Nasopharyngeal colonization dynamics with *Streptococcus pneumoniae* and associated antimicrobial resistance in a South African birth cohort

By

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TO MY FAMILY AND FRIENDS, WITH LOVE.

Philippians 3:7-9.

But whatever were gains to me I now consider loss for the sake of Christ. What is more, I consider everything a loss because of the surpassing worth of knowing Christ Jesus my Lord, for whose sake I have lost all things. I consider them garbage that I may gain Christ and be found in him, not having a righteousness of my own that comes from the law, but that which is through faith in Christ – the righteousness that comes from God on the basis of faith.

Declaration

I, **Rendani Innocent Manenzhe**, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. The research described here was carried out under the supervision of **Dr Clinton Moodley**, **Prof Mark Nicol** and **Dr Felix Dube**.

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Publication 1:

Manenzhe RI, Dube FS, Wright M, Lennard K, Mounaud S, Lo SW, Zar HJ, Nierman WC, Nicol MP, Moodley C. A novel approach to studying pneumococcal nasopharyngeal colonization dynamics and antimicrobial resistance using shotgun metagenomic sequencing. (2019) (Submitted, in review).

Publication 2:

Manenzhe RI, Moodley C, Abdulgader SM, Robberts FJL, Zar HJ, Nicol MP and Dube FS. (2019) Nasopharyngeal Carriage of Antimicrobial-Resistant Pneumococci in an Intensively Sampled South African Birth Cohort. *Front. Microbiol.* 10:610.

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ABSTRACT

Introduction: Nasopharyngeal (NP) colonization by *Streptococcus pneumoniae* (the pneumococcus) precedes the development of respiratory tract infection. Colonization by antimicrobial-resistant pneumococci, especially in infants, is a major public health concern as pneumococcus is a frequent cause of bacterial acute respiratory tract infections among children. This study longitudinally investigated antimicrobial resistance amongst pneumococci colonizing the nasopharynx of South African infants immunized with the 13-valent pneumococcal conjugate vaccine (PCV13). Furthermore, the study explored strain-level pneumococcal colonization patterns and associated antimicrobial resistance determinants as well as the composition of the NP antibiotic resistome using shotgun metagenomic sequencing.

Methods: NP swabs were collected every second week from birth through the first year of life from 137 infants who were immunized with 2+1 doses of PCV13. These were the first 137 infants enrolled in the cohort who had the most complete fortnightly NP sampling (defined as at least 23-26 fortnightly collected NP swabs). Pneumococci were identified and serotyped using conventional techniques, and their antibiotic susceptibility profiles determined by disc diffusion and E-test. A subset of 196 NP samples from 23 infants were selected based on changes in serotype or antimicrobial resistance. These were subjected to broth enrichment, total nucleic acid extraction and subsequent shotgun metagenomic sequencing. Sequence reads were assembled and aligned to reference pneumococcal genomes. *In-silico* pneumococcal capsular, multilocus sequence typing, and antimicrobial resistance determinants were described. Finally, antibiotic resistance genes were identified from all bacterial contigs, to determine the NP resistome.

Results: 1520 pneumococcal (760 non-duplicate) isolates were recovered from 137 infants; including non-typeable (n = 99), PCV13 (n = 133), and non-PCV13 serotypes (n = 528). The prevalence of penicillin, erythromycin, and cotrimoxazole non-susceptibility was 19% (147/760; 95% CI 17-22%) (3% resistant), 18% (136/760; 95% CI 15-21%) (14% resistant) and 45% (344/760; 95% CI 42-49%) (36% resistant), respectively. The predominant

penicillin-non-susceptible serotypes included 15B/15C (n = 20), 19A (n = 13), 15A (n = 10), 19F (n = 8), and 21 (n = 8). Multi-drug resistance (MDR) was observed in 9% (68/760; 95% CI 7-11%) of the isolates. PCV13 serotypes were more likely to be non-susceptible, compared to non-PCV13 serotypes, to penicillin (26% *vs*. 16%, $p = 0.007$), erythromycin (23% *vs*. 15%, $p = 0.027$) and cotrimoxazole (62% *vs*. 41%, $p < 0.001$). Non-susceptibility to penicillin, erythromycin, and cotrimoxazole remained relatively constant through the first year of life (X^2 test for trend: $p = 0.184$, range 0 – 25%; $p = 0.171$, range 0 – 27%; and $p = 0.572$, range 0 – 55%, respectively). Overall, penicillin or erythromycin-non-susceptible pneumococci were carried for a shorter duration than susceptible pneumococci (penicillin [mean days, 18 *vs*. 21, $p = 0.013$] and erythromycin [mean days, 18 *vs*. 21, $p = 0.035$]). Forty-five percentage (61/137) of infants carried the same serotype which acquired or lost resistance over time, and these changes were predominantly for penicillin (76%, 79/104).

Of the 196 NP samples sequenced, 174 had corresponding positive cultures for pneumococci and, of these, 152 were assigned an *in-silico* serotype. Metagenomic sequencing detected a single pneumococcal serotype in 85% (129/152), and co-colonization in 15% (23/152) of NP samples, respectively. In total, 22 different pneumococcal serotypes were identified, with 15B/15C (n = 49) and 16F (n = 21) being the most common non-PCV13 serotypes, while 23F (n = 9) and 19A (n = 8) were the most common PCV13 serotypes. Twenty-six different sequence types (STs), including 4 novel STs were identified. Mutations in the *fol*A and *fol*P genes, associated with cotrimoxazole resistance, were detected in 89% (87/98) of cotrimoxazole-non-susceptible pneumococci and mutations in the *pbp*1a and *pbp*2x genes, known to confer beta-lactam resistance, were identified in penicillin non-susceptible $ST7052^{15B/15C}$ isolates. A total of 329 antimicrobial resistance (AMR) genes were detected in 64% (125/196) of the sequenced samples, including 36 non-redundant genes ranging from 1 to 14 genes per sample. The predominant AMR genes detected were those conferring resistance to beta-lactams (52%, 172/329), macrolide-lincosamide-streptogramin (17%, 56/329), and tetracycline (12%, 38/329). The *msr*D, *erm*B, and *mef*A genes were only

detected from pneumococcal reads. The predominant resistance genes detected from non-pneumococcal reads included *bla*$_{\text{OXA-60}}$, *bla*$_{\text{OXA-22}}$, and *bla*$_{\text{BRO-1}}$.

Conclusion: NP carriage of antibiotic-non-susceptible pneumococci was relatively constant throughout the first year of life. Despite high vaccine coverage levels, PCV13 serotypes were identified and were more commonly non-susceptible to penicillin, erythromycin, and cotrimoxazole. Overall, penicillin or erythromycin-non-susceptible pneumococci were carried for a shorter duration than susceptible pneumococci, however, non-susceptible PCV13 serotypes were carried for a longer duration than non-susceptible non-PCV13 serotypes. Direct shotgun sequencing from enriched NP samples was shown to be a powerful technique for a detailed description of the pneumococcal component of the NP microbiome and resistome, and its use should be explored similarly for other bacteria in this niche.

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Scientific contributions

Peer-reviewed articles

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Manenzhe RI, Moodley C, Abdulgader SM, Robberts FJL, Zar HJ, Nicol MP and Dube FS. (2019) Nasopharyngeal Carriage of Antimicrobial-Resistant Pneumococci in an Intensively Sampled South African Birth Cohort. *Front. Microbiol.* 10:610.

Peer-reviewed conference proceedings

Manenzhe, R.I; Moodley, C; Dube, F.S; Wright, M; Zar, H.J; Nierman, W.C; Nicol, M.P. Shotgun sequencing to elucidate pneumococcal strain level nasopharyngeal colonization patterns and antimicrobial resistance in a South African birth cohort. Abstract oral presentation at the Molecular Cell Biology Research Day, 13 September 2018, Cape Town, South Africa.

Manenzhe, R.I; Moodley, C; Dube, F.S; Wright, M; Zar, H.J; Nierman, W.C; Nicol, M.P. Shotgun sequencing to elucidate pneumococcal strain level nasopharyngeal colonization patterns and antimicrobial resistance in a South African birth cohort. Abstract oral presentation at the 11th International Symposium on Pneumococci and Pneumococcal Diseases (ISPPD), 15 - 19 April 2018, Melbourne, Australia.

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presentation at the 11th Human Heredity and Health in Africa (H3Africa) Consortium, 21 - 25 March 2018, Entebbe, Uganda.

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Manenzhe, R.I; Moodley, C; Dube, F.S; Wright, M; Zar, H.J; Nierman, W.C; Nicol, M.P. Characterizing antibiotic-resistant pneumococci in the nasopharynx of South African infants using shotgun sequencing and conventional typing. Abstract oral presentation at the University of Cape Town, Child and Adolescent Health Research Days, 31 October – 1 November 2017, Red Cross War Memorial Children's Hospital, Cape Town.

Manenzhe, R.I; Moodley, C; Dube, F.S; Wright, M; Zar, H.J; Nierman, W.C; Nicol, M.P. Characterizing antibiotic-resistant pneumococci in the nasopharynx of South African infants using shotgun sequencing and conventional typing. Abstract oral presentation at the University of Cape Town's Faculty of Health Sciences Research Day, 27 September 2017.

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CHAPTER 1

General introduction and thesis outline

1.1 General introduction

Streptococcus pneumoniae (the pneumococcus) is a commensal that also causes diseases.[1] Pneumococcus resides in the human nasopharynx where it can be carried asymptomatically and transmitted from person to person.[2] It is a Gram-positive, alpha-haemolytic facultative anaerobic bacterium,[3] first identified simultaneously and independently by George Sternberg and Louis Pasteur in 1881.[1] Pneumococcus is optochin-sensitive which generally distinguishes it from viridans streptococci which are also alpha-haemolytic; it can also be differentiated from other related species based on its sensitivity to lysis by bile using the bile solubility test.[4] Pneumococci are classified by serotype, which is defined by an antigenically distinct polysaccharide capsule. The capsule biosynthesis is encoded by the capsular polysaccharide (*cps*) locus within the pneumococcal genome. Due to high levels of genetic diversity within the *cps* locus, there have been more than 100 pneumococcal serotypes described thus far.[5,6]

Nasopharyngeal (NP) carriage of antimicrobial-resistant pneumococci is more common in children than the elderly.[7] Colonization by resistant pneumococcus, especially in children, is a significant problem worldwide, since NP carriage of pneumococci precedes the development of disease.[2] Pneumococcus is a frequent cause of bacterial acute respiratory tract infections which are the leading cause of mortality among children.[8] Penicillin has been the most commonly used drug in treating pneumococcal diseases, however, its widespread use has resulted in the development of penicillin-resistant pneumococci.[9] This has led to the introduction of beta-lactams with extended spectrum of activity and other classes of antibiotics for the treatment of pneumococcal diseases; and due in part to high antibiotic selective pressure, pneumococci with resistance to various classes of antibiotics have emerged worldwide.[10]

The introduction of the pneumococcal conjugate vaccines (PCVs), which only include a subset of serotypes, has successfully reduced the carriage and attributable disease caused by vaccine serotypes worldwide.[11] This has however led to an increase in the carriage and

levels of disease caused by serotypes not targeted by the PCVs, a phenomenon known as serotype replacement.[11,12] PCVs have significantly reduced overall antibiotic resistance among pneumococci by targeting serotypes included in the vaccine which are commonly resistant to various antibiotics.[13,14] However, there has been an increase in resistance among serotypes not included in the vaccine.[15] NP colonization with antibiotic-resistant pneumococci is therefore concerning as this may lead to the development of pneumococcal diseases which are hard to treat with first-line antibiotics.[16]

NP colonization by antibiotic-resistant pneumococci among children has continuously increased over the years.[17] Despite this, there are limited data in the currently available literature on the NP carriage of resistant pneumococci among infants in early life and the prevalence of resistance is likely to be underestimated especially in low income community settings where there are limited resources and poor active surveillance.[18] Additionally, there are no longitudinal cohort studies describing antimicrobial resistance among pneumococci in early life; therefore, there is a need for community-based studies to better understand the resistance patterns among pneumococci circulating in the community.

1.2 Thesis outline

This study describes the NP carriage of pneumococci including antimicrobial-resistant pneumococci among PCV13-vaccinated children participating in a South African birth cohort study. In addition, the study describes the use of a novel approach to studying pneumococcal NP colonization dynamics and antimicrobial resistance, using shotgun metagenomic sequencing. Furthermore, this study investigated the composition of the NP antibiotic resistome in *Streptococcus*-enriched samples in a South African longitudinal birth cohort.

Chapter 2: Asymptomatic NP colonization is an important step for the development of pneumococcal diseases and also a reservoir for person-to-person transmission of antibiotic-resistant pneumococci circulating in the community. To better understand the global

distribution and emergence of antibiotic-non-susceptible pneumococci, a review of the literature was conducted, reporting on the proportion as well as the mechanisms of resistance in pneumococci. This chapter presents a systematic review describing the global distribution of antibiotic-non-susceptible pneumococci colonizing the nasopharynx of apparently healthy children ≤12 years of age. This chapter summarizes the data on the proportions of antibiotic-non-susceptible pneumococci, with focus on the most commonly used antibiotics for treatment of pneumococcal infections. Chapter 2 also describes the identified limitations of the currently available data and highlights areas which need further research.

Chapter 3: Pneumococcus is an important bacterial cause of pneumonia among children, therefore, NP colonization with antimicrobial-resistant pneumococci is of major public health concern because pneumococcal diseases are preceded by NP colonization. To better understand pneumococcal resistance patterns, among carriage isolates, we used culture-based methods to investigate antimicrobial resistance amongst pneumococci colonizing the nasopharynx of 137 vaccinated South African infants during the first year of life. This chapter describes the penicillin, erythromycin, and cotrimoxazole resistance patterns among pneumococci, as well as the trends of resistance over time.

Chapter 4: Pneumococci are most commonly identified using bacterial culture followed by both phenotypic and genotypic characterization of the isolates. Individuals may be colonized by multiple different serotypes at the same time, but currently used methods are unable to provide a detailed characterization of strain-level pneumococcal colonization. We used shotgun metagenomic sequencing, a novel approach, to study pneumococcal NP colonization dynamics and antimicrobial resistance determinants. This chapter describes the pneumococcal component of the NP microbiota, including colonization with multiple pneumococcal serotypes and genetic resistance determinants.

Chapter 5: The nasopharynx is a reservoir for many respiratory pathogens including pneumococcus, and may also serve as a source for the transfer of antimicrobial resistance

genes from non-pathogenic to pathogenic bacteria. Culture-based methods are unable to comprehensively characterize the antibiotic resistome of the NP microbiota. As a continuation of chapter 4, we investigated the composition of the NP antibiotic resistome, using shotgun metagenomic sequencing. This chapter therefore describes the NP antibiotic resistome (pneumococcal and non-pneumococcal resistomes).

Chapter 6: This section presents the general conclusions of the investigations conducted in this thesis. This chapter summarizes the key findings from these studies and also highlights future prospects.

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CHAPTER 2

Global distribution of antibiotic-resistant pneumococci in the nasopharynx of apparently healthy children: a systematic review

Summary

Antimicrobial resistance in *Streptococcus pneumoniae* (the pneumococcus) is a growing public health concern. Nasopharyngeal (NP) colonization with antibiotic-non-susceptible pneumococcus (ANSP) is therefore of importance, since the NP is the major reservoir for transmission of pneumococci and facilitates exchange of antibiotic resistance genes. This systematic review summarizes data on the global distribution of antimicrobial resistance in pneumococci colonizing the nasopharynx of healthy children. A literature search was conducted in PubMed, Scopus, Web of Science, and EBSCOhost to identify articles reporting on ANSP in the nasopharynx of apparently healthy children ≤12 years old. We only included articles published in English up to 30 April 2018. Ninety studies were included and analysed. Most of these studies were conducted in Europe (41%, 37/90), followed by Asia (31%, 28/90), Africa (12%, 11/90), South America (8%, 7/90), North America (6%, 5/90), and Australia/Oceania (2%, 2/90). Non-susceptibility to penicillin and cotrimoxazole were high in studies from Asia and Africa. Cefotaxime-non-susceptibility was uncommon in most countries except in South Korea and China. Levofloxacin non-susceptibility was very rare. Few studies (n = 9) reported the genetic resistance determinants in pneumococci, and *erm*B was the most commonly described gene in those studies. Although there are limited data on the carriage of resistant pneumococci in children from developing countries, this review showed that resistant pneumococci have a worldwide distribution. There is a need to conduct further studies to monitor the trends of resistance and this should be coupled with the description of the genetic determinants of resistance associated with phenotypic resistance.

2.1 Introduction

Streptococcus pneumoniae (the pneumococcus) is a major cause of morbidity and mortality in young children and adults worldwide. Those at the extremes of age are particularly vulnerable to life-threatening invasive diseases including pneumonia, sepsis and meningitis.[1–4] The treatment of pneumococcal disease has traditionally relied on beta-lactams and macrolides, however, resistance to these agents is increasing globally.[5,6] Increasingly, antibiotic resistance poses a threat to the effective treatment of pneumococcal infections. Emerging antimicrobial resistance is exacerbated by excessive use of antimicrobial therapy in primary care, particularly in children.[7,8]

Asymptomatic NP colonization by pneumococci precedes invasive disease and is a reservoir for community transmission of drug-resistant pneumococci.[9,10] To better understand the global distribution and potential for emergence of antibiotic-non-susceptible pneumococcus (ANSP) infections, we conducted a review of the currently available literature, reporting on the proportion and/or underlying mechanisms of resistance in pneumococci carried in the NP of apparently healthy children.

2.2 Methods

2.2.1 Eligibility criteria

Peer-reviewed articles reporting on antibiotic susceptibility profiles among pneumococci in the nasopharynx of children ≤12 years, published in English prior to 30 April 2018, were considered. Studies that enrolled children who were reported to be healthy, or had no reported underlying illness at the time of enrolment were included. NP samples included specimens obtained from the nasopharynx, with a sterile swab or by NP aspiration. Studies where there was no clear differentiation of illness, sample type, age, or antibiotic susceptibility results were excluded. Reviews, clinical trials and intervention studies were also excluded. References from reviews and included studies were not checked for eligibility.

A literature search was conducted in PubMed, Scopus, Web of Science and EBSCOhost for published articles using the keywords in Table 2.1. The search strategy was designed to include all studies reporting on any antibiotic-non-susceptible bacteria colonizing the nasopharynx of children. There was no limit on publication year.

Table 2.1: Keywords search

Database	Search period	Search strategy
PubMed EBSCOhost * ISIweb of Science	Until 30 April 2018	(neonat* OR infant* OR child* OR baby OR babies) AND (nasopharyn*) AND (bacteria* OR bacterium OR pathogen* OR microb*) AND (antibiotic* OR antimicrobial* OR drug*) AND (resistome OR resistan* OR non-susceptib* OR nonsusceptib* OR non susceptib*) NOT (fungi OR fungus OR fungal OR virus OR viral OR parasite OR parasitic OR parasites)
Scopus	Until 30 April 2018	(neonat* OR infant* OR child* OR baby OR babies) AND (nasopharyn*) AND (bacteria* OR bacterium OR pathogen* OR microb*) AND (antibiotic* OR antimicrobial* OR drug*) AND (resistome OR resistan* OR non-susceptib* OR nonsusceptib*)

*(Academic Search Premier, Africa-Wide Information, and CINAHL)

2.2.2 Data analysis

Studies meeting the inclusion criteria were reviewed by two independent reviewers, data were extracted and summarized (the student conducted the systematic review and was responsible for data analyses and write-up, however, a second independent reviewer was required to go through the same process of selecting articles using the defined criteria thus avoiding biases). The following information was extracted: the year and country in which the study was conducted, age group of the participants, methods used to determine antibiotic susceptibility profiles, categorical antibiotic susceptibility data, and antibiotic resistance genes. The proportion of ANSP was calculated based on the total number of isolates tested in each of the eligible studies. Penicillin, cefotaxime, erythromycin, tetracycline, cotrimoxazole, ciprofloxacin, levofloxacin, and chloramphenicol susceptibility data were extracted. Non-meningitis breakpoints were considered in studies that used both non-

meningitis and meningitis breakpoints for interpretation of penicillin and cefotaxime susceptibilities. Resistant and intermediate isolates were collectively regarded as non-susceptible. Multi-drug resistance (MDR) was defined as non-susceptibility to three or more classes of antibiotics.

2.3 Results

2.3.1 Study selection

A total of 2133 articles were identified from the four databases: PubMed (n = 91), Scopus (n = 372), Web of Science (n = 732) and EBSCOhost (n = 938). After removing duplicates, the titles and abstracts of 2072 articles were screened for inclusion. Of 2072 articles screened, 1680 did not meet the specified inclusion criteria and were subsequently excluded. Of the remaining 392 full-text articles assessed for eligibility, 90 full-text articles were included in this review. A PRISMA flow diagram of studies reviewed is shown in Figure 2.1.

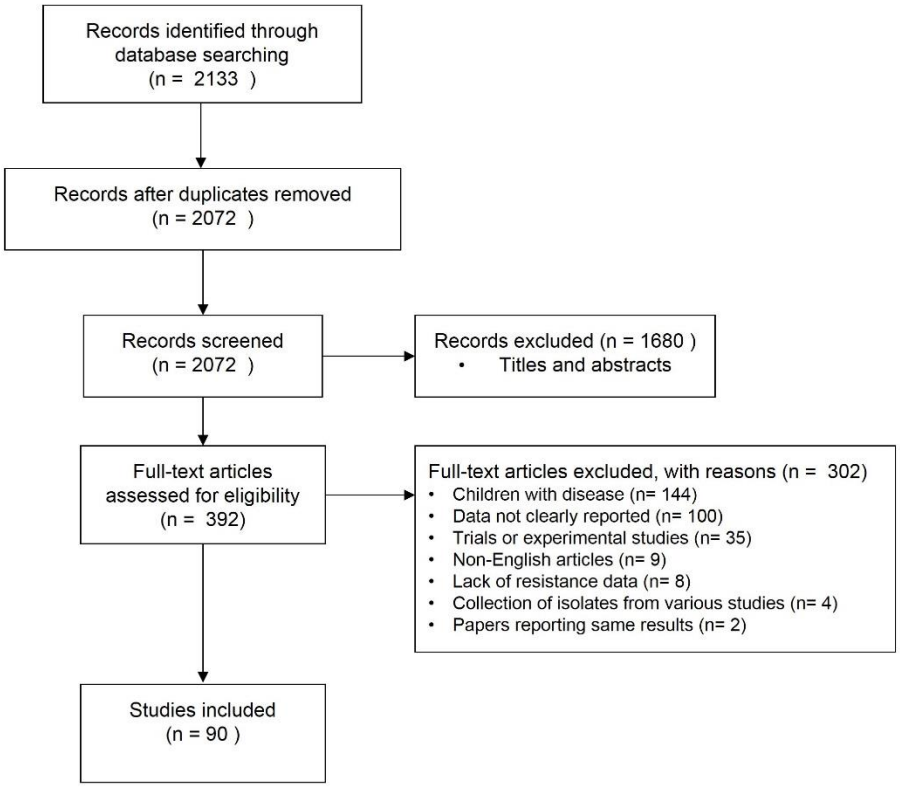

Figure 2.1: Flow diagram showing selection of studies reviewed according to Preferred Reporting Items for Systematic Reviews and Meta-Analysis.

2.3.2 Study characteristics

A summary of articles included is shown in Tables 2.2, 2.3 and 2.4. Ninety studies (80 cross-sectional and 10 repeated cross-sectional studies) conducted in 44 countries were included. The majority of the studies originated from Europe (41%, 37/90), followed by Asia (31%, 28/90), Africa (12%, 11/90), South America (8%, 7/90), North America (6%, 5/90), and Australia/Oceania (2%, 2/90). All 90 studies were conducted between 1990 and 2015: 30 studies (1990-1999), 38 studies (2000-2009), and 18 studies (2010-2015). The year of study was not reported in four studies.

The age group of participants included ranged from 0-120 months (Tables 2.2 and 2.3). The extracted data could not be stratified according to age since age grouping was reported as overlapping age ranges. Sample collection procedures were described in 94% (85/90) of the studies. NP samples were obtained using sterile swabs (n = 82), NP aspiration (n = 2), or using both techniques (n = 1) (data not shown). The methods used to determine antimicrobial susceptibility patterns were reported in 99% (89/90) of the studies, with E-test being the most commonly used (71%, 63/89). E-test was used alone in 18% (16/89) of the studies, and in combination with disc diffusion and agar dilution in 52% (46/89) and 1% (1/89) of the studies, respectively. Susceptibility breakpoints used for interpretation of the results were described in 94% (85/90) of the studies; Clinical Laboratory Standards Institute (CLSI) breakpoints were most commonly used (87%, 74/85) (data not shown).

2.3.3 Distribution of ANSP

The proportion of ANSP isolated from the NP of healthy children reported from each study is shown in Tables 2.2 and 2.3. The distribution of the overall proportion of penicillin, erythromycin, and cotrimoxazole-non-susceptible pneumococci by country is shown in Figures 2.2, 2.3, and 2.4, respectively.

