Addis Ababa University College of Health Sciences, Department of Microbiology, Immunology and Parasitology

THE IMPACT OF TEN-VALENT PNEUMOCOCCAL CONJUGATE VACCINE (PCV10) ON *STREPTOCOCCUS PNEUMONIAE NASOPHARYNGEAL* CARRIAGE RATE IN HEALTHY CHILDREN: PHENOTYPIC AND GENETIC DIVERSITY OF ISOLATES FROM ADDIS ABABA, ETHIOPIA

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A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Medical Microbiology

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Dedication

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Abbreviations

ABC	ATP-binding cassette
AHRI	Armauer Hansen Research Institute
AFLP	Amplified fragment length polymorphism
AOM	Acute Otitis Media
ATCC	American Type Culture Collection
BBB	Blood-brain barrier
BLAST	Basic Local Alignment Search Tool
BURST	Based Upon Related Sequence Type
CBP	Choline-binding proteins
CC	Clonal Complexes
CGH	Comparative Genomic Hybridization
CI	Confidence Interval
CLSI	Clinical and Laboratory Standards Institute
CPS	Capsular Polysaccharide
CSF	Cerebrospinal Fluid
CWPS	Cell wall Polysaccharide
DCC	Day-care centres
DNA	Deoxyribonucleic Acid
Erm	Erythromycin methylase
HIV	Human Immunodeficiency Virus
IDP	Invasive Disease Potential
IPD	Invasive Pneumococcal Disease
IRB	Institutional Review Board
Hyl	Hyaluronate lyase
LTA	Lipoteichoic Acid
MDR	Multi Drug Resistant
Mef	Macrolide efflux
MIC	Minimum Inhibitory Concentrations
MILST	Multi-invasive Locus Sequence Typing
MLST	Multilocus Sequence Typing

MR	Macro-Restriction
NCBI	The National Centre for Biotechnology Information
NP	Nasopharyngeal
NT	Non-typeable
NVT	Non-vaccine types
PAMP	Pathogen Associated Molecular Pattern
PavA	Pneumococcal adhesion and virulence A
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PCV	Pneumococcal Conjugate Vaccine
PiaA	Pneumococcal iron acquisition A
PiuA	Pneumococcal iron uptake A
Ply	Pneumolysin
PpmA	Putative proteinase maturation protein A
PPV	Pneumococcal polysaccharide Vaccine
PRSP	Penicillin Resistant *Streptococcus Pneumoniae*
PS	Polysaccharide
PsP	Pneumococcal Surface Protein
PsA	Pneumococcal surface Antigen
RFEL	Restriction Fragment End Labelling
PFGE	Pulsed Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
ST	Sequence Types
STGG	Skim Milk-Tryptone-Glucose Glycerin
SlrA	Streptococcal lipoprotein rotamase A
TA	Teichoic acid
TNF	Tumor Necrosis Factor
TIGR	The Institute for Genomic Research
TLR	Toll Like Receptor
UPGMA	Unweighted Pair group Methods with Arithmetic Averages
VT	Vaccine Type

Abstract

Background: *Streptococcus pneumoniae* is among the most important human pathogens responsible for high morbidity and mortality rates. Nasopharyngeal colonisation is the necessary first step in the pathogenesis of associated invasive pneumococcal diseases. Ethiopia, introduced the ten-valent pneumococcal conjugate vaccine (PCV10) in October 2011, and the vaccine is currently given at a "3+0" schedule: at the age of 6, 10 and 14 weeks without booster dose. However, there is lack of adequate baseline information on epidemiological factors for comparison in subsequent impact assessment. The aim of this study was to determine the impact of PCV10 vaccine on nasopharyngeal carriage and analyze the phenotypic and genetic diversity of pneumococcal isolates identified.

Method: A total of 789 infants were enrolled at the age of six weeks when they came for the first PCV10 vaccine; and 206 were re-sampled at the age of nine months; and 201 at two years of age after the final dose of PCV10. Nasopharyngeal swab samples were taken for bacteriological analysis before vaccination at the age of six weeks, and after completion at the age of nine months and two years. Isolates were tested for commonly used antibiotics by the disc diffusion method, and the minimum inhibitory concentration was determined by E-test for those isolates that showed reduced susceptability to penicillin and resistance to erythromycin. A total of 422 pneumococcal isolates were serotyped and 325 isolates characterized by Pulsed Field Gel Electrophoresis (PFGE) and twelve isolates were analysed by multilocus sequence typing (MLST).

Results: The carriage rate of *S. pneumoniae* at the age of six weeks, nine months and two years was 26.6%, 56.8% and 48.3% respectively. A total of 59 serotypes of *S. pneumoniae were* identified from 422 isolates, and serotype 6A, 34, 10A, 11A, 19F, 15B, 23F, and 15A dominated in decreasing order. The proportion of serotypes covered by PCV10 vaccine among the isolates at six weeks, nine months and two years were 20.2%, 11.1% and 10.3% respectively. Molecular typing of six-week and nine-month isolates further showed a presence of a high genetic diversity. Resistance rates ranged from none

to ceftriaxone and 4.3% for chloramphenicol to 27.7% for trimethoprim/sulfamethoxazole.

Conclusion: This study highlights the presence of very diverse serotypes in the country, and PFGE and MLST results indicate a case of possible capsular switching event. A vaccine impact has been observed on with a reduction of carriage of vaccine types; but, like in similar other studies, non-vaccine type replacement were also demonstrated. The use of PCV13 would have an added value by increasing the coverage over additional nasopharyngeal carried isolates by at least 10% more. This work was conducted in Addis Ababa only and it is necessary to extend the investigation to other parts of the country and include clinical cases to have a complete picture of the impact of PCV on pneumococcal epidemiology.

Key words: *Steptococcus pneumoniae*, Serotypes, Genetic diversity, Pneumococcal conjugate vaccine, Addis Ababa

CHAPTER ONE

1.1 Introduction

Streptococcus pneumoniae (Pneumococcus) is among the most important human pathogens with high morbidity and mortality rates, and has one of the largest public health and economic impacts of any bacterial infectious agent in both developing and industrialized countries (Darboe *et al.,* 2010, Yoshioka *et al.,* 2011). There are about one million new pneumococcal infections every year, majority of which occur in the developing world where children under 5 years are most affected; and the organism is responsible for 10–20% of all deaths in this age group (O'Brien *et al.,* 2009). Each year, approximately half of the 2.6 million deaths due to acute respiratory infections in children under five are due to pneumococcal pneumonia (Obaro and Adegbola 2002a). In Ethiopia, and in other similar countries in sub-Saharan Africa, respiratory tract infection and pneumonia are the leading causes of hospital admission and mortality for children under 5 years and cause an estimated 112,000 deaths per year. Apart from being the main cause of community-acquired pneumonia, the pneumococci are also responsible for other diseases such as meningitis, septicaemia, otitis media and sinusitis (Rudan *et al.,* 2008, Usuf *et al.,* 2014, FMOH 2015).

The pneumococcus is a Gram positive α-haemolytic capsulated bacterium. It may act both as a commensal bacterium colonising the nasopharyngeal epithelium, mainly of young children (Bogaert *et al.,* 2004a). Nasopharyngeal (NP) carriage in children plays a major role in its spread within the family, at school, and in day-care centres, kindergarten and orphanage populations. NP colonisation is the necessary first step in the pathogenesis of associated invasive pneumococcal disease (IPD) and non-invasive diseases (Wattal *et al.,* 2007). NP carriage studies provide insight into the local prevalence of circulating pneumococcal serotypes (Kandasamy *et al.,* 2015). The rate of asymptomatic carriage of pneumococcus increases during the first two years of life and then levels off, peak prevalence ranging from 43% to 70% (Vestrheim *et al.,* 2008a). Although all age groups may be affected, the highest rate of pneumococcal disease occurs in young children and in the elderly population. In addition, persons suffering from a wide range of chronic

conditions and immune deficiencies are at increased risk (Girard *et al.,* 2005). Invasive pneumococcal disease and non-invasive infections such as acute otitis media (AOM), pneumonia, meningitis and sepsis, tend to develop soon after the acquisition of new pneumococcal serotypes in the respiratory tract rather than after prolonged carriage (Prymula *et al.,* 2011).

So far, there are 97 serotypes of pneumococcus and the distribution of clinically relevant serotypes varies with time, geographical areas and age of the population (Bogaert *et al.,* 2004b, Zartler *et al.,* 2009, Rodgers and Klugman 2011, Oliver *et al.,* 2013, Lin *et al.,* 2014). The distribution and prevalence of serotypes also differ significantly among isolates from asymptomatic carriers versus those from individuals with IPD; while some serotypes are found frequently in both conditions, others are rarely isolated from either carriage or invasive disease (Hausdorff *et al.,* 2005). Analysis of distribution of the leading serotypes and disease incidence in a particular area is important to evaluate the efficacy of vaccines (Obaro 2002, Bogaert *et al.,* 2004a, Bogaert *et al.,* 2004c, WHO 2007).

Currently, there are two types of vaccines, pneumococcal polysaccharide (PPV) and pneumococcal conjugate (PCV) vaccines. The pneumococcal polysaccharide 23 valent vaccine (PPV23) protects against 23 serotypes and the pneumococcal conjugate vaccines (PCV) may protect against 7, 10 or 13 serotypes. PCV is the recommended effective vaccine for under five children since it induces T-cell dependent immune response that is important for immunoglobulin class switching, affinity maturation and memory (Gertz *et al.,* 2003). The first conjugate vaccine available was the 7-valent PCV (PCV7), which offered protection against 7 serotypes (4, 6B, 9V, 14, 18C, 19F, 23F). In 2010, PCV10 (including all serotypes in PCV7 plus 1, 5, 7F) was made available and PCV7 was replaced by PCV13 (including all serotypes in PCV10 plus 3, 6A, and 19A) (CDC 2010, 2012, Andrade *et al.,* 2014).

Several studies have reported the effectiveness of PCV vaccines in reducing both invasive and non-invasive disease in children and adults (Jansen *et al.,* 2009, Pilishvili *et*

al., 2010). Following first PCV7 introduction in 2000 in the US, a reduction in pneumococcal carriage of PCV7 serotypes was described, as well as an increase in colonization by non-PCV7 serotypes (Spijkerman *et al.,* 2011, Tocheva *et al.,* 2011, Andrade *et al.,* 2014). As a result of the emergence of non-PCV7 serotypes causing invasive and non-invasive diseases, all European countries and the USA are currently using either PCV10 or PCV13 (Prymula *et al.,* 2011, Ansaldi *et al.,* 2012, Lee *et al.,* 2014, van der Linden *et al.,* 2015). The high disease burden in sub-Saharan Africa combined with limited access to health services and obstacles to providing appropriate treatment make vaccination against pneumococcal disease a priority (Valles *et al.,* 2006).

Ethiopia started using 10 valent (PCV10) vaccine in October 2011 to be given in routine vaccination programme at the age of six weeks, ten weeks and fourteen weeks (3+0) without catch-up campaign for older children. Nevertheless, there is lack of adequate baseline information on epidemiological factors (such as the rate of carriage and transmission, serotypes, genetic relatedness of isolates, antibiotic susceptibility profile for commonly used antibiotics) for subsequent impact assessment. The objective of this study was to determine the impact of PCV10 vaccine on nasopharyngeal carriage and analyze the phenotypic and genetic diversity of pneumococcal isolates identified.

1.2 Literature Review

1.2.1 The History of *S. pneumoniae* (Pneumococcus)

Pneumococcus, as it is commonly known, continues to be an important cause of mortality and morbidity in all age groups, from children to the elderly, both in the developed and developing nations worldwide and it is one of the most common bacterial pathogens encountered in medicine. Benjamin White, 1938 *described* Pneumococcus *as* "*pneumococcus is altogether an amazing cell. Tiny in size, simple in structure, frail in make-up, it possesses physiological functions of great variety, performs biochemical feats of extraordinary intricacy and, attacking man, sets up a stormy disease so often fatal that it must be reckoned as one of the foremost causes of human death.*" In the pre-antibiotic era, pneumococcal pneumonia was so common and fatal that it was termed as the ‘‘old

man's friend'' and the ''captain of the men of death'' by William Osler (Sa-Leao and Tomasz 2009)

Since many people suffered and died from the disease during 19th and 20th centuries, an intense research was initiated to gain an understanding of this bacterium. The original observations of bacteria that we now believe were pneumococci are attributed to Klebs (1875), Eberth and Ma´tray (1880), the latter having coined the term Pneumoniekokken (White *et al.* 1938). The pneumococcus was first isolated independently by Louis Pasteur and George Miller Sternberg in 1881. In 1886, Abert Frankel showed that the organism caused pneumonia (Rosenow 2004) but often confused with other pathogens causing pneumonia until Gram staining was developed in 1884, by Christian Gram. Soon after its discovery, the pneumococcus was recognized as a major pathogen responsible for pneumonia, which was later determined to have caused much of the mortality during the 1918 influenza pandemic (Morens *et al.*,2008, Geno *et al.,* 2015). Between 1915 and 1945, the chemical structure of the capsule and its antigenic and virulence properties, and its influence on disease were descried. By 1940, more than 80 different serotypes had been identified (CDC 2015).

At the beginning of twentieth century, Cooper *et al* 1932 described the first 32 pneumococcal types and named them consecutively (Beeson and Goebel 1939). In the 1940s, Kauffmann and his co-workers described groups of related types (Henrichsen 1999), such as group 19, with type 19A and 19F. In the seventies, the letter F was added to the first type of the group, with the exception for group 6 and 9. In 1995, 90 different serotypes had been described (Henrichsen 1995), and recently, at least 97 types have been identified (Bentley *et al.,* 2006, Park *et al.,* 2007, Calix and Nahm 2010, Lin *et al.*, 2014). In 2006, the genetic transformation of 90 different capsule loci were analyzed, showing a huge variety, suggesting that these genes had been transformed into the pneumococcal genomes on multiple occasions and from different sources (Bentley *et al.*, 2006).

Serotype switching was first described by Griffith in 1928 (Griffith 1928), and has since been observed to occur randomly. The first evidence of transformation was done by Avery and his colleagues. Griffith, in 1944, showed that the pneumococcus was able to transform its capsule by injecting mice with rough (non-encapsulated) bacteria as well as heat killed smooth (encapsulated) pneumococci. Live non-encapsulated pneumococci were transformed and got a capsule vital for virulence, and were then able to cause disease in mice (Avery *et al.,* 1944)

Since the discovery and development of antibiotics and the advent of treatment of infectious diseases with, for example penicillin, the death rate caused by pneumococci has significantly decreased. The first report of penicillin-resistant pneumococci was published in the 1960s (Schrag *et al.,* 2004). Austrian et al., 1976 recongized that despite antibiotics there was residual mortality of at least 10%. This phenomenon gave the impetus for researchers to develop ever newer and more effective vaccines and antibiotics to keep pace and necessitating intensive research into vaccines and antibiotics to win the race for effective control of the disease (Austrian *et al*., 1976).

1.2.2 Morphology and Identification of *S. pneumoniae*

Pneumococcus is a Gram-positive encapsulated, oval or spherical coccus of 0.5-1.25 μm in diameter, lancet-shaped bacterium that is often seen in pairs and shorter chains. The bacteria are facultative anaerobes and α-haemolytic. Pneumococcus is identified based on colony morphology, α-haemolytic activity (they form macroscopic colonies that are characterized by their surrounding greenish halo or that tend to show a depressed centre upon prolonged incubation on blood agar plates), catalase negative, additionally sensitivity to optochin and bile solubility (Chandler *et al.,* 2000). However, some pneumococcal isolates are non-typeable with regard to the capsule. They either do not express any capsule or they express yet unidentified capsules. In a study done by Sa-Leao *et al* as many as 7.4% of the isolates collected from healthy children in Portugal between 1997 to 2003, were found to be non-typeable isolates (Sa-Leao *et al.,* 2006). Since as many as 8% of the strains may require it for growth, culture of pneumococci is frequently

performed in media supplemented with blood, under an atmosphere enriched in carbon dioxide. Recently, two genes were identified as being responsible for the capacity of pneumococci to grow under ambient air: pneumococcal carbonic anhydrase gene *pca* (encoding a carbonic anhydrase) and *folC* (encoding a dihydrofolate/ folylpolyglutamate syntheses) (Burghout *et al.,* 2010, Burghout *et al.,* 2013). The pneumococcal colonies vary in size and phenotype depending on what serotypes they belong, specifically serotype 3 and 37 are mucoid (Browall 2015, Geno *et al.,* 2015).

In many cases, atypical *S. pneumoniae* has been mistaken for non-typeable (NT) pneumococci. They are not able to react with capsule antibodies, and in recent study, some were identified as *Streptococcus pseudopneumoniae* (Simoes *et al.,* 2010). Smaller in size than encapsulated pneumococci, they were first described by Arbique *et al* (Arbique *et al.,* 2004) and belongs to *Streptococcus mitis*/*orlais* group of viridans Streptococci (Keith *et al.,* 2006). *S. pseudopneumoniae* and NT pneumococci were observed to cause disease, even invasive disease, but the bacteria are more commonly found as asymptomatic carrier in the upper respiratory tract (Rolo *et al.,* 2013).

1.2.3 Serotypes

Serotyping of pneumococci is based on difference in the capsular structure, and so far, at least 97 capsule types, known as serotype can be distinguished (Bentley *et al.,* 2006, Calix and Nahm 2010, Calix *et al.,* 2012, Oliver *et al.,* 2013). They are distributed over 46 different serogroups, of 25 single types and 21 groups. Each group consists of 2-5 serotypes which are very similar (SSI 2016). A serogroup was defined to include serotypes that share many serologic properties (i.e., cross-reactive antibodies). The polysaccharide capsule is an essential virulence factor for IPD, defined as the isolation of pneumococcal isolates from normally sterile body sites e.g blood and cerebrospinal fluid (Tan 2012). As serotype-specific antisera were widely used for treating patients in the early 20th century, a large number of serotypes were discovered. In 1932, Cooper *et al*. described 32 serotypes, which were sufficient to serotype most clinical isolates of pneumococci (Cooper *et al.,* 1932). Type-specific sera were not available for all

serotypes, however, and antisera were not always effective. The limitation was illustrated by the death of Danish Prince Valdemar in 1939, which provided a strong stimulus for studying pneumococcal serotypes. He was found to have pneumococcal pneumonia caused by serogroup 9, but he was unresponsive to treatment with antiserum 9L from Lederle Laboratories, Inc., and antiserum 9N from the New York State Laboratory. Additional studies after his death showed that his pneumonia was caused by a new serotype in serogroup 9, which was then named 9V after him (Vammen 1939). Prince Valdemar's case clearly indicated the need to accurately serotype pneumococci and distinguish all serotypes within a serogroup. To facilitate antiserum therapy, many serotyping procedures were developed, including the precipitin test, agglutination, and the Quellung reaction. The Quellung reaction was described by Neufeld in 1902 and was later widely adopted as the preferred capsular typing method (Beckler and Macleod 1934, Lund 1960). Further studies in America led to the description of 75 serotypes by Eddy during World War II, which were simply numbered in the order of their discovery. However, contemporary studies in Denmark led to a system that distinguished serogroups from serotypes, perhaps spurred by the experience with Prince Valdemar (Eddy 1944).

The majority of all serotypes are synthesised and exported through the Wzy-dependent pathway, where extracellular polymerization of component lipid-linked repeat unit is preceded by flippase transfer across the cell membrane. The proteins involved in capsule synthesis are encoded by the capsule polysaccharide synthesis (*cps*) genes, located between dexB and aliA on the chromosome. The two capsular types, 3 and 37, are synthesized through independent biochemical pathways (Bentley *et al.,* 2006, Calix and Nahm 2010, Calix *et al.,* 2012).

The *cps* genes (10-30kb) are exchanged via transformation and recombination, and it is of highest concern that vaccine-type in this way can switch to non-vaccine serotype (NVT). A study by Wyres *el al* (Wyres *et al.,* 2013) showed that capsular switching has been occurring regularly over the last 70 years. As an example, showed an occurrence of a capsular switch for clonal complex, CC156, of serotype 9V (Sjostrom *et al.,* 2007).

Studies have suggested that serotype switching is an event of selection due to antibiotic treatment and pneumococcal vaccination. As a result of extensive vaccination, there has been a selection for NVTs and serotype replacement among carrier and in patient with invasive disease. It has also been shown that no significance decrease has been observed in antibiotic resistance in some countries after vaccine introduction. It has also been suggested that recombination of *cps* locus and flanking region might be a normal biological process, and a regular occurrence among the pneumococcal population. Recombination of large DNA fragment (>30kb), sometimes included in the capsule locus and penicillin-binding protein genes, pre-dated both vaccine introduction and widespread use (Wyres *et al.*, 2013).

The presence of difference caspsular types has been shown to vary with time point, geographical region, and age of the host (Hausdorff *et al.,* 2005). Nevertheless, there were about 20 common serotypes identified throughout the world, widely associated with more than 80% of the IPD in all age groups before PCVs were introduced (WHO 2007). The thirteen most prevalent serotypes, also included in PCV13, are responsible for 70-75% of all invasive disease in children, suggesting a high coverage by the pneumococcal vaccines available today in the world (Tan 2012).

Among the 97 different serotypes recognised today, such as serotype 6B, 9V, 14, 19F and 23F are more common among young children in the world. Studies have shown that some serotypes are more of associated with invasive diseases (Sandgren *et al.,* 2004). Pneumococcal serotypes can be divided into 3 groups according to their capacity for colonization. The "prime league" players (i.e., those most often carried and found globally) comprise serotypes 6A, 6B, 14, 19A, 19F and 23F (Weinberger *et al.,* 2009, Niedzielski *et al.,* 2012, Valente *et al.,* 2012, Sharma *et al.,* 2013). Serotypes that are important "second league" players, such as serotypes 10A, 11A, 15A, 15B/C, 33F and 35B, have a somewhat lower capacity to colonize but are still more common than others (Weinberger *et al.,* 2009, Sharma *et al.,* 2013). Furthermore, when the carriage of "prime league" serotypes is reduced by vaccination, the "second league" serotypes become unmasked with these becoming the dominant colonizing serotypes (Weinberger

et al., 2009). A third group of serotypes, such as serotypes 1, 4, 5, 7F, 8 and 12F are highly invasive and associated with severe disease, mainly in outbreaks (Brueggemann *et al.,* 2003, Kronenberg *et al.,* 2006, Shouval *et al.,* 2006, Lagos *et al.,* 2008). However, they are not often carried and constitute a "league of their own" (i.e., poorly carried but highly invasive) (Azzari *et al.,* 2014). In addition, a correlation between invasive disease potential (IDP) and serotype behaviour has been showed. Serotype with high IDP seem to be behave as primary pathogen, while serotypes with lower relative risk of causing invasive disease are more opportunistic, and mainly infect patient with underlying disease or patient already infected with other bacteria or virus (Brueggemann *et al.,* 2003, Sjostrom *et al.,* 2006, Browall *et al.,* 2014).

The underlying factors are huge contributing factor to pneumococcal mortality (Naucler *et al.,* 2013). A study showed that some serotypes are responsible for fatal outcome of pneumococcal infection (Sjostrom *et al.,* 2006). A Danish study of IPD reported that risk of fatal outcome was dependent on the serotype even after adjusting for age and co-morbidities that are known to be confounding factors (Harboe *et al.,* 2009). A meta-analysis looking into the association of serotype with risk of death due to pneumococcal pneumonia showed a stable serotype associated property (Weinberger *et al.,* 2010).

Host factors appear to be more important than specific serotype as determinant of mortality in patient with pneumococcal pneumonia (Naucler *et al.,* 2013). On the other hand, some serotypes have been associated with severe IPD. Serotype 12F is rarely found among carriers, but it is known to cause outbreak of severe IPD. This occurred twice in Alaska, and since 12F is not covered by currently available vaccine it has increased in causing IPD. Whether or not this is due to normal fluctuation or due selection is still not clear (Zulz *et al.,* 2013). Other serotypes with normal fluctuation are serotype 1 and 5 (Harboe *et al.,* 2010).

Serotype 11A is a common colonizer of the nasopharynxand can cause IPD, and has been shown to acquire a mutation in wcjE leading to capsular switching from11A to 11E. The diversity of pneumococal capsules appear to be much greater than previously recognized.

Recently, two serologically and biochemically distinct subtypes, 11A alpha and 11A beta, were discovered among serotype 11A isolates of pneumococci (Calix and Nahm 2010). 11A is a rarely isolated from the middle ear, although it has been found to be a common nasopharyngeal isolate in Aboriginal children (Leach *et al.,* 2009). It has been associated with AOM, and clinical isolates with MLST 62 (ST62) have been shown to have a high IDP in patient with underlying medical conditions (Sjostrom *et al.,* 2006).

Not all pneumococci are able to express a capsule. A small fraction of non-capsulated pneumococci has been observed among children in Portugal. Due to the absence of a polysaccharide capsule they cannot be assigned a serotype and have therefore been named non-typable (NT) pneumococci (Sa-Leao *et al.,* 2006). NT Strains has been associated with resistance and multi-drug resistance (Andrade *et al.,* 2010).

Further differentiation of serotypes into clones may be achieved using molecular techniques such as PFGE and MLST (Browall *et al.,* 2014).

1.2.4 The Genome of Pneumococci

In 1944, Avery *el al* identified as DNA harbouring the genetic information (Avery *et al.,* 1944) and the experiment was performed using pneumococci. In 2001, the complete sequences of the three pneumococcal genome were published (Dopazo *et al.,* 2001, Hoskins *et al.,* 2001, Tettelin *et al.,* 2001); and since then, the number of sequenced pneumococcal strains has steadily increased. The genome consists of single circular chromosome that has a GC content of approximately 40%. The Institute for Genomic Research (TIGR) 4, genome originates from serotype 4 strains and contains more than two million base pairs encoding 2236 genes (Tettelin *et al.,* 2001). Sixty four percent of the predicted proteins were assigned a biological function. There are many repeated sequences, such as inserted sequence, present in the genome, contributing the genetic diversity of the bacterium. Another feature influencing the heterogeneity is the natural transformability of the pneumococcus, the ability to pick DNA from the surrounding and incorporate it into its genome. This was discovered in 1928 by Griffith (Griffith 1928)

and has since then been widely used in the molecular biology applications of pneumococcal research.

1.2.5 Pneumococcal Carriage Rate

The human nasopharynx is an important ecological niche for many bacterial species, including the pneumococcus. For the pneumococcus, however, only the human nasopharynx provides the environment necessary for survival and therefore the pneumococcus depends on successful transmission between humans to stay alive. The phenomenon collectively described as 'carriage' is actually divided into three distinct phases, namely, acquisition, carriage and termination of carriage. Acquisition refers to the point when pneumococci gains access into the host nasopharynx and establishes itself leading to carriage, which refers to a period during which the organism is resident in the host nasopharynx. Loss of the organism from the human nasopharynx is referred to as termination of carriage (Murray 2007).

Healthy children commonly carry at least one serotype of pneumococci in their nasopharynx without developing disease (Obaro and Adegbola 2002a). Asymptomatic carriage of pneumococci is central to the organism's transmission, and colonization is frequently followed by horizontal spread of the pneumococci through an individual's immediate environment and the surrounding community (Bogaert *et al.,* 2004a). It is known that pneumococcal disease will not occur without preceding nasopharyngeal colonization with the homologous strain, although the precise mechanism(s) by which colonizing pneumococci travels to other anatomical sites and becomes pathogenic is poorly understood (Obaro and Adegbola 2002a, Bogaert *et al.,* 2004a).

The epidemiology of pneumococcus carriage differs between developed and developing nations, primarily due to differences in crowding, family size, socioeconomic status, underlying immune compromise and chronic conditions, and antibiotic usage. The pneumococcal NP carriage rates in children in developing countries are generally two to three times higher than those found in children from industrialized countries. Similarly, in some European countries, there are reports of high rate of carriage in normal children (Table 1.1).

