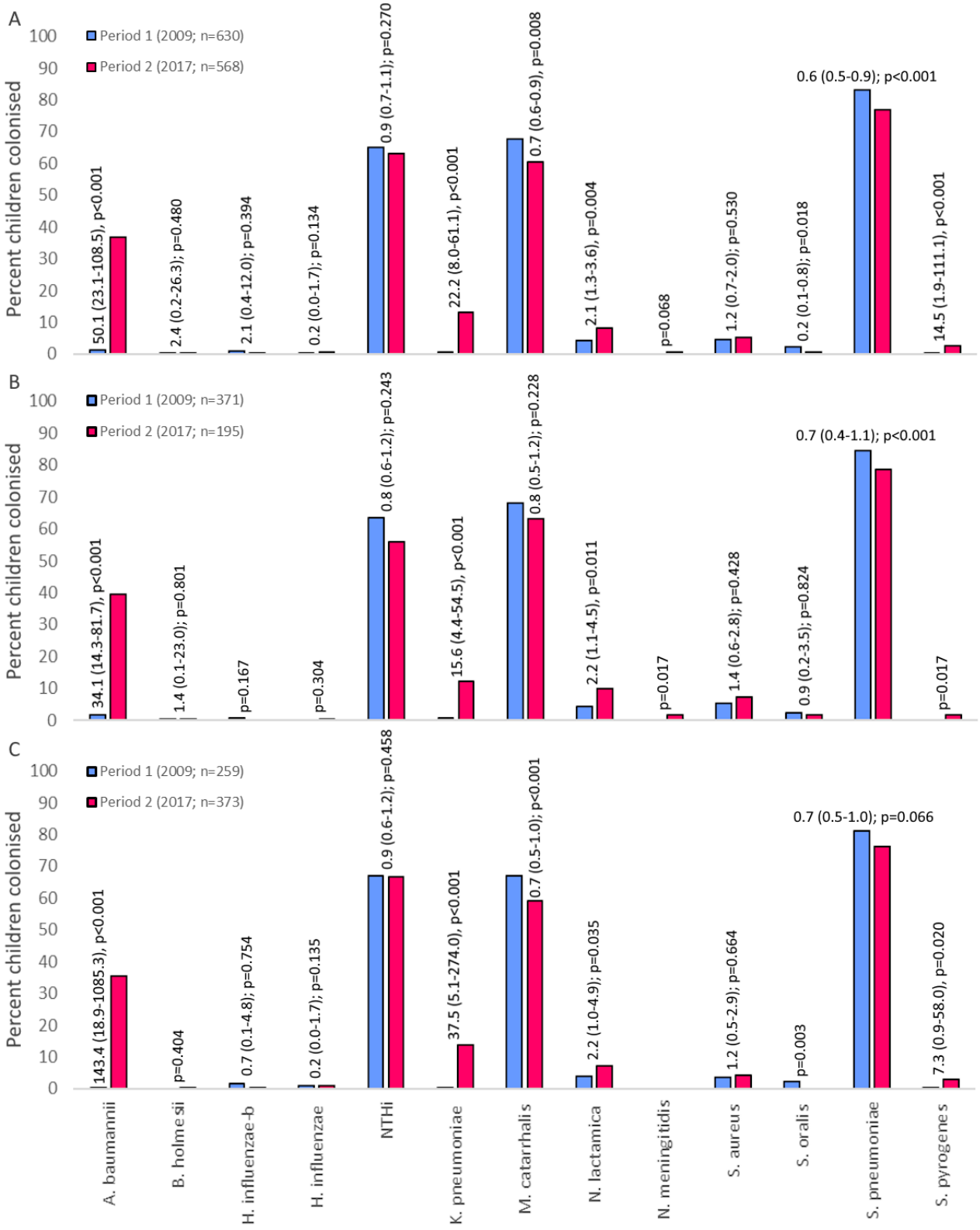

Figure 5: Colonisation prevalence of bacterial colonisers in children aged ≤60 months, stratified by age (0-24 months and 25-60 months).

Panel A includes all children ≤60 months, Panel B includes children 0-24 months and Panel C includes children 25-60 months; Adjusted Odds Ratios (aOR) with 95% Confidence

Intervals (CI) calculated using logistic regression and p-values are included. For reference, all aOR, p-values and proportions, see supplementary Table 5.

being detected as a co-coloniser to other bacterial species in Period-2 (*S. pneumoniae,* non-typeable and *Haemophilus influenzae* type b, *A. baumannii*, *N. lactamica* and *S. oralis*) compared with Period-1 (*S. pneumoniae,* non-typeable and *Haemophilus influenzae* type b). Conversely, non-typeable *Haemophilus influenzae* had a 0.64 (95%CI: 0.44-0.94) lower odds of detection as a co-coloniser in Period-2 (77.9%, 279/358) than Period-1 (82.6, 338/409). In Period-2, Non-typeable *Haemophilus influenzae* was detected as a co-coloniser to *M. catarrhalis*, *S. pneumoniae*, *K. pneumoniae*, *A. baumannii*, *N. lactamica* and *S. oralis*, while in Period-1, was a co-colonizer to *M. catarrhalis*, *S. pneumoniae and S. aureus.* In both periods, *N. lactamica, N. meningitidis, H. influenzae, K. pneumoniae, S. aureus, S. oralis* and *S. pyogenes* were mostly detected as a co-colonising bacteria to other bacterial species (Figure 6, Supp. Table 8 and 9, Supp. Figure 5).

4.0 Discussion

Routine childhood immunization of PCV was temporally associated with an 80% reduction in PCV13-VT colonisation among children after eight years in a rural South African setting. Nevertheless, we observed high residual prevalence of PCV13-VT colonization (14.3%), particularly serotypes 19F (5.3%) and 6B (4.8%), in our setting compared with that observed in high-income countries (<4% for all VT) after routine childhood PCV immunization [22, 31-35]. The high residual prevalence of PCV13-VT in our setting occurred despite a high coverage of PCV vaccination in our study (>90%), as well as a high coverage in the general sampled population (82.2% in 2013) [10]. Our findings in a rural African setting, are similar to observations in an urban South Africa setting in 2018; however, there was a higher prevalence of serotype 6B colonisation in our rural (4.8%) compared with the urban setting (1.6%; 9/571; p=0.002), whilst residual serotype 19F colonization was marginally higher in the urban setting (8.1%; 46/571) compared to the rural setting (5.3%; p=0.061), albeit, not significant [15].

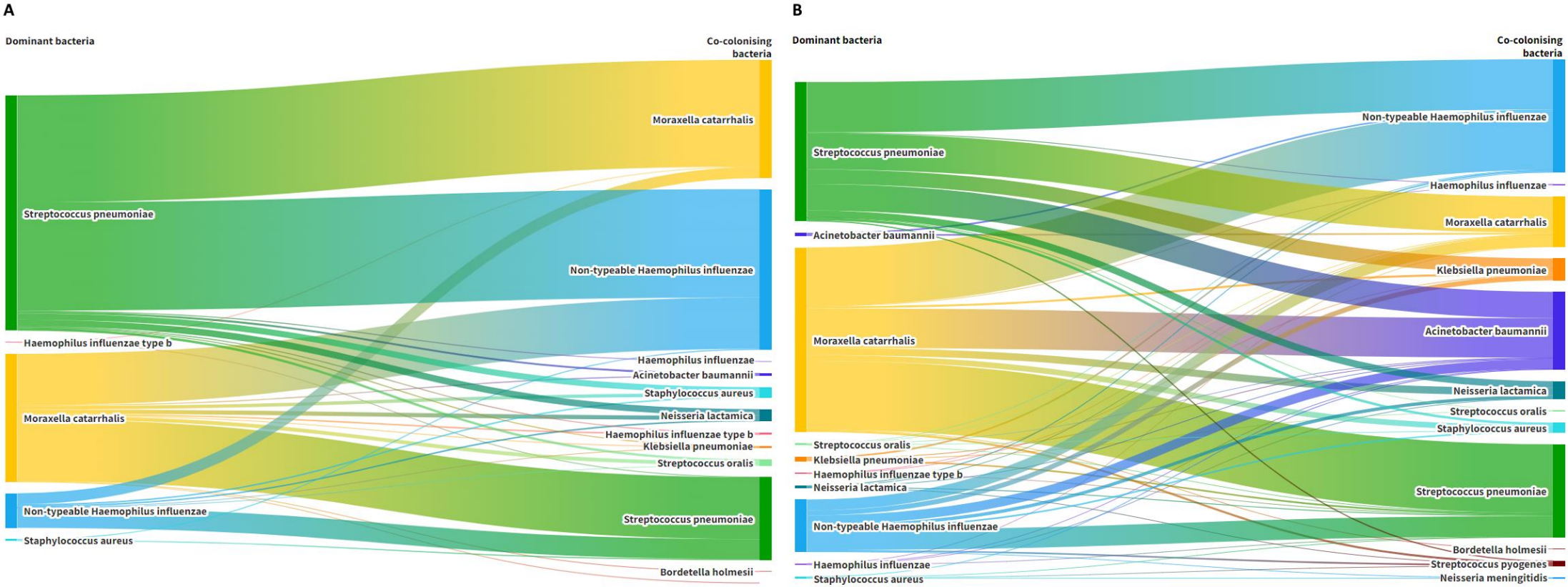

Figure 6: Alluvial Sankey diagram of bacterial co-colonizing events in children 0-60 months of age in Agincourt, Period-1 and Period-2.

Panel A: Period-1 (2009), and Panel B: Period-2 (2017). The bar nodes represent dominant (highest density) bacteria on the left and co-colonising (lower density relative to the dominant species) bacteria on the right. Each dominant bacteria is paired with a co-colonizing bacteria through a flow line and the thickness is proportional to number of co-colonising events between each bacteria. The node thickness relates to the number of colonizing relationships between each of the bacterium rather than the prevalence. Where more than two bacteria were detected in co-colonization, these were paired separately with the dominant bacterium, i.e.: Bacterium 1 > Bacterium 2 > Bacterium 3 would be Bacterium 1 > Bacterium 2, and Bacterium 1 > Bacterium 3. An interactive version of each Sankey diagram that includes the number of times bacteria are involved in each co-colonisation relationship is available at https://public.flourish.studio/visualisation/16708392/ and https://public.flourish.studio/visualisation/16722899/. The numbers of interactions are detailed in Supp. Table 8.

The differing effectiveness of PCV on individual VT colonization may be due serotype-specific differences in immunogenicity of vaccine, and likely differing antibody concentrations (including functional activity) required to reduce the risk of colonization acquisition [36]. Serotypes 1, 4, 6A, and 18C were completely eradicated in Period-2 compared with Period-1 where these serotypes accounted for 20.2% (65/321) of all VT.

Conversely, there was no difference in the colonisation prevalence of VT 3 (1.4 vs 1.6%) and 5 (1.2 vs. 1.0%) between the study periods. This ongoing circulation and colonization by serotype 3 in South Africa corroborates with the absence of marked decrease in serotype 3 IPD in South Africa between 2009 and 2017 [13], supporting the absence of PCV13 effectiveness against serotype 3 [37].

The 62-91% reduction in the prevalence of vaccine serotypes 14, 6B, 6A, 19A and 23F is encouraging, given that these were the most predominant serotypes associated with IPD in children in 2009 [13]. Moreover, the elimination of 4, 18C and 6A reported here in 2017 is likely to contribute to indirect protection in older, unvaccinated age-groups.

The increase in NVT serotype 16F carriage requires monitoring, as this serotype was amongst the top five most common serotypes causing IPD in South Africa in 2018 [13], and 16F lineages are associated with antibiotic resistance in South Africa [38].

The serotype-specific correlate of protection against IPD for serotype 3 and 19F (2.83 µg/ml and 1.17 µg/ml) exceeds the purported threshold of 0.35 µg/ml established to protect against other serotype disease and are used to evaluate PCV immunogenicity [37], whereas higher IgG concentrations are required to prevent the acquisition of colonisation [39]. Consequently, PCVs may not fully protect against serotype 19F acquisition [40]. This translated to a lower impact of PCV immunization on carriage of serotypes 3 and 19F in our study, which is consistent with studies from other African settings [10, 18-20, 41, 42].

Studies conducted in rural African settings consistently documented high residual prevalence of VT carriage in children ≤60 months at least two years post-PCV [10, 18-21]. Our residual VT carriage estimate (14.3%) eight years after routine PCV childhood immunization demonstrates the continued decrease in VT carriage compared with 17.9% reported in children aged 9-59 months in the same setting four years after the start of the PCV immunization program in South Africa [10]. Other studies have reported prevalence of PCV13-VT carriage of 18.0% in Cameroon among children 24-36 months old, four years after routine PCV immunisation [18] and 15.1% in Malawian children aged six weeks to four years at three years post-PCV13 introduction[19]. A study in rural and urban areas in Mozambique after 2-3 years of PCV10-immunisation identified much higher carriage of PCV13-VT (31.9%) than our estimate (14.3%), whereas carriage of PCV10-VT (14.5%, 82/564) was similar [21].

We employed a highly sensitive molecular technique for detecting and serotyping pneumococcus, while previous studies conducted in rural African settings [10, 19-21] predominantly relied on culture-based detection methods that don't effectively identify lower-density or concurrent colonisers [15], presenting a limitation to comparing prevalence between studies. Here, we report that 19.7% of all identified serotypes corresponded to co-colonisation events, with 17.3% and 8.1% of the detected VT being co-colonising serotypes to NVT in Period-2 and Period-1 respectively. This suggests the potential oversight of co-colonisation events resulting in an under-estimation of serotype carriage prevalence when utilizing culture-based, compared with molecular methodologies [26]. The use of more sensitive molecular techniques in our study allowed a better estimation of carriage prevalence and mitigates our finding of only modestly lower carriage prevalence compared with studies conducted only 2-4 years after the introduction of PCVs that relied on culture-based detection and serotyping.

Along with a shift to a lower mean density for VT in Period-2 compared with Period-1, the odds of VT being co-colonisers to NVT, and not the highest density colonisers were also increased in Period-2, indicating a shift in density dynamics. Specifically, although 19F remained a prominent VT during Period-2 (5.3%), there was a reduction in the density compared with Period-1. This has not translated to a marked impact on disease, as 19F was

the second most predominant serotype identified in IPD in children ≤5 years old between 2018 and 2020 [13, 43].

The overall prevalence of pneumococcal carriage in Period-2 was 76.9%, showing a small reduction from 83.2% observed in Period-1. The carriage prevalence in both time periods are consistent with those reported from other rural African settings [44].

We found higher prevalence of *A. baumannii* and *K. pneumoniae* and a concomitant increase in the density of *K. pneumoniae* in the nasopharynx of children in Period-2. Few studies have assessed the upper respiratory tract carriage in asymptomatic children of these two bacterial species with studies conducted in low-resource settings reporting moderate nasopharyngeal colonisation by *A. baumannii* and *K. pneumoniae* [45, 46]. The prevalence of *K. pneumoniae* colonisation increased in Period-2 [46, 47]. In the urban township of Soweto, South Africa, there was a similar increase in the prevalence of *K. pneumoniae* from 6.7% to 14.2% between 2010/11 and 2018, however *A. baumannii* prevalence remained the same (4.6-6.0%) between the two time periods [15]. These two highly transmissible pathogens are often involved in antibiotic-resistant and nosocomial infections [48-50]. Asymptomatic bacterial carriage in isolation may not be alarming, however an increase in both *K. pneumoniae* and *A. baumannii* disease cases have been reported in enhanced surveillance of invasive bacterial disease in 2017-18 in South Africa [13]. Furthermore, in 42% of children aged <59 months that demised with *K. pneumoniae* in the causal pathway in the multi-site Child Health and Mortality Prevention Surveillance (CHAMPS) study (Dec 2016 to Dec 2021) that included South Africa, *K. pneumoniae* was likely acquired in community settings prior to admission. Together with the 15% of deaths in community settings in South Africa involving *K. pneumoniae*, this suggests that community transmission also contributes to disease burden [51]. It will be important to further characterise the virulence factors and antibiotic resistance profile associated with colonisation in healthy children, including the relevance of co-colonisation with other bacterial species, as these two bacteria are listed as WHO priority pathogens [48].

This study was limited in that, although the sample size was calculated to adequately detect changes in the most prevalent VT among children aged ≤60 months between the two periods, we were not powered to detect differences in less common events, e.g.: colonisation, density, or the multiple concurrent colonisation of less prevalent serotypes or bacterial species, particularly when stratified into age categories. In light of this, the findings related to less frequent colonisers should be approached with caution. Enrolment in Period-1 was conducted in the winter and autumn months (May-October 2009), whereas in Period-2 enrolments continued into the summer months (July 2017-February 2018, and seasonal variation in transmission pf pneumococcus could have resulted in an underestimated impact, due to higher transmission in the summer months [52]. Other limitations included that non-typeable pneumococcus were determined by the detection of pneumococcus without ascribing a specific capsular type, or where the sum of the density of assigned serotypes was lower than the total pneumococcal density using our qPCR method. It is thus plausible that a subset of these non-typeable samples were actually newly identified serotypes for which capsular targets were not incorporated into our qPCR serotyping scheme, for example, serotype 33G which was recently described in two isolates from South Africa [53]. Nevertheless, these were not expected to be prevalent at this time-point in our setting.

In conclusion, excluding serotypes 6B and 19F, there was a large overall reduction in PCV13-VT colonisation in children ≤60 months-old in Period-2 relative to Period-1. Nonetheless eight years after routine PCV immunisation, there was still residual carriage (14.3%) of PCV13-VT in 2017.

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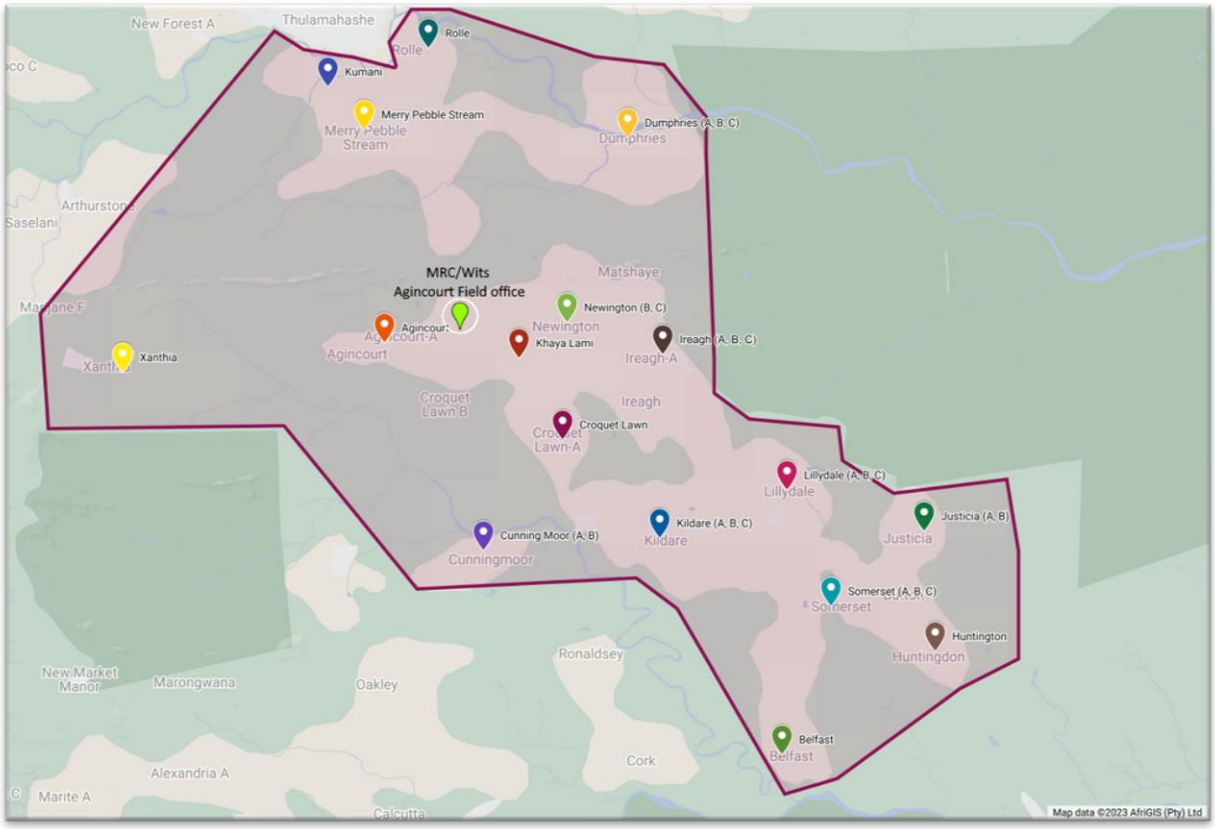
Supplementary Tables and Figures



Supplementary Figure 1: Villages included within the Agincourt Health and Demographic Surveillance Site (HDSS)

The MRC/Wits Agincourt field office, and the laboratory are indicated by a circled green marker. Where a group of villages have the same name but are sub-divided into sections and denoted alphabetically, the central point is indicated with one marker, e.g. the three villages: Ireagh A, Ireagh B and Ireagh C are marked as Ireagh (A, B, C).

Supplementary Table 1: Pneumococcal conjugate vaccine coverage in Agincourt HDSS in Period-2 (2017/18)

Age (weeks)	1 dose	2 doses	3 doses
Period-2: 6-14, % (n/N)	95.5 (21/22)	-	-
Period-2: 14-40, % (n/N)	100 (34/34)	100 (34/34)	-
Period-2: >40, % (n/N)	99.6 (501/503)	99.2 (499/503)	94.4 (475/503)
Period-1: >40, % (n/N)			61.1 (385/630); p<0.001

Note: In Period-1, data on PCV immunisation was not collected per dose, and only fully immunized children (3 doses) could be compared with Period-2.

Supplementary Table 2: Prevalence of *Streptococcus pneumoniae* colonisation in Period-1 (2009) and Period-2 (2017/8) in Agincourt children 0-60 months-of-age.

	Period-1, % (n/N)	Period-2, % (n/N)	OR (95% CI), p-value	aOR (95% CI), p-value
All pneumococcus	83.2 (524/630)	76.9 (437/568)	0.67 (0.51-0.90), p=0.007	0.65 (0.48-0.87), p<0.001
PCV13-VT	51.0 (321/630)	14.3 (81/568)	0.16 (0.12-0.21), p<0.001	0.16 (0.12-0.21), p<0.001
NVT	35.6 (224/630)	63.2 (359/568)	3.11 (2.46-3.94), p<0.001	3.12 (2.45-3.97), p<0.001
NT	12.5 (79/630)	21.8 (124/568)	1.95 (1.43-2.65), p<0.001	1.94 (1.42-2.67), p<0.001
1	0.6 (4/630)	0 (0/568)	p=0.057	
3	1.6 (10/630)	1.4 (8/568)	0.89 (0.35-2.26), p=0.799	0.88 (0.34-2.25), p=0.785
4	1.3 (8/630)	0 (0/568)	p=0.007	
5	1.0 (6/630)	1.2 (7/568)	1.30 (0.43-3.88), p=0.640	1.20 (0.40-3.66), p=0.743
6A	7.6 (48/630)	0 (0/568)	p<0.001	
6B	15.2 (96/630)	4.8 (27/568)	0.28 (0.18-0.43), p<0.001	0.26 (0.16-0.41), p<0.001
9A/V	0.8 (5/630)	0.2 (1/568)	0.22 (0.03-1.89), p=0.131	0.19 (0.02-1.64), p=0.130
14	6.2 (39/630)	0.9 (5/568)	0.13 (0.05-0.34), p<0.001	0.14 (0.05-0.35), p<0.001
18C	0.8 (5/630)	0 (0/568)	p=0.033	
19A	3.7 (23/630)	1.4 (8/568)	0.38 (0.17-0.85), p=0.015	0.37 (0.16-0.86), p=0.020
19F	10.3 (65/630)	5.3 (30/568)	0.48 (0.31-0.76), p=0.001	0.52 (0.33-0.82), p=0.005
23F	7.9 (50/630)	0.7 (4/568)	0.08 (0.03-0.23), p<0.001	0.08 (0.03-0.23), p<0.001
2	0.3 (2/630)	0 (0/568)	p=0.179	
6D	0.2 (1/630)	0.9 (5/568)	5.59 (0.65-47.96), p=0.077	5.31 (0.61-46.17), p=0.130
7B/C/40	0.3 (2/630)	0.4 (2/568)	1.11 (0.16-7.90), p=0.917	1.15 (0.16-8.17), p=0.892
8	0.6 (4/630)	1.6 (9/568)	2.52 (0.77-8.23), p=0.113	2.33 (0.71-7.68), p=0.165
9L/N	0.6 (4/630)	0.9 (5/568)	1.39 (0.37-5.20), p=0.623	1.33 (0.35-5.01), p=0.677
9-like	1.6 (10/630)	0.7 (4/568)	0.44 (0.14-1.41), p=0.156	0.39 (0.12-1.29), p=0.123
10A	0.6 (4/630)	0.7 (4/568)	1.11 (0.28-4.46), p=0.883	1.15 (0.28-4.61), p=0.848
10B	0.2 (1/630)	0 (0/568)	p=0.342	
10C/F	0.2 (1/630)	0 (0/568)	p=0.342	
11A/D	2.4 (15/630)	3.7 (21/568)	1.57 (0.80-3.08), p=0.183	1.37 (0.69-2.72), p=0.362
12A/F/44	0.2 (1/630)	0.4 (2/568)	2.22 (0.20-24.58), p=0.504	1.80 (0.16-20.85), p=0.638
13	2.4 (15/630)	3.4 (19/568)	1.42 (0.71-2.82), p=0.316	1.44 (0.73-2.88), p=0.296
15A/F	0.3 (2/630)	2.8 (16/568)	9.10 (2.08-39.76), p<0.001	8.86 (2.02-38.86), p=0.004
15B/C	2.5 (16/630)	0.9 (5/568)	0.34 (0.12-0.94), p=0.029	0.34 (0.12-0.93), p=0.035
15-like	5.9 (37/630)	7.0 (40/568)	1.21 (0.76-1.93), p=0.410	1.20 (0.75-1.92), p=0.436
16A	0.2 (1/630)	0 (0/568)	p=0.342	
16F	0.3 (2/630)	8.1 (46/568)	27.67 (6.69-114.53), p<0.001	27.33 (6.59-113.33), p<0.001
17F	1.4 (9/630)	1.4 (8/568)	0.99 (0.38-2.57), p=0.977	1.09 (0.40-2.94), p=0.867
18A	0.3 (2/630)	0 (0/568)	p=0.179	
18B	0.2 (1/630)	0 (0/568)	p=0.342	
19B	0.5 (3/630)	0.5 (3/568)	1.11 (0.22-5.52), p=0.899	0.98 (0.19-4.98), p=0.983
20	1.0 (6/630)	0.4 (2/568)	0.37 (0.07-1.83), p=0.203	0.39 (0.08-1.94), p=0.248
21	0.2 (1/630)	3.7 (21/568)	24.15 (3.24-180.11), p<0.001	22.42 (3.00-167.71), p=0.002
22F	0 (0/630)	0.2 (1/568)	p=0.292	
23A	3.2 (20/630)	3.9 (22/568)	1.23 (0.66-2.28), p=0.512	1.38 (0.73-2.61), p=0.323
23B	3.5 (22/630)	5.1 (29/568)	1.49 (0.84-2.62), p=0.167	1.61 (0.89-2.89), p=0.112
24A	0 (0/630)	0.2 (1/568)	p=0.292	
31	0.3 (2/630)	0.9 (5/568)	2.79 (0.54-14.43), p=0.202	2.34 (0.44-12.36), p=0.318
33A/F	0 (0/630)	0.2 (1/568)	p=0.292	
33B	0 (0/630)	0.2 (1/568)	p=0.292	
33D	0 (0/630)	0.2 (1/568)	p=0.292	
34	2.5 (16/630)	3.0 (17/568)	1.18 (0.59-2.37), p=0.632	1.21 (0.60-2.43), p=0.591
35A/C	0.3 (2/630)	1.4 (8/568)	4.49 (0.95-21.21), p=0.038	4.42 (0.93-21.04), p=0.062
35B	0.8 (5/630)	2.5 (14/568)	3.16 (1.13-8.83), p=0.021	3.03 (1.08-8.52), p=0.036
35F	0.2 (1/630)	0.7 (4/568)	4.46 (0.50-40.03), p=0.144	4.16 (0.46-37.95), p=0.206
43	0.2 (1/630)	0 (0/568)	p=0.342	
45	0.2 (1/630)	0.4 (2/568)	2.22 (0.20-24.58), p=0.504	1.80 (0.16-20.85), p=0.638
46	0.2 (1/630)	0.2 (1/568)	1.11 (0.07-17.78), p=0.942	1.19 (0.07-19.03), p=0.904
47A	0.3 (2/630)	0 (0/568)	p=0.179	

OR: Odds Ratio

aOR: adjusted Odds Ratio; adjusted for breastfeeding, HIV infection, antibiotics, co-trimoxazole prophylaxis, and tuberculosis treatment. The OR or aOR could not be calculated where there were too few, or zero detected colonisation events in either timepoint zero. Where OR or aOR could not be calculated, the p-value was calculated using X^2.

Overall pneumococcus includes all samples positive for pneumococcal reference genes. PCV13-VT: vaccine serotypes including serotypes/groups 1, 3, 4, 5, 6A, 6B, 7A/F, 9A/V, 14, 18C, 19A, 19F and 23F. NVT: serotypes/serogroups not included in PCV13. NT: non-typeable pneumococci.

Supplementary Table 3: Prevalence of *Streptococcus pneumoniae* colonisation in Period-1 (2009) and Period-2 (2017/8) in Agincourt children 0-24 months-of-age.