2.3.3.1 Non-susceptibility to beta-lactams

The proportion of penicillin-non-susceptible *Streptococcus pneumoniae* (PNSP) differed substantially between countries and regions. The proportions of PNSP exceeded 80% in studies in Kenya, Uganda, South Korea, and Taiwan, but were lower than 2% in Norway (Tables 2.2 and 2.3). Three South Korean studies in 2008 (92%),[11] 2009-2010 (95%),[12] and 2010 (91%),[13] reported the highest proportions of PNSP. Low proportions of PNSP were mainly reported in India (Table 2.2), ranging from 0% in 1994-1995,[14] to 17% in 2010-2013.[15] No PNSP were reported in two studies in Indonesia in 1997,[16] and in England in 2003.[17] Cefotaxime-non-susceptible pneumococci were uncommon, with proportions less than 10% reported mainly in European and African countries (Table 2.2 and 2.3). However, the proportions of cefotaxime-non-susceptible pneumococci were high in China in 1999-2000 (43%),[18] and Korea in 2014 (31%).[19]

Repeated cross-sectional studies from 8 countries reported varying changes in penicillin or cefotaxime non-susceptibility over time (Table 2.3). There was an increase in the proportions of PNSP in studies conducted in Greece (12% in 2000 to 29% in 2003,[20] and 20% in 2005 to 35% in 2009),[21] Singapore (28% in 1998, to 70% in 2007-2008),[22] and Israel (33% in 1993 to 77% in 1994).[23] In other studies, the change in the proportions of PNSP over time were minimal including in Iceland (11% in 2009-2012 to 10% in 2013-2015),[24] Norway (1.7% in 2006 to 1.9% in 2008),[25] and Portugal (24% in 2001 to 22% in 2006).[26] Only one study, conducted in the USA, reported a notable decrease in the proportions of PNSP (44% in 2006-2010 to 32% in 2010-2015) and cefotaxime-non-susceptible pneumococci over time (16% in 2006-2010 to 7% in 2010-2015).[27]

2.3.3.2 Non-susceptibility to macrolides

The highest proportions of erythromycin-non-susceptible pneumococci were described in studies in China, South Korea, and Taiwan (Table 2.2). Four studies in China reported high proportions of erythromycin-non-susceptible pneumococci: 1997-1998 (99%),[28] 1999-2000 (77%),[18] 2012 (51%),[29] and 2013-2014 (80%), respectively.[30] Three South Korean studies conducted in 2008 (85%),[11] 2009-2010 (93%),[12] and 2014 (91%),[19] as well as one study in

Taiwan (87%),[31] also reported high erythromycin-non-susceptible pneumococci. High proportions of erythromycin-non-susceptibility were also reported in non-Asian countries including in Italy (64% in 1995-1996,[32] 61% in 1996-1997,[33] and 60% in 1997),[34] Portugal (69% in 1997-2003),[35] Belgium (62% in 2000-2001),[36] and Morocco (62% in 2007-2009).[37] Three studies in Africa, one from Lesotho,[38] and two from Uganda,[39,40] did not detect any erythromycin-non-susceptible pneumococci. Repeated cross-sectional studies in Portugal (26% in 2001 to 24% in 2006),[26] Norway (6% in 2006 to 3% in 2008),[25] the USA (30% in 2006-2010 to 16% in 2010-2015),[27] and Iceland (13% in 2009-2012 to 9% in 2013-2015),[24] showed a decrease in the proportions of erythromycin non-susceptibility over time.

2.3.3.3 Non-susceptibility to cotrimoxazole

Studies in Asia, Africa, and South America reported the highest overall proportions of cotrimoxazole non-susceptible pneumococci (Figure 2.4). Four studies in Kenya in 2009-2010 (99%),[41] Uganda in 1995 and 2008 (85%,[39] and 99%,[40] respectively), and Tanzania in 2010 (83%),[42] reported the highest proportions of cotrimoxazole-non-susceptibility on the African continent (Figure 2.4). Non-susceptibility to cotrimoxazole was also common in Asia with proportions over 65% reported in India, Iran, South Korea, Taiwan, and China (Table 2.2). Studies in India recorded high proportions of cotrimoxazole-non-susceptible pneumococci: 81% in 1998-1999,[43] 82% in 2000-2002,[44] 53% in 2004,[45] 91% in 2010-2011,[15] and 78% in 2012-2013.[46] Repeated cross-sectional studies reported a notable drop in the proportions of cotrimoxazole-non-susceptibility over time: in Portugal (26% in 2001 to 12% in 2006),[26] Norway (5% in 2006 to 2% in 2008),[25] the USA (27% in 2006-2010 to 13% in 2010-2015),[27] and Iceland (22% in 2009-2012 to 12% in 2013-2015).[24]

2.3.3.4 Non-susceptibility to fluoroquinolones

Reports on ciprofloxacin or levofloxacin susceptibility were uncommon. Among ten studies that reported ciprofloxacin susceptibility, five studies in Bulgaria (in 1999),[47] China (in 1999-2000),[18] Russia (in 2001-2002),[48] Greece (in 2005-2009),[49] and Turkey (year not specified),[50]

reported the proportion of ciprofloxacin non-susceptibility of less than 2%. Alarming proportions of ciprofloxacin-non-susceptible pneumococci were reported in Morocco (71%),[37] and Greece (51%),[51] in 2007-2009 and 2010-2012, respectively. Non-susceptibility to levofloxacin was rare; no levofloxacin-non-susceptible pneumococci were detected in 18 out of 25 studies that reported results (Table 2.2). Seven studies in Singapore (2% in 2007-2008),[22] Senegal (2% in 2007-2008),[52] South Korea (1% in 2009-2010),[12] Greece (1% in 2010-2012),[51] India (2% in 2012-2013),[46] and Egypt (4% in 2013-2014),[53] and South Korea (1% in 2014),[19] reported low proportions of levofloxacin-non-susceptible pneumococci.

2.3.3.5 Non-susceptibility to other antibiotics

Similar to cotrimoxazole-non-susceptibility, high proportions of non-susceptibility to tetracycline of over 65% were reported in studies conducted in Taiwan (97%),[31] China (99% in 1997-1998),[28] and South Korea (89% in 2009-2010,[12] and 79% in 2014).[19] Chloramphenicol susceptibility was not determined in many studies included in this review (Tables 2.2 and 2.3). However, the proportions of chloramphenicol-non-susceptible pneumococci exceeding 30% were only reported in Italy (34% in 1997),[34] China (34% in 1999-2000),[18] Taiwan (42%),[31] Morocco (38% in 2007-2009),[37] and South Korea (38% in 2009-2010).[12]

2.3.3.6 Multi-drug resistant pneumococci

Data on the proportions of MDR were available in 52 of the 90 studies included in this review; three studies in South Korea recorded the highest proportions of MDR (62% in 2008,[11] 92% in 2009-2010,[12] and 82% in 2014).[19] High proportions of MDR were also reported in other Asian countries including in China in 1999-2000 (39%),[18] Singapore in 2007-2008 (75%),[22] India in 2012-2013 (51%),[46] and Taiwan (87%).[31] Studies in Africa and southern Europe also reported high proportions of MDR: in Italy in 1996-1997 and 1997 (45%,[33] and 53%,[34] respectively), Turkey 2007-2008 (50%),[54] Morocco 2007-2009 (43%),[37]

and Egypt in 2013-2014 (41%).[53] MDR proportions were low in Norway in 2006 and 2008 (3%,[55] and 1%,[25] respectively).

2.3.4 Antimicrobial resistance determinants

Ten percent (9/90) of the studies reported testing for genetic resistance determinants using targeted PCR (Table 2.4). The *erm*B gene was the most commonly detected macrolide-resistance gene among macrolide-non-susceptible pneumococci, followed by *mef*A and *mef*E genes (Table 2.4). In an Italian study, pneumococci carrying the *erm*B gene were resistant to both erythromycin and clindamycin whereas those harbouring *mef*A had low level (intermediate) of resistance to erythromycin.[56] Another Italian study showed that all 82 tetracycline-resistant pneumococcal isolates carried the *tet*M gene.[33]

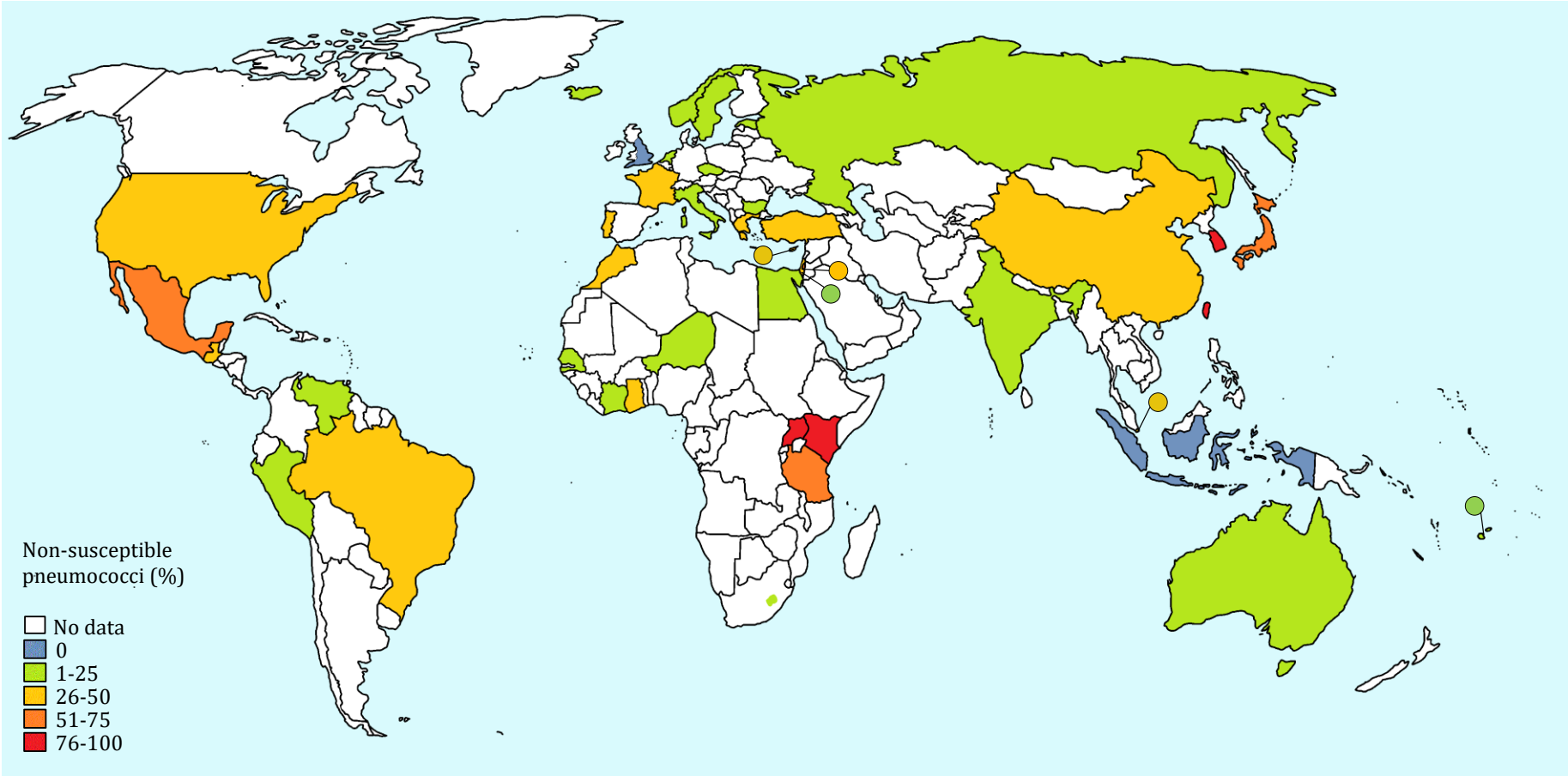

Figure 2.2. Distribution of the overall proportion of penicillin-non-susceptible pneumococci (%), by country, in the NP of apparently healthy children younger than 12 years of age (included eligible studies until 30 April 2018). Note: the map does not represent national surveillance data and does not take into account the temporal variation in antibiotic-resistant pneumococci.

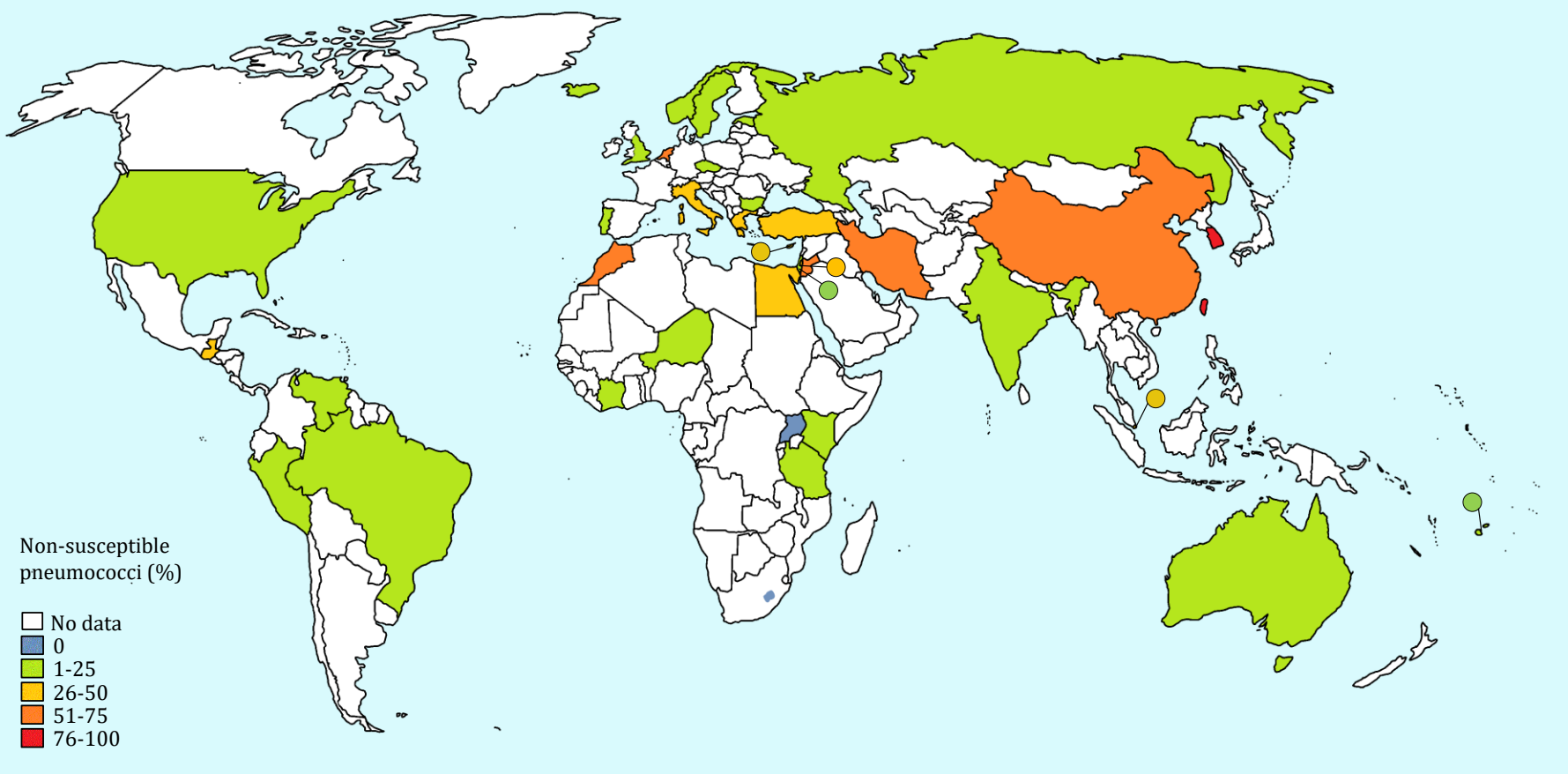

Figure 2.3. Distribution of the overall proportion of erythromycin-non-susceptible pneumococci (%), by country, in the NP of apparently healthy children younger than 12 years of age (included eligible studies until 30 April 2018). Note: the map does not represent national surveillance data and does not take into account the temporal variation in antibiotic-resistant pneumococci.

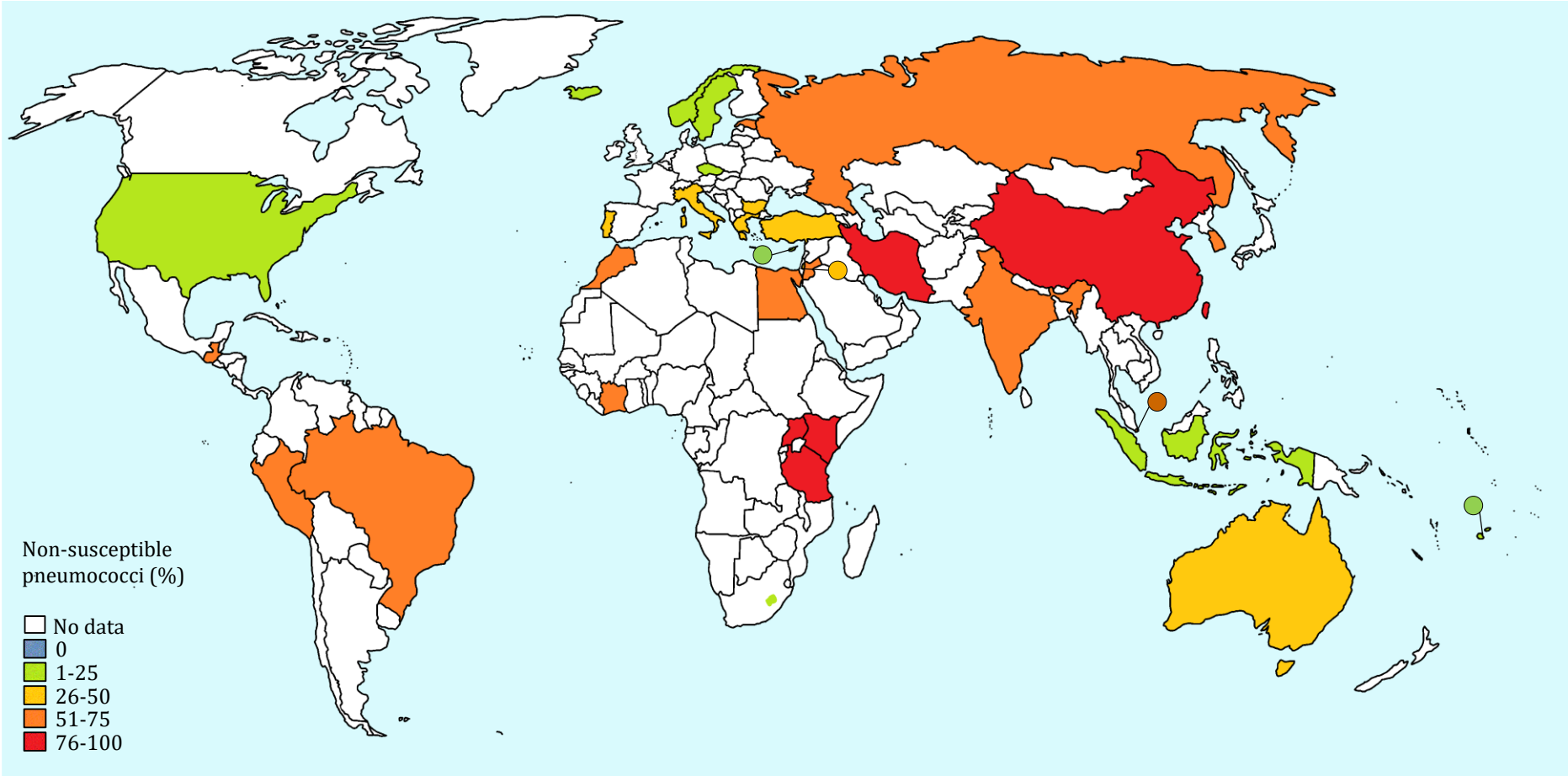

Figure 2.4. Distribution of the overall proportion of cotrimoxazole-non-susceptible pneumococci (%), by country, in the NP of apparently healthy children younger than 12 years of age (included eligible studies until 30 April 2018). Note: the map does not represent national surveillance data and does not take into account the temporal variation in antibiotic-resistant pneumococci.

Table 2.2. Characteristics of eligible cross-sectional studies reporting ANSP.

Country [Ref]	Year of study	Age group (months)	AST methods	MDR	Antibiotic, no. of non-susceptible isolates (% of non-susceptible isolates)							
					PEN	CTX	ERY	TET	SXT	CIP	LVX	CHL
Africa												
Côte d'Ivoire[57]	1997-98	3-60	E-test/DD	13 (9)	11 (8)	3 (2)	16 (12)	79 (57)	72 (52)	-	-	4 (3)
Egypt[53]	2013-14	2-60	Broth/DD	41 (41)	15 (15)	1 (1)	43 (43)	55 (55)	62 (62)	-	4 (4)	0
Ghana[58]	2011	≤72	E-test/DD	-	13 (46)	-	-	-	-	-	-	-
Kenya[41]	2009, 2010	<60	Broth	105 (16)	515 (82)	-	8 (1)	122 (19)	619 (99)	-	0	12 (2)
Lesotho[38]	1995-96	<60	E-test/DD	-	11 (4)	-	0	6 (2)	17 (6)	-	-	2 (1)
Morocco[37]	2007-09	1-24	E-test/DD	65 (43)	58 (39)	11 (7)	93 (62)	75 (50)	86 (57)	106 (71)	0	57 (38)
Niger[59]	2007-08	0-24	E-test/DD	-	23 (11)	-	9 (4)	148 (71)	-	-		24 (11)
Senegal[52]	2007-08	0-24	DD/Agar	-	20 (15)	1 (1)	-	-	-	32 (24)	3 (2)	-
Tanzania[42]	2010	<60	E-test/DD	19 (17)	78 (68)	-	7 (6)	12 (10)	95 (83)	-	-	4 (4)
Uganda[39]	1995	0-36	DD/Agar	32 (28)	96 (84)	0	0	37 (32)	98 (85)	-	-	12 (10)
Uganda[40]	2008	2-59	E-test/DD	-	72 (81)	-	0	-	88 (99)	-	-	-
Asia												
China[28]	1997-98	<60	E-test/Agar	-	42 (40)	-	103 (99)	103 (99)	90 (87)	-	-	30 (29)
China[18]	1999-00	24-72	E-test/DD	151 (39)	223 (58)	164 (43)	295 (77)	-	309 (81)	0	0	129 (34)
China[29]	2012	12-18	E-test	-	6 (6)	-	52 (51)	-	-	-	0	-
China[30]	2013-14	2-18	Broth	8 (10)	6 (7)	11 (13)	67 (80)	-	-	-	-	-
India[60]	-	3-36	E-test/DD	2 (15)	2 (15)	0	1 (8)	-	13 (100)	2 (15)	-	-
India[14]	1994-95	0-18	DD	-	0	-	5 (3)	-	11 (5)	-	-	13 (6)
India[43]	1998-99	2-6	DD	3 (1)	11 (3)	-	120 (37)	-	262 (81)	-	-	-
India[44]	2000-02	60-120	DD	194 (15)	42 (3)	-	140 (11)	782 (61)	1053 (82)	-	-	137 (11)
India[45]	2004	72-120	-	-	43 (6)	35 (6)	5 (1)	230 (30)	413 (53)	-	-	12 (2)
India[15]	2010-11	3-60	E-test/DD	10 (19)	9 (17)	2 (4)	5 (10)	19 (36)	48 (916)	5 (9)	-	-
India[46]	2012-13	6-50	E-test/DD	23 (51)	5 (11)	-	22 (49)	19 (42)	35 (78)	-	1 (2)	6 (13)
Indonesia[16]	1997	0-25	E-test/DD	-	0	-	-	-	20 (9)	-	-	13 (6)
Iran[61]	2013-14	6-72	DD	-	-	0	42 (57)	45 (61)	69 (93)	-	0	12 (16)
Israel[62]	1996-97	12-35	E-test/DD	65 (11)	256 (43)	-	-	-	-	-	-	-
Japan[63]	1999	1-60	E-test/DD	-	57 (61)	-	-	-	-	-	-	-
Jordan[64]	2012-13	1-60	E-test/DD	-	-	-	31 (59)	-	36 (68)	-	0	0

Table 2.2. Continued.

Country [Ref]	Year of study	Age group (months)	AST methods	MDR	Antibiotic, no. of non-susceptible isolates (% of non-susceptible isolates)							
					PEN	CTX	ERY	TET	SXT	CIP	LVX	CHL
South Korea[11]	2008	18-59	Broth	58 (62	86 (92)	-	80 (85)	-	-	-	-	-
South Korea[12]	2009-10	<60	E-test	139 (92)	144 (95)	24 (16)	140 (93)	134 (89)	98 (65)	-	1 (1)	57 (38)
South Korea[13]	2010	6-60	Broth	-	122 (91)	-	-	-	-	-	-	-
South Korea[19]	2014	6-71	E-test	181 (82)	191 (86)	68 (31)	201 (91)	176 (79)	108 (49)	-	2 (1)	66 (30)
Palestine[65]	2012-13	1-23	E-test/DD	72 (34)	141 (67)	0	64 (30)	-	97 (46)	-	-	
Russia[48]	-	<84	E-test	6 (2)	23 (8)	-	14 (5)	-	163 (53)	-	-	-
Russia[66]	2001-02	<60	Broth	195 (10)	397 (19)	18 (1)	143 (7)	1070 (52)	1327 (65)	35 (2)	0	-
Taiwan[31]	-	48-78	Broth	27 (87)	27 (87)	2 (7)	27 (87)	30 (97)	28 (90)	-	0	13 (42)
Europe												
Bulgaria[47]	1999	12-72	DD	19 (22)	19 (22)	0	18 (21)	32 (38)	36 (42)	0	-	9 (11)
Belgium[36]	2000-01	3-36	E-test/DD	-	14 (14)	-	61 (62)	48 (49)	-	-	-	-
Czech Republic[67]	2004-05	36-72	Broth	-	5 (3)	0	2 (1)	9 (6)	26 (16)	-	-	9 (6)
Cyprus[68]	2013-14	6-36	E-test/DD	37 (13)	78 (28)	10 (4)	79 (28)	41 (15)	68 (24)	-	-	0
England[17]	2003	6-60	DD	-	0	-	21 (185)	9 (8)	-	-	-	-
Estonia[69]	1999-00	24-84	DD	-	16 (9)	-	7 (4)	62 (34)	130 (71)	-	-	-
Estonia[70]	1999-00,03	12-84	E-test/DD	14 (4)	18 (6)	-	18 (6)	-	200 (67)	-	-	-
France[71]	2006-09	6-24	Agar	-	85 (32)	-	-	-	-	-	-	-
Greece[72]	1995-96	12-73	E-test/DD	41 (31)	38 (29)	16 (12)	25 (19)	39 (29)	58 (43)	-	-	36 (27)
Greece[73]	1997	8-74	E-test/DD	15 (11)	13 (10)	-	18 (14)	15 (11)	30 (23)	-	-	11 (8)
Greece[74]	1997-98	2-23	E-test/DD	-	70 (17)	24 (6)	79 (19)	72 (17)	76 (18)	-	-	52 (12)
Greece [75]	1997-99	2-23	E-test/DD	110 (14)	122 (16)	-	137 (18)	136 (17)	151 (19)	-	-	86 (11)
Greece[76]	2000-04	30-54	E-test	-	20 (12)	0	32 (19)	-	12 (7)	-	-	-
Greece[49]	2005-09	13-76	E-test/DD	-	229 (87)	-	263 (24)	158 (60)	174 (66)	0	0	42 (16)
Greece[51]	2010-12	21-78	E-test	32 (22)	43 (29)	0	42 (28)	3 (2)	45 (30)	76 (51)	1 (1)	0
Iceland[77]	1999	14-76	E-test/DD	-	30 (7)	-	25 (6)	62 (15)	181 (45)	-	-	31 (8)
Iceland[78]	2009	14-76	E-test/DD	-	58 (15)	-	-	-	-	-	-	-
Italy[32]	1995,96	8-36	DD	-	11 (21)	3 (6)	34 (64)	17 (32.1)	34 (64)	-	-	2 (4)
Italy[33]	1996-97	-	E-test/DD	77 (45)	22 (13)		106 (61)	82 (47.4)	-	-	-	-
Italy[34]	1997	36-60	E-test/DD	64 (53)	17 (14)	1 (1)	73 (60)	66 (54.5)	89 (73)	-	-	41 (34)

Table 2.2. Continued.