Table 1.1 Summary of Some of Higher Carriage Rate of *Streptococcus pneumoniae* reported from different countries

Country	**Age group of study participant**	**Carriage rate**	**Authors and Year**
Africa			
The Gambia	<14-years	90%	Hill *et al.*, 2008
Ghana	43-48 months	48.9%	Mills *et al.*, 2015
Senegal	<2 -years	50%	Ba *et al*., 2014
Kenya	< 5-years	76%	Abdullahi *et al*., 2008
Tanzania	1-15 - years	81%	Anthony *et al*., 2012
Uganda	2-59 -months	58.6%	Rutebemberwa *et al*., 2015
Malawi	<13 years	61%	Kamng'ona *et al*., 2015
South Africa	<2years	83%	Nzenze *et al.*, 2013
Bostwana	<5years	69%	Huebner *et al*., 1998
Asia			
Nepal	3-6 months	69.2%	Hanieh *et al*., 2014
Indonesia	<2years	48%	Soewignjo *et al*., 2001
Japan	<5 year	60.9%	Otsuka *et al.*, 2013
India	<5 years	53.2%	Jain *et al*., 2005
China	2-3 years	47.1%	Yao and Yang 2008
Latin America			
Brazil	<5years	55%	Menezes *et al*., 2016
Colombia	<5years	57.5%	Parra *et al*., 2013
Europe			
United Kingdom	<4year	69%	Tocheva *et al*., 2011
Norway	<5years	78%	Vestrheim *et al.*, 2008a
The Netherlands	<2years	64%	Bogaert *et al.*, 2001
Czech Republic	<5years	62.8%	Zemlickova *et al.*, 2006
United States	<7years	76%	Bruce *et al.*, 2015
Australia	2-15years	74%	Mackenzie *et al*., 2010

Low carriage rate also reported 16.2% in US healthy children (Cheng Immergluck *et al.*, 2004), 13.2% in Calgary, Canada children, 12.9% in Turkey in children age of less five years (Ercan *et al.*, 2011) like 14.1% in Taiwan (Kuo *et al.*, 2011), 27% in Italy under five children (Zuccotti *et al.*, 2014), 21% in Belgian infants attending at DCC (Malfroot *et al.*, 2004), 27.9% in India (Kumar *et al.*, 2014), 29.4% in Greece (Katsarolis *et al.*, 2009), 29.9% in Mexico of healthy children attending at DCC (Espinosa-de Los Monteros *et al.*, 2007).

In Ethiopia, the carriage of pneumococci has been reported of higher rate of pneumococci, in a study done in Gonder Northern part of Ethiopia reported over-all carriage of 41% in children up to the age of 10 years, with a downward trend with increasing age and maximum carriage of 50% in children whose age less than three years (Assefa *et al.*, 2013); 76.8% of carriage was reported in children involved in trail of mass azithromycin treatment trial for control of trachoma in Gurage zone southern part of Ethiopia (Haug *et al.*, 2010), in another similar study, it was reported 69.1% (Skalet *et al.*, 2010), and the review done by Usuf *el al* indicates also the highest carriage rate of 93.3% (Usuf *et al.*, 2014). However, there is still paucity of information on carriage rate of pneumococci for the country.

Based on a longitudinal carriage study of children in the United States, Gray, *et al.*, reported the mean age of acquisition of the first pneumococci serotype to be six months; 95% of children were colonized at some point during the first two years of life, and 73% had acquired at least two serotypes during the first 24 months, usually on different occasions (Gray *et al.*, 1980). The other studies in Africa in the acquisition of pneumococci reported at the age of 38.5 days in Kenyan study (Tigoi *et al.*, 2012) and with median age of 59 days in Malawi (Heinsbroek *et al.*, 2016).

In the carriage rate of pneumococci is higher in under-five children (Usuf *et al.*, 2014) and HIV positive children (Anthony *et al.*, 2012, Heinsbroek *et al.*, 2016). Two or three serotypes were present simultaneously in 4% and 0.3% of specimens, respectively. The duration of carriage was found to be serotype dependent and was between 3-5 months. A

Finnish cohort investigation of healthy children and adolescents 1-19 years old yielded a peak carriage prevalence at approximately three years of age (~55% colonized), followed by a steady decline until a stable carriage prevalence of ~8% was achieved by about 10 years of age (Bogaert *et al.*, 2004a). Another Finnish study of the frequency of nasopharyngeal carriage in children 2-24 months old demonstrated an increase in carriage prevalence from 13% for children less than six months old to 43% in children more than 19 months of age. The prevalence of carriage increased to 22- 45% during respiratory infections, lending support to the hypothesis of increased pneumococcal adherence during viral infections (Syrjanen *et al.*, 2001).

It is known that if carriage occurs shortly after birth, the prevalence of carriage is much higher, and the prevalence of simultaneous colonization with multiple serotypes is greater. The prevalence of carriage is nearly 95% among healthy children less than three years old in developing nations, and simultaneous carriage of up to four serotypes for several months has been documented (Obaro and Adegbola 2002b). This high frequency of multiple carriages among children in less developed countries and the dynamics and competition of species and serotypes colonizing the nasopharynx are particularly salient issues, given the potential for the pneumococcal conjugate vaccines to alter the balance between vaccine and non-vaccine serotypes within the nasopharynx (Browall 2015).

1.2.6 Risk factors for nasopharyngeal carriage

Host factors that have been consistently associated with an increased risk of IPD across geographic locales include: young age, day-care attendance, lower socioeconomic status, underlying immunodeficiency and other chronic medical conditions, preceding or coincident respiratory viral infection, and recent antibiotic usage. The incidence of pneumococcal disease is up to 50 times higher in children < 2 years of age and in adults > 65 years of age, than in adolescents (Davidson *et al.*, 1994, Mehr and Wood 2012). Previous studies have shown the incidence of IPD is greatest among children less than two years old. Young patients, especially those younger than two years, are at particular

risk because of their lack of immunoglobulin (Ig) G2 (Janoff and Rubins 1997, Ash and Sheffield 2013).

Colonisation rates are higher (i.e. > 50%) in situations of overcrowding such as in DCC, orphanages, slums and in indigenous populations. Day-care center attendance has specifically been cited as a predisposing factor for colonization due to resistant pneumococci. Owing to under five sibling, close interactions between children, it has been proposed that pneumococci are more efficiently transmitted from the nasopharynx of one child to another in DCCs. The higher rate of disease in DCCs results in increased antibiotic usage, selecting for the carriage of resistant pneumococci (Mehr and Wood 2012). Indicators of socioeconomic deprivation, such as low household income, minimal education, and crowding, have been identified as risk factors for higher carriage. Within-household crowding and exposure to children/siblings, in particular, have been utilized as markers of increased transmission of pneumococcal serotypes. Maternal pneumococcal carriage, and passive smoke (Cardozo *et al.,* 2008, Bosch *et al.,* 2016) have been reported by some, but not by others (Neto *et al.,* 2003, Sulikowska *et al.,* 2004) to be associated with higher rates of colonisation.

1.2.7 Risk factors for invasive pneumococcal disease (IPD)

In particular, the highest rates of disease occur among children 6-11 months of age; neonatal infections are not uncommon in developing countries (Thaver and Zaidi 2009,) . The increased risk of IPD among children less than two years old is postulated to be due to an immature immunologic response to the polysaccharide capsule of pneumococci and the high prevalence of high among this age group (Ueno *et al.,* 2013). Individuals with functional or anatomic asplenia (e.g., sickle cell disease or splenectomy) are at a higher risk for pneumococcal infection due to reduced clearance of encapsulated bacteria from the bloodstream. Asplenia is associated with the highest risk, with an overall incidence of invasive pneumococcal disease of ~500 per 100,000 per year (Bisharat *et al.,* 2001). The presence of other chronic medical conditions, such as HIV infection, chronic obstructive pulmonary disease, asthma, diabetes mellitus, cancer, and people on

immunosuppressive therapies, individuals with congenital or traumatic cerebrospinal fluid fistulae and children who have cochlear implants are associated with a higher risk of IPD. The risk of pneumococcal pneumonia is 25-fold higher among HIV-infected people compared to HIV-uninfected people. The incidence of pneumococcal invasive disease among HIV-positive children is 9–43 times higher than among HIV-negative children and the rates of IPD among HIV-positive adults are 6–343 times higher than among HIV-negative adults (Andiman *et al*., 1994; Bliss *et al*., 2007). These medical conditions are characterized by a state of immune compromise, anatomical abnormalities, and/or altered physiology of the respiratory tract, which facilitate opportunistic infection with pneumococci (Mehr and Wood 2012)

Cases of IPD are also more common during colder weather months, and IPD tends to follow the annual cyclic pattern of influenza and other viral respiratory infections (Kim *et al.,* 1996). In particular, an association between IPD and influenza A, parainfluenza, and respiratory syncytial viruses has been observed. Pneumococcal has multiple neuraminidases that cleave terminal sialic acid, potentially exposing cell-surface receptors and promoting adherence for pneumococci. Viruses with neuraminidase activity, such as influenza and parainfluenza, may act synergistically with the pneumococcus to promote adherence to the respiratory epithelium. Increased risk of pneumococcal infection during respiratory viral infection may also be due to inhibition of the normal clearance mechanisms of the respiratory tract, epithelial barrier damage, or serve as a marker of increased transmission due to indoor crowding during cooler weather (Klugman *et al.,* 2009).

Finally, previous antibiotic usage has been associated with an increased risk of IPD caused by a resistant strain of pneumococcus. Specifically, prior treatment with β- lactam antibiotics has been associated with IPD due to a drug-resistant strain. In a case-control study of children 2-59 months old, IPD due to a penicillin-resistant pneumococcal strain was independently associated with at least one course of antibiotics in the previous three months. Prior antibiotic usage may alter an individual's nasopharyngeal flora, and

increase the likelihood of carrying resistant pneumococci that can cause IPD under opportunistic conditions (O'Brien *et al.,* 2009).

1.2.8 Pneumococcal virulence factors

The role played by virulence factors allows an understanding of the pathogenesis of infection and can be used to identify possible points of intervention for treatment or vaccination. Understanding the distribution of virulence factors among strains may also allow associations to be made between these factors and the ability to cause different types of disease. Pathogenicity of pneumococci has been attributed to various structures, most of which are situated on its surface, although the list of virulence factors is probably far from complete. The high morbidity caused by this microorganism is still poorly understood, but it seems to be triggered primarily by the host response to infection rather than the production of bacterial toxins (Dobay *et al.,* 2004, Mitchell and Mitchell 2010).

1.2.8.1 The pneumococcal capsular polysaccharide

Pneumococci are encased by a capsular polysaccharide (CPS) and they are distinguished by chemical differences in their capsular polysaccharides (Henrichsen 1995). The importance of the capsule in pneumococcal virulence is known since decades. CPS is the most important virulence determinant of pneumococci, protecting the bacterium against complement-mediated phagocytosis and bacterial killing by neutrophil extracellular traps (Wartha *et al.,* 2007, Kadioglu *et al.,* 2008, Hyams *et al.,* 2010a, Hyams *et al.,* 2010b). Only encapsulated pneumococci have been isolated from clinical materials such as blood (Avery and Dubos 1931) and non-encapsulated pneumococci are unable to cause disease in animal models, which illustrates the importance of capsule in pneumococcal virulence (Avery *et al.,* 1944, Morona *et al.,* 2006). Capsule changed on fixed genotypic background that shows clear capsular performance independent of genotype (Malley and Anderson 2011). However, not all capsule types appear to be equally effective in shielding. Only 20 to 30 serotype of the more than 97 show significant invasiveness; and there is more than 100-fold variability in the invasiveness of a given serotype (i.e., the

ratio of the rate of IPD to the rate of carriage for that serotype) in children (Brady *et al.*, 2014, Geno *et al.*, 2015).

1.2.8.2 The pneumococcal cell wall

The important structural components of pneumococcal cell wall are peptidoglycan, teichoic acid, (TA), lipoteichoic acid, (LTA), choline binding proteins and Leu-Pro-X-Thr-Gly (LPxTG)- anchored proteins (Bergmann and Hammerschmidt 2006, Kadioglu *et al.*, 2008). Peptidoglycan is linked by stem peptides to form a complex three dimensional structure and about half of the cell wall mass is composed of peptidoglycan. The other pneumococcal cell wall structure LTA, also referred to as pneumococcal Forssman antigen, is composed of chain structures and identical repeats. TA and LTA are composed of extended repeats of carbohydrates and differ only in their attachment to pneumococcal cell surface. In contrast to capsular polysaccharides, cell wall polysaccharide (PS) can cause pneumococcal infections such as pneumonia, otitis media and meningitis in animal models similar to those infections caused by intact pneumococci (Tuomanen *et al.*, 1987, Carlsen *et al.*, 1992). Such virulence consequences of cell wall PS may be due to the induction of cytokines and activation of the complement system (Heumann *et al.*,1994). Interestingly, this inflammation resembled the inflammation caused by intact pneumococci, but seems to be more efficient. Consequently, antibiotics inducing lysis of pneumococci during invasive diseases boost pro-inflammatory reactions. Furthermore, purified cell walls are a stronger stimulus compared to endotoxin when the production of interleukin-1 and tumor necrosis factor (TNF) in host cells is monitored (Riesenfeld-Orn *et al.*, 1989). Moreover, none encapsulated pneumococcal cell walls are capable to promote pneumococcal adherence to human endothelial cells; and on late stages of pneumococcal infections they have a cytopathic effect on host cells (Geelen *et al.*, 1993).

1.2.8.3 Pneumococcal proteins

Bacterial virulence factors and host susceptibility are the major determinants for bacterial infections. Often these proteins are involved in direct interactions with host tissues or

protect the pathogens against host defense mechanisms. Pneumococci are not an exception in this regard. Earlier in the 1970s, the capsule was considered as the primary virulence factor of pneumococci. However, later on, several studies suggested that also a numerous number of pneumococcal proteins (Fig 1.1) including pneumococcal hyaluronate lyase (Lopez *et al.,* 1992), pneumolysin, two neuraminidases (NanA and NanB), the major autolysin (LytA), and pneumococcal surface protein A and C (PspA and PspC) (Sampson *et al.,* 1994, Dave *et al.,* 2001, Dave *et al.,* 2004a) have also an important role in pneumococcal pathogenesis and could be used as a potential candidates for a protein-based vaccine against pneumococci. Pneumolysin is an intracellular protein of pneumococci while the surface exposed pneumococcal proteins are classified as LPxTG anchored proteins, lipoproteins, non-classical proteins and choline-binding proteins (Dave *et al.,* 2004b).

1.2.8.3.1 Pneumolysin

Pneumolysin (Ply) is a member of the bacterial pore-forming toxin family that has long been recognized as a key virulence factor of pneumococci (Los *et al.,* 2013). The two major activities of Ply are its ability to form pores on cholesterol-containing cell membranes, resulting in cytolysis and potent induction of inflammation, and to activate complement via the classical pathway (Cassidy and O'Riordan 2013). Although pore-forming is expected to cause cell death, it is becoming increasingly clear that the extent to which this occurs is a function of both toxin and host cell properties. Ply can bind both the Fc portion of immunoglobulin and C1q, activating the classical pathway of complement, but it also decreases C3 opsonization (Marriott *et al.,* 2008). Moreover, the activities of the cholesterol-dependent toxins, including Ply, as potent regulators of signalling and immunity are becoming more apparent, as well as their consequences for pathogenesis (Cassidy and O'Riordan 2013, Los *et al.,* 2013). In spite of limited genetic variation (3.3% sequence variation in ply), there are at least 15 different alleles (Jefferies *et al.,* 2007, Marriott *et al.,* 2008). These include non haemolytic variants in sequence type (ST) 306 expressing serotype 1 as well as low-haemolytic variants in serotypes 7F and 8, all serotypes identified as having a high IDP. These observations are in agreement with virulence studies that showed that mutants lacking both haemolytic and

complement-activating activities were still more virulent than pneumolysin-deficient mutants, reinforcing the importance of Ply's other functions (Marriott *et al.,* 2008).

The presence of Ply is also sensed directly by Toll Like Receptor 4 (TLR4) (leading to enhanced cytokine production (Los *et al.,* 2013). These pro-inflammatory effects of Ply, together with direct cellular damage to the epithelial and endothelial cells, contribute to Ply-induced barrier dysfunction. Even at sub-lytic concentrations, Ply was shown to interfere with Na^+ and Ca^{2+} fluxes or to act indirectly by modulating the activity of cellular factors (Lucas *et al.,* 2013) that can result in significant barrier-disruptive effects. It is clear that the host tries to detect the presence and effects of Ply to fight off pneumococcal infection, while this toxin plays multiple roles at different stages of infection and in different tissues that enhance the pathogenicity of pneumococci (Los *et al.,* 2013).

1.2.8.3.2 Sortase-anchored pneumococcal surface proteins

The role of sortase-anchored surface proteins possessing LPxTG anchorage motif in the C-terminal part of the proteins in pneumococcal virulence has been investigated for the neuraminidase,IgA1-protease, PrtA, hyaluronidase and HtrA. The enzyme neuraminidase is conserved in all pneumococcal strains and represents a virulence factor of pneumococci. Neuraminidase cleaves sialic acid from host cell surface glycans such as mucin, glycolipids and glycoproteins, which probably cause damage to host cell glycans and ultimately to host cells (King *et al.,* 2004). This property of neuraminidase causes changes in the glycosylation patterns of the host cell. Then it may expose host surface receptors for possible interaction with pneumococci, resulting in increased adhesion of the pathogen. Pneumococci encode three neuraminidases: NanA, NanB and NanC. NanA and NanB are almost conserved in all strains while only 50% pneumococcal strains harbour NanC (Pettigrew *et al.,* 2006).

IgA1-protease is zinc metalloprotease produced by pneumococci along with other three zinc metalloproteases named as ZmpB, ZmpC and ZmpD. These proteases are anchored to the pneumococcal cell wall by an N-terminal LPxTG motif. The IgA1-protease is

conserved in all pneumococcal strains and demonstrated significant role in pneumococcal lung infections, bacteremia and adherence of pneumococci to host cells (Weiser *et al.*, 2003).

Serine protease PrtA is a major pneumococcal cell wall-associated serine protease. Recently, it has been suggested that PrtA knockout strains lost their ability to degrade apolactoferrin and were relatively resistance to killing by apolactoferrin (Mirza *et al.*, 2011). PrtA was shown to be important for pneumococcal virulence in a mouse peritoneal challenge model (Bethe *et al.*, 2001).

Hyaluronate lyase (hyaluronidase; Hyl) facilitates pneumococcal penetration into host cells by hydrolyzing the hyaluronan of the extracellular matrix (Berry *et al.*, 1994). The pneumococci deficient in hyaluronate lyase demonstrated significant reduced virulence compared to its parent strain in animal infection model (Berry and Paton 2000, Chapuy-Regaud *et al.*, 2003).

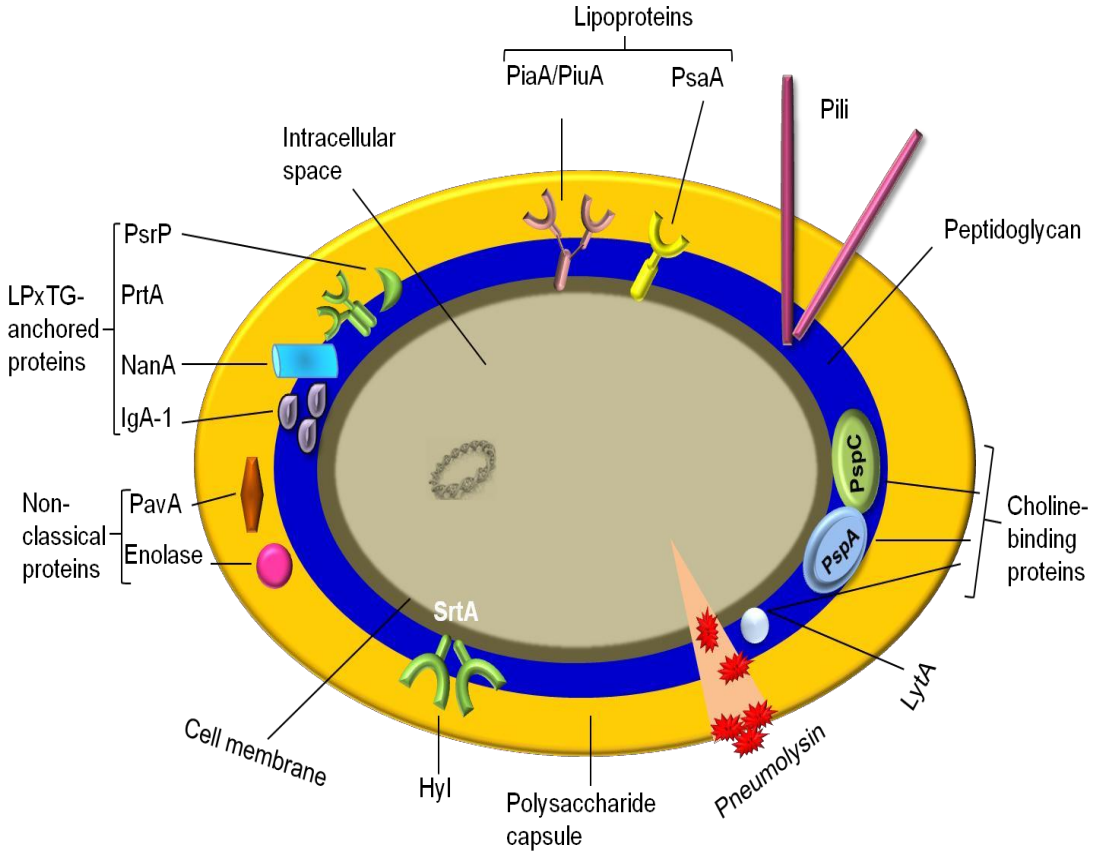

Figure 1.1 Schematic diagram of the pneumococcal virulence factors (Important pneumococcal virulence factors)

Include: the capsule, the cell wall, choline-binding proteins, pneumococcal surface proteins A and C (PspA and PspC), the LPXTG-anchored neuraminidase proteins, hyaluronate lyase (Hyl), pneumococcal adhesion and virulence A (PavA), enolase (Eno), pneumolysin (Ply), autolysin A (LytA), and the metal- binding proteins pneumococcal surface antigen A (PsaA), pneumococcal iron acquisition A (PiaA) and pneumococcal iron uptake A (PiuA**)**

(Adapted from (Jedrzejas 2001))

1.2.8.3.3 Pneumococcal surface exposed lipoproteins

Bacterial lipoproteins perform several functions such as signal transduction, adhesion, nutrient uptake, colonization, invasion, evasion of host defence and immunomodulation (Sutcliffe and Russell 1995, Kovacs-Simon *et al.,* 2011). Pneumococcal surface antigen A (PsaA), Putative proteinase maturation protein A (PpmA) and streptococcal lipoprotein rotamase A (SlrA), pneumococcal iron uptake A (PiuA) pneumococcal iron acquisition A (PiaA) have been suggested to play role in pneumococcal virulence. PspA binds lactoferrin on the surface of the bacteria, to evade the immune system. Lactoferrin is a secreted iron binding glycoprotein that is released to bind iron, which is needed for bacterial growth. PspA can also inhibit cytokines activation and complement activation (Hakansson *et al.,* 2001).

PiuA and PiaA are iron uptake by ATP-binding cassette (ABC) transporters and seem to be important for pneumococcal virulence (Brown *et al.,* 2001). (PpmA) and SlrA belong to the family of chaperones, the peptidyl-prolyl cis/trans isomerases and these two surface exposed lipoproteins are conserved in pneumococci. SlrA has been shown to contribute to pneumococcal colonization but not to invasiveness and was suggested to play role in the first stage of pneumococcal infections such as colonization of the upper airways, most likely by modulating the biological functions of the important virulence proteins (Hermans *et al.,* 2006).

1.2.8.4 Pneumococcal adherence and virulence factor A (PavA)

PavA is localized on the pneumococcal outer cell surface and has been suggested to serve as a pneumococcal adhesin for fibronectin (Holmes *et al.,* 2001). Moreover, PavA has been suggested to protect pneumococci against recognition, and actin cytoskeleton-dependent phagocytosis and killing by innate immune cells, indicating that PavA is essential for pneumococci to escape phagocytosis and for the optimal induction of immune response in dendritic cells (Noske *et al.,* 2009).

1. 2.8.5 Choline-binding proteins

Several Gram-positive bacteria including pneumococci display surface proteins by anchoring them covalently to the cell wall using the sortase and LPxTG-motifs of the proteins as the key players. In addition to these, the pneumococcal surface is decorated with non-covalently attached proteins named choline-binding proteins (CBPs). The family of CBPs consists of 13-16 different proteins and the number of produced CBPs depends on the pneumococcal strain. CBPs are highly homologous in their C-terminal parts whereas the N-terminal parts are non homologous and display the biological function. These so called CBPs includes several important molecules such as for example, the pneumococcal surface protein A (PspA), the pneumococcal surface protein C (also described initially as CbpA) and four cell wall hydrolases. The cell walls for hydrolases are the major autolysin LytA, the autolysins LytB, and LytC (Novak *et al.*, 1998).

PspC, due to its functional properties (Janulczyk *et al.,* 2000), is highly polymorphic protein important for virulence, adherence, and a binding to human factor H (Dave *et al.*, 2001, Dave *et al.,* 2004a, Dave *et al.,* 2004b). It is known to have antigenic properties important for control of complement system, binding secretory IgA, and has been suggested as a vaccine candidate. PspC is an inhibitor of complement deposition through its binding to negative regulator Factor H of the complement component C3 (Yuste *et al.,* 2010). Different pneumococcal strains have shown different binding potential that could

be related to their difference in ability to avoid complement-mediated opsonisation (Iannelli *et al.,* 2002).

Pneumococcal choline-binding protein A (PcpA), has been suggested to be a vaccine candidate, but is unlikely to protect against colonization due its manganese-dependent regulation, i.e. the expression of PcpA protein is high in blood where the concentration of manganese is low; where in the nasopharynxwhere manganese concentration is high, the expression of PcpA is low. It has a C-terminal choline binding domain, which is used to anchor the protein to the cell wall (Glover *et al.,* 2008). Due to its leucine rich repeat in the N-terminal of the protein, it has been suggested to be involved in adhesion of pneumococci to epithelial cells (Sanchez-Beato *et al.,* 1998).

1.2.9 Pathogenesis

Although colonization with pneumococci is mostly asymptomatic, it can progress to respiratory or systemic disease. An important feature is that pneumococcal disease will not occur without preceding nasopharyngeal colonization with the homologous strain (Bogaert *et al.,* 2004a). The bacteria enter the nasal cavity and attach to the nasopharyngeal epithelial cells and may then either stay as a colonizer or spread further to other organs, such as the ears, sinuses, or via bronchi down to the lungs and then potentially penetrate the mucosal barrier to enter the blood stream and/or cross the blood–brain barrier to cause meningitis (Fig.1.2).

Many of the characteristics or factors that increase the likelihood of pneumococcal colonization (e.g., young age, underlying immunodeficiency, crowding, and exposure to young children) are the same characteristics that increase individual risk for pneumococcal disease. An individual who is susceptible to colonization with a pneumococcus may become colonized upon exposure and can carry the strain for weeks to months. Invasive disease most commonly occurs following acquisition of a new serotype, typically after an incubation period of 1-3 days (Henriques-Normark and Tuomanen 2013). However, the mechanisms by which colonizing pneumococci become pathogenic and the processes involved in the translocation of the pneumococcus from the

nasopharynx to other anatomic sites are poorly understood (Johnston 1991, Obaro and Adegbola 2002a, Mook-Kanamori *et al.*, 2011).