	Period-1, % (n/N)	Period-2, % (n/N)	OR (95% CI), p-value	aOR (95% CI), p-value
All pneumococcus	84.6 (314/371)	78.5 (153/195)	0.66 (0.42-1.03), p=0.066	0.69 (0.43-1.10), p<0.001
PCV13-VT	52.6 (195/371)	12.3 (24/195)	0.13 (0.08-0.20), p<0.001	0.12 (0.07-0.19), p<0.001
NVT	34.8 (129/371)	65.6 (128/195)	3.58 (2.49-5.16), p<0.001	3.98 (2.70-5.87), p<0.001
NT	13.8 (51/371)	18.5 (36/195)	1.42 (0.89-2.27), p=0.139	1.64 (0.99-2.71), p<0.001
1	0.8 (3/371)	0 (0/195)		p=0.208
3	0.8 (3/371)	1.0 (2/195)	1.27 (0.21-7.67), p=0.793	1.26 (0.19-8.19), p=0.810
4	1.6 (6/371)	0 (0/195)		p=0.074
5	0.5 (2/371)	2.6 (5/195)	4.86 (0.93-25.26), p=0.038	3.65 (0.70-19.03), p=0.125
6A	7.6 (28/371)	0 (0/195)		p<0.001
6B	17.8 (66/371)	3.6 (7/195)	0.17 (0.08-0.38), p<0.001	0.15 (0.07-0.33), p<0.001
9A/V	0.3 (1/371)	0 (0/195)		p=0.468
14	7.3 (27/371)	0.5 (1/195)	0.07 (0.01-0.49), p<0.001	0.06 (0.01-0.47), p=0.007
18C	0.5 (2/371)	0 (0/195)		p=0.304
19A	4.3 (16/371)	1.5 (3/195)	0.35 (0.10-1.20), p=0.082	0.42 (0.12-1.52), p=0.187
19F	10.2 (38/371)	3.6 (7/195)	0.33 (0.14-0.75), p=0.005	0.36 (0.15-0.84), p=0.019
23F	7.3 (27/371)	0.5 (1/195)	0.07 (0.01-0.49), p<0.001	0.07 (0.01-0.51), p=0.009
2	0.5 (2/371)	0 (0/195)		p=0.304
6D	0.3 (1/371)	0.5 (1/195)	1.91 (0.12-30.66), p=0.643	1.43 (0.09-23.01), p=0.801
7B/C/40	0.3 (1/371)	0 (0/195)		p=0.468
8	0.8 (3/371)	1.0 (2/195)	1.27 (0.21-7.67), p=0.793	1.12 (0.17-7.41), p=0.909
9L/N	0.8 (3/371)	1.0 (2/195)	1.27 (0.21-7.67), p=0.793	0.95 (0.16-5.75), p=0.956
9-like	1.6 (6/371)	0.5 (1/195)	0.31 (0.04-2.62), p=0.259	0.39 (0.04-3.51), p=0.402
10A	0.5 (2/371)	1.0 (2/195)	1.91 (0.27-13.68), p=0.511	3.58 (0.40-31.97), p=0.254
10B	0.3 (1/371)	0 (0/195)		p=0.468
10C/F	0.3 (1/371)	0 (0/195)		p=0.468
11A/D	1.6 (6/371)	3.6 (7/195)	2.27 (0.75-6.84), p=0.137	1.94 (0.60-6.22), p=0.268
12A/F/44	0.3 (1/371)	0.5 (1/195)	1.91 (0.12-30.66), p=0.651	1.43 (0.09-23.01), p=0.643
13	2.7 (10/371)	4.6 (9/195)	1.75 (0.70-4.37), p=0.228	1.61 (0.62-4.19), p=0.330
15A/F	0 (0/371)	4.1 (8/195)		p<0.001
15B/C	1.6 (6/371)	1.0 (2/195)	0.63 (0.13-3.15), p=0.571	0.77 (0.14-4.16), p=0.759
15-like	5.9 (22/371)	7.7 (15/195)	1.32 (0.67-2.61), p=0.420	1.56 (0.75-3.23), p=0.231
16A	0 (0/371)	0 (0/195)		-
16F	0.3 (1/371)	6.2 (12/195)	24.26 (3.13-188.03), p<0.001	27.17 (3.27-226.06), p=0.002
17F	1.6 (6/371)	1.0 (2/195)	0.63 (0.13-3.15), p=0.571	0.77 (0.14-4.16), p=0.759
18A	0.3 (1/371)	0 (0/195)		p=0.468
18B	0 (0/371)	0 (0/195)		-
19B	0.5 (2/371)	0.5 (1/195)	0.95 (0.09-10.55), p=0.967	2.17 (0.15-30.65), p=0.566
20	0.8 (3/371)	1.0 (2/195)	1.27 (0.21-7.67), p=0.793	1.26 (0.19-8.19), p=0.810
21	0 (0/371)	4.1 (8/195)		p<0.001
22F	0 (0/371)	0.5 (1/195)		p=0.167
23A	2.7 (10/371)	6.2 (12/195)	2.37 (1.00-5.58), p=0.043	2.40 (0.95-6.06), p=0.064
23B	3.5 (13/371)	6.2 (12/195)	1.81 (0.81-4.04), p=0.145	2.03 (0.84-4.91), p=0.115
24A	0 (0/371)	0.5 (1/195)		p=0.167
31	0 (0/371)	1.0 (2/195)		p=0.051
33A/F	0 (0/371)	0 (0/195)		-
33B	0 (0/371)	0.5 (1/195)		p=0.167
33D	0 (0/371)	0 (0/195)		-
34	1.6 (6/371)	5.6 (11/195)	3.64 (1.32-9.99), p=0.008	3.88 (1.32-11.39), p=0.014
35A/C	0.5 (2/371)	2.6 (5/195)	4.86 (0.93-25.26), p=0.060	4.98 (0.87-28.43), p=0.038
35B	1.1 (4/371)	2.1 (4/195)	1.92 (0.48-7.77), p=0.351	2.17 (0.49-9.65), p=0.309
35F	0.3 (1/371)	0.5 (1/195)	1.91 (0.12-30.66), p=0.643	1.43 (0.09-23.01), p=0.801
43	0.3 (1/371)	0 (0/195)		p=0.468
45	0.3 (1/371)	0.5 (1/195)	1.91 (0.12-30.66), p=0.643	1.43 (0.09-23.01), p=0.801
46	0.3 (1/371)	0.5 (1/195)	1.91 (0.12-30.66), p=0.643	1.43 (0.09-23.01), p=0.801
47A	0.3 (1/371)	0 (0/195)		p=0.468

OR: Odds Ratio

aOR: adjusted Odds Ratio; adjusted for breastfeeding, HIV infection, antibiotics, co-trimoxazole prophylaxis, and tuberculosis treatment.

The OR or aOR could not be calculated where there were too few, or zero detected colonisation events in either timepoint zero. Where OR or aOR could not be calculated, the p-value was calculated using X^2.

Overall pneumococcus includes all samples positive for pneumococcal reference genes. PCV13-VT: vaccine serotypes including serotypes/groups 1, 3, 4, 5, 6A, 6B, 7A/F, 9A/V, 14, 18C, 19A, 19F and 23F. NVT: serotypes/serogroups not included in PCV13. NT: non-typeable pneumococci.

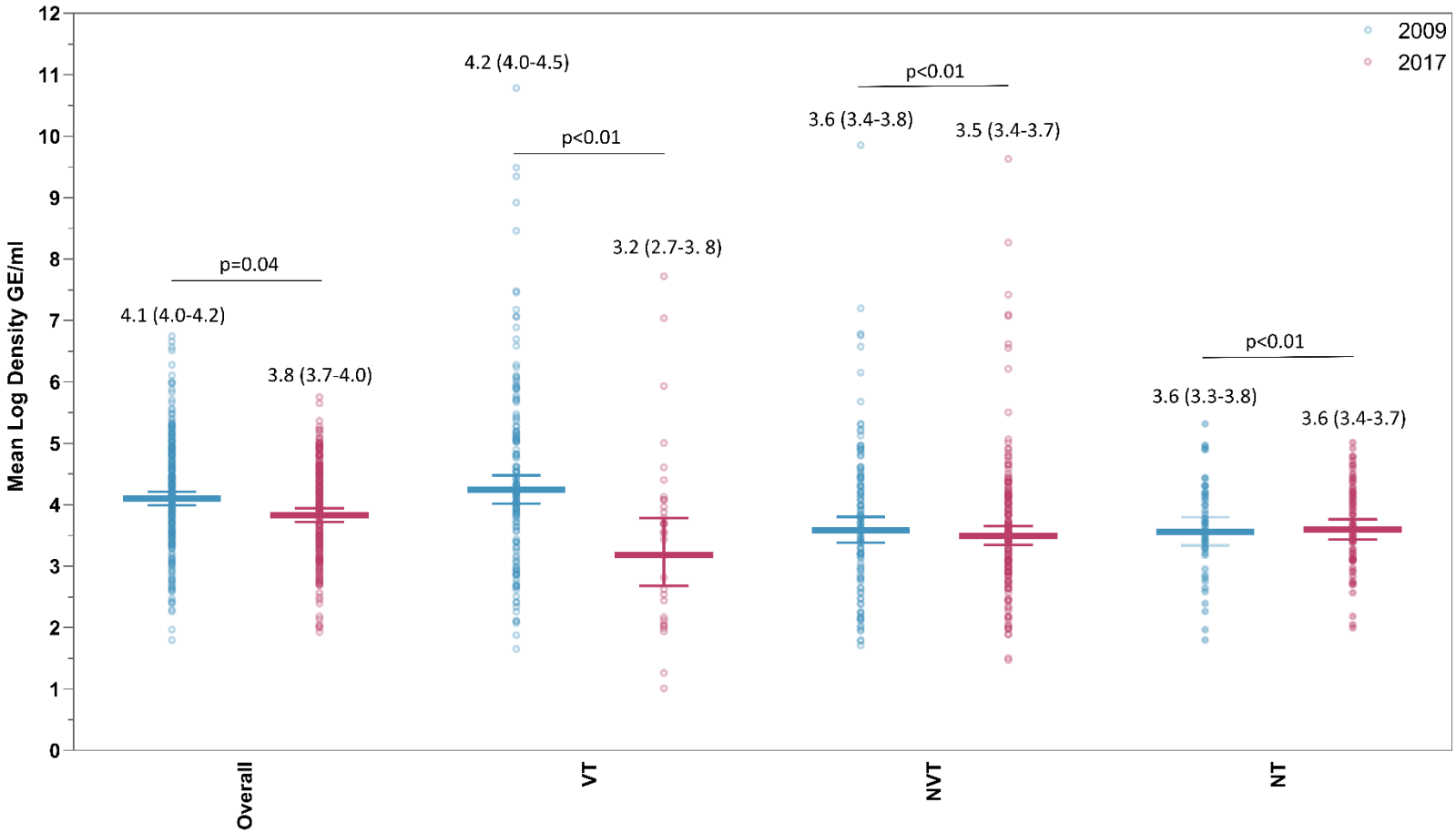
Supplementary Table 4: Prevalence of *Streptococcus pneumoniae* colonisation in Period-1 (2009) and Period-2 (2017/8) in Agincourt children 25-60 months-of-age.

	Period-1, % (n/N)	Period-2, % (n/N)	OR (95% CI), p-value	aOR (95% CI), p-value
All pneumococcus	81.1 (210/259)	76.1 (284/373)	0.74 (0.50-1.10), p=0.139	0.68 (0.45-1.03), p=0.066
PCV13-VT	48.7 (126/259)	15.3 (57/373)	0.19 (0.13-0.28), p<0.001	0.18 (0.12-0.27), p<0.001
NVT	36.7 (95/259)	61.9 (231/373)	2.81 (2.02-3.90), p<0.001	2.67 (1.90-3.74), p<0.001
NT	10.8 (28/259)	23.6 (88/373)	2.55 (1.61-4.03), p<0.001	2.43 (1.52-3.88), p<0.001
1	0.4 (1/259)	0 (0/373)		p=0.230
3	2.7 (7/259)	1.6 (6/373)	0.59 (0.20-1.77), p=0.341	0.58 (0.19-1.77), p=0.338
4	0.8 (2/259)	0 (0/373)		p=0.089
5	1.5 (4/259)	0.5 (2/373)	0.34 (0.06-1.89), p=0.199	0.22 (0.04-1.39), p=0.107
6A	7.7 (20/259)	0.0 (0/373)		p<0.001
6B	11.6 (30/259)	5.4 (20/373)	0.43 (0.24-0.78), p=0.004	0.35 (0.19-0.66), p=0.001
9A/V	1.5 (4/259)	0.3 (1/373)	0.17 (0.02-1.54), p=0.075	0.11 (0.01-1.11), p=0.062
14	4.6 (12/259)	1.1 (4/373)	0.22 (0.07-0.70), p=0.005	0.23 (0.07-0.75), p=0.014
18C	1.2 (3/259)	0 (0/373)		p=0.037
19A	2.7 (7/259)	1.3 (5/373)	0.49 (0.15-1.56), p=0.217	0.58 (0.16-2.12), p=0.410
19F	10.4 (27/259)	6.2 (23/373)	0.56 (0.32-1.01), p=0.051	0.63 (0.34-1.16), p=0.138
23F	8.9 (23/259)	0.8 (3/373)	0.08 (0.02-0.28), p<0.001	0.09 (0.03-0.30), p<0.001
2	0 (0/259)	0 (0/373)		-
6D	0 (0/259)	1.1 (4/373)		p=0.095
7B/C/40	0.4 (1/259)	0.5 (2/373)	1.39 (0.13-15.42), p=0.787	1.40 (0.12-15.65), p=0.787
8	0.4 (1/259)	1.9 (7/373)	4.93 (0.60-40.35), p=0.099	4.65 (0.56-38.62), p=0.155
9L/N	0.4 (1/259)	0.8 (3/373)	2.09 (0.22-20.22), p=0.514	1.53 (0.15-15.19), p=0.716
9-like	1.5 (4/259)	0.8 (3/373)	0.52 (0.11-2.33), p=0.382	0.39 (0.08-1.96), p=0.253
10A	0.8 (2/259)	0.5 (2/373)	0.69 (0.10-4.95), p=0.713	0.74 (0.10-5.34), p=0.764
10B	0 (0/259)	0 (0/373)		-
10C/F	0 (0/259)	0 (0/373)		-
11A/D	3.5 (9/259)	3.8 (14/373)	1.08 (0.46-2.54), p=0.854	0.97 (0.40-2.34), p=0.940
12A/F/44	0 (0/259)	0.3 (1/373)		p=0.404
13	1.9 (5/259)	2.7 (10/373)	1.40 (0.47-4.14), p=0.542	1.41 (0.47-4.20), p=0.540
15A/F	0.8 (2/259)	2.1 (8/373)	2.82 (0.59-13.37), p=0.174	2.88 (0.60-13.84), p=0.187
15B/C	3.9 (10/259)	0.8 (3/373)	0.20 (0.06-0.74), p=0.008	0.19 (0.05-0.72), p=0.015
15-like	5.8 (15/259)	6.7 (25/373)	1.17 (0.60-2.26), p=0.644	1.12 (0.57-2.21), p=0.741
16A	0.4 (1/259)	0 (0/373)		p=0.230
16F	0.4 (1/259)	9.1 (34/373)	25.88 (3.52-190.28), p<0.001	26.73 (3.62-197.21), p=0.001
17F	1.2 (3/259)	1.6 (6/373)	1.40 (0.35-5.63), p=0.638	1.94 (0.38-9.93), p=0.426
18A	0.4 (1/259)	0 (0/373)		p=0.230
18B	0.4 (1/259)	0 (0/373)		p=0.230
19B	0.4 (1/259)	0.5 (2/373)	1.39 (0.13-15.42), p=0.787	0.94 (0.08-10.90), p=0.963
20	1.2 (3/259)	0 (0/373)		p=0.037
21	0.4 (1/259)	3.5 (13/373)	9.32 (1.21-71.67), p=0.009	7.85 (1.00-61.37), p=0.050
22F	0 (0/259)	0 (0/373)		-
23A	3.9 (10/259)	2.7 (10/373)	0.69 (0.28-1.67), p=0.405	0.83 (0.33-2.08), p=0.686
23B	3.5 (9/259)	4.6 (17/373)	1.33 (0.58-3.02), p=0.500	1.33 (0.58-3.08), p=0.501
24A	0 (0/259)	0 (0/373)		-
31	0.8 (2/259)	0.8 (3/373)	1.04 (0.17-6.28), p=0.964	0.68 (0.10-4.41), p=0.682
33A/F	0 (0/259)	0.3 (1/373)		p=0.404
33B	0 (0/259)	0 (0/373)		-
33D	0 (0/259)	0.3 (1/373)		p=0.404
34	3.9 (10/259)	1.6 (6/373)	0.41 (0.15-1.13), p=0.076	0.41 (0.14-1.16), p=0.093
35A/C	0 (0/259)	0.8 (3/373)		p=0.148
35B	0.4 (1/259)	2.7 (10/373)	7.11 (0.90-55.87), p=0.030	6.15 (0.77-49.07), p=0.087
35F	0 (0/259)	0.8 (3/373)		p=0.148
43	0 (0/259)	0 (0/373)		-
45	0 (0/259)	0.3 (1/373)		p=0.404
46	0 (0/259)	0 (0/373)		-
47A	0.4 (1/259)	0 (0/373)		p=0.230

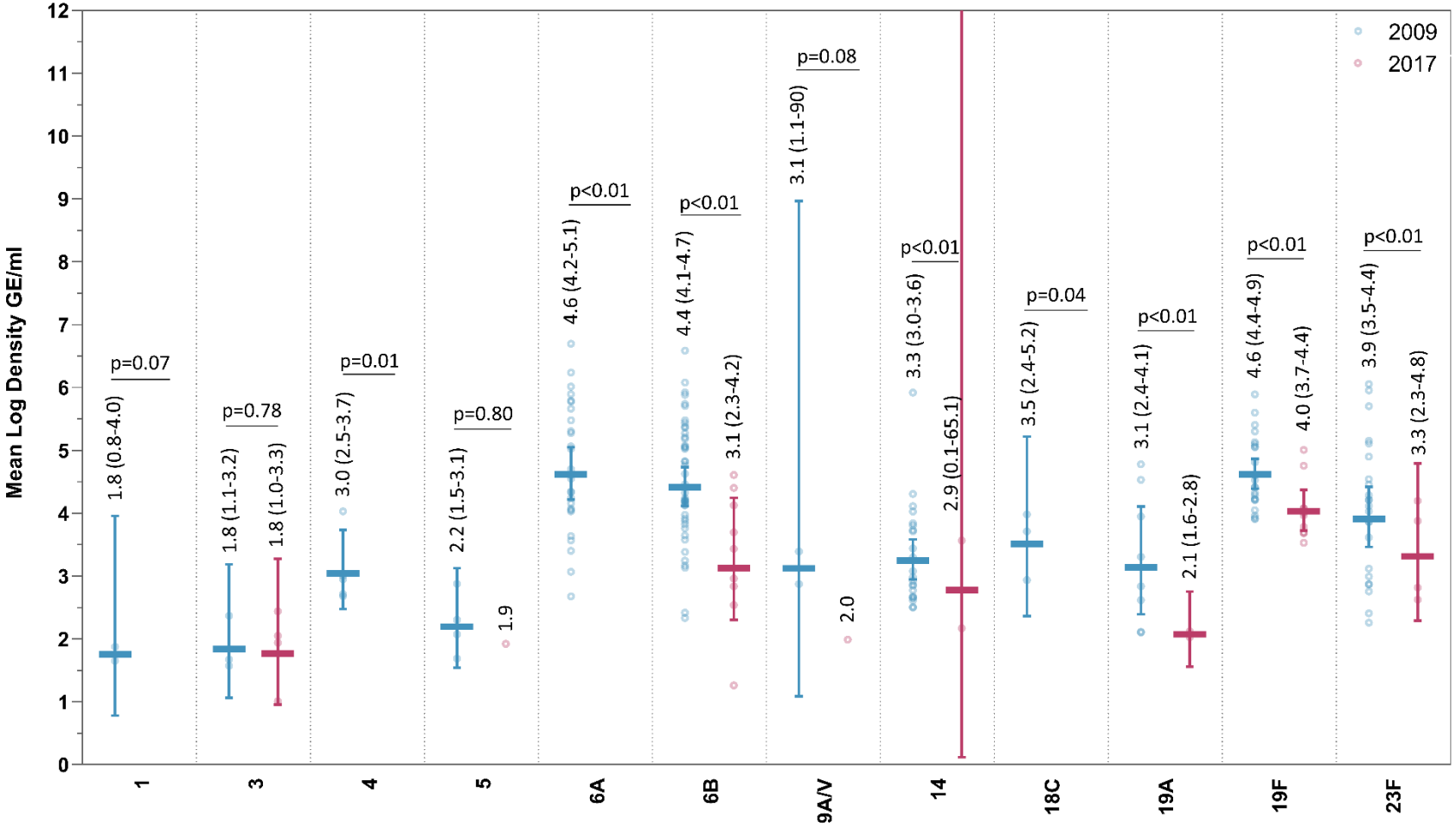
OR: Odds Ratio

aOR: adjusted Odds Ratio; adjusted for breastfeeding, HIV infection, antibiotics, co-trimoxazole prophylaxis, and tuberculosis treatment. The OR or aOR could not be calculated where there were too few, or zero detected colonisation events in either timepoint zero. Where OR or aOR could not be calculated, the p-value was calculated using X^2.

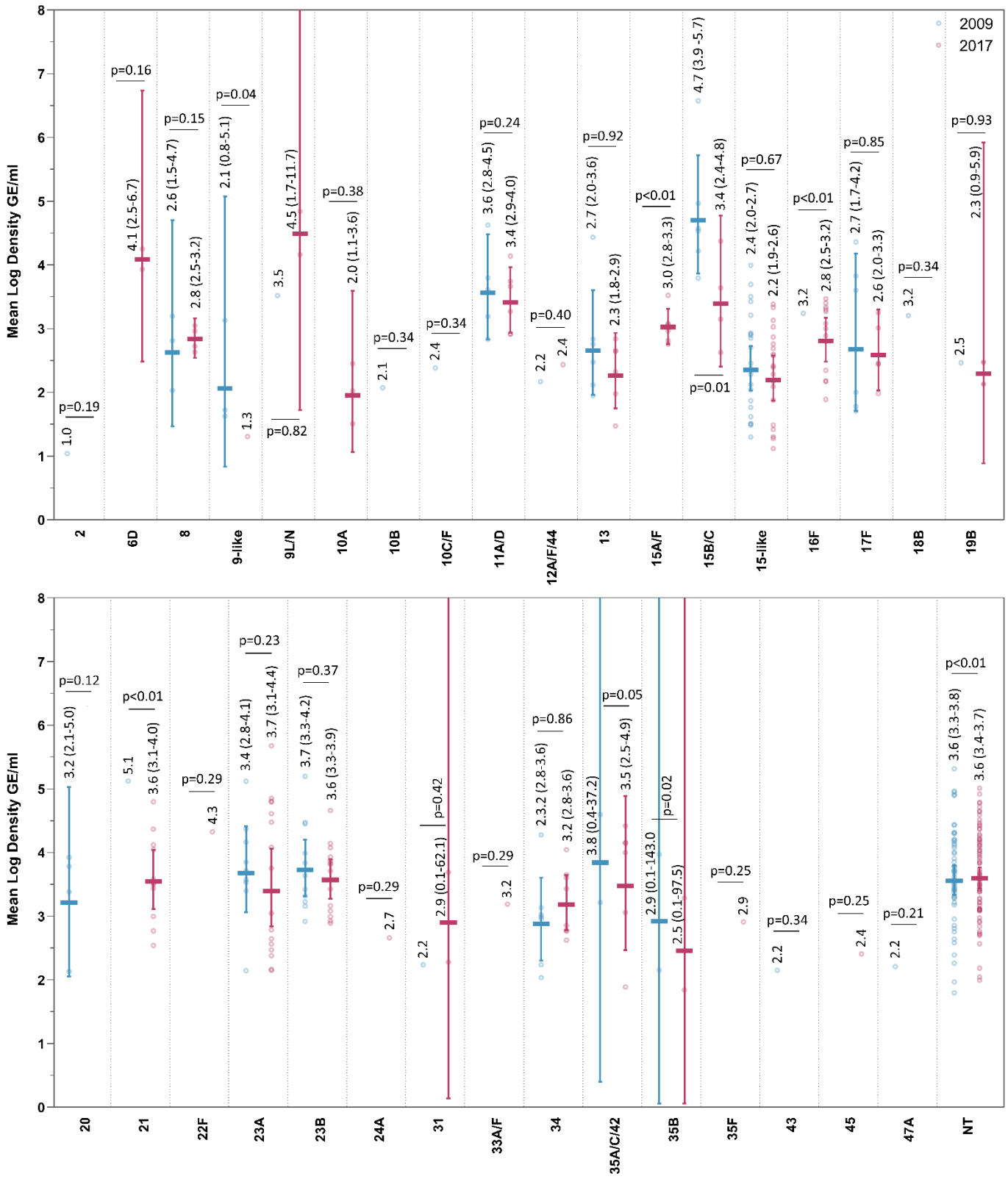
Overall pneumococcus includes all samples positive for pneumococcal reference genes. PCV13-VT: vaccine serotypes including serotypes/groups 1, 3, 4, 5, 6A, 6B, 7A/F, 9A/V, 14, 18C, 19A, 19F and 23F. NVT: serotypes/serogroups not included in PCV13. NT: non-typeable pneumococci.



Supplementary Figure 2: Mean log density (log_{10} Genomic Equivalents per mL [GE/mL]) of *Streptococcus pneumoniae* in children 0-60 months of age in Period 1 compared with Period 2.
Overall includes all pneumococcus; VT include PCV13 vaccine serotypes serotypes/serogroups 1, 3, 4, 5,6A, 6B, 7A/F, 9A/V, 14, 18C, 19A, 19F and 23F; NVT, all serotypes/serogroups not included in PCV13. NT, non-typeable S. pneumoniae. Mean log_{10} density (95%Confidence Interval) values shown.



Supplementary Figure 3: Mean log density (log_{10} Genomic Equivalents per mL [GE/mL]) of colonising PCV13 vaccine serotypes in children 0-60 months-of-age.
Mean log_{10} density (95%Confidence Interval) values shown.



Supplementary Figure 4: Mean log density (log_{10} Genomic Equivalents per mL [GE/mL]) of non-PCV13 serotypes (NVT) in children 0-60 months of age.
Mean log_{10} density (95%Confidence Interval) values shown.

Supplementary Table 5: Carriage mean log density of *Streptococcus pneumoniae* from Nasopharyngeal Swab samples collected from children 0-60 months of age in Period 1 (2009, n=630) and Period 2 (2017/18, n=568).

Serotype/ group	Period	0-60 months n†	0-60 months Mean log density (95% CI) log_{10} GE/ml‡	p-value
Overall	1	261	4.1 (4.0-4.2)	
	2	210	3.8 (3.7-4.0)	0.035
NVT	1	120	3.6 (3.4-3.8)	
	2	173	3.5 (3.4-3.7)	<0.001
PCV13-VT	1	151	4.2 (4.0-4.5)	
	2	30	3.2 (2.7-3.8)	<0.001
NT	1	51	3.6 (3.3-3.8)	
	2	75	3.6 (3.4-3.7)	<0.001
1	1	2	1.8 (0.8-4.0)	
	2	-	-	0.067
3	1	3	1.8 (1.1-3.2)	
	2	4	1.8 (1.0-3.3)	0.775
4	1	5	3.0 (2.5-3.7)	
	2	-	-	0.009
5	1	4	2.2 (1.5-3.1)	
	2	1	1.9	0.799
6A	1	27	4.6 (4.2-5.1)	
	2	-	-	<0.001
6B	1	44	4.4 (4.1-4.7)	
	2	9	3.1 (2.3-4.2)	<0.001
9A/V	1	2	3.1 (1.1-90)	
	2	1	2.0	0.079
14	1	22	3.3 (3.0-3.6)	
	2	2	2.9 (0.1-65.1)	<0.001
18C	1	3	3.5 (2.4-5.2)	
	2	-	-	0.035
19A	1	8	3.1 (2.4-4.1)	
	2	2	2.1 (1.6-2.8)	0.004
19F	1	23	4.6 (4.4-4.9)	
	2	10	4.0 (3.7-4.4)	<0.001
23F	1	23	3.9 (3.5-4.4)	
	2	4	3.3 (2.3-4.8)	<0.001
2	1	1	1.0	
	2	-	-	0.185
6D	1	-	-	
	2	2	4.1 (2.5-6.7)	0.158
8	1	3	2.6 (1.5-4.7)	
	2	4	2.8 (2.5-3.2)	0.146
9-like	1	3	2.1 (0.8-5.1)	
	2	1	1.3	0.044
9L/N	1	1	3.5	

Serotype/ group	Period	0-60 months n†	0-60 months Mean log density (95% CI) log_{10} GE/ml‡	0-60 months p-value
	2	2	4.5 (1.7-11.7)	0.817
10A	1	-	-	
	2	3	2.0 (1.1-3.6)	0.375
10B	1	1	2.1	
	2	-	-	0.343
10C/F	1	1	2.4	
	2	-	-	0.343
11A/D	1	5	3.6 (2.8-4.5)	
	2	6	3.4 (2.9-4.0)	0.241
12A/F/44	1	1	2.2	
	2	1	2.4	0.395
13	1	6	2.7 (2.0-3.6)	
	2	6	2.3 (1.8-2.9)	0.922
15A/F	1	-	-	
	2	6	3.0 (2.8-3.3)	0.002
15B/C	1	6	4.7 (3.9 -5.7)	
	2	4	3.4 (2.4-4.8)	0.012
15-like	1	22	2.4 (2.0-2.7)	
	2	20	2.2 (1.9-2.6)	0.672
16F	1	1	3.2	
	2	13	2.8 (2.5-3.2)	0.000
17F	1	6	2.7 (1.7-4.2)	
	2	5	2.6 (2.0-3.3)	0.847
18B	1	1	3.2	
	2	-	-	0.343
19B	1	1	2.5	
	2	2	2.3 (0.9-5.9)	0.932
20	1	4	3.2 (2.1-5.0)	
	2	-	-	0.120
21	1	1	5.1	
	2	11	3.6 (3.1-4.0)	<0.001
22F	1	-	-	
	2	1	4.3	0.292
23A	1	16	3.4 (2.8-4.1)	
	2	9	3.7 (3.1-4.4)	0.230
23B	1	11	3.7 (3.3-4.2)	
	2	14	3.6 (3.3-3.9)	0.373
24A	1	-	-	
	2	1	2.7	0.292
31	1	1	2.2	
	2	2	2.9 (0.1-62.1)	0.424
33A/F	1	-	-	
	2	1	3.2	0.292
34	1	7	2.9 (2.3-3.6)	
	2	8	3.2 (2.8-3.6)	0.857

Serotype/ group	Period	0-60 months n†	0-60 months Mean log density (95% CI) log_{10} GE/ml‡	p-value
35A/C/42	1	2	3.8 (0.4-37.2)	
	2	6	3.5 (2.5-4.9)	0.053
35B	1	2	2.9 (0.1-143.0)	
	2	2	2.5 (0.1-97.5)	0.019
35F	1	-	-	
	2	1	2.9	0.253
43	1	1	2.2	
	2	-	-	0.343
45	1	-	-	
	2	1	2.4	0.252
47A	1	1	2.2	
	2	-	-	0.209

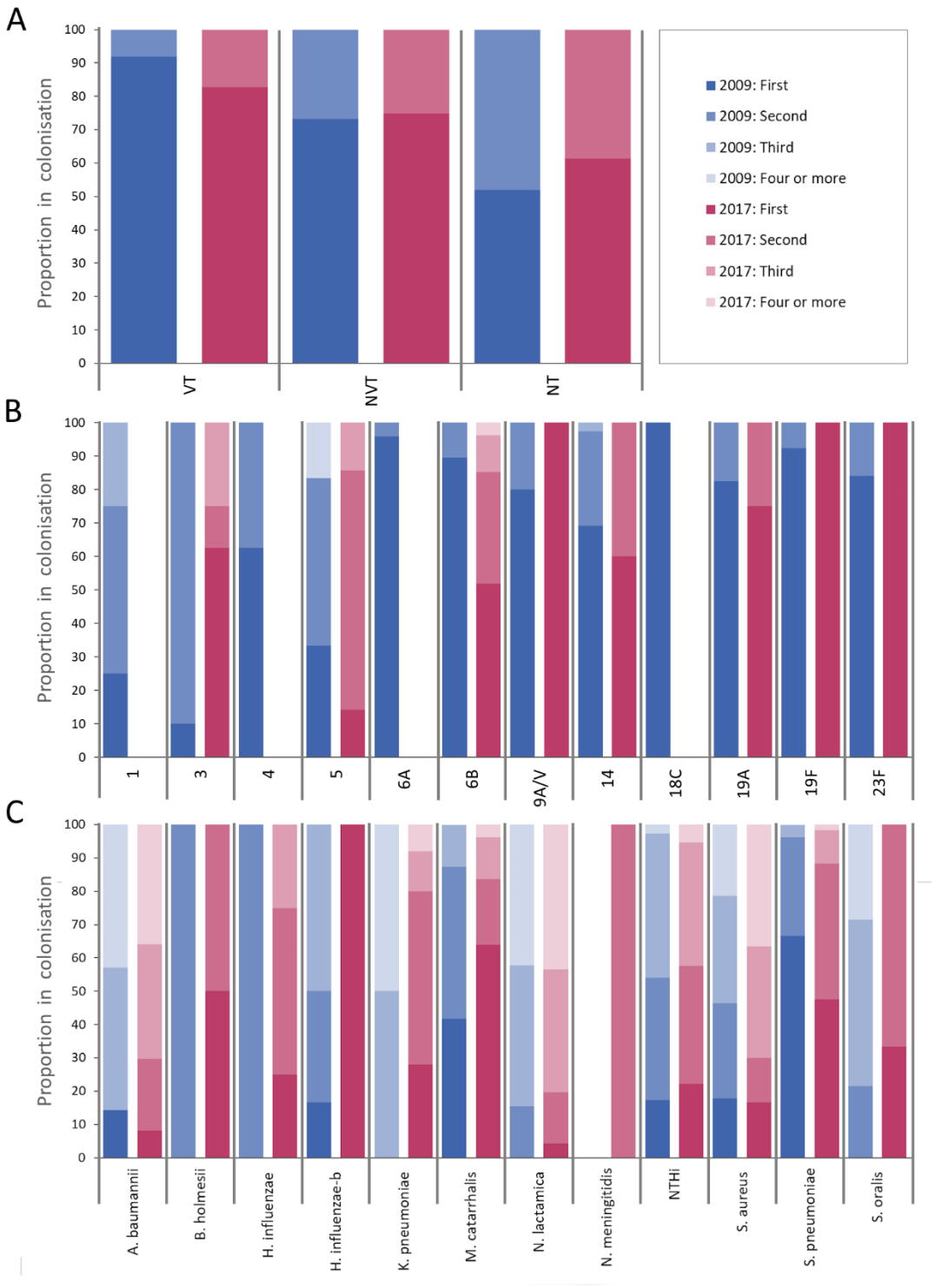
P: study Period
†n: number of isolates.
‡ 95% CI is Confidence Interval and GE: Genomic Equivalents p-values were considered significant if ≤0.01.
Density of carriage was determined through quantitative real-time nanofluidic PCR in the Fluidigm.
Overall pneumococcus includes all samples positive for pneumococcal reference genes.
PCV13-VT: vaccine serotypes including serotypes/groups 1, 3, 4, 5, 6A, 6B, 7A/F, 9A/V, 14, 18C, 19A, 19F and 23F. NVT: serotypes/serogroups not included in PCV13. NT: non-typeable pneumococci.