Country [Ref]	Year of study	Age group (months)	AST methods	MDR	Antibiotic, no. of non-susceptible isolates (% of non-susceptible isolates)							
					PEN	CTX	ERY	TET	SXT	CIP	LVX	CHL
Italy[56]	1999	2-65	E-test	-	16 (19)	1 (1)	44 (52)	41 (48)	43 (51)	-	-	4 (5)
Italy[79]	2011	3-59	E-test	58 (17)	106 (31)	-	122 (36)	100 (29)	50 (15)	-	0	6 (2)
Italy[80]	2011-12	0-60	E-test	-	56 (30)	-	78 (42)	68 (37)	-	-	-	3 (2)
Norway[55]	2006	10-69	E-test	14 (3)	9 (2)	-	30 (6)	28 (6)	67 (13)	-	-	-
Portugal[81]	1996	6-72	E-test/DD	-	66 (24)	-	46 (17)	61 (22)	92 (33)	-	-	22 (8)
Portugal[35]	1997-03	-	E-test/DD	-	177 (83)	-	146 (69)	141 (66)	155 (73)	-	-	0
Portugal[82]	2007	6-72	E-test/DD	-	48 (16)	2 (1)	48 (16)	35 (12)	31 (10)	-	0	4 (1)
Sweden[83]	2004-05	13-24	DD	-	12 (4)	-	13 (4)	16 (5)	29 (10)	-	-	-
Turkey[50]	-	72-120	Agar	-	24 (25)	26 (27)	40 (42)	39 (41)	51 (53)	1 (1)	-	-
Turkey[84]	2004	0-67	E-test/DD	20 (18)	44 (39)	-	18 (16)	18 (16)	51 (46)	-	-	
Turkey[85]	2004-05	12-72	DD	-	7 (28)	-	1 (4)	2 (8)	5 (20)	-	-	-
Turkey[54]	2007-08	12-59	E-test/DD	15 (50)	26 (87)	-	12 (40)	10 (33)	19 (63)	-	0	4 (13)
Turkey[86]	2012	48-72	E-test/DD	-	3 (50)	-	-	-	-	-	-	-
North America												
Guatemala[87]	2001-06	5-60	E-test	113 (20)	149 (27)	18 (3)	155 (28)	-	347 (63)	-	-	-
Mexico[88]	2002	2-72	Broth	-	528 (64)	-	-	-	-	-	-	-
USA[89]	1998	≤96	E-test	19 (14)	35 (25)	-	19 (14)	-	39 (28)	-	-	-
USA[90]	1998-99	≤60	E-test/DD	-	3 (6)	-	2 (4)	0	9 (19)	-	-	-
South America												
Brazil[91]	2005	2-59	E-test/DD	170 (25)	178 (26)	-	9 (6)	15 (11)	116 (82)	-	0	20 (14)
Brazil[92]	2008	3-72	E-test/DD	9 (10)	32 (35)	-	8 (9)	6 (6)	67 (73)	-	0	1 (1)
Brazil[93]	2008-09	1-59	Broth	27 (7)	153 (38)	8 (2)	12 (3)	74 (19)	231 (58)	-	-	-
Brazil[94]	2010	<72	E-test/DD	-	33 (27)	-	2 (2)	2 (2)	62 (51)	-	0	4 (3)
Peru[95]	2000	3-38	E-test	9 (6)	22 (15)	-	10 (7)	-	83 (57)	-	-	3 (2)
Venezuela[96]	2004-05	0-72	DD	8 (5)	21 (13)	-	16 (10)	12 (8)	-	-	0	-
Australia/Oceania												
Australia[97]	1997	<48	E-test/DD	172 (17)	256 (25)	-	176 (17)	175 (17)	468 (46)	-	-	139 (14)
Fiji[98]	2003-04	3-13	E-test/DD	3 (1)	28 (11)	-	4 (2)	-	50 (20)	-	-	0

Ref- Reference, AST- Antibiotic susceptibility testing, MDR- Multi-drug resistance, PEN- Penicillin, CTX- Cefotaxime, ERY- Erythromycin, TET- Tetracycline, SXT- Cotrimoxazole, CIP- Ciprofloxacin, LVX- Levofloxacin, CHL- Chloramphenicol, * AST data available for non-PCV7 serotypes only. CLSI- Clinical Laboratory Standards Institute, CASFM- Committee of the Antibiogram of the French Society of Microbiology, EUCAST- European Committee on Antimicrobial Susceptibility Testing, BSAC- British Society for Antimicrobial Chemotherapy, (-)- data not specific or missing, DD- Disc diffusion.

Table 2.3. Characteristics of eligible repeated cross-sectional studies reporting ANSP.

Country [Ref]	Year of study	Age group (months)	AST methods	MDR	Antibiotic, no. of non-susceptible isolates (% of non-susceptible isolates)							
					PEN	CTX	ERY	TET	SXT	CIP	LVX	CHL
Asia												
Israel[23]	1993	6-42	E-test/DD	42 (15)	91 (33)	-	44 (16)	44 (16)	96 (34)	-	-	-
	1994	6-17	E-test/DD	9 (11)	63 (77)	-	9 (11)	9 (11)	66 (81)	-	-	-
Singapore[22]	1998	17-78	E-test/DD	34 (33)	28 (28)	18 (18)	34 (33)	49 (48)	-	-	-	-
	2007-08	4-103	E-test/DD	44 (75)	41 (70)	4 (7)	46 (78)	40 (68)	39 (66)	-	1 (2)	
Europe												
Greece[20]	2000	12-72	E-test	-	77 (20)	-	89 (23)	-	-	-	-	-
	2003	12-72	E-test	-	160 (35)	-	140 (31)	-	-	-	-	-
Greece[99]	2000	12-72	E-test	3 (1)	31 (8)	-	89 (23)	-	-	-	-	-
	2003	12-72	E-test	77 (17)	106 (23)	-	106 (30)	-	-	-	-	-
Greece*[21]	2005	13-76	E-test/DD	6 (3)	26 (12)	0	16 (8)	7 (3)	47 (22)	-	-	1 (1)
	2006	13-76	E-test/DD	3 (3)	19 (18)	0	14 (13)	3 (3)	16 (15)	-	-	0
	2007	13-76	E-test/DD	11 (5)	33 (15)	0	31 (14)	9 (4)	32 (14)	-	-	0
	2009	13-76	E-test/DD	21 (8)	29 (29)	2 (1)	36 (14)	19 (7)	86 (32)	-	-	3 (1)
Iceland[24]	2009-12	13-76	E-test/DD	82 (9)	97 (11)	-	114 (13)	95 (11)	192 (22)	-	-	-
	2013-15	13-76	E-test/DD	16 (4)	38 (10)	-	33 (9)	31 (9)	44 (12)	-	-	-
Norway[25]	2006	-	E-test	24 (5)	9 (2)	-	32 (6)	32 (6)	25 (5)	-	-	-
	2008	-	E-test	8 (1)	11 (2)	-	18 (3)	38 (7)	11 (2)	-	-	-
Portugal[26]	2001	4-72	E-test/DD	114 (25)	110 (24)	-	122 (26)	107 (23)	119 (26)	-	0	35 (8)
	2006	4-72	E-test/DD	80 (20)	86 (22)	-	93 (24)	72 (18)	47 (12)	-	0	1 (0)
North America												
USA[27]	2006-10	6-30	Vitek 2	22 (11)	93 (44)	33 (16)	63 (30)	40 (19)	56 (27)	-	0	3 (1)
	2010-15	6-30	Vitek 2	12 (3)	138 (32)	29 (7)	69 (16)	26 (6)	56 (13)	-	0	0
South America												
Peru[100]	1996-7	6-24	E-test/DD	6 (8)	4 (5)	-	14 (19)	-	39 (52)	-	-	17 (23)
	2001	6-24	E-test/DD	17 (23)	15 (20)	-	12 (16)	-	55 (73)	-	-	20 (27)
	2003	6-24	E-test/DD	10 (8)	40 (37)	-	17 (14)	-	74 (61)	-	-	13 (11)

Ref- Reference, AST- Antibiotic susceptibility testing, MDR- Multi-drug resistance, PEN- Penicillin, CTX- Cefotaxime, ERY- Erythromycin, TET- Tetracycline, SXT- Cotrimoxazole, CIP- Ciprofloxacin, LVX- Levofloxacin, CHL- Chloramphenicol, * AST data available for non-PCV7 serotypes only. CLSI- Clinical Laboratory Standards

Institute, CASFM- Committee of the Antibiogram of the French Society of Microbiology, EUCAST- European Committee on Antimicrobial Susceptibility Testing, BSAC- British Society for Antimicrobial Chemotherapy, (-)- data not specific or missing, DD- Disc diffusion.

Table 2.4. The distribution of antibiotic resistance genes by country.

Country	**Number of isolates carrying resistance gene (percentage of isolates carrying resistance gene)**										
	Macrolides							Fluoroquinolones			Tetracycline
	***erm*(B)**	***mef*(A)**	***mef*(E)**	***mef*(A/E)**	**L4[#]**	**L22[#]**	**23S rRNA[#]**	***gyrA***	***parC***	***parE***	***tet*(M)**
Russia[66]	46 (35)	40 (31)	-	-	30 (23)	3 (2)	2 (2)	1 (3)	6 (17)	19 (54)	-
Jordan[64]	21 (40)	-	20 (38)	-	-	-	-	-	-	-	-
China[28]	100 (96)*	-	-	11 (11)*	-	-	-	-	-	-	-
Greece[51]	1 (2)	14 (30)	-	-	-	-	-	-	-	-	-
Greece[75]	93 (12)	40 (5)	-	-	-	-	-	-	-	-	-
Greece[99]	82 (39)**	-	-	127 (60)	-	-	-	-	-	-	-
Greece[49]	139 (53)	14 (5)	174 (66)	-	-	-	-	-	-	-	-
Italy[33]	78 (45)*	-	28 (16)	-	-	-	-	-	-	-	82 (47)
Italy [56]	40 (47)	4 (5)	-	-	-	-	-	-	-	-	-

[#] - Mutation in ribosomal proteins, * - *erm* or *mef* not characterized, ** - *erm* (AM)

2.4 Discussion

This review summarizes data on NP carriage of ANSP among apparently healthy children under the age of 12 years. There was marked variation in the proportion of resistance geographically. The proportions of pneumococci non-susceptible to penicillin and cotrimoxazole were greater than 50% in most studies conducted in Africa and Asia. Studies conducted in Asia (South Korea, China, and Taiwan) also reported high proportions of MDR, erythromycin, and tetracycline-non-susceptible pneumococci. Cefotaxime non-susceptibility was uncommon in most countries, but with important exceptions in South Korea and China. Levofloxacin non-susceptibility was very rare.

NP colonization by pneumococcus is a vital step for the development of respiratory tract infection.[101] However, not all NP carriage isolates are associated with invasive disease but may approximate the proportions of antibiotic-non-susceptibility found in invasive isolates.[102] There was heterogeneity in the methods used to conduct studies included in this review which made comparison of the data from various studies difficult. Despite this limitation, some antibiotic-non-susceptibility trends were evident; the proportions of penicillin-non-susceptible pneumococci were high in studies conducted in Asia and Africa.

Beta-lactams, including penicillin and cefotaxime, are the most commonly used antibiotics in treating pneumococcal infections.[6] Beta-lactam resistance may result in pneumococcal infection treatment failure; it is therefore important to evaluate the level of resistance to these drugs as infections with intermediate pneumococci may be treatable with higher doses.[103] Antibiotic non-susceptibility data could not be separated into intermediate and resistant due to inconsistency of data presentation in various studies and these were regarded as non-susceptible.

Erythromycin-non-susceptible pneumococci were not detected in two studies from Uganda, a country where penicillin and cotrimoxazole-non-susceptibility were high.[39,40] These studies reported that erythromycin was the least frequently prescribed antibiotic among the

participants, which may explain the lack of resistance.[39,40] Macrolides remain an alternative to beta-lactams for the treatment of pneumococcal infections.[104] Similar to penicillin-non-susceptibility, erythromycin-non-susceptibility was common in various studies included in the current review (Tables 2.2 and 2.3). High proportions of erythromycin-non-susceptibility in carriage pneumococcal isolates are concerning, especially in South Korea, Taiwan, China, Iran, Jordan, Belgium, and Morocco (Figure 2.3). To make any conclusions on the usage of erythromycin for pneumococcal diseases in these countries, sufficient surveillance data on invasive isolates are needed as macrolide resistance may be of limited clinical relevance in areas where beta-lactams remain effective.[104]

Striking proportions of non-susceptibility to cotrimoxazole were described in studies conducted in Asian and African countries (Table 2.2 and Figure 2.4). Cotrimoxazole is a widely used antibiotic for the treatment of respiratory tract infections in many African and Asian countries.[40,105] In several sub-Saharan countries, cotrimoxazole is used as a prophylaxis to prevent opportunistic infections in HIV-infected or exposed individuals, and its widespread use may drive cotrimoxazole resistance in this region.[40,106] Therefore, resistance to cotrimoxazole needs further monitoring as this drug is also used, in many sub-Saharan countries, as first-line treatment for non-severe pneumonia.[40] In India, the proportions of penicillin and erythromycin-non-susceptibility were very low but non-susceptibility to cotrimoxazole was high. A review from India has shown that cotrimoxazole is more readily available than beta-lactams or erythromycin; this may drive the emergence of cotrimoxazole resistance in this setting.[105]

Studies from Asian countries reported the highest proportions of MDR compared to those in African and European countries. The emergence and spread of multi-drug-resistant pneumococci threaten the use of commonly prescribed antibiotics for pneumococcal infections as the carriage isolates are likely to cause invasive diseases.[107] Fluoroquinolones, including levofloxacin, are recommended for the treatment of MDR pneumococcal infections.[108] Resistance to levofloxacin has emerged but the proportions of non-

susceptibility were low (Table 2.2 and 2.3). Further surveillance studies for monitoring levofloxacin resistance are warranted.

There are limited longitudinal studies describing the NP pneumococcal antibiotic resistance patterns among children. This review only identified 10 repeated cross-sectional studies from eight countries (Table 2.3). Studies in Israel,[23] Singapore,[22] and Greece,[20,21,99] reported an increase in the proportion of ANSP; this observation could be due to lack of vaccination as these studies were conducted between 1993 and 2008, a period where PCV was not available in many countries.[109] In Israel, the rate of non-susceptibility to penicillin and cotrimoxazole was more than twice as high in 1994 as compared to 1993 and this was attributed to previous antibiotic exposure.[23] Three studies in Greece that reported an increase in ANSP were conducted in two different regions, two in Athens metropolitan area (2000-2003)[20,99] and one in central Greece (2005 -2009).[21] Although there was an increase in non-susceptibility, temporal fluctuations in antibiotic non-susceptibility observed may be due to sampling of participants in various communities within Greece (not vaccine-induced as PCV7 was introduced in 2006).[110] Serotypes included in the PCV are known to be highly resistant and the introduction of PCV has resulted in the reduction of resistance in PCV serotypes.[111] A study conducted in the USA reported a notable decrease in the carriage of ANSP between 2006 and 2015 and this was likely due to the effects of PCV7.[27]

Nine studies reported testing for genetic resistance determinants responsible for resistance (Table 2.4). For beta-lactam resistance, a combination of several penicillin-binding protein mutations is required to confer resistance to beta-lactams, this is complex, making it difficult to characterise all mutations responsible for resistance.[112] *erm*B was the most commonly detected resistance gene among erythromycin-non-susceptible pneumococci; this gene confers resistance not only to macrolides but also to lincosamides and streptogramin B, characterized as the MLS_B phenotype.[113] *erm*B is associated with high-level resistance to both erythromycin and clindamycin whereas *mef* genes confer low or moderate levels of resistance to erythromycin but not clindamycin.[114] The detection of *tet*M-mediated resistance

to tetracycline was only described in Italy.[33] *tet*M, which encodes a protein blocking the binding of tetracyclines to the bacterial 30S ribosomal subunit, is the most common tetracycline resistance gene described in pneumococcal isolates.[115]

Several limitations exist in the current review; the non-susceptibility data described were based on carriage isolates and cannot be used directly for treatment guidelines for invasive disease causing isolates. Studies included in this review used various antibiotic susceptibility testing methods, antibiotic panels and guidelines; for better comparison of resistance data from different countries, standardized methods for conducting surveillance studies are required. Antibiotic susceptibility data could not be linked to specific pneumococcal serotypes due to insufficient data reported in various studies. The design of the studies included in the review did not allow the evaluation of the impact of vaccines on the NP carriage of ANSP (there was insufficient data to tease this out and generate a significant analysis). This review did not evaluate the risk factors for carriage of resistant pneumococci, but studies reviewed here found that prior use of antibiotics, day-care attendance, winter season, and multiple physician visits were key risk factors.[18,20,23,56,70,84]

Although there are limited data on the carriage of resistant pneumococci in children from developing countries, this review showed that resistant pneumococci have a worldwide distribution.[116,117] Non-susceptibility to penicillin and cotrimoxazole were high in studies from Asia and Africa. To inform treatment guidelines and monitor the trends of resistance, routine and standardised surveillance studies for resistant NP carriage and invasive isolates are needed. To determine how resistance in pneumococci develops and spreads within regions, the genetic resistance determinants should be investigated.[118]

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CHAPTER 3

Nasopharyngeal carriage of antimicrobial-resistant pneumococci in an intensively sampled South African birth cohort

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Summary

Nasopharyngeal (NP) colonization by *Streptococcus pneumoniae* (pneumococcus) precedes the development of respiratory tract infection. Colonization by antimicrobial-resistant pneumococci, especially in infants, is a major public health concern. We longitudinally investigated antimicrobial resistance amongst pneumococci colonizing the nasopharynx of South African infants immunized with the 13-valent pneumococcal conjugate vaccine (PCV13). NP swabs were collected every second week from birth through the first year of life from 137 infants. Pneumococci were identified and serotyped using conventional microbiological techniques, and their antibiotic susceptibility profiles determined by disc diffusion and E-test. All infants were immunized with 3 doses of PCV13. 1520 pneumococcal (760 non-duplicate) isolates were recovered from 137 infants; including non-typeable (n = 99), PCV13 (n = 133) and non-PCV13 serotypes (n = 528). The prevalence of penicillin, erythromycin, and cotrimoxazole non-susceptibility was 19% (147/760; 95% CI 17-22%) (3% resistant), 18% (136/760; 95% CI 15-21%) (14% resistant) and 45% (344/760; 95% CI 42-49%) (36% resistant) respectively. The predominant penicillin-non-susceptible serotypes included 15B/15C (n = 20), 19A (n = 13), 15A (n = 10), 19F (n = 8), and 21 (n = 8). Multi-drug resistance (MDR) was observed in 9% (68/760; 95% CI 7-11%) of the isolates. PCV13 serotypes were more likely to be non-susceptible, compared to non-PCV13 serotypes, to penicillin (26% *vs*. 16%, p = 0.007), erythromycin (23% *vs*. 15%, p = 0.027) and cotrimoxazole (62% *vs*. 41%, $p < 0.001$). Non-susceptibility to penicillin, erythromycin, and cotrimoxazole remained relatively constant through the first year of life (X^2 test for trend: p = 0.184, range 0 – 25%; p = 0.171, range 0 – 27%; and p = 0.572, range 0 – 55%, respectively). Overall, penicillin or erythromycin-non-susceptible pneumococci were carried for a shorter duration than susceptible pneumococci (penicillin [mean days, 18 *vs*. 21, p = 0.013] and erythromycin [mean days, 18 *vs*. 21, p = 0.035]). Within individual infants carrying the same serotype longitudinally, changes in antibiotic susceptibility were observed over time in 45% (61/137) of infants and these changes were predominantly for penicillin (76%, 79/104). Prevalence of NP carriage with antibiotic-non-susceptible pneumococci was

relatively constant throughout the first year of life. PCV13 serotypes were more commonly non-susceptible to penicillin, erythromycin, and cotrimoxazole.

3.1 Introduction

Nasopharyngeal (NP) colonization by antimicrobial-resistant *Streptococcus pneumoniae* (pneumococcus) is a global public health concern.[1] The pneumococcus is an important bacterial cause of childhood pneumonia, meningitis, and sepsis.[2,3] NP colonization with pneumococci is a prerequisite for development of pneumococcal disease.[4] The nasopharynx of children serves as a natural reservoir for pneumococci and the source for person to person transmission.[5] Vaccination with pneumococcal conjugate vaccine (PCV) is effective in reducing both vaccine type pneumococcal carriage and invasive disease.[6] Despite this, carriage prevalence has remained unchanged due to an increase in carriage of non-vaccine serotypes which also contribute to resistance.[7]

A rise in antibiotic resistance among pneumococci has reduced the effectiveness of empiric antibiotics used to treat pneumococcal infections.[8] Beta-lactam antibiotics are commonly used for the treatment of infections caused by pneumococci.[9] Widespread beta-lactam prescription and routine immunization exert selective pressures on the pneumococcal population structure which contribute to the emergence of beta-lactam resistant-pneumococci.[5,9] This has in turn resulted in the increased use of other classes of antibiotics such as macrolides and fluoroquinolones. Transmission of pneumococci that are resistant to these classes of antibiotics is also increasing in certain parts of the world, more so following the widespread PCV use.[10]

Detection of antibiotic resistance is important for successful therapy in the individual child, as well as for tracking antibiotic resistance patterns, which inform empiric treatment guidelines, and antimicrobial stewardship.[11] Most studies on the NP carriage of antibiotic-resistant pneumococci are cross sectional, and there are few longitudinal studies describing pneumococcal resistance patterns, including duration of carriage of susceptible and resistant pneumococci especially in low and middle income country settings where there is the greatest burden of disease.[12,13] This study aimed to investigate antimicrobial resistance patterns in pneumococci colonizing the nasopharynx of PCV13 vaccinated South African infants, from birth through the first year of life.

3.2 Material and methods

3.2.1 Study population and sampling

NP swabs were collected fortnightly from 137 infants enrolled between May 29th 2012 and May 31st 2014 as part of intensive cohort of the Drakenstein Child Health Study (DCHS), a longitudinal, prospective birth-cohort study in the Drakenstein sub-district, Cape Town, South Africa.[14] The study population is a stable, semi-urban community with a low socioeconomic status. This area also has a high incidence of childhood pneumonia (incidence 0.29 episodes/child year).[15] Pregnant women (>18 years) were enrolled in public sector health clinics during the second trimester, and followed until childbirth. Thereafter, infants were enrolled at birth and followed through their first year of life.[14] All births occurred at Paarl Hospital, a single public hospital serving this area. All 137 infants received a 2+1-dosing schedule of PCV13 at 6 weeks, 14 weeks and 9 months of age, according to the South African Expanded Program on Immunization (EPI-SA, 2015). For this study we included the first 137 infants enrolled in the cohort who had the most complete fortnightly NP sampling (defined as at least 23-26 fortnightly collected NP swabs).

3.2.2 Bacterial isolates

The NP sample collection, transportation, culture and storage have been described previously.[16] Briefly, the NP swabs were placed into 1ml skim milk-tryptone-glucose-glycerol (STGG) immediately after collection and transported at 4°C to the laboratory within two hours of collection (if not cultured immediately, the samples were stored at -80°C for later batch culture). Pneumococcal isolates were previously identified by colony morphology, alpha-hemolysis, optochin disc susceptibility (Oxoid, Basingstoke, UK) and confirmed by using the lytA polymerase chain reaction (PCR).[17] The isolates were stored in 1 ml skim milk-tryptone-glucose-glycerol (STGG) medium at -80°C for further analyses. The isolates were resuscitated by inoculating 20 µl of thawed STGG onto 2% sheep blood agar (Green Point Media Laboratory, National Health Laboratory Service, Cape Town, South Africa) and incubated for 24-48 hours at 37°C, in 5% CO_2.

3.2.3 Antibiotic susceptibility testing (AST)

Susceptibility testing of the isolates to oxacillin (1 µg), erythromycin (5 µg) and cotrimoxazole (1.25-23.75 µg) (bioMérieux, Marcy l'Etoile, South Africa) was performed using the disc diffusion method and interpreted according to CLSI 2017 guidelines (Clinical Laboratory Standards Institute, 2017). The isolates that were non-susceptible to oxacillin on disc diffusion underwent minimum inhibitory concentrations (MIC) testing. This was determined by using benzyl penicillin/penicillin G E-test (bioMérieux, Marcy l'Etoile, South Africa) according to the manufacturer's instructions, and interpreted using CLSI 2017 guidelines (Clinical Laboratory Standards Institute, 2017). A subset of 243 randomly selected isolates were screened for resistance to ciprofloxacin (5 µg) and levofloxacin (5 µg) (bioMérieux, Marcy l'Etoile, South Africa). This subset was tested to check if there was any fluoroquinolone non-susceptible isolate; these antibiotics are not recommended as the primary antibiotics to be tested (Clinical Laboratory Standards Institute, 2017). *S. pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 25923 were used as quality control strains. Oral penicillin breakpoints were used to interpret penicillin results (susceptible: ≤ 0.06 µg/ml, intermediate: 0.12 – 1 µg/ml, resistant: ≥ 2 µg/ml). Intermediate (low-level resistance) and resistant (high-level resistance) isolates were all considered as non-susceptible isolates. Multi-drug resistance was defined as non-susceptibility to the three classes of antibiotics (penicillin, erythromycin, and cotrimoxazole) tested and dual-resistance as non-susceptibility to two classes of antibiotics. To calculate the prevalence of non-susceptible isolates, longitudinal isolates from a single infant with the same antibiogram and serotype were classified as a single isolate (duplicate isolates). The acquisition of a non-susceptible pneumococcal isolate was defined as the detection of a non-susceptible isolate for the first time in an infant or the detection of a non-susceptible isolate that was initially susceptible. Loss of a non-susceptible isolate was similarly defined. The acquisition of a non-susceptible pneumococcal isolate was presumed to start at the mid-point between the last sampling time-point before which the non-susceptible isolate was detected and the time-point at which the non-susceptible isolate was first identified, whilst a loss of a non-susceptible isolate was considered as the mid-point

between the time at which the non-susceptible isolate was last identified and the next time-point. The carriage duration was determined by the difference between the loss date and acquisition date.[16]

3.2.4 Ethical considerations

This study was carried out in accordance with the recommendations of the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town. The protocol was approved by the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (HREC refs: 401/2009 and 740/2013) and the Western Cape Provincial Child Health Research Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

3.2.5 Statistical analysis

Statistical analyses were performed using STATA software (Stata Corporation, College Station, TX). Chi-square and Fisher's exact tests were used where applicable to compare the differences in the prevalence of carriage of non-susceptible pneumococci. Unpaired t-test (mean-comparison test) was used to compare the mean carriage durations. A two-tailed *p*-value of <0.05 was considered statistically significant.