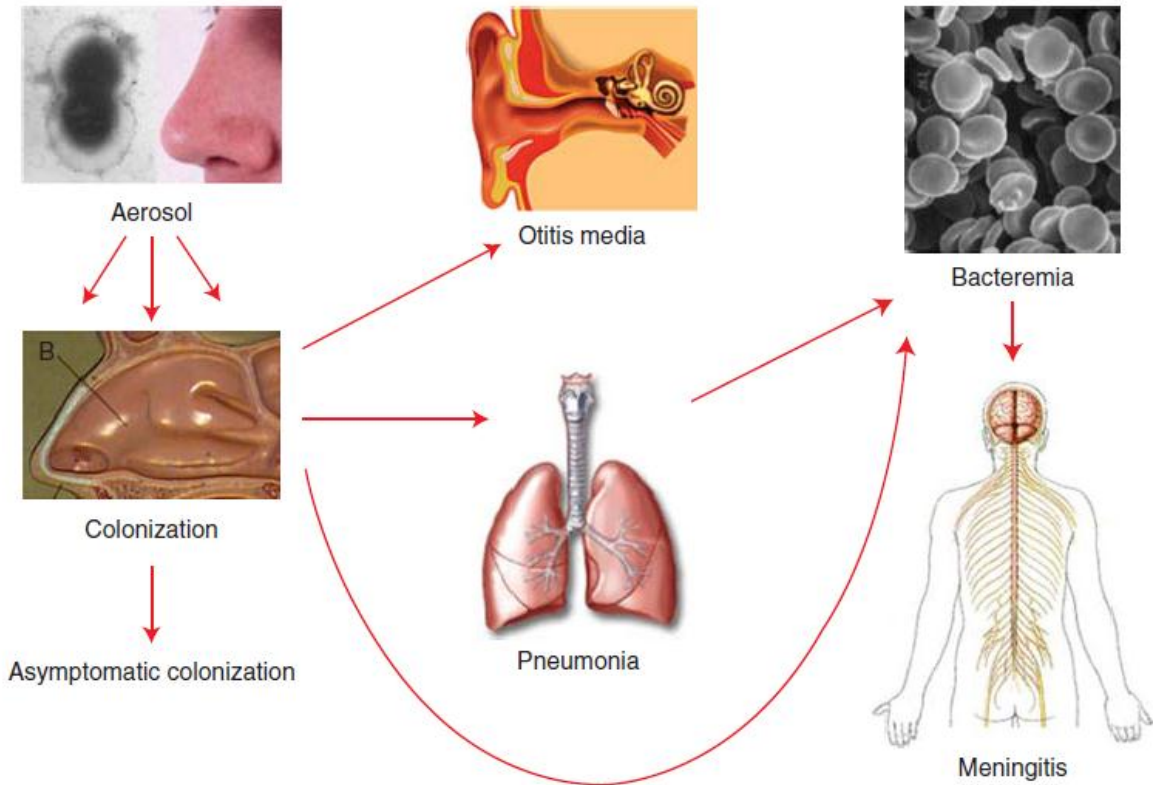

Figure 1.2 Progression of pneumococcal disease.
(Adapted from Henriques-Normark and Tuomanen 2013)

This interaction most commonly leads to clearance and serotype-specific immunity. Progression to otitis media is very common in children. Invasive disease involves spread to the lungs and bloodstream. The most serious development is meningitis. This series of invasive steps is a pattern shared by the three major bacterial pathogens of children (Pneumococcus, Haemophilus, and Meningococcus) and is driven by a very basic interplay between innate invasion and innate immunity (Henriques-Normark and Tuomanen 2013).

Colonization by pneumococci requires adherence to the epithelial lining of the respiratory tract. It has been proposed that attachment of pneumococci to the respiratory epithelium is mediated by a disaccharide receptor on fibronectin, which is present on all epithelial

cells (van der Poll and Opal 2009). Additionally, adherence of pneumococci to tracheal epithelial cells may be enhanced by prior influenza or parainfluenza virus infection, most likely mediated by viral neuraminidase (Henriques-Normark and Tuomanen 2013). This enzyme cleaves sialic acid from glycosphingolipids, which are found in substantial amounts in human lung tissue. Thus, neuraminidase is thought to expose other structures that function as receptors to which pneumococci can adhere (Obaro and Adegbola 2002a).

Three interchangeable variants of pneumococci have been described, based on colony morphology: opaque, semi-transparent, and transparent; these phenotypes appear to have different abilities to colonize the nasopharynx and cause different types of disease, but the biochemical basis for the phenotypic variation is unknown. The bacterium interacts with the glycoconjugate that serves as a receptor on eukaryotic cells in the nasopharynx (Obaro and Adegbola 2002a). Phase variation appears to play a role in the adaptation of pneumococci to changes in receptors presented on activated host cells (Weiser *et al.,* 1994). The transparent phenotype appears to have an advantage in colonization of the nasopharynx, due to its ability to recognize the cognate eukaryotic ligand (G1cNAcβ1-3Gal) (Cundell *et al.,* 1995).

More adhesive strains cause localized infections and less adhesive strains cause invasive disease, such as bacteremia and meningitis. Translocation of the organism by either aspiration or penetration of the mucosa results in bacteremia or sepsis, or both, and seeding in different anatomic body parts may cause disease, such as meningitis and arthritis (Obaro and Adegbola 2002a). Once pneumococci attach to and invade host tissues, disease is primarily caused by replication within host tissues and the subsequent inflammatory response (Spreer *et al.,* 2004). Unlike many other pathogens (e.g., *Staphylococcus aureus*, *Corynebacterium diphtheriae*, *Escherichia coli*, and *Clostridium difficile*), pneumococci produce relative few exotoxins (Tonnaer *et al.,* 2006). The host responds to cell wall components, such as TA that are released after bacteriolysis and to pneumolysin that is released during bacterial growth and lysis (Ginsburg 2002, Spreer *et al.,* 2004). Certain proteins or enzymes displayed on the surface of pneumococci (e.g.,

hyaluronate lyase, autolysin, neuraminidases, PsaA, and other choline binding proteins) may contribute to the pathogenesis and disease symptoms (Mitchell and Mitchell 2010)

It is generally thought that pneumonia results from the aspiration of pneumococci from the upper respiratory tract, although a blood-born route of dissemination from the upper respiratory tract is also possible (Busse 1991). Pneumococci are presumably aerosolized from the nasopharynx to the alveolus, bypassing the ciliated epithelium, to which they cannot attach (Henriques-Normark and Tuomanen 2013). Bronchopneumonia, which involves airways more than alveoli, is promoted by antecedent injury to the ciliated mucosa, providing conditions for pneumococcal attachment (Dobay *et al.,* 2004, Mook-Kanamori *et al.,* 2011). This tropism of pneumococci for damaged epithelium was shown in autopsy materials from patients dying of pneumonia during the influenza pandemic of 1957-1958 (van der Poll and Opal 2009). Damaged epithelium can also arise by smoking or infection with other respiratory pathogens. Exposure of the endobronchial submucosa allows pneumococci to attach to basement membrane components, such as fibronectin and collagen. Once localized to the airway, release of pneumolysin exacerbates the mucosal damage, since epithelial cells are particularly sensitive to toxin-induces cytolysis and inhibition of ciliary beat frequency (Mook-Kanamori *et al.,* 2011).

Pneumococcal meningitis usually arises in the context of sustained bacteremia that permits bacterial localization to and transit across the blood-brain barrier (BBB) into the subarachnoid space. The central pathologic event in meningitis is breakdown of the BBB. Bacteria penetrate from the blood across the tight junctions of the cerebral vessels and multiply upon entry into the cerebrospinal fluid (CSF), a site sequestered from host defenses (Mook-Kanamori *et al.,* 2011). Once in the CSF space, multiplication of bacteria results in release of cell wall components and toxins (pneumolysin and hydrogen peroxide). Bacterial surface fragments, including cell wall, incite direct host cell toxicity and the release of a broad range of cytokines that serve to increase inflammation, alter vascular permeability, and induce influx of leukocytes (van Furth *et al.,* 1996, Freyer *et al.,* 1999). Over a period of hours, gaps are opened between endothelial cells,

pinocytotic activity increases, leukocytes are recruited to the CSF, and cytochemical parameters in CSF change. Injury to the cerebral microvasculature promotes brain edema that in turn leads to intracranial hypertension. In addition, oxygen and nitrogen intermediates released by the inflammatory process causes loss of autoregulation of cerebral vascular perfusion pressure with resultant ischemia (van der Poll and Opal 2009). The precise events that lead to neuronal damage remains unclear, but both direct toxicity and injury from host-derived inflammation are involved (Mook-Kanamori *et al.*, 2011).

1.2.10 The immune system

The human immune system can be divided into two categories: the innate and adaptive immunity. Human are born with innate immunity, while the adaptive immune system develops and matures during the first years of life. By meeting pathogens such as viruses, bacteria and fungi the immune cells are trained (CDC 2010).

Epithelial cells from barriers (i.e. the skin and epithelial cells inside a body) that protect us from infection when exposed to contaminated food and air (Hornef *et al.,* 2002). The innate immunity is always active, and it is the first response triggered by pathogen. In addition to epithelial cells, neutrophils, dendritic cells, and macrophages are part of the innate immunity. Macrophages are capable of phagocytosing encountered pathogens. The recognition of foreign particles is partly due to its expression of TLRs by these immune cells (Hornef *et al.,* 2002). There are ten known human TLRs today; they recognized common structure, pathogen-associated molecular pattern (PAMPs) that are present on, or produced by pathogens. Some TLRS have been suggested to be involved in the innate immune response against pneumococci, such as TLR1, TLR2, TLR4, TLR6 and TLR9. TLR2 has been shown to recognize cell wall component of pneumococci, while TLR4 has been suggested to be involved in recognising pneumolysin. TLR9 recognise CpG-DNA and has been suggested to affect pneumococcal pathogenesis (Albiger *et al.,* 2007). Macrophages secret cytokines that attract other immune cells to help to clear the body from pathogens (Kadioglu and Andrew 2004, Kadioglu *et al.,* 2008).

The adaptive immune response which includes lymphocytes, B and T cells is much slower than the innate immune response. It can take up to a week before it is fully active. Lymphocytes circulate in the bloodstream and in the lymph nodes, where they meet antigens presented on dendritic cells, macrophages and antibody producing B cells. Upon encountering a specific antigen, they will undergo clonal expansion, bind more antigens and produce antibody against it. The antibodies that are produced bind pathogens and neutralise the pathogen by blocking the binding of the pathogen to its ligand. Antibodies can also activate the complement system, leading to a stronger immune defence (Kadioglu *et al.,* 2008).

When bacteria enters to our body the innate immune response, including complement, interferons, cytokines, natural killer cells, neutrophils, and macrophages, is activated to prevent the invasion (Kadioglu *et al.,* 2008).

The complement system, involving around 30 serum protein and membrane proteins, is an important host defence mechanism against encapsulated bacteria, such as pneumococci. The complement system can be activated in three different ways; through the classical, the alternative, or the lectin pathways. The dominant pathway to defend the host against pneumococci is the classical pathway. It is activated when antibodies bind to an antigen, while the alternative pathway is constantly active at a lower frequency. The alternative pathway can be regulated in response to foreign surface, by binding of C3b to these surfaces. The lecin pathway is activated by recognition of carbohydrate, such as mannose, on the surface of microbes, by mannose binding receptor (Paterson and Orihuela 2010).

The complement system, independent on what pathways is activated, is to produce C3b. It is an opsonin that binds to the bacteria and gives a signal for binding to phagocytic cells, such as neutrophils and macrophages, which express receptors for C3b. The complement system leads to phagocytosis of the bacteria (Murphy *et al.,* 2012).

As many other bacteria, pneumococci have developed mechanism to avoid the innate and adaptive immunity. The expression capsule is one of these strategies. By expressing capsule, the complement mediated opsonophagocytosis is inhibited due to less C3b binding to the capsule. The capsule is the major virulence determinant for invasive pneumococcal disease (Mac and Kraus 1950). The expression of capsule has been associated with the invasive potential, since it inhibits complement and protects the bacteria from neutrophil-mediated killing (Weinberger *et al.,* 2009). Encapsulated pneumococci are more often found to cause invasive disease, while bacteria expressing less capsule are more prone to be found in children with asymptomatic carriage (Kadioglu *et al.,* 2008, Weinberger *et al.,* 2009).

The pneumococcal surface protein C (PspC), which is also called CbpA, which recruit human factor H to the bacterial surface and thereby negatively regulates the complement activation by cleavage of C3b (Hornef *et al.,* 2002). Pneumococcal surface protein A (PspA) has also been shown to have the ability to down regulate the complement activation, but the mechanism is unclear (Moffitt and Malley 2011, Vadesilho *et al.,* 2012).

1. 2.11 Typing of Pneumococci

1.2.11.1 Serotyping

Based on the structural diversity of the capsular polysaccharide, pneumococci can be divided into 97 different serotypes. Two types of nomenclature have been developed: the Danish (Quellung reaction) and the American (Geno *et al*., 2015), but the Danish nomenclature is the most widely accepted. Unlike the American, which assigns the serotype number in the sequence of the first isolations, the Danish method classifies serotypes according to structural and antigenic relatedness, reflecting the cross reactivity of similar serotypes, e.g. 6A and 6B. This system involves 97 antisera, developed in the Statens Serum Institute, Copenhagen (Henrichsen 1995), and can identify 46 main serogroups using capsule swelling test, or in some other cases a simpler agglutination test (e.g. Mast Diagnostics). A serogroup was defined to include serotypes that share many

serologic properties (i.e., cross-reactive antibodies).These serogroups in many cases can be divided into further serotypes (altogether 97), e.g. 19A, 19B, 19C or 19F with the help of factor sera. For others, such as 1, 3 or 14, no further typing is possible and hence these numbers are referred as serotypes (Geno *et al.,* 2015).

1.2.11.2 Genotyping

Phenotyping methods, such as serotyping or the determination of the antimicrobial resistance pattern, have limited discriminatory power; so to get detailed epidemiological information at both local and international levels, molecular typing methods are essential (Overweg *et al.,* 2000, McGee *et al.,* 2001). Many different methods are used to track pneumococci. These include pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), ribotyping, BOX-PCR, restriction fragment end labeling (RFEL), amplified fragment length polymorphism (AFLP), protein A typing, or penicillin binding protein (PBP) gene finger printing and microarrays (Hermans *et al.,* 1995, Neeleman *et al.,* 2004, Wang *et al.,* 2007). These methods differ in discriminatory power, ease of interpretation, requirement of special knowledge, level of complicatedness, length of time and finances. Generally, PFGE with its fragment pattern provides increased level of discrimination and follows the rapid evolutionary changes more closely, therefore better for local epidemiological studies, while MLST is more rigorous with its allelic profile of the housekeeping genes, hence more applicable for international comparisons (McGee *et al.,* 2001).

1.2.12 Treatment and Antibiotic Resistance

Antibiotic resistance is a major challenge to modern medicine. Bacteria constantly adjusting to their environment and could lead to increased resistance, due to selection. It is well known that some pneumococcal serotypes and clones carry mores resistance determinants than others. Penicillin G resistance, for example, can be less associated with some clonal complexes (CCs) (Doern *et al.,* 1998). Non-susceptible isolates are often found among carriers and are more genetically diverse, i.e. belonging to CCs, since they are residing in the nasopharynx where they exchange DNA with other pneumococci or

with other bacterial species. Invasive isolates are generally more clonally related, since they are transmitted to the blood and are not able to exchange DNA with each other at that site of infection (Robinson *et al.,* 2001, Ardanuy *et al.,* 2009).

For decades, the standard treatment for pneumococcal infection has been penicillin but resistance to penicillin as well as to erythromycin is increasing even in developed countries with overall low frequency of resistance (Browall 2015).

Penicillin is a β-lactam antibiotic and, has been in use since it was discovered by Alexander Fleming in 1928, been used to treat infections caused by Gram positive bacteria such as pneumococci. In 1967, not many years after the introduction of penicillin, the first resistant pneumococcal isolates were found. Penicillin resistant pneumococcal strain was first recovered in Papua New Guinea and Australia and it is now prevalent throughout the world and the first multi-resistant clinical strain was isolated in 1977. Between 40–50% of pneumococcal isolates from Spain, Eastern Europe, South Africa, South America, New Guinea and South Korea are penicillin resistant (Kadioglu and Andrew 2004).

Pneumococci constantly remodel their peptidoglycan cell wall, building and breaking down part of the cell wall as they are growing and dividing. The genetics of penicillin resistance is complex, involving mosaic forms of pbp1a, pbp2b, pbp2x, and pbp2a. These mosaic PBP gene alleles are stably inherited in the presence or absence of selection, and strains carrying these alleles serve as resistance-gene reservoirs for the horizontal transfer of β-lactam resistance to other pneumococcal strains (Gherardi *et al.,* 2000). There is a claim that the global increase of Penicillin Resistant *S. pneumoniae* (PRSP) instead of the increased emergence of unrelated PRSP strains is attributable to the global dissemination and propagation of a small number of clones (Davies *et al.,* 2004). Recent studies have shown modifications of several PBPs among pneumococcal strains, causing resistance among non-vaccine types (NVTs) such as 15A and 33B, while resistance was decreasing among vaccine types (VTs) (Chiba *et al.,* 2014, Lin *et al.,* 2014).

In post-vaccination study done in 2009, by Dagan *et al*, was shown that the observed decrease in penicillin resistance was due to a reduced carriage of VTs. However, NVTs, that were initially only intermediate resistant to penicillin, also increased and overall penicillin resistance post-vaccination was first lower than before PCV introduction, but then increased (Dagan *et al.,* 2009). An important serotype post-vaccination was 19A. Serotype 19A has been shown to be responsible for the high frequency of penicillin resistance, including for resistance spread worldwide (Pelton *et al.,* 2004, Hicks *et al.,* 2007).

In a study done by Isaacman *et al*, the cause of some phenomena was observed. Antibiotic resistant strains of VTs decreased, while serotype replacement led to an increase of other serotypes carrying antibiotic resistance. They also observed difference in antibiotic resistance between geographical regions (Isaacman *et al.,* 2010).

Macrolides are a group of antibiotics whose activity is due to the inhibition of protein synthesis via binding to the 23s rRNA of the 50S ribosomal subunit (Murray 2007). Erythromycin belongs to the macrolide group and is commonly used to treat pneumococcal infection, especially among patients that are allergic to penicillin. Resistance to macrolides in pneumococcus has increased in many parts of the world, resistance is mediated by two main mechanisms: target modification due to a ribosomal methylase encoded by the *erm(B)* gene, which confers high-level resistance to macrolides, lincosamides, and streptogramin B (Leclercq and Courvalin 2002) and an efflux transport system associated with the mef gene (*mef(A) and mef(E)*), which confers resistance to 14- and 15-membered macrolides only (Clancy *et al.,* 1996, Tait-Kamradt *et al.,* 1997). Molecular epidemiology studies have shown that the prevalence of these two mechanisms among macrolide-resistant pneumococci varies in different geographical areas. Conducting periodical surveys on serotype distribution and drug susceptibility pattern, on a regular basis is useful to detect changes in resistance and serotype distribution in a population. Determining the molecular bases for resistance might be helpful for appropriate empirical antibiotic therapy and to design new strategies in vaccine preparation and new drug development (Bayraktar *et al.,* 2005b).

Trimethoprime-sulphametoxazole is a broad spectrum antibiotic used to treat a variety of bacterial, fungal, and protozoal infections. It inhibits the biosynthesis of folic acid (Murray 2007). Resistance was first observed in the seventies, and today it is commonly found among pneumococcal strains, and especially among serotypes 9V and 14 (Sjostrom *et al.,* 2007).

Quinolones have an antibacterial effect by preventing pneumococcal DNA from unwinding, due to its inhibition of bacterial topoisomerase type IV. Levofloxacin is also used to treat pneumococcal infections and a newer versions of quinolones, fluoroquninolones. Chromosomal mutation in topoisomerase IV, results in resistance to fluroquinolones (Murray 2007).

Vancomycin is a glycopeptide that disrupts the synthesis of peptidoglycan in the growing Gram positive bacterial cells. It interferes with the production of cross linkage between peptidoglycan chains. Vancomycin can be used when strains are resistant to penicillin and other antibiotics (Murray *et al.,* 2013).

Clindamycin blocks protein elongation by binding to the 50S ribosomal subunit, as macrolides and chloramphenicol do. Resistance to clindamycin can occur due to methylation of 23S ribosomal RNA, also observed in erythromycin resistance. Therefore cross resistance is often observed between erythromycin and clindamycin (Murray *et al.*, 2013)

The worldwide escalation of Multi drug resistant (MDR) strains could largely be attributed to the spread of a small number of MDR clones. Currently, the Pneumococcal Molecular Epidemiology Network (PMEN) has documented 43 international clones, 26 of which are MDR. The first report was Spain^{23F}-1 or PMEN1, after then a number of reports from diferent countires (Colombia^{5}-19, South Africa^{19A}-7, England^{14}-9, South Africa^{19A}-13, Taiwan^{23F}-15, Sweden^{15A}-25 etc) (Cornick and Bentley 2012)

1.2.13 Pneumococcal vaccines

As pneumococcal vaccines provide serotype-specific protection, it is important that vaccines prevent disease caused by the most clinically relevant serotypes. Thus, vaccines provide the greatest impetus for recognizing capsular diversity and serotype epidemiology. Pneumococcal vaccines are an important public health tool and have undergone dramatic changes in recent years, and two major group of pneumococcal vaccines, pneumococcal polysaccharide vaccines (PPVs) and pneumococcal conjugate vaccine (PCV) (Torres *et al.,* 2015).

1.2.13.1 Polysaccharide vaccines (PS)

Studies in 1930s and 1940s led to clinical uses of two hexavalent PPVs in 1946 (one for children and one for adults), but they were withdrawn due to therapeutic successes with antibiotics. PPV was again introduced in 1977 as a 14-valent form. In 1983, PPV14 was expanded to a 23-valent vaccine (PPV23) (Robbins *et al.,* 1983, Grabenstein and Klugman 2012).

The only currently available PS vaccine, pneumococcal polysaccharide vaccine 23 (PPV23), is composed of 25µg PS per each of 23 serotypes (Table 1.1) (Robbins *et al.,* 1983). It also contains a significant amount of cell wall polysaccharide (CWPS) (Sorensen and Henrichsen 1984, Musher *et al.,* 1990). PPV23 covers a wider array of serotypes than current PCVs (Table 1.1) and could thus theoretically represent at least 85 to 90% of the serotypes that cause invasive infections (Pilishvili *et al.,* 2010); however, it has some limitations. First, PPV23 is poorly immunogenic in infants younger than 2 years of age (Song *et al.,* 2013). Second, it shown poor effectiveness against non bacteraemic or community-acquired pneumonia and against IPD in chronically ill patients (Moberley *et al.,* 2013) and individuals aged 75 years or older (Andrews *et al.,* 2012). Third, it may not prevent NP colonization or mucosal infections (non bacteraemic pneumonia and otitis media) (O'Brien and Santosham 2004). Finally, antibody concentrations after subsequent doses of PPV23 appear to be lower than those after primary vaccination (Dagan *et al.,* 2010a); this hyporesponsiveness is related to the large

amount of PS in PPV23, which is thought to exhaust the memory B-cell pool without replenishment (Clutterbuck *et al.,* 2012).

1.2.13.2 Pneumococcal conjugate vaccines

The poor immunogenicity of PPV in infants younger than 2 years led to the development of PCVs (CDC 2012, Song *et al.,* 2013). PCVs are made by conjugating PSs to proteins, but their immunogenicity may be determined by the protein carrier, the method of conjugation, the ratio of PS to carrier, the length of the saccharide chains (PS versus oligosaccharide), and the quantity of PS (Poolman *et al.,* 2013). Excessive amounts of PS can reduce the immune response, while excessive amounts of carrier protein may lead to immune interference known as carrier-induced epitope suppression (Dagan *et al.*, 2010b). All licensed PCVs are summarized in Table 1.1. PCV10 and PCV13 differ in terms of the carrier proteins and conjugation methods. In the case of PCV10 (Synflorix; Glaxo- SmithKline (GSK) Biologicals Belgium), each serotype is conjugated to one of three carrier proteins: serotype 18C to tetanus toxoid, serotype 19F to diphtheria toxoid (DT), and the remaining serotypes to protein D (a recombinant, highly conserved 42-kDa cell surface lipoprotein of nontypeable *Haemophilus influenzae*). PCV10 is conjugated by a bifunctional spacer (cyanylation using 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate) increasing side chain but ay have minimal alteration in PS structure (Porat *et al.,* 2012).

In comparison, all serotypes of PCV7 (Prevnar; Pfizer) and PCV13 (Prevnar13; Pfizer) are conjugated to cross-reactive mutant of diphtheria toxin (CRM197) (DT variant). PCV7 and PCV13 are conjugated to CRM197 through reductive amination, which may modify structural PS conformations or create new epitopes (Porat *et al.,* 2012). Such conjugation induced epitope changes may explain the poorer immunogenicity of PCV13 for serotype 19F and the absence of cross-reactivity between serotypes 19F and 19A after PCV7 vaccination (Poolman *et al.,* 2011). In addition, PCV7 was composed of natural PS antigens to 6 pneumococcal serotypes (4, 6B, 9V, 14, 19F, and 23F) but of a size reduced PS for serotype 18C (Obaro 2002, Feldman and Anderson 2014). The rationale for PCV10 is that 19F in PCV7 did not cross react with 19A but PCV10 does, but the

evidence for PCV10 cross protection against 19A is not good (Hammitt *et al*., 2014, Domingues *et al*., 2014, Farkouh *et al*., 2015). Currently, countries are using two different WHO approved vaccination schedules: a 6-week, 10-week, 14-week (3+0) schedule; and a 2- month, 4-month, 6-month schedule, which is followed by a booster dose at 12–15 months of age (3+1), and studies are undergoing of other option two primary doses with a booster (2+1) (Walter and Clements 2013).

Table 1.2 Comparison of pneumococcal vaccine compositions

Vaccine (commercial name)	**Year licensed**	**Conjugation method**	**Carrier protein (content, µg)**	**Serotypes**	**Polysaccharide (content, µg)**	**Adjuvant**
PPV23 (Pneumo 23)	1983	None	None	1, 2, 3, 4, 5, 6B, 7F,8,9V, 9N, 10A,11A,12, 14, 15B,17F, 18C, 19F, 19A, 20, 22F, 23F, 33F	25 per serotype	None
PCV7 (Prevnar)	2000	Reductive amination	CRM197	4, 6B, 9V, 14, 18C, 19F, 23F	6B, 4; others, 2	Alum
PCV10 (Synflorix)	2008	Bifunctional spacer	NTHi protein D (9–16); tetanus toxoid (5–10); diphtheria toxoid (3–6)	PCV7+1, 5, 7F	4, 18C, 19F, 3; others, 1	Alum

PCV13 (Prevnar 13)	2009	Reductive Amination	CRM197	PCV10 + 3, 6A, 19A	6B, 4.4; others, 2	Alum

PCV7 was first licensed in the United States in 2000, and included the seven serotypes most isolated in children under-five years in the United States (Pilishvili *et al.,* 2010). To cover a broader range of serotypes and reflect the altered serotype epidemiology following PCV7 introduction, PCV10 and PCV13 were developed soon after. PCV10 was approved first in Canada in 2008 and then approved by the European Medicines Agency in 2009. However, PCV10 is not yet licensed in the United States but is widely being used in Latin America, Europe and Africa. PCV13 was approved first in Chile in 2009 and then in the United States in 2010. Both PCV10 and PCV13 were approved for infants and children to protect against IPD and acute otitis media (CDC 2010). In December 2011, the U.S. FDA also licensed PCV13 for adults aged 50 years (CDC 2012). Merck has performed phase I/II clinical trials with a fifteen-valent PCV, which has all the serotypes in PCV13 (Table 1.1) as well as 22F and 33F (Feldman and Anderson 2014, Geno *et al.,* 2015).

1.2.13.3 PCV vaccines impact

PCV7 has significantly reduced the burden of pneumococcal diseases in children, after the introduction PCV7 in the USA in 2000. The Centers for Disease Control and Prevention (CDC) reported a 77 % reduction in overall IPD rates and a 98 % reduction in PCV7 serotype disease in children aged <5 years for 2005 compared with pre-PCV7 years (1998– 1999), based on an analysis of laboratory and population surveillance data (CDC 2008). Reductions in overall and/or PCV7 serotype IPD cases have also been documented in children aged <2 or <5 years in many other countries, including Australia, Canada, France, Norway and Spain, following the introduction of PCV7 (Kellner *et al.*,

2005, Roche *et al.,* 2006, Dubos *et al.,* 2007, Vestrheim *et al.,* 2008b, Perez-Trallero *et al.,* 2009, Ruckinger *et al.,* 2009). Furthermore, reductions in hospitalisation rates for all-cause pneumonia (39 %) and pneumococcal pneumonia (65 %) in children <2 years of age have been observed in an US analysis of admissions data (Grijalva *et al.,* 2007). Similarly, a Polish study found that pneumonia admission rates significantly declined, following the introduction of PCV7 in children <5 years of age in the city of Kielce (Patrzalek *et al.,* 2010). PCV7 has also reduced otitis media in children aged <2 years, as demonstrated by a ≥28% reduction in recurrent otitis media (Poehling *et al.,* 2007) and a ≥43 % reduction in AOM outpatient visits or prescriptions (Zhou *et al.,* 2008).

Antibiotic resistance in vaccine serotypes and carriage of pneumococcus has declined since the introduction of PCV7. Kyaw *et al*. reported an 81 % decrease in penicillin-resistant IPD (almost all caused by vaccine or vaccine-related serotypes) among children aged <2 years in the USA (Kyaw *et al.,* 2006). In addition, reductions in the carriage of vaccine serotypes and antibiotic resistant serotypes have been observed in Greece and the USA (O'Brien *et al.,* 2007, Grivea *et al.,* 2008).