Supplementary Table 6: Hierarchy of co-colonising pneumococcal serotypes detected in nasopharyngeal swab samples collected from children 0-60 months-of-age in Period 1 (2009, n=630) and Period 2 (2017, n=568).

Serotype/ group	P	Primary Coloniser % (n/N)	Co-Colonisation % (n/N)	aOR of co-colonisation (95% CI) ‡	Second Serotype % (n/N)	Third Serotype % (n/N)	≥ Four Serotypes % (n/N)
PCV13-VT	1	91.9 (295/321)	8.1 (26/321)		-	-	-
	2	82.7 (67/81)	17.3 (14/81)	2.46 (1.20-5.04)	-	-	-
NVT	1	73.2 (164/224)	26.8 (60/224)		-	-	-
	2	74.9 (269/359)	25.1 (90/359)	0.91 (0.62-1.35)	-	-	-
NT	1	51.9 (41/79)	48.1 (38/79)		-	-	-
	2	61.3 (76/124)	38.7 (48/124)	0.67 (0.37-1.21)	-	-	-
1	1	25.0 (1/4)	75.0 (3/4)		50.0 (2/4)	25.0 (1/4)	0 (0/4)
	2	-	-	-	-	-	-
3	1	10.0 (1/10)	90.0 (9/10)		90.0 (9/10)	0 (0/10)	0 (0/10)
	2	62.5 (5/8)	37.5 (3/8)	0.04 (0.00-0.66)	12.5 (1/8)	25.0 (2/8)	0 (0/8)
4	1	62.5 (5/8)	37.5 (3/8)		37.5 (3/8)	0 (0/8)	0 (0/8)
	2	-	-	-	-	-	-
5	1	33.3 (2/6)	66.7 (4/6)		50.0 (3/6)	0 (0/6)	16.7 (1/6)
	2	14.3 (1/7)	85.7 (6/7)	9.00 (0.54-149.07)	71.4 (5/7)	14.3 (1/7)	0 (0/7)
6A	1	95.8 (46/48)	4.2 (2/48)		4.2 (2/48)	0 (0/48)	0 (0/48)
	2	-	-	-	-	-	-
6B	1	89.6 (86/96)	10.4 (10/96)		10.4 (10/96)	0 (0/96)	0 (0/96)
	2	51.9 (14/27)	48.1 (13/27)	7.91 (2.80-22.36)	33.3 (9/27)	11.1 (3/27)	3.7 (1/27)
9A/9V	1	80.0 (4/5)	20.0 (1/5)		20.0 (1/5)	0 (0/5)	0 (0/5)
	2	100 (1/1)	0 (0/1)	p=0.624	0 (0/1)	0 (0/1)	0 (0/1)
14	1	69.2 (27/39)	30.8 (12/39)		28.2 (11/39)	2.6 (1/39)	0 (0/39)
	2	60.0 (3/5)	40.0 (2/5)	p=0.677	40.0 (2/5)	0 (0/5)	0 (0/5)
18C	1	100 (5/5)	0 (0/5)		0 (0/5)	0 (0/5)	0 (0/5)
	2	-	-	-	-	-	-
19A	1	82.6 (19/23)	17.4 (4/23)		17.4 (4/23)	0 (0/23)	0 (0/23)
	2	75.0 (6/8)	25.0 (2/8)	p=0.639	25.0 (2/8)	0 (0/8)	0 (0/8)
19F	1	92.3 (60/65)	7.7 (5/65)		7.7 (5/65)	0 (0/65)	0 (0/65)
	2	100 (30/30)	0 (0/30)	p=0.119	0 (0/30)	0 (0/30)	0 (0/30)
23F	1	84.0 (42/50)	16.0 (8/50)		16.0 (8/50)	0 (0/50)	0 (0/50)
	2	100 (4/4)	0 (0/4)	p= 0.386	0 (0/4)	0 (0/4)	0 (0/4)
2	1	0 (0/2)	100 (2/2)		0 (0/2)	0 (0/2)	100 (2/2)
	2	-	-	-	-	-	-
6D	1	100 (1/1)	0 (0/1)		0 (0/1)	0 (0/1)	0 (0/1)
	2	100 (5/5)	0 (0/5)	-	0 (0/5)	0 (0/5)	0 (0/5)
7B/7C/40	1	100 (2/2)	0 (0/2)		0 (0/2)	0 (0/2)	0 (0/2)
	2	100 (2/2)	0 (0/2)	-	0 (0/2)	0 (0/2)	0 (0/2)
8	1	50.0 (2/4)	50.0 (2/4)		50.0 (2/4)	0 (0/4)	0 (0/4)
	2	44.4 (4/9)	55.6 (5/9)	0.98 (0.07-13.63)	55.6 (5/9)	0 (0/9)	0 (0/9)
9like	1	40.0 (4/10)	60.0 (6/10)		40.0 (4/10)	20.0 (2/10)	0 (0/10)
	2	25.0 (1/4)	75.0 (3/4)	1.61 (0.10-25.38)	75.0 (3/4)	0 (0/4)	0 (0/4)
9LN	1	100 (4/4)	0 (0/4)		0 (0/4)	0 (0/4)	0 (0/4)
	2	80.0 (4/5)	20.0 (1/5)	p= 0.343	20.0 (1/5)	0 (0/5)	0 (0/5)
10A	1	100 (4/4)	0 (0/4)		0 (0/4)	0 (0/4)	0 (0/4)
	2	100 (4/4)	0 (0/4)	-	0 (0/4)	0 (0/4)	0 (0/4)
10B	1	0 (0/1)	100 (1/1)		0 (0/1)	0 (0/1)	100 (1/1)
	2	-	-	-	-	-	-
10C/10F	1	0 (0/1)	100 (1/1)		0 (0/1)	100 (1/1)	0 (0/1)
	2	-	-	-	-	-	-
11A/11D	1	73.3 (11/15)	26.7 (4/15)		20.0 (3/15)	6.7 (1/15)	0 (0/15)
	2	76.2 (16/21)	23.8 (5/21)	1.35 (0.26-7.02)	19.0 (4/21)	4.8 (1/21)	0 (0/21)
12A/12F/44	1	100 (1/1)	0 (0/1)		0 (0/1)	0 (0/1)	0 (0/1)
	2	100 (2/2)	0 (0/2)	-	0 (0/2)	0 (0/2)	0 (0/2)
13	1	73.3 (11/15)	26.7 (4/15)		26.7 (4/15)	0 (0/15)	0 (0/15)
	2	78.9 (15/19)	21.1 (4/19)	0.78 (0.16-3.87)	10.5 (2/19)	5.3 (1/19)	5.3 (1/19)
15A/15F	1	50.0 (1/2)	50.0 (1/2)		50.0 (1/2)	0 (0/2)	0 (0/2)
	2	75.0 (12/16)	25.0 (4/16)	0.50 (0.02-11.09)	18.8 (3/16)	6.3 (1/16)	0 (0/16)
15B/15C	1	100 (16/16)	0 (0/16)		0 (0/16)	0 (0/16)	0 (0/16)
	2	80.0 (4/5)	20.0 (1/5)	p= 0.067	20.0 (1/5)	0 (0/5)	0 (0/5)
15like	1	54.1 (20/37)	45.9 (17/37)		35.1 (13/37)	8.1 (3/37)	2.7 (1/37)
	2	70.0 (28/40)	30.0 (12/40)	0.42 (0.15-1.14)	17.5 (7/40)	10.0 (4/40)	2.5 (1/40)
16A	1	0 (0/1)	100 (1/1)		100 (1/1)	0 (0/1)	0 (0/1)
	2	-	-	-	-	-	-
16F	1	50.0 (1/2)	50.0 (1/2)		50.0 (1/2)	0 (0/2)	0 (0/2)
	2	78.3 (36/46)	21.7 (10/46)	0.20 (0.01-3.91)	13.0 (6/46)	8.7 (4/46)	0 (0/46)
17F	1	55.6 (5/9)	44.4 (4/9)		33.3 (3/9)	0 (0/9)	11.1 (1/9)
	2	62.5 (5/8)	37.5 (3/8)	0.22 (0.02-3.00)	37.5 (3/8)	0 (0/8)	0 (0/8)

Serotype/ group	P	Primary Coloniser % (n/N)	Co-Colonisation % (n/N)	aOR of co-colonisation (95% CI) ‡	Second Serotype % (n/N)	Third Serotype % (n/N)	≥ Four Serotypes % (n/N)
18A	1	0 (0/2)	100 (2/2)		50.0 (1/2)	50.0 (1/2)	0 (0/2)
	2	-	-	-	-	-	-
18B	1	100 (1/1)	0 (0/1)		0 (0/1)	0 (0/1)	0 (0/1)
	2	-	-	-	-	-	-
19A	1						
	2			2.69 (0.33-22.09)			
19B	1	33.3 (1/3)	66.7 (2/3)		66.7 (2/3)	0 (0/3)	0 (0/3)
	2	100 (3/3)	0 (0/3)	-	0 (0/3)	0 (0/3)	0 (0/3)
20	1	100 (6/6)	0 (0/6)		0 (0/6)	0 (0/6)	0 (0/6)
	2	0 (0/2)	100 (2/2)	p=0.005	100 (2/2)	0 (0/2)	0 (0/2)
21	1	100 (1/1)	0 (0/1)		0 (0/1)	0 (0/1)	0 (0/1)
	2	90.5 (19/21)	9.5 (2/21)	p=0.746	9.5 (2/21)	0 (0/21)	0 (0/21)
22F	1	-	-		-	-	-
	2	100 (1/1)	0 (0/1)	-	0 (0/1)	0 (0/1)	0 (0/1)
23A	1	65.0 (13/20)	35.0 (7/20)		30.0 (6/20)	5.0 (1/20)	0 (0/20)
	2	95.5 (21/22)	4.5 (1/22)	0.07 (0.01-0.72)	4.5 (1/22)	0 (0/22)	0 (0/22)
23B	1	86.4 (19/22)	13.6 (3/22)		13.6 (3/22)	0 (0/22)	0 (0/22)
	2	79.3 (23/29)	20.7 (6/29)	2.34 (0.34-0.41)	17.2 (5/29)	3.4 (1/29)	0 (0/29)
24A	1	-	-		-	-	-
	2	100 (1/1)	0 (0/1)	-	0 (0/1)	0 (0/1)	0 (0/1)
31	1	100 (2/2)	0 (0/2)		0 (0/2)	0 (0/2)	0 (0/2)
	2	80.0 (4/5)	20.0 (1/5)	p=0.495	20.0 (1/5)	0 (0/5)	0 (0/5)
33A/33F	1	-	-		-	-	-
	2	100 (1/1)	0 (0/1)	-	0 (0/1)	0 (0/1)	0 (0/1)
33B	1	-	-		-	-	-
	2	0 (0/1)	100 (1/1)	-	100 (1/1)	0 (0/1)	0 (0/1)
33D	1	-	-		-	-	-
	2	0 (0/1)	100 (1/1)	-	100 (1/1)	0 (0/1)	0 (0/1)
34	1	87.5 (14/16)	12.5 (2/16)		0 (0/16)	12.5 (2/16)	0 (0/16)
	2	82.4 (14/17)	17.6 (3/17)	1.50 (0.21-10.88)	17.6 (3/17)	0 (0/17)	0 (0/17)
35A/35C/42	1	50.0 (1/2)	50.0 (1/2)		50.0 (1/2)	0 (0/2)	0 (0/2)
	2	62.5 (5/8)	37.5 (3/8)	0.81 (0.03-23.59)	25.0 (2/8)	12.5 (1/8)	0 (0/8)
35B	1	80.0 (4/5)	20.0 (1/5)		20.0 (1/5)	0 (0/5)	0 (0/5)
	2	85.7 (12/14)	14.3 (2/14)	0.85 (0.06-12.62)	7.1 (1/14)	7.1 (1/14)	0 (0/14)
35F	1	100 (1/1)	0 (0/1)		0 (0/1)	0 (0/1)	0 (0/1)
	2	100 (4/4)	0 (0/4)	-	0 (0/4)	0 (0/4)	0 (0/4)
43	1	0 (0/1)	100 (1/1)		0 (0/1)	0 (0/1)	100 (1/1)
	2	-	-	-	-	-	-
45	1	0 (0/1)	100 (1/1)		100 (1/1)	0 (0/1)	0 (0/1)
	2	50.0 (1/2)	50.0 (1/2)	p=0.386	50.0 (1/2)	0 (0/2)	0 (0/2)
46	1	0 (0/1)	100 (1/1)		100 (1/1)	0 (0/1)	0 (0/1)
	2	100 (1/1)	0 (0/1)	p=0.157	0 (0/1)	0 (0/1)	0 (0/1)
47A	1	0 (0/2)	100 (2/2)		50.0 (1/2)	0 (0/2)	50 (1/2)
	2	-	-	-	-	-	-

P: study Period.
n: number of isolates in each category for each period.
N: total isolates identified as each serotype/group.
‡ Where an OR could not be calculated, Fisher exact test was used to generate a p-value, OR (or p-value) compares the proportion of co-colonising events out of all events detected in Period-2 compared with Period-1. The rank was determined according to colonization density.
PCV13-VT: vaccine serotypes including serotypes/groups 1, 3, 4, 5, 6A, 6B, 7A/F, 9A/V, 14, 18C, 19A, 19F and 23F.
NVT: serotypes/serogroups not included in PCV13.
NT: non-typeable pneumococci.



Supplementary Figure 5: Hierarchy of pneumococcal and other bacterial colonisers based on relative density in the nasopharynx of children aged 0-60 months.

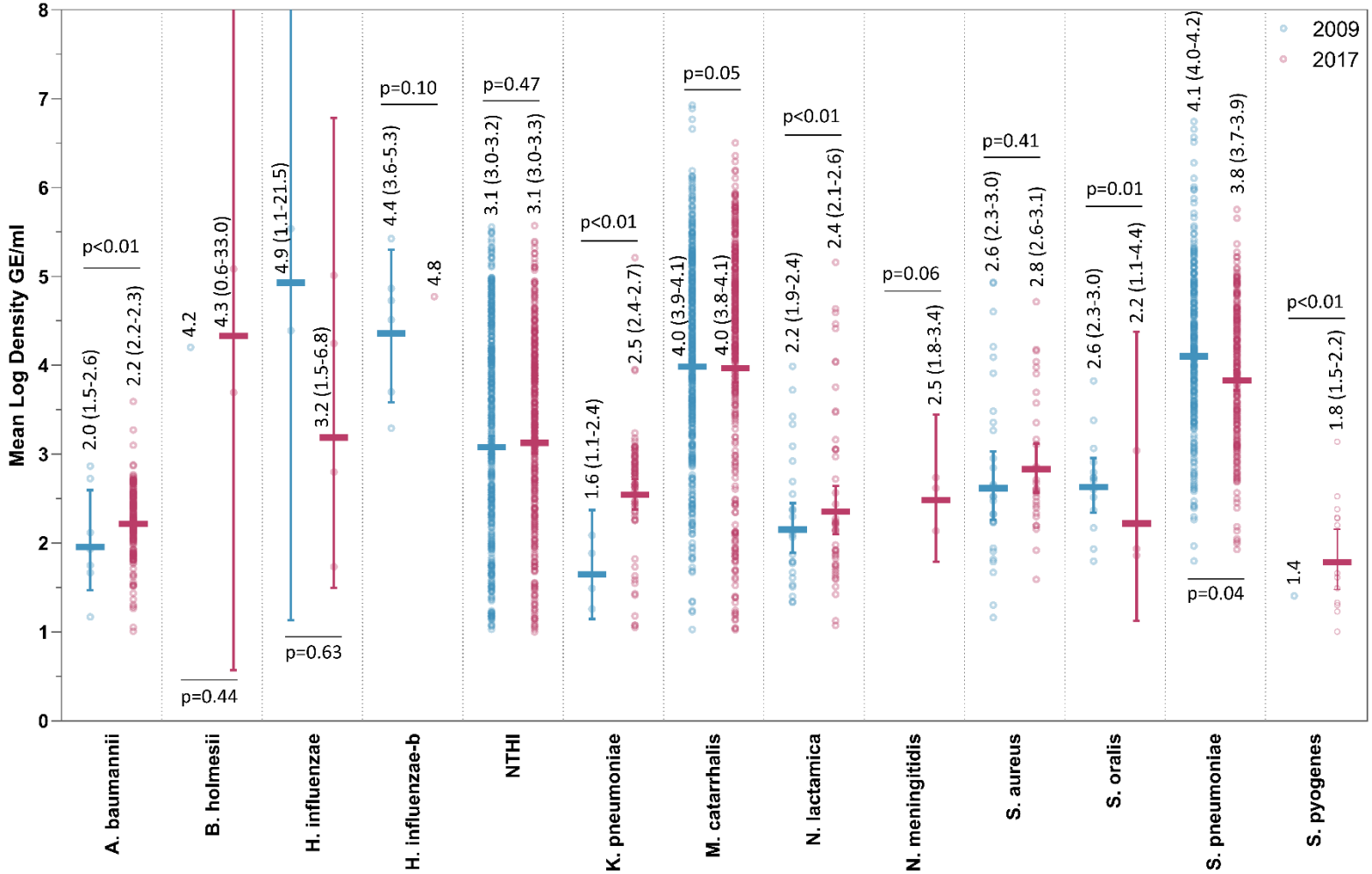
Panel A: relative density of VT (PCV13 vaccine serotypes: 1, 3, 4, 5,6A, 6B, 7A/F, 9A/V, 14, 18C, 19A, 19F and 23F); NVT (non-PCV13-vaccine serotypes) and NT=non-typeable S. pneumoniae, Panel B: individual VT, Panel C: bacterial colonisers. The proportions are calculated from the number of times a pneumococcal serotype or bacterial species was detected as the dominant or the second, third or fourth density coloniser out of the total times the pneumococcal serotype or bacterial species was detected. The hierarchy in colonisation is further detailed in supplementary Table 4 and 7.

Supplementary Table 7: Prevalence of bacterial colonisation from Nasopharyngeal Swab samples in Period-1 (2009) and Period-2 (2017/8) in Agincourt children 0-60 months of age**.**

	Age Group (Months)	Period-1 % (n); N=630	Period-2 % (n); N=568	OR (95% CI) p-value	aOR (95% CI) P-value
Acinetobacter baumannii	0-60	1.1 (7/630)	36.8 (209/568)	51.81 (24.13-111.26); p<0.001	50.11 (23.14-108.50); p<0.001
	0-24	1.6 (6/371)	39.5 (77/195)	39.70 (16.86-93.46); p<0.001	34.15 (14.26-81.75); p<0.001
	25-60	0.4 (1/259)	35.4 (132/373)	141.31 (19.60-1018.57); p<0.001	143.37 (18.94-1085.31); p<0.001
Bordetella holmesii	0-60	0.2 (1/630)	0.4 (2/568)	2.22 (0.20-24.58); p=0.504	2.38 (0.21-26.35); p=0.480
	0-24	0.3 (1/371)	0.5 (1/195)	1.91 (0.12-30.66); p=0.643	1.43 (0.09-23.01); p=0.801
	25-60	0.0 (0/259)	0.3 (1/373)		p=0.404
Haemophilus influenzae	0-60	0.3 (2/630)	0.7 (4/568)	2.23 (0.41-12.20); p=0.356	2.13 (0.38-12.04); p=0.394
	0-24	0.0 (0/371)	0.5 (1/195)		p=0.304
	25-60	0.8 (2/259)	0.8 (3/373)	1.04 (0.17-6.28); p=0.964	0.74 (0.11-4.82); p=0.754
Haemophilus influenzae type b	0-60	1.0 (6/630)	0.2 (1/568)	0.18 (0.02-1.53); p=0.117	0.19 (0.02-1.67); p=0.134
	0-24	0.5 (2/371)	0.0 (0/195)		p=0.167
	25-60	1.5 (4/259)	0.3 (1/373)	0.17 (0.02-1.54); p=116	0.17 (0.02-1.74); p=0.135
Non-typeable Haemophilus influenzae	0-60	64.9 (409/630)	63.0 (358/568)	0.92 (0.73-1.17); p=0.496	0.87 (0.68-1.11); p=0.270
	0-24	63.3 (235/371)	55.9 (109/195)	0.73 (0.52-1.04); p=0.085	0.80 (0.55-1.16); p=0.243
	25-60	67.2 (174/259)	66.8 (249/373)	0.98 (0.70-1.37); p=0.911	0.88 (0.62-1.24); p=0.458
Klebsiella pneumoniae	0-60	0.6 (4/630)	13.2 (75/568)	23.81 (8.65-65.54); p<0.001	22.16 (8.03-61.11); p<0.001
	0-24	0.8 (3/371)	12.3 (24/195)	17.22 (5.11-57.96); p<0.001	15.55 (4.44-54.46); p<0.001
	25-60	0.4 (1/259)	13.7 (51/373)	40.86 (5.61-297.70); p<0.001	37.47 (5.13-273.96); p<0.001
Moraxella catarrhalis	0-60	67.8 (427/630)	60.4 (343/568)	0.73 (0.57-0.92) ; p=0.008	0.72 (0.56-0.91); p=0.007
	0-24	68.2 (253/371)	63.1 (123/195)	0.80 (0.55-1.15); p=0.221	0.79 (0.54-1.16); p=0.228
	25-60	67.2 (174/259)	59.0 (220/373)	0.70 (0.50-0.98); p=0.036	0.69 (0.49-0.97); p<0.001
Neisseria lactamica	0-60	4.1 (26/630)	8.1 (46/568)	2.05 (1.25-3.36); p=0.004	2.14 (1.28-3.57); p=0.004
	0-24	4.3 (16/371)	9.7 (19/195)	2.40 (1.20-4.77); p=0.011	2.18 (1.05-4.53); p=0.036
	25-60	3.9 (10/259)	7.2 (27/373)	1.94 (0.92-4.09); p=0.075	2.22 (1.01-4.87); p=0.035
Neisseria meningitidis	0-60	0.0 (0/630)	0.5 (3/568)		p=0.068
	0-24	0.0 (0/371)	1.5 (3/195)		p=0.017
	25-60	0.0 (0/259)	0.0 (0/373)		-
Staphylococcus aureus	0-60	4.4 (28/630)	5.3 (30/568)	1.20 (0.71-2.03); p=0.500	1.19 (0.69-2.03); p=0.530
	0-24	5.1 (19/371)	7.2 (14/195)	1.43 (0.70-2.92); p=0.321	1.35 (0.64-2.84); p=0.428
	25-60	3.5 (9/259)	4.3 (16/373)	1.24 (0.54-2.86); p=0.605	1.22 (0.50-2.94); p=0.664
Streptococcus pneumoniae	0-60	83.2 (524/630)	76.9 (437/568)	0.67 (0.51-0.90), p=0.007	0.65 (0.48-0.87), p<0.001
	0-24	84.6 (314/371)	78.5 (153/195)	0.66 (0.42-1.03), p=0.066	0.69 (0.43-1.10), p<0.001

	Age Group (Months)	Period-1 % (n); N=630	Period-2 % (n); N=568	OR (95% CI) p-value	aOR (95% CI) P-value
	25-60	81.1 (210/259)	76.1 (284/373)	0.74 (0.50-1.10), p=0.139	0.68 (0.45-1.03), p=0.066
Streptococcus oralis	0-60	2.2 (14/630)	0.5 (3/568)	0.23 (0.07-0.82); p=0.023	0.21 (0.06-0.77); p=0.018
	0-24	2.2 (8/371))	1.54 (3/195)	0.71 (0.19-2.70); p= 0.615	0.85 (0.21-3.49); p=0.824
	25-60	2.32 (6/259)	0 (0/373)	p=0.003	
Streptococcus pyogenes	0-60	0.2 (1/630)	2.5 (14/568)	15.90 (2.08-121.27); p<0.001	14.49 (1.89-111.09); p=0.010
	0-24	0.0 (0/371)	1.5 (3/195)	p=0.017	
	25-60	0.4 (1/259)	3.0 (11/373)	7.84 (1.01-61.10); p=0.020	7.34 (0.93-57.96); p=0.059

OR: Odds Ratio; aOR: adjusted Odds Ratio, calculated using logistic regression; adjusted for breastfeeding status, HIV infection, antibiotic usage, co-trimoxazole prophylaxis, and tuberculosis treatment. The OR or aOR could not be calculated where there were too few, or zero detected colonisation events in either timepoint zero. Where OR or aOR could not be calculated, the p-value was calculated using X^2.



Supplementary Figure 6: Mean log density (log_{10} Genomic Equivalents per mL [GE/mL]) of bacterial colonisers in children 0-60 months of age across two study periods.

Mean log_{10} density (95%Confidence Interval) values shown.

Supplementary Table 8: Bacterial carriage density from Nasopharyngeal Swab samples collected from children 0-60 months of age in Period 1 (2009, n=630) and Period 2 (2017, n=568).

Serotype/group	Period	0-60 months n†	0-60 months GMD (95% CI) log_{10} GE/ml‡	p-value
Acinetobacter baumannii	1	7	2.0 (1.5-2.6)	
	2	209	2.2 (2.2-2.3)	<0.001
Bordetella holmesii	1	1	4.2	
	2	2	4.3 (0.6-33.0)	0.438
Haemophilus influenzae	1	2	4.9 (1.1-21.5)	
	2	4	3.2 (1.5-6.8)	0.634
Haemophilus influenzae type b	1	6	4.4 (3.6-5.3)	
	2	1	4.8	0.099
Non-typeable Haemophilus influenzae	1	409	3.1 (3.0-3.2)	
	2	358	3.1 (3.0-3.3)	0.471
Klebsiella pneumoniae	1	4	1.6 (1.1-2.4)	
	2	75	2.5 (2.4-2.7)	<0.001
Moraxella catarrhalis	1	427	4.0 (3.9-4.1)	
	2	343	4.0 (3.8-4.1)	0.053
Neisseria lactamica	1	26	2.2 (1.9-2.4)	
	2	46	2.4 (2.1-2.6)	0.002
Neisseria meningitidis	1	-	-	
	2	3	2.5 (1.8-3.4)	0.055
Staphylococcus aureus	1	28	2.6 (2.3-3.0)	
	2	30	2.8 (2.6-3.1)	0.407
Streptococcus oralis	1	14	2.6 (2.3-3.0)	
	2	3	2.2 (1.1-4.4)	0.008
Streptococcus pyogenes	1	1	1.4	
	2	14	1.8 (1.5-2.2)	0.001

P denotes the study Period.
† n is the number of isolates.
‡ 95% CI is Confidence Interval and GE: Genomic Equivalents.
Density of carriage was determined through quantitative real-time nanofluidic PCR in the Fluidigm and considered significant if the confidence intervals did not overlap.

Supplementary Table 9: Multiple concurrent bacterial colonisers detected in nasopharyngeal swab samples collected from children 0-60 months-of-age in Period 1 (2009, n=630) and Period 2 (2017, n=568)

Bacteria	P	Primary Coloniser % (n/N)	Co-Colonisation % (n/N)	OR of co-colonisation (95% CI)‡	Second Coloniser % (n/N)	Third Coloniser % (n/N)	≥ Four Bacteria % (n/N)
A. baumannii	1	14.3 (1/7)	85.7 (6/7)	1.88 (0.21-17.10)	0 (0/7)	42.9 (3/7)	42.9 (3/7)
	2	8.1 (17/209)	91.9 (192/209)		21.5 (45/209)	34.4 (72/209)	35.9 (75/209)
B. holmesii	1	0 (0/1)	100 (1/1)		100 (1/1)	0 (0/1)	0 (0/1)
	2	50.0 (1/2)	50.0 (1/2)	p=0.386	50.0 (1/2)	0 (0/2)	0 (0/2)
H. influenzae	1	0 (0/2)	100 (2/2)		100 (2/2)	0 (0/2)	0 (0/2)
	2	25.0 (1/4)	75.0 (3/4)	p=0.439	50.0 (2/4)	25.0 (1/4)	0 (0/4)
-H. influenzae. type b	1	16.7 (1/6)	83.3 (5/6)		33.3 (2/6)	50.0 (3/6)	0 (0/6)
	2	100 (1/1)	0 (0/1)	p=0.088	0 (0/1)	0 (0/1)	0 (0/1)
-NTHI	1	17.4 (71/409)	82.6 (338/409)		36.7 (150/409)	43.3 (177/409)	2.7 (11/409)
	2	22.1 (79/358)	77.9 (279/358)	0.64 (0.44-0.94)	35.5 (127/358)	37.2 (133/358)	5.3 (19/358)
K. pneumoniae	1	0 (0/4)	100 (4/4)		0 (/4)	50.0 (2/4)	50.0 (2/4)
	2	28.0 (21/75)	72.0 (54/75)	p=0.217	52.0 (39/75)	12.0 (9/75)	8.0 (6/75)
M. catarrhalis	1	41.7 (178/427)	58.3 (249/427)		45.7 (195/427)	12.4 (53/427)	0.2 (1/427)
	2	63.8 (219/343)	36.2 (124/343)	0.40 (0.30-0.55)	19.8 (68/343)	12.5 (43/343)	3.8 (13/343)
N. lactamica	1	0 (0/26)	100 (26/26)		15.4 (4/26)	42.3 (11/26)	42.3 (11/26)
	2	4.3 (2/46)	95.7 (44/46)	p=0.281	15.2 (7/46)	37.0 (17/46)	43.5 (20/46)
N. meningitidis	1	-	-		-	-	-
	2	0 (0/3)	100 (3/3)	-	100 (3/3)	0 (0/3)	0 (0/3)
S. aureus	1	17.9 (5/28)	82.1 (23/28)		28.6 (8/28)	32.1 (9/28)	21.4 (6/28)
	2	16.7 (5/30)	83.3 (25/30)	0.97 (0.24-3.92)	13.3 (4/30)	33.3 (10/30)	36.7 (11/30)
S. oralis	1	0 (0/14)	100 (14/14)		21.4 (3/14)	50.0 (7/14)	28.6 (4/14)
	2	33.3 (1/3)	66.7 (2/3)	p=0.026	66.7 (2/3)	0 (0/3)	0 (0/3)
S. pneumoniae	1	66.6 (349/524)	33.4 (175/524)		29.6 (155/524)	3.6 (19/524)	0.2 (1/524)
	2	47.6 (208/437)	52.4 (229/437)	2.32 (1.78-3.04)	40.7 (178/437)	10.1 (44/437)	1.6 (7/437)
S. pyrogenes	1	0 (0/1)	100 (1/1)		0 (0/1)	0 (0/1)	100 (1/1)
	2	0 (0/14)	100 (14/14)	p=0.464	0 (0/14)	35.7 (5/14)	64.3 (9/14)

NTHI: non-typeable *Haemophilus influenzae*.
P: study Period.
n: number of isolates in each category for each period.
N: total number of isolates identified as each bacterial species.
‡ Where an OR could not be calculated, Fisher exact test was used to generate a p-value, OR (or p-value) compares the proportion of co-colonising events out of all events detected in Period-2 compared with Period-1. The rank was determined according to colonization density.