3.3 Results

3.3.1 Participant demographics

Of 137 infants included in this study, 56% (77/137) were female and 6% (8/137) were preterm. The majority of infants were born via vaginal delivery (80%, 109/137), followed by emergency caesarean section (13%, 18/137), and elective caesarean section (7%, 10/137). Only 7% (10/137) of the infants were admitted to a ward after birth whereas 93% (127/137) roomed with their mother. Overall, 25% (34/137) of infants were born to HIV-infected mothers, with only one infant being HIV-infected (Table 3.1). PCV13 immunization coverage was 100% at 6 weeks, 14 weeks, and 9 months scheduled visit, respectively. Despite this,

immunization was delayed by at least 2 weeks in 6% (8/137) and 18% (24/137) of the infants at 6 weeks and 9 months, respectively.

Table 3.1: Characteristics of infants included in this study

Characteristics	% (Total), N = 137
Gender:	
Female	56 (77)
Mode of delivery:	
Vaginal	80 (109)
Emergency caesarean	13 (18)
Elective caesarean	7 (10)
Preterm (< 37 weeks)	6 (8)
Low birth weight (< 2500g)	13 (18)
HIV exposure[#]	24 (33)
Admission after birth:	
Ward	7 (10)
Roomed with the mother	93 (127)
Breastfed before discharge	89 (119)
PCV13 immunization	
6 weeks[*]	100 (137)
14 weeks	100 (137)
9 months[**]	100 (137)

[#]One of the 33 HIV-exposed infants was HIV-infected.
*Immunization delayed by at least 2 weeks in 8 infants.
**Immunization delayed by at least 2 weeks in 24 infants.

3.3.2 Antibiotic susceptibility patterns among non-duplicate isolates

Fifty percent (760/1520) of isolates were non-duplicate pneumococcal isolates. Of these, 49% (376/760, [95% confidence interval, CI 46-53%]) were susceptible to all three antibiotics tested (penicillin, erythromycin, and cotrimoxazole). In total, 19% (147/760, [95% CI 17-22%]), 18% (136/760, [95% CI 15-21%]) and 45% (344/760, [95% CI 42-49%]) were non-

susceptible to penicillin, erythromycin and cotrimoxazole, respectively (Table S3.1). Three percent (21/760, [95% CI 2-4%]), 14% (108/760, [95% CI 12-17%]), and 36% (274/760, [95% CI 33-40%]) of the non-susceptible isolates were fully resistant to penicillin, erythromycin, and cotrimoxazole, respectively. None of the 243 randomly selected isolates were non-susceptible to ciprofloxacin or levofloxacin.

3.3.3 Serotype distribution and antibiotic susceptibility patterns

Non-PCV13 serotypes (n = 528) were more commonly isolated than PCV13 serotypes (n = 133). However, PCV13 serotypes were more likely to be non-susceptible than non-PCV13 serotypes to penicillin, erythromycin and cotrimoxazole (Figure 3.1, Table 3.2 and Table S3.1). The rates of dual resistance and multi-drug resistance (MDR) were low, ranging from 3% to 9% for penicillin-erythromycin dual resistance and MDR, respectively (Table 3.2 and Table S3.1). A significantly higher proportion of PCV13 serotypes had dual penicillin-cotrimoxazole resistance (12%; 16/133 vs. 5%, 28/528, *P* = 0.003) and dual erythromycin-cotrimoxazole resistance (11%, 14/133 vs. 4%, 20/528, *P* = 0.001), compared to non-PCV13 serotypes. The overall prevalence of MDR was 9% (68/760, [95% CI 7-11%]), which was higher (but not significant) for PCV13 than non-PCV13 serotypes (10%, 13/133 vs. 7%, 39/528, *P* = 0.244) (Table 3.2 and Table S3.1). The most frequent MDR PCV13 serotypes were 19F (6%, 4/68) and 19A (4%, 3/68), while non-PCV13 serotypes included 15B/15C (15%, 10/68), 16F (6%, 4/68), 13 (6%, 4/68), and 15A (6%, 4/68) (Table S3.1). The predominant penicillin-non-susceptible pneumococci were non-typeable isolates (19%, 28/147), serotypes 15B/15C (14%, 20/147), 19A (9%, 13/147), 15A (7%, 10/147), 19F (5%, 8/147), and 21 (5%, 8/147). Of note, penicillin-non-susceptible isolates were frequently also non-susceptible to erythromycin (61%, 89/147) or cotrimoxazole (80%, 118/147).

Table 3.2: Proportion of antibiotic-non-susceptible pneumococcal isolates, obtained from 137 infants, by vaccine type

Antibiotic(s)	% non-susceptible isolates (95% confidence interval)		*P*-value[a]
	PCV13 serotypes (n = 133)	Non-PCV13 serotypes (n = 528)	
Penicillin	26 (19-34)	16 (13-19)	0.007
Erythromycin	23 (16-30)	15 (12-18)	0.027
Cotrimoxazole	62 (53-70)	41 (37-46)	<0.001
Penicillin + erythromycin	2 (0-5)	3 (2-4)	0.532
Penicillin + cotrimoxazole	12 (8-19)	5 (4-8)	0.003
Erythromycin + cotrimoxazole	11 (6-17)	4 (2-6)	0.001
MDR	10 (6-16)	7 (5-10)	0.244

[a] Difference between PCV13 *vs*. non-PCV13 serotypes. PCV13 serotypes- Serotypes included in the 13-pneumococcal conjugate vaccine, Non-PCV13 serotypes- Serotypes not included in the 13-pneumococcal conjugate vaccine. MDR- Multi-drug resistance.

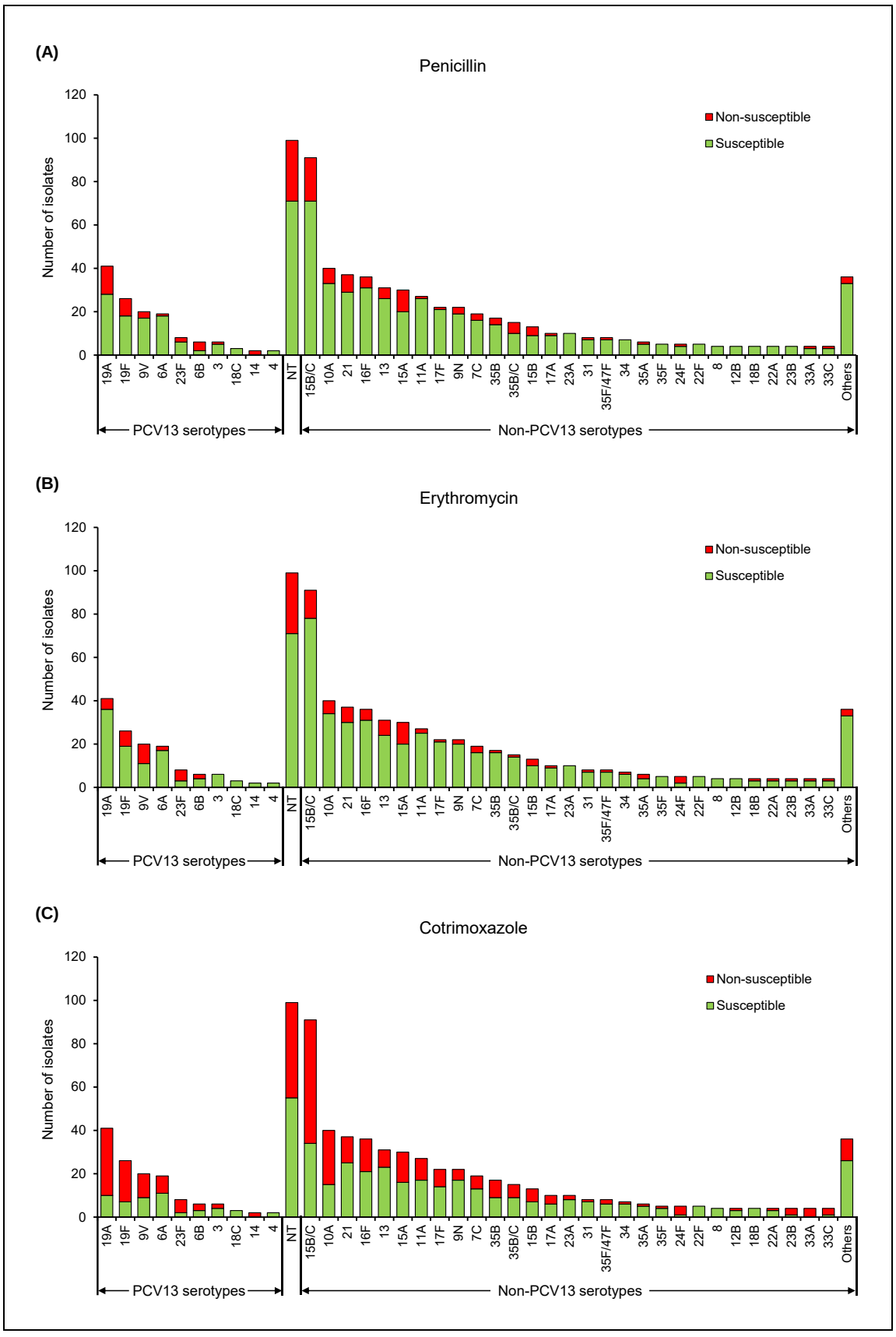

Figure 3.1: Serotype frequency and susceptibility profiles of 760 non-duplicate pneumococcal isolates obtained from 137 infants, by serotype. Susceptibility to (A) penicillin, (B) erythromycin, and (C) cotrimoxazole. Others include serotypes 20, 38, 11F, 18A, 19B, 19C, 22A/F, 23B, 25A/38, 33B/35C, 33F, 47F, and 6C/D. PCV13 serotypes- Serotypes included in the 13-pneumococcal

conjugate vaccine, Non-PCV13 serotypes- Serotypes not included in the 13-pneumococcal conjugate vaccine, NT- Non-typeable.

3.3.4 Antibiotic non-susceptibility trends over time

None of the infants were colonized by pneumococci at birth, thereafter, colonization with any pneumococci was detected from 2 weeks of which 20% (95% CI 0-72%) were non-susceptible to penicillin (Figure 3.2). NP carriage of penicillin-non-susceptible pneumococci was observed as early as 2 weeks of age, however, erythromycin- and cotrimoxazole-non-susceptibility were only observed at week 4; 13% (95% CI 4-29%) and 41% (95% CI 24-59%), respectively. Although there was a slight decline in penicillin-non-susceptibility between weeks 22 to 32 (≤ 10%), the levels of non-susceptibility to penicillin, erythromycin, and cotrimoxazole remained relatively constant through the first year of life (X^2 test for trend: p = 0.184, range 0 – 25%; p = 0.171, range 0 – 27%; and p = 0.572, range 0 – 55%, respectively) (Figure 3.2). Non-susceptibility to more than one antibiotic class among the pneumococcal serotypes is shown in Supplementary Figure S3.1. Although prevalence of non-susceptibility to more than one antibiotic class was generally low, the frequency of dual resistance to penicillin-cotrimoxazole was higher during the first six months of life (range 0 – 13%) and then declined over the next six months of life (range 0 – 6%) (X^2 test for trend, p < 0.001). There were no other clear trends for changes in the prevalence of dual or multi-drug resistance over time.

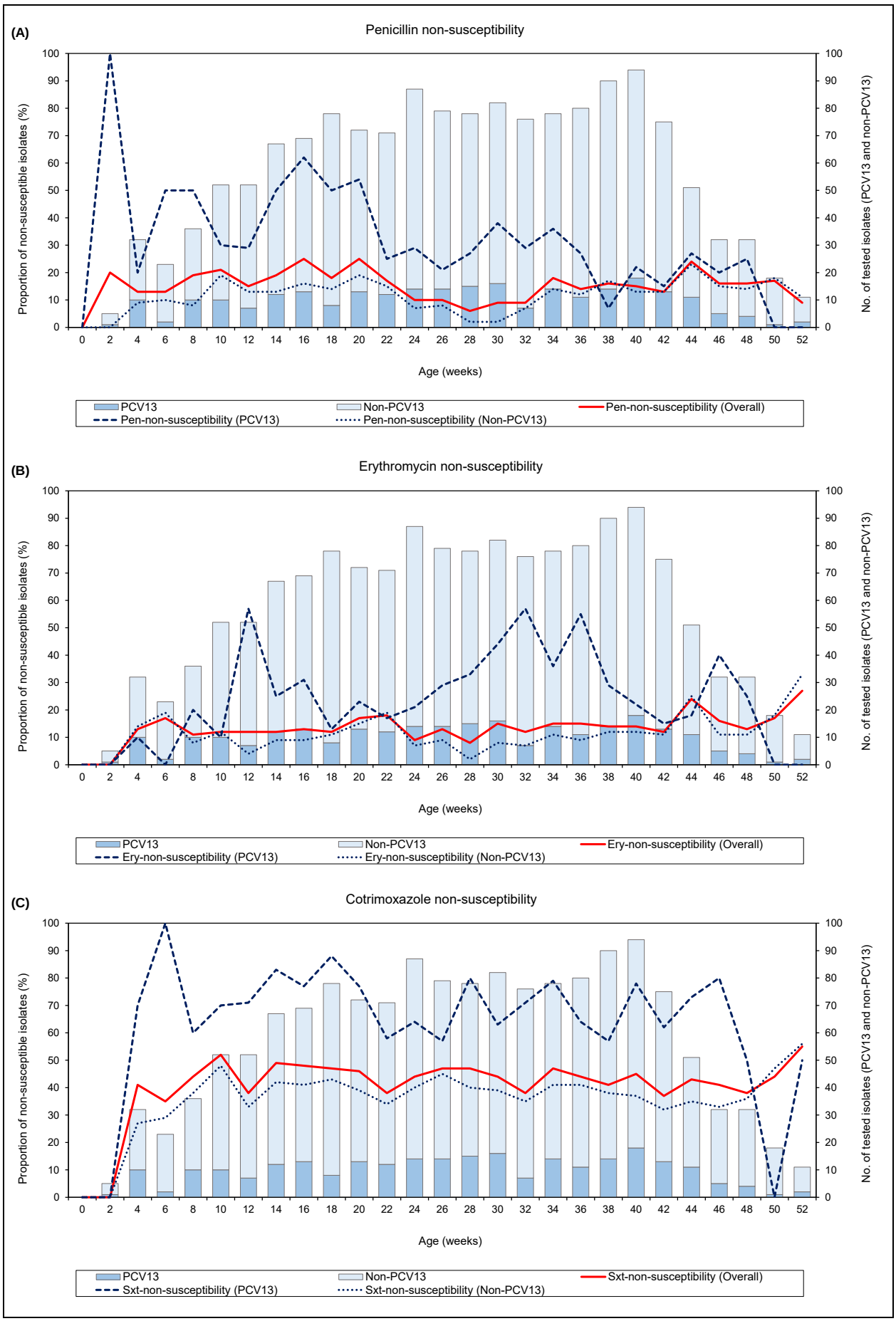
(A)
Penicillin non-susceptibility
Proportion of non-susceptible isolates (%)
No. of tested isolates (PCV13 and non-PCV13)
Age (weeks)
PCV13
Non-PCV13
Pen-non-susceptibility (Overall)
Pen-non-susceptibility (PCV13)
Pen-non-susceptibility (Non-PCV13)
(B)
Erythromycin non-susceptibility
Proportion of non-susceptible isolates (%)
No. of tested isolates (PCV13 and non-PCV13)
Age (weeks)
PCV13
Non-PCV13
Ery-non-susceptibility (Overall)
Ery-non-susceptibility (PCV13)
Ery-non-susceptibility (Non-PCV13)
(C)
Cotrimoxazole non-susceptibility
Proportion of non-susceptible isolates (%)
No. of tested isolates (PCV13 and non-PCV13)
Age (weeks)
PCV13
Non-PCV13
Sxt-non-susceptibility (Overall)
Sxt-non-susceptibility (PCV13)
Sxt-non-susceptibility (Non-PCV13)

Figure 3.2: Point-prevalence of antibiotic-non-susceptible pneumococci (n = 1520) obtained from 137 infants throughout the first year of life, (A) penicillin-non-susceptibility, (B) erythromycin-non-susceptibility, and (C) cotrimoxazole-non-susceptibility. Pen- Penicillin, Ery- Erythromycin, Sxt- Cotrimoxazole, No- Number, PCV13 serotypes- Serotypes included in the 13-pneumococcal conjugate vaccine, Non-PCV13 serotypes- Serotypes not included in the 13-pneumococcal conjugate vaccine.

3.3.5 Relationship between antibiotic non-susceptibility and duration of carriage

The mean carriage duration for all pneumococcal serotypes ranged from 13 to 65 days; the mean carriage duration for individual serotypes has been previously described.[16] Overall, penicillin or erythromycin-non-susceptible pneumococci were carried for a shorter duration than susceptible pneumococci, and the difference in the mean carriage duration was significant: penicillin-non-susceptible (18 days, 95% CI 17-20) *vs.* penicillin-susceptible (21 days, 95% CI 20-22), $p = 0.013$ and erythromycin-non-susceptible (18 days, 95% CI 16-21) *vs*. erythromycin-susceptible (21 days, 95% CI 20-22), $p = 0.035$. Cotrimoxazole-non-susceptible and -susceptible pneumococci were carried for similar durations (21 days, 95% CI 19-22, and 20 days, 95% CI 19-22, respectively, $p = 0.766$) (Table 3.3).

When stratified according to serotype, penicillin or erythromycin-non-susceptible non-PCV13 serotypes were carried for a shorter duration than susceptible non-PCV13 serotypes: penicillin (mean days, 18 *vs*. 22 respectively, $p = 0.019$) and erythromycin (mean days, 17 *vs*. 22 respectively, $p = 0.007$), (Table 3.3). These findings were reversed for PCV13 serotypes; non-susceptible PCV13 serotypes were carried for a longer duration than susceptible PCV13 serotypes but this was not statistically significant for penicillin or erythromycin: penicillin (mean days, 20 *vs*. 19 respectively, $p = 0.621$), erythromycin (mean days, 23 *vs*. 20 respectively, $p = 0.122$), and cotrimoxazole (mean days, 22 *vs*. 17 respectively, $p = 0.027$). These difference were largely due to serotypes 19F and 9V (PCV13 serotypes) which were carried for a longer duration if non-susceptible: serotype 19F, for penicillin and both serotypes 19F and 9V, for erythromycin (Supplementary Figure S3.2).

Table 3.3: Carriage duration of antibiotic-non-susceptible pneumococcal isolates, obtained from 137 infants, by vaccine type

Antibiotic susceptibility	Carriage duration, mean number of days (95% confidence interval)				P-value[a]
	Any serotype	Non-typeable	PCV13 serotypes	Non-PCV13 serotypes	
Penicillin					
Non-susceptible	18 (17-20)	16 (13-23)	20 (18-23)	18 (15-19)	0.313
Susceptible	21 (20-22)	17 (15-20)	19 (17-21)	22 (21-23)	0.033
Erythromycin					
Non-susceptible	18 (16-21)	15 (14-16)	23 (17-29)	17 (14-20)	0.037
Susceptible	21 (20-22)	17 (15-18)	20 (18-22)	22 (21-24)	0.086
Cotrimoxazole					
Non-susceptible	21 (19-22)	16 (14-17)	22 (19-25)	21 (19-23)	0.649
Susceptible	20 (19-22)	17 (15-18)	17 (14-20)	21 (20-23)	0.030

[a] Difference between PCV13 *vs*. non-PCV13 serotypes. PCV13 serotypes- Serotypes included in the 13-pneumococcal conjugate vaccine, Non-PCV13 serotypes- Serotypes not included in the 13-pneumococcal conjugate vaccine.

3.3.6 Changes in antibiotic susceptibility pattern within the same infant

Changes in antibiotic susceptibility profiles within the same pneumococcal serotype carried by an infant were observed in 45% (61/137) of infants (Figure 3.3). Of the 104 shifts in susceptibility profiles observed in these infants, 76% (79/104) were for penicillin, including changes from susceptible to non-susceptible (n = 41) and non-susceptible to susceptible phenotypes (n = 38) (Figure 3.3).

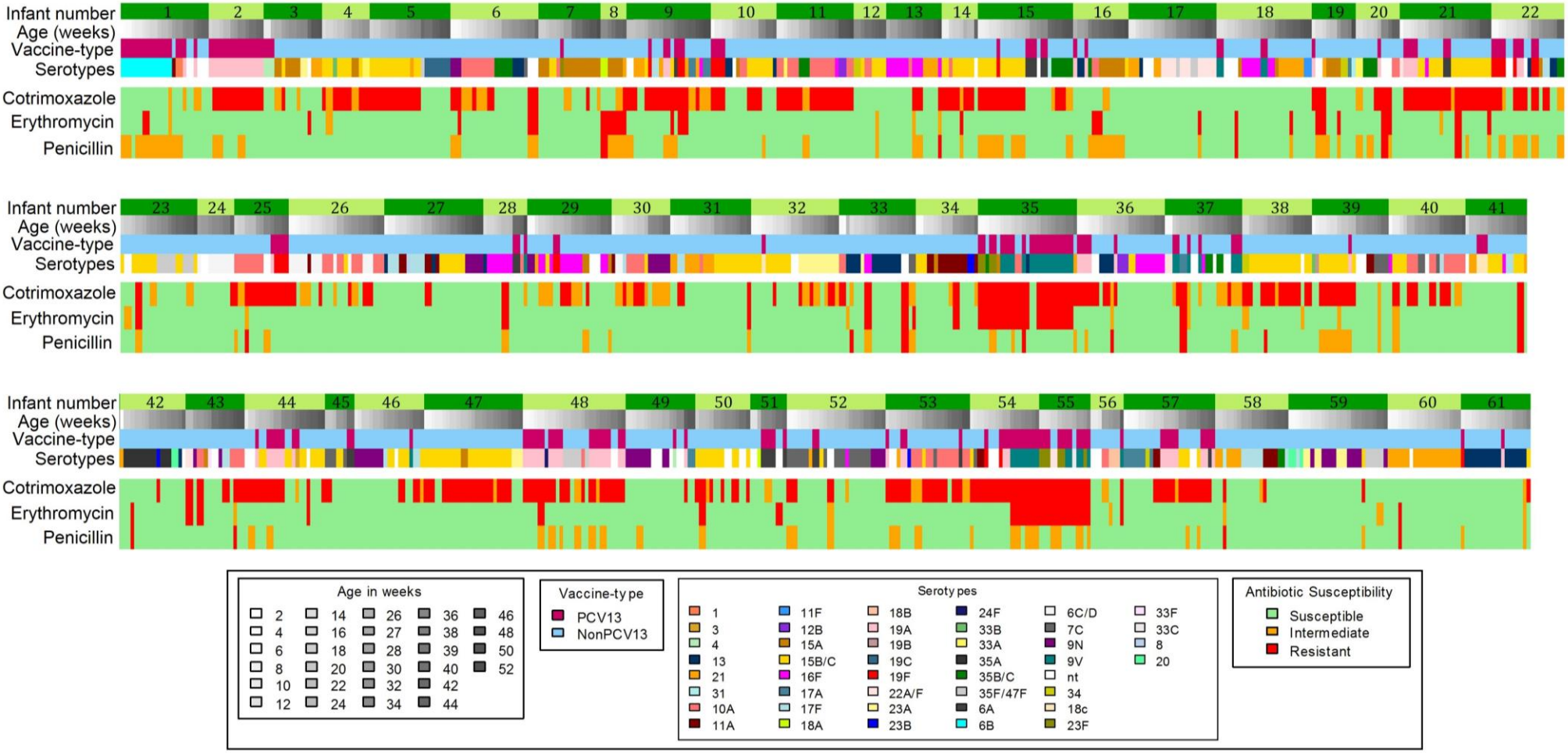

Figure 3.3: Longitudinal carriage of pneumococci showing shifts in antibiotic-non-susceptibility profiles which occurred in 61 out of 137 infants. PCV13 serotypes- Serotypes included in the 13-pneumococcal conjugate vaccine, Non-PCV13 serotypes- Serotypes not included in the 13-pneumococcal conjugate vaccine.

3.4 Discussion

This study reports the antimicrobial-non-susceptibility patterns of pneumococci colonizing the nasopharynx of PCV13 vaccinated South African infants. Despite high vaccine coverage, PCV13 serotypes were identified in 54% of children and these were more commonly non-susceptible to penicillin, erythromycin, and cotrimoxazole than non- PCV13 serotypes. The point-prevalence of antibiotic-non-susceptible pneumococci to penicillin, erythromycin, and cotrimoxazole was relatively constant from week 4 through the first year of life. Overall, penicillin or erythromycin-non-susceptible pneumococci, irrespective of serotype, were carried for a shorter duration than penicillin or erythromycin-susceptible pneumococci.