PCV7 has demonstrated an indirect effect in unvaccinated populations (herd effect), as exemplified by declines in IPD cases within adults aged ≥65 years and infants ≤60 days of age in Canada and the USA (Kellner *et al.,* 2005, Poehling *et al.,* 2006). A review of data from 14 countries, most of which were developed countries, also reported consistent and significant declines in both vaccine-type IPD and vaccine-type pneumococcal carriage following PCV introduction in individuals not targeted for PCV vaccination (Davis *et al.,* 2013). These decreases were found to be contemporaneous in studies assessing both vaccine-type carriage and vaccine-type IPD, and longitudinal data demonstrated continued declines, with the greatest declines occurring in the first few years following PCV introduction (Torres *et al.,* 2015).

Since the availability of PCV7 in 2000/2001, there have been changes in the overall serotype distribution of pneumococci, in particular, a rise in serotype 19A has been observed globally. The estimated proportion of IPD caused by serotype 19A has ranged

from 22 % reported in a Spanish study (2001–2005) in 85 vaccinated and unvaccinated individuals <5 years of age to 40 % in a US study (2005) in 1.26 million individuals <5 years of age (Barricarte *et al.,* 2007, CDC 2008). A rate of 27 % has also been reported in cases of pneumococcal pneumonia in a French study (Bekri *et al.,* 2007). This changing serotype epidemiology has led to the development and introduction of higher-valent pneumococcal conjugate vaccines, including PCV13, which includes serotype 19A, to provide improved serotype coverage against pneumococcal diseases (Torres *et al.,* 2015).

By 2013, 2–3 years after the widespread use of PCV13, there were emerging data concerning the impact of PCV13 on the rate of vaccine serotype-specific IPD in children. There has been a decline in IPD in the UK, due to the 6 additional serotypes in PCV13 following the introduction of PCV13 and a sustained decline in IPD due to shared serotypes in PCV7 and PCV13 with the exception of serotype 3 (Flasche *et al.,* 2011). Furthermore, reductions in IPD due to PCV13 serotypes have been reported in children <2 years of age in Germany following the introduction of PCV10 and PCV13 (van der Linden *et al.,* 2012). Temporal trends in nasopharyngeal carriage have also been monitored in several studies following the introduction of PCV13. In a study of 448 healthy children aged ≤6 years attending day-care centres in Portugal, there were reductions of 8% and 10%, respectively, in the incidence of NP carriage of serotypes 19A and 6C from 2010 to 2011 following the introduction of PCV13 (Félix *et al.,* 2012).

Similar to the PCV7 experience, in countries where there has been a high uptake of PCV13 within childhood immunisation programmes, there has been a decline in vaccine-type IPD cases in adults, indicating a possible early herd effect. For example, in Norway, which had a vaccine coverage rate of 92 % for children <2 years of age in 2012, IPD cases caused by PCV13 serotypes in individuals ≥65 years old declined from 27/100,000 in 2010 to 18/100,000 in 2012 (Steens *et al.,* 2013). There have also been declines in the number of IPD cases caused by the additional PCV13 serotypes (1, 3, 5, 6A, 7F and 19A) in individuals aged ≥65 years in adults in Germany since the introduction of paediatric PCV13 vaccination (van der Linden *et al.,* 2012). These data have led to a debate about

whether there will be a reduced requirement for adult vaccination as the indirect effect of PCV13 increases. However, despite this potential herd effect, a significant burden of pneumococcal disease in adults' remains and direct vaccination of adults is the optimal way to provide individual protection to those at risk and to significantly reduce the pneumococcal disease burden in the adult population (Torres *et al.,* 2015).

There is also evidence that PCV10 has reduced the burden of pneumococcal diseases in children. Palmu *et al*. reported point estimates for PCV10 efficacy against vaccine-type IPD of 100 (95 % CI 83–100) for the 3+1 schedule and 92 (58–100) for the 2+1 schedule in a cluster-randomised, double-blind trial, involving Finnish children aged <19 months, vaccinated with PCV10 or hepatitis vaccines (control group) (Palmu *et al.,* 2013). A PCV10 study in Canada has shown some evidence of reduced vaccine-type IPD in infants, with a reduction in IPD cases caused by the additional three PCV10 serotypes (1, 5 and 7F) observed, following the introduction of PCV10 (Lim *et al.,* 2013). A reduction in hospitalisation rates due to pneumonia has also been reported in Brazil, one year after the introduction of PCV10 into the national immunisation programme (Afonso *et al.,* 2013). PCV10 has been shown to be immunogenic in a number of primary and booster vaccination studies (Dicko *et al.,* 2011, Kim *et al.,* 2011, Ruiz-Palacios *et al.,* 2011, van den Bergh *et al.,* 2011), and also to reduce vaccine-type pneumococcal carriage (Hammitt *et al.,* 2014b).

Studies done in subsaharan such as a Kenyan study in Kilifi, the carriage rate was 34% for vaccine-serotype pneumococci, 41% for non-vaccine-serotype pneumococci, and 54% for non-typeable *Haemophilus influenzae* among children younger than 5 years at the baseline (2009-10). Carriage prevalence was reduced to 13%, 57%, and 40%, respectively after PCV10 introduction (2011-12) (Hammitt *et al.,* 2014a). Similarly, studies conducted in The Gamibia , South Africa and Malawi showed a significant reduction in carriage and IPD of vaccine serotypes after introduction of PCV13 in cluded in PCV13 (Mackenzie *et al*., 2016, Nzenze *et al*., 2013, Heinsbroek et al., 2015).

1.2.13.4 Other vaccine candidates

The observation of serotype replacement, only a few years following PCV introduction, raises concern about the long term usage of capsular based vaccine that includes a limited amount of serotypes alone. One potential strategy to reduce serotype replacement could be a protein-based vaccine targeting all pneumococci. The ultimate goal with pneumococcal vaccine would be to eradicate all disease-causing strains, leading to a further decrease in mortality and disease incidence. To achieve this, a common protein present in all clinical strains could be included in the vaccine. Another option could be more polysaccharides, but the costs are high; and the question is how many different serotypes the immune system can learn to recognize (Vadesilho *et al.,* 2012).

Pneumococcal surface protein A (PspA) is a surface protein and inhibits the classical complement pathway, specifically by competing with binding by C-reactive protein to block complement deposition. PspA elicits antibody mediated protection against invasive infections through its alpha-helical regions. It has been suggested as a candidate in protein-based vaccine against pneumococcal infections (Moffitt and Malley 2011, Vadesilho *et al.,* 2012).

Another important vaccine candidate for pneumococcal vaccines is pneumococcal surface protein C (PspC) also known as CbpA. It has similar structural features as PspA. It binds factor H, thus inhibiting the alternative pathways of complement deposition. It has also been shown to be important for adherence through interaction with the polymeric Ig receptors, and binding secretory IgA (Dave *et al.,* 2001, Dave *et al.,* 2004a). Croney *el al* showed that the virulence proteins PspA, PsPC and one of the pili structural proteins, Rrg, have potential to cover a wider number of strains than currently available with higher valence serotype PCV13. The efficacy of these proteins as vaccine in man remains to be shown (Croney *et al.,* 2013).

1.3 Statement of the problem

The worldwide disease burden from *S. pneumoniae* remains both significant and widespread. In developing countries, nearly 2.6 million preschool children die from

pneumonia and 151.8 million new cases, 11-20 million of which require hospitalization, occur annually. Almost half of these deaths are attributable to pneumococcus either exclusively or in conjunction with viral infections and/or malnutrition (Shann 2001, O'Brien *et al.,* 2009, Darboe *et al.,* 2010, Yoshioka *et al.,* 2011). This bacterium causes more deaths than does any other vaccine-preventable organism. It is an important cause of bacteremia, bacterial meningitis, and pneumonia in children worldwide. In the African meningitis belt, pneumoccoccus is as important a cause of meningitis as *Neisseria meningitidis*, particularly among older children and working age adults (Gessner *et al.*, 2010). Pneumococcal diseases are not limited to invasive infections, but are also responsible for localised infection with an estimated 7 million episodes of acute otitis media and 500 000 episodes of pneumonia per year in the United States alone for example (Myers and Gervaix 2007).

In 2005, the world Health Organization (WHO) estimated that every year 1.6 million people die due to pneumococcal infections around the world. This estimate includes the death of 0.7-1 million children younger than five years of age, most of who live in developing countries. In 2009, the WHO published that, in year 2000 the global death due to a pneumococcal infection in HIV-negative children younger than five years of age, was around 700,000. In 2012, the WHO published that the estimate of year 2008 had decreased to 500,000 in the same population. This decrease of the pneumococcal burden and resulting death has been suggested to be due to pneumococcal vaccinations (WHO 2007, WHO 2012).

Pneumococcal disease occurs worldwide, but the infection rate is high in developing countries in comparison to industrialized countries. It is a seasonal pathogen, common during winter and early spring, especially in European countries, when respiratory viruses are circulating more frequently. Outbreaks are uncommon but can occur in closed populations such as within prisons, or armies, often presented by specific serotypes, such as serotype 1 (Dagan *et al.,* 2000), 4 (Gleich *et al.,* 2000), 5 (Tyrrell *et al.,* 2012) and 8 (Vanderkooi *et al.,* 2011).

The pneumococcal disease can range from mild mucosal respiratory tract infection, such as AOM and sinusitis, to more severe disease such as pneumonia, septicaemia, and meningitis. Although pneumococci can cause lethal diseases, it is more often that they asymptomatically colonize the upper respiratory tract among children (Regev-Yochay *et al.,* 2004a, Henriques-Normark and Tuomanen 2013). It is also known that pneumococci are the most common cause of pneumonia, being responsible for 40-50% of all community-acquired pneumonia (CAP) (Michelow *et al.,* 2004, de Roux *et al.,* 2006)

The nasopharynx is the major ecological niche of pneumococci, serving as the reservoir of pathogenic strains and a place where transformation of strains occurs. It is therefore important to monitor and decrease asymptomatic carriage to prevent pathogenic strains accumulating in individuals. NP colonization is a prerequisite (pre-state) for spread from the nasopharynx to the lower respiratory tract or other sites and may lead to IPD. There is a high prevalence of carriage among children living in close communities such as day care centres (DCCs) often reaching up to 60-75% (Bogaert *et al.,* 2004a, Hussain *et al.,* 2005, Adetifa *et al.,* 2012). The highest prevalence is also found among children in endemic areas with carriage rates of up to 90% (Hill *et al.,* 2008, Abdullahi *et al.,* 2012).

The burden of pneumococcal diseases is further compounded by the trend of increasing antibiotic resistance exhibited by the organism, especially multiple drug resistance. Of more concern, up to 40% of pneumococci display multi-drug resistant phenotypes, again with highly variable prevalence among countries (Van Bambeke *et al.,* 2007, Donkor *et al.,* 2013b). The huge number of circulating pneumococcal serotypes remains the main challenge in developing strategies to control this global pathogen (Henriques-Normark and Tuomanen 2013).

Molecular typing of the pneumococcus has played a crucial role in understanding the epidemiology of the organism. However, most of what is known about molecular epidemiology of the pneumococcus pertains to the developed world. The brunt of pneumococcal infections is borne by sub-Saharan African countries, which makes

epidemiological monitoring of the pneumococcus essential in this region of the world (Donkor 2013).

In Ethiopia, upper respiratory tract infection and pneumonia are among the first top 10 leading causes of hospital admission for children under 5 years (FMOH 2009), and an estimate of 112, 000 (84.6 per 10,000) deaths occur per year (Rudan *et al.,* 2008). Pneumococcal pneumonia is the leading cause of death among children in the country (Assefa *et al.,* 2013). However, little is known on the patterns of carriage and transmission, serotype distribution, genetic relatedness, antibiotic susceptibility profile for commonly used antibiotics and other critical epidemiological parameters of interest in pneumococcal infection. To our knowledge, only few published data are available that describe serotypes/serogroups and antibiotic resistance patterns of pneumococcal isolates from meningitis cases and other clinical samples and reported prevalent serotypes that are isolated from IPD (Muhe and Klugman 1999, Dinku 2013) in Ethiopia.

1.4 Significance of the study

Pneumococcus is the most important cause of life-threatening pneumonia in young children globally (Mulholland 2007). It is estimated that pneumococcus accounts for 30-50% of the two million deaths from pneumonia that occur annually in children <5 years of age (Wardlaw *et al.,* 2006, Rudan *et al.,* 2008). The effectiveness of empiric therapy for pneumococcal infections is being threatened by the spread of strains resistant to first-line antibiotics. In poor settings, second-line antibiotics are often unavailable, which may increase treatment failures and facilitate disease transmission (Jacobs 2003).

NP colonisation is the necessary first step in the pathogenesis of pneumococcus-associated invasive respiratory tract infection (Garcia-Rodriguez and Fresnadillo-Martinez 2002, Bogaert *et al.,* 2004a). Results from observational studies have shown rapid acquisition of pneumococcus in early infancy among populations with a high burden of infections (Coles *et al.,* 2001).

Pneumococcal conjugate vaccines are highly efficacious in the prevention of invasive disease in young children caused by vaccine serotypes (Klugman 2001, Greenwood *et al.*, 2007) and in lowering disease transmission by reducing NP carriage of these strains, which, in turn, may stem the spread of antibiotic-resistant strains (Klugman 2001, Käyhty *et al.,* 2006). Surveillance of NP pneumococcal carriage strains is frequently used to approximate the prevalent phenotypes causing invasive disease and has an important role in the design of multivalent vaccines (Kellner *et al.,* 1998).

A licensed seven-valent pneumococcal conjugate vaccine was in routine use in the USA and Europe and this reduced prevalence of IPD cases. However, in parallel, it was observed that there was a rise in diseases caused by non-vaccine types of pneumococci. The advent of the pneumococcal conjugate vaccines holds promise for reducing the pneumococcal disease burden, especially for children less than two years old. However, no studies in Ethiopia have systematically characterized the incidence of IPD in children, and assessed the trend of circulating pneumococcal carriage serotypes as well as possible replacement of types as observed elsewhere after vaccination. Such basic information upon which health policymakers can make decisions regarding strategies for pneumococcal vaccination is currently lacking.

Studies on the distribution of pneumococccal serotypes associated with colonization of the upper respiratory tract in young children are currently of general interest because of the impact of the new PCV on carriage of pneumococci and the incidence of pneumococcal disease. Furthermore, studies of the prevalence of pneumococci, their resistance patterns, and possible risk factors can provide useful indications for more rational therapeutic and preventive strategies. In African countries, children are suffering from an extremely high burden of pneumococcal diseases, and therapy for pneumococcal disease remains empirical because of the lack of rapid, sensitive, and specific diagnostic tests. In many African countries and, particularly in Ethiopia there is in general paucity of genotypic data on pneumococci including on antibiotic resistance trends and on clonal diversity

Therefore, this study will provide a baseline for future monitoring of trends, including interpretation of any changes arising from using PCV10 vaccine, antibiotic resistance pattern and clonality of the isolates. Furthermore, the findings from this study will also provide guidance for the rational use of antimicrobial agents in clinical management of IPD.

1.5 Hypotheses

- Introduction of the ten-valent PCV will decrease carriage rates of vaccine types, but will increase the carriage rate of non-vaccine serotypes of pneumococci in Ethiopia
- Serotypically and genetically diverse clones of pneumococcal populations are circulating in Ethiopia

1.6 Objectives

1.6.1 General Objective

The overall aim of this work was to analyze the impact of PCV10 on the carriage rate of the pneumococcus and other bacteria in the nasopharynx, and to determine the phenotypic and genotypic characteristics of pneumococcal isolates from nasopharyngeal swabs.

1.6.2 Specific objectives

- To determine the prevalence of nasopharyngeal carriage of pneumococci in infants prior to the start of PCV10 vaccination at the age of 6 weeks and after completion of the vaccine (at 9 months and 2 years)
- To determine the carriage rate of other bacteria carried in the nasopharynx before and after completion of the PCV10 vaccine
- To analyze the risk factors associated with carriage of pneumococci
- To determine the susceptibility pattern of isolates to commonly used antibiotics for treatment of pneumococcal diseases
- To describe the serotype and molecular characteristics of pneumococcal isolates before taking the vaccine and after completion of vaccination

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 Study area

Ethiopia is a federal state divided into 11 administrative regions and two city administrations covering about 1.1 million sq kilometres. The total population of the country is estimated to be 92.2 million according to a projection by the national Central Statistical Agency (CSA) (CSA 2013).

This study was conducted in Addis Ababa city administration which had an estimated population of 3.35 million in during the study period, of which 1.76 million were female (CSA 2013). There were more than 100 public and private health facilities providing the PCV10 vaccine when we started the study, and all of them reported the number of children they vaccinated to the City administration regional health bureau. The average number of children vaccinated since the introduction of the vaccine in October 2011 was in the range of 22,000- 23,000 children per month (Annex 3) (Addis Ababa City Administration Health Bureau Monthly report).

A total of six sub-cities were randomly selected at the initial stage of the study out of all 10 sub-cities, and then one health centre from each and two health centres from one sub-city were selected as the recruitment sites for the study participants (Fig 2.1).

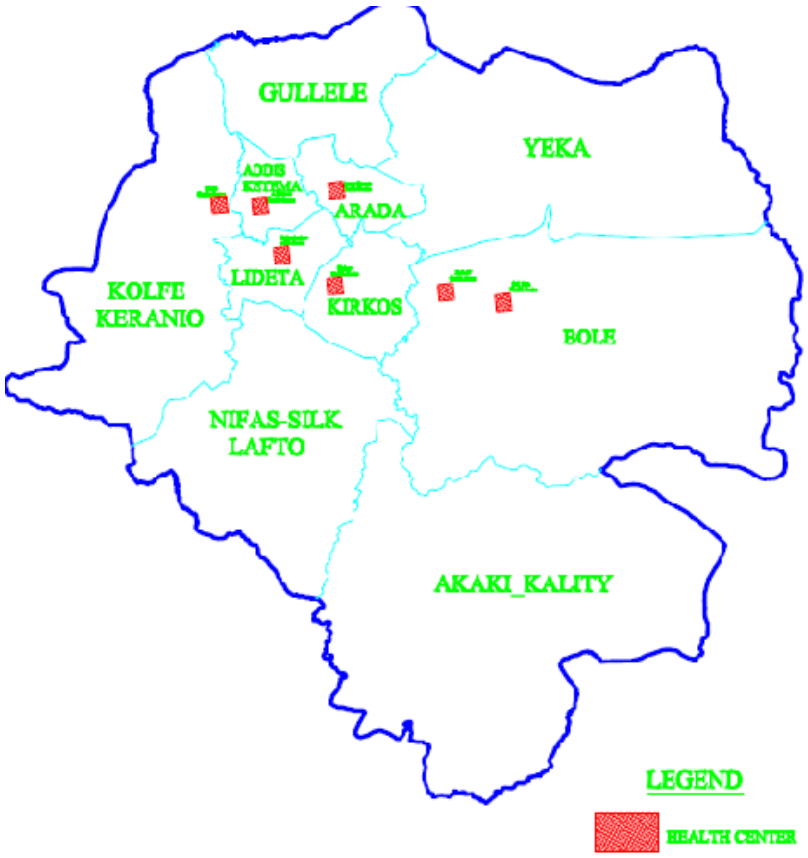

Figure 2.1 Map of Addis Ababa city Administration showing sample collection areas

2.2 Study Design

An observational longitudinal (follow up) study was conducted to determine the impact of PCV10 on the carriage rate of pneumococci. Infants were recruited for the study when they came to a health facility at the age of 6 weeks for the first PCV10 vaccine, and were then followed up. Nasopharyngeal samples were taken at the age of 6 weeks, 9 months and 2 years to determine the carriage rates. Sample and data collection was started in February 2013, and the last sample was collected in November 2015.

2.3 Sample size and sampling strategy

The sample size was determined based on the single proportion method for the first enrolment of infants at the age of six weeks. To calculate the sample size, since there were no prior data in the country on the prevalence of pneumococcal NP colonization that could be used for this study, we used estimates based on data from a carriage study that was done in Kenya, a neighbouring country with similar socio-demographic conditions, where carriage rate was nearly 80% (Abdullahi *et al.,* 2012). We calculated that 789 participants would be sufficient to determine the carriage rate in our study population at 95% confidence interval ($Z_{1-\alpha/2}$ = 1.96) and 3% marginal error (d) with the formula n=[(Zα/2)^2 * p (1-p)]/d^2 assuming 10% refusal to participate. Then sample size for follow up was calculated based on the actual finding of 6 weeks carriage rate, with the power of 90% to observe the change in serotype distribution in pneumococcal carriage after completion of the vaccine. Through follow up, 206 participants at 9 months and 201 at the age of 2 years and the same 116 children were sampled consecutively in the three periods.

The PCV10 vaccine was given at the age of six weeks, 10 weeks and the last dose at 14 weeks following the schedule of the extended programme of Immunization (EPI). Samples were taken at the age of six weeks before taking the first dose of PCV10, and at nine months and two years of age after completing the vaccination (Fig 2.2). Seven health centres were randomly selected using lottery method from those 36 health centres involved in giving PCV10 vaccine in the city administration; and the study participants whose parents or guardians gave consent were recruited sequentially until the initial sample size was achieved. Parents and guardians who consented to participate in the study and were enrolled at six weeks were requested to bring their children back for follow up at the age of nine months and two years.

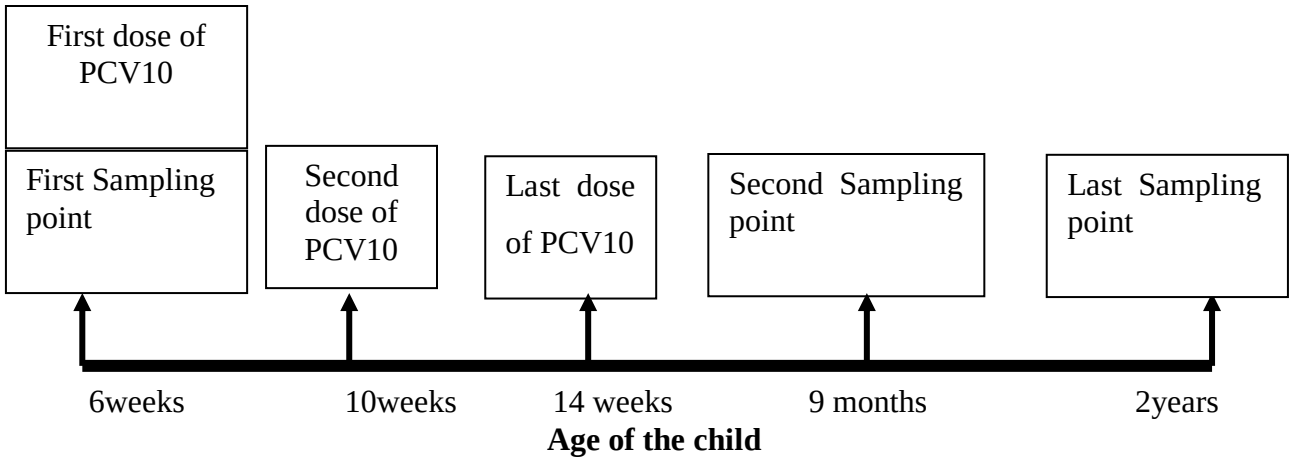

Figure 2.2 PCV10 vaccination schedule and sampling points

2.4 Inclusion and exclusion criteria

Inclusion criteria were: any infant who was eligible to and was going to receive PCV10 vaccine at a health facility in Addis Ababa as part of the routine immunization schedule, whose parents/guardians were willing to continue for the full course and were willing to provide informed consent for participation in the study.

Exclusion criteria were any signs of respiratory tract infection in the child and antibiotic exposure within 2 weeks at the time of sampling.

2.5 Sample collection and processing

Samples and data were collected by nurses working in the vaccination unit at each selected health centre who were trained on study procedures, including on how to take nasopharyngeal swab samples and on data collection using a structured questionnaire. Trained nurses practiced nasopharyngeal swab sample collection on each other until they proved competent, and were approved by the trainers (principal investigator, paediatrician and a senior nurse who has rich experience with paediatric patients) to start sample collection from study participants.

Nasopharyngeal swab specimens were collected following standard operating procedures (WHO 2003) by the trained nurses. A flexible calcium alginate-tipped swab (Fisher Scientific, UK) was inserted through the nostril to the posterior nasopharynx and was kept there for a minimum of five seconds before removal. The swab tip was then inserted in a cryotube containing 1ml skim milk-tryptone-glucose glycerin medium (STGG) (O'Brien *et al.,* 2003), and was transported in an icebox to the Bacteriology Laboratory of the Armauer Hansen Research Institute (AHRI). Each swab was plated within six hours of collection on blood agar, chocolate agar with X and V factor supplement and on pneumococcal selective media (trypticase soy agar (TSA) plates supplemented with 5% sheep blood containing 5µg/ml gentamicin) (Fisher Scientific, UK). All plates were incubated at 37 °C for 18-24 hrs. Chocolate and trypticase soy agar plates were incubated in a 5% CO_2 atmosphere.

2.6 Identification of *S. pneumoniae*

Colonies on TSA plates with macroscopic appearance characteristic for pneumococci (small, greyish, and mucoid colonies demonstrating α-hemolysis and gram positive diplococci), single colony from primary culture was streaked on TSA to get single pure colonies (Annex 4). A pure colonies were sub-cultured on sheep blood agar plates, and incubated in a 5% CO_2 atmosphere at 37°C for 18-24 hours to get sufficient colonies. The suspect α-hemolytic pneumococcal colony was then streaked on a blood agar plate and an optochin or "P" disk with a diameter of 6 mm (and containing 5µg ethylhydrocupreine) (Fisher Scientific, UK) was aseptically placed and the plates were incubated at 37°C for 18-24 hours in a 5% CO_2 incubator. The result was interpreted as follows: isolates with a zone of inhibition of growth greater than 14 mm in diameter were determined as pneumococci (Figure 2.3) (WHO 2003, Murray 2007); isolates with no zones of inhibition were considered resistant to optochin, and thus determined as viridans streptococci; α-hemolytic isolates with zones of inhibition ranging from 9 to 13 mm showing intermediate resistance to optochin were tested for bile solubility.

The bile solubility test was performed using the tube method: a bacterial cell suspension in 0.5 ml of sterile saline was prepared from fresh growth of suspected isolates on a blood agar and the suspension of cells was measured to that of a 0.5 McFarland density standard. The suspension was divided into two equal amounts (0.25 ml per tube) 0.25 ml of saline was added to one tube and 0.25 ml of 2% sodium desoxycholate (bile salts) tubes were gently shaken and incubated at 37°C for 2 hours. The result was interpreted as positive if there was clearing of the tube, or a loss in turbidity (WHO 2003, Murray 2007). Confirmed pneumococcal isolates were stored in duplicate in 1.5 ml STGG broth cryotube at -80^{o}C, one for drug sensitivity testing and the other one to be transported to Karolinska Institutet, Sweden for serotyping and molecular characterization. Identification of other bacteria in the nasopharynx such as *Haemophilius influenzae*, *Moraxella catarrhalis*, Group A streptococci and *Staphylococcus aureus* was made using standard bacteriological procedures and we used a flow chart (Annex 5 and 6) (Murray 2007). The two year samples were analyzed to the step of identification of isolates and serotyping.