Supplementary Table 10: Bacterial co-colonizing events in children 0-60 months of age in Agincourt, in Period 1 (2009, n=630) and Period 2 (2017, n=568).

		Number of events		aOR of association	
Dominant bacteria	**Co-colonising bacteria**	**Period-1**	**Period-2**	**Period-1**	**Period-2**
A. baumannii	*M. catarrhalis*	0	4	-	1.11 (0.35-3.46)
	Non-typeable H. influenzae	0	4	-	0.29 (0.09-0.90)
H. influenzae	*A. baumannii*	0	1	-	-
	M. catarrhalis	0	1	-	-
	N. lactamica	0	1	-	-
	S. pneumoniae	0	1	-	-
H. influenzae type b	*K. pneumoniae*	0	1	-	-
	M. catarrhalis	1	1	-	-
	S. pneumoniae	1	1	-	-
K. pneumoniae	*A. baumannii*	0	2	-	0.19 (0.04-0.84)
	Non-typeable H. influenzae	0	4	-	0.23 (0.08-0.69)
	S. oralis	0	1	-	25.66 (1.52-434.58)
	S. pneumoniae	0	4	-	0.33 (0.11-1.00)
M. catarrhalis	*A. baumannii*	2	97	1.21 (0.22-6.72)	2.12 (1.48-3.04)
	B. holmesii	1	1	-	-
	H. influenzae type b	3	0	3.82 (0.63-23.25)	-
	H. influenzae	0	1	-	0.83 (0.07-9.24)
	K. pneumoniae	1	5	0.85 (0.09-8.25)	0.14 (0.06-0.37)
	N. lactamica	9	18	1.38 (0.60-3.17)	1.07 (0.57-2.02)
	Non-typeable H. influenzae	108	144	1.49 (1.04-2.14)	3.30 (2.30-4.74)
	S. aureus	7	14	1.13 (0.46-2.81)	2.14 (0.95-4.81)
	S. oralis	8	0	3.40 (1.16-10.00)	-
	S. pneumoniae	130	169	25.88 (16.32-41.05)	15.56 (10.22-23.67)
	S. pyogenes	1	5	-	1.37 (0.36-5.20)
N. lactamica	*A. baumannii*	0	1	-	2.04 (0.12-33.68)
	M. catarrhalis	0	1	-	2.89 (0.17-48.68)
	Non-typeable H. influenzae	0	2	-	-
	S. pneumoniae	0	2	-	-
	S. pyogenes	0	1	-	42.16 (2.06-861.69)
Non-typeable H. influenzae	*A. baumannii*	0	23	-	0.79 (0.47-1.33)
	K. pneumoniae	1	12	2.61 (0.27-25.65)	1.91 (0.96-3.83)
	M. catarrhalis	23	27	0.69 (0.41-1.17)	2.12 (1.27-3.56)
	N. lactamica	3	8	0.95 (0.28-3.28)	1.47 (0.65-3.32)
	N. meningitidis	0	1	-	-
	S. aureus	3	4	1.21 (0.35-4.21)	1.20 (0.40-3.59)
	S. oralis	1	0	0.60 (0.08-4.76)	-
	S. pneumoniae	42	50	4.76 (2.83-8.01)	2.93 (1.79-4.80)
	S. pyogenes	0	4	-	3.31 (0.80-13.66)
S. aureus	*Non-typeable H. influenzae*	2	0	0.58 (0.10-3.52)	-
	A. baumannii	0	2	-	-
	N. meningitidis	0	1	-	-
	S. pneumoniae	2	1	-	-
	S. pyogenes	0	1	-	-

S. oralis	*A. baumannii*	0	1	-	-
	M. catarrhalis	0	1	-	-
	Non-typeable H. influenzae	0	1	-	-
	S. aureus	0	1	-	-
	S. pneumoniae	0	1	-	-
S. pneumoniae	*A. baumannii*	4	65	1.72 (0.31-9.54)	0.85 (0.59-1.23)
	H. influenzae	2	2	-	3.26 (0.29-36.52)
	H. influenzae type b	2	0	0.59 (0.09-3.66)	-
	K. pneumoniae	2	36	0.82 (0.11-5.87)	3.92 (2.16-7.13)
	M. catarrhalis	225	89	20.84 (12.85-33.79)	7.13 (4.55-11.18)
	N. lactamica	14	17	0.97 (0.44-2.15)	1.19 (0.63-2.27)
	Non-typeable H. influenzae	228	124	2.87 (2.07-4.00)	1.87 (1.32-2.66)
	S. aureus	13	6	1.02 (0.44-2.38)	0.52 (0.20-1.32)
	S. oralis	5	1	0.45 (0.15-1.37)	1.99 (0.12-32.18)
	S. pyogenes	0	3	-	-

1. STATSSA. Census 2011 Statistical release – P0301.4 / Statistics South Africa. Pretoria: Statistics South Africa, 2012 2012.

Chapter 5: *Streptococcus pneumoniae* and other bacterial nasopharyngeal colonization seven years post-introduction of 13-valent pneumococcal conjugate vaccine in South African children. – Published Manuscript

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Streptococcus pneumoniae and other bacterial nasopharyngeal colonization seven years post-introduction of 13-valent pneumococcal conjugate vaccine in South African children

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ABSTRACT

Objectives: Pneumococcal conjugate vaccines (PCVs) reduce pneumococcal-associated disease by reducing vaccine-serotype (VT) acquisition in vaccinated children, thereby interrupting VT transmission. The 7-valent-PCV was introduced in the South African immunization program in 2009 (13-valent-PCV since 2011) using a 2+1 schedule (at 6, 14, and 40 weeks of age). We aimed to evaluate temporal changes in VT and non-vaccine-serotype (NVT) colonization after 9 years of childhood PCV immunization in South Africa.

Methods: Nasopharyngeal swabs were collected from healthy children <60-month-old (n = 571) in 2018 (period-2) and compared with samples (n = 1135) collected during early PCV7-introduction (period-1, 2010-11) in an urban low-income setting (Soweto). Pneumococci were tested for using a multiplex quantitative-polymerase chain reaction serotyping reaction-set.

Results: Overall pneumococcal colonization in period-2 (49.4%; 282/571) was 27.5% lower than period-1 (68.1%; 773/1135; adjusted odds ratio [aOR]: 0.66; 95% confidence interval [CI]: 0.54-0.88). Colonization by VT was reduced by 54.5% in period-2 (18.6%; 106/571) compared with period-1 (40.9%; 465/1135; aOR: 0.41; 95% CI: 0.3-0.56). Nevertheless, serotype 19F carriage prevalence was higher (8.1%; 46/571) in period-2 compared with period-1 (6.6%; 75/1135; aOR: 2.0; 95% CI: 1.09-3.56). NVT colonization prevalence was similar in period-2 and period-1 (37.8%; 216/571 and 42.4%; 481/1135).

Conclusion: There remains a high residual prevalence of VT, particularly 19F, colonization nine years post-introduction of PCV in the South African childhood immunization program.

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Background

Streptococcus pneumoniae remains a leading cause of morbidity and mortality, with the prevalence of nasopharyngeal colonization and burden of invasive pneumococcal disease (IPD) being highest in low-middle-income settings (LMIS) [1]. Routine immunization of children with pneumococcal conjugate vaccines (PCVs) has led to a reduction in vaccine-serotype (VT) IPD globally, including in South Africa [1,2]. The introduction of PCV in South Africa (along with HIV interventions) resulted in a rate reduction of 1277 IPD cases per 100 000 child-years comparing the pre- (2005–2008) and post-PCV introduction (2012-2013) periods [2]. Nevertheless, the overall prevalence of pneumococcal nasopharyngeal (NP) colonization has remained unchanged compared with prior to routine childhood immunization with PCV, due to replacement by non-vaccine serotypes (NVT) and residual colonization by some VT, especially in African countries [3–6]. Similarly, the burden of IPD in the PCV immunization era is dominated by NVT and some residual disease from VT [7]. Therefore, surveillance of PCV impact on colonization

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at a community level can detect longer-term changes in the NP niche, and signal a risk of an increase in IPD by residual VT or replacement NVT [8].

Colonization by VT in South Africa was reduced by 62% [9] in an urban setting three years after the introduction of PCV7, and by 68% in a rural setting four years post-PCV7 introduction [3]. Only three studies in sub-Saharan Africa (Malawi [4], The Gambia [5], and Kenya [6]) have assessed NP carriage in healthy children, more than five years after the implementation of PCVs. In addition, Malawi, The Gambia, and Kenya use a '3+0' PCV dosing schedule, administering PCV at 6, 10, and 14 weeks of age without a booster dose [4–6]. South Africa differs from other sub-Saharan African countries and uses a '2+1' schedule involving two priming doses administered at 6 and 14 weeks of age and a booster (40 weeks of age). The booster dose is key for the durability of protection against VT acquisition, disease, and transmission [10,11]. Waning mucosal immunity in settings using primary series only has limited the impact of PCV on reducing colonization and transmission [4,10,11]. Furthermore, studies in South Africa, The Gambia, Kenya, and Malawi investigated colonization in NP samples using culture-based methods, which hamper the detection of co-colonization. Real-time polymerase chain reaction (PCR) can be optimized to quantify both concurrent and lower-density colonization by VT, NVT, and other bacterial species [12].

Studies investigating the long-term impact of PCV '21' in South Africa using comprehensive and sensitive typing schemes are warranted to monitor changes in pneumococcal serotype epidemiology and vaccine coverage in a setting with a mature PCV immunization program [13]. This will be important as South Africa considers transitioning to a reduced dosing schedule that involves one primary dose and one booster ('1+1') in order to alleviate the high costs of PCV [14]. Further, community-based surveillance could serve as a benchmark against which future changes in pneumococcal colonization profiles can be compared if childhood immunization schedules transition to PCV 1+1 doses.

The aim of this study was to evaluate the temporal changes of serotype-specific pneumococcal and other bacteria colonization in the nasopharynx of children ≤5 years of age, 9 years after the routine implementation of PCV (7 years after PCV13) into the childhood immunization program in South Africa.

Methods

Study population

In South Africa, routine PCV immunization of children at 6, 14, and 40 weeks (2+1 schedule) commenced in April 2009 (7-valent PCV [PCV7]) and transitioned to 13-valent PCV (PCV13) in May 2011 [9]. Serotype-specific colonization was evaluated in the early PCV7-era (May 2010 to February 2011; period-1) among children living with HIV (CLWH) and HIV-uninfected children, as described [9]. Briefly, children were enrolled in Soweto, at HIV treatment clinics within Chris Hani Baragwanath Academic Hospital, and through community state clinics. For the current analysis, archived nasopharyngeal (NP) swabs (aluminum-shafted, Dacron swab, Medical Wire and Equipment Co. Ltd., Corsham, Wiltshire, England) collected from children <60 months of age and stored in skim milk-tryptone-glucose-glycerol (STGG) transport media at -70°C, were retrospectively analyzed (period-1) and served as baseline for comparison. Samples collected from period-1 had previously been investigated by standard culture methods and may have undergone multiple (up to four) freeze-thaw cycles before this study [9]. Here, we used a comprehensive quantitative real-time PCR (qPCR) that is capable of detecting pneumococcus and other NP bacterial colonizers with high sensitivity and specificity [12].

To assess the changes in pneumococcal colonization, a further prospective carriage survey was undertaken in Soweto from June to December 2018 (period-2). In period-2, healthy children <60 months of age were enrolled through household-level surveys, selected using the World Health Organization (WHO) EPI (Expanded Program on Immunization) probability proportional to size cluster survey method detailed in the supplementary text. All healthy children <60 months of age within the selected households were sampled, with parental consent.

Sample collection

In period-2, the nasopharynx of children <60 months of age were sampled using FLOQSwabs (Copan Diagnostics, Murrieta, CA, USA) and placed in 1.5 ml of STGG, on ice and transferred to the Vaccines, and Infectious Diseases Analytics Research Unit (Wits-VIDA) within 6 hours of collection. Samples (NP swabs) were then stored at -70°C, according to WHO recommendations [15]. On the day of NP sampling, information was collected about the child's health, and other co-variates, detailed in supplementary text.

Total nucleic acid extraction and quantitative real-time polymerase chain reaction

Stored NP samples were thawed and vortexed (30 seconds). Nucleic acids were extracted using the NucliSens® easyMAG® extraction system (BioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions and stored at –20°C until tested. Nucleic acid extracts were tested using the Standard BioTools™ nanofluidic qPCR on the Biomark HD System within the 96.96 Dynamic Array integrated fluidic circuit (96.96 IFC) as described previously [12]. The qPCR could detect 92 pneumococcal serotypes and differentiate these into 35 serotypes in 16 groups, 57 individual types, and identify 15 other bacterial species.

Statistical analysis

Statistical analysis was performed with STATA Version 13.1 (StataCorp, Texas, USA). The cycle of quantification (Cq) values were converted to density (Log_{10} genomic equivalents [GE]/ml), and the relevant algorithms were applied to interpret pneumococcal serotypes and bacterial colonizers, detailed previously [12]. Samples were considered positive for a target if the density was above the limit of detection for that assay set [16] and were considered positive for pneumococcus if at least three out of four included pneumococcal reference genes (Lyt*A*, Pia*B, Ply,* and Xisco) were detected. Samples positive for pneumococcus but without a designated serotype, were interpreted as non-typeable (NT) *S. pneumoniae*. Further, we compared the difference in the average density of Lyt*A* and Pia*B* and the cumulative density of detected serotypes using Bland Altman (BAT) plots. If the average Lyt*A* and Pia*B* density exceeded the upper 95% limit of agreement of the BAT plot, these were considered co-colonizing NT pneumococci. Similarly, where *Haemophilus influenzae* was detected using the IgA1 gene, but the capsular loci (Bex*A* and Bex*B*) and the type-b capsule were not detected, these were designated as NT *H. influenzae* (NTHi).

PCV immunization coverage was assessed in period-2 only, due to differences in the data collection methods. Children were categorized into age groups (6-14; >14-40; and >40 weeks of age) according to the South African PCV immunization schedule. The demographic characteristics were compared between the two periods using the Student's *t*-test for continuous variables or the Pearson X^2 test for categorical variables. The multivariate analysis included *P*-values <0.1.

Table 1
Baseline characteristics of children 0-60 months of age enrolled in two cross-sectional surveys undertaken in Soweto, South Africa.

	Period 1 (2010; N = 1135)	Period 2 (2018; N = 571)	*P*-value
0-60 months: mean age in months (SD)	25.9 (17.8)	25.9 (16.0)	0.934
≤ 24 months: mean age in months (SD)	11.2 (6.0); N=616	12.3 (6.6); N=289	0.009
> 24 months: mean age in months (SD)	43.4 (9.3); N=519	39.7 (9.8); N=282	<0.001
Currently breastfed. n/N (%)	310/1134 (27.3)	137 (24%)	0.14
Ever breastfed n/N (%)	521/882 (59)	454 (79.5)	<0.001
Attendance at daycare n/N (%)	409/1134 (36.1)	115 (20.1)	<0.001
Children living with HIV n/N (%)	538 (47.4)	3 (0.5)	<0.001
Co-trimoxazole prophylaxis. n/N (%)	257 (22.6)	2 (0.4)	<0.001
Treated for Tuberculosis in the past year n/N (%)	45/1124 (4)	2 (0.4)	<0.001
Current antibiotic treatment n (%)	105 (9.3)	1 (0.2)	<0.001
Hospitalized in last 3 months n (%)	52 (4.6)	0	<0.001

Student's *t*-test or Pearson X^2 test was used to compare demographic and risk factors for colonization between the cohorts and those with *P*-values <0.01 and known to affect pneumococcal colonization were included in multivariate analyses; n: number of individuals with investigated outcomes; N: total number of individuals with available information on the characteristic. N was only included in rows where N was different from that indicated in the column header.

Multivariate logistic regression models were used to assess the temporal association of colonization prevalence of pneumococcal (overall, VT, and NVT) and other bacteria NP colonization in period-2 compared with period-1. We calculated adjusted odd ratios (aOR), adjusting for factors known to impact colonization including if the child was ever breastfed, HIV infection, antimicrobial use, co-trimoxazole prophylaxis, and tuberculosis treatment. We did not stratify children according to their HIV status, as the number of CLWH was expected to be low in period-2. When aOR could not be calculated, Fisher's exact test was used to test for statistical significance. Regression analysis was used to calculate the difference between the geometric mean density (GMD) in period-2 and period-1. Further, we used density to rank co-colonizers (serotypes or bacterial targets). To explore the interrelationship between co-colonizers, we constructed a colonization hierarchy matrix based on a relative target density. We considered the highest-density colonizer as the primary colonizer and where lower-density colonizers were present, these were classified as secondary colonizers. The matrix was translated into paired relationships and chord diagrams were constructed with flourish.studio (available at https://flourish.studio). Logistic regression was used to compare co-colonization events. We did not adjust for multiple comparisons as this is a descriptive study, and the analysis was pre-planned. Nevertheless, to account for multiplicity, we considered *P*-values of ≤0.01 as significant.

Results

A total of 1135 NP swabs collected during period-1 and 571 during period-2 were available for qPCR analysis. Overall, children were of a similar mean age (25.9 months) in period-1 and period-2, however, those <24 months and >24 months of age were 1 month younger and approximately 4 months older, respectively, in period-1, Table 1. Since some children were enrolled through HIV clinics in period-1, there were more CLWH and children taking co-trimoxazole prophylaxis in period-1 (47.4% and 22.6%) compared with period-2 (0.5%; *P* <0.001 and 0.4%; *P* <0.001), and more children were on tuberculosis treatment in period-1 (4%) compared with period-2 (0.4%; *P* <0.001). More children attended day-care in Period-1 (36.1%) compared with period-2 (20.1%; *P* <0.001), and the proportion of children who have ever breastfed was lower in period-1 (59%) compared with period-2 (79.6%; *P* <0.001). Since antibiotic usage was not an exclusion criterion for enrollment in period-1, 9.3% of the enrolled children used antibiotics within three weeks of sample collection. The coverage of three doses of PCV for children >40 weeks of age was 93.2% in 2018 (period-2), and 99.4% of the enrolled children had received at least one dose of PCV, Supplementary Table 1. The WHO/UNICEF Estimates of National Immunization Coverage (WUENIC) for PCV was 58% in 2010 for South Africa (https://immunizationdata.who.int/pages/coverage/PCV.html). The WUENIC estimates per year since 2009 are included in Supplementary Figure 1.

Pneumococcal colonization prevalence

The overall prevalence of pneumococcal colonization was lower in period-2 (49.4%; 282/571) compared with period-1 (68.1%; 773/1135; aOR: 0.66; 95% confidence intervals [CI]: 0.54-0.88); Figure 1, Supplementary Table 2). This was driven by the 59% lower VT prevalence in period-2 (18.6%; 106/571) compared with period-1 (40.9%; 465/1135; aOR: 0.41; 95% CI: 0.3-0.56). The net reduction in VT colonization in period-2 compared with period-1 was due to a lower prevalence of colonization by serotypes 6A; 6B; 19A; and 23F (Figure 2, Supplementary Table 2). Nevertheless, the prevalence of serotype 19F was unchanged in period-2 (8.1%; 46/571) compared with period-1 (6.6%; 75/1135; aOR: 2.0; 95% CI: 1.09-3.56). The prevalence of NVT colonization was similar in period-2 (37.8%; 216/571) and period-1 (42.4%; 481/1135); Figure 1; Supplementary Table 2 and Supplementary Figure 2.

Density of pneumococcal carriage

There was an increase in the overall pneumococcal GMD in the NP samples in period-2 (4.50 $\log_{10}$ GE/ml) compared with period-1 (4.32 $\log_{10}$ GE/ml; $P = 0.01$) Supplementary Figure 3; Supplementary Table 3. The GMD of VT was, however, lower in period-2 (3.92 $\log_{10}$ GE/ml) compared with period-1 (4.32 $\log_{10}$ GE/ml; $P < 0.001$). Specifically, the GMD of VT 6A and 6B were lower in period-2 compared with period-1 ($P < 0.001$ and $P = 0.002$, respectively); whereas 19A and 23F were higher ($P = 0.003$ and $P = 0.005$, respectively), and the other VT were unchanged; Supplementary Figure 4, Supplementary Table 3. The GMD of NVT were similar between periods ($P = 0.089$), Supplementary Figure 3; Supplementary Table 3, however the GMD 15A/F and 35B increased in period-2 compared with period-1 ($P = 0.008$ and $P = 0.01$, respectively; Supplementary Figure 5, Supplementary Table 3).

Co-colonization of pneumococcal serotypes

There was no statistically significant difference in the number of individual circulating serotypes/serogroups between period-2 (n = 59) and period-1 (n = 63) or the percentage of children co-colonized ($P = 0.48$). In period-1 and period-2, 40.6% (314/773) and 37.9% (107/282) of children carried two or more, 13.5% (104/773) and 15.6% (44/282) at least three, and 4.9% (38/773) and 6.4% (18/282) carried at least four serotypes concurrently, respectively.

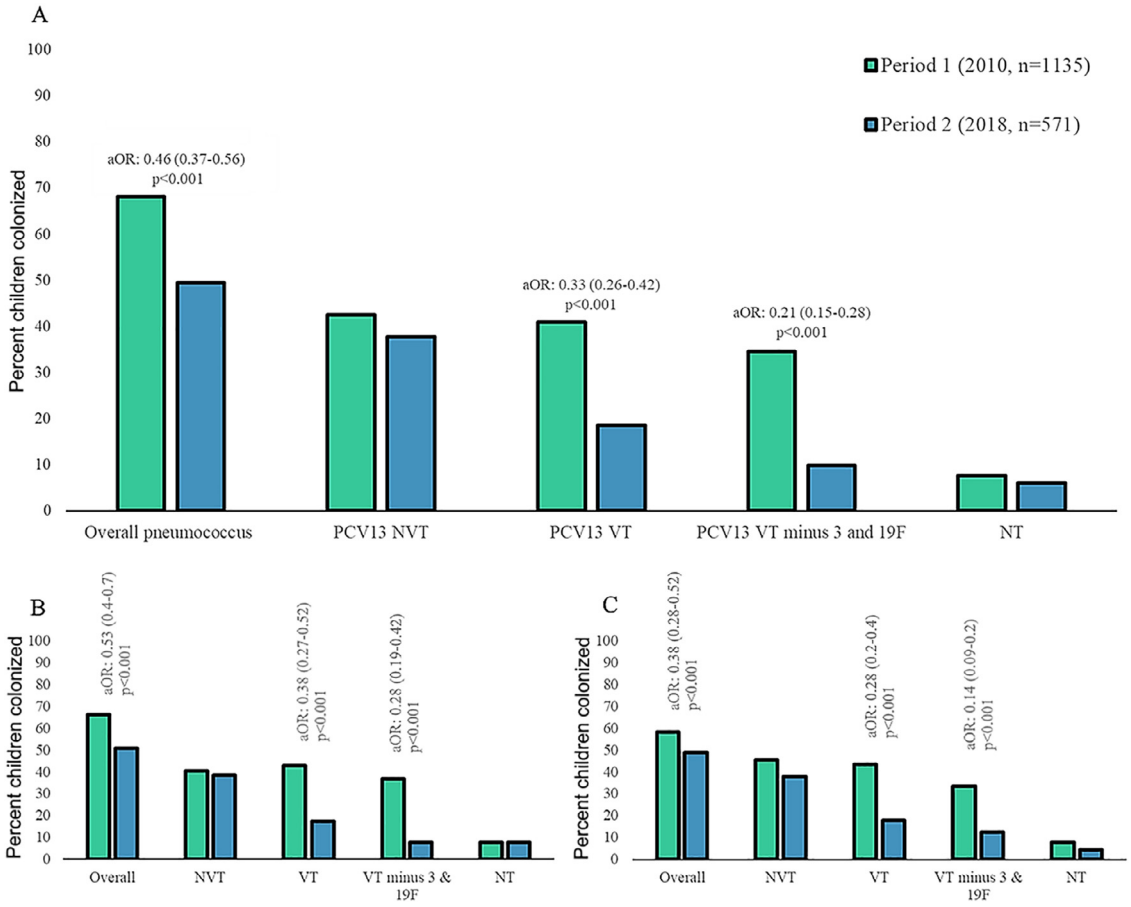

Figure 1. Colonization prevalence of *Streptococcus pneumoniae* in children aged 0-60 months. Panel A includes all children 0-60 months-of-age, Panel B includes children 0-24 months of age, and Panel C includes children 25-60 months of age. PCV13 NVT; PCV13 VT (1, 3, 4, 5,6A, 6B, 7A/F, 9A/V, 14, 18C, 19A, 19F, and 23F); NT *S. pneumoniae*. *P*-values <0.01 were considered significant, and calculated using logistic regression. In Panel B–C significant values are indicated by * and are detailed in Supplementary Table 2. aOR, adjusted odds ratio; NT, non-typeable; NVT, non-vaccine serotypes; VT, vaccine serotypes.

In co-colonized children, the detection of VT as the dominant colonizer (highest density or only) as a proportion of all the times VT was detected, was lower in period-2 (73.6%; 78/106) compared with period-1 (84.3%; 391/464; OR: 0.52; 95% CI: 0.32-0.86), whereas a higher proportion of NVT were identified as dominant colonizers in period-2 (87.0%; 180/207) compared with period-1 (70.1%; 331/472; OR: 2.84; 95% CI: 1.81-4.45).

When detected together with other pneumococcal serotypes in period-1, VT 6A, 19F, 23F, 18C, 19A, and 14 were more frequently the dominant colonizing serotype (Figure 3a, Supplementary Table 6). This is demonstrated by the arc size for the respective serotypes in Figure 3, which is linked to the number of times they are the dominant co-colonizing serotype. Notably, serotypes 19F and 6A were detected as the dominant serotype in 92.0% (69/75) and 92.5% (74/80), respectively, of all occasions they were found co-colonizing with any other serotype in period-1. In period-2, VT 6A, 14, and 19F persisted as the dominant colonizers when carried concurrently with other serotypes, with 19F being the dominant colonizer in 93.5% (43/46) of cases where it was detected in period-2, shown also by the similar arc size in both periods (Figure 3b; Supplementary Table 6). Serotype 6C increased in dominance relative to other co-colonizing serotypes in period-2 (Figure 3b), despite the cross-protection against serotype 6C afforded by the inclusion of 6A in PCV13. In period-2, NVT 8, 9LN, 10A, 10CF, 13, 15AF, 23A, 23B, 35B, 35F, 43, and 47A increased in dominance in co-colonization compared with period-1 and the arc size for these serotypes becomes larger in Figure 3b, representing replacement in co-colonization.

Colonization prevalence by other bacteria

The only difference in colonization by investigated bacteria in period-2 compared with period-1 was the higher prevalence of *N. Lactamica* (8.9%; 51/571 vs 6.8%; 77/1135; aOR: 2.39; 95% CI: 1.29-4.43); Supplementary Figure 5, Supplementary Table 4. Also, there were no differences in the GMD of non-pneumococcal bacterial colonizers between period-2 and period-1 (Supplementary Figure 7, Supplementary Table 5).

Co-colonization of other bacterial pathogens

A lower percentage of children had more than one bacterial species identified in period-2 (78.1%; 446/571) compared with in period-1 (83.7%; 950/1135; OR: 0.69; 95% CI: 0.5-0.9). In both study periods, *M. catarrhalis* and *S. pneumoniae* were more frequently detected as dominant colonizing bacterium than sub-dominant in co-colonization events, as shown by the large arc size in Figure 4. In contrast, *A. baumannii, H. influenzae, K. pneumoniae, N. lactamica, N. meningitidis, S. aureus, S. oralis,* and *S. pyogenes* were mostly detected as sub-dominant colonizers (Supplementary Table 7) when identified with another bacterial species.

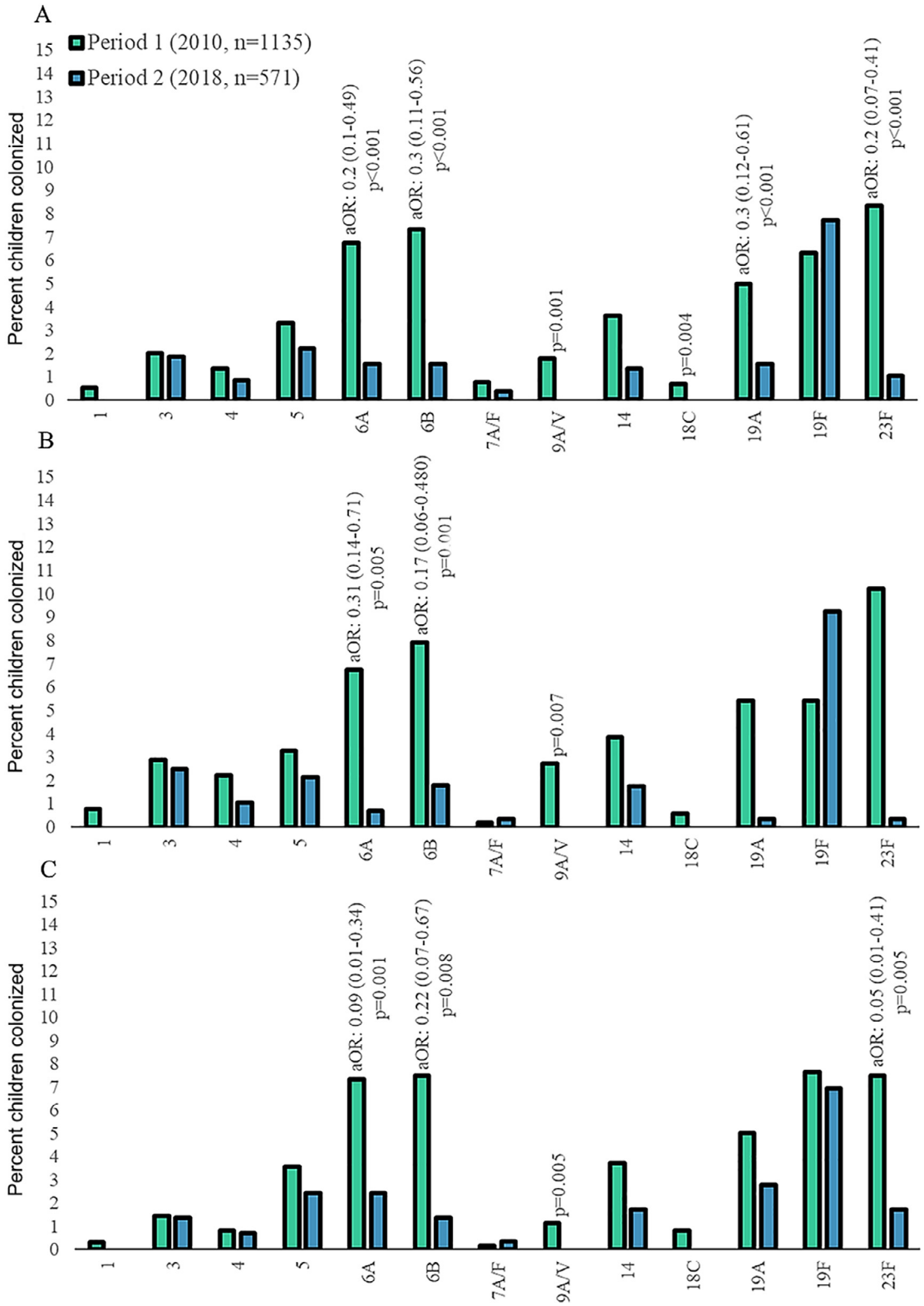

Figure 2. Colonization prevalence of pneumococcal vaccine serotypes in children 0-60 months of age. Panel A includes all children 0-60 months of age, Panel B includes children 0-24 months of age, and Panel C includes children 25-60 months of age. Only significant *P*-values shown, $P < 0.01$ were considered significant, calculated using logistic regression. All other *P*-values are presented in Supplementary Table 2.
aOR, adjusted odds ratio.