There are limited data on the NP carriage of antibiotic-non-susceptible pneumococci among vaccinated children during the first year of life.[19–21] The prevalence of penicillin-non-susceptible pneumococci was 19% in this study. The rate of non-susceptibility was higher compared to that reported among infants in India (3.4%),[19] Fiji (11.4%),[21] and The Gambia (13.3%).[20] The majority of the isolates in the present study displayed intermediate resistance to penicillin, in line with other studies.[22] Serotypes 15B/15C, 19A, and 15A showed the highest non-susceptibility to penicillin and this finding has been documented in other studies.[23,24] The prevalence of erythromycin-non-susceptible pneumococci in the current study was 18%, which was higher than that reported in The Gambia (0%),[20] and in Fiji (11.6%),[21] but lower than 37% reported in India.[19] Factors associated with high antibiotic-non-susceptibility in the current study could not be determined due to the limited sample size.

Compared to penicillin and erythromycin, the prevalence of cotrimoxazole-non-susceptible isolates in this study was high (45%). Russell *et al.* reported a relatively low cotrimoxazole non-susceptibility level of 20.3% among Fijian children within the same age group.[21] However, high cotrimoxazole non-susceptibility rates have been reported among Gambian (60%),[20] and Indian (81%),[19] children less than one year of life. In South Africa, cotrimoxazole is given as prophylaxis to individuals who are HIV-infected or exposed. Infants are administered cotrimoxazole prophylaxis from 4 or 6 weeks of life until the child is

confirmed HIV-uninfected.[25] Twenty-four percent (33/137) of the infants in this study were born to mothers who were HIV-infected, which may have contributed to the high levels of cotrimoxazole non-susceptibility observed. There was no difference in the proportion of carriage of cotrimoxazole-non-susceptible pneumococci between infants born to HIV-infected vs. HIV-uninfected mothers. However, this analysis was not included due to the small sample size, and inability to test significance. The acquisition of cotrimoxazole-non-susceptible pneumococci from the mother or other sources cannot be disregarded since cotrimoxazole is widely used in low and middle income countries including South Africa.[26]

None of the selected pneumococcal isolates were non-susceptible to ciprofloxacin or levofloxacin. Fluoroquinolones, particularly, levofloxacin, gatifloxacin, and moxifloxacin are increasingly used for the treatment of community-acquired pneumonia,[27] especially in high income countries where tuberculosis is less prevalent.[28] These antibiotics are uncommonly used in the public health sector, including the clinics and hospital that our population accessed. However, non-susceptibility to levofloxacin has emerged in several countries and this poses a threat to its ability to treat resistant pneumococcal infections.[29–33]

This study showed that PCV13 serotypes were still circulating and had higher proportions of antibiotic-non-susceptibility than non-PCV13 serotypes, and this has been observed elsewhere.[34,35] In the current study, this might be attributable to the time elapsed between PCV13 implementation in 2011 (in South Africa) and the commencement of the study in 2012. However, a study in The Gambia reported the persistence carriage of vaccine serotypes even five years after the introduction of PCV13.[36] Interestingly, antibiotic-non-susceptibility among non-typeable pneumococci was also frequent. Although non-typeable pneumococci have limited potential for disease, they may act as a reservoir for the transfer of antibiotic resistance elements to other bacteria. Monitoring of resistance levels and colonization rates of non-typeable pneumococci is therefore important.[37] In addition, the current study did not investigate whether the capsule gene among non-typeable pneumococci was down regulated or switched, therefore, further investigation is warranted.

Few studies have reported on the carriage duration of antibiotic-non-susceptible pneumococci, with most focused on individuals with respiratory tract infection.[12,38,39] The carriage duration of penicillin-non-susceptible pneumococci (mean 18 days) in this study was lower than that reported among Swedish infants with respiratory tract infection (mean 49 days).[12] In this study, duration of carriage was related to both serotype (PCV13 *vs*. non PCV13) and antibiotic non-susceptibility. Firstly, we observed that penicillin or erythromycin-non-susceptible pneumococci were carried for a shorter duration than penicillin or erythromycin-susceptible pneumococci. This may also have been likely due to the serotypes that were carried or may suggest that a fitness cost could be associated with expression of antibiotic resistance.[40,41] Our finding was however dependent on serotype since penicillin, erythromycin or cotrimoxazole-non-susceptible PCV13 serotypes were carried for a longer duration than susceptible PCV13 serotypes, while the inverse was true for non-PCV13 serotypes. Penicillin-non-susceptible serotype 19F and erythromycin-non-susceptible serotypes 19F and 9V were carried for a longer duration than the other PCV13 serotypes. One possible explanation for our findings is that, in pneumococci, the relative fitness cost of particular non-susceptible genotypes may depend on the genetic background of the strains in which the resistance-conferring mutations or genes are found.[40] Further work, using whole genome sequencing to identify the resistance-conferring genes and strain genetic background is needed.

Dual resistance and MDR were generally low, however, significantly higher rates of dual resistance were observed among PCV13 serotypes compared to non-PCV13 serotypes for penicillin-cotrimoxazole (12% *vs*. 5%, $p = 0.003$) and erythromycin-cotrimoxazole (11% *vs*. 4%, $p = 0.001$). Penicillin-non-susceptible pneumococci have been shown to be frequently non-susceptible to other classes of antibiotics.[42] In the current study, penicillin-non-susceptible pneumococcal isolates were frequently non-susceptible to erythromycin and cotrimoxazole, as described elsewhere.[9,43,44]

This study documented shifts in the antibiotic susceptibility profiles over time within the same pneumococcal serotype carried by an infant. Seventy-six percent (79/104) of the shifts

observed were for penicillin susceptibility profiles, however, since genetic resistance determinants were not investigated, we are unable to confirm whether switches in resistance occurred within the same strain, or identify the genetic basis of resistance acquisition or loss, and this should be addressed in future studies.

There were several limitations to this study. Firstly, pneumococcal isolates were only screened against five commonly used antibiotics. Secondly, the infants enrolled were from the same geographic district, therefore the results may not be generalizable. This cohort included only vaccinated infants, and no case control of unvaccinated children could be made. The methods used in this study were unable to detect co-colonization with multiple serotypes or confirm or characterise genetic resistance determinants. A further limitation is the inability to determine risk factors associated with antibiotic non-susceptibility and carriage duration of non-susceptible pneumococci due to the small sample size. This study does however provide baseline data on the prevalence, trends, and carriage duration of antibiotic-non-susceptible pneumococci among infants enrolled in a PCV13 immunized birth cohort in a low and middle income country setting with a high incidence of lower respiratory tract infection in infants.[15]

In conclusion, this study showed that the NP carriage of antibiotic-non-susceptible pneumococci was relatively constant through the first year of life. Despite a high vaccine coverage, PCV13 serotypes were identified and were more commonly non-susceptible to penicillin, erythromycin, and cotrimoxazole. Overall, penicillin or erythromycin-non-susceptible pneumococci were carried for a shorter duration than susceptible pneumococci, however, non-susceptible PCV13 serotypes were carried for a longer duration than non-susceptible non-PCV13 serotypes.

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CHAPTER 4

A novel approach to studying pneumococcal nasopharyngeal colonization dynamics and antimicrobial resistance using shotgun metagenomic sequencing

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Summary

Shotgun metagenomic sequencing of nasopharyngeal (NP) samples, from children enrolled in a PCV13-vaccinated South African birth cohort was used to explore strain-level pneumococcal colonization patterns and associated antimicrobial resistance determinants. NP swabs were collected at two week intervals from birth through the first year of life from 137 infants. Pneumococcal isolates were serotyped and tested for phenotypic antimicrobial resistance. 196 NP samples from a subset of 23 infants were then selected based on changes in serotype or antimicrobial resistance. DNA extraction and shotgun metagenomic sequencing were performed directly from enriched NP samples. Reads were assembled and aligned against reference pneumococcal genomes. *In-silico* pneumococcal capsular, multilocus sequence typing, and resistome analyses were performed. Of the 196 samples sequenced, 174 had corresponding positive cultures for pneumococci and, of those 152 were assigned an *in-silico* serotype. Metagenomic sequencing detected a single pneumococcal serotype in 85% (129/152), and co-colonization in 15% (23/152) of the samples, respectively. The concordance between Quellung/sequetyping and shotgun *in-silico* typing was 86% (127 out of 148 NP samples that were assigned a serotype by both methods). Twenty-two pneumococcal serotypes were identified, with 15B/15C (n = 49) and 16F (n = 21) being the most common non-PCV13 serotypes, while 23F (n = 9) and 19A (n = 8) were the most common PCV13 serotypes. 26 different sequence types (STs), including 4 novel STs were identified *in-silico*. Mutations in the *fol*A and *fol*P genes, associated with cotrimoxazole resistance, were detected in 89% (87/98) of cotrimoxazole-non-susceptible pneumococci, as well as in the *pbp*1a and *pbp*2x genes, in penicillin non-susceptible ST7052$^{15B/15C}$ isolates. Metagenomic sequencing of nucleic acid from NP samples is a valuable culture-independent technique for a detailed evaluation of the pneumococcal component of the NP microbiome.

4.1 Introduction

Streptococcus pneumoniae (the pneumococcus) is a frequent bacterial cause of infections such as bacterial pneumonia, otitis media, meningitis, sinusitis, and bacteraemia in young children, and is a major cause of morbidity and mortality.[1–4] Globally, pneumococcal pneumonia was responsible for an estimated 393,000 deaths in children less than five years of age in 2015.[5] Asymptomatic pneumococcal nasopharyngeal (NP) carriage is common among infants, a reservoir for transmission and precedes the development of disease.[6] Pneumococci are classified into more than 100 serotypes, based on the antigenic specificity of the polysaccharide capsule.[7,8] Children are often sequentially colonized by multiple different serotypes,[9,10] and may be co-colonized by different pneumococcal serotypes at the same time.[11]

Immunization with the pneumococcal conjugate vaccine (PCV) has substantially reduced NP carriage and invasive pneumococcal disease caused by, serotypes represented in the vaccine.[12,13] In addition, the introduction of PCV has also resulted in a reduction in antimicrobial resistance amongst circulating pneumococci due to the inclusion of serotypes associated with high antibiotic resistance in the conjugate vaccine.[14] However, non-vaccine-serotypes have emerged among both carriage and disease-causing isolates, and are increasingly associated with antimicrobial resistance.[15,16] Increasing pneumococcal resistance to different classes of antibiotics, including beta-lactams, macrolides, tetracyclines, and cotrimoxazole, has compromised the effectiveness of antibiotics to treat pneumococcal infections.[17]

Pneumococci are most commonly identified from samples using bacterial culture followed by phenotypic and genotypic characterization of the isolates.[18,19] The Quellung reaction, which is based on antigen-antibody reaction, is currently the gold standard for pneumococcal serotyping, but requires viable isolates in pure culture. To detect colonization with multiple serotypes, typing of many individual (often identical) pneumococcal colonies from each NP sample is often required.[20] Sequential multiplex PCR assays are increasingly used for serotyping, and can be done directly on the NP sample, however, this is relatively laborious,

costly, and limited to serotypes targeted by the PCR.[21] None of these methods are able to provide a more detailed, strain-level characterization, important for pneumococci, as capsular switching often results in different serotypes within the same lineage.[22]

Metagenomic approaches, where the collective genomes of all organisms recovered directly from a sample are sequenced, is a high-throughput approach to investigate the members of a microbial community in an ecological niche, and represents an alternative to culture-dependent methods for microbial characterization. In this study, we explored the use of shotgun metagenomic sequencing to characterize the pneumococcal component of the NP, including identification of co-colonization with multiple serotypes and antimicrobial resistance, among PCV-vaccinated children participating in a South African birth cohort.

4.2 Materials and methods

4.2.1 Study population and sampling

Children were enrolled in a longitudinal birth-cohort study, the Drakenstein Child Health Study,[23] in the Western Cape Province of South Africa. NP swabs were collected every second week from birth through the first year of life from 137 infants.[10] Infants received 2+1 doses of 13-valent pneumococcal conjugate vaccine (PCV13) at 6 weeks, 14 weeks, and 9 months according to the national immunization program in South Africa. Details of the study population and sampling have been previously described.[23] The study was approved by the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee (Reference numbers 401/2009 and 235/2016). Written informed consent was obtained from mothers.

4.2.2 Identification and antimicrobial characterization of pneumococci

The collected NP swabs were immediately placed into 1 ml skim milk-tryptone-glucose-glycerol (STGG) medium, transported on ice, and stored at -80°C until further batch processing. A 10 µl aliquot of the NP-STGG was inoculated onto a blood agar plate (Oxoid Columbia base with 5% sheep blood) containing gentamicin (5 µg/ml) and incubated overnight at 37°C, in 5% CO_2. Presumptive pneumococcal isolates were identified as

previously described;[24] only a single pneumococcal isolate was selected for characterization from each sample. The isolates were serotyped using sequetyping and confirmed by Quellung as previously described.[25,26]

Susceptibility to oxacillin, erythromycin, and cotrimoxazole were assessed using the disc diffusion method, and interpreted using Clinical and Laboratory Standards Institute (CLSI) guidelines.[27] Penicillin minimum inhibitory concentrations (MICs) were determined using the E-test method (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Resistant- and intermediate-resistant pneumococcal isolates were collectively regarded as non-susceptible.

4.2.3 Broth enrichment of NP samples for pneumococci

For this exploratory study, in order to explore the utility of metagenomic sequencing to detect colonization with multiple serotypes and identify antimicrobial resistance determinants, a total of 196 NP-STGG samples were purposively selected from a subset of 23 of the 137 infants described above, based on changes in serotype and antibiogram over time identified using phenotypic methods (The selection of isolates based on changes in serotypes or antimicrobial resistance applies to 61 infants in total as described in Chapter 3. However, it was not possible to select all 61 infants and all their samples for shotgun sequencing). The NP-STGG samples were enriched as previously described, with minor modifications.[28] Briefly, 200 µl of an NP-STGG sample was transferred to 6 ml Todd-Hewitt Broth (without antibiotics), containing 0.5% yeast extract and 17% foetal bovine serum. The liquid culture was incubated at 37°C with 5% CO_2, without shaking for 6 hours. The culture was then centrifuged at 12,000 x g for 10 minutes at 4°C. Total nucleic acid extraction was performed on the collected pellet using the QIAsymphony SP automated platform (Qiagen, Hilden, Germany) with the QIAsymphony Virus/Bacteria Mini Kit (Cat. No. 931036) following the manufacturer's instructions. Nucleic acid concentrations and purity were determined using the NanoDrop® ND-100 (Thermo Fishers Scientific, Waltham, USA).

4.2.4 Metagenomic DNA sequencing, assembly and *in-silico* typing

Total nucleic acid was subjected to shotgun sequencing on the MiSeq platform using the MiSeq Reagent Kit v3 (600-cycle) (Illumina, San Diego, USA) at the J. Craig Venter Institute, Rockville, USA. Metagenomic DNA sequencing protocols and the pipeline used to assemble the reads and evaluate the assembly have been previously described.[28] Reads were assembled using metaSPAdes,[29] and aligned to the created serotype nucleotide database using BLASTn, with an identity over 98% considered a match.[28] In addition, assembly-based *in-silico* multi-locus sequence typing (MLST) was performed using LOCUST as previously described.[30,31]

Serotypes identified by Quellung and sequetyping on isolates,[26] were compared to *in-silico* serotypes identified from metagenomic sequences obtained directly from NP samples. Culture-based and metagenomic-assigned serotypes were considered concordant if the serotype detected by Quellung/sequetyping was detected by shotgun sequencing (either as a single serotype or amongst co-colonizing serotypes detected).

4.2.5 Phylogenetic tree construction

A phylogenetic tree of pneumococcal genomes was constructed to assess strain relatedness. Assembly-based (metaSPAdes assembler) variant calling,[29] was performed using The Northern Arizona Single Nucleotide Polymorphism Pipeline (NASP).[32] All completed pneumococcal genomes available on the NCBI database were included in the NASP run and the *S. pneumoniae* R6 genome (Accession number AE007317) was used as a reference genome. The aligned base calls for each of the core variant positions identified by NASP were used for the construction of the maximum likelihood tree.[33]

4.2.6 Analysis of genetic determinants of antimicrobial resistance

Pneumococcal contigs (contigs that mapped to reference pneumococcal genome R6) were compared to the Antibiotic Resistance Gene-Annotation (ARG-ANNOT) database by BLAST alignment.[34] Results were filtered using ≥ 90% sequence identity over 80% of the sequence length of the reference antibiotic resistance gene as cut-offs. The *pbp*1a, *pbp*2x, *pbp*2b, *fol*A (encoding dihydropteroate synthase (DHPS)), and *fol*P (encoding dihydrofolate reductase

(DHFR)) gene mutations associated with resistance to beta-lactams, trimethoprim, and sulfonamides respectively, were investigated by manual local alignment, as the ARG-ANNOT database does not automatically detect these mutations. The ARG-ANNOT database only included the *pbp*1a (JN645776) and *pbp*1b (AF101781) genes of wild-type pneumococcal strains. Therefore, local alignments of the *pbp*1a (JN645776.1), *pbp*2x (JN645706.1), *pbp*2b (DQ056780.1), *fol*A_R6 (Gene ID 4442919), and *fol*P_R6 (Gene ID 4443057) were performed using BLASTn (80% identity and 60% gene length coverage).

For each individual, and for each of the genes, sequences from the longitudinal samples were aligned with Multiple Alignment using Fast Fourier Transform (MAFFT).[35] Aligned sequences were viewed and translated to amino acids in AliViewer (version 1.18.1) for active site mutation analysis. The transpeptidase domains of the *pbp*1a, 2x, and 2b, from the wild type *S. pneumoniae* R6 strain were used as references. Phylogenetic trees were constructed with Molecular Evolutionary Genetic Analysis software (MEGA version 7.0.26) using a neighbour-joining method and bootstrapping 1000 replicates. *pbp*1a (JN645776.1), *pbp*2x (JN645706.1), and *pbp*2b (DQ056780.1) were used as reference gene sequences in the construction of the phylogenetic trees.

4.3 Results

4.3.1 Participant characteristics

Shotgun sequencing was used to study the pneumococcal population structure in the selected subset of 196 NP samples from 23 infants. The number of NP samples selected for sequencing ranged from 4 – 21 samples per infant (average of 8.5 samples); selected samples and age at sampling (average 14.9 weeks) for each of the infants is shown in Figure 4.1. Thirteen of the infants were males (57%). The mean birth weight was 3.0 kg (range, 2.4 – 3.8 kg) with only one preterm infant. Eight infants were HIV exposed (born to HIV-infected mothers) but were HIV-uninfected (more detailed clinical characteristics of 23 infants are described in Chapter 5 in Table 5.1).

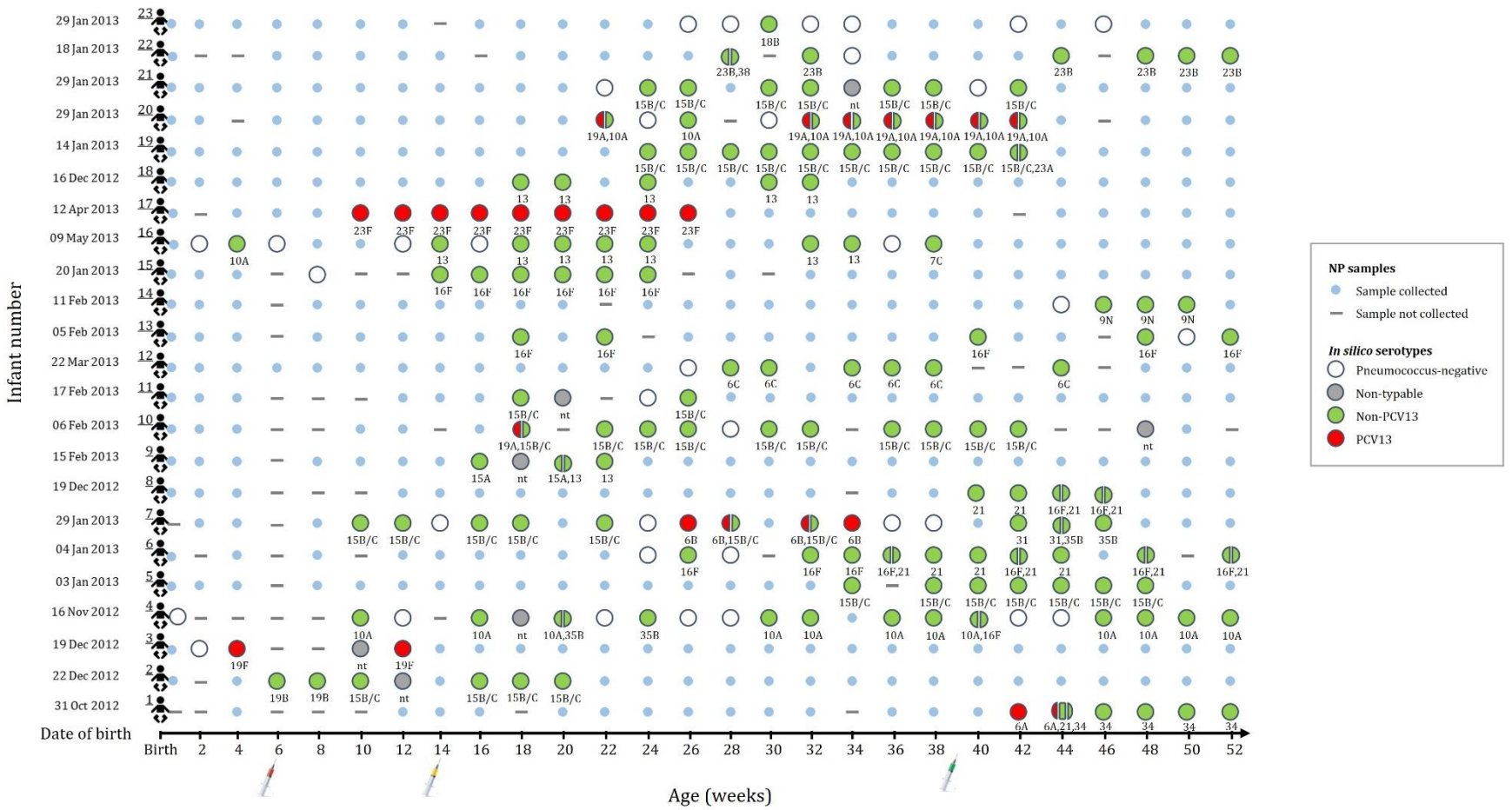

Figure 4.1: This figure indicates the number of samples selected for metagenomic sequencing and serotype assignments for the 196 nasopharyngeal (NP) samples selected from 23 infants (shown in rows 1 to 23). None of the infants were colonized until 4 weeks of age. Small dots indicate that samples were collected. Large circles (colours represent serotype group) represent the collected NP samples selected for shotgun metagenomic sequencing. Split circles represent samples with co-colonization with multiple serotypes. *In-silico* serotypes are displayed for samples selected for shotgun metagenomic sequencing. (-) Indicates that no sample was collected at that time point. (nt) Non-typeable.

4.3.2 Metagenomic sequencing results

The average number of reads per sample was 13 million (ranging from 80 thousand – 93 million reads per sample) despite low input DNA concentrations (data not shown). The average number of microbial reads that mapped to the R6 pneumococcal reference genome, per sample, was 4.5 million (range, 141 reads – 32 million reads per sample) with an average coverage of 308.53X (range, 0.01X – 2239.71X).

4.3.3 In-silico serotypes and sequence types

Of the 196 samples sequenced, 174 had a corresponding positive pneumococcal culture. Shotgun sequencing detected pneumococcal reads in all 174 samples, however, 15 samples had no reads covering the desired regions (cps, housekeeping genes, and core variant positions identified by the NASP) of the pneumococcal genomes and were excluded from further analyses. *In-silico* serotypes from shotgun sequencing were assigned in 96% (152/159) of the remaining samples and serotypes were assigned in 93% (148/159) of samples by both Quellung/sequetyping and shotgun sequencing. The concordance between Quellung/sequetyping and shotgun *in-silico* typing was 86% (127/148) (Table S4.1). Co-colonization with two (n = 22) or three (n = 1) different serotypes was detected in 15% (23/152) of the samples from 10 infants using shotgun sequencing. Since Quellung/sequetyping typing was only done on a single colony per sample, co-colonization was not detected using this method.

Non-PCV13 serotypes were more commonly detected than PCV13 serotypes (Figure 4.1). Twenty-two different pneumococcal serotypes were identified *in-silico*, with 15B/15C (n = 49), 16F (n = 21), 10A (n = 21), 13 (n = 14), and 21 (n = 12) being the most common non-PCV13 serotypes, and 23F (n = 9) and 19A (n = 8) being the most common PCV13 serotypes. Serotypes and STs identified are shown in Table S4.2. MLST was assigned in 89% (142/159) of the samples representing 26 different MLST profiles (Figure 4.2). The common STs detected include ST8687$^{15B/C}$ (n = 28/142), ST5647^{13} (n = 13/142), ST2068^{10A}

(n = 12/142), and ST7052$^{15B/C}$ (n = 10/142). Four novel STs (ST13795$^{15B/15C}$, ST13797^{34}, ST13798^{23B}, and ST13799^{21}) were also identified (Figure 4.2 and Table S4.2).