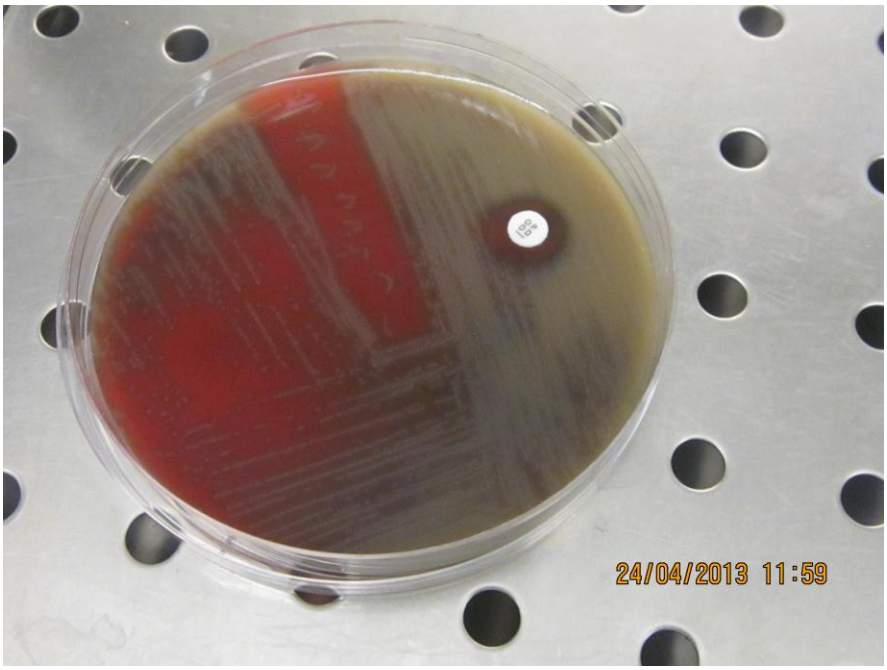

Figure 2.3. Optochin sensitive pneumococcal isolate from nasopharyngeal sample

2.7 Drug susceptibility testing

A total of 327 pneumococcal isolates from six-week and nine-month-old children were tested for sensitivity to thirteen antibiotics, 1µg Oxacilin (for penicillin), Ceftriaxone (30µg), Erythromycin (15µg), Telithromycin (15µg) trimethoprim- Trimethoprim-Sulfamethoxazole (1.25-23.75µg), chloramphenicol (30 µg), clindamycin (10µg), Ofloxacin (5µg), Levofloxacin (5µg), Vancomycin (30µg), Rifampicin (5µg), Tetracycline (30µg), and Linezolid (10µg), (Fisher Scientific, UK) by the Bauer and Kirby disk diffusion method (Bauer *et al.,* 1966) on Mueller Hinton agar with 5% sheep blood. Results were interpreted according to the criteria of the Clinical and Laboratory Standards Institute (CLSI 2012). Those isolates that were resistant to Oxacillin and Erythromycin by disc diffusion were subjected to Epsilometer-test (E-test) to Penicillin and Erythromycin (Etest® bioMérieux, France) respectively to determine minimum inhibition (MIC) concentrations on Mueller Hinton agar with 5% sheep blood, and results were interpreted according Clinical and Laboratory Standards Institute criteria (CLSI 2012).

Quality control strains (*S. pneumoniae* ATCC 49619, *Staphylococcus aureus* ATCC 29213, and *Escherichia coli* ATCC 25922) were included with each run for quality assurance.

2.8 Serotyping

A total of 424 isolates (210 of six-week, 117 of nine- months and 97 of two-year isolates) were transported to Sweden and serotyping was done at the Public Health Agency of Sweden. The pneumococcal isolates were sub-cultured on blood agar and incubated at 35-37^{o}C in 5% CO_2 atmosphere for 18-24 hours and the next day colonies from each isolate were inoculated in 5 ml (1% dextrose bouillon with 10% inactivated horse serum (DS)), and in parallel on blood plates, for propagation before freezing and to have fresh cultures for the Quellung reaction. The tubes and plates were incubated in a 5% CO_2 incubator overnight (18-24 hours). Antigen for gel diffusion was prepared through

centrifugation of the DS-tube at about 1600g for 8-10 minutes, re-suspending the pellet by adding about 400µL of distilled water. Drop of samples of Antigen were loaded around antisera in 1% agarose gel and kept overnight at room temperature. The following day, the gel plate was read/examined for precipitation (Figure 2.4). Isolates belonging to serogroups including more than one serotype were further analyzed with type and factor sera using the already prepared antigen (Henriqus Normark *et al.*, 2003). Quellung reaction was used to confirm doubtful results and for factor typing of isolates which were not possible to type by gel precipitation. The antisera we used were purchased from Statens Serum Institute in Copenhagen, Denmark.

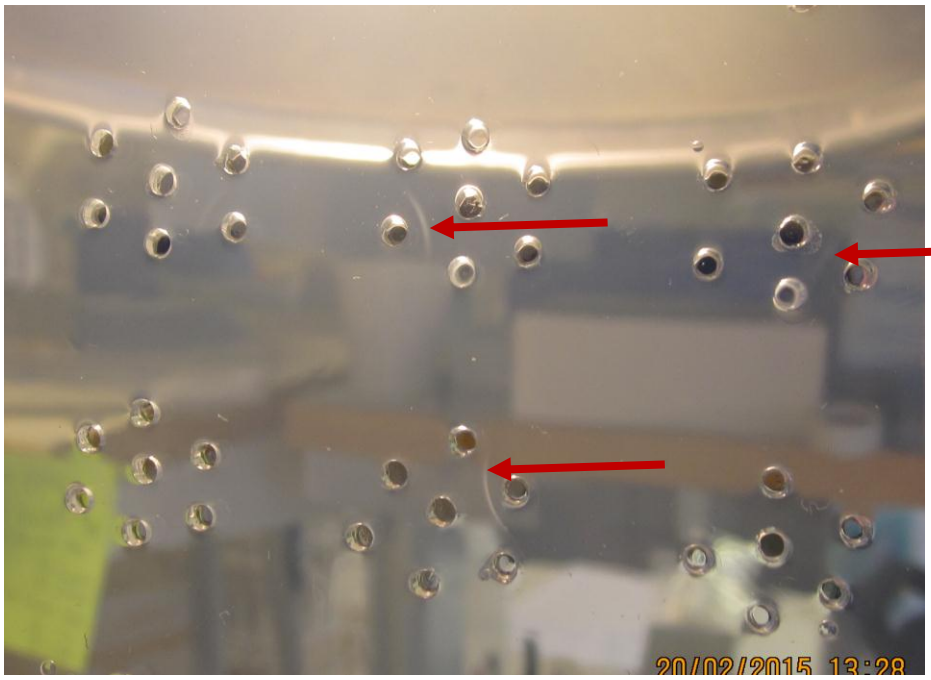

Figure 2.4 Serotyping by gel diffusion method

In the centre of each flower was antiserum and all other five holes had sample antigen

It was possible to identify serotype 3 with its typical mucoid colony and big zone of inhibition after testing some of the isolates with optochin test (Figure 2.5)

Figure 2.5 Typical colony morphology of Serotype 3

2.9 Molecular typing of the isolates

2.9.1 Pulsed Field Gel Electrophoresis (PFGE)

PFGE analysis was carried out for 325 six-week and nine-month isolates as previously described (Hermans *et al.,* 1995, Descheemaeker *et al.,* 1999). Briefly, isolates were sub-cultured onto blood agar plates and incubated at 37°C in 5% CO_2 for not more than 16 hours to prevent autolysis and then washed, adjusted to a density of $4x10^4$ cfu/ml in EET buffer (100mM EDTA, 10mM Ethylene glycol-bis (β aminoethyl ether)-N,N,N', N' tetra acetic acid (EGTA), 10mM Tris HCl pH 8.0) and mixed with an equal volume of 1.6% low-melting agarose (Sigma-Aldrich) and allowed to solidify in plug mold. The plug was prepared in duplicate. The block was incubated for 3hrs at 37°C in 2 mL of lysis solution [10 mM Tris-HCl pH 7.6, 100 mM EDTA, 1 mM NaCl, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine, 0.5 mg/mL of lysozyme]. Following this, the lysis buffer was then substituted by 2 mL of proteolysis buffer [1% sodium lauryl sarcosine, 0.5M EDTA (pH 9.5), and 500µg/mL of proteinase K, and incubated overnight at 56°C. Lysed bacterial material and proteinase K activity was eliminated by three washes in TE buffer (10 mM Tris-HCl pH 7.5, 10 mM EDTA), containing 1mM

phenylmethylsulfony fluoride for 10 min at 4°C. Before DNA digestion, the agarose plugs were washed three times with TE buffer. After cell wall and protein digestion, one of the plugs was incubated for 2 hours at 37 ^{o}C in 150µl fresh restricted buffer, containing *Apa I* enzyme (Promega, USA), and the restriction enzyme action stopped by washing with 200µl 0.5xTBE in 20min. The electrophoresis was performed in a contour-clamped homogeneous electric field (CHEF) apparatus (Bio-Rad Laboratories, Inc, USA) with ramped pulse times from 2 to 30 seconds for 22 hours at 6V/cm at 14ºC in 1.2% agarose. The reference strain was included in the 1^{st,} 10th, 20th and last lanes of each gel as a molecular weight standard for gel-to-gel comparisons (Tenover *et al.,* 1995).

The DNA banding profiles was stained with GelRed™ (Biotium, USA) and by the Gel Doc 1000 documentation system. The gel picture was grouped as indigestible, empty or weak due to absence of enough DNA to be digested and complete for analysis (Figure 2.6). The empty and indigestible samples were re-processed starting from the initial step again for confirmation. Conversion, normalization and further analysis of patterns were carried out with the GelCompare software version 4. The level of similarity between the PFGE patterns was calculated by using the Dice coefficient, and correlation coefficient was calculated with the UPGMA (unweighted pair group methods with arithmetic averages) as previously described (Descheemaeker *et al.,* 2000). The Dice coefficient of similarity is 2x number of matching bands/total number of bands in both strains). An isolate was considered to be within a cluster if band matching tolerance was 1%, optimization 1% and dendrogram created using Bionumerics ver. 7.1 (Applied Maths, Belgium) with the coefficient of similarity > 80%.

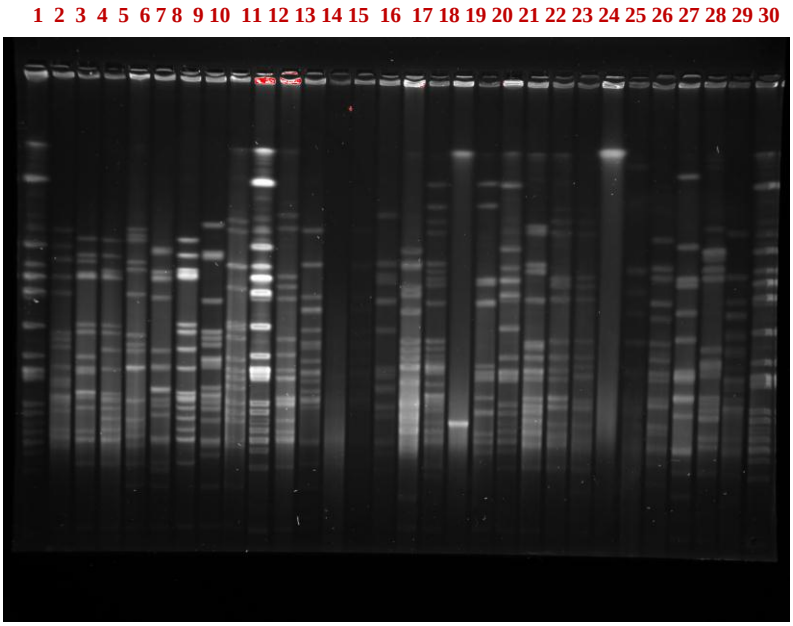

Figure 2.6 Gel picture of PFGE of *S. pneumoniae* isolates.
Lane 1, 10, 20 and 30 were reference strains, the rest are sample isolates (lane 14, 15 and 25 empty and weak bands).

2.9.2 Multilocus Sequence Typing (MLST)

Twelve selected six-week and nine-month pneumococcal isolates from 11 PFGE genotypes were analysed by MLST, as described previously (Enright and Spratt 1998)), according to the instructions on the MLST website (http://spneumoniae mlst.net/). Briefly, PCR was performed to amplify seven pneumococcal housekeeping genes (aroE, ddl, gdh, gki, recP, spi, and xpt) (Figure 2. 7) and primers used to amplify genes indicated in Table 2.1. These genes were then sequenced and aligned using the Basic Local Alignment Search Tool (BLAST) from NCBI. The resulting allelic profiles were concatenated (or seen together) to confirm pneumococcal identity (Hanage *et al.*, 2005) and analyzed to determine sequence type. Correlation was assessed between the sequence types (ST) of novel *S. pneumoniae* clones with all ST existent in the database, using the eBURST (electronic Based upon Related Sequence Type) software available at the MLST website (http://pubmlst.org/spneumoniae). The combination of the seven allele

numbers provides sequence type (ST) and the STs were compared between the typed isolates.

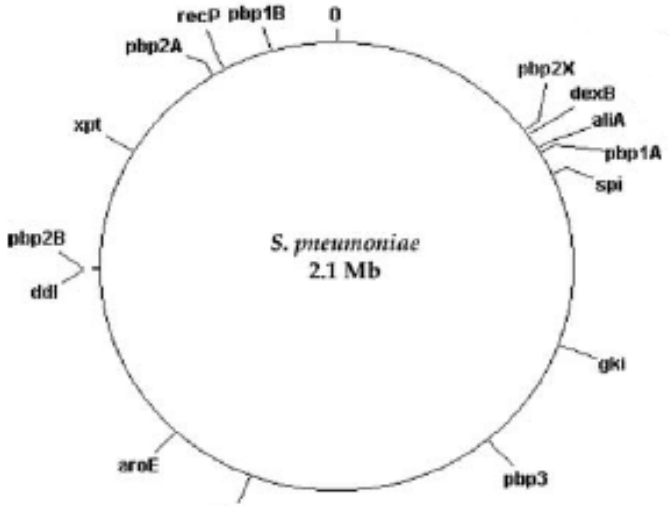

Figure 2.7 Schematic picture showing the approximate genome location of seven house-keeping genes used in MLST cps locus, encoding the capsule Adapted from Brueggermann (Brueggemann *et al.*, 2007)

Table 2.1 Primers used for MLS

(Enright and Spratt 1998)

Gene	Name	Primer Name	Primer sequence
aroE	shikimate dehydrogenase	***aroE*-up**	**5'-GCC TTT GAG GCG ACA GC 3'**
		***aroE*-**	**5'-TGC AGT TCA (G/A)AA ACA T(A/T)T**
gdh	glucose-6-phosphate dehydrogenase	***gdh*-up**	**5'-ATG GAC AAA CCA GC(G/A/T/C) AG(C/T) T 3'**
		***gdh*-dn**	
gki	glucose kinase	***gki*-up**	**5'-GGC ATT GGA ATG GGA TCA CC 3'**

recP	Transketolase	***recP*-up** ***rec*P-dn**	**5'-GCC AAC TCA GGT CAT CCA GG 3'** **5'- TGC AAC CGT AGC ATT GTA AC 3'**
spi	signal peptidase I	***spi*-up** ***spi*-dn**	**5'-TTA TTC CTC CTG ATT CTG TC 3'** **5'-GTG ATT GGC CAG AAG CGG AA 3'**
xpt	Xanthine Phosphoribosyltransferase	***xpt*-up** ***xpt*-dn**	**5'-TTA TTA GAA GAG CGC ATC CT 3'** **5'-AGA TCT GCC TCC TTA AAT AC 3'**
ddl	D-alanine-D-alanine Ligase	***ddl*-up** ***ddl*-dn**	**5'-TGC (C/T)CA AGT TCC TTA TGT GG 3'** **5'-CAC TGG GT(G/A) AAA CC(A/T) GGC A' 3**

2.10 Data management and statistical analysis

All data collected with the study questionnaire (Annex 2) that was designed to gather information on risk factors, and results of laboratory investigations were double-entered in an Excel spreadsheet at the AHRI Data Management unit, checked for errors and cleaned.

All statistical analyses were performed using Windows Statistical Package for the Social Sciences (SPSS), v.21.0 and STATA version 11. The association between nasopharyngeal (NP) carriage, and all other variables, including socio-demographic characteristics of study participants and microbiological and molecular characteristics of the pneumococcal isolates were determined using univariate and multivariate statistical analyses. The magnitude of association between the different variables in relation to the outcome variable was measured by odds ratio, with 95% confidence interval and level of significanceat $p<0.05$. Binary logistic regression analysis was done to obtain odds ratio and the confidence interval of statistical associations. Student's t-test was employed to determine the variation in proportion of carriage rate and serotype difference at six weeks, nine months and two years. Pearson's Chi-square statistical analysis was also applied to determine the association of socio-demographic characteristics with nasopharyngeal carriage categories. Acquisition was defined as an event where a subject whose nasopharyngeal swab became positive for a specific bacterium or serotype, after a

previously negative swab for that specified bacterium or serotype. All statistical tests were considered significant at p-values<0.05. The sequence type and clonality were analyzed from the databases using e-BURST and Basic Local Alignment Search Tool (BLAST).

2.11 Ethical consideration

Ethical clearance was obtained from the Institutional Review Board (IRB) of the College of Health Sciences, Addis Ababa University (protocol number 029/12/DMIP), from the Armauer Hansen Research institute (AHRI)/All-Africa Leprosy Rehabilitation and Training Centre (AHRI/ALERT) Ethics Review Committee (Reference number P021/21,), and from the National Health Research Ethics Review Committee (NERC) (reference number 3.10.1820/05).

Support letters were written from the Department of Microbiology, Immunology and Parasitology of Addis Ababa University and/or AHRI to the selected health institutes to get permission for taking samples from study participants. All parents/guardians of eligible children were informed about the study and any questions they had were explained to them clearly. They were given sufficient time to consider and discuss participation in the study. Written informed consent (Annex 1.1 and 1.2) was then obtained from all parents/guardians of the children who were recruited into the study before enrolment.

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CHAPTER THREE

3. RESULTS

3.1 Socio-demographic data

Socio-demographic analysis was done for 789 study participants who were involved in the initial stage when the children came to selected health centers for the first PCV10 vaccine at age of six weeks. All children were from different mothers, and there were no twins in our study samples. The proportion of children by gender was almost equal with 404 (51.2%) male and 385 (48.8%) female study participants. The family size ranged from 2 to 12 and 446 children (56.5%) came from a family size of 4 to 6. A quarter of the study participants (197) had under five-year-old siblings. The educational status of the majority of mothers was up to the level of primary or secondary education. The economic status of the family in monetary value indicates that most participants were earning monthly income less than 1500 birr or 66 USD. More than 90% of the mothers were breast-feeding their infants. Only 32 children had reported illness, 6 and 26 children had otitis media and respiratory tract infection, respectively. Antibiotic use in the first six weeks of age was reported for only 21 children or 2.7% of the participants (Table 3.1).

Table 3.1 Socio-demographic characteristics and risk assessment for *S. pneumoniae nasopharyngeal* colonization in 6 week old children not yet vaccinated with PCV10 (n=789)

Characteristics	Number(%)	Positive	Percent	AOR*(95%CI)	p-value
Sex					
Male	404 (52.2)	112	27.7	1.1(0.70-1.51)	0.363
Female	385 (48.2)	98	25.5	1	
Educational Status of the Mother					
Illiterate	112 (14.2)	34	30.4	0.90 (0.45-1.3)	0.776
Primary education	217 (27.5)	63	29.0	0.86(0.45-1.62)	0.648

Secondary education	316 (45.6)	91	25.3	0.87(0.49-1.54)	0.633
Certificate and University	100 (12.7)	22	22.0	1	
Family size					
≤ 3	304 (38.5)	80	26.3	0.95(0.41-2.19)	0.905
4-6	446 (56.5)	119	26.7	0.91(0.42-1.97)	0.802
≥ 7	39 (4.9)	11	28.2	1	
Presence of under 5 year siblings					
Yes	197 (25)	59	29.9	1.54(1.01-2.36)	0.046
No	592 (75)	151	25.9	1	
Breast feeding					
Yes	736 (93.3)	199	27.0	0.80(0.40-1.62)	0.543
No	53(6.7)	11	20.7	1	
Level of Crowding (Number of rooms in household/Family size)					
0.1-0.25	191 (24.2)	132	30.9	0.84(0.39-1.81)	0.657
0.26-0.50	391(49.6)	102	26.1	0.94(0.47-1.89)	0.859
0.51-0.75	143 (18.1)	36	25.2	0.87(0.41-1.84)	0.723
≥0.76	64(8.1)	13	20.2	1	
Presence of a separate cooking room					
Yes	390 (49.4)	100	25.6	1.09(0.75-1.57)	0.655
No	399 (50.6)	110	27.6	1	
Source of energy for household cooking					
Kerosene	370 (46.9)	98	26.5	1.43(0.97-2.12)	0.074
Charcoal	611(77.4)	170	27.8	0.89(0.56-1.39)	0.599
Electric Stove	331(42)	71	21.5	0.55(0.34-0.88)	0.013

Firewood	56 (7.1)	20	35.7	0.75(0.40-1.38)	0.351
Cylinder (gas) Stove	11 (1.4)	4	36.4	0.58(0.15-2.16)	0.414
Presence of Smoker in the house					
Yes	77(9.8)	18	23.4	0.92(0.52-1.65)	0.787
No	712(90.2)	192	27.0	1	
Monthly family income level in Birr					
<=500	84(10.6)	29	34.5	2.25(1.10-4.59)	0.026
501-1500	357(45.3)	113	31.7	1.93(1.07-3.51)	0.027
1501-3000	239(30.3)	47	19.7	0.98(0.54-1.79	0.954
>=3001	109(13.8%)	21	19.3	1	
Antibiotic use of child within the last 6 weeks					
Yes	21	2	9.5	NA	
No	768	208	27.1		
History of hospitalization of child					
Yes	11	2	18.2	NA	
No	778	208	26.7		
History of respiratory infection in the child					
Yes	26	3	11.5	NA	
No	763	207	27.1		
History of otitis media in the child					
Yes	6	-	-	NA	
No	783	210	26.8		

AOR= Adjusted odds ratio

3.2 Nasopharyngeal carriage rate of *S. pneumoniae*

The overall nasopharyngeal carriage rate of *S. pneumoniae was* 26.6% (95% CI 23.6-29.8), 56.8% (95% CI 49.7-63.6) and 47.3% (95% CI, 40.2-54.4%) at the age of six weeks, nine months and two years, respectively. As depicted in Table 3.2, the carriage rate among children recruited in the seven health centers ranged from 20% to 33.3% before vaccination at the age of six weeks, and there was no statistically significant variation in proportion of carriage rate (p=0.113) among health centers.

Table 3.2 Nasopharyngeal carriage rate of *S. pneumoniae in* infants before the first dose of PCV10 at the age of 6 weeks: distribution by site of recruitment (n=789).

Health Centers	**Total sample**	**Positive**	**Percent (95 %CI)**	χ^2 (p-value)
Addis Ketema	117	39	33.3 (24.9-42.6)	
Arada	59	16	27.1 (16.4-40.3)	
Beletshachew	102	21	20.6 (13.2-29.7)	
Bole-17	110	31	28.2 (20.0-37.6)	10.28(0.113)
Dil Fire	141	38	27.0 (19.8-35.1)	
Kirkos	140	28	20.0 (13.7-27.6)	
Kolfe	120	37	30.8 (22.7-39.9)	
Total	**789**	**210**	**26.6 (23.6-29.8)**	

The second round of nasopharyngeal samples were taken from 206 children at the age of nine months at six health centres from those who had been screened earlier at the age of six weeks as part of the first carriage study. It was not possible to do the follow up study in Arada health centre because the trained study nurse resigned from his job. As showed in Table 3.3, the total carriage rate in nine-month-old children who had been fully vaccinated with PCV10 was 56.8% (95% CI, 49.7-63.6), and the carriage rate at health

centre level ranged from 48.1% (95% CI, 28.7-68.0) to 65.2% (42.7-83.6) in Kirkos and Addis Ketema health centers. As was observed in the first round samples, there was no statistically significant variation in the carriage rate among the health centres (p=0.112) in the second round as well. However, the carriage rate at the age of nine months was significantly higher than at six weeks of age with carriage rates of 26.6% (p<0.001).

Table 3.3 Nasopharyngeal carriage rate of *S. pneumoniae in* 9 months old infants after completion of PCV10 vaccination: distribution by site of recruitment (n=206)

Health Centers	**Total sample**	**Positive**	**Percent (95% CI)**	χ^2 (p-value)
Addis Ketema	47	34	65.2 (57.4-84.4)	
Beletshachew	20	10	50.0 (27.2-72.8)	
Bole-17	45	23	51.1 (34.7-65.4)	
Dil Fire	44	22	50.0 (34.6-65.4)	8.92(0.112)
Kirkos	27	13	48.1 (28.7-68.0)	
Kolfe	23	15	65.2 (42.7-83.6)	
Total	**206**	**117**	**56.8 (49.7-63.6)**	

The last samples were collected when the children were two years old, and an overall carriage rate of 48.3 % (95%CI, 40.2-54.4) was found. Similar to previous sampling time points, there was no statistically significant difference in the carriage rate among health centres. The carriage rate ranged from 36.4 (10.9-69.2) to 62.5 (43.2-78.9) in Kolfe and Beletshachew Health centres, respectively (Table 3.4). The carriage rate of pneumococci at the age of nine months and two years showed statistically significant variation (P=0.022) between the two sampling periods. Overall, a total of 1196 nasopharyngeal samples were collected from all the three sampling time points, and 27.4% of the children were found to carry pneumococci at least once within the two years of follow up.

Table 3.4 Nasopharyngeal carriage rate of *S. pneumoniae in* 2 year old children after completion of PCV10 vaccination: distribution by site of recruitment (n=201).

Health Centers	Total sample	Positive	Percent (95% CI)	χ^2 (p-value)
Addis Ketema	39	22	56.4 (39.6-72.2)	
Beletshachew	32	20	62.5 (43.2-78.9)	
Bole-17	38	22	57.9 (40.1-73.7)	
Dil Fire	34	12	35.3 (19.7-53.5)	1.64(0.897)
Kirkos	47	17	36.2 (22.8-51.5)	
Kolfe	11	4	36.4 (10.9-69.2)	
Total	**201**	**97**	**48.3 (40.2-54.4)**	

3.3 Risk factors analysis for nasopharyngeal carriage of *S. pneumoniae*

The economic status of the family and the presence of under-five siblings in the household were the only risk factors associated with a higher carriage rate of *S. pneumonia*e. The carriage rate in families with monthly income category of less than or equal to 500 birr (approximately 22 USD) was 34.5%, and this was associated with a 2.3 times higher risk of children acquiring the bacteria in their nasophaynx, AOR 2.31 (95% CI, 1.12-4.8). Infants living with one or more under-five siblings in the household had a carriage rate of 29.9%. These children were 1.5 times more likely to carry pneumococcal bacteria in the nasophaynx, AOR 1.54 (95% CI, 1.01-2.36). Carriage rates were relatively lower (21.5%) among infants where families used an electric stove as energy source for cooking food at home. The risk of acquiring carriage was reduced 0.5 times in these children, AOR 0.55 (95% CI, 0.34-0.88). Other risk factors investigated in this study, i.e. gender, crowding (family size and number of rooms in the house), breast feeding and presence of smokers in the household were not found to be associated with carriage rate in these infants ($p>0.05$). The risk factors for cases of otitis media, history of

respiratory tract infection, history of hospitalization and antibiotic use were not computed because the number of cases was too low to allow for comparison (Table 3.1).

3.4 The serotype distribution of *S. pneumoniae strains*

A total of 424 strains of *S. pneumoniae were* isolated from the nasopharynx of children in this study, with 210 isolates from unvaccinated children at the age of six weeks and 117 at the age of nine months, and 97 at the age of two years from vaccinated children. However, two nasopharyngeal isolates from the six-week-old infants identified as *S. pneumoniae* could not be recovered during sub-culturing and therefore the serotype remains unknown. From the total of 422 isolates, 59 different serotypes were identified, and a total of 10 isolates were non-typable (NT). Except for two serotypes (7F and 18C), all serotypes included in PPV23 and currently available PCVs were represented among the isolates. Interestingly, we managed to identify two different colonies in the same sample on three different occasions. As a result, two different pneumococcal strains were isolated in three separate cultures of nasopharyngeal samples taken from three different children all aged two years which, when typed, proved to belong to two different serotypes. The serotypes were 3 and 23A, 3 and 20, and 19A and NT in the three different children.

Serotyping of strains obtained from six-weeks-old unvaccinated children nine-months and two-years-old vaccinated children showed that there were 54 different serotypes among 208 from six-weeks pre-vaccination isolates, and 43 different serotypes among 117 isolates from nine-month, and 37 different serotypes among two-year old post-vaccination isolates. Serotypes 6A (5.0% (21/422)), 34 (4.5%(19/422)), 10A (4.0% (17/422)), 11A (4.0% (17/422)), 19F (3.8% (16/422)), 15B (3.8% (16/422)), 23F (3.6% (15/422)), and 15A (3.6% (15/422)) were dominant in decreasing order, covering nearly 32% of all isolates. Only three of the predominant serotypes (19F, 23F and 6A) are included in currently available PCV vaccines, in PCV7 (19F and 23F), PCV10 (19F and 23F) and PCV13 (19F, 23F and 6A). Total potential coverage rates for by PCV7, PCV10, and PCV13 were 13.7%, 15.4% and 26.3% of the 59 serotypes to which all the isolates

belonged, respectively. Among vaccine types, serotypes 5, 14, 23F and 19F occurred more frequently before vaccination than after. Serotypes 6A, 34, 10A and 11A were more predominant serotypes after vaccination. Serotypes 5, 9V and 14, were detected in pre-vaccination samples, but were not present among strains isolated after vaccination both in nine month and two year isolates. Serotype 6B was isolated at the age of nine month only and serotype 1, 4, 19F and 23F were detected both at the age of nine months and two-year isolates after vaccine completion. The number of isolates with serotype 34 and NT were significantly higher in number at the age of two years when compared to the age of six weeks and nine months.