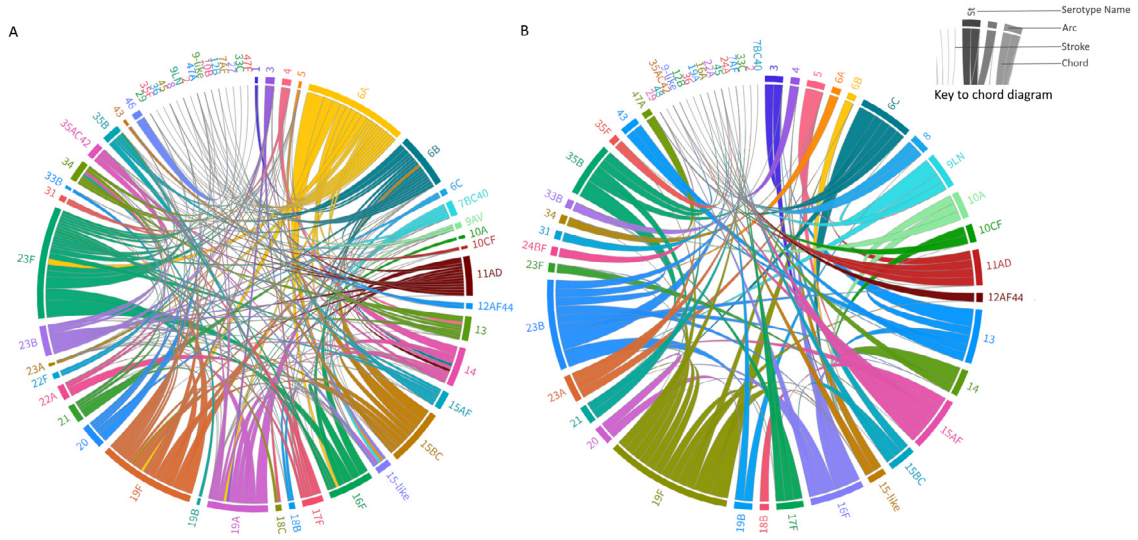

Figure 3. Chord diagram of co-colonizing events by pneumococcal serotypes in children 0-60 months of age in period-1 and period-2. Panel A includes period-1 (2010), and Panel B includes period-2 (2018). The right inset describes features of the diagram. Colors are assigned randomly. The outer circle contains arcs that represent each serotype. Each arc is paired with a co-colonizing serotype through a directional chord. The chord thickness at the arc represents the number of co-colonization events in which that serotype is dominant. The thickness of the arc is related to the relative dominance of the serotype rather than the prevalence. Where more than two serotypes were in co-colonization, these were paired separately with the dominant serotype, e.g., 6A>23F>15BC would be 6A>23F; 6A>15BC. Where the chord terminates in a stroke, e.g., serotype 29 in both panels, this indicates the serotype is dominant in zero cases of co-colonization. The flow of dominance is also represented by a color gradient, where the more often dominant serotype determines the color of the chord. If a chord color is different from the color of the terminating arc, this represents the serotype at the base color of the chord being mostly dominant. An interactive version of each chord diagram is available at https://public.flourish.studio/visualisation/12162597/ and https://public.flourish.studio/visualisation/12182182/.

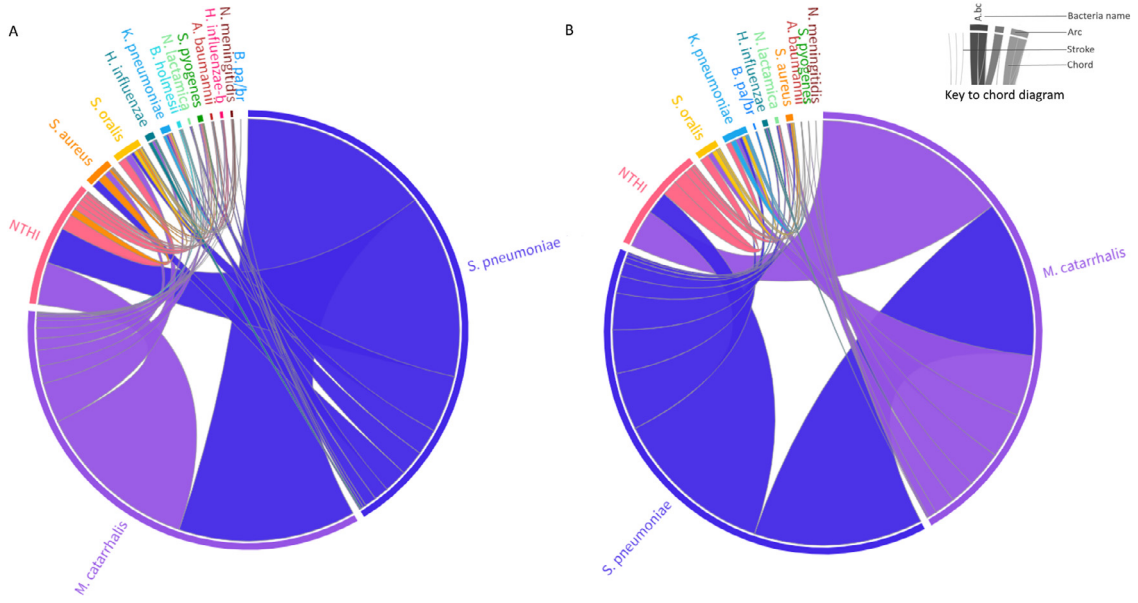

Figure 4. Chord diagram of bacterial co-colonizing events in children 0-60 months of age in Soweto, period-1 and period-2. Panel A includes period-1 (2010), and Panel B includes period-2 (2018). The right inset describes features of the diagram. The outer circle contains arcs that represent each bacterial species. Each arc is paired with a co-colonizing bacterium through a directional chord. The chord thickness at the arc represents the number of co-colonization events in which that bacterium is dominant. The thickness of the arc is related to the relative dominance of the bacterium rather than the prevalence. Where more than two bacteria were in co-colonization, these were paired separately with the dominant bacterium, e.g., Spn>Mca>Hin would be Spn>Mca; Spn>Hin. Where the chord terminates in a stroke, this indicates the bacterium is dominant in zero cases of co-colonization. The flow of dominance is also represented by a color gradient, where the more often dominant bacterium determines the color of the chord. If a chord color is different from the color of the terminating arc, this represents the bacterium at the base color of the chord being mostly dominant. An interactive version of each chord diagram is available at https://public.flourish.studio/visualisation/12186193/ and https://public.flourish.studio/visualisation/12583513/.

Discussion

Our study reports a modest decrease in VT nasopharyngeal colonization (54.5%) in children <5 years of age, almost nine years since PCV was first introduced into the South African immunization program, despite 93-100% of the sampled children having been fully vaccinated for their age. When we excluded serotypes 19F and three from the analysis, there was a 73.6% lower prevalence of colonization by the remaining VT in period-2 relative to period-1, with the main circulating serotypes being 5 (2.3%), 6A, 6B, and 19A (all 1.6%).

Despite the reduction in VT, the high carriage prevalence of serotype 19F (8.1%) is a concern, as it accounted for 43.5% of residual VT colonization (18.6%). Furthermore, 19F was more likely to

be identified as the highest-density serotype (>80% of the time) than sub-dominant in co-colonized children in both study periods. This is concerning as higher carriage density has been linked to increased transmission and progression to disease [17,18]. Also, 19F is typically carried for longer (212 days) compared with other serotypes (median 60 days) [19], increasing potential for 19F transmission.

The high residual carriage of 19F is corroborated by other colonization studies (1.4-4.5%) in urban settings in sub-Saharan Africa [5,20] and warrants further surveillance and genomic investigation, especially as 19F continues to be associated with ongoing VT IPD in South Africa [21]. Although PCVs reportedly prevent the acquisition of serotype 19F [22], the conjugation method used for PCV13 (reductive amination) may induce less productive opsonophagocytic antibodies against 19F [23]. Coupled with a higher force of infection from a high baseline prevalence, PCVs may not fully interrupt the transmission of 19F in our setting. Similarly, PCV13 had limited effectiveness against serotype 3 IPD [24]. Higher antibody concentrations have been correlated with protection against IPD for serotypes 3 (2.83 μg/ml) and 19F (1.17 μg/ml) than the putative threshold of 0.35 μg/ml when aggregating across all serotypes [24]. Generally, a higher antibody concentration is required to protect against mucosal disease and carriage compared with protection against IPD [25].

Consistently in sub-Saharan Africa, persistent VT colonization after the routine use of PCVs has been documented in urban settings similar to ours, including 9.6% (Congo, 2 years post-PCV) [20]; 11.4% (Gambia, 7 years post-PCV) [5] and 17.5% (Malawi, 4-7 years post-PCV) [4] for PCV13-VT and 6% (Kenya, 5 years post-PCV) for PCV10-VT [6], compared with large reductions in VT (to <3%) typically observed in higher income settings [26]. It is, however, difficult to directly compare these estimates to ours, due to the disparate detection and serotyping schemes used to assess VT and NVT pneumococcal colonization in each study. All four studies were culture-based or utilized an initial culture step. Typically, culture-based typing is not sensitive in the detection of co-colonizing serotypes [12]. Here, we used a sensitive qPCR that could detect 92 serotypes with a limit of detection of 10-100GE/ml, maximizing the ability to detect and serotype pneumococcus, including in co-colonization, and at low density.

In our setting, enhancing protection against the acquisition of 19F, which could translate to further reductions in 19F IPD, is warranted. Of interest, we recently reported that a one-dose primary series of PCV13 and booster dose (14 and 40 weeks of age) yielded higher anti-19F IgG compared with the two-dose and booster schedule currently used in South Africa (6, 14, and 40 weeks of age). Furthermore, the 1+1 schedule was associated with 2.89-fold lower prevalence of 19F colonization at 15 months of age compared with the 2+1 schedule, suggesting that transitioning to a 1+1 schedule could assist in reducing the residual prevalence of 19F colonization in settings such as South Africa [27].

The proportion of colonized children that carried more than one serotype was similar in both periods, ranging from 37.9-40.4%. We detected increases in the frequency of NVT (6C, 8, 9LN, 10A, 10CF, 13, 15AF, 23A, 23B, 35B, 35F, 43, and 47A) being the dominant serotype in co-colonization episodes in period-2, likely due to unmasking when dominant VT was reduced in prevalence. Assessing co-colonization is important in surveillance, as transformation, the ability to acquire genetic elements from other serotypes or bacteria, requires physical proximity afforded by co-colonization [28]. This has implications for antimicrobial resistance genes and capsule switching, the latter to evade serotype-specific vaccine-derived immunity. Indeed, in The Gambia, Multi-Locus-Sequence-Typing, revealed higher proportions of children under-2-years-old carrying multiple serotypes, and genetic transformation (34%) compared with those in higher income settings (2-5%), which the authors attribute to higher carriage prevalence [29]. It will therefore be important to further interrogate episodes of co-colonization for antimicrobial resistance genes.

Limitations of our study, include that our study was not powered to detect changes in less-prevalent individual serotypes, temporally linked with vaccination. As a result, trends in the detection of individual serotypes and density were exploratory. Different swab consumables were used in period-1 and period-2. Nevertheless, this should not have impacted our estimation of bacterial colonization prevalence, as the recovery of *S. pneumoniae* from FLOQSwabs (used in period-2) is comparable to that of Dacron swabs (period-1) [30]. In addition, good diagnostic sensitivity (average 89.1%) has been demonstrated for the qPCR method used here, compared with the culture-based Quellung method, previously used to serotype pneumococcus in period-1 [12]. Still, FLOQSwabs have been shown to yield a higher density [30] and thus comparisons of density (GMD) of colonizing bacteria between period-1 (Dacron swabs) and period-2 (FLOQSwabs) should be interpreted with caution. Furthermore, the previous storage time (10 years) and freeze-thaw cycles for the period-1 samples may have impacted the yield of the NP swabs in our study, meaning we may have underestimated the prevalence or density of some lower-density colonizers. In a comparison between the qPCR method used here and the Quellung method performed soon after sample collection, Quellung detected an additional 9.3% of serotypes [12]. Regardless, when compared with Quellung, qPCR detected almost 40% more serotypes, around 72-90% of which were co-colonizing serotypes [12]. This translates to an overall higher sensitivity to detect co-colonizing and lower-density serotypes, minimizing the effects of storage or freeze-thaw cycles on our conclusions. Although we used the presence of multiple pan-pneumococcal genes to confirm pneumococcus, in the case of co-colonization, some non-pneumococcal species with homologous genetic regions to the targeted pneumococcal capsular regions, may be typed as pneumococcal serotypes, overestimating their prevalence.

This study is important in our setting as South Africa considers the transition to a reduced PCV dosing schedule. Here, we find that in children targeted for routine vaccination (<60 months old) have a reduced risk of disease from VT compared with the baseline period (2010, period-1) and with vaccination coverage above 80% and the possibility that a 1+1 schedule compared with 2+1 schedule could reduce 19F colonization, transitioning to the reduced dosing schedule should be considered.

Declarations of competing interest

The authors have no competing interests to declare.

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Ethical considerations

Ethical approval was obtained from the Medical Human Research Ethics Committee of the University of Witwatersrand, certificate numbers M090015 and M170314. Sample collection and laboratory experiments were all performed following the relevant guidelines and regulations. Written informed consent was ob-

tained from the guardians of all study participants at the time of enrollment.

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Authors contributions

SLD, CPO, SAM, and MCN conceived and designed the study. SN was responsible for sample collection in period-1 and SLD for sample collection in period-2. Fluidigm data was generated by SLD and LVDM. SLD analyzed the Fluidigm data. Funding acquisition was undertaken by SLD, SAM, CPO, and MCN. SLD and CPO conducted the statistical analysis and wrote the first draft of the manuscript. All authors contributed to subsequent drafts, read, and approved the final version of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2023.05.016.

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Streptococcus pneumoniae and other bacterial nasopharyngeal colonisation eight years post-introduction of 13-valent pneumococcal conjugate vaccine in South African children.

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Methods:

This study was conducted through the South African Medical Research Council and University of the Witwatersrand Vaccines and Infectious Diseases Analytics Research Unit (SAMRC/Wits-VIDA). SAMRC/Wits-VIDA is located at Chris Hani Baragwanath Academic Hospital (CHBAH) in Soweto, a settlement/township southwest of Johannesburg, Gauteng, SA. Soweto is one of the largest and oldest 'urban' townships within South Africa covering 200 Km^2, with a population of 1 271 628 (6 357.29 per km^2) in 355 331 (1776.42

per km²) households [1]. A sample size of 408 would allow us to detect an 80%-90% individual reduction in the most prevalent PCV13-serotypes in 2009 (19F; 6B; 6A; 23F; 19A and 14) among the ≤5-year-olds.

We used WHO EPI (Expanded Program on Immunisation) two-stage systematic random sampling probability proportional to size cluster survey method (PPS) to select households (https://www.who.int/publications/i/item/WHO-IVB-18.09) from the population census data collected by Statistics South Africa (STATSSA, 2011). The Soweto community structure being diverse, ranging from high-density informal settlements to suburban neighbourhoods, was accounted for by including sociodemographic strata (informal settlements; high-density mixed-income housing; middle-class suburbs, and mixed high-density and informal settlements) in the selection process. The first stage involved categorizing enumeration areas (EAs) into the four sociodemographic strata. Subsequently, 45 EAs were selected as clusters. The second stage involved random selection of co-ordinates for ten dwelling units (DU; households) in each of the 45 EAs. DUs were first approached and if they were not eligible (no child 0-59 months in the household) the next nearest front door was approached. Households were approached once in traditional hours (Monday to Friday, 09:00-17:00) and if no one was home, they were re-approached outside of traditional hours (Saturday, 09:00-17:00), and if the second visit was unsuccessful the DU was replaced with the next nearest front door. All age-eligible children within the household were sampled. Children were excluded if they had a febrile illness (fever ≥37.5°C), used antibiotics in the three weeks before sampling, the primary caregiver was unwilling to discuss their own or their child's HIV status (caregiver unwilling to undergo an HIV rapid test, or provide documented HIV status within 6 months of the sampling date), or if the caregiver did not provide informed

consent for their child to participate.

On the day of NP sampling, information was collected about the child's health (respiratory symptoms, fever, HIV-status and treatment, antibiotic treatment, cotrimoxazole prophylaxis, TB exposure and/or treatment, hospitalisation within three months, and medical conditions), and other co-variates (daytime social contact and breastfeeding status). When possible, the child's HIV and PCV immunisation status was transcribed from the child's health records, ie: Road to Health Card; RTHC. Where the RTHC was unavailable, the child's immunisation and HIV status information were collected verbally from the caregiver.

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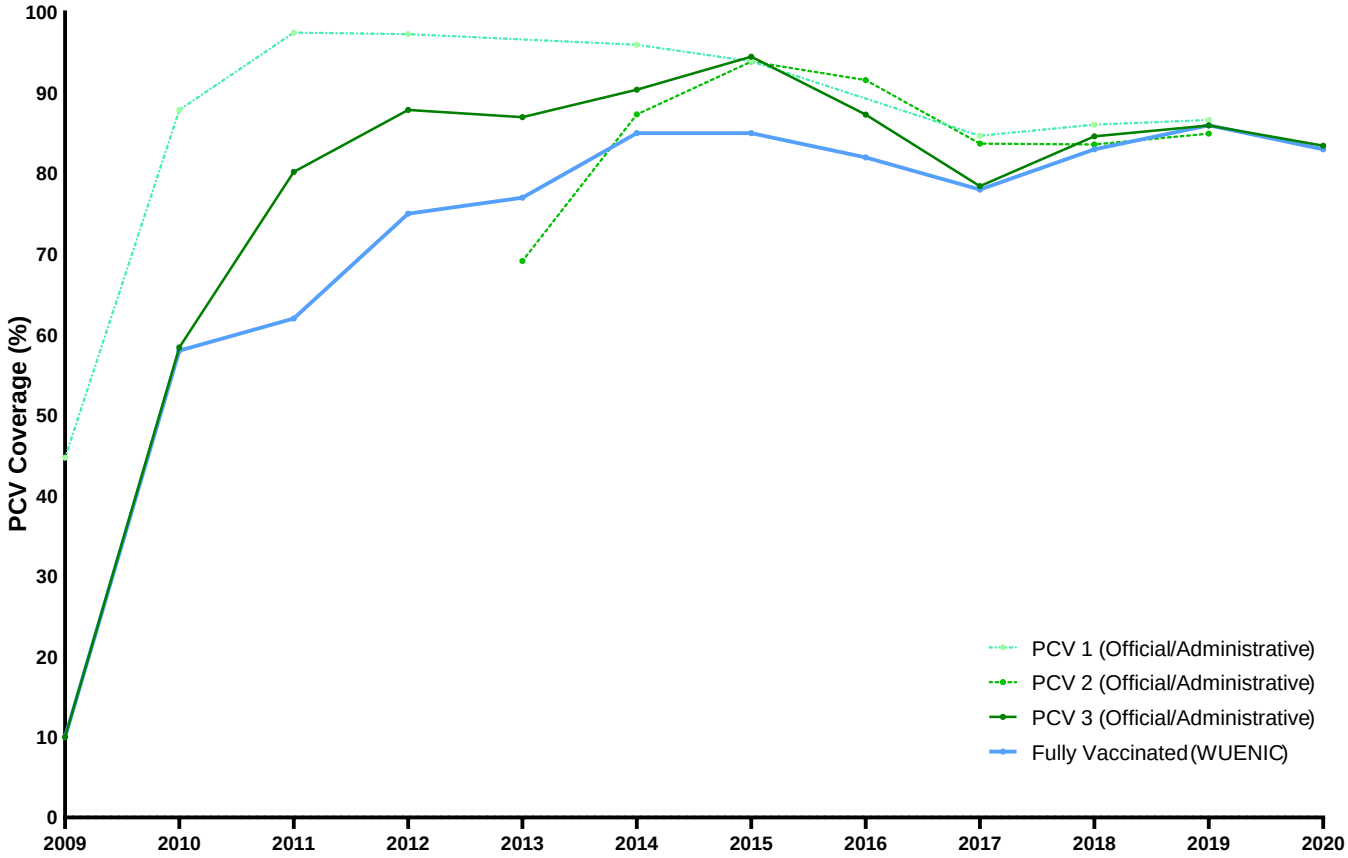
1. STATSSA. Census 2011 Statistical release – P0301.4 / Statistics South Africa. Pretoria: Statistics South Africa, 2012 2012.



Supplementary Figure 1: Administrative and Official Pneumococcal vaccination coverage in South Africa reported annually through the World Health Organization (WHO)/ The United Nations Children's Fund (UNICEF) Joint Reporting Form on Immunization (JRF) and the WHO and UNICEF Joint Estimates of National Immunization Coverage (WUENIC) [1, 2].

WUENIC estimates include the percentage of surviving infants who received the 3rd dose of pneumococcal conjugate vaccine or infants that received at least 2 doses of PCV prior to their first birthday. Official estimates are usually based on data from the administrative method. Administrative data is calculated based on the number of doses recorded as administered as part of the national immunization schedule. The national Department of Health may provide official estimates from other sources. Official and Administrative data been combined here. For the Official/Administrative data, PCV 1; PCV 2 and PCV 3 is the percentage in the target population who have received one; two and three doses of PCV respectively in each year.

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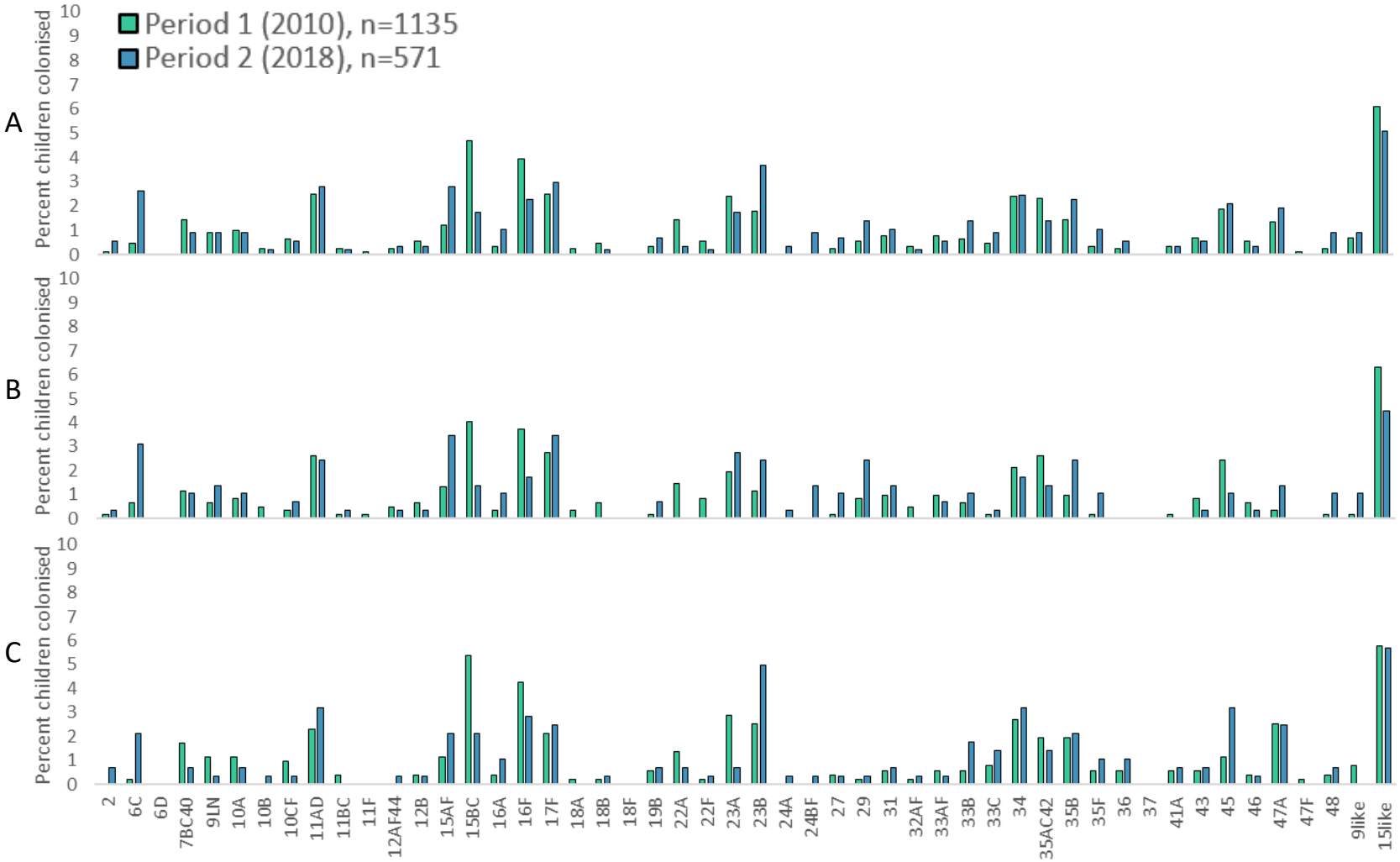
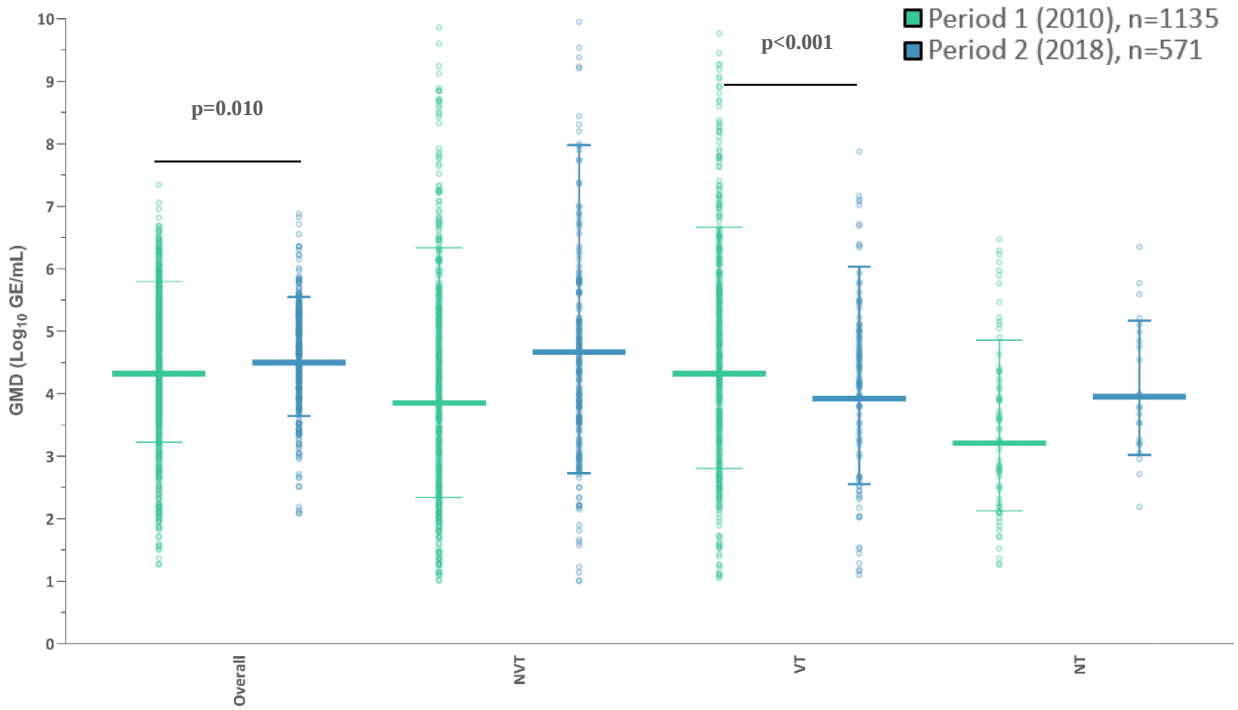


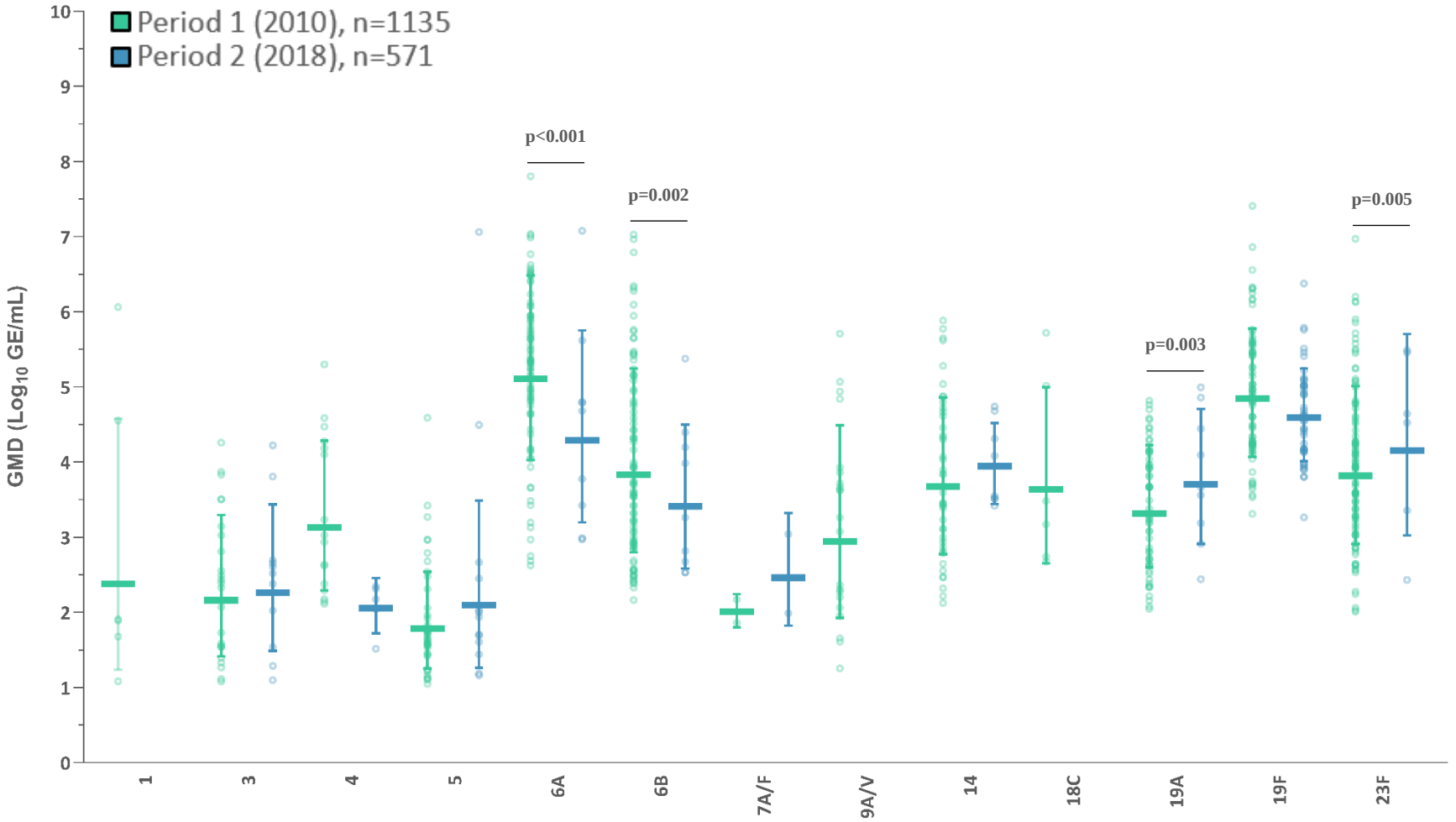
Supplementary Figure 2: Prevalence of non-vaccine serotypes in children 0-60 months-of-age.