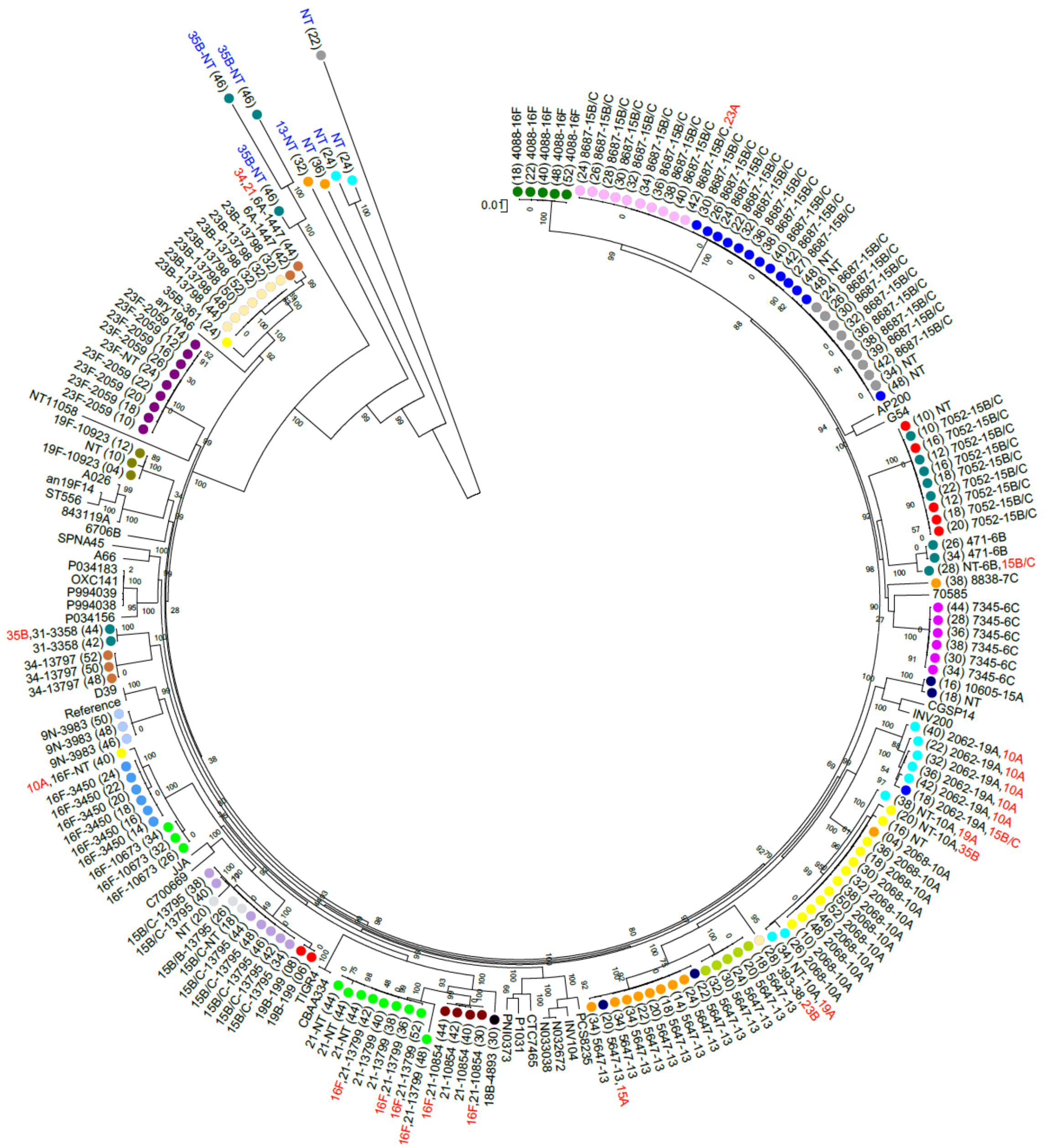

Figure 4.2: A maximum likelihood whole genome phylogenetic tree of pneumococcal isolates recovered from 23 infants. Bootstrap values are shown on each branch and the scale bar represents the number of single-nucleotide polymorphisms (SNPs). Circles with the same colour represent longitudinal samples from the same infant. Numbers in brackets indicate the age in weeks at each time-point. The sequence type (ST) detected in each sample is shown as a number, followed by the associated serotype. Serotypes indicated in red were among co-colonizing strains and had genomes with lower coverage than the other co-detected strain. Five out of 15 samples that had low reads mapping to pneumococcal genomes are indicated in blue text. NT- Non-typeable. Completed pneumococcal genomes available on the NCBI database were included and the R6 genome was used as a reference. The samples clustered according to ST but not serotype. Persistent colonization with the same genotype was common.

4.3.4 Antimicrobial resistance determinants

Phenotypic antibiotic susceptibility profiles were determined for the 159 pneumococcal isolates obtained from samples included for this analysis.[36] Overall, 18% (29/159), 17% (28/159), and 61% (98/159) of the isolates were non-susceptible to penicillin, erythromycin, and cotrimoxazole respectively.

Using *in-silico* analyses, a total of 48 acquired antimicrobial resistance (AMR) genes were identified from pneumococcal contigs in 20 samples collected from 10 infants (Figure 4.3). The AMR genes detected included macrolide-lincosamide-streptogramin B resistance (MLS_B) (*msr*D, *mef*A and *erm*B), and tetracycline resistance (*tet*M) genes (Figure 4.3). The average sequence length coverage across the reference genes was 99% (range, 81-100%) while the average sequence depth was 160X (range, 1-411X).

Phenotypic non-susceptibility to erythromycin was detected in 18% (28/159) of isolates (Figure 4.3). Among the 28 samples from which these isolates were obtained, genes predicted to confer macrolide non-susceptibility were detected in 39% (11/28) of samples [*msr*D and *mef*A (n = 9), *mef*A (n = 1), and *erm*B (n = 1)] from three infants. In addition, macrolide resistance genes were detected in a further six samples (from five infants) that were culture negative for pneumococci (Figure 4.3); two of these samples had co-colonization with multiple serotypes.

Pneumococcal *pbp* genes were analysed for mutations associated with beta-lactam resistance. No indels were identified in any of the extracted *pbp* gene sequences, but multiple amino acid changes were identified at various positions within the transpeptidase domains. Pneumococcal isolates with penicillin MICs between 0.064 and 8.0 µg/ml commonly carried mutations within the transpeptidase domains of the *pbp*1a and *pbp*2b genes, but not in *pbp*2x (Figure S4.1), compared to the wild-type pneumococcal strain R6. *Pbp* point mutations known to contribute to beta-lactam resistance are shown in Table S4.3. The S351A and P432T point mutations (known to confer beta-lactam resistance) which are

within or close to the conserved motifs of the *pbp*1a gene were identified in penicillin-non-susceptible ST7052$^{15B/15C}$ (n = 9), ST361^{35B} (n = 1), and ST2068^{10A} (n = 1) isolates from four infants (Figure 4.3 and Table S4.3). Amino acid substitution H394L (known to confer beta-lactam resistance), in the *pbp*2x gene, was only identified among PCV13 serotypes 23F (n = 9), 19A (n = 4), and 6A (n = 2), from four infants, irrespective of penicillin susceptibility (Table S4.4). These serotypes (23F, 19A, and 6A) had identical amino acid sequences of the transpeptidase domain of the *pbp*2x gene despite having different STs (Figure S4.3). Pneumococcal strains encountered in this study had a higher divergence in the transpeptidase domain sequences of the *pbp*1a and *pbp*2x genes than in the *pbp*2b gene (Figure S4.2, S4.3, and S4.4).

Ninety-eight out of 159 (62%) isolates were phenotypically non-susceptible to cotrimoxazole (Figure 4.3). The *fol*A (I100L) and *fol*P (6-bp insertion in the region encoding amino acid 58 to 67) gene mutations, known to confer resistance to cotrimoxazole,[37,38] were detected in 89% (87/98) of the samples from which the non-susceptible isolates were obtained (Figure 4.3 and Table S4.4). Combinations of resistance mutations including I100L plus $R_{59}P_{60}$ (n = 45), and I100L plus $S_{62}Y_{63}$ (n = 24) were commonly detected (Table S4.4).

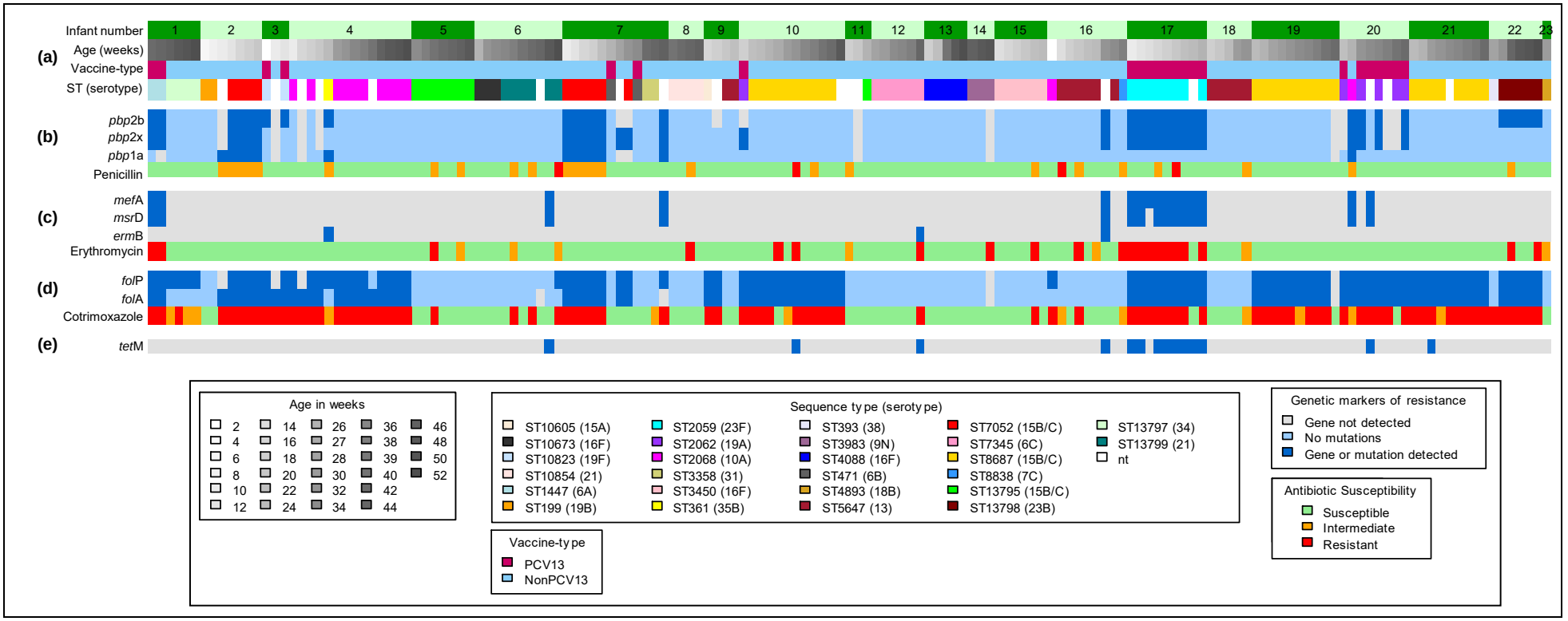

Figure 4.3: Shotgun sequence-derived molecular sequence type and resistome (from direct sequencing of NP swabs), and phenotypic antimicrobial susceptibility results (from pneumococcal isolates) in 159 out of 196 NP samples obtained from 23 infants (assigned 1 to 23, top row). (a) *In-silico* sequence types (STs) and associated serotypes detected in longitudinal NP samples from each infant. (b) *pbp* gene mutations and associated penicillin phenotypic susceptibility profiles; light blue colour indicates wild-type *pbp* genes; the dark blue colour indicates detected mutations within or close to the conserved motifs for the pbp genes. (c) Macrolide-resistance genes and associated erythromycin phenotypic susceptibility profiles; the dark blue colour indicates detected resistance genes. (d) *fol*A and *fol*P gene mutations and associated cotrimoxazole phenotypic susceptibility profiles; light blue colour indicates wild-type *fol*A and *fol*P genes; dark blue colour indicates detected mutations, in *fol*A and *fol*P, that reduce the affinity of trimethoprim or sulfamethoxazole. (e) Detected tetracycline resistance gene; dark blue colour indicates detected resistance gene. Grey colour in (b) to (e) indicates that no genes were detected.

4.4 Discussion

This study explored the use of shotgun metagenomic sequencing as an alternative approach to culture-based testing to study pneumococcal NP colonization and associated antimicrobial resistance determinants, in a South African birth cohort. We were able to derive pneumococcal serotypes and sequence types and to identify co-colonisation and antimicrobial resistance determinants directly from shotgun sequence data. Since NP samples from apparently healthy individuals generally have low numbers of bacterial cells, we used short-term broth enrichment, which has previously been shown to successfully enrich streptococci.[28]

There was complete concordance between detection of pneumococcal sequences and positive culture for pneumococci. However, of the 174 samples sequenced, which were also culture positive, 15 samples produced poor sequence read mapping to the reference pneumococcal genomes, despite good overall read counts. These 15 samples were excluded from further analyses as they are likely other *Streptococcus species*. There was good correlation (86%) between conventional typing methods and shotgun sequencing in assigning pneumococcal serotypes. Discordant serotype results were predominantly from samples where only one serotype was identified by shotgun sequencing, and were therefore not due to detectable co-colonization. Both Quellung and sequetyping are not infallible in assigning pneumococcal serotypes.[21] Shotgun metagenomic sequencing produced robust serotyping results, based on sequence coverage and depth, but this technique is, at present, relatively expensive, time consuming, and computationally intensive for routine typing.[39] Whole-genome sequencing of cultured pneumococcal isolates produces reliable serotyping results and can generate additional genetic information, but it is mostly performed on single isolates, and may thus fail to detect co-colonization, or exclude samples with non-viable bacteria.[40] On the other hand, microarray techniques which may or may not require a culture-enrichment step has been shown to have the potential to detect multiple serotypes within a sample, however, this technique can only detect serotypes included on the array.[41]

Eleven percent (17/159) of the samples could not be assigned a multilocus sequence type due to a lack of resolution at all the loci necessary for typing. This was frequently observed in samples where co-colonization with multiple serotypes was detected, or in samples with low estimated sequencing coverage.[28] In samples where sequence types were assigned, we observed a strong association between certain pneumococcal serotypes and multilocus sequence types. However, serotypes 15B/15C (ST7052, ST8687, and ST13795) and 16F (ST4088 and ST3450/ST10673) were associated with multiple STs. Serotypes 15B/15C and 16F were the predominant serotypes detected in our cohort, and highly prevalent serotypes tend to be more diverse.[42] No other ST in our collection was associated with more than one serotype, in line with previous observations.[43]

Shotgun sequencing detected co-colonization with multiple serotypes in 15% of the samples; these would have been difficult or laborious to detect when using Quellung method, since multiple colonies would have to be tested individually, or pooled colonies tested with multiple reagents.[40] This rate of co-colonization was lower than that reported in Malawi among children aged 0 – 13 years (40%, 46/116),[44] but comparable to that reported among children less than 2 years of age in Tehran (17%, 225/1302).[45] In the current study, serotype 19A isolates (from two infants) were only detected using shotgun sequencing (and not culture), in samples with multiple serotypes. Detection of circulating serotype 19A is important in epidemiological studies, since 19A is included in the PCV13 administered to infants. Culture-dependent techniques are biased to detect the most abundant serotype in a sample and are likely to miss co-colonization with less abundant serotypes.[46] Carriage of multiple pneumococcal serotypes is also important since it provides an opportunity for horizontal gene transfer, which is one of the most common mechanisms driving pneumococcal evolution.[47]

To date, 11 STs identified in this study, including ST2059^{23F}, ST2062^{19A}, and ST10823^{19F}, matched other STs which have only been described among isolates from South Africa (https://pubmlst.org), and may therefore be unique to South Africa. These strains are

associated with serotypes included in the PCV13, which is currently administered in the South African routine immunization schedule, and which all infants in this study received. Five STs (ST2068[10A], ST3450[16F], ST3358[31], ST199[19B], and ST393[38]) identified here are reported for the first time in an African country (https://pubmlst.org). Additionally, four novel STs were identified, namely: ST13795[15B/15C], ST13797[34], ST13798[23B], and ST13799[21].

The AMR genes identified by pneumococcal resistome analysis were *msr*D, *mef*A, *erm*B, and *tet*M. Macrolide-resistance genes (*msr*D, *mef*A, or *erm*B), predicted to confer resistance, were detected in 11/28 NP samples with erythromycin-non-susceptible pneumococci. Acquired AMR genes were not detected in the remaining samples, and the resistance observed could be due to other mechanisms of macrolide resistance not investigated here.[48] The frequency of *msr*D or *mef*A was higher than that of *erm*B, and this is in contrast to what has been reported in other studies, including South Africa.[49,50] *Tet*M gene was detected in 8 samples but this could not be associated with phenotypic resistance as isolates in this study were not tested for susceptibility to tetracycline, since it is not routinely used to treat pneumococcal infections.

A proportion of 62% (98/159) of cotrimoxazole non-susceptibility was observed in pneumococcal isolates contained in samples included for shotgun sequencing. Cotrimoxazole, which is a combination of trimethoprim and sulfamethoxazole, inhibits folic acid biosynthesis, and non-susceptibility to this drug is conferred by the acquisition of mutations in *fol*A and *fol*P.[37] The majority of cotrimoxazole-non-susceptible isolates (81%) in the current study possessed the I100L amino acid substitution in DHFR, and this mutation has been shown to be sufficient to confer high-level cotrimoxazole resistance.[37,38] The most common insertions detected in DHPS led to the duplication of $R_{58}P_{59}$ and $S_{62}Y_{63}$ in 56% and 27% of cotrimoxazole-non-susceptible isolates, respectively. These insertions have been shown to confer low-level cotrimoxazole resistance.[37,38] Most instances (71/86) of high-level cotrimoxazole resistance observed were due to both *fol*A I100L substitution and *fol*P insertion, and this has been previously described.[51] Lack of associations in other isolates

might be due to other mechanisms of resistance or loss of expression of the detected mutations.[51]

Pbp genes code for the penicillin-binding proteins (PBPs) which are essential for cell envelope bio-synthesis and are the target for beta-lactam antibiotics.[52] Gene mutations occurring within or close to the *pbp*-conserved motifs within the transpeptidase domain are known to confer resistance to beta-lactams.[53] Amino acid alterations in *pbp*1a, *pbp*2b, and *pbp*2x have been shown to be the most reliable markers for beta-lactam-resistance in pneumococci.[54] A higher level of variation in the transpeptidase domain sequences of *pbp*1a and *pbp*2x, than in *pbp*2b was observed (Figure S4.2, S4.3, and S4.4). The *pbp*1a and *pbp*2x genes flank the capsule (*cps*) locus, which is prone to frequent recombination events,[55] and recombination events involving the *cps* locus and one or both *pbp*1a and/or *pbp*2x genes have been observed.[56] This could account for the higher level of variation observed in the *pbp*1a and *pbp*2x genes among the strains here.

In this study, penicillin non-susceptibility was observed mainly in isolates carrying the P432T (in *pbp*1a) and T338P (in *pbp*2x) mutations. The P432T and T338P mutations were detected in all 9 penicillin-non-susceptible, ST7052$^{15B/15C}$ isolates, from two infants. The P432T mutation, which is close to the ^{428}SRN^{430} conserved motif,[54] and the T338P mutation, occurring within the active ^{337}STMK^{340} motif,[57] decrease beta-lactam-binding affinity of PBP1a and PBP2x, respectively. The contribution of other mutations to penicillin non-susceptibility among our strains was unclear (Table S4.3).

This study was limited to a small sample size and the use of metagenomic sequencing in samples obtained from a larger population is warranted. NP samples were enriched using broth without antibiotics and this may have favoured other bacteria more than pneumococci thus leading to inability to assemble the pneumococcal genomes in samples with low sequence reads. The description of antibiotic use and detection of antibiotic resistance genes has bee described in Chapter 5 and no intensive analyses were done due to small sample size, and incomplete data on antibiotic usage in this cohort.

Using shotgun sequencing, we were able to derive pneumococcal serotype and sequence type, and to detect co-colonisation, and antimicrobial resistance determinants directly from enriched NP samples. Direct shotgun sequencing from enriched NP samples is therefore a promising technique for detailed evaluation of the pneumococcal component of the NP microbiome, and its use should be explored similarly for other bacteria in this niche.

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Supplementary materials

Table S4.1: Comparison between shotgun sequencing and conventional methods for pneumococcal serotyping

No. of infants	Detected pneumococcal serotypes (no. of samples)		Comment
	Shotgun sequencing	Conventional methods	
3	13 (13)	13 (11)	Concordant
		23A (1)	x
		16F (1)	x
2	21 (5)	21 (4)	Concordant
		16F (1)	x
1	31 (1)	31 (1)	Concordant
1	34 (4)	34 (2)	Concordant
		21 (1)	x
		16F (1)	x
3	10A (12)	10A (11)	Concordant
		22F (1)	x
1	15A (1)	15A (1)	Concordant
7	15B/15C (45)	15B/15C (42)	Concordant
		10A (1)	x
		35B/C (1)	x
		nt (1)	x
3	16F (14)	16F (11)	Concordant
		9V (2)	x
		6C (1)	x
1	18B (1)	18B (1)	Concordant
1	19B (2)	19B (2)	Concordant
1	19F (2)	19F (2)	Concordant
1	23B (5)	23B (3)	Concordant
		11A (1)	x
		nt (1)	x
1	23F (9)	23F (1)	Concordant
		9V (6)	x
		10A (1)	x
		13 (1)	x
2	35B (2)	35B (2)	Concordant
1	6A (1)	6A (1)	Concordant
1	6B (2)	6B (2)	Concordant
1	6C (6)	6C (6)	Concordant
1	7C (1)	7C (1)	Concordant
1	9N (3)	9N (3)	Concordant
1	19A, 10A (7)	10A (6)	Concordant
		19A (1)	Concordant
1	6A, 21, 34 (1)	6A (1)	Concordant
1	10A, 35B (1)	19C (1)	x
1	10A, 16F (1)	16F (1)	Concordant
2	21, 16F (6)	21 (3)	Concordant
		16F (3)	Concordant
1	6B, 15B/15C (2)	Nt (2)	x
1	31, 35B (1)	35B (1)	Concordant
1	13, 15A (1)	13 (1)	Concordant

1	15B/15C, 23A (1)	23A (1)	Concordant
1	23B, 38 (1)	23B (1)	Concordant
1	19A, 15B/15C (1)	19A (1)	Concordant
7	nt (7)	*	x
12	Negative[1] (22)	Negative[2] (22)	Concordant

* 15B/15C (4), 10A (1), 19F (1), and 15A (1). [1] No pneumococcal reads detected, [2] Culture-negative for pneumococcus. (x) Discordant results. Conventional and metagenomic-assigned serotypes were considered concordant if the serotype detected by conventional methods was among the co-colonizing serotypes.

Table S4.2: Distribution of *in-silico* serotypes, associated STs, and MLST profiles.

Serotype	Sequence type (no. of samples)	No. of infants	MLST allelic profile							Other serotypes associated with the ST described[1]
			*aro*E	*gdh*	*gki*	*rec*P	*spi*	*xpt*	*ddl*	
15B/15C	ST8687 (28)	3	1	32	97	5	10	88	18	6A
	ST7052 (10)	2	7	43	47	16	6	14	17	none
	ST13795* (8)	2	2	13	14	1	17	4	14	none
13	ST5647 (13)	3	15	5	54	1	197	1	168	none
10A	ST2068 (12)	3	2	40	4	19	10	1	27	none
23F	ST2059 (8)	1	15	29	4	21	30	27	1	6A, 19A, 19F
19A	ST2062 (6)	2	1	5	53	32	14	20	199	none
16F	ST3450 (6)	1	1	5	14	5	15	1	1	none
	ST4088 (5)	1	50	8	1	16	6	88	14	none
	ST10673 (3)	1	1	5	14	4	15	1	1	none
6C	ST7345 (6)	1	1	10	9	43	15	1	17	none
21	ST13799* (6)	1	50	10	2	16	1	834	1	none
	ST10854 (4)	1	8	10	2	138	1	26	1	none
23B	ST13798* (5)	1	7	608	8	6	25	6	8	none
34	ST13797* (4)	1	12	5	54	38	27	14	18	none
9N	ST3983 (3)	1	8	5	7	12	15	16	6	9V
19B	ST199 (2)	1	8	13	14	4	17	4	14	19A, 15B/15C, 3
6A	ST1447 (2)	1	6	13	8	6	25	6	8	23F, 35B
31	ST3358 (2)	1	1	2	29	1	43	14	8	none
19F	ST10823 (2)	1	10	25	9	10	6	4	168	none
6B	ST471 (2)	1	7	25	4	4	15	1	28	6A
35B	ST361 (1)	1	7	13	8	6	6	6	8	1, 23F, 6A, 6B, 19A, 23B
38	ST393 (1)	1	10	43	41	18	13	49	6	25A, NT
18B	ST4893 (1)	1	2	5	2	16	1	26	1	18C
7C	ST8838 (1)	1	7	8	146	15	25	20	5	none
15A	ST10605 (1)	1	2	18	6	10	17	12	19	none

[1] Other serotypes associated with sequence types described in this study as listed in the PubMLST global database. * Novel STs. MLST profile could not be assigned for serotype 23A, which is not shown in the table.

Table S4.3: Deduced amino acid substitutions in penicillin-binding proteins (PBPs)

Infant no.[1]	Sequence type	Serotype	Penicillin phenotype	Amino acid substitution at position:[a]									
				PBP1a			PBP2x			PBP2b			
				351	371	432	338	394	546	451	614	624	629
Ref	R 6		S	S	T	P	T	H	L	T	L	A	A
	NT	NT	I	A	.	T	x	x	x	x	x	x	x
	ST7052	15B/15C	I	A	.	T	P	.	.	A	A	.	N
2	ST7052	15B/15C	I	A	.	T	P	.	.	A	A	.	N
	ST7052	15B/15C	I	A	.	T	P	.	.	A	A	.	N
	ST7052	15B/15C	I	A	.	T	P	.	.	A	A	.	N
	ST7052	15B/15C	I	A	.	T	P	.	.	A	A	.	N
	ST7052	15B/15C	I	A	.	T	P	.	.	A	A	.	N
7	ST7052	15B/15C	I	A	.	T	P	.	.	A	A	.	N
	ST7052	15B/15C	I	A	.	T	P	.	.	A	A	.	N
	ST7052	15B/15C	I	A	.	T	P	.	.	A	A	.	N
	nt	35B	S	A	A	T	A	.	V	A	T	G	.
	nt	15B/15C	S	x	x	x	P	.	.	x	x	x	x
	ST7052	15B/15C	S	x	x	x	P	.	.	x	x	x	x
4	ST361	35B	I	A	.	T	.	.	.	A	S	.	E
1	ST1447	6A	S	.	.	.	.	L	.	A	S	.	E
	ST1447	6A	S	x	x	x	.	L	.	A	S	.	E
3	ST10823	19F	S	.	.	.	.	.	.	A	.	.	.
	ST10823	19F	S	.	.	.	.	.	.	A	.	.	.
	ST2059	23F	S	.	.	.	.	L	.	A	.	.	.
	ST2059	23F	S	.	.	.	.	L	.	A	.	.	.
	ST2059	23F	S	.	.	.	.	L	.	A	.	.	.
17	ST2059	23F	I	.	.	.	.	L	.	A	.	.	.
	ST2059	23F	S	.	.	.	.	L	.	A	.	.	.
	ST2059	23F	R	.	.	.	.	L	.	A	.	.	.
	ST2059	23F	S	.	.	.	.	L	.	A	.	.	.
	nt	23F	S	.	.	.	.	L	.	A	.	.	.
	ST2059	23F	S	.	.	.	.	L	.	A	.	.	.
10	ST2062	19A	S	.	.	.	.	L	.	x	x	x	x
20	ST2068	10A	I	A	S	T	A	.	V	A	.	.	.
	ST2062	19A	S	.	.	.	.	L	.	A	.	.	.
	ST2062	19A	S	.	.	.	.	L	.	A	.	.	.
	ST2062	19A	S	.	.	.	.	L	.	A	.	.	.
22	ST13798	23B	S	.	.	.	.	.	.	A	S	.	E
	ST13798	23B	I	.	.	.	.	.	.	A	S	.	E
	ST13798	23B	S	.	.	.	.	.	.	A	S	.	E
	ST13798	23B	S	.	.	.	.	.	.	A	S	.	E
	ST13798	23B	S	.	.	.	.	.	.	A	S	.	E
16	nt	13	S	.	.	.	A	.	V	A	T	G	.