Among serotypes included in the PCV10 vaccine, 13 isolates of serotypes 1, 4, 6B, 19F, and 23F from the age of nine months, and 10 isolates of serotype 1, 4, 19F and 23F from the age of two years were identified in the samples collected after vaccination, even though these serotypes were included in PCV10. Nearly one third of the isolates, belonging to serotype 34, 11A, 10A, 15B, 15A, 19B and 22F in decreasing frequency, were strains with serotypes not included in any of the currently available PCV vaccines. Serotypes 3, 6A and 19A, which are not included in PCV10, but in PCV13 is covered 10.9% (46/422) of the isolates. The frequency of serotype 3 which appeared frequently in unvaccinated children at six weeks of age was much more reduced in vaccinated children both at the age nine months and two years. There were also serotypes that were not identified in vaccinated children (but present before vaccination) although these serotypes are not included in the vaccine (Figure 3.1).

The most frequently found global serotypes 6A, 6B, 14, 19A, 19F and 23F were all present, and together covered 18.2% of the total number of isolates. From the second group, serotypes that have a somewhat lower capacity to colonize but are still rather common (10A, 11A, 15A, 15B/C, 33F and 35B), were also detected and covered 15.6% of the total number of isolates. Among serotypes such as 1, 4, 5, 7F, 8 and 12F, which are less frequently carried but are highly invasive and mainly associated with outbreaks, all of them except serotype 7F were identified and covered 7.1% of the total isolates.

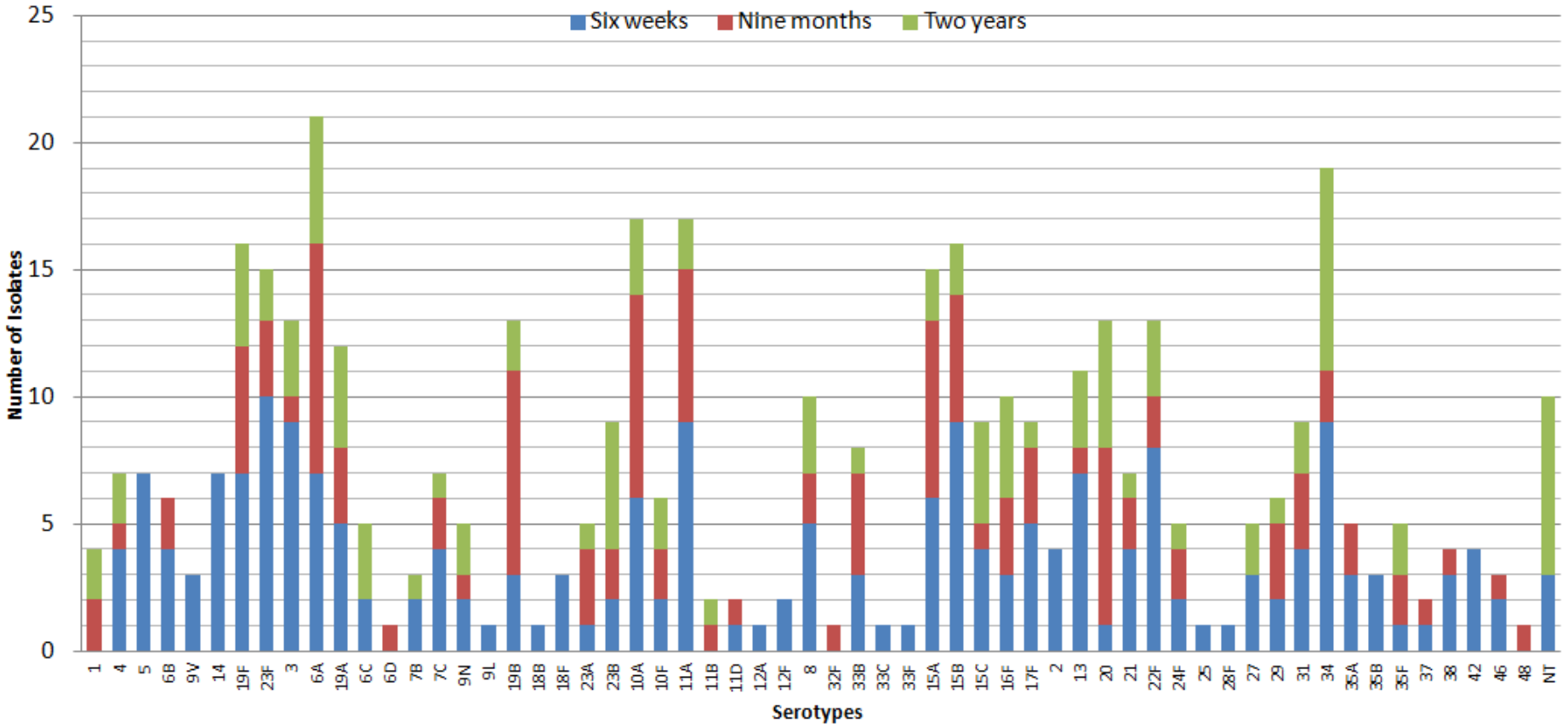


Figure 3.1 Serotype distribution of *S. pneumoniae isolates* from infants at the initiation (6 weeks) and after completion of PCV10, at 9 months and at 2 years of age

Separate analysis of 116 children, who were sampled at each of the three sampling points, shows that 37 (31.9%) were carriers at six weeks of age, 69 (59.4%) at nine months and 57 (49.1%) two years of age. Only 15 children were carriers of *S. pneumoniae* in all three sampling periods. We did not identify any child carrying similar serotype persistently throughout the two year period. Serotypes 1, 19F and 23F, which are included in PCV10, were detected after full vaccination, at both nine months and at two years. Similarly, serotype 6B was present at the age of nine months and serotype 4 at the age of two years (Figure 3.2).

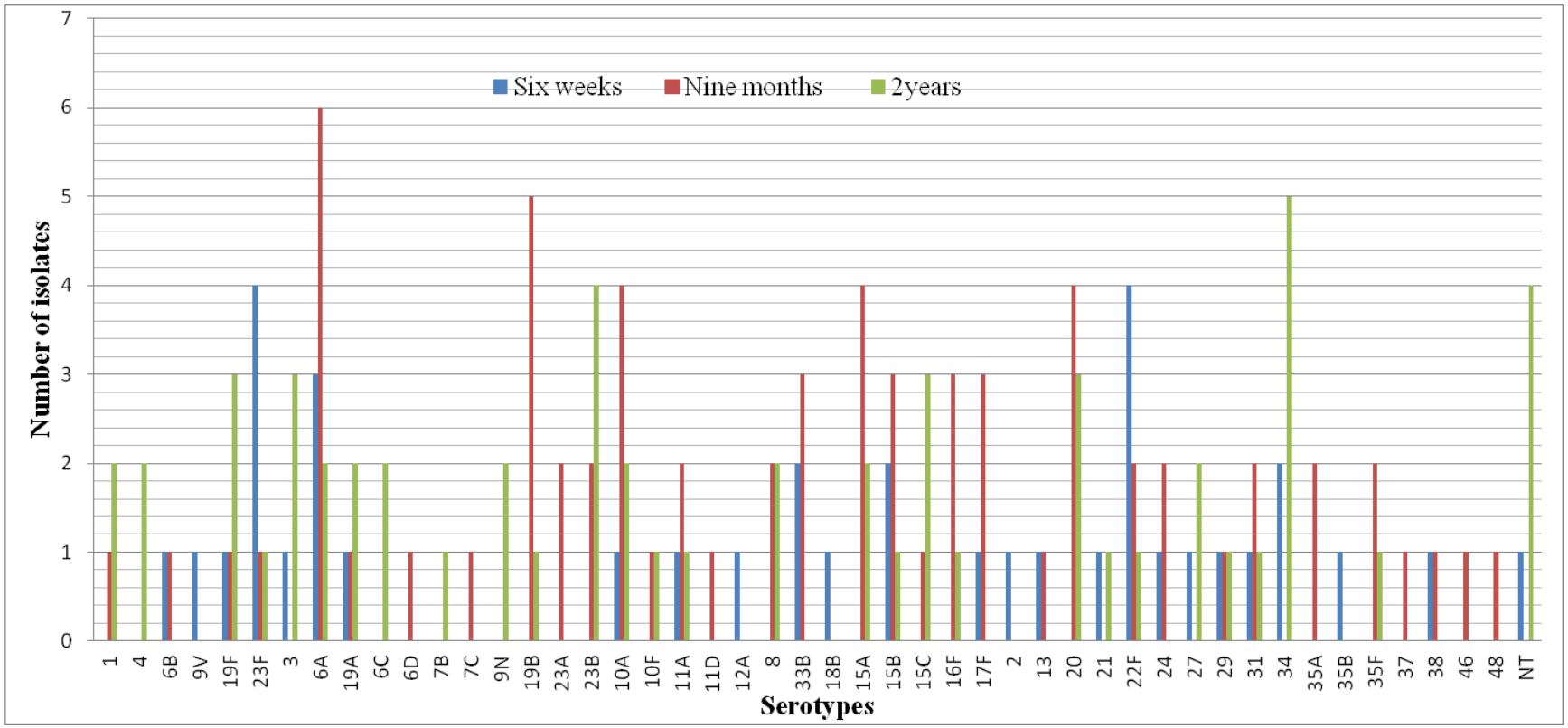


Figure 3.2 Serotype distribution of *S. pneumoniae* isolates from the same children at the age of 6 weeks, 9 months and 2 years

3.5 Serotype variation among carriers at the initiation and after completion of PCV10

Out of 208 isolates from six-week-old unvaccinated children that were serotyped, 42 (20.2%), 135 (64.9%) and 31 (14.9%) were vaccine, non-vaccine and vaccine-related serotypes (such as 6A, 6C, 7C, 9L, 19A, 19B, etc.), respectively. After completion of vaccination at the age of nine months, the percentage of vaccine serotypes was significantly reduced to 13 (11.1%). Overall, there was a statistically significant variation in serotype frequency distribution before and after vaccination (P=0.037) (Table 3.5). Similarly, as showed in Table 3.6, the vaccine types were significantly less frequent at the age of two years compared to at the age of six weeks (p=0.034). Out of the total of 97 children at the age of two year who carried *S. pneumoniae* in their nasopharynx, 10 (10.3%) had vaccine types and among the vaccine types, 4 isolates were serotype 19F.

Table 3.5 Comparison of serotypes of nasopharyngeal isolates of *S. pneumoniae* before vaccination with PCV10 at the age of 6 weeks and after completion of vaccination at age of 9 months

Isolate Types	At the age of 6 weeks (n=208)			At the of 9 months (n=117)			P-value
	Total isolates	%	95% CI	Total isolates	%	95% CI	
Vaccine types	42	20.2	15.0-26.3	13	11.1	6.0-18.2	
Non-vaccine types	135	64.9	58.0-71.3	75	64.1	54.7-72.7	0.037
Vaccine related types	31	14.9	10.3-20.4	29	24.8	17.3-33.6	
Total	**208**	**100**		**117**	**100**		

Table 3.6 Comparison of serotypes of nasopharyngeal isolates of *S. pneumoniae before* vaccination with PCV10 at the age of 6 weeks and after completion of vaccination at age of 2 years

Isolate Types	At the age of 6 weeks (n=208)			At 2 years (n=97)			P-value
	Total isolates	%	95% CI	Total isolates	%	95% CI	
Vaccine types	42	20.2	15.0-26.3	10	10.3	5.0-18.1	
Non-vaccine types	135	64.9	58.0-71.3	62	63.9	53.5-73.4	0.034
Vaccine related types	31	14.9	10.3-20.4	25	25.8	17.4-35.7	
Total	**208**	**100**		**97**	**100**		

Among 117 positive cases at the age of 9 months, 44 (37.6%) were positive for *S. pneumoniae* in the nasopharynx at the age of six weeks and only 4 (9.1%) children were carrying the same serotype similar to what they had at the age of six weeks. These serotypes were 6A in two children and 23F and 33B in two other children, while the remaining 40 (90.9%) were colonized by totally different serotypes. Seventy three or 62.4% of children who were colonized at the age of six months were previously negative at the age of six weeks (Table 3.7). Out of the total number of 97 children who carried of *S. pneumoniae* at the age of two years (as indicated in Table 3.8), 29 (29.9%) were also positive at the age of six weeks and all children were colonized with different serotypes. The comparison between the age of nine months and two years is depicted in Table 3.9. Among 57 carrier children, 37 (64.9%) were carrying *S. pneumoniae* both at the age of nine months and at two years of age and only three children carried the same serotypes.

Table 3.7 Nasopharyngeal carriage of *S. pneumoniae* serotypes in children at the age of 9 months (vaccinated) compared to type and frequency at the age of 6 weeks (pre-vaccination).

Carriage status	Positive at the age of 9 months		Colonizing Serotypes				Negative at the age of 9 months	
			Similar		Different			
	No.	%	No.	%	No.	%	No.	%
Positive at the age of 45days	44	37.6	4*	9.1	40	90.9	24	27
Negative at the age of 45 days	73	62.4	-	-	73	-	65	73
Total	**117**	**100**	**4**		**113**		**89**	**100**

Serotype: *6A(2), 23F(1), 33B(1)

Table 3.8 Nasopharyngeal carriage of *S. pneumoniae serotypes* in children at the age of 2 years (vaccinated) (n=201) compared to type and frequency at the age of 6 weeks (pre-vaccination).

Carriage status	Positive at the age of 2 years		Colonizing Serotypes				Negative at the age of 2 years	
			Similar		Different			
	No.	%	No.	%	No.	%	No.	%
Positive at the age of 6 weeks	29	29.9	-	-	29	100	23	27
Negative at the age of 6 weeks	68	70.1	-	-	68	-	81	73
Total	**97**	**100**	**-**	**-**	**97**		**104**	**201**

Table 3.9 Nasopharyngeal carriage of *S. pneumoniae serotypes* in children at the age of 2 years (n=116) compared to type and frequency at the age of 9 months

Carriage status	Positive at the age of 2 years		Colonizing Serotypes				Negative at the age of 2 years	
			Similar		Different			
	No.	%	No.	%	No.	%	No.	%
Positive at the age of 9 months	37	64.9	3*	8.1	34	100	32	27
Negative at the age of 9 months	20	35.1	-	-	20	-	27	73
Total	**57**	**100**	**3**	**-**	**54**		**59**	**116**

* serotype 19F, 8, and 23B

3.6 Antibiotic susceptibility testing

3.6.1 Antibiotic resistance of *S. pneumoniae nasopharyngeal* isolates before and after vaccination of children

Both six-week and nine-month *S. pneumoniae* isolates were tested for antibiotic susceptibility against a total of 13 antibiotics, while two-year isolates were not tested because of logistic problem. Penicillin susceptibility was determined using E-test for all isolates that were resistant to oxacillin with the disk diffusion test. All isolates were susceptible to Ceftriaxone, Telithromycin, Ofloxacin, Levofloxacin, Vancomycin, Rifampicin and Linezolid. Out of 210 six-week of age isolates, 97 (46.2%) and out of 117 nine-month isolates, 62 (53%) were resistant at least to one antibiotic tested. As depicted in Table 3.10, resistance and intermediate resistance was observed against 6 antibiotics. A higher rate of resistance of 25.7% was observed for Trimethoprim-Sulfamethoxazole for isolates collected at six weeks, and 33.3% for isolates at nine months of age. The

second higher resistance was detected against tetracycline. Nineteen, or 9% of isolates from six week and 12 or 10.3% of those at nine months were resistant to erythromycin; and only one strain, of serotype 19F (isolated at nine months), was resistant to penicillin with an MIC level of 4µg/ml. The comparison of antimicrobial resistance between six-week and nine-month isolates showed no statistically significant variation ($p>0.05$). Intermediate or reduced susceptibility to penicillin was 16% and 25.6% for six-week and nine-month isolates, respectively. For both chloramphenicol and clindamycin, 4.3% resistance (9 isolates) was detected in samples from unvaccinated children at six weeks of age (Table 3.10).

Table 3.10 Antibiotic susceptibility pattern of *S. pneumoniae nasopharyngeal* isolates obtained before (age of 6 weeks) and after completion (age of 9 months) of PCV10 vaccination

Antibiotics	At the age of 6 weeks (n=210)			At the of 9 months (n=117)		
	Resistant n(%)	Intermediate n(%)	Susceptible n(%)	Resistant n(%)	Intermediate n(%)	Susceptible n(%)
SXT	54 (25.7)	2 (1)	154 (73.3)	39 (33.3)	7 (6.7)	71 (60.4)
E	19 (9.0)	-	188 (89.5)	12 (10.3)	1 (0.9)	104 (88.8)
Penicillin*	-	35 (16.7)	175 (83.3)	1 (0.01)‡	29 (25.6)	87 (74.4)
TE	40 (19.1)	4 (1.9)	166 (79)	9(7.7)	9 (7.7)	99 (84.6)
DA	9 (4.3)	-	201(95.7)	8(6.8%)	-	109 (93.3)
C	9 (4.3)	-	201(95.7)	5 (4.3)	-	112 (95.7)

*Susceptible, $\leq$0.12 mg/L; Intermediate (Reduced susceptibility), 0.12–2mg/L; Resistant, $\geq$2 mg/L,); SXT = Trimethoprim-Sulfamethoxazole, E=Erythromycin, TE=Tetracycline, DA=Clindamycin , C= Chloramphenicol
‡= MIC, 4 and serotype of the isolates is 19F

3.6.2 Serotypes with reduced susceptibility to penicillin

The most frequent six week isolates that showed reduced susceptibility to penicillin were strains that belonged to serotypes 14 and 6A followed by strains that belonged to

serotypes 23F, 10A, 6B, 5 and 42 in decreasing order of frequency (Figure 3.3). Nine month isolates (those cultured after completion of vaccine) that showed reduced susceptibility to penicillin belonged to serotypes 6A, 10A and 16F (Figure 3.4).

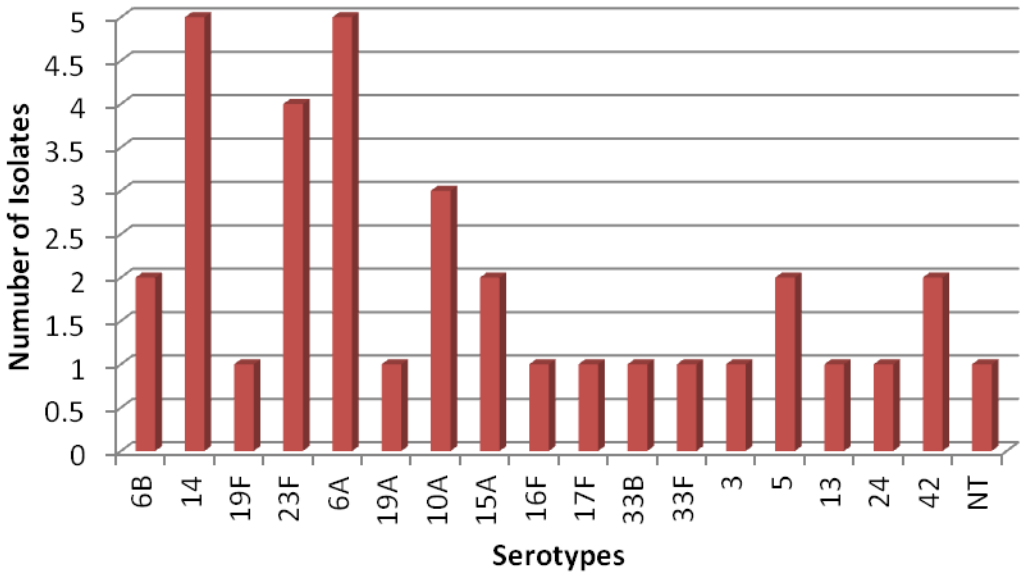


Figure 3.3 Nasopharyngeal *S. pneumoniae isolates* from infants aged 6 weeks: serotypes with reduced susceptibility to penicillin

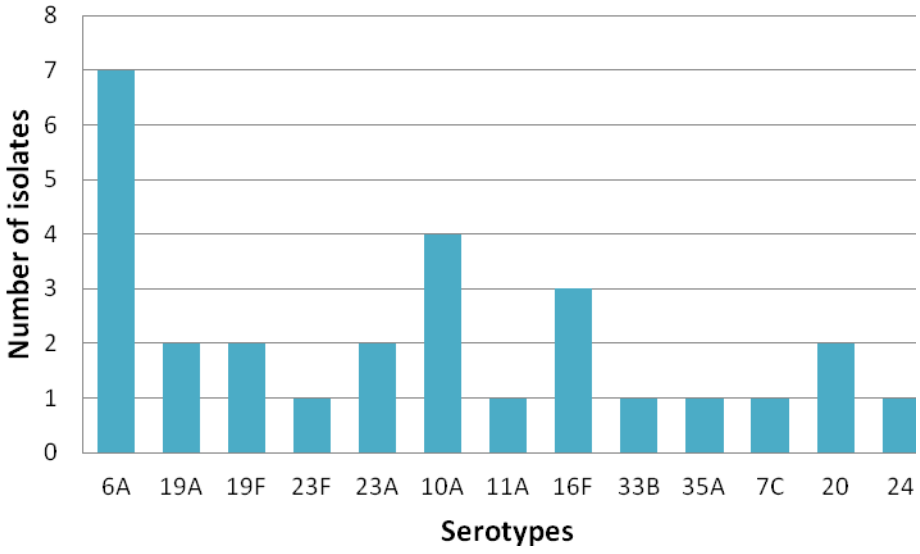


Figure 3.4 Nasopharyngeal *S. pneumoniae isolates* from infants at the age of 9 months: serotypes that showed reduced susceptibility to penicillin

3.6.3 Erythromycin resistance of *S. pneumoniae* isolates: MIC and serotypes

Minimum inhibitory concentrations (MIC) for erythromycin ranged from < 0.5-256 µg/mL. Three (15.8 %) resistant isolates from pre-vaccination samples (collected at six week of age) had an MIC level of 256µg/mL (Figure 3.5); and the isolates belonged to serotypes 14, 15B and 21. From total of 19 resistant isolates from infants at six weeks of age, 6(31.6%) isolates belonged to serotypes 2, 4, 13, 11A and 24 had a MIC of 4 µg/mL. Out of 12 resistant isolates obtained from nine-month-old children, 6(50%) had MIC of 256 and the isolates belonged to serotypes 10A, 15A, 15B and 19A (Table 3.11).

Table 3.11 Nasopharyngeal *S. pneumoniae isolates* from 6-week and 9-month old children: Erythromycin resistance and minimum inhibition concentration (MIC).

Minimum Inhibition Concentration	**At the age of 6 weeks**			**At the age of 9 months**		
	Number of Isolates	**Percent**	**Serotypes (n)**	**Number of Isolates**	**Percent**	**Serotypes (n)**
0.5- 2	-	-	-	3	25	17F(1),19B(1), 20(1)
2	1	5.3	35A	1	8.3	19F
3	2	10.5	6A(1), 34(1)	1	8.3	6A
4	6	31.6	2(1), 4(1), 13(1) 11A(2), 24(1)	-		-
6	1	5.3	34	-		-
8	1	5.3	6B	-		-
12	1	5.3	31	-		-
16	2	10.5	19F(1), 35B(1)	-		-
24	1	5.3	7C	1	8.3	33B
32	1	5.3	18F			-
256	3	15.8	14(1), 15B(1), 21(1)	6	50	10A(1), 15A(1), 15B(2), 19A(2)
Total	**19**			**12**		

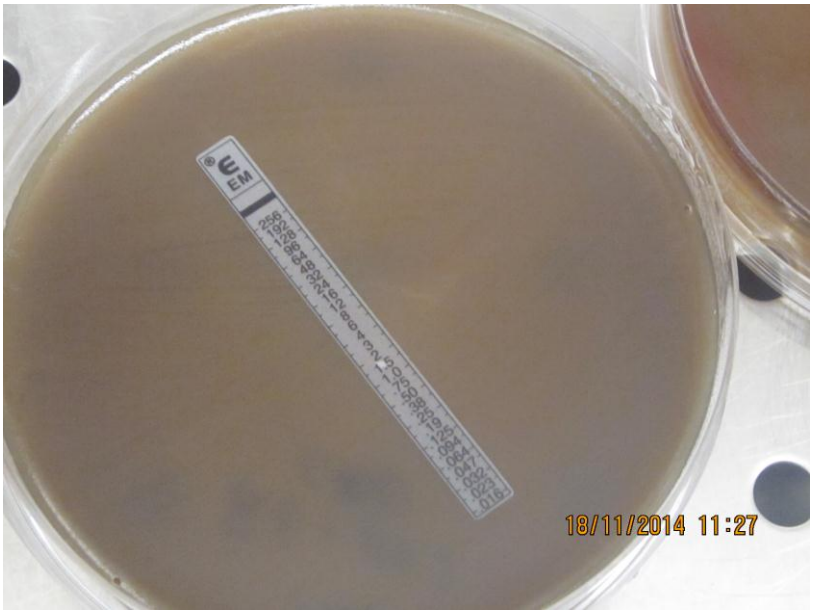


Figure 3.5 *S. pneumoniae* isolates resistant to Erythromycin at MIC 256 µg/mL

3.6.4 Multidrug resistance pattern and serotype of isolates

The antibiogram pattern and associated serotypes of nasopharyngeal *S. pneumoniae* isolates (with intermediate resistance considered as resistant) is summarized in Table 3.12. The highest frequency of mono-drug resistance was observed for trimethoprim-sulfamethoxazole in both six-week and nine-month isolates; and the dominant serotypes were 14, 6A, 23F, and 19F in the six-week groups, and 6A, 10A, 19F and 15A in the nine-month group of isolates. Twenty five (11.9% of six-week) and 24 (20.5% of nine-month) isolates were resistant to trimethoprim-sulfamethoxazole and penicillin, and serotype 6A was the most frequent serotype carrying penicillin resistance in both cases. Twenty three (11.0%) of six-week and 12 (10.3%) nine-month isolates were resistant to both trimethoprim-sulfamethoxazole and tetracycline, and the predominant resistant serotypes were serotype 14 from six-week and serotype 10A from nine-month isolates. Twenty one (10%) of six-week and 17 (14.5%) of nine-month isolates were resistant to more than three drugs. Multidrug resistance, that is resistance to three or more antibiotic,

was detected in vaccine types such as serotype 6B, 14, 19F, and 23F, and non-vaccine types 19A and 10A of six-week isolates. However, non-PCV10 types 6A, 19A, and 10A were observed with multidrug resistance among post-vaccination isolates. Two isolates of serotype 6B from six-week and serotype 19A, from nine-month isolates were resistant to five antibiotics (Table 3.12).