Panel A includes all children 0-60 months-of-age, Panel B includes children 0-24 months-of-age and Panel C includes children 25-60 months-of-age; p-values <0.01 were considered significant. Adjusted Odds Ratios and p-values calculated using logistic regression in Supp table 2.



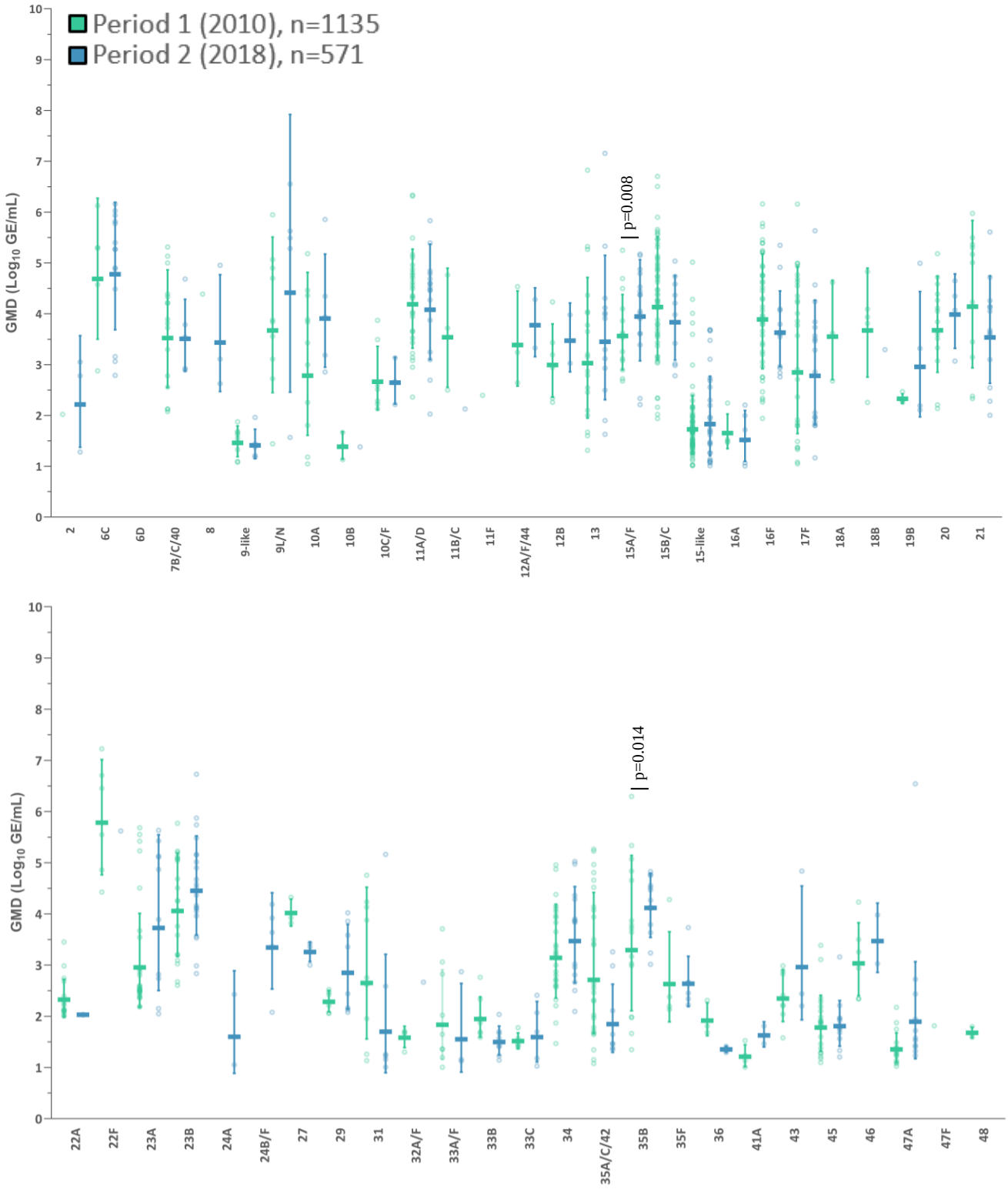
Supplementary Figure 3: Geometric mean density (GMD, log_{10} Genomic Equivalents per mL [GE/mL]) of *Streptococcus pneumoniae* in children 0-60 months of age.

Overall includes all pneumococcus; VT include PCV13 vaccine serotypes serotypes/serogroups 1, 3, 4, 5,6A, 6B, 7A/F, 9A/V, 14, 18C, 19A, 19F and 23F; NVT, all serotypes/serogroups not included in PCV13. NTSP, non-typeable S. pneumoniae. Only significant p-values shown, p-values <0.01 were considered significant. All other p-values presented in supp table 3.

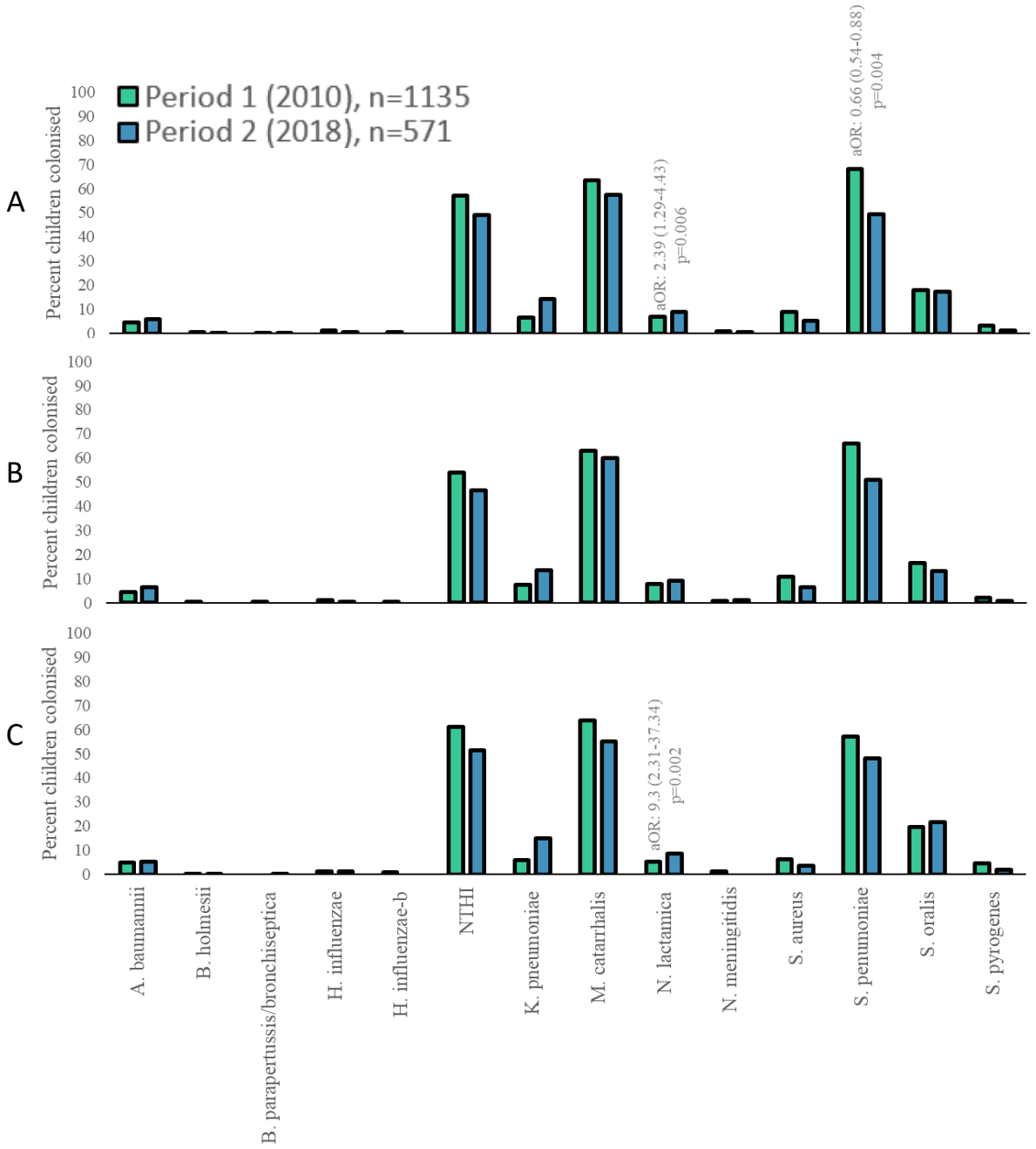


Supplementary Figure 4: Geometric mean density (GMD $\log_{10}$ Genomic Equivalents per mL [GE/mL]) of colonising PCV13 vaccine serotypes in children 0-60 months-of-age.

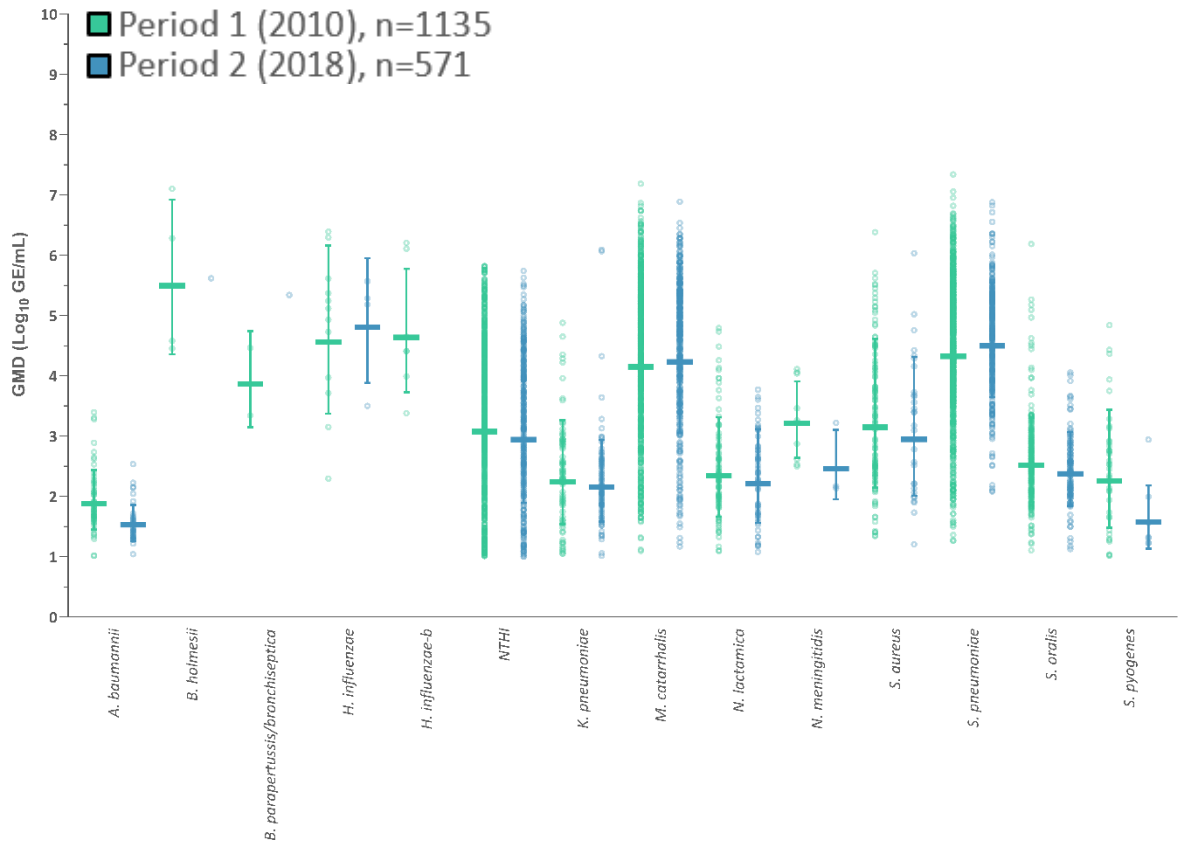
Only significant p-values shown, p-values <0.01 were considered significant. All other p-values presented in supp table 3.



Supplementary Figure 5: Geometric mean density (GMD $\log_{10}$ Genomic Equivalents per mL [GE/mL]) of non-PCV13 serotypes (NVT) in children 0-60 months of age.
Only significant values (<0.01) presented. All p-values in Supp table 3.



Supplementary Figure 6: Prevalence of bacterial colonisers in children 0-60 months-of-age. *Panel A includes all children 0-60 months-of-age, Panel B includes children 0-24 months-of-age and Panel C includes children 25-60 months-of-age. Only significant p-values shown, p-values <0.01 were considered significant. All other p-values presented in supp table 4.*



Supplementary Figure 7: Geometric mean density (GMD $\log_{10}$ Genomic Equivalents per mL [GE/mL]) of bacterial colonisers in children 0-60 months of age across three study periods.

Only significant p-values shown, p-values <0.01 were considered significant. All other p-values presented in supp table 5.

Supplementary Table 1: Pneumococcal conjugate vaccine coverage in Soweto, Gauteng in Period-2 (2018)

Age (weeks)	1 dose	2 doses	3 doses
6-14 % (n/N)	100 (24/24)	-	-
14-40 % (n/N)	98.6 (68/69)	100 (69/69)	-
>40 % (n/N)	99.2 (475/479)	98.3 (471/479)	93.3 (477/479)

Supplementary Table 2: Prevalence of *Streptococcus pneumoniae* colonisation in Period-1 (2010) and Period-2 (2018) in Sowetan children 0-60 months-of-age.

	Age Group Months	Period-2 % (n); N=571	Period-1 % (n); N=1135	OR (95% CI); p-value	aOR (95% CI); p-value
Overall pneumococcus	0-60	49.4 (282)	68.1 (773)	0.46 (0.37-0.56); p<0.001	0.66 (0.54-0.88); p=0.004
	0-24	50.9 (147)	66.1 (407)	0.53 (0.4-0.7); p<0.001	0.69 (0.47-1); p=0.05
	25-60	47.9 (135)	57.2 (366)	0.38 (0.28-0.52); p<0.001	0.58 (0.38-0.9); p=0.015
PCV13-VT	0-60	18.6 (106)	40.9 (465)	0.33 (0.26-0.42); p<0.001	0.41 (0.3-0.56); p<0.001
	0-24	17.4 (49)	42.6 (221)	0.38 (0.27-0.52); p<0.001	0.41 (0.27-0.62); p<0.001
	25-60	17.4 (49)	42.6 (221)	0.28 (0.2-0.4); p<0.001	0.37 (0.23-0.6); p<0.001
NVT	0-60	37.8 (216)	42.4 (481)	0.83 (0.67-1.01); p=0.07	0.96 (0.72-1.26); p=0.75
	0-24	38.4 (111)	40.4 (249)	0.92 (0.69-1.22); p=0.56	1.06 (0.72-1.55); p=0.79
	25-60	37.2 (105)	44.7 (232)	0.73 (0.54-0.98); p=0.04	0.87 (0.57-1.32); p=0.53
NT	0-60	5.95 (34)	7.58 (86)	0.77 (0.51-1.16); p=0.22	0.99 (0.57-1.71); p=0.98
	0-24	7.6 (22)	7.5 (46)	1.02 (0.6-1.74); p=0.94	1.29 (0.62-2.71); p=0.49
	25-60	4.3 (12)	7.7 (40)	0.53 (0.27-1.03); p=0.06	0.69 (0.28-1.68); p=0.41
1	0-60	0 (0)	0.53 (6)	p=0.08	-
	0-24	0 (0)	0.32 (2)	p=0.35	-
	25-60	0 (0)	0.77 (4)	p=0.15	-
3	0-60	1.93 (11)	2.11 (24)	0.91 (0.44-1.87); p=0.8	2.3 (0.63-8.19); p=0.21
	0-24	1.38 (4)	1.46 (9)	0.94 (0.29-3.1); p=0.93	2.49 (0.2-6.24) p=0.43
	25-60	2.48 (7)	2.89 (15)	0.86 (0.34-2.12); p=0.74	2.09 (0.42-10.38) p=0.37
4	0-60	0.88 (5)	1.41 (16)	0.62 (0.23-1.7); p=0.35	0.6 (0.19-2.16); p=0.47
	0-24	0.69 (2)	0.81 (5)	0.85 (0.16-4.42); p=0.85	1.29 (0.12-14.29) p=0.84
	25-60	1.06 (3)	2.21 (11)	0.5 (0.14-1.8); p=0.29	0.53 (0.11-2.53) p=0.43
5	0-60	2.28 (13)	3.44 (39)	0.65 (0.35-1.24); p=0.19	0.5 (0.23-1.06); p=0.07
	0-24	2.42 (7)	3.57 (22)	0.67 (0.28-1.59); p=0.36	0.51 (0.18-1.46) p=0.21
	25-60	2.13 (6)	3.28 (17)	0.64 (0.25-1.65); p=0.36	0.47 (0.14-1.57) p=0.22
6A	0-60	1.58 (9)	7.05 (80)	0.21 (0.11-0.42); p<0.001	0.2 (0.1-0.49); p<0.001
	0-24	2.42 (7)	7.31 (45)	0.31 (0.14-0.71); p=0.005	0.37 (0.14-0.99) p=0.048
	25-60	0.71 (2)	6.74 (35)	0.1 (0.02-0.41); p=0.002	0.09 (0.01-0.34) p=0.001
6B	0-60	1.58 (9)	7.67 (87)	0.19 (0.1-0.39); p<0.001	0.3 (0.11-0.56); p<0.001
	0-24	1.38 (4)	7.47 (46)	0.17 (0.06-0.48); p=0.001	0.26 (0.08-0.87) p=0.029
	25-60	1.77 (5)	7.90 (41)	0.21 (0.08-0.54); p=0.001	0.22 (0.07-0.67) p=0.008
7A/F	0-60	0.35 (2)	0.18 (2)	1.99 (0.28-14.18); p=0.49	1.1 (0.1-12.6); p=0.93
	0-24	0.35 (1)	0.16 (1)	2.13 (0.13-34.26); p=0.59	0.64 (0.04-10.51) p=0.75
	25-60	0.35 (1)	0.19 (1)	1.84 (0.11-29.58); p=0.66	9.2 (0.03-272.48) p=0.46
9A/V	0-60	0 (0)	1.85 (21)	p=0.001	-
	0-24	0 (0)	1.14 (1)	p=0.07	-

	Age Group Months	Period-2 % (n); N=571	Period-1 % (n); N=1135	OR (95% CI); p-value	aOR (95% CI); p-value
	25-60	0 (0)	2.7 (14)	p=0.005	-
14	0-60	1.4 (8)	3.79 (43)	0.36 (0.17-0.77); p=0.009	0.4 (0.15-0.92); p=0.03
	0-24	1.73 (5)	3.73 (23)	0.45 (0.17-1.2); p=0.11	0.44 (0.14-1.43) p=0.17
	25-60	1.73 (5)	3.85 (20)	0.27 (0.08-0.91); p=0.04	0.32 (0.07-1.39) p=0.13
18C	0-60	0 (0)	0.7 (8)	p=0.004	-
	0-24	0 (0)	0.81 (5)	p=0.13	-
	25-60	0 (0)	0.58 (3)	p=0.2	-
19A	0-60	1.58 (9)	5.2 (59)	0.29 (0.14-0.59); p=0.001	0.3 (0.12-0.61); p<0.001
	0-24	2.77 (8)	5.03 (31)	0.54 (0.24-1.19); p=0.12	0.35 (0.14-0.87) p=0.02
	25-60	0.35 (1)	5.39 (28)	0.06 (0.008-0.46); p=0.007	0.11 (0.01-0.91) p=0.04
19F	0-60	8.06 (46)	6.61 (75)	1.23 (0.85-1.81); p=0.27	2 (1.09-3.56); p=0.03
	0-24	6.92 (20)	7.63 (47)	0.9 (0.52-1.55); p=0.7	0.23 (0.5-72.8) p=0.56
	25-60	9.22 (26)	5.39 (28)	1.78 (1.0 -3.1); p=0.04	2.45 (0.93-6.41) p=0.07
23F	0-60	1.05 (6)	8.72 (99)	0.11 (0.05-0.25); p<0.001	0.2 (0.07-0.41); p<0.001
	0-24	1.73 (5)	7.47 (46)	0.22 (0.09-0.56); p=0.001	0.27 (0.09-0.78) p=0.02
	25-60	0.35 (1)	10.21 (53)	0.03 (0.004-0.23); p=0.001	0.05 (0.01-0.41) p=0.005
2	0-60	0.53 (3)	0.09 (1)	5.99 (0.62-57.71); p=0.12	-
	0-24	0.35 (1)	0.16 (1)	2.14 (0.13-34.26); p=0.59	1.16 (0.79-1.72); p=0.45
	25-60	0.71 (2)	0 (0)	-	-
6C	0-60	2.63 (15)	0.44 (5)	6.1 (2.2-16.86); p<0.001	4.57 (1.05-19.93); p = 0.04
	0-24	3.11 (9)	0.65 (4)	4.92 (1.5-16.1); p=0.008	2.76 (0.58-13.01); p=0.2
	25-60	2.13 (6)	0.19 (1)	11.26 (1.35-94.01); p=0.025	47.97 (0.77-2996.83); p=0.07
7B/7C/40	0-60	0.88 (5)	1.41 (16)	0.62 (0.23-1.7); p=0.35	1.58 (0.31-8); p = 0.58
	0-24	1.04 (3)	1.14 (7)	0.91 (0.23-3.55); p=0.9	40.27 (0.04-37553.68); p=0.29
	25-60	0.71 (2)	1.73 (9)	0.4 (0.09-1.89); p=0.25	0.61 (0.08-4.68); p=0.63
9L/9N	0-60	0.88 (5)	0.88 (10)	0.99 (0.34-2.92); p=0.99	0.86 (0.22-3.44); p = 0.83
	0-24	1.38 (4)	0.65 (4)	2.15 (0.53-8.65); p=0.28	1.96 (0.21-18.23); p=0.56
	25-60	0.35 (1)	1.16 (6)	0.3 (0.04-2.54); p=0.27	0.37 (0.03-3.91); p=0.41
10A	0-60	0.88 (5)	0.97 (11)	0.9 (0.31-2.61); p=0.85	0.9 (0.23-3.62); p = 0.89
	0-24	1.04 (3)	0.81 (5)	1.28 (0.3-5.4); p=0.74	1.45 (0.14-14.77); p=0.75
	25-60	0.71 (2)	1.16 (6)	0.61 (0.12-3.05); p=0.55	0.74 (0.11-5.19); p=0.76
10B	0-60	0.18 (1)	0.26 (3)	0.66 (0.07-6.38); p=0.72	0.2 (0.02-2.47); p = 0.21
	0-24	0 (0)	0.49 (3)	-	-
	25-60	0.35 (1)	0 (0)	-	-
10CF	0-60	0.53 (3)	0.62 (7)	0.85 (0.22-3.3); p=0.82	0.62 (0.12-3.29); p = 0.57
	0-24	0.69 (2)	0.32 (2)	2.14 (0.3-15.26); p=0.45	-
	25-60	0.35 (1)	0.96 (5)	0.37 (0.04-3.15); p=0.36	0.11 (0.01-1.49); p=0.1
11AD	0-60	2.80 (16)	2.47 (28)	1.14 (0.61-2.12); p=0.68	0.95 (0.43-2.08); p = 0.9
	0-24	2.42 (7)	2.6 (16)	0.93 (0.38-2.29); p=0.88	0.62 (0.21-1.85); p=0.39
	25-60	3.19 (9)	2.31 (12)	1.39 (0.58-3.35); p=0.46	1.87 (0.57-6.15); p=0.3
11BC	0-60	0.18 (1)	0.26 (3)	0.66 (0.07-6.38); p=0.72	-

	Age Group Months	Period-2 % (n); N=571	Period-1 % (n); N=1135	OR (95% CI); p-value	aOR (95% CI); p-value
	0-24	0.35 (1)	0.16 (1)	2.14 (0.13-34.26); p=0.59	-
	25-60	0.00 (0)	0.39 (2)	-	-
11F	0-60	0 (0)	0.09 (1)	p=0.48	-
	0-24	0 (0)	0.16 (1)	-	-
	25-60	0 (0)	0 (0)	-	-
12A/12F/44	0-60	0.35 (2)	0.26 (3)	1.33 (0.22-7.96); p=0.76	0.94 (0.08-10.88); p = 0.96
	0-24	0.35 (1)	0.49 (3)	0.71 (0.07-6.85); p=0.77	0.27 (0.01-5.2); p=0.38
	25-60	0.35 (1)	0 (0)	-	-
12B	0-60	0.35 (2)	0.53 (6)	0.66 (0.13-3.29); p=0.61	0.56 (0.07-4.27); p = 0.58
	0-24	0.35 (1)	0.65 (4)	0.53 (0.06-4.77); p=0.57	0.39 (0.02-6.82); p=0.52
	25-60	0.35 (1)	0.39 (2)	0.92 (0.08-10.19); p=0.95	1.25 (0.06-24.85); p=0.89
15A/15F	0-60	2.80 (16)	1.23 (14)	2.31 (1.12-4.76); p=0.02	5.16 (1.27-20.88); p=0.02
	0-24	3.46 (10)	1.30 (8)	2.72 (1.06-6.98); p=0.037	6.07 (0.84-44.11); p=0.08
	25-60	2.13 (6)	1.16 (6)	1.86 (0.59-5.82); p=0.29	5.54 (0.69-44.7); p=0.11
15B/15C	0-60	1.75 (10)	4.67 (53)	0.36 (0.18-0.72); p=0.004	0.37 (0.16-0.83); p=0.02
	0-24	1.38 (4)	4.06 (25)	0.33 (0.11-0.96); p=0.042	0.3 (0.09-1.02); p=0.05
	25-60	2.13 (6)	5.39 (28)	0.38 (0.16-0.93); p=0.034	0.4 (0.13-1.22); p=0.11
16A	0-60	1.05 (6)	0.35 (4)	3 (0.84-10.68); p=0.09	44.43 (0.28-6987.84); p=0.14
	0-24	1.04 (3)	0.32 (2)	3.22 (0.54-19.38); p=0.2	78.53 (0.02-665994.7); p=0.347
	25-60	1.06 (3)	0.39 (2)	2.78 (0.46-16.73); p=0.26	-
16F	0-60	2.28 (13)	3.96 (45)	0.56 (0.3-1.05); p=0.07	0.83 (0.35-1.98); p = 0.67
	0-24	1.73 (5)	3.73 (23)	0.45 (0.17-1.21); p=0.11	0.52 (0.16-1.75); p=0.29
	25-60	2.84 (8)	4.24 (22)	0.66 (0.29-1.5); p=0.32	1.07 (0.27-4.31); p=0.92
17F	0-60	2.98 (17)	2.47 (28)	1.21 (0.66-2.24); p=0.54	1.17 (0.5-2.75); p = 0.72
	0-24	3.46 (10)	2.76 (17)	1.26 (0.57-2.79); p=0.56	1.25 (0.42-3.68); p=0.69
	25-60	2.48 (7)	2.12 (11)	1.18 (0.45-3.07); p=0.74	1.27 (0.3-5.38); p=0.74
18A	0-60	0.00 (0)	0.26 (3)	p=0.22	-
	0-24	0 (0)	0.32 (2)	-	-
	25-60	0.00 (0)	0.19 (1)	-	-
18B	0-60	0.18 (1)	0.44 (5)	0.4 (0.05-3.4); p=0.38	1.08 (0.06-18.79); p = 0.96
	0-24	0 (0)	0.65 (4)	-	-
	25-60	0.35 (1)	0.19 (1)	1.84 (0.11-29.58); p=0.67	2.14 (0.12-38.14); p=0.6
19B	0-60	0.70 (4)	0.35 (4)	1.99 (0.50-8.01); p=0.33	9.2 (0.34-249.32); p = 0.19
	0-24	0.69 (2)	0.16 (1)	4.29 (0.39-47.46); p=0.24	-
	25-60	0.71 (2)	0.58 (3)	1.23 (0.2-7.4); p=0.82	-
22A	0-60	0.35 (2)	1.41 (16)	0.25 (0.06-1.07); p=0.06	0.22 (0.04-1.15); p = 0.07
	0-24	0 (0)	1.46 (9)	-	-
	25-60	0.71 (2)	1.35 (7)	0.52 (0.11-2.53); p=0.42	0.42 (0.05-3.45); p=0.42
22F	0-60	0.18 (1)	0.53 (6)	0.33 (0.04-2.75); p=0.31	0.31 (0.02-5.33); p = 0.42
	0-24	0 (0)	0.81 (5)	-	-
	25-60	0.35 (1)	0.19 (1)	1.84 (0.11-29.58); p=0.67	0.22 (0.01-4.93); p=0.34