[1] Assigned infant number. Only mutations at positions within or close to the conserved motifs in the transpeptidase domain in PBP1a (^{370}STMK^{373}, ^{428}SRN^{430}), PBP2x (^{337}STMK^{340}, ^{395}SSN^{397}, ^{547}KSG^{549}), and PBP2b (^{448}SSN^{450}, ^{619}KTG^{621}) are shown. [a] Identity with the amino acid from *S. pneumoniae* strain R6 is indicated by the dot. (x)- The gene was not detected. Pen- penicillin, Ery- erythromycin, Sxt- cotrimoxazole, NT- non-typable, S- Susceptible, I- Intermediate, R- Resistant.

Table S4.4: Amino acid variation of DHFR (*fol*A) and DHPS (*fol*P) associated with cotrimoxazole non-susceptibility

Cotrimoxazole phenotype (no. of isolates)	No. of isolates (no. of infants)	Amino acid mutations	
		*fol*A substitution	*fol*P insertion
Resistant (n = 86)			
	45 (8)	I100L	$R_{58}P_{59}$
	24 (4)	I100L	$S_{62}Y_{63}$
	2 (1)	I100L	$S_{61}S_{62}$
	3 (3)	I100L	-
	1 (1)	I100L	wt
	2 (2)	wt	$R_{58}P_{59}$
	1 (1)	wt	$S_{62}Y_{63}$
	1 (1)	-	$R_{58}P_{59}$
	7 (6)	wt	wt
Intermediate (n = 12)			
	3 (2)	I100L	$R_{58}P_{59}$
	1 (1)	I100L	$S_{62}Y_{63}$
	4 (2)	wt	$R_{58}P_{59}$
	4 (3)	wt	wt
Susceptible (n = 5)			
	3* (2)	I100L	$S_{62}Y_{63}$
	2 (2)	I100L	$R_{58}P_{59}$

Wt –Wild type gene, * Samples had co-colonization with multiple serotypes.

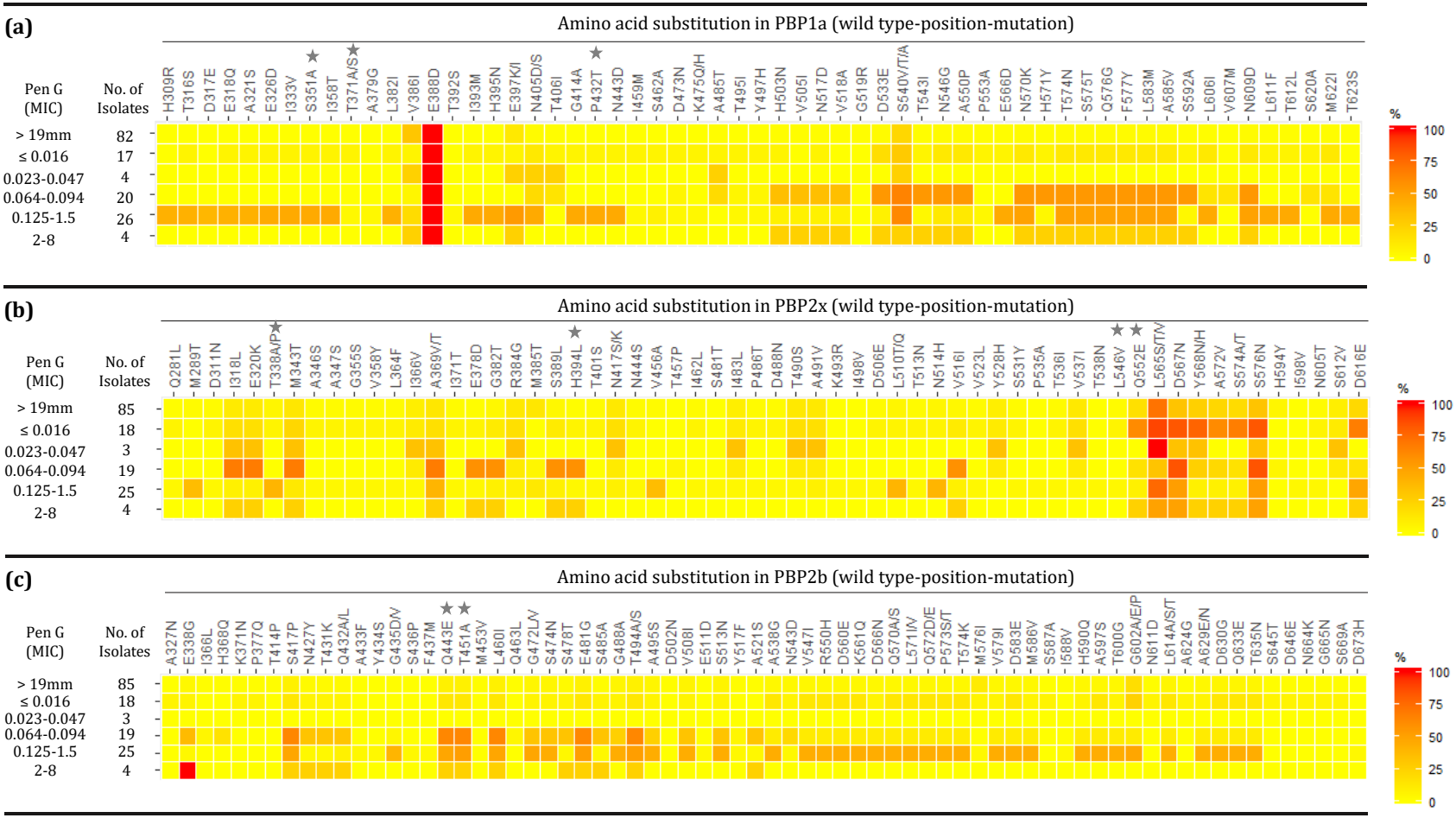

Figure S4.1: Codons with mutations within the transpeptidase domains of penicillin-binding proteins (PBPs). (a) PBP1a; (b) PBP2x; (c) PBP2b. Only amino acids that differ from the R6 wild-type strain (GenBank accession no. AE007317.1) are shown. Mutations at positions within or close to each of the conserved penicillin-binding motifs (^{370}STMK^{373} and ^{428}SRN^{430} in PBP1a, ^{337}STMK^{340}, ^{395}SSN^{397}, and ^{547}KSG^{549} in PBP2x, and ^{448}SSN^{450} in PBP2b) are indicated by the asterisks.

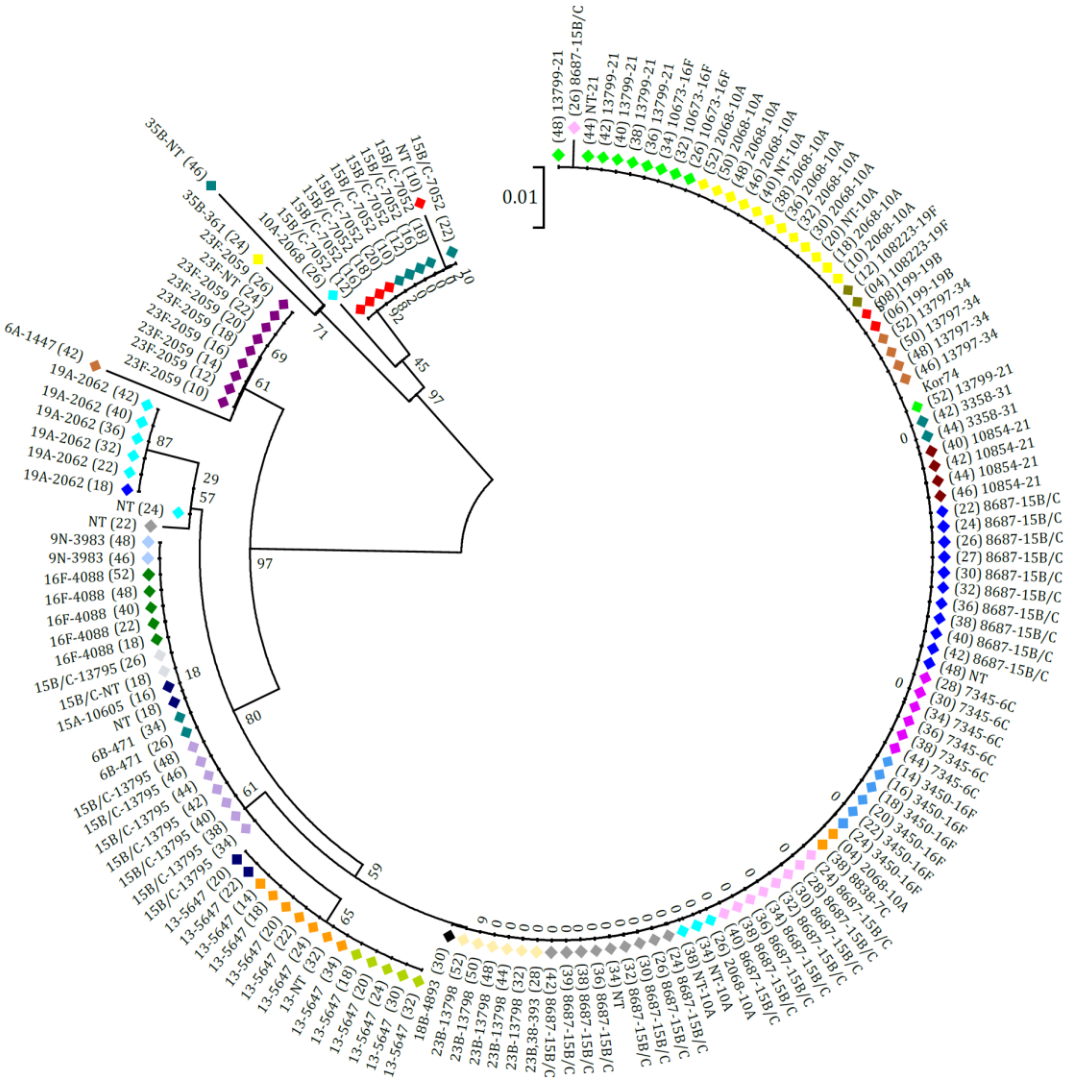

Figure S4.2: Phylogenetic tree, based on the transpeptidase domain of the *pbp*1a gene constructed, by MEGA using the neighbour-joining method. Bootstrap values (percentage) are based on 1,000 replications. The scale bar represents the genetic divergence as calculated by the MEGA software. Same colour represents the longitudinal samples from the same infant. The number in brackets indicate the age in weeks at each time-point. The ST detected in each sample is shown as a number, followed by the associated serotype.

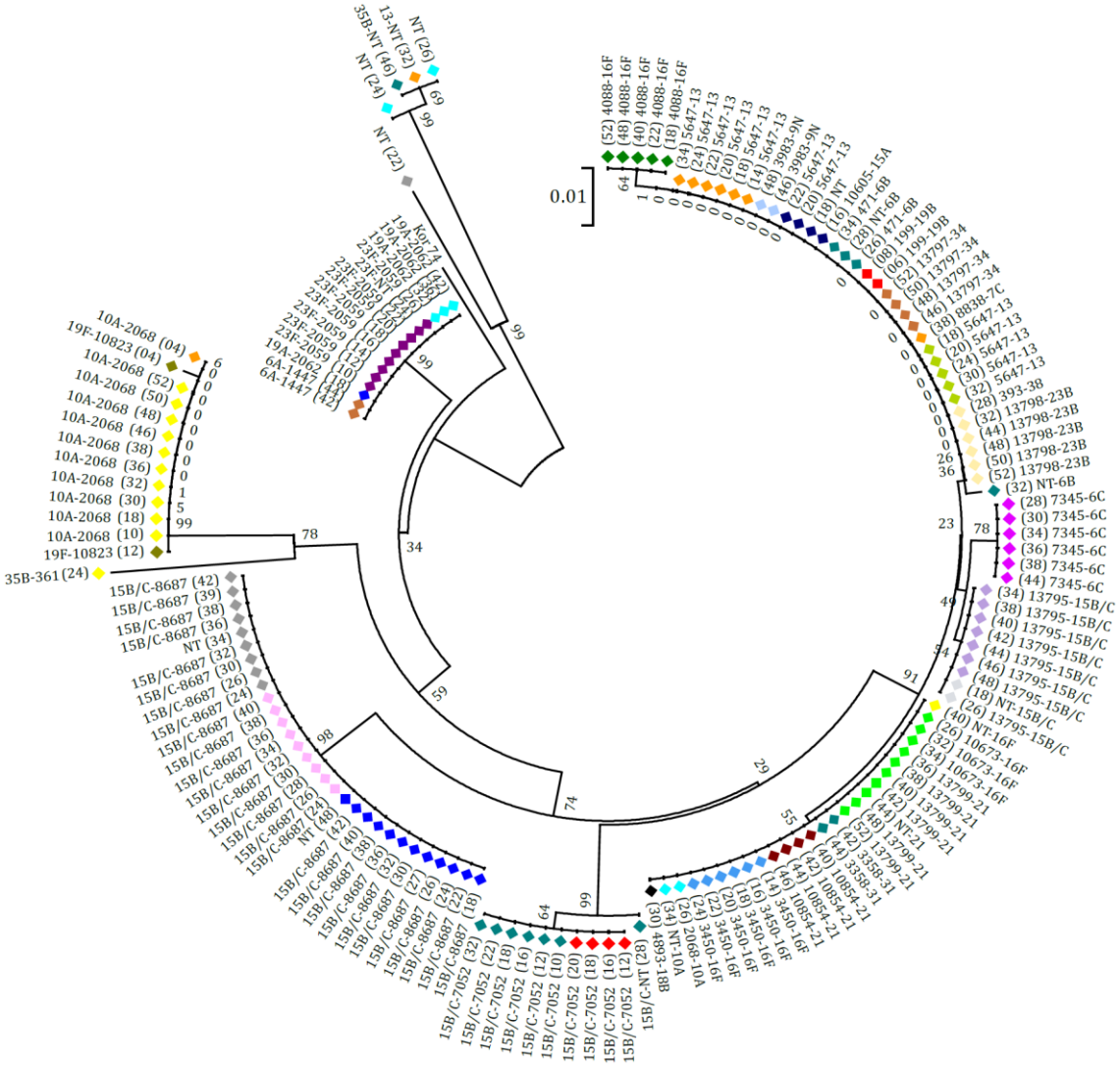

Figure S4.3: Phylogenetic tree, based on the transpeptidase domain of the *pbp*2x gene, constructed by MEGA using the neighbour-joining method. Bootstrap values (percentage) are based on 1,000 replications. The scale bar represents the genetic divergence as calculated by the MEGA software. Same colour represents the longitudinal samples from the same infant. The number in brackets indicate the age in weeks at each time-point. The ST detected in each sample is shown as a number, followed by the associated serotype.

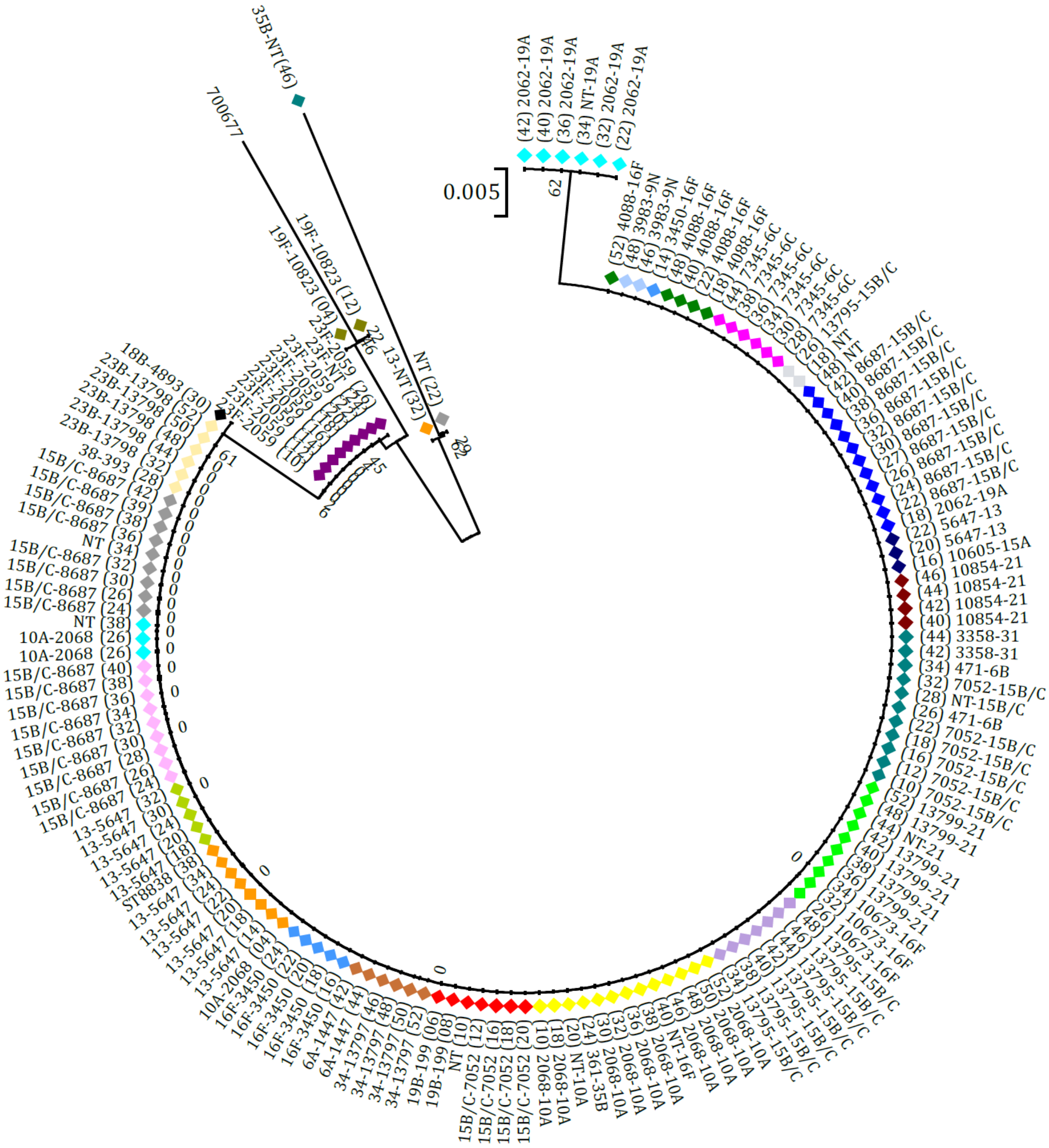

Figure S4.4: Phylogenetic tree, based on the transpeptidase domain of the *pbp*2b gene, constructed by MEGA using the neighbour-joining method. Bootstrap values (percentage) are based on 1,000 replications. The scale bar represents the genetic divergence as calculated by the MEGA software. Same colour represents the longitudinal samples from the same infant. The number in brackets indicate the age in weeks at each time-point. The ST detected in each sample is shown as a number, followed by the associated serotype.

CHAPTER 5

Longitudinal changes in the nasopharyngeal antibiotic resistome in South African infants using shotgun metagenomic sequencing

Summary

Nasopharyngeal (NP) colonization with antimicrobial-resistant bacteria is a global public health concern. Antimicrobial resistance (AMR) genes carried by the resident NP microbiota may serve as a reservoir for transfer of resistance elements to opportunistic pathogens. Little is known about the NP antibiotic resistome. This study longitudinally investigated the composition of the NP antibiotic resistome in *Streptococcus*-enriched samples in a South African birth cohort. NP swabs were obtained fortnightly from 137 infants from birth through the first year of life. Samples were cultured for pneumococci and isolates serotyped and antimicrobial susceptibility profiles determined. 196 longitudinal NP samples from a subset of 23 infants were selected based on changes in serotype and antibiogram over time. NP samples underwent short-term enrichment for streptococci, and total nucleic acid was extracted for whole metagenome shotgun sequencing (WMGS). Reads were assembled and aligned to pneumococcal reference genomes for the extraction of pneumococcal and non-pneumococcal bacterial reads. Contigs were aligned to the Antibiotic Resistance Gene-ANNOTation database of acquired AMR genes. A total of 329 AMR genes were detected in 64% (125/196) of the NP samples, including 36 non-redundant genes, ranging from 1 to 14 genes per sample. The predominant AMR genes detected encoded resistance mechanisms to beta-lactam (52%, 172/329), macrolide-lincosamide-streptogramin (17%, 56/329), and tetracycline antibiotics (12%, 38/329). *Msr*D, *erm*B, and *mef*A genes were only detected from pneumococcal reads. The predominant genes detected from non-pneumococcal reads included $bla_{\text{OXA-60}}$, $bla_{\text{OXA-22}}$, and $bla_{\text{BRO-1}}$. Different patterns of carriage of AMR genes were observed, with only one infant demonstrating stable carriage of *mef*A, *msr*D, and *tet*M over a long period. This study demonstrates that WMGS can provide a broad snapshot of the NP resistome and has the potential to provide a comprehensive assessment of all resistance elements present in this niche.

5.1 Introduction

Infection with antibiotic-resistant bacteria is a major public health concern due to the limited availability of new treatment options.[1] Increasing antibiotic resistance has been noted in respiratory tract commensal bacteria which are capable of causing life-threatening infections.[2,3] The upper respiratory tract, including the nasopharynx, is the reservoir for many respiratory pathogens and may also serve as a source for the horizontal transfer of antimicrobial resistance (AMR) genes from non-pathogenic to pathogenic bacteria.[4]

Commensal and potentially pathogenic bacteria which commonly colonize the upper airways include *Streptococcus pneumoniae* (the pneumococcus), *Staphylococcus aureus*, *Haemophilus influenzae*, and several Gram-negative bacilli.[5–7] The pneumococcus and *H. influenzae* are among the leading causes of bacterial respiratory tract infections in young children.[8,9] Asymptomatic NP carriage of pneumococci is prevalent among infants and often precedes the development of disease.[4,10] Drug-resistant pneumococci may cause difficult-to-treat infections, associated with increased morbidity and mortality.[4,10] In many cases, antibiotic resistance results from of horizontal gene transfer (HGT) of a mobile genetic element, or the uptake of free DNA from the surrounding environment,[11,12] which is of particular importance as pneumococci are naturally competent, and can therefore readily take up exogenous DNA.[13]

Culture-based methods only allow for the detection of certain AMR genes in viable, culturable bacteria, and are therefore unable to completely characterise the resistome in a particular niche.[14] An alternative approach for the detection of all AMR genes is whole metagenome shotgun sequencing (WMGS) of the total DNA extracted directly from samples.[15,16] The majority of WMGS studies of the antibiotic resistome have focused on the human gut resistome,[16–20] however to the best our knowledge there are no published studies of the NP antibiotic resistome.

We have previously reported changes in NP pneumococcal antibiotic resistance in infants studied longitudinally over the first year of life, using culture-based susceptibility testing.[21] Here, we further characterise a subset of these samples using WMGS, to demonstrate proof of concept for resistome analysis of upper respiratory tract samples.

5.2 Material and Methods

This study was nested within a longitudinal birth-cohort study, the Drakenstein Child Health Study (DCHS).[22] The first 137 infants enrolled for the DCHS were selected and had NP swabs collected every second week from birth through the first year of life. The collected NP swabs were immediately placed into 1 ml skim milk-tryptone-glucose-glycerol (STGG) medium. The NP sample collection, storage, and culture for pneumococci have previously been described.[23] The study was approved by the Faculty of Health Sciences Human Research Ethics Committee of the University of Cape Town (reference numbers: 235/2016 and 401/2009) and informed consent obtained from all mothers.

A total of 196 NP samples from a subset of 23 out of the 137 infants were purposively selected for shotgun metagenomic sequencing. The selection of NP samples was based on changes in pneumococcal serotype or in penicillin, and/or erythromycin susceptibility profiles over time.[21] The NP-STGG samples were enriched as previously described, with minor modifications.[24] Briefly, 200 µl of an NP-STGG sample was transferred to 6 ml Todd-Hewitt Broth (without antibiotics), containing 0.5% yeast extract and 17% fetal bovine serum. The broth was incubated at 37°C with 5% CO_2, without shaking for 6 hours, then centrifuged at 9000 rpm for 10 minutes at 4°C. Total nucleic acid extraction was performed on the collected pellet using the QIAsymphony SP automated platform (Qiagen, Hilden, Germany) with the QIAsymphony Virus/Bacteria Mini Kit (Cat. No. 931036) following the manufacturer's instructions. Nucleic acid concentrations and purity were determined using the NanoDrop® ND-100 (Thermo Fishers Scientific, Waltham, USA).

Total nucleic acid was subjected to shotgun sequencing on the MiSeq platform using the MiSeq Reagent Kit v3 (600-cycle) (Illumina, San Diego, USA) at the J. Craig Venter Institute, Rockville, USA. Metagenomic DNA sequencing and assembly protocols have previously been described.[24] Reads were assembled using metaSPAdes,[25] and aligned to a database containing *Streptococcus pneumoniae* complete genomes in order to re-construct the pneumococcal genomes and extract all the pneumococcal contigs. Bacterial contigs not mapping to pneumococcal genomes were regarded as non-pneumococcal contigs and were separately extracted for further analysis.[24]

Screening for AMR genes present in the selected NP samples was performed on the assembled contigs for both pneumococcal and non-pneumococcal contig datasets. Contigs were aligned to the Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) database of acquired AMR genes. To increase sensitivity for identifying novel genes or genotypes with low levels of similarity to the reference genes, less stringent criteria were used.[26] A sequence with ≥ 90% identity,[27] with an alignment coverage length of ≥ 25% to the reference gene sequence was designated as an AMR gene.[26] The AMR genes were manually confirmed.