Table 3.12 Antibiogram of nasopharyngeal isolates of *S. pneumoniae before* (age of 6 weeks) and at completion (age of 9 months) of PCV10 vaccine

	At the age of 6 week (n=210)			**At the of 9 months (n=117)**		
Antibiotics	**Number of Resistant Isolates**	**%**	**Serotypes (n)**	**Number of Resistant Isolates**	**%**	**Serotypes (n)**
SXT	56	26.7	5(3), 14(7) 6A(6), 6B(3),19A(2),19F(5), 2(1),23F(6),10A(2), 12F(1),15A(2),15B(1), 15C(1),22F(2),33F(1), 33C(1),35A(1), 9N(1), 2(1), 3(2), 13(2), 21(1), 24(1), 31(1), 34(1), 38(1), NT(1)	46	39.3	1(2),6B(2), 6A(7), 23F(2) 19F(4),19A(2), 19B(2),10A(6), 11A(2), 15A(4), 15B(1),23B(2), 33B(1),35F(1) ,7C(1), 16F(1), 13(1), 20(3), 24(1), 48(1)
SXT+P	19	9.1	5(2), 14(4) 6A(3), 6B(2),19A(1),19F(1), 23F(3),10A(2), 13(1),	16	13.7	6B(2),6A(3), 23F(1), 19F(1), 19A(1),10A(3), 11A(1), 7C(1), 20(2), 24(1),
SXT+E	10	4.8	14(1), 6A(1),6B(1), 19F(1),15B(1),33F(1),	6	5.2	6A(1),19F(1) 19A(2), 19B(1),

			13(1),21(1),24(1), NT(1)			33B(1)
SXT+TE	23	11.0	14(5),6A(1),6B(1), 19A(2)19F(1),23F(4) 10A(1),15A(1),15B(1) ,22F(1),3(2),13(1), 21(1), NT(1)	12	10.3	6A(2), 19F(1), 19A(2),19B(1), 10A(5), 35F(1)
SXT+DA	4	1.9	6B(1),14(1),19F(1), 15B(1)	4	3.4	19F(1),19A(2), 33B(1)
SXT+C	1	0.5	21(1)	1	0.9	20
DA+E*	9	4.3	6B(1),19F(1),14(1), 3(1),2(1),7C(1), 15B(1),34(2)	8	6.8	19F(1),19A(2), 10A(1),15A(1), 15B(2),33B(1)
SXT+P+TE	6	2.9	6B(1),14(2),23F(1), 19A(1),10A(1),	5	4.2	6A(1),19A(1), 10A(3),
SXT+P+E	2	1.0	6B(1), 14(1)	1	0.9	19A(1)
SXT+P+E+DA	2	1.0	6B(1), 14(1)	1	0.9	19A(1)
SXT+P+E+TE	1	0.5	6B(1)	1	0.9	19A(1)
SXT+P+TE+E+DA	1	0.5	6B(1)	1	0.9	19A(1)

SXT = Trimethoprim-Sulfamethoxazole, E=Erythromycin, TE=Tetracycline, DA=Clindamycin, C= Chloramphenicol, P=penicillin

3.7 Molecular characterization

3.7.1 Pulsed Field Gel Electrophoresis (PFGE)

A total of 325 isolates (208 from infants at six-weeks and 117 from infants at nine-months of age) were analyzed using PFGE. Among these, 23 isolates were indigestible by the restriction enzyme *ApaI*. As showed in Figure 3.6, eleven PFGE genotypes were identified which comprised three and more isolates per PFGE genotype. The remaining 252 PFGE genotypes contained only one isolate each. Out of 11 PFGE genotypes, six

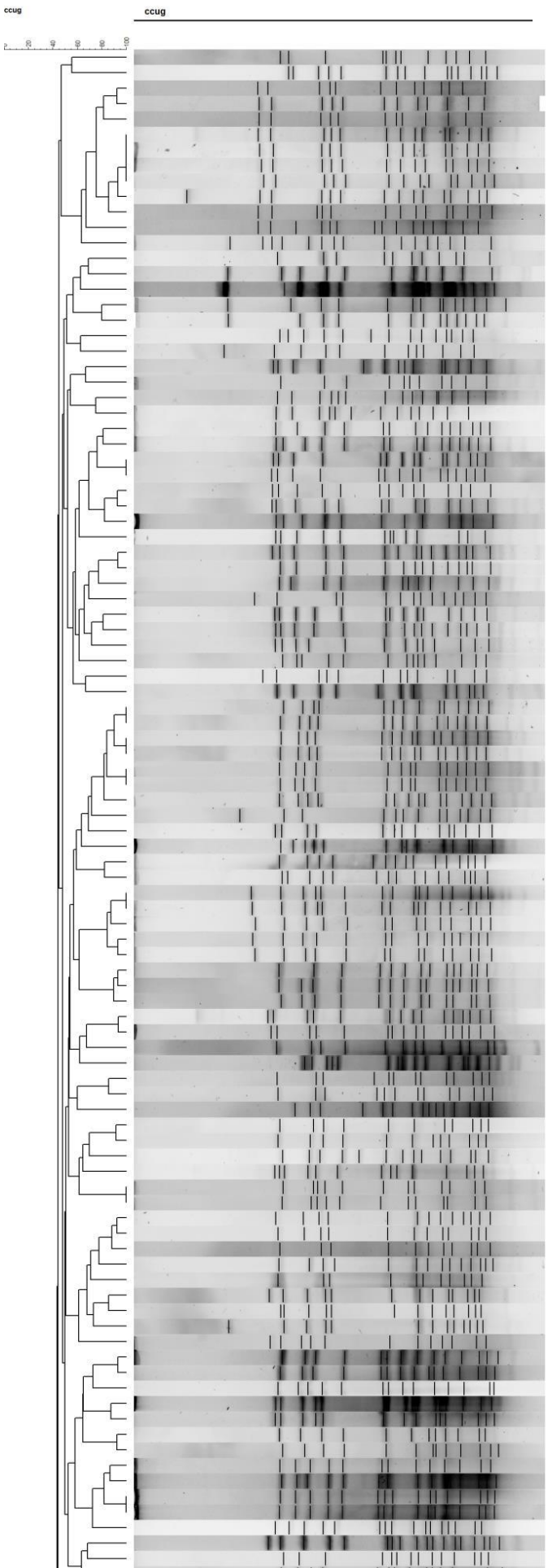
PFGE genotypes comprised the same serotypes in their PFGE genotype and the PFGE genotype was named A to F. The six PFGE genotypes that comprised the same serotypes in their PFGE genotype were genotype A of serotype 31 isolates, PFGE genotype B of serotype 46 isolates, PFGE genotype C of serotype 8 isolates, PFGE genotype D of serotype 3 isolates, PFGE genotype E with serotype 6A isolates and PFGE genotype F of serotype 14. Out of the total of seven isolates of serotype 31, four isolates belonged to PFGE genotype A. They originated from three sub-cities and two of them were before-vaccine isolates. All three isolates of serotype 46 belonged to PFGE genotype B originated from three sub-cities of which two isolates were after-vaccine strains. From the total of seven isolates of serotype 8, five isolates belonged to PFGE genotype C, of which 3 isolates were before-vaccine isolates originating from three sub-cities. Out of ten isolates of serotype 3, seven isolates originated from five sub-cities and belonged to PFGE genotype D, and all of them were before-vaccine isolates. From the total of sixteen isolates with serotype 6A, four isolates belonged to PFGE genotype E and originated from two sub-cities, and all of them were before-vaccine isolates. Only three out of the seven isolates with serotype 14 belonged to PFGE genotype F, and all of them were before-vaccine isolates (Table 3.13 and Figure 3.6).

The other five PFGE genotypes contained a combination of serotypes and PFGE genotypes were named G to K. PFGE genotype G comprised four isolates of serotype 10A and one isolate each of serotype 5 and 6A. PFGE genotype H, contained three isolates of serotype 16F, one isolate of serotype 7B and one isolate of serotype 33B. PFGE genotype I, comprised two isolates of serotype 13 and one isolate each of serotype 12A, 1, 20, 42, and 23F. PFGE genotype J contained three isolates of serotype 6A and two isolates of serotype 10A. PFGE genotype K comprised one isolate each of serotype 23F, 18F and 13 (Table 3.13 and Figure 3.6).

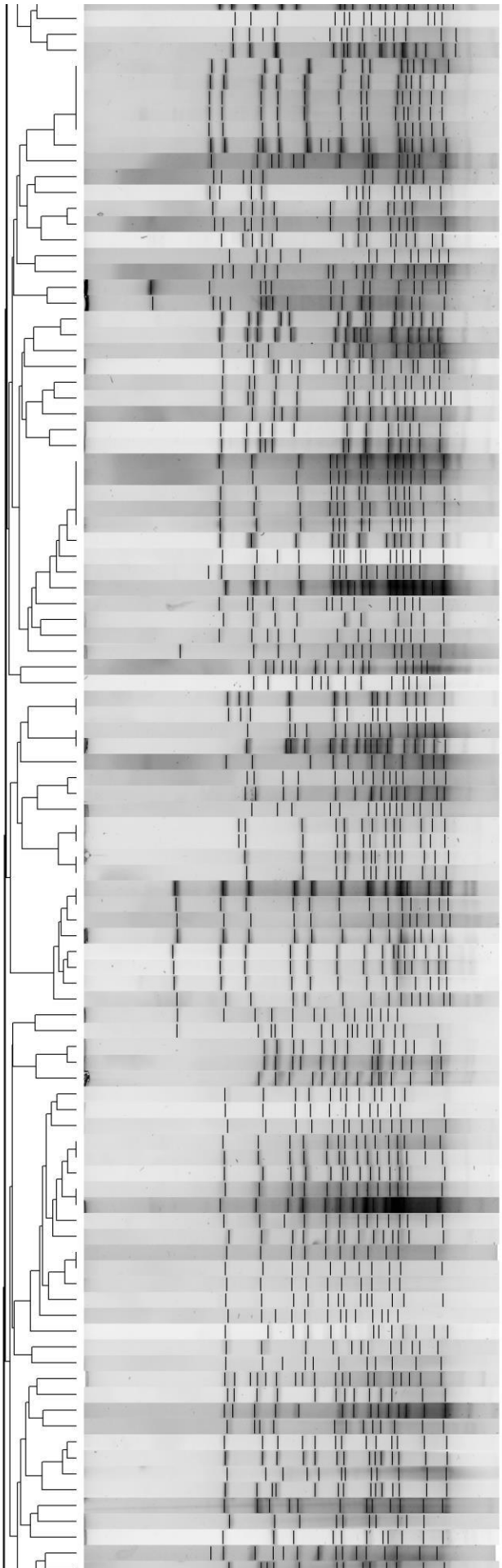
Table 3.13 PFGE genotypes and serotypes of 6 weeks and 9 months *S. pneumoniae* isolates

PFGE Genotype	Serotypes of isolates (n)	Time of isolation		Number of isolation sites	Number of isolates with similar serotype not belonging to the genotype
		Before vaccine(n)	After vaccine(n)		
A	31(4)	2	2	3	3
B	46(3)	2	1	3	-
C	8(5)	3	2	4	2
D	3(7)	7	-	3	3
E	6A (4)	4	-	2	12
F	14(3)	3	-	3	4
G	10A (4)	2	2	4	10
	5(1),	1	-	1	6
	6A(1)	1	-	1	-
H	16F(3)	1	2	3	3
	7B (1),	1	-	1	1
	33B(1)	1	-	1	2
I	13(2)	1	1	2	6
	12A(1)	1	-	1	-
	1(1)	-	1	1	1
	20(1)	1	-	1	7
	42(1)	1	-	1	3
	23F(1)	1	-	1	12
J	6A(2)	-	2	1	-
	10A(1)	-	1	1	-
K	23F(1)	1	-	1	-
	18F(1)	1	-	1	2
	13(1)	1	-	1	-

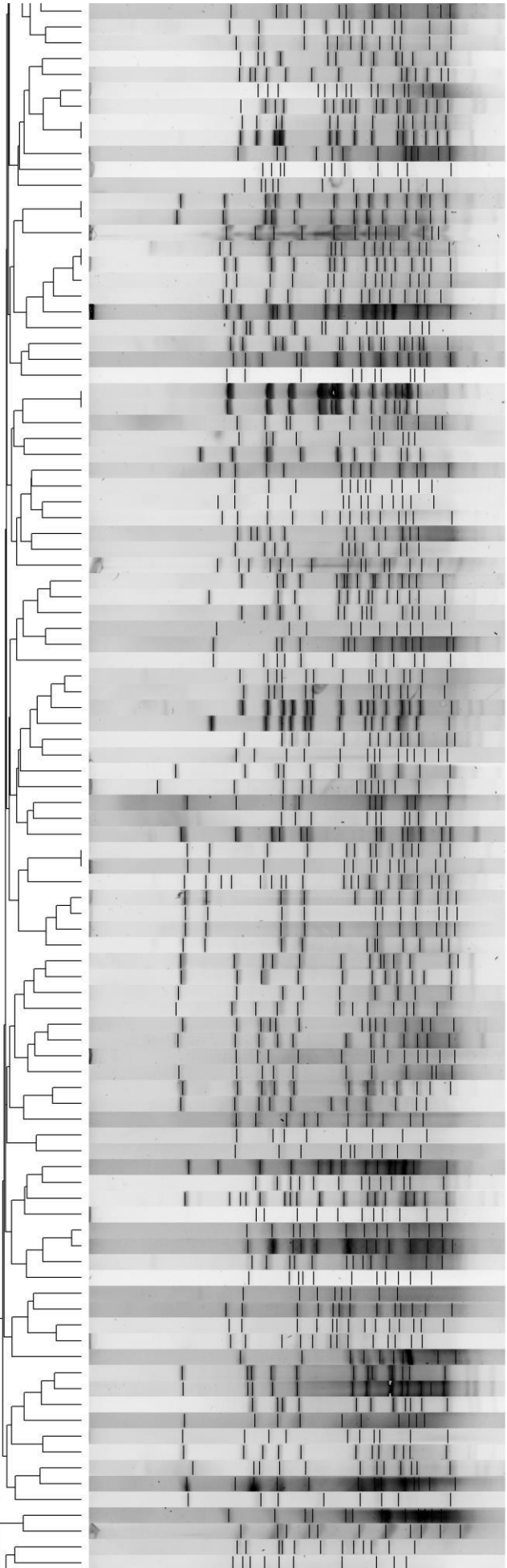
A reduced susceptibility to penicillin was observed in PFGE genotype F in all three isolates and in PFGE genotype E in three of four isolates.



Sample code	Serotype	Heath centre	Period of isolation
AD-036	48	Addis ketema	after vaccination
DE-002	17F	Dilefre	before vaccination
BO-006	10A	Bole-17	after vaccination
KO-030	10A	Kolefe	after vaccination
AD-064	10A	Addis ketema	before vaccination
■ BE-025	6A	Beleteshachew	before vaccination
■ DE-080	10A	Dilefre	after vaccination
■ BO-063	10A	Bole-17	after vaccination
■ BE-027	10A	Beleteshachew	before vaccination
■ BE-092	5	Beleteshachew	before vaccination
■ KO-118	10A	Kolefe	before vaccination
DE-120	10A	Dilefre	before vaccination
AR-039	5	Arada	before vaccination
DE-103	15B	Dilefre	before vaccination
AD-010	24	Addis ketema	after vaccination
KI-115	9N	Kirikos	before vaccination
KI-076	9N	Kirikos	before vaccination
BO-061	9N	Bole-17	after vaccination
BO-065	4	Bole-17	after vaccination
KI-079	19B	Kirikos	before vaccination
DE-020	15A	Dilefre	after vaccination
DE-073	33F	Dilefre	before vaccination
KI-035	7C	Kirikos	before vaccination
KO-040	17F	Kolefe	before vaccination
TE-001	17F	Beleteshachew	before vaccination
DE-072	16F	Dilefre	after vaccination
KO-112	15A	Kolefe	before vaccination
AD-099	15A	Addis ketema	before vaccination
DE-054	15A	Dilefre	after vaccination
DE-089	15A	Dilefre	after vaccination
AD-046	11A	Addis ketema	before vaccination
KO-032	19F	Kolefe	after vaccination
AD-066	29	Addis ketema	after vaccination
AD-070	29	Addis ketema	after vaccination
KI-088	29	Kirikos	before vaccination
AD-052	23A	Addis ketema	before vaccination
BO-026	15A	Bole-17	after vaccination
KO-057	29	Kolefe	before vaccination
DE-023	29	Dilefre	after vaccination
BE-088	31	Beleteshachew	before vaccination
BO-097	19A	Bole-17	before vaccination
AR-014	15A	Arada	before vaccination
♦ KO-081	31	Kolefe	before vaccination
♦ AD-023	31	Addis ketema	after vaccination
♦ AR-025	31	Arada	before vaccination
♦ AD-054	31	Addis ketema	after vaccination
AD-059	23A	Addis ketema	after vaccination
BO-062	23A	Bole-17	after vaccination
DE-007	20	Dilefre	after vaccination
AD-004	31	Addis ketema	before vaccination
BO-022	15B	Bole-17	before vaccination
KI-077	10F	Kirikos	before vaccination
DE-028	17F	Dilefre	after vaccination
KI-026	17F	Kirikos	after vaccination
⊙ KO-022	7B	Kolefe	before vaccination
⊙ KO-021	16F	Kolefe	before vaccination
⊙ KI-013	16F	Kirikos	after vaccination
⊙ BO-019	33B	Bole-17	before vaccination
⊙ AD-035	16F	Addis ketema	after vaccination
☆ KO-064	46	Kolefe	before vaccination
☆ DE-090	46	Dilefre	after vaccination
☆ BE-026	46	Beleteshachew	before vaccination
BO-017	19A	Bole-17	before vaccination
DE-054	11A	Dilefre	before vaccination
AD-064	1	Addis ketema	after vaccination
KO-003	19F	Kolefe	before vaccination
BO-060	20	Bole-17	after vaccination
AD-049	20	Addis ketema	after vaccination
DE-008	35A	Dilefre	before vaccination
BE-035	34	Beleteshachew	before vaccination
BO-072	34	Bole-17	after vaccination
AD-016	19A	Addis ketema	after vaccination
DE-039	23F	Dilefre	before vaccination
BO-069	23B	Bole-17	after vaccination
BO-070	23B	Bole-17	after vaccination
BE-014	14	Beleteshachew	before vaccination
BE-029	34	Beleteshachew	after vaccination
DE-043	35F	Dilefre	after vaccination
KO-108	34	Kolefe	before vaccination
KO-068	23F	Kolefe	before vaccination
DE-023	34	Dilefre	before vaccination
KI-118	34	Kirikos	before vaccination
DE-030	34	Dilefre	before vaccination
AD-094	23B	Addis ketema	before vaccination
BE-072	11A	Beleteshachew	after vaccination
BO-032	11D	Bole-17	after vaccination
KO-050	32F	Kolefe	after vaccination
DE-006	11A	Dilefre	before vaccination
DE-004	11D	Dilefre	before vaccination
KO-035	15B	Kolefe	before vaccination
AR-030	11A	Arada	before vaccination
AD-051	11A	Addis ketema	after vaccination
BE-052	11A	Beleteshachew	after vaccination
AD-002	11A	Addis ketema	before vaccination
BO-002	11A	Bole-17	after vaccination
AR-002	23F	Arada	before vaccination
KO-111	22F	Kolefe	before vaccination
KO-023	2	Kolefe	before vaccination



	.KO-023	2	Kolefe	before vaccination
	.AD-042	23A	Addis ketema	after vaccination
	.BO-011	23F	Bole-17	after vaccination
●	.BO-008	8	Bole-17	before vaccination
●	.KO-066	8	Kolefe	before vaccination
●	.DE-116	8	Dilefre	before vaccination
●	.AD-004	8	Addis ketema	after vaccination
●	.AD-005	8	Addis ketema	after vaccination
	.BO-014	8	Bole-17	before vaccination
	.BE-013	35F	Beleteshachew	after vaccination
	.KA-008	35B	Kirikos center	before vaccination
	.DE-057	18F	Dilefre	before vaccination
	.BO-075	15A	Bole-17	after vaccination
	.BO-072	35B	Bole-17	before vaccination
	.KA-011	15B	Kirikos center	before vaccination
	.KI-047	22F	Kirikos	after vaccination
	.AD-078	35B	Addis ketema	before vaccination
	.AR-023	11A	Arada	before vaccination
	.AR-032	11A	Arada	before vaccination
	.BO-067	15B	Bole-17	before vaccination
	.KI-060	15B	Kirikos	after vaccination
	.KO-116	19F	Kolefe	before vaccination
	.AD-055	10F	Addis ketema	before vaccination
	.AD-037	22F	Addis ketema	before vaccination
	.BO-043	22F	Bole-17	before vaccination
	.BO-102	NT	Bole-17	before vaccination
	.DE-037	22F	Dilefre	before vaccination
	.KI-008	22F	Kirikos	before vaccination
★	.AD-061	12A	Addis ketema	before vaccination
★	.KO-006	1	Kolefe	before vaccination
★	.BE-090	13	Beleteshachew	before vaccination
★	.BO-090	20	Bole-17	before vaccination
★	.KI-109	42	Kirikos	before vaccination
★	.BE-013	23F	Beleteshachew	before vaccination
★	.AD-038	13	Addis ketema	after vaccination
	.AD-051	4	Addis ketema	before vaccination
	.KI-048	22F	Kirikos	after vaccination
	.DE-019	19F	Dilefre	after vaccination
	.BO-071	13	Bole-17	before vaccination
	.AD-024	10F	Addis ketema	after vaccination
	.BE-063	33B	Beleteshachew	before vaccination
	.AD-021	37	Addis ketema	after vaccination
	.DE-042	6B	Dilefre	before vaccination
	.AR-015	21	Arada	before vaccination
	.DE-108	21	Dilefre	after vaccination
	.AD-O74	8	Addis ketema	before vaccination
	.AD-065	3	Addis ketema	before vaccination
	.KI-073	12F	Kirikos	before vaccination
	.DE-092	15A	Dilefre	before vaccination
	.DE-078	12F	Dilefre	before vaccination
	.AR-047	33C	Arada	before vaccination
	.AD-079	2	Addis ketema	before vaccination
	.AD-075	2	Addis ketema	before vaccination
	.DE-053	5	Dilefre	before vaccination
	.KI-064	5	Kirikos	before vaccination
▲	.KI-026	3	Kirikos	before vaccination
▲	.KI-084	3	Kirikos	before vaccination
▲	.BO-003	3	Bole-17	before vaccination
▲	.DE-106	3	Dilefre	before vaccination
▲	.KO-104	3	Kolefe	before vaccination
▲	.BE-015	3	Beleteshachew	before vaccination
▲	.KO-063	3	Kolefe	after vaccination
	.KO-037	3	Kolefe	before vaccination
	.BO-058	38	Bole-17	after vaccination
	.AR-018	38	Arada	before vaccination
	.DE-105	5	Dilefre	before vaccination
	.KO-033	5	Kolefe	before vaccination
	.BE-075	5	Beleteshachew	before vaccination
	.AD-012	14	Addis ketema	before vaccination
	.BO-017	19A	Bole-17	before vaccination
	.KO-089	6A	Kolefe	before vaccination
Ⅱ	.AR-028	6A	Arada	before vaccination
Ⅱ	.AD-012	6A	Addis ketema	before vaccination
Ⅱ	.AD-011	6A	Addis ketema	before vaccination
	.AD-020	6A	Addis ketema	before vaccination
	.KO-080	6A	Kolefe	after vaccination
Ⅱ	.AD-100	6A	Addis ketema	before vaccination
	.KI-045	19F	Kirikos	after vaccination
○	.BO-013	10A	Bole-17	after vaccination
○	.AD-020	6A	Addis ketema	after vaccination
○	.AD-012	6A	Addis ketema	after vaccination
	.DE-083	6A	Dilefre	after vaccination
	.KI-014	6A	Kirikos	after vaccination
	.BO-039	6A	Bole-17	after vaccination
	.DE-061	15B	Dilefre	after vaccination
	.KO-025	6A	Kolefe	after vaccination
	.DE-038	22F	Dilefre	before vaccination
	.AR-021	14	Arada	before vaccination
	.BO-009	35A	Bole-17	before vaccination
	.AD-025	22F	Addis ketema	before vaccination
✪	.BO-098	14	Bole-17	before vaccination
✪	.AD-042	14	Addis ketema	before vaccination
✪	.DE-010	14	Dilefre	before vaccination
	.AD-031	14	Addis ketema	before vaccination
	.AD-031	20	Addis ketema	after vaccination
	.AD-058	42	Addis ketema	before vaccination
	.AD-043	6B	Addis ketema	before vaccination
	.KI-043	10A	Kirikos	after vaccination
	.KO-010	42	Kolefe	before vaccination



.AD-044	35A	Addis ketema	after vaccination	
.DE-078	3	Dilefre	after vaccination	
.BO-039	13	Bole-17	before vaccination	
.KO-008	23F	Kolefe	before vaccination	
.BE-057	14	Beleteshachew	before vaccination	
.AD-050	6B	Addis ketema	after vaccination	
.KO-045	6B	Kolefe	after vaccination	
.KI-017	23F	Kirikos	before vaccination	
.KI-016	23F	Kirikos	before vaccination	
.KO-104	11B	Kolefe	after vaccination	
.DE-015	6B	Dilefre	before vaccination	
.BE-067	10F	Beleteshachew	after vaccination	
.KI-053	15B	Kirikos	before vaccination	
.AD-035	38	Addis ketema	before vaccination	
.DE-094	16F	Dilefre	before vaccination	
✧ .BO-033	23F	Bole-17	1. before vaccination	
✧ .BO-058	18F	Bole-17	1. before vaccination	
✧ .BE-055	13	Beleteshachew	1. before vaccination	
.DE-099	15B	Dilefre	before vaccination	
.AD-055	11A	Addis ketema	after vaccination	
.KO-097	21	Kolefe	before vaccination	
.KO-034	19B	Kolefe	after vaccination	
.BO-052	35A	Bole-17	before vaccination	
.DE-064	15A	Dilefre	before vaccination	
.KO-031	34	Kolefe	before vaccination	
.KI-031	7B	Kirikos	before vaccination	
.BO-051	31	Bole-17	after vaccination	
.DE-035	27	Dilefre	before vaccination	
.AD-008	38	Addis ketema	before vaccination	
.BO-002	20	Bole-17	before vaccination	
.KI-009	42	Kirikos	before vaccination	
.AD-016	21	Addis ketema	before vaccination	
.BE-019	13	Beleteshachew	before vaccination	
.BE-038	19B	Beleteshachew	after vaccination	
.KI-090	9L	Kirikos	before vaccination	
.BO-037	16F	Bole-17	before vaccination	
.DE-075	15B	Dilefre	before vaccination	
.AD-045	7C	Addis ketema	after vaccination	
.DE-067	15A	Dilefre	after vaccination	
.KO-110	19F	Kolefe	before vaccination	
.AD-104	7C	Addis ketema	before vaccination	
.KI-052	19F	Kirikos	after vaccination	
.AD-061	19B	Addis ketema	after vaccination	
.BE-025	19B	Beleteshachew	after vaccination	
.BO-052	19B	Bole-17	after vaccination	
.AD-009	19B	Addis ketema	after vaccination	
.KO-056	19B	Kolefe	before vaccination	
.KO-045	27	Kolefe	before vaccination	
.BE-003	15B	Beleteshachew	before vaccination	
.AR-043	15C	Arada	before vaccination	
.KO-058	1	Kolefe	after vaccination	
.KI-002	NT	Kirikos	before vaccination	
.BO-030	20	Bole-17	after vaccination	
.KO-105	4	Kolefe	before vaccination	
.AD-014	23B	Addis ketema	before vaccination	
.KO-106	19A	Kolefe	before vaccination	
.KI-052	9V	Kirikos	before vaccination	
.BO-066	9V	Bole-17	before vaccination	
.AD-001	9V	Addis ketema	before vaccination	
.DE-022	19A	Dilefre	before vaccination	
.KI-005	4	Kirikos	before vaccination	
.KI-061	4	Kirikos	before vaccination	
.KO-065	15B	Kolefe	after vaccination	
.KO-068	15B	Kolefe	after vaccination	
.DE-087	19B	Dilefre	after vaccination	
.KO-103	6C	Kolefe	before vaccination	
.KO-034	11A	Kolefe	before vaccination	
.KO-004	6C	Kolefe	before vaccination	
.BE-040	24	Beleteshachew	before vaccination	
.BE-064	24	Beleteshachew	before vaccination	
.AD-015	15A	Addis ketema	after vaccination	
.AD-040	6A	Addis ketemaAddis ket.	after vaccination	
.KO-019	15A	Kolefe	before vaccination	
.BO-085	10A	Bole-17	before vaccination	
.BE-027	7C	Beleteshachew	after vaccination	
.KI-095	3	Kirikos	before vaccination	
.AD-113	6B	Addis ketema	before vaccination	
.AD-034	19F	Addis ketema	before vaccination	
.BO-041	19F	Bole-17	before vaccination	
.DE-032	19F	Dilefre	after vaccination	
.KO-050	19B	Kolefe	before vaccination	
.KA-002	15C	Kirikos center	before vaccination	
.KI-041	15C	Kirikos	after vaccination	
.BE-021	15B	Beleteshachew	after vaccination	
.AD-026	17F	Addis ketema	after vaccination	
.AD-106	19F	Addis ketema	before vaccination	
.DE-051	23F	Dilefre	before vaccination	
.DE-039	23F	Dilefre	after vaccination	
.DE-074	23F	Dilefre	before vaccination	
.BE-079	7C	Beleteshachew	before vaccination	
.DE-037	6D	Dilefre	after vaccination	
.KI-054	7C	Kirikos	after vaccination	
.KI-067	23F	Kirikos	after vaccination	
.BE-022	15C	Beleteshachew	before vaccination	
.BE-030	24	Beleteshachew	after vaccination	
.KO-071	15C	Kolefe	before vaccination	
.KO-038	20	Kolefe	after vaccination	
.AD-065	19B	Addis ketema	after vaccination	
.DE-088	21	Dilefre	before vaccination	

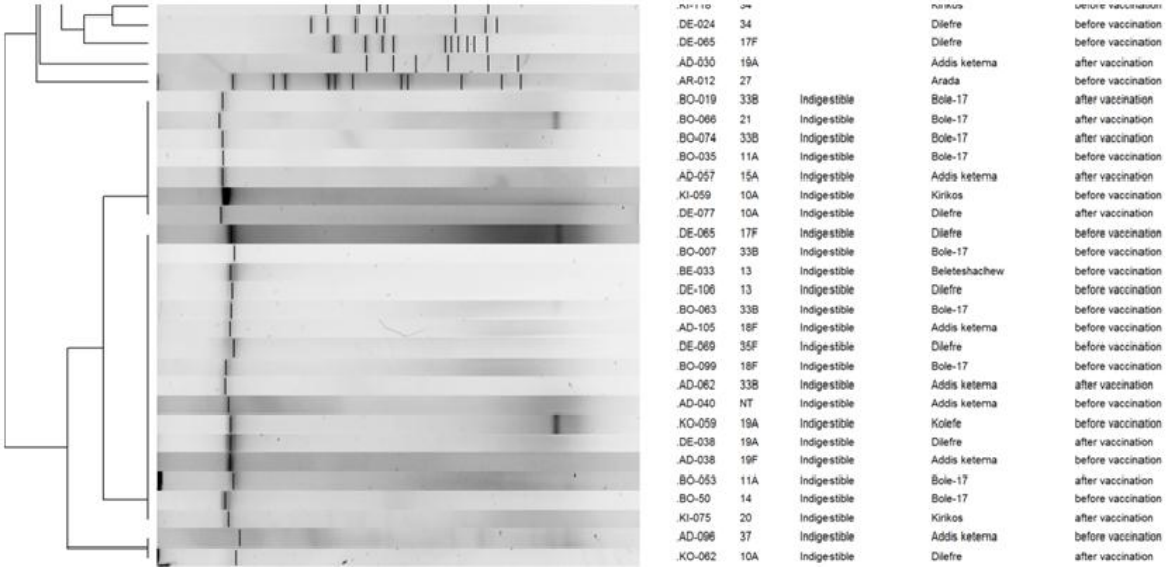


Figure 3.6 PFGE analysis of 325 isolates (208 pre-vaccination and 117 post vaccination isolates)

3. 7.2 Multilocus Sequencing Typing (MLST)

A total of twelve isolates, two isolates from PFGE genotype I and one isolate each from the rest of the10 PFGE genotypes were subjected to Multilocus Sequence Typing (MLST). As depicted in Table 3.14 all the sequence types were not new sequence types. However, ST 6489 of serotype 31, ST8930 of serotype 23F and ST3460 of serotype 6A were different from the already reported sequence types in only one allele, allele (*gki*), allele (*spi*) and allele (*xpiI*), respectively. Serotypes 1 and 23F, which were analyzed by MSLT from PFGE genotype I, revealed they do have similar sequence types though they belonged to different serotypes. The two 6A serotype types which were from two different PFGE genotypes gave the same sequence type.