	Age Group Months	Period-2 % (n); N=571	Period-1 % (n); N=1135	OR (95% CI); p-value	aOR (95% CI); p-value
23A	0-60	1.75 (10)	2.38 (27)	0.73 (0.35-1.52); p=0.4	0.6 (0.25-1.43); p = 0.25
	0-24	2.77 (8)	1.95 (12)	1.43 (0.58-3.54); p=0.44	0.71 (0.23-2.15); p=0.54
	25-60	0.71 (2)	2.89 (15)	0.24 (0.05-1.06); p=0.06	0.28 (0.05-1.54); p=0.14
23B	0-60	3.68 (21)	1.76 (20)	2.13 (1.14-3.96); p=0.02	2.8 (1.05-7.49); p = 0.04
	0-24	2.42 (7)	1.14 (7)	2.16 (0.75-6.22); p=0.15	4.43 (0.53-37.35); p=0.17
	25-60	4.96 (14)	2.50 (13)	2.03 (0.94-4.39); p=0.07	2.46 (0.77-7.87); p=0.13
24A	0-60	0.35 (2)	0.00 (0)	p=0.05	-
	0-24	0.35 (1)	0 (0)	-	-
	25-60	0.35 (1)	0.00 (0)	-	-
24B/24F	0-60	0.88 (5)	0.00 (0)	p=0.02	-
	0-24	1.38 (4)	0 (0)	-	-
	25-60	0.35 (1)	0.00 (0)	-	-
27	0-60	0.70 (4)	0.26 (3)	2.66 (0.59-11.93); p=0.2	31.94 (0.4-2525.83); p = 0.12
	0-24	1.04 (3)	0.16 (1)	6.45 (0.67-62.29); p=0.11	-
	25-60	0.35 (1)	0.39 (2)	0.92 (0.08-10.19); p=0.95	-
29	0-60	1.40 (8)	0.53 (6)	2.67 (0.92-7.74); p=0.07	4.58 (0.71-29.58); p = 0.11
	0-24	2.42 (7)	0.81 (5)	3.03 (0.95-9.64); p=0.06	4.33 (0.61-360.46); p=0.14
	25-60	0.35 (1)	0.19 (1)	1.84 (0.11-29.58); p=0.67	-
31	0-60	1.05 (6)	0.79 (9)	1.33 (0.47-3.75); p=0.59	0.81 (0.21-3.11); p = 0.76
	0-24	1.38 (4)	0.97 (6)	1.43 (0.4-5.1); p=0.58	0.98 (0.17-5.59); p=0.98
	25-60	0.71 (2)	0.58 (3)	1.23 (0.2-7.4); p=0.82	0.79 (0.08-7.7); p=0.84
32A/32F	0-60	0.18 (1)	0.35 (4)	0.5 (0.06-4.45); p=0.53	3.87 (0.07-208.35); p = 0.51
	0-24	0 (0)	0.49 (3)	-	-
	25-60	0.35 (1)	0.19 (1)	1.84 (0.11-29.58); p=0.67	-
33A/33F	0-60	0.53 (3)	0.79 (9)	0.66 (0.18-2.45); p=0.54	1.27 (0.15-10.5); p = 0.83
	0-24	0.69 (2)	0.97 (6)	0.71 (0.14-3.53); p=0.67	1.02 (0.1-10.76); p=0.99
	25-60	0.35 (1)	0.58 (3)	0.61 (0.06-5.91); p=0.67	4.19 (0.04-474.85); p=0.55
33B	0-60	1.40 (8)	0.62 (7)	2.29 (0.83-6.35); p=0.11	5.32 (0.79-35.28); p = 0.09
	0-24	1.04 (3)	0.65 (4)	1.6 (0.36-7.22); p=0.54	8.36 (0.22-311.17); p=0.25
	25-60	1.77 (5)	0.58 (3)	3.1 (0.74-13.09); p=0.12	5.99 (0.66-54.59); p=0.11
33C	0-60	0.88 (5)	0.44 (5)	2 (0.58-6.92); p=0.28	2.56 (0.31-21.23); p = 0.38
	0-24	0.35 (1)	0.16 (1)	2.14 (0.13-34.26); p=0.59	1155454 (0-.); p=0.996
	25-60	1.42 (4)	0.77 (4)	1.85 (0.46-7.46); p=0.39	1.56 (0.15-16.52); p=0.71
34	0-60	2.45 (14)	2.38 (27)	1.03 (0.54-1.98); p=0.93	1.56 (0.58-4019); p = 0.38
	0-24	1.73 (5)	2.11 (13)	0.82 (0.29-2.31); p=0.7	0.76 (0.17-3.44); p=0.72
	25-60	3.19 (9)	2.70 (14)	1.19 (0.51-2.78); p=0.69	2.1 (0.54-8.24); p=0.29
35A/35C/42	0-60	1.40 (8)	2.29 (26)	0.61 (0.27-1.35); p=0.22	0.38 (0.15-0.96); p = 0.04
	0-24	1.38 (4)	2.60 (16)	0.53 (0.17-1.59); p=0.26	0.35 (0.1-1.22); p=0.1
	25-60	1.42 (4)	1.93 (10)	0.73 (0.23-2.36); p=0.6	0.37 (0.08-1.72); p=0.2
35B	0-60	2.28 (13)	1.41 (16)	1.63 (0.78-3.41); p=0.2	1.4 (0.53-3.68); p = 0.5
	0-24	2.42 (7)	0.97 (6)	2.52 (0.84-7.58); p=0.1	5.18 (0.62-43.21); p=0.13

	Age Group Months	Period-2 % (n); N=571	Period-1 % (n); N=1135	OR (95% CI); p-value	aOR (95% CI); p-value
	25-60	2.13 (6)	1.93 (10)	1.11 (0.4-3.08); p=0.85	0.57 (0.15-2.16); p=0.41
35F	0-60	1.05 (6)	0.35 (4)	3 (0.84-10.68); p=0.09	1.75 (0.34-8.97); p = 0.51
	0-24	1.04 (3)	0.16 (1)	6.45 (0.67-62.29); p=0.11	2.02 (0.21-19.78); p=0.55
	25-60	1.06 (3)	0.58 (3)	1.85 (0.37-9.22); p=0.45	1.98 (0.19-21.09); p=0.57
36	0-60	0.53 (3)	0.26 (3)	1.99 (0.4-9.91); p=0.4	17.62 (0.4-772.89); p = 0.14
	0-24	0 (0)	0 (0)	-	-
	25-60	1.06 (3)	0.58 (3)	1.85 (0.37-9.22); p=0.45	12.08 (0.31-467.95); p=0.18
41A	0-60	0.35 (2)	0.35 (4)	0.99 (0.18-5.44); p=0.99	4.66 (0.19-116.16); p = 0.35
	0-24	0 (0)	0.16 (1)	-	-
	25-60	0.71 (2)	0.58 (3)	1.23 (0.20-7.4); p=0.82	3.89 (0.07-206.77); p=0.5
43	0-60	0.53 (3)	0.70 (8)	0.74 (0.2-2.82); p=0.66	0.58 (0.12-2.73); p = 0.49
	0-24	0.35 (1)	0.81 (5)	0.42 (0.05-3.65); p=0.44	0.53 (0.03-8.29); p=0.65
	25-60	0.71 (2)	0.58 (3)	1.23 (0.20-7.4); p=0.82	0.62 (0.07-5.58); p=0.67
45	0-60	2.10 (12)	1.85 (21)	1.14 (0.56-2.33); p=0.72	3.41 (0.79-14.65); p = 0.1
	0-24	1.04 (3)	2.44 (15)	0.42 (0.12-1.46); p=0.17	1.27 (0.13-12.62); p=0.84
	25-60	3.19 (9)	1.16 (6)	2.82 (0.99-8.0); p=0.05	7.94 (1.14-55.3); p=0.04
46	0-60	0.35 (2)	0.53 (6)	0.66 (0.13-3.29); p=0.61	0.56 (0.07-4.27); p = 0.58
	0-24	0.35 (1)	0.65 (4)	0.53 (0.06-4.77); p=0.57	0.39 (0.02-6.82); p=0.52
	25-60	0.35 (1)	0.39 (2)	0.92 (0.08-10.19); p=0.95	1.25 (0.06-24.85); p=0.89
47A	0-60	1.93 (11)	1.32 (15)	1.47 (0.67-3.21); p=0.34	1.26 (0.44-3.62); p = 0.67
	0-24	1.38 (4)	0.32 (2)	4.31 (0.78-23.66); p=0.09	43.74 (0.01-210690.1); p=0.38
	25-60	2.48 (7)	2.50 (13)	0.99 (0.39-2.51); p=0.98	0.83 (0.24-2.87); p=0.77
47F	0-60	0 (0)	0.09 (1)	p=0.48	-
	0-24	0 (0)	0 (0)	-	-
	25-60	0 (0)	0.19 (1)	-	-
48	0-60	0.88 (5)	0.26 (3)	3.33 (0.79-14); p=0.1	12.25 (0.37-409.43); p = 0.16
	0-24	1.04 (3)	0.16 (1)	6.45 (0.67-62.29); p=0.11	121.81 (0-232000000); p=0.52
	25-60	0.71 (2)	0.39 (2)	1.85 (0.26-13.18); p=0.54	10.53 (0.15-744.76); p=0.28
9like	0-60	0.88 (5)	0.7 (8)	1.24 (0.41-3.82); p=0.7	2.1 (0.38-11.5); p=0.40
	0-24	1,73 (5)	0,65 (4)	2.69 (0.72-10.2); p=0.14	20.9 (0.54-803.82); p=0.1
	25-60	0 (0)	0,77 (4)	-	-
15like	0-60	5.08 (29)	6.08 (69)	0.83 (0.53-1.29); p=0.4	0.95 (0.51-1.75); p=0.86
	0-24	4,5 (13)	6,33 (39)	0.69 (0.37-1.33); p=0.27	0.76 (0.33-1.75); p=0.52
	25-60	5,67 (16)	5,78 (30)	0.98 (0.52-1.83); p=0.95	1.06 (0.4-2.79); p=0.9

The total number of children 0-60 months in Period-1: N=1135; and Period-2: N=571; children 0-24 months in Period-1: N=616 and Period-2: N=289; and children 25-60 months in Period-1: N=519 and in Period-2: N=282. OR: Odds Ratio; aOR: adjusted Odds Ratio, calculated using logistic regression; adjusted for breastfeeding status, HIV infection, antibiotic usage, co-trimoxazole prophylaxis, and tuberculosis treatment. Overall pneumococcus includes all samples positive for pneumococcal reference genes. PCV13-VT: vaccine serotypes including serotypes/groups 1, 3, 4, 5, 6A, 6B, 7A/F, 9A/V, 14, 18C, 19A, 19F and 23F. NVT: serotypes/serogroups not included in PCV13. NT: non-typeable pneumococci.

Supplementary Table 3: Carriage density of *Streptococcus pneumoniae* from Nasopharyngeal Swab samples collected from children 0-60 months of age in Period 1 (2010, n=1135) and Period 2 (2018, n=572).

Serotype/ group	Period	0-60 months			0-24 months			25-60 months		
		n†	GMD (95% CI)‡ log_{10} GE/ml	p-value	n†	GMD (95% CI)‡ log_{10} GE/ml	p-value	n†	GMD (95% CI)‡ log_{10} GE/ml	p-value
Overall	1	773	4.32 (4.23-4.41)		407	4.46 (4.34-4.59)		366	4.17 (4.04-4.30)	
	2	282	4.50 (4.39-4.61)	0.010	147	4.61 (4.45-4.77)	0.247	135	4.38 (4.23-4.54)	0.025
NVT	1	472	3.85 (3.68-4.03)		245	4.03 (3.77-4.29)		227	3.67 (3.45-3.91)	
	2	207	4.67 (4.34-5.02)	0.089	106	4.95 (4.48-5.48)	0.070	101	4.38 (3.93-4.88)	0.317
VT	1	464	4.32 (4.16-4.50)		243	4.47 (4.24-4.71)		221	4.17 (3.93-4.42)	
	2	106	3.92 (3.61-4.26)	0.000	57	3.95 (3.55-4.40)	0.001	49	3.89 (3.40-4.45)	0.000
NTSP	1	70	3.21 (2.91-3.54)		36	3.49 (2.99-4.06)		34	2.94 (2.60-3.33)	
	2	23	3.95 (3.52-4.44)	0.692	15	4.28 (3.68-4.98)	0.117	8	3.40 (2.89-4.00)	0.247
1	1	6	2.38 (1.20-4.73)		6	2.38 (1.20-4.73)		4	2.21 (0.70-6.98)	
	2	0	-	0.040	0	-	0.097	0	-	0.191
3	1	24	2.16 (1.81-2.58)		24	2.16 (1.81-2.58)		15	2.10 (1.61-2.75)	
	2	11	2.26 (1.71-3.00)	0.217	11	2.26 (1.71-3.00)	0.323	7	2.23 (1.53-3.25)	0.529
4	1	16	3.13 (2.65-3.70)		16	3.13 (2.65-3.70)		11	3.39 (2.74-4.19)	
	2	5	2.06 (1.65-2.57)	0.143	5	2.06 (1.65-2.57)	0.804	3	2.18 (1.86-2.54)	0.112
5	1	39	1.79 (1.59-2.00)		39	1.79 (1.59-2.00)		17	1.63 (1.40-1.90)	
	2	13	2.10 (1.54-2.85)	0.615	13	2.10 (1.54-2.85)	0.776	6	1.99 (1.02-3.91)	0.984
6A	1	80	5.11 (4.85-5.39)		80	5.11 (4.85-5.39)		35	5.00 (4.58-5.45)	
	2	9	4.29 (3.42-5.38)	0.000	9	4.29 (3.42-5.38)	0.056	2	4.05 (0.47-34.71)	0.002
6B	1	87	3.83 (3.59-4.10)		87	3.83 (3.59-4.10)		41	3.83 (3.48-4.21)	
	2	9	3.41 (2.76-4.22)	0.002	9	3.41 (2.76-4.22)	0.055	5	3.37 (2.24-5.06)	0.010
7A/7F	1	2	2.01 (0.75-5.38)		2	2.01 (0.75-5.38)		1	2.18	
	2	2	2.46 (0.17-36.56)	0.706	2	2.46 (0.17-36.56)	0.966	1	1.99	0.501
9A/9V	1	21	2.94 (2.43-3.57)		21	2.94 (2.43-3.57)		14	2.58 (2.05-3.25)	
	2	0	-	0.099	0	-	0.052	0	-	0.476
14	1	43	3.67 (3.37-4.00)		43	3.67 (3.37-4.00)		20	3.40 (2.97-3.89)	

Serotype/ group	Period	0-60 months			0-24 months			25-60 months		
		n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value	n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value	n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value
	2	8	3.95 (3.52-4.42)	0.060	8	3.95 (3.52-4.42)	0.303	3	3.71 (3.01-4.57)	0.209
18C	1	6	3.64 (2.61-5.08)		6	3.64 (2.61-5.08)		3	3.49 (1.56-7.83)	
	2	0	-	0.565	0	-	-	0	-	0.353
19A	1	59	3.32 (3.11-3.53)		59	3.32 (3.11-3.53)		28	3.05 (2.74-3.39)	
	2	9	3.70 (3.08-4.45)	0.003	9	3.70 (3.08-4.45)	0.023	1	4.45	0.092
19F	1	75	4.85 (4.66-5.05)		75	4.85 (4.66-5.05)		28	4.69 (4.45-4.94)	
	2	46	4.59 (4.41-4.78)	0.039	46	4.59 (4.41-4.78)	0.739	26	4.66 (4.44-4.90)	0.009
23F	1	99	3.82 (3.62-4.03)		99	3.82 (3.62-4.03)		53	3.80 (3.54-4.08)	
	2	6	4.16 (2.98-5.80)	0.005	6	4.16 (2.98-5.80)	0.091	1	5.49	0.014
6D	1	0	-		0	-			-	
	2	0	-	-	0	-	-		-	-
7B/7C/40	1	16	3.52 (2.97-4.18)		7	3.74 (2.72-5.15)		9	3.36 (2.64-4.28)	
	2	5	3.51 (2.74-4.50)	0.726	3	3.72 (2.04-6.77)	0.220	2	3.22 (0.96-10.81)	0.445
8	1	1	4.39		1	4.39		0	-	
	2	3	3.43 (1.52-7.77)	0.230	3	3.43 (1.52-7.77)	0.178	0	-	-
9-like	1	8	1.46 (1.23-1.73)		4	1.38 (1.04-1.83)		4	1.54 (1.05-2.27)	
	2	5	1.41 (1.10-1.81)	0.516	5	1.41 (1.10-1.81)	0.064	0	-	0.148
9L/9N	1	10	3.67 (2.75-4.91)		4	3.25 (1.28-8.24)		6	3.99 (3.02-5.26)	
	2	5	4.42 (2.14-9.12)	0.706	4	4.18 (1.46-11.96)	0.159	1	5.49	0.431
10A	1	11	2.78 (1.93-4.02)		5	2.24 (0.93-5.38)		6	3.34 (2.35-4.75)	
	2	5	3.91 (2.76-5.54)	0.882	3	4.19 (1.94-9.03)	0.831	2	3.52 (0.25-50.59)	0.883
10B	1	3	1.39 (0.86-2.24)		3	1.39 (0.86-2.24)		0	-	
	2	1	1.38	0.189	0	-	0.098	1	1.38	0.666
10C/10F	1	7	2.66 (2.15-3.30)		2	2.87 (0.07-126.70)		5	2.59 (2.07-3.23)	
	2	3	2.65 (1.72-4.07)	0.657	2	2.89 (1.04-8.07)	0.182	1	2.22	0.085
11A/11D	1	28	4.19 (3.83-4.58)		16	4.55 (4.08-5.08)		12	3.75 (3.26-4.31)	
	2	16	4.08 (3.53-4.72)	0.839	7	4.30 (3.65-5.06)	0.394	9	3.92 (3.02-5.08)	0.336
11B/11C	1	3	3.54 (1.58-7.93)		1	4.76		2	3.05 (0.24-37.96)	

Serotype/ group	Period	0-60 months			0-24 months			25-60 months		
		n†	GMD (95% CI) ‡ log10 GE/ml	p-value	n†	GMD (95% CI) ‡ log10 GE/ml	p-value	n†	GMD (95% CI) ‡ log10 GE/ml	p-value
	2	1	2.13	0.466	1	2.13	0.344	0	-	0.868
11F	1	1	2.40		1	2.40		0	-	
	2	0	-	-	0	-	-	0	-	-
12A/12F/44	1	3	3.39 (1.72-6.67)		3	3.39 (1.72-6.67)		0	-	
	2	2	3.77 (0.76-18.70)	0.751	1	4.28	0.881	1	3.33	0.133
12B	1	6	2.99 (2.33-3.84)		4	3.16 (2.12-4.71)		2	2.69 (0.30-24.30)	
	2	2	3.47 (0.61-19.77)	0.874	1	3.98	0.936	1	3.03	0.916
13	1	19	3.03 (2.45-3.75)		12	3.04 (2.23-4.16)		7	3.01 (2.12-4.27)	
	2	13	3.45 (2.71-4.40)	0.757	7	3.47 (2.39-5.04)	0.704	6	3.43 (2.17-5.42)	0.739
15A/15F	1	14	3.56 (3.16-4.01)		8	3.65 (2.93-4.56)		6	3.45 (3.10-3.83)	
	2	16	3.95 (3.46-4.51)	0.008	10	4.00 (3.37-4.75)	0.041	6	3.86 (2.87-5.20)	0.039
15B/15C	1	53	4.13 (3.82-4.48)		25	4.52 (4.10-4.98)		28	3.82 (3.38-4.31)	
	2	10	3.83 (3.29-4.47)	0.017	4	3.37 (2.62-4.35)	0.024	6	4.17 (3.33-5.23)	0.186
15-like	1	69	1.73 (1.60-1.87)		39	1.72 (1.54-1.93)		30	1.73 (1.54-1.93)	
	2	29	1.83 (1.56-2.14)	0.898	13	1.95 (1.50-2.54)	0.550	16	1.74 (1.40-2.15)	0.744
16A	1	4	1.65 (1.20-2.29)		2	1.85 (0.16-21.58)		2	1.48 (1.32-1.65)	
	2	6	1.52 (1.08-2.13)	0.118	3	1.64 (0.57-4.73)	0.266	3	1.40 (0.76-2.59)	0.230
16F	1	45	3.89 (3.57-4.24)		23	4.09 (3.67-4.55)		22	3.69 (3.21-4.25)	
	2	13	3.63 (3.21-4.10)	0.714	5	3.43 (2.81-4.18)	0.272	8	3.76 (3.11-4.56)	0.757
17F	1	28	2.85 (2.30-3.52)		17	3.16 (2.41-4.16)		11	2.42 (1.66-3.52)	
	2	17	2.78 (2.23-3.47)	0.677	10	2.77 (1.96-3.91)	0.590	7	2.80 (1.98-3.96)	0.891
31	1	9	2.65 (1.76-4.00)		6	2.42 (1.30-4.52)		3	3.18 (1.11-9.08)	
	2	6	1.70 (0.87-3.32)	0.068	4	1.74 (0.55-5.52)	0.454	2	1.62 (0.00-644.70)	0.593
32A/32F	1	4	1.58 (1.29-1.95)		3	1.69 (1.64-1.74)		1	1.30	
	2	1	2.67	0.984	0	-	0.908	1	2.67	0.157
33A/33F	1	9	1.84 (1.29-2.62)		6	1.74 (1.12-2.68)		3	2.05 (0.44-9.64)	
	2	3	1.55 (0.41-5.83)	0.424	2	1.14 (0.94-1.38)	0.846	1	2.87	0.814
33B	1	7	1.95 (1.62-2.34)		4	1.98 (1.36-2.87)		3	1.91 (1.18-3.08)	

Serotype/ group	Period	0-60 months			0-24 months			25-60 months		
		n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value	n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value	n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value
	2	8	1.50 (1.28-1.75)	0.947	3	1.60 (0.94-2.70)	0.341	5	1.44 (1.15-1.82)	0.198
33C	1	5	1.52 (1.34-1.72)		1	1.57		4	1.50 (1.26-1.80)	
	2	5	1.59 (1.02-2.50)	0.144	1	1.18	0.753	4	1.72 (0.95-3.10)	0.436
34	1	27	3.14 (2.81-3.52)		13	2.96 (2.52-3.47)		14	3.33 (2.79-3.97)	
	2	14	3.47 (2.97-4.05)	0.305	5	3.97 (3.15-5.01)	0.897	9	3.22 (2.59-4.00)	0.172
35A/35C/42	1	26	2.71 (2.23-3.30)		16	3.18 (2.55-3.96)		10	2.10 (1.46-3.03)	
	2	8	1.85 (1.37-2.48)	0.257	4	1.88 (1.05-3.35)	0.061	4	1.82 (0.97-3.41)	0.315
35B	1	16	3.29 (2.60-4.18)		6	3.17 (2.19-4.58)		10	3.37 (2.34-4.86)	
	2	13	4.12 (3.76-4.51)	0.014	7	4.15 (3.59-4.79)	0.070	6	4.09 (3.46-4.83)	0.368
35F	1	4	2.63 (1.56-4.43)		1	4.28		3	2.23 (1.94-2.57)	
	2	6	2.64 (2.17-3.20)	0.521	3	2.69 (1.32-5.47)	0.614	3	2.59 (2.30-2.91)	0.614
36	1	3	1.92 (1.27-2.90)		0	-		3	1.92 (1.27-2.90)	
	2	3	1.36 (1.21-1.51)	0.605	0	-	-	3	1.36 (1.21-1.51)	0.605
37	1	0	-		0	-		0	-	
	2	0	-	-	0	-	-	0	-	-
41A	1	4	1.21 (0.92-1.60)		1	1.24		3	1.20 (0.71-2.05)	
	2	2	1.63 (0.42-6.26)	0.403	0	-	0.988	2	1.63 (0.42-6.26)	0.436
43	1	8	2.35 (1.97-2.81)		5	2.60 (2.16-3.13)		3	1.98 (1.17-3.36)	
	2	3	2.96 (1.02-8.58)	0.714	1	4.84	0.937	2	2.32 (1.21-4.45)	0.539
45	1	21	1.78 (1.55-2.04)		15	1.70 (1.44-2.02)		6	1.98 (1.46-2.70)	
	2	12	1.81 (1.55-2.11)	0.089	3	1.73 (0.99-3.01)	0.908	9	1.84 (1.50-2.25)	0.021
46	1	6	3.03 (2.38-3.87)		4	3.18 (2.13-4.75)		2	2.76 (0.34-22.25)	
	2	2	3.47 (0.61-19.77)	0.869	1	3.98	0.937	1	3.03	0.908
47A	1	15	1.35 (1.20-1.52)		2	1.28 (1.03-1.61)		13	1.37 (1.19-1.57)	
	2	11	1.90 (1.37-2.62)	0.192	4	1.86 (1.15-3.00)	0.163	7	1.92 (1.12-3.28)	0.396
47F	1	1	1.81		1	1.59		1	1.81	
	2	0	-	0.118	0	-	-	0	-	-
48	1	3	1.68 (1.44-1.96)		0	-		2	1.73 (1.02-2.91)	

Serotype/ group	Period	0-60 months			0-24 months			25-60 months		
		n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value	n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value	n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value
	2	5	1.86 (1.29-2.68)	0.692	3	1.63 (1.09-2.43)	0.248	2	2.27 (0.06-87.79)	0.224

The total number of children 0-60 months in Period-1: N=1135; and Period-2: N=571; children 0-24 months in Period-1: N=616 and Period-2: N=289; and children 25-60 months in Period-1: N=519 and in Period-2: N=282. †n is the number of isolates. ‡GMD is the Geometric Mean Density (95% Confidence Interval). p-values were considered significant if ≤0.01. Density of carriage was determined through quantitative real-time nanofluidic PCR in the Fluidigm.

Supplementary Table 4: Prevalence of bacterial colonisation from Nasopharyngeal Swab samples in Period-1 (2010) and Period-2 (2018) in Sowetan children 0-60 months of age.

	Period-2 % (n); N=571	**Period-1 % (n); N=1135**	**OR (95% CI) p-value**	**aOR (95% CI) P-value**
A. Baumannii	6 (34)	4.6 (52)	1.32(0.85-2.06); p=0.22	0.84(0.49-1.46); p=0.539
B. Holmesii	0.2 (1)	0.4 (4)	0.5(0.06-4.45); p=0.53	0.36(0.03-4.17); p=0.41
B. Parapertussis/bronchiseptica	0.2 (1)	0.2 (2)	0.99(0.09-10.98); p=0.99	0.18(0.02-2.23); p=0.18
H. influenzae	0.7 (4)	1.1 (12)	0.66(0.21-2.06); p=0.47	0.46(0.13-1.66); p=0.24
- H. influenzae-b	0 (0)	0.62 (7)	p=0.06	-
- NTHI	49 (280)	57.1 (648)	0.72(0.59-0.88); p<0.001	0.86(0.65-1.13); p=0.27
K. pneumoniae	14.2 (81)	6.7 (76)	2.3(1.65-3.21); p<0.001	1.69(1.07-2.66); p=0.02
M. catarrhalis	57.4 (328)	63.3 (719)	0.78(0.64-0.96); p=0.02	0.8(0.6-1.06); p=0.11
N. Lactamica	8.9 (51)	6.8 (77)	1.35(0.93-1.95); p=0.11	2.39(1.29-4.43); p=0.006
N. meningitidis	0.5 (3)	0.9 (10)	0.59(0.16-2.17); p=0.43	0.38(0.08-1.76); p=0.22
S. aureus	5.1 (29)	8.8 (100)	0.55(0.36-0.85); p=0.01	0.67(0.38-1.17); p=0.16
S. pneumoniae	49.4 (282)	68.1 (773)	0.46(0.37-0.56); p<0.001	0.66 (0.54-0.88); p=0.004
S. oralis	17.3 (99)	17.8 (202)	0.97(0.74-1.26); p=0.81	0.9(0.63-1.27); p=0.54
S. pyogenes	1.2 (7)	3.3 (37)	0.37(0.16-0.83); p=0.02	0.79(0.28-2.24); p=0.65

The total number of children 0-60 months in Period-1: N=1135; and Period-2: N=571; OR: Odds Ratio; aOR: adjusted Odds Ratio, calculated using logistic regression; adjusted for breastfeeding status, HIV infection, antibiotic usage, co-trimoxazole prophylaxis, and tuberculosis treatment.

Supplementary Table 5: Bacterial carriage density from Nasopharyngeal Swab samples collected from children 0-60 months of age in Period 1 (2010, n=1135) and Period 2 (2018, n=572).

Bacterial species	P*	All Ages			Under 24 months			Over 24 months		
		n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value	n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value	n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value
Acinetobacter baumannii	1	52	1.88 (1.75-2.02)		27	2.16 (1.96-2.38)		25	1.62 (1.50-1.75)	
	2	34	1.53 (1.43-1.64)	0.273	19	1.53 (1.41-1.66)	0.747	15	1.52 (1.34-1.73)	0.048
Bordetella holmesii	1	4	5.49 (3.80-7.94)		2	5.29 (0.60-47.01)		2	5.70 (0.35-92.77)	
	2	1	5.62	0.356	0	-	0.054	1	5.62	0.644
Bordetella parapertussis/bronchiseptica	1	2	3.86 (0.61-24.33)		2	3.86 (0.61-24.33)		0	-	
	2	1	5.34	0.207	0	-	0.040	1	5.34 -	0.666
Haemophilus influenzae	1	12	4.56 (3.77-5.52)		6	5.06 (4.29-5.96)		6	4.11 (2.74-6.17)	
	2	4	4.81 (3.42-6.75)	0.236	1	3.50	0.028	3	5.34 (4.87-5.86)	0.489
Haemophilus influenzae type b	1	7	4.64 (3.79-5.68)		3	5.33 (2.86-9.93)		4	4.18 (3.32-5.26)	
	2	0	-	0.133	0	-	0.402	0	-	0.231
Non-typeable Haemophilus influenzae	1	648	3.07 (2.97-3.18)		332	3.12 (2.96-3.29)		316	3.03 (2.89-3.17)	
	2	280	2.94 (2.79-3.10)	0.133	135	3.09 (2.88-3.32)	0.158	145	2.81 (2.61-3.04)	0.350
Klebsiella pneumoniae	1	76	2.24 (2.06-2.44)		46	2.32 (2.06-2.62)		30	2.13 (1.89-2.41)	
	2	80	2.16 (2.01-2.31)	0.019	38	2.11 (1.89-2.36)	0.075	42	2.20 (2.02-2.40)	0.086
Moraxella catarrhalis	1	719	4.15 (4.05-4.25)		388	4.18 (4.03-4.32)		331	4.12 (3.98-4.25)	
	2	329	4.23 (4.09-4.38)	0.429	174	4.39 (4.20-4.60)	0.720	155	4.06 (3.84-4.29)	0.582
Neisseria lactamica	1	77	2.35 (2.17-2.54)		49	2.31 (2.07-2.57)		28	2.42 (2.16-2.71)	
	2	51	2.21 (2.00-2.43)	0.023	27	2.32 (2.05-2.63)	0.407	24	2.09 (1.78-2.44)	0.005
Neisseria meningitidis	1	10	3.21 (2.79-3.70)		4	3.12 (2.57-3.80)		6	3.27 (2.53-4.23)	
	2	3	2.46 (1.38-4.39)	0.100	3	2.46 (1.38-4.39)	0.624	0	-	0.005
Staphylococcus aureus	1	100	3.15 (2.92-3.40)		67	3.17 (2.89-3.49)		33	3.10 (2.70-3.55)	
	2	29	2.95 (2.55-3.41)	0.156	19	2.94 (2.43-3.56)	0.247	10	2.95 (2.26-3.85)	0.678
Streptococcus pneumoniae	1	773	4.32 (4.23-4.41)		407	4.46 (4.34-4.59)		366	4.17 (4.04-4.30)	

Bacterial species	P*	All Ages			Under 24 months			Over 24 months		
		n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value	n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value	n†	GMD (95% CI) ‡ log_{10} GE/ml	p-value
	2	282	4.50 (4.39-4.61)	0.010	147	4.61 (4.45-4.77)	0.247	135	4.38 (4.23-4.54)	0.025
Streptococcus oralis	1	202	2.52 (2.42-2.61)		101	2.53 (2.38-2.68)		101	2.50 (2.39-2.63)	
	2	99	2.38 (2.26-2.50)	0.401	38	2.29 (2.09-2.51)	0.204	61	2.44 (2.29-2.59)	0.815
Streptococcus pyogenes	1	37	2.26 (1.96-2.60)		13	2.30 (1.81-2.91)		24	2.23 (1.85-2.70)	
	2	7	1.57 (1.17-2.13)	0.542	2	1.90 (0.01-503.00)	0.410	5	1.46 (1.15-1.86)	0.226

*P denotes the study Period. †n is the number of isolates. ‡ GMD is the Geometric Mean Density (95% Confidence Interval). p-values were considered significant if ≤0.01. Density of carriage was determined through quantitative real-time nanofluidic PCR in the Fluidigm.

Supplementary Table 6: Hierarchy of co-colonising pneumococcal serotypes detected in Nasopharyngeal Swab samples collected from Sowetan children 0-60 months-of-age in Period-1 (2010, n=1135) and Period-2 (2018, n=572).