Statistical analyses were performed using STATA (Stata Corporation, College Station, TX). Chi-square and Fisher's exact tests were used to compare the differences in the proportion of samples with AMR genes. A *p*-value of <0.05 was considered statistically significant.

5.3 Results

5.3.1 Participants and metagenomic sample characteristics

A total of 196 longitudinal NP samples were selected from 23 infants, with an average of 9 selected NP samples per infant (range, 4 – 21 samples). The age at which the NP samples were collected spanned the first year of life with an average of 15 weeks. Four of the 23 infants were born via caesarean section (Table 5.1). Eight infants were born to HIV infected mothers, but none of the infants were infected. Antibiotics were administered to 6 out of 7

infants who had severe or non-severe lower respiratory tract infection (LRTI) during the first year of life (ages, 0 – 52 weeks) (Table 5.1).

Table 5.1: Clinical characteristics of the participants

Infant no.	Mode of delivery	HIV exposed	LRTI case, age (weeks)	Age antibiotic(s) given (weeks)	Antibiotic for LRTI treatment (days)	Admission for suspected LRTI
1	Normal vaginal	No	0	0	Erythromycin-PO (7 days) Amikacin-IV (5 days) Cefotaxime-IV (4 days) Gentamycin-IV (3 days) Meropenem-IV (3 days)	-
2	Normal vaginal	No	8	8	Amoxicillin-PO (5 days)	-
3	Normal vaginal	No	-	-	-	-
4	Normal vaginal	No	-	-	-	Ambulatory
5	Emergency Caesarean section	No	-	-	-	Ambulatory
6	Normal vaginal	Yes	-	-	-	Both
7	Normal vaginal	Yes	-	-	-	Unknown
8	Elective Caesarean section	No	-	-	-	-
9	Normal vaginal	No	-	-	-	-
10	Normal vaginal	No	25	28	Amoxicillin-PO	Ambulatory
11	Normal vaginal	Yes	-	-	-	Ambulatory
12	Normal vaginal	Yes	8	-	none	Ambulatory
13	Normal vaginal	Yes	-	-	-	-
14	Normal vaginal	No	-	-	-	Ambulatory
15	Normal vaginal	No	-	-	-	-
16	Emergency Caesarean section	Yes	52	52	Amoxicillin-PO (5 days) Ampicillin-IV (1 day) Gentamycin-IV (1 day)	Both
17	Normal vaginal	Yes	36	36	Amoxicillin-PO (8 days) Ampicillin-IV (1 day) Gentamycin-IV (1 day)	Ambulatory
18	Normal vaginal	No	-	-	-	Ambulatory
19	Normal vaginal	No	-	-	-	Ambulatory
20	Normal vaginal	Yes	-	-	-	Hospitalized
21	Normal vaginal	No	-	-	-	Ambulatory
22	Normal vaginal	No	24	24	Amoxicillin-PO (5 days)	Hospitalized
23	Elective Caesarean section	No	-	-	-	Ambulatory

LRTI- Lower respiratory tract infection, PO- Oral antibiotic, IV-Intravenous antibiotic, Both- Ambulatory and acute care

5.3.2 NP resistome characteristics

The average depth of coverage of the detected AMR genes from all contigs was 26X (range 1 – 862X). A total of 329 AMR genes were detected in 64% (125/196) of the selected NP samples. Among these, 57% (188/329) were detected at ≥ 90% identity and ≥ 25% gene

coverage while only 30% (97/329) were detected at the more stringent cut-off of ≥ 90% identity and ≥ 80% gene coverage (Table 5.2). The number of resistance genes detected per sample ranged from 1 – 14 genes (Figure 5.1), and included 36 non-redundant genes (Figure 5.2). AMR genes were detected in at least one sample from each of the 23 selected infants (Figure 5.3). The most common resistance genes detected were those conferring resistance to beta-lactams (52%, 172/329), macrolide-lincosamide-streptogramin antibiotics (MLS) (17%, 56/329), and tetracyclines (12%, 38/329) (Table 5.2 and Figure 5.2). A high number of AMR genes conferring resistance to MLS (n = 38), tetracyclines (n = 25), aminoglycosides (n = 17), fluoroquinolones (n = 4), and trimethoprim (n = 3) were detected at a cut-off of 90% identity over 50% gene coverage (Table 5.2). Different patterns of carriage of AMR genes were observed, with only one infant having a stable carriage of *mef*A, *msr*D and *tet*M over a long period (Figure 5.3).

Table 5.2: AMR genes conferring resistance to various classes of antibiotics from 196 NP samples using different stringency criteria.

Antibiotic class	Number of genes			Total number (%)
	≥ 90% ID ≥ 25% Cov	≥ 90% ID ≥ 50% Cov	≥ 90% ID ≥ 80% Cov	
Bla	125	29	18	172 (52.3)
MLS	18	4	34	56 (17.0)
Tet	13	2	23	38 (11.6)
AGly	11	8	9	28 (8.5)
Phe	8	0	5	13 (4.0)
Sul	10	1	1	12 (3.6)
Flq	2	0	4	6 (1.8)
Tmt	0	0	3	3 (0.9)
Rif	1	0	0	1 (0.3)
Total	188	44	97	329 (100.0)

AMR- Antimicrobial resistance, Bla- Beta-lactam, MLS- Macrolide-lincosamide-streptogramin, Tet- Tetracycline, AGly- Aminoglycoside, Phe- Chloramphenicol, Sul- Sulfonamide, Flq- Fluoroquinolone, Tmt- Trimethoprim, Rif- Rifamycin, ID- identity, Cov- coverage.

5.3.3 Pneumococcal resistome

Shotgun sequencing detected pneumococcal reads in all 174 samples that were culture positive for *S. pneumoniae*. Seventy AMR genes (four non-redundant genes) were detected from pneumococcal contigs; the average depth of coverage was 103X (range 1 – 411X). MLS and tetracycline resistance genes were the only genes detected from pneumococcal contigs. The most commonly detected gene was *tet*M (n = 23), followed by *msr*D (n = 22), *mef*A (n = 21), and *erm*B (n = 4). *msr*D, *erm*B, and *mef*A genes were only identified from pneumococcal contigs. The combination of *msr*D, *mef*A and *tet*M genes was detected in 10 samples from 3 infants and all were identified on the same contig in 9 out of 10 samples.

5.3.4 Non-pneumococcal resistome

A total of 259 AMR genes were detected from non-pneumococcal contigs with an average coverage depth of 10X (range, 1 – 862X). Nine types of AMR genes were detected from non-pneumococcal contigs (Figure 5.3), with beta-lactam resistance genes (66%, 172/259) being the most commonly detected genes (Table 5.2 and Figure 5.2). The most commonly detected beta-lactam resistance gene was $bla_{\text{OXA-60}}$, followed by $bla_{\text{OXA-22}}$, $bla_{\text{BRO-1}}$, bla_{TEM}, $bla_{\text{BRO-2}}$, *cfx*A, and *bla*Z (Figure 5.2). The fluoroquinolone resistance gene *nor*A was detected in six samples from four infants (Figure 5.3).

5.3.5 Association between antibiotic use and the NP resistome

No significant difference was observed between the presence of AMR genes in samples collected before and after the treatment of LRTI (Figure 5.3). A large proportion of AMR genes (69% 227/329) was detected in samples from a subset of eight infants (Figure 5.3).

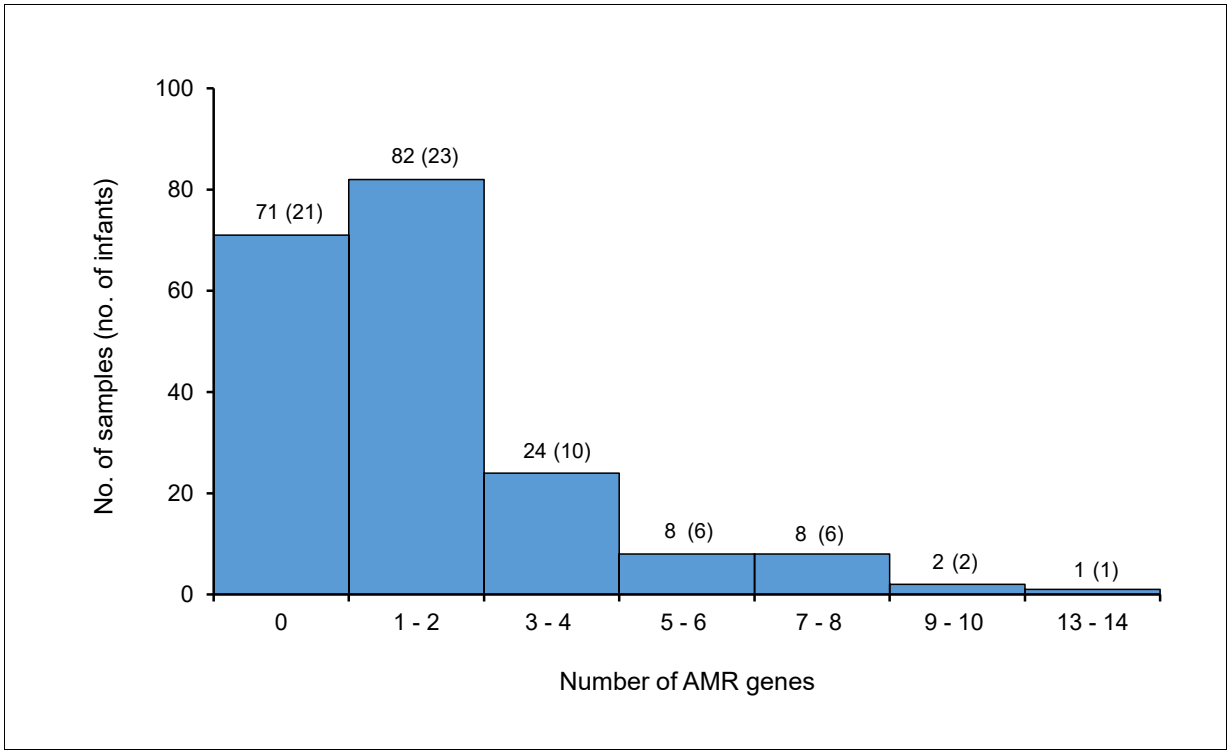

Figure 5.1: Distribution of antimicrobial resistance (AMR) genes within 196 longitudinal NP samples selected from 23 infants.

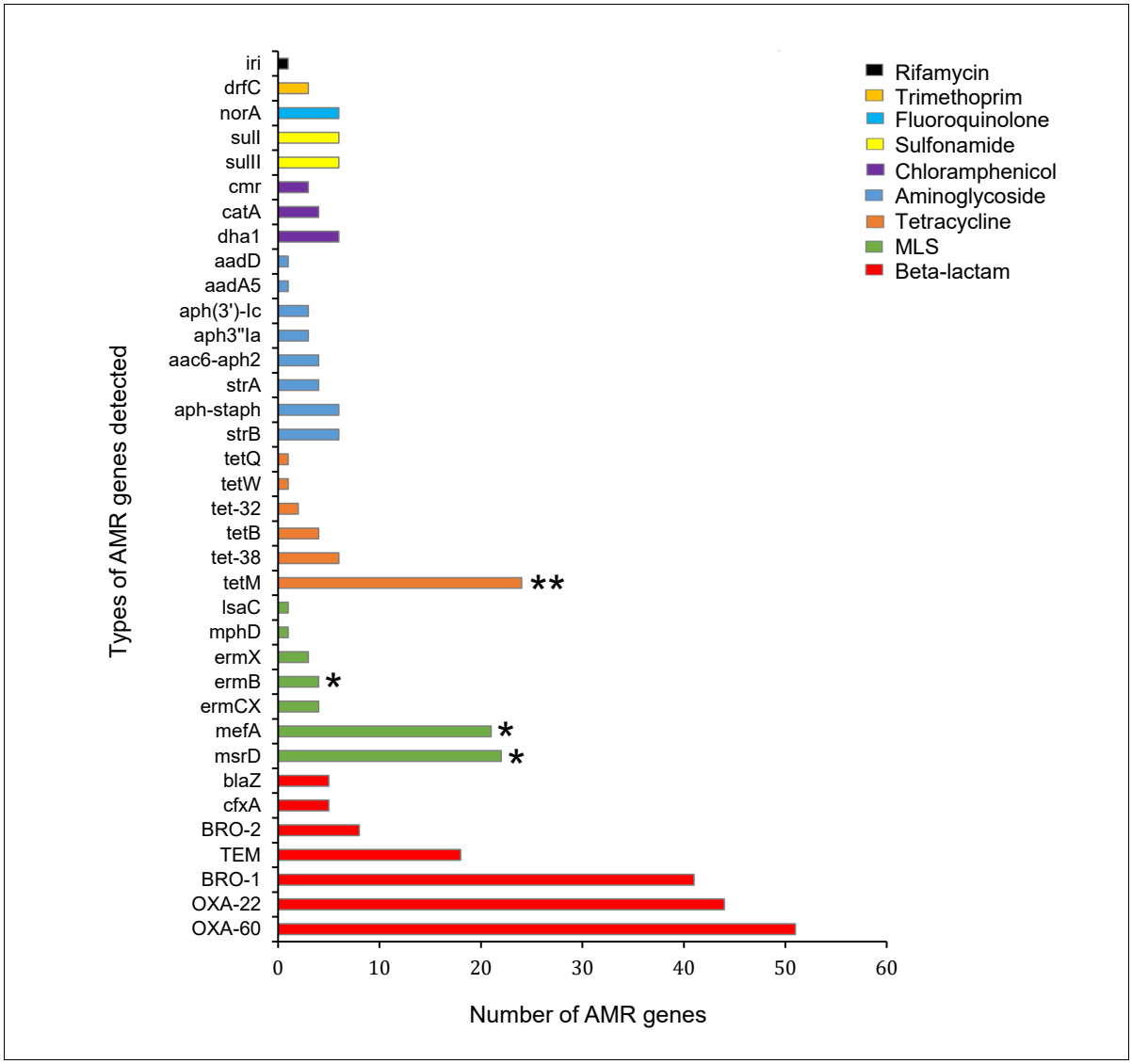

Figure 5.2: Frequency of antimicrobial resistance (AMR) genes within 196 longitudinal NP samples selected from 23 infants. MLS-Macrolide-lincosamide-streptogramin. (*) AMR genes detected from pneumococcal contigs. (**) AMR gene detected from pneumococcal contigs in 23 out of 24 samples.

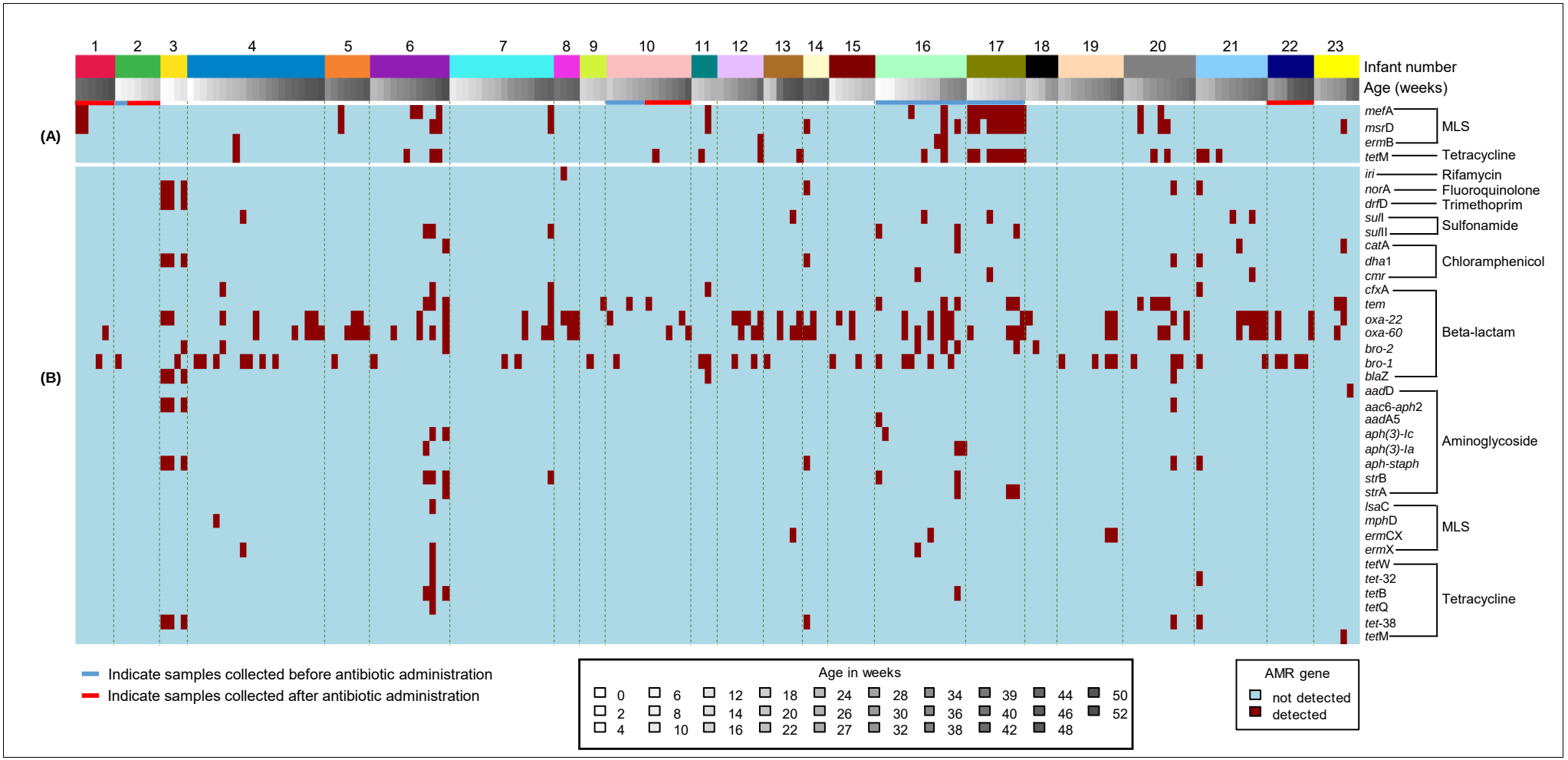

Figure 5.3: Antibiotic resistance genes detected from 196 longitudinal NP samples selected from 23 infants (assigned 1 to 23). (A) Pneumococcal resistome. (B) Non-pneumococcal resistome. Blue line indicates samples collected before antibiotic administration, for lower respiratory tract infection. Red line indicates samples collected after antibiotic administration, for lower respiratory tract infection. MLS- Macrolide-lincosamide-streptogramin, AMR- Antimicrobial resistance.

5.4 Discussion

This proof-of-concept study investigated the composition of the NP antibiotic resistome in an intensively sampled South African birth cohort. 329 AMR genes were detected across 64% of the selected NP samples using targeted enrichment culture and shotgun metagenomic sequencing. The average depth of coverage for the resistance genes from pneumococcal contigs (103X) was higher than that from non-pneumococcal contigs (10X). This observation is likely due to the short streptococcal enrichment culture step using Todd Hewitt broth (without antibiotics).[24]

We detected more resistance genes using a lower stringency criterion of ≥ 90% identity over 25% coverage of the reference gene, which has been shown to be more reliable than the more stringent criteria, in detecting AMR genes.[27] Yang *et al*., reported a high accuracy (99%) for detecting AMR genes using these less stringent parameters in metagenomic analysis.[28] In the current study, at least 25% gene coverage was used,[26] and this cut-off was higher than the suggested coverage of ≥ 25 amino acids.[28] Only 30% of the AMR genes were detected using ≥ 90% identity over 80% coverage of the reference gene.[26] The more stringent parameters detected mainly MLS, tetracycline, and aminoglycoside resistance genes which are frequently carried by *Streptococcus* species, presumably due to the higher depth of coverage as a result of the enrichment step.[29,30]

We observed differences in the types and numbers of AMR genes identified from pneumococcal and non-pneumococcal contigs. Except for one non-pneumococcal sample in which *tet*M was detected (possibly from a different species of *Streptococcus*); *tet*M, *msr*D, *erm*B, and *mef*A genes were only detected from pneumococcal contigs (contigs mapping to reference pneumococcal genomes) (Figure 5.3). The *msr*D, *erm*B, and *mef*A genes are most frequently detected among pneumococcal isolates.[31] Pneumococci which are resistant to MLS antibiotics are commonly also resistant to tetracycline due to the insertion of an MLS gene into the conjugative transposons of the Tn*916* family, which typically carry the *tetM* gene.[32,33] Although transposon analyses were not evaluated in the current study, *msr*D and

*tet*M genes were commonly identified on the same contig (9/10 samples) suggesting they could be carried on the same transposon.[33]

The predominant AMR genes detected from non-pneumococcal contigs were beta-lactamase genes, specifically *bla*$_{OXA-60}$, *bla*$_{OXA-22}$, *bla*$_{BRO-1}$, and *bla*$_{TEM}$. All *bla*$_{TEM}$ gene variants detected in the current study codes for narrow spectrum beta-lactamase enzymes, and these have previously been detected in the *Enterobacteriaceae*, *H. influenzae*, and *Neisseria gonorrhoea*.[34] The *bla*$_{BRO-1}$ gene was more commonly detected than *bla*$_{BRO-2}$ (Figure 5.2), both are typically found among *Moraxella catarrhalis* isolates, with *bla*$_{BRO-1}$ more prevalent than *bla*$_{BRO-2}$ in *M. catarrhalis*.[35]

The *bla*$_{OXA-60}$ and *bla*$_{OXA-22}$ genes, encoding the chromosomal and inducible class D beta-lactamases have only been described in *Ralstonia pickettii* or *R. mannitolilytica*.[36–40] *Ralstonia* sp. are Gram-negative, non-fermentative bacilli, commonly isolated from the respiratory tract and their carriage among infants in this study warrants further investigation.[41] OXA-22 is an oxacillinase with the ability to hydrolyse narrow-spectrum beta-lactams.[40] Unlike OXA-22, the hydrolysis spectrum of OXA-60, although narrow, includes carbapenems. Whilst *R. pickettii* infrequently causes infections, the potential for transfer of this gene to other NP bacteria should be studied.[36,39]

The *nor*A gene, which encodes a fluoroquinolone efflux transporter protein, has been described mainly in *Staphylococcus aureus* and can render resistance to both fluoroquinolones and other classes of antibiotics with dissimilar structures.[42,43]

Beta-lactamase genes were the most commonly detected resistance genes in the current study. Amoxicillin, a beta-lactam antibiotic, was the most commonly prescribed antibiotic for both acute and ambulatory care in children in this study, which could explain the high number and types of beta-lactamase genes detected in this study.[44]

There were several limitations to the current study. Firstly, the enrichment culture for pneumococci altered the composition of the NP microbiome and the prevalence of the

different AMR genes detected may therefore differ from that found in tested samples without enrichment. Secondly, the purposively selected sample set is unlikely to be broadly representative of infants in this study, since only samples with changes in resistance or serotype were selected for this investigation. Thirdly, the reference database used for the resistome analysis is not comprehensive, and excludes chromosomal mutations associated with resistance. Penicillin resistance associated with *pbp* gene mutations, such as *pbp*1a and *pbp*2x, and trimethoprim sulphamethoxazole resistance, associated with *fol*A I100L substitutions and *fol*P insertions, would not be detected using this database, and these analyses must be done manually.

This study demonstrates that WMGS can provide a broad snapshot of the NP resistome. Recent work has highlighted that the nasopharynx is a conducive environment for the exchange of AMR genes between related *Streptococcus* species responsible for respiratory tract infections in children.[45] WMGS has the potential to provide a comprehensive assessment of all resistance elements present in this niche.

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CHAPTER 6

General conclusions

We longitudinally investigated antimicrobial resistance amongst pneumococci colonizing the nasopharynx of PCV13-vaccinated South African infants. We also explored strain-level pneumococcal colonization patterns and associated antimicrobial resistance determinants, and the composition of the NP antibiotic resistome using shotgun metagenomic sequencing.

Carriage of NP antibiotic-non-susceptible pneumococci was relatively constant throughout the first year of life. Despite a high vaccine coverage, PCV13 serotypes were identified, and were more commonly non-susceptible to penicillin, erythromycin, and cotrimoxazole. These observations might be attributable to the time elapsed in South Africa between PCV13 implementation in 2011 and the commencement of the study in 2012. Therefore, an extended follow-up period of more than a year may be necessary in order to observe significant changes in the carriage of antibiotic-non-susceptible pneumococci.

Risk factors associated with the high antibiotic-non-susceptibility observed in this study could not be determined due to the small sample size and purposive sample selection strategy, therefore, future studies with a larger sample size, and unbiased selection strategy would allow for a detailed risk factor analysis.

Antibiotic resistance in bacteria may be caused by different mechanisms which may not be detected using conventional methods. To identify the presence of additional resistance elements, more discriminatory genetic methods are needed to characterize the composition of the NP resistome. Our resistome analyses using shotgun metagenomic sequence data allowed for the differentiation of AMR genes identified from pneumococcal and non-pneumococcal sequences.

A limitation of our resistome analyses was that the results were based on shotgun metagenomic data and not on individual isolates. It was therefore difficult to link resistance genes identified in the clinical sample to individual isolates present, and future work will address this by employing WGS on individual isolates.

Children may be sequentially or concurrently colonized by different pneumococcal strains, yet current methods for the detection of co-colonization are limited. We explored the use of shotgun metagenomic sequencing to characterize the complete pneumococcal component of the NP microbiota in children. We were able to derive pneumococcal serotype, sequence type, co-colonisation, and antimicrobial resistance determinants directly from shotgun sequence data. We identified four novel sequence types and varying colonization patterns, per infant, over time. Direct shotgun sequencing from enriched NP samples is therefore a valuable technique for the detailed evaluation of the pneumococcal component of the NP microbiota. Our findings highlight the power of this technique to obtain a comprehensive assessment of the pneumococcal component of the NP microbiota, including colonization with multiple and novel genotypes.

THE END