Serotype/ group	P*	Primary Coloniser % (n/N) †	Co-Colonisation % (n/N) †	Second Serotype % (n/N) †	Third Serotype % (n/N) †	≥ Four Serotypes % (n/N) †
VT	1	84.3% (391/464)	15.7% (73/464)	-	-	-
	2	73.6% (78/106)	26.4% (28/106)	-	-	-
NVT	1	70.1% (331/472)	29.9% (141/472)	-	-	-
	2	87.0% (180/207)	13.0% (27/207)	-	-	-
NTSP	1	100% (70/70)	-	-	-	-
	2	100% (23/23)	-	-	-	-
1	1	33.3% (2/6)	66.7% (4/6)	33.3% (2/6)	33.3% (2/6)	-
	2	-	-	-	-	-
3	1	37.5% (9/24)	62.5% (15/24)	45.8% (11/24)	12.5% (3/24)	4.2% (1/24)
	2	54.5% (6/11)	45.5% (5/11)	36.4% (4/11)	-	9.1% (1/11)
4	1	50.0% (8/16)	50.0% (8/16)	31.3% (5/16)	12.5% (2/16)	6.3% (1/16)
	2	40.0% (2/5)	60.0% (3/5)	60.0% (3/5)	-	-
5	1	20.5% (8/39)	79.5% (31/39)	38.5% (15/39)	23.1% (9/39)	17.9% (7/39)
	2	23.1% (3/13)	76.9% (10/13)	38.5% (5/13)	30.8% (4/13)	7.7% (1/13)
6A	1	92.5% (74/80)	7.5% (6/80)	7.5% (6/80)	-	-
	2	77.8% (7/9)	22.2% (2/9)	-	22.2% (2/9)	-
6B	1	65.5% (57/87)	34.5% (30/87)	26.4% (23/87)	6.9% (6/87)	1.1% (1/87)
	2	55.6% (5/9)	44.4% (4/9)	44.4% (4/9)	-	-
7A/7F	1	50.0% (1/2)	50.0% (1/2)	50.0% (1/2)	-	-
	2	-	100% (2/2)	100% (2/2)	-	-
9A/9V	1	42.9% (9/21)	57.1% (12/21)	42.9% (9/21)	14.3% (3/21)	-
	2	-	-	-	-	-
14	1	69.8% (30/43)	30.2% (13/43)	27.9% (12/43)	2.3% (1/43)	-
	2	62.5% (5/8)	37.5% (3/8)	25.0% (2/8)	12.5% (1/8)	-
18C	1	83.3% (5/6)	16.7% (1/6)	16.7% (1/6)	-	-
	2	-	-	-	-	-
19A	1	67.8% (40/59)	32.2% (19/59)	25.4% (15/59)	5.1% (3/59)	1.7% (1/59)
	2	44.4% (4/9)	55.6% (5/9)	22.2% (2/9)	-	33.3% (3/9)
19F	1	92.0% (69/75)	8.0% (6/75)	6.7% (5/75)	1.3% (1/75)	-
	2	93.5% (43/46)	6.5% (3/46)	4.3% (2/46)	-	2.2% (1/46)
23F	1	82.8% (82/99)	17.2% (17/99)	14.1% (14/99)	2.0% (2/99)	1.0% (1/99)
	2	66.7% (4/6)	33.3% (2/6)	33.3% (2/6)	-	-
2	1	-	100% (1/1)	100% (1/1)	-	-
	2	-	100% (3/3)	33.3% (1/3)	33.3% (1/3)	33.3% (1/3)
6C	1	100% (5/5)	-	-	-	-
	2	86.7% (13/15)	13.3% (2/15)	13.3% (2/15)	-	-
7B/7C/40	1	62.5% (10/16)	37.5% (6/16)	31.3% (5/16)	6.3% (1/16)	-
	2	80.0% (4/5)	20.0% (1/5)	20.0% (1/5)	-	-
8	1	-	100% (1/1)	100% (1/1)	-	-
	2	66.7% (2/3)	33.3% (1/3)	33.3% (1/3)	-	-
9like	1	-	100% (8/8)	37.5% (3/8)	50.0% (4/8)	12.5% (1/8)
	2	-	100% (5/5)	40.0% (2/5)	40.0% (2/5)	20.0% (1/5)
9LN	1	50.0% (5/10)	50.0% (5/10)	40.0% (4/10)	10.0% (1/10)	-
	2	80.0% (4/5)	20.0% (1/5)	20.0% (1/5)	-	-
10A	1	72.7% (8/11)	27.3% (3/11)	-	9.1% (1/11)	18.2% (2/11)
	2	80.0% (4/5)	20.0% (1/5)	-	-	20.0% (1/5)
10B	1	-	100% (3/3)	66.7% (2/3)	-	33.3% (1/3)
	2	-	100% (1/1)	-	-	100% (1/1)
10C/10F	1	14.3% (1/7)	85.7% (6/7)	71.4% (5/7)	14.3% (1/7)	-
	2	66.7% (2/3)	33.3% (1/3)	33.3% (1/3)	-	-
11A/10D	1	78.6% (22/28)	21.4% (6/28)	17.9% (5/28)	3.6% (1/28)	-
	2	68.8% (11/16)	31.3% (5/16)	12.5% (2/16)	18.8% (3/16)	-
11B/11C	1	66.7% (2/3)	33.3% (1/3)	-	33.3% (1/3)	-
	2	-	100% (1/1)	-	100% (1/1)	-
11F	1	-	100% (1/1)	-	100% (1/1)	-
	2	-	-	-	-	-
12A/12F/44	1	66.7% (2/3)	33.3% (1/3)	33.3% (1/3)	-	-
	2	50.0% (1/2)	50.0% (1/2)	50.0% (1/2)	-	-
12B	1	-	100% (6/6)	66.7% (4/6)	33.3% (2/6)	-
	2	-	100% (2/2)	50.0% (1/2)	-	50.0% (1/2)
13	1	73.7% (14/19)	26.3% (5/19)	21.1% (4/19)	5.3% (1/19)	-
	2	92.3% (12/13)	7.7% (1/13)	7.7% (1/13)	-	-
15A/15F	1	57.1% (8/14)	42.9% (6/14)	35.7% (5/14)	7.1% (1/14)	-
	2	87.5% (14/16)	12.5% (2/16)	12.5% (2/16)	-	-
15B/15C	1	83.0% (44/53)	17.0% (9/53)	15.1% (8/53)	1.9% (1/53)	-
	2	80.0% (8/10)	20.0% (2/10)	20.0% (2/10)	-	-

Serotype/ group	P*	Primary Coloniser % (n/N) †	Co-Colonisation % (n/N) †	Second Serotype % (n/N) †	Third Serotype % (n/N) †	≥ Four Serotypes % (n/N) †
15like	1	20.3% (14/69)	79.7% (55/69)	46.4% (32/69)	26.1% (18/69)	7.2% (5/69)
	2	37.9% (11/29)	62.1% (18/29)	41.4% (12/29)	6.9% (2/29)	13.8% (4/29)
16A	1	-	100% (4/4)	-	50.0% (2/4)	50.0% (2/4)
	2	-	100% (6/6)	33.3% (2/6)	33.3% (2/6)	33.3% (2/6)
16F	1	77.8% (35/45)	22.2% (10/45)	20.0% (9/45)	2.2% (1/45)	-
	2	92.3% (12/13)	7.7% (1/13)	7.7% (1/13)	-	-
17F	1	50.0% (14/28)	50.0% (14/28)	32.1% (9/28)	14.3% (4/28)	3.6% (1/28)
	2	41.2% (7/17)	58.8% (10/17)	23.5% (4/17)	11.8% (2/17)	23.5% (4/17)
18A	1	66.7% (2/3)	33.3% (1/3)	-	33.3% (1/3)	-
	2	-	-	-	-	-
18B	1	60.0% (3/5)	40.0% (2/5)	40.0% (2/5)	-	-
	2	100% (1/1)	-	-	-	-
19B	1	25.0% (1/4)	75.0% (3/4)	25.0% (1/4)	25.0% (1/4)	25.0% (1/4)
	2	50.0% (2/4)	50.0% (2/4)	25.0% (1/4)	25.0% (1/4)	-
20	1	93.3% (14/15)	6.7% (1/15)	-	6.7% (1/15)	-
	2	75.0% (3/4)	25.0% (1/4)	25.0% (1/4)	-	-
21	1	72.7% (8/11)	27.3% (3/11)	27.3% (3/11)	-	-
	2	46.2% (6/13)	53.8% (7/13)	38.5% (5/13)	7.7% (1/13)	7.7% (1/13)
22A	1	43.8% (7/16)	56.3% (9/16)	37.5% (6/16)	18.8% (3/16)	-
	2	-	100% (2/2)	100% (2/2)	-	-
22F	1	100% (6/6)	-	-	-	-
	2	100% (1/1)	-	-	-	-
23A	1	29.6% (8/27)	70.4% (19/27)	63.0% (17/27)	3.7% (1/27)	3.7% (1/27)
	2	70.0% (7/10)	30.0% (3/10)	30.0% (3/10)	-	-
23B	1	80.0% (16/20)	20.0% (4/20)	20.0% (4/20)	-	-
	2	95.2% (20/21)	4.8% (1/21)	4.8% (1/21)	-	-
24A	1	-	-	-	-	-
	2	-	100% (2/2)	50.0% (1/2)	-	50.0% (1/2)
24B/24F	1	-	-	-	-	-
	2	20.0% (1/5)	80.0% (4/5)	-	40.0% (2/5)	40.0% (2/5)
27	1	33.3% (1/3)	66.7% (2/3)	33.3% (1/3)	33.3% (1/3)	-
	2	-	100% (4/4)	-	25.0% (1/4)	75.0% (3/4)
29	1	-	100% (6/6)	100% (6/6)	-	-
	2	-	100% (8/8)	62.5% (5/8)	12.5% (1/8)	25.0% (2/8)
31	1	66.7% (6/9)	33.3% (3/9)	-	33.3% (3/9)	-
	2	16.7% (1/6)	83.3% (5/6)	-	33.3% (2/6)	50.0% (3/6)
32A/32F	1	-	100% (4/4)	-	25.0% (1/4)	75.0% (3/4)
	2	-	100% (1/1)	-	-	-
33A/33F	1	33.3% (3/9)	66.7% (6/9)	-	-	66.7% (6/9)
	2	-	100% (3/3)	-	33.3% (1/3)	66.7% (2/3)
33B	1	14.3% (1/7)	85.7% (6/7)	57.1% (4/7)	14.3% (1/7)	14.3% (1/7)
	2	25.0% (2/8)	75.0% (6/8)	37.5% (3/8)	12.5% (1/8)	25.0% (2/8)
33C	1	-	100% (5/5)	20.0% (1/5)	20.0% (1/5)	60.0% (3/5)
	2	-	100% (5/5)	20.0% (1/5)	40.0% (2/5)	40.0% (2/5)
34	1	63.0% (17/27)	37.0% (10/27)	37.0% (10/27)	-	-
	2	78.6% (11/14)	21.4% (3/14)	14.3% (2/14)	7.1% (1/14)	-
35A/35C/42	1	46.2% (12/26)	53.8% (14/26)	26.9% (7/26)	15.4% (4/26)	11.5% (3/26)
	2	-	100% (8/8)	50.0% (4/8)	37.5% (3/8)	12.5% (1/8)
35B	1	50.0% (8/16)	50.0% (8/16)	43.8% (7/16)	6.3% (1/16)	-
	2	84.6% (11/13)	15.4% (2/13)	7.7% (1/13)	-	7.7% (1/13)
35F	1	50.0% (2/4)	50.0% (2/4)	25.0% (1/4)	-	25.0% (1/4)
	2	66.7% (4/6)	33.3% (2/6)	33.3% (2/6)	-	-
36	1	-	100% (3/3)	33.3% (1/3)	66.7% (2/3)	-
	2	-	100% (3/3)	33.3% (1/3)	-	66.7% (2/3)
41A	1	-	100% (4/4)	-	25.0% (1/4)	75.0% (3/4)
	2	50.0% (1/2)	50.0% (1/2)	-	-	50.0% (1/2)
43	1	25.0% (2/8)	75.0% (6/8)	62.5% (5/8)	-	12.5% (1/8)
	2	66.7% (2/3)	33.3% (1/3)	33.3% (1/3)	-	-
45	1	19.0% (4/21)	81.0% (17/21)	52.4% (11/21)	19.0% (4/21)	9.5% (2/21)
	2	-	100% (12/12)	41.7% (5/12)	25.0% (3/12)	33.3% (4/12)
46	1	50.0% (3/6)	50.0% (3/6)	16.7% (1/6)	16.7% (1/6)	16.7% (1/6)
	2	-	100% (2/2)	-	100% (2/2)	-
47A	1	6.7% (1/15)	93.3% (14/15)	40.0% (6/15)	26.7% (4/15)	26.7% (4/15)
	2	18.2% (2/11)	81.8% (9/11)	36.4% (4/11)	18.2% (2/11)	27.3% (3/11)
47F	1	-	100% (1/1)	100% (1/1)	-	-
	2	-	-	-	-	-
48	1	-	100% (3/3)	-	-	100% (3/3)
	2	-	100% (5/5)	20.0% (1/5)	20.0% (1/5)	60.0% (3/5)

*P denotes the study Period. †n is the number of isolates in each category for each period and N is the total isolates identified as each serotype/group. The rank was determined according to colonization density.

Supplementary Table 7: Multiple concurrent bacterial colonisers detected in Nasopharyngeal Swab samples collected from Sowetan children 0-60 months-of-age in Period-1 (2010, n=1135) and Period-2 (2018, n=572).

Bacteria	p	Primary Coloniser % (n/N) †	Co-Colonisation % (n/N) †	Second Serotype % (n/N) †	Third Serotype % (n/N) †	≥ Four Serotypes % (n/N) †
A. baumannii	1	9.6% (5/52)	90.4% (47/52)	9.6% (5/52)	28.8% (15/52)	51.9% (27/52)
	2	11.8% (4/34)	88.2% (30/34)	29.4% (10/34)	11.8% (4/34)	47.1% (16/34)
S. pneumoniae	1	58.2% (450/773)	41.8% (323/773)	33.1% (256/773)	7.5% (58/773)	1.2% (9/773)
	2	61.3% (173/282)	38.7% (109/282)	33.7% (95/282)	4.6% (13/282)	0.4% (1/282)
B. holmesii	1	50.0% (2/4)	50.0% (2/4)	25.0% (1/4)	25.0% (1/4)	
	2	100% (1/1)				
B. parapertussis/ bronchiseptica	1		100% (2/2)	50.0% (1/2)	50.0% (1/2)	
	2	100% (1/1)				
H. influenzae	1	41.7% (5/12)	58.3% (7/12)	41.7% (5/12)	16.7% (2/12)	
	2	25.0% (1/4)	75.0% (3/4)	75.0% (3/4)		
-H. influenzae. type b	1	28.6% (2/7)	71.4% (5/7)	71.4% (5/7)		
	2					
-NTHI	1	21.6% (140/648)	78.4% (508/648)	36.7% (238/648)	35.8% (232/648)	5.9% (38/648)
	2	23.5% (66/281)	76.5% (215/281)	36.7% (103/281)	32.7% (92/281)	7.1% (20/281)
K. pneumoniae	1	15.8% (12/76)	84.2% (64/76)	25.0% (19/76)	34.2% (26/76)	25.0% (19/76)
	2	22.2% (18/81)	77.8% (63/81)	30.9% (25/81)	21.0% (17/81)	25.9% (21/81)
M. catarrhalis	1	52.2% (375/719)	47.8% (344/719)	33.5% (241/719)	12.1% (87/719)	2.2% (16/719)
	2	61.1% (201/329)	38.9% (128/329)	27.7% (91/329)	10.3% (34/329)	0.9% (3/329)
N. lactamica	1	3.9% (3/77)	96.1% (74/77)	13.0% (10/77)	40.3% (31/77)	42.9% (33/77)
	2	5.9% (3/51)	94.1% (48/51)	15.7% (8/51)	35.3% (18/51)	43.1% (22/51)
N. meningitidis	1	10.0% (1/10)	90.0% (9/10)	20.0% (2/10)	60.0% (6/10)	10.0% (1/10)
	2		100% (3/3)		66.7% (2/3)	33.3% (1/3)
S. aureus	1	30.0% (30/100)	70.0% (70/100)	30.0% (30/100)	24.0% (24/100)	16.0% (16/100)
	2	24.1% (7/29)	75.9% (22/29)	27.6% (8/29)	24.1% (7/29)	24.1% (7/29)
S. oralis	1	17.3% (35/202)	82.7% (167/202)	31.2% (63/202)	23.3% (47/202)	28.2% (57/202)
	2	21.2% (21/99)	78.8% (78/99)	26.3% (26/99)	23.2% (23/99)	29.3% (29/99)
S. pyrogenes	1	10.8% (4/37)	89.2% (33/37)	8.1% (3/37)	45.9% (17/37)	35.1% (13/37)
	2		100% (7/7)	28.6% (2/7)	28.6% (2/7)	42.9% (3/7)

*P denotes the study Period. †n is the number of isolates in each category for each period and N is the total isolates identified as each serotype/group. The rank was determined according to colonization density. NTHI is non-typeable *Haemophilus influenzae*

Chapter 6: Integrated Discussion

This study reports on an optimized and validated, comprehensive high throughput nanofluidic qPCR method able to detect and quantify 92 pneumococcal serotypes together with 15 other nasopharyngeal bacterial species. The method showed good concordance and discrimination compared with the gold standard Quellung method for serotyping pneumococcus, with the ability to detect additional serotypes, often at lower bacterial densities. In previous studies, the nanofluidic qPCR system was compared to standard real-time qPCR, achieving a sensitivity exceeding 92% and a specificity greater than 99% for a subset (48) of the assay sets used here [211]. The qPCR method also successfully differentiated difficult-to-distinguish PCV13-VT serotypes using modified primers and probes and shows potential for further assay development for higher valency PCVs, including PCV15 and PCV20, where additional assays will be needed to distinguish serotypes 11A, 12F, 15B, and 33F from their respective serogroups.

A limitation of real-time PCR, is that non-pneumococcal mitis-group *Streptococcus* can acquire capsule genes from pneumococci, which can lead to false-positive results due to cross-reactivity and non-specific binding [409]. Nevertheless, the nanofluidic qPCR panel included serogroup assay sets that were previously validated against non-pneumococcal *Streptococcus* species [166]. Furthermore, algorithms were designed to assign serotypes to a sample if it was positive for both the serogroup assay sets and two pan-pneumococcal reference genes. The collective specificity of pan-pneumococcal reference genes lyt*A* and pia*B* has been demonstrated (99.5%) when evaluated in 150 pneumococci, 433 non-pneumococci, and 240 polymicrobial samples [207, 371, 409], and thus the detection of non-pneumococcal species was unlikely.

The detection of short pneumococcal DNA targets by PCR translates to an inability to differentiate between viable, transmissible bacteria and non-viable, non-transmissible nucleic acid fragments after living bacteria have been cleared from the nasophrynx.

However, PCR's high sensitivity still provides valuable epidemiological data, particularly in assessing circulating VT or serotype replacement by NVT. The PCR developed in this study assessed nasopharyngeal colonization and cannot be used for diagnosis of pneumococcal disease (mucosal or invasive). Quantitative PCR can mitigate the detection of non-viable bacteria, as bacterial density that correlates with culturable isolates or disease isolates could be used to discern active colonization or infection with further research and validation [193, 259, 410].

The study evaluated the impact of PCV vaccination 8-9 years after routine PCV immunization [59, 113] in children under 5 years of age living in both rural (Agincourt, Mpumalanga) and urban (Soweto, Gauteng) settings of South Africa [59, 113]. The overall prevalence of pneumococcus was lower in both Agincourt (35% reduction) and Soweto (34% reduction), which was driven by a lower prevalence of PCV13-VT (84% and 59% reduction, respectively). Nevertheless, there was a high residual prevalence of PCV13-VT detected in both setting (14.3-18.6%), despite >90% of children aged over 40 weeks receiving three doses of PCV. These reports are similar to other African countries who have also reported a high residual colonization of PCV13 VT (pooled estimate: 20%; 95% CI: 17-23), 2-7 years post-PCV implementation).

A high residual PCV13 VT after PCV immunization may be a result of incomplete ‘herd immunity’ and high baseline colonization prior to PCV introduction. A Kenyan household transmission model estimated that it would take >more than 10 years for the full indirect benefit of PCV to actualize in high colonization settings, including many LMIC such as South Africa [73]. Furthermore, in settings with high VT colonization prior to the introduction of PCV, the decay in VT prevalence is more protracted than in lower colonization settings [141]. While children under 5 years of age account for most transmission, unvaccinated older children and adults may still act as reservoirs of PCV13-VT if indirect benefit of routine PCV immunization is incomplete [39, 73, 82].

Nevertheless, there was a 4.3% higher residual colonization of PCV13-VT in children living in Soweto compared with Agincourt. In part, the lower measured impact on PCV13-VT in Soweto may be explained by the one-year difference in the baseline timepoint compared with Agincourt. In May 2010–February 2011 in Soweto, PCV7 had been implemented routinely for 12-22 months, whereas in Agincourt, baseline sampling (May to October 2009) occurred during the initial six months of PCV7 rollout. The reported (WUENIC) coverage for three doses of PCV in South Africa was 10% in 2009, and 58% in 2010 [153], and thus PCV had likely reduced the VT colonization in Soweto prior to sampling in 2010/11 and may explain the lower relative reduction detected in Soweto in 2018. This is corroborated by findings from Nzenze *et al*. (2013), that reported a 50% reduction in PCV7-VT colonization in children aged under 2 years of age by May-October of 2011 relative to 2009 in Soweto [113]. The early impact of PCV7 is consistent with the lower PCV13-VT prevalence detected in 2010/11 in Soweto (40.9%) compared with children enrolled in 2009 in Agincourt (51.0%; $p<0.001$).

Despite there being a higher proportion of children sleeping with >2 people in the same room (32% vs. 22%) and of children aged ≤24 months in Soweto compared with Agincourt (51% vs. 34%), differences did not account for the higher residual colonization of PCV13-VT in Soweto. The higher PCV13-VT colonization in Soweto may also be due to environmental factors or other secular trends that influence colonization prevalence, such as air pollution, that differ between urban and rural locales [411, 412], but not characterized in this study.

While there were large reductions in PCV13-VT, serotypes 19F and 6B remained prevalent in Agincourt in 2017/18 (5.3% and 4.8%, respectively) whereas 19F increased (8.1% vs 6.6%) in Soweto in 2018 relative to 2010/11. Serotype 19F accounted for 37-44% of residual PCV13-VT in 2017-2018 in Agincourt and Soweto. High 19F colonization after routine PCV implementation has been demonstrated globally in children under 5 years of age using culture-based detection methods in both HIC and

LMIC (2.8-8.3%) [224, 225, 403, 413-415]. Residual 19F colonization may be due to: i) sub-optimum immune responses where 19F acquisition precedes, or coincides with PCV-immunization (hyporesponsiveness) [416]; ii) 19F colonization duration exceeds other VT, increasing both transmission and sampling opportunity [73, 329, 336]; iii) higher antibody concentrations are correlated with protection against 19F acquisition [417, 418]; iv) 19F polysaccharide-protein conjugation by reductive amination (PCV7/13) elicits additional antigenic determinants allochthonous to circulating 19F, reducing functional responses (opsonophagocytic antibodies) against 19F [419].

It is plausible that PCV13 does not adequately protect against 19F acquisition. A clinical trial undertaken in South African that aimed to investigate reduced PCV dosing schedules reported higher anti-19F IgG in children receiving a single priming dose of PCV13 administered at 14 weeks followed by a booster dose at 40 week of age compared with two priming doses (6, 14 and 40 weeks) as well as a 2.89-fold lower prevalence of 19F colonization at 6 months post-booster dose [420, 421], that may be due to reduced hyporesponsiveness to 19F. In addition, African settings that utilize PCV10 where 19F polysaccharide-protein is conjugated through cyanylation, have demonstrated reductions in 19F colonization [226, 228, 422-424]. Lastly, a systematic review identified 19F as the predominant serotype associated with vaccine failure [425] and 19F is persistent in IPD in South Africa [154]. These findings warrant further investigation into the genomic and immunogenic determinants of persistent colonization (and risk of disease) of 19F.

In Agincourt there was a 3-fold higher increase in the prevalence of NVT in 2017/18 compared with 2009, which was driven by an increase in serotypes 16F, 21, 15A/F, 35B, and non-typeable pneumococcus. In contrast, the prevalence of NVT remained similar in 2018 compared with 2010/11 in Soweto, despite an increase in serotypes 6C, 15A/F and 23B reported in 2018. While serotypes 6C, 15A/F, 23B, 16F, and 35B have lower invasive potential compared with 19A as a reference [426], serotypes 16F, 23B, and 15A were the predominant NVT involved in IPD in all age groups in South Africa in 2009-

2018 [154]. In addition to contributing to residual IPD in 2017/18, 16F was also the most prevalent serotype carried in Agincourt in 2017 and predominant in IPD, compared with 2009 where 16F was not prevalent in colonization or IPD [154, 155]. Serotype 16F is not targeted by licensed PCVs, including those in trial phase (e.g.: PCV24) [427], whereas prevalent 16F lineages identified from South Africa were associated with disease and resistance to penicillin and cotrimoxazole [428]. The estimated pooled prevalence of NVT across African colonization surveys conducted 2-7 years post-PCV-introduction was 47% (95% CI: 41-54) but ranged between 31-74%. Nevertheless, the reported difference in NVT epidemiology between Agincourt and Soweto, and the pooled estimate is not unusual, as serotype replacement has been heterogenous in different settings globally [389].

In Agincourt and Soweto, the cumulative colonization prevalence of serotypes covered by SII-PCV10 (14.5-17.5%), PCV15 (16.3-20.7%) and PCV20 (23.6-26.5%) in 2017-2018 indicate that PCVs that are affordable and sustainable (i.e.: SII-PCV10) [158], or that interrupt the circulation of a higher proportion of invasive serotypes (PCV15/20), could be considered as alternative vaccines in South Africa. The SII-PCV10 will be introduced in South Africa in 2024 [429], and contains PCV13-VT excluding serotypes 3, 4 and 18C (Table 1.1). A further benefit of SII-PCV10 is the conjugation of 19F to CRM_{197} using cyanylation [430], which may translate to improved functional antibody responses to 19F [419]. It will be important to monitor the circulation of serotypes 3, 4, 18C and 19F following the programmatic change to SII-PCV10, and the 2017-2018 colonization surveys may serve as a baseline for comparison.

Across the study periods, *S. pneumoniae*, *M. catarrhalis* and non-typeable *H. influenzae* remained the most prevalent colonizing bacteria at both study sites and were found to be positively associated with each other in multiple concurrent colonization, that is consistent with previously described bacterial dynamics [40, 275, 283-288, 297, 303]. In Agincourt, a 50-fold increase in the prevalence of *Acinetobacter baumannii* and a 22-fold

increase in the prevalence of *Klebsiella pneumoniae* were detected in 2017/18 compared to 2009. The relevance of the increased prevalence of colonization by *A. baumanii* and *K. pneumoniae* in relation to risk of disease is unknown and thus further characterization of these bacteria is needed to determine the antimicrobial susceptibility profile and disease risk of lineages involved in colonization.

In conclusion, this study demonstrated that nanofluidic PCR is both sensitive and specific for the detection of 92 pneumococcal serotypes and 15 bacterial species. The routine use of PCV13 achieving three-dose coverage >90% has resulted in a 59-80% reduction in PCV13-VT colonization in one rural and one urban setting in South Africa, although PCV13-VT persist in colonization (14.3-18.6%). Colonization surveys are useful as an early warning system for the circulation of serotypes known to have invasive potential [(431)] and the continued surveillance of VT: 3, 4, 18C and 19F; and NVT: 16F will be important.

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Appendix 1: Ethical Clearance

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R49 Ms SL Downs

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M220488

NAME: (Principal Investigator)	Ms SL Downs
DEPARTMENT:	School of Pathology Wits VIDA - Vaccines and Infectious Diseases Analytics Research Unit Medical School University
PROJECT TITLE:	*Temporal changes in Streptococus pneumoniae colonization in children and adults following routine childhood immunization with pneumococcal conjugate vaccine in South Africa*
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Includes two additional objectives, as specified in applicant's letter of 2022/04/17 Renewal of M17/03/14 - expired on 2022/05/02
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Professors SA Madhi and M Nunes; Dr CP Olwagen
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2022/05/17

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

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 submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **2022/04/01** and therefore reports and re-certification will be due in the month of **2022/04/01** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

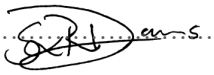
Signature of Principal Investigator

18-Mar-22
Date

Appendix 2: Agreement for Published Papers Submitted for Examination Purpose

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Sarah Downs, student number 1668863, declare that this Thesis/Dissertation/Research Report is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis/Dissertation/Research Report.

Signature of Student ……  ……**Date**……26-March-2024……

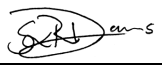
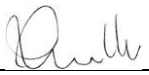
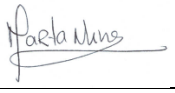
Name of Primary Supervisor……Prof Shabir A. Madhi……

Signature of Primary Supervisor ……  ……**Date**……27-March-2024……

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his/her Thesis/Dissertation/Research Report. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

Article 1: Title: High-throughput nanofluidic real-time PCR to discriminate Pneumococcal Conjugate Vaccine (PCV)-associated serogroups 6, 18, and 22 to serotypes using modified oligonucleotides

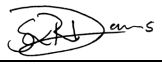
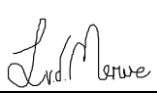
Journal name, year, volume and page numbers: Scientific Reports (11): 1; 23728; 2021/12/09

Authors	Name	Signature	Date
1st author	Downs, S. L.		26-March-2024
2nd author	Madhi, S. A.		27-March-2024
3rd author	Van der Merwe, L.		26-March-2024
4th author	Nunes, M. C.		26-March-2024
5th author	Olwagen, C. P.		26-March-2024

Comments by primary supervisor: N/A

Article 2: **Title:** Optimization of a high-throughput nanofluidic real-time PCR to detect and quantify 15 bacterial species and 92 Streptococcus pneumoniae serotypes

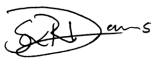
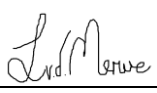
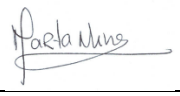
Journal name, year, volume and page numbers: Scientific Reports (13): 1; 4588; 2023/03/21.

Authors	Name	Signature	Date
1st author	Downs, S. L.		26-March-2024
2nd author	Madhi, S. A.		27-March-2024
3rd author	Van der Merwe, L.		26-March-2024
4th author	Nunes, M. C.		26-March-2024
5th author	Olwagen, C. P.		26-March-2024

Comments by primary supervisor: N/A

Article 3: **Title**: Streptococcus pneumoniae and other bacterial nasopharyngeal colonization seven years post-introduction of 13-valent pneumococcal conjugate vaccine in South African children

Journal name, year, volume and page numbers: International Journal of Infectious Diseases (134): 1; 45-52; 2023.

Authors	Name	Signature	Date
1st author	Downs, Sarah L.		26-March-2024
2nd author	Olwagen, Courtney P.		26-March-2024
3rd author	Van Der Merwe, Lara		26-March-2024
4th author	Nzenze, Susan A.		26-March-2024
5th author	Nunes, Marta C.		26-March-2024
6th author	Madhi, Shabir A.		27-March-2024

Comments by primary supervisor: N/A

Appendix 3: Originality Report

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ORIGINALITY REPORT

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