Table 3.14 MLST analysis of selected *S. pneumoniae* isolates.

PFGE genotype	Number of isolates	Serotypes(n)	MLST typed Isolate origin	MLST typed isolate serotype	Sequence type
G	6	10A(4), 6A(1), 5(1)	After vaccine	10A	ST3135
A	4	31 (4)	Before vaccine	31	ST6489‡
H	5	16F(3), 7B(1), 33B(1)	Before vaccine	16F	ST6882
B	3	46(3)	After vaccine	46	ST6450
C	5	8(5)	After vaccine	8	ST3500
I	7	1,12A,13(2), 20, 23F, 42	After vaccine	1	ST8930*
			Before vaccine	23F	ST8930*
D	7	3(7)	Before vaccine	3	ST458
E	4	6A(4)	Before vaccine	6A	ST3460
F	3	14(3)	Before vaccine	14	ST63
K	3	13, 18F, 23F	Before vaccine	23F	ST988
J	3	6A(2),10A(1)	After vaccine	6A	ST3460ŧ

‡ one allele (gki) different, * One allele (spi) different, ŧ one allele (xpiI) different

3.8 Nasopharyngeal carriage rate of other colonizers

The proportion of carriage for *Staphylococcus aureus* was 21.7% (95%CI, 18.8- 24.6), 4.4% (95% CI, 10.8-15.6) and 7.8% (95% CI, 4.6-12.6) at the age of six weeks, nine months and two years respectively, with statistically significant variation between the three periods. The carriage rate of *Moraxella catarrhalis* was 13.2% (95% CI 10.8-15.6) at the age of six weeks, 6.8% (95% CI 5.6-7.0) at the of nine months and 9% at the age of two years; and there was statistically significant differences between the periods ($p<0.001$). However, no significant difference was observed for the carriage rate of *Haemophilus infulenzae* at the age of six-weeks, nine-months and two-years with the carriage rate of 3.9%, 6.3% and 4% , respectively (P=0.346) (Table 3.15).

Table 3.15 Nasopharyngeal colonizers other than *S. pneumoniae* isolated at the age of 6 weeks, 9 months and 2 years.

Bacterial Species	Before vaccine at age of 6 week (n=789)		After completion of vaccine			
			At age of 9 months (n=206)		At the age of 2 year (n=201)	
	Positive	Percent (95% CI)	Positive	Percent (95% CI)	Positive	Percent (95% CI)
Staphylococcus aureus	171	21.7 18.8- 24.6	9	4.4 10.8-15.6	16	7.8 4.6-12.6
Moraxella catarrhalis	104	13.2 10.8-15.6	14	6.8 3.8-11.1	18	9.0 5.4-13.8
Haemophilus influenzae	31	3.9 2.7-5.5	13	6.3 3.4-10.5	8	4.0 1.7-7.8
Streptococcus pyogenes	2	0.3	-	-	-	-
Total	**308**					

3. 9 *S. pneumoniae and* other co-colonizers: mixed carriage

Mixed carriage of *S. pneumoniae* with *Staphylococcus aureus* was 22.4% (47/210), 0.9% (1/117), and 5.3% (5/97) respectively in the six-week, nine-month and two-year old children. Similarly, mixed carriage rate of *S. pneumoniae* with *Moraxella catarrhalis* and *Haemophilus influenzae* was high at the age of six weeks. In five infants, the three bacteria were detected together at the age of six weeks and in one child at the age of two years. Mixed carriages with other bacteria were significantly reduced at the age of nine months and two years (Table 3.16).

Table 3.16 Nasopharyngeal carriage rate of *S. pneumoniae mixed* with other co-colonizers in children at the age of 6 weeks, 9 months and 2 years of age

Species of Bacteria	6 weeks of age n=210		9 months of age n=117		2 years of age n=97	
	Positive	**Percent**	**Positive**	**Percent**	**Positive**	**Percent**
Pneumococci + *Staphylococcus aureus*	47	22.4	1	0.9	5	5.1
Pneumococci + *Moraxella catarrhalis*	34	16.2	6	5.9	6	6.2
Pneumococci + *Haemophilus influenzae*	12	5.7	9	7.7	7	7.2
Pneumococci + *Staphylococcus aureus* + *Moraxella catarrhalis*	5	2.4	-	-	1	1.0

CHAPTER FOUR

4.1 Discussion

4.1.1 Carriage rate and risk factors

Pneumococcus is the leading cause of infection in humans and frequently colonises the nasopharynx. Apart from causing non-invasive diseases such as sinusitis, otitis media, and community-acquired pneumonia, which result from direct spread from the nasopharynx, the pneumococcus can invade the bloodstream and cause septicaemia, meningitis, and septicemic pneumonia (Flasche *et al.,* 2011, Ozdemir *et al.,* 2013). Thus, the pneumococcus is responsible for great mortality and morbidity, and is an important cause of vaccine-preventable deaths in children (Cohen *et al.,* 2016).

Individual nasopharyngeal carriage is a necessary step for pneumococcal disease and is the only source for transmission and invasive infection. Asymptomatic carriers infect other individuals through the respiratory tract. Nasopharyngeal (NP) colonization of pneumococci starts during early infancy and the median age at first acquisition of carriage was reported as 33 days in The Gambia (Hill *et al.,* 2008), 38.5 days in Kenya (Tigoi *et al.,* 2012), 8 weeks in Bangladesh (Granat *et al.,* 2007) and 45.5 days in Thailand-Myanmar (Turner *et al.,* 2012). In our study, we found a carriage rate of 26.6% (210/789) at the age of six weeks when the infants came for the first dose of the PCV10 vaccine, which is in agreement with these findings. This early exposure in infancy is likely to play an important role in the development of infection at an early age.

Nasopharyngeal carriage rates of pneumococci vary according to geographic region and population (Bayraktar *et al.,* 2005a). In African and Asian countries, NP pneumococcal carriage rates were often reported to be high. In Africa, these included carriage rates of nearly 90% in The Gambia in children (Hill *et al.,*2008), and other reports from the continent range from 18.6% to as high as 93.4 % (Abdullahi *et al.,* 2008, Mills *et al.,* 2015 Huebner *et al.,* 1998, Heinsbroek *et al.,* 2015, Anthony *et al.,* 2012, Rutebemberwa *et al.,* 2015, Usuf *et al.,* 2014).

Similarly, a high carriage rate was also reported from Asian countries such as Nepal, (Hanieh *et al.,* 2014), Indonesia (Soewignjo *et al.,* 2001), Taiwan (Kuo *et al.,* 2011), China (Yao and Yang 2008) and India (Jain *et al.,* 2005) for different age groups. Reported carriage rates in Latin America included, 55% in < 5 children in Brazil (Menezes *et al.,* 2016), and 55.7% in Colombia (Parra *et al.,* 2013). These high carriage rates were in part attributed to the overcrowded living condition in most of these developing countries (Adegbola *et al.,* 2014).

In industrialized countries, in comparison to African and Asian countries, most countries reported a lower carriage rate ranging from 21 to 30% (Leino *et al.,* 2001, Tocheva *et al.,* 2011, Malfroot *et al.,* 2004, Dunne *et al.,* 2012, Luminos *et al.,* 2014, Zuccotti *et al.,* 2014). However, higher carriage rates have also been reported for developed countries, such as 43% in < 2-year-old children in the United States (Finkelstein *et al.,* 2003) and 74% in Australia among Aborigian children aged 2-15 years (Mackenzie *et al.,* 2010).

Our finding of a carriage rate of 56.8% at nine months and of 47.3% at two years is in agreement with the high rate of carriage reported from most African and Asian countries. The fact that our study population had been fully vaccinated but still shows a relatively high carriage rate suggests that the impact of the vaccine on the overall carriage rate was minimal.

Previous studies done in Ethiopia have reported differing rates of carriage probably due to differences among study participants and in study areas. A study conducted at Gonder University Hospital (Assefa *et al.,* 2013) reported an overall carriage prevalence of 41.3% in children below 10 years of age seen in the outpatient department. This is similar to our finding in the two year olds. In an earlier study in children < 5 years of age done in three different study areas, Buatajira, Shershera-Bido village and Addis Ababa revealed an overall prevalence of 90% (Ringertz *et al.,* 1993). A similarly high carriage rate of 93% was reported more recently in a rural district of Gurage zone in Southern Ethiopia in one-to ten- year old children included in a study on antibiotic resistance after mass treatment with azithromycin for the prevention of trachoma (Haug *et al.,* 2010). Our

finding is different from these studies that reported much higher carriage rates in Ethiopia, probably due to differences in the study population and in period as well as time point of sampling. The other reports which a merge of age groups are difficult to compare with ours because we determined carriage rates for exact ages of sampling. Similar trend of carriage prevalence was reported from Kenya where the maximum peak was seen in 6–11 months old children which then declined (Abdullahi *et al.,* 2012). The higher carriage rate at early age might be a risk for developing invasive and non-invasive pneumococcal diseases at an early infancy.

A variety of demographic and clinical characteristics have been explained to be related with an increase in pneumococcal colonization, for example age (a progressive decrease of carriage rate with increasing age of children after the age of two years), society/ ethnicity, family size, crowding, presence of under-five siblings, attendance of day-care centre, smoking at home, recent antibiotic use, and socioeconomic status of study populations; although variations in sampling and isolation techniques might confound the results (Roche *et al.,* 2007, Abdullahi *et al.,* 2008, Prymula *et al.,* 2009, Ozdemir *et al.,* 2013, Devine *et al.,* 2015, Geno *et al.,* 2015). In this study, we found that only living with siblings with the age of less than five years and low income of the family of less than 500 birr equivalent to 22 USD) were associated with a higher rate of carriage of pneumococci. This finding is in line with other studies that showed the presence of under-five sibling and low socioeconomic status as predisposing risk factors for the acquisition of pneumococci (Kuo *et al.,* 2011, Ansaldi *et al.,* 2012, Kumar *et al.,* 2014). Other risk factors investigated in this study, i.e. gender, crowding (family size and number of rooms in the house) and presence of smokers in the household were not found to be associated with carriage rate.

In our finding, 93% of mothers breastfed their children; however, active breastfeeding did not appear to play a protective role in pneumococcal carriage in children in our study group. A similar situation has been observed in another study where it is discussed that antibodies passively transferred by breastfeeding may provide protection to infants against IPD but that the type and target of the antibodies may not protect against carriage

since the level of antibody required to protect against carriage is higher than against IPD (Scott *et al.*, 2012).

The impact of PCVs on nasopharyngeal carriage is considered to be a predictor of vaccine effectiveness. Carriage studies can be used to predict the potential impact of different formulations of PCVs (Roca *et al.*, 2015). To our knowledge, this is the first study after the introduction of PCV10 in Ethiopia to determine the carriage rate of pneumococci and analyze the impact of PCV10 on pneumococcal carriage rate in vaccinated children in Ethiopia. The carriage rate of pneumococci increased significantly from the age of six weeks (26.6% before the vaccine to 56.8%) to the age of nine months when they completed vaccination. This could probably be due to increased exposure as children grew up. Then carriage rate slightly declined to 47.3% when the children reached age two. Similar situations have been reported from other studies in Malawi (Heinsbroek *et al.*, 2016), Kenya (Tigoi *et al.*, 2012) and The Gambia (Hill *et al.*, 2008). A number of studies have showed that introduction of PCVs into routine vaccination programs did not affect overall pneumococcal carriage rate, but rather commonly led to non-vaccine type replacement (Vestrheim *et al.*, 2010, Grivea *et al.*, 2011, Ansaldi *et al.*, 2012, Scott *et al.*, 2012, Spijkerman *et al.*, 2012, van der Linden *et al.*, 2015, Bosch *et al.*, 2016). The overall carriage rate may not be affected and the effect of the vaccine seems to more reflected on serotype distribution than on the carriage rate. This seems to have been the case in our study as well.

4.1.2 Serotype distribution and the impact of the vaccine

A total of 422 pneumococcal isolates were serotyped in this study (208 at six week before, and 117 at nine months and 97 at two years of age after vaccination) and among these ten isolates were non-typeable. The serotypes we identified were of 59 different serotypes and nearly 80% were non-vaccine types. From all six week (before vaccine) isolates, we identified 54 different serotypes of which vaccine types included in PCV7, PCV10 and PCV13 comprised only 16.8%, 20.2% and 30.3%, respectively. These findings revealed a high diversity of circulating serotypes in the study area, constituting

predominantly serotypes not included in the vaccines (non-vaccine types). This highlights the presence of a sizable pool of strains with the potential for replacement of VT strains that might cause invasive and non-invasive diseases.

Several studies done elsewhere in Africa on the relative proportion of vaccine and non-vaccine types have showed diverse results. For instance, in Tanzania from the total carriage isolates 37%, were PCV7, 37%, PCV10 and 49% were PCV13 types (Anthony *et al.,* 2012). In Kenya, in one study the 43% of isolates were of PCV10 types (Abdullahi *et al.,* 2012), and in another study 47% of the isolates were of PCV7 types (Abdullahi *et al.,* 2008). In Malawi, 60% of the isolates were covered by PCV13 (Kamng'ona *et al.,* 2015). In two different studies in The Gambia, 63% of the carriage isolates were of PCV7 types (Hill *et al.,* 2006) and 46% were of PCV10 types (Antonio *et al.,* 2008), while a study done in Senegal indicated that PCV7 covered 36.4%, PCV10 covered 39.4% and PCV13 covered 53% of the identified serotypes in the nasopharynx of children (Ba *et al.,* 2014). A pre-vaccine carriage study in Nigeria indicated that 43.8%, 45.0% and 62% of strains were PCV-7, PCV-10 and PCV-13 types respectively (Adetifa *et al.,* 2012). In a Thailand carriage study, 55.8% of the isolates from infants were of PCV13 serotypes (Turner *et al.,* 2012). The difference of our finding with these studies probably stems from the fact that our study was carried out one year and four months after the vaccine was already introduced into routine vaccination schedule. That variation might have impacted carriage rate of the vaccine types in the community, even if it is very difficult to truly justify the herd immunity effect. To fully observe herd immunity a majority of the target population of under-five children should be vaccinated, even if it is possible to see an effect of herd immunity also after 1-3 doses with an infant vaccine (Stephens 2008)

Out of 117 isolates from nine-month-old children who have completed the vaccination, we have identified 43 different serotypes of which 89% were non-vaccine type of PCV10. The vaccine types were reduced from 20% before vaccine to 11.1% after completion of the vaccine at the age of nine months. Similarly, out of 97 isolates of two years, 89.7% were non-vaccine, and we did not find a reduction of vaccine type at this age, when we compared this with age at nine months. Our finding is in agreement with

the Kenyan study where the carriage rate of PCV10 vaccine serotypes were significantly reduced from 34% before the vaccine to 13% after two years of completion of the vaccine (Hammitt *et al.*, 2014a). The proportion of PCV10 serotypes such as 23F, 14 and 5 were in relatively higher number before the vaccine, and we did not detect any serotype 5, 14 and 9V after completion of the vaccine at the age of nine months and two-years. Serotype 14 and 6B have been implicated as common causes of IPD in young children globally (Johnson *et al.*, 2010). In this study, we identified seven isolates of serotype 14 and four isolates of serotype 6B among isolates obtained before vaccination.

We did not identify any serotype 7F and 18C strains which are constituted in PCV10 among both six-week, nine-month and two-year isolates, but we rather detected 7C and 18F. It is important to note that serotypes 7C and 18F were the serotypes identified in clinical specimens (CFS, blood, ear discharge, throat, pleural fluid, and sputum) from Gondar and Addis Ababa (Dinku 2013). This suggests that the prevalent serotypes might be 7C from serogroup 7 and 18F from serogroup 18, causing different clinical diseases in the country. However, this needs further investigation both from the carriage and clinical aspects of the epidemiology of the strains.

Among non-vaccine types of this study, 13% of the isolates were serotype 9N, 20, 11A, 8, 16F, and these isolates were previously reported in a study done in children with meningitis in Tikur Anbassa Hospital, Ethiopia (Muhe and Klugman 1999). In another study in Ethiopia, serotypes 8, 10A, 13, 20, 22A, 27 and 15C had been isolated from different clinical samples (Dinku 2013). We detected all these serotypes in the nasopharynx of children except for serotype 22A. Serotype 10A was detected at a higher frequency than the others.

A limited number of serotypes cause most IPD worldwide. Even if serotypes included in existing PCV formulations account for 49%–88% of deaths in Africa and Asia where pneumococcal disease morbidity and mortality are the highest (Johnson et al, 2010), there is a report from a study done in Denmark, among individuals aged 5 years and older, that serotypes 31, 11A, 35F, 17F, 3, 16F, 19F, 15B and 10A were associated with higher

mortality as compared with serotype 1 (Harboe *et al.,* 2009), which a common cause of invasive diseases in African settings. In this study, we identified all uncommon serotypes in carriage that were reported in Denmark as the causes of invasive diseases. This suggests a potential for these serotypes to cause diseases with high mortality in older children in our setting as well.

Several strains belonging to vaccine serotypes were detected in vaccinated children. These included 13 strains that belonged to PCV10 serotypes 1, 4, 6B, 19F, 23F isolated from children at the age of nine months, and ten strains that belonged to serotypes 1, 4, 19F and 23F detected at the age of two years. For example, there were five 19F strains isolated from nine-month-old children, and four from children aged two years. Why children are still colonized by these vaccine types after completion of the vaccination schedule could be due to several reasons. One possibility is that the concentration of antibodies produced as a result of carriage may not be sufficient to prevent colonization. Higher concentrations of IgG are required to avoid colonisation than those necessary to prevent IPD (Jokinen *et al.,* 2004, Prymula *et al.,* 2011, Principi and Esposito 2015). Currently available conjugate vaccines are based on capsular antigens and when the polysaccharides are conjugated with carrier proteins, they induce a strong immune response against the polysaccaride capable of preventing both colonisation and disease, but this response is neither quantitatively nor qualitatively uniform for all serotypes. Moreover, the antibody concentrations needed to eradicate pathogens migh vary from serotype to serotype, and from site to site of infection.

Our findings show high frequency of the PCV13 vaccine types, 6A and 19A, in both before and after vaccine isolates. In countries currently using the PCV13 vaccine, such as America, Spain, Canada, Australia, Germany and other European countries earlier reports showed an increase of serotype 19A as the causative agent of IPD after the introduction of PCV7 in these countries, a serotype associated with antibiotic resistance (Kaplan *et al.,* 2004, Hanage *et al.,* 2010, Richter *et al.,* 2013, Guevara *et al.,* 2009, Kellner *et al.,* 2009, Williams *et al.,* 2011, van der Linden *et al.,* 2013, van der Linden *et al.,* 2015, Weil-Olivier *et al.,* 2012). An increased involvement of serotype 6C in acute otitis media was

also observed in the years after PCV7 implementation in The Netherlands (Bosch *et al.*, 2016). Therefore, these serotypes might be potential causes of IPD in Ethiopia in the future too.

In the current study, among the infants who were positive for *S. pneumoniae carriage* at the age of six weeks, 44 (37.6%) were still positive at the age of nine months. However, at nine months, only 4 children were still colonized with a similar serotype as that at the age of six weeks. The serotypes were 6A in two children and 23F and 33B in the others. The PFGE analysis proved that these nine month isolates are different strains from those obtained at six weeks of age. This finding indicates that there is a natural fluctuation in carriage rates of different serotypes rather than a persistence of the first colonizer. Presence of the same serotype at two different time points does not necessarily mean that the same strain is there throughout. Colonization by different members of the same serotype replacing each other over time is therefore possible.

A high rate of multiple serotype carriage has been reported from several regions including 40% in Malawi (Kamng'ona *et al.,* 2015), 48.8% among refugee infants born in the Thailand-Myanmar border (Turner *et al.*, 2011) and 43.6% in Spain (Ercibengoa *et al.*,2012). Multiple carriage is reported to promote genetic recombination through horizontal gene transfer of antibiotic resistance and virulence genes, which may contribute to pathogen adaptation and increased risk of disease in the host (Hanage *et al.*, 2009, Donkor *et al.*, 2011). A recent report suggests that multiple carriage may promote co-infection with two or more pneumococcal serotypes (Rodrigues *et al.*, 2013). In this study, we did not conduct a thorough multiple serotype carriage rate determination due to the required work load and the cost of detecting multiple serotype carriages in the laboratory. We limited our investigation to identify a single serotype per swab. However, we have observed that some of the samples had more than one morphologically different colonies, which could be seen as indirect indication of the presence of multiple serotypes in nasopharynx.

Introduction of PCV into the routine immunisation schedule of developed countries over the past 13 years has resulted in a dramatic reduction in the incidence of invasive pneumococcal disease caused by vaccine serotypes (Lehmann *et al.,* 2010, Flasche *et al.*, 2011, Miller *et al.,* 2011, Feikin *et al.,* 2013, Pinchas *et al.,* 2015). Additionally, vaccinated individuals are less likely to be carriers of vaccine-serotype pneumococci, and are therefore less likely to transmit the infection than non-vaccinated individuals. At a population level, vaccination leads to a reduction in the carriage prevalence of vaccine-serotype pneumococci in both vaccinated and unvaccinated individuals, and a reduction in the incidence of invasive pneumococcal disease, caused by vaccine-serotype pneumococci in the whole population, known as the "herd effect," or " herd immunity" (Hammitt *et al.,* 2014a, Hammitt *et al.,* 2014b).

According to a Federal Ministry of Health (FMOH) report on the health and health indicators of 2014/2015 of the country, the overall country wide vaccine coverage for PCV10 was 93.9%, which is high enough to achieve herd immunity to those serotypes included in the vaccine. This effect needs to be verified both in clinical cases and in carriage in subsequent years, to analyze the impact of the vaccine on the whole population. Our finding also shows that the majority of the isolates were non-vaccine types and indicates the existence of very diverse serotypes in the country. These serotypes might replace vaccine types in causing invasive and non invasive disease. Furthermore, our study also identified that some of the children are carriers of serotypes that are included in the vaccine and this needs further investigation to determine the reason for this.

4.1.3 Antibiotic susceptibility pattern of isolates: rate and risk factors

Treatment of infections due to pneumococci has become a complicated global problem, due to antibiotic resistance development. Antibiotic resistance patterns found among isolates from carriers provide a rough estimate of those found among isolates causing invasive diseases (Lehmann *et al.,* 1997) which indicates that pneumococcal carriage studies may be used for monitoring resistance patterns (Cizman 2003). Significant

geographical variations in the frequency of antibiotic resistance have been observed both between countries and within the same country. The risk factors for development of antibiotic resistance in pneumococci include use of antibiotics particularly at low levels for prolonged periods, easy availability over the counter and wider, indiscriminate use in the hospital as well as in the community (Klugman 2007). The emergence of resistance to penicillin in the 1980s led to the increased use of macrolides, flouroquinolones and other drugs. While these antibiotics provided effective alternate therapy for pneumococcal infections, selective pressure associated with the widespread use of these antibiotics has resulted in resistance to various classes of antimicrobial agents (Kumar *et al.,* 2014).

In our study, 46.2% of six-week and 53% of nine-month pneumococcal isolates were resistant or intermediate resistant to at least one antibiotic from the total of 13 antibiotics tested. A higher rate of resistance was observed to Trimethoprim-Sulfamethoxazole in 25.7% from the total six-week and 33.3% from nine-month isolates. This finding is nearly similar to the rate of resistance reported in earlier studies in Ethiopia, 27% of clinical isolates of pneumococci and 29.1% of nasopharyngeal isolates in Gonder (Erqou *et al.,* 2008, Assefa *et al.,* 2013). Our findings are lower than reports from India 41% (Kumar *et al.,* 2014), 35.% in Cambodia (Parra *et al.,* 2013), 74% from Malawi (Everett *et al.,* 2011), and 98.9% in Uganda (Rutebemberwa *et al.,* 2015). This variation could be a reflection of the level of inappropriate antibiotic use, level of controls on drug prescribing; compliance level with treatment regimens, poor dosing and the variation of resistant strains; lack of infection control in the countries. The resistance rate to Trimethoprim-Sulfamethoxazole is relatively lower in our study as compared to other countries but very high in comparison to other antibiotics tested in this study. Therefore, empirical use of this drug is not justifiable with this rate of resistance.

Antibiotic resistance has developed primarily in pneumococcal strains belonging to the serotypes that are most prevalent in children, serogroup 6, 14, 19, and 23. The probable reason is the common use of antibiotic therapy in small children and hence a frequent exposure of strains of these serotypes to antimicrobial drugs, providing a selective advantage to resistant mutants (Klugman and Friedland 1995). In our finding also, we

have identified that the majority of the resistant strains from both six-week and nine-month isolates belonged to these serogroups.

Reduced susceptibility to penicillin was found in 16.7% of isolates at the age of six-week, and this increased significantly to 25.6% at the age of nine-months among isolates, as it might be a result of prolonged carriage that gives chance to acquire resistance gene. The Gondar study also reported that 31% of the nasopharyngeal isolates were intermediate resistant (Assefa *et al.,* 2013). Such level of intermediate-resistance is of great concern as it might add to the accumulation of resistance and spread of resistant strains in the community. Moreover, it is also known that strains showing penicillin resistance may carry other genes responsible for resistance to other antibiotics (Lehmann *et al.,* 1997). Resistance, particularly of high-level penicillin resistance (i.e., in PRSP - penicillin resistant pneumococci) and of multidrug resistance (MDRP)) is mainly associated with seven serotypes commonly carried in children: 6A, 6B, 9V, 14, 19A, 19F, and 23F (Dagan and Klugman 2008). Similarly, in our study we identified MDRP of serotype 6B and 14 among pre vaccination isolates and 19A among those carried after completion of the PCV10.

The level of Erythromycin resistance rate in this study at the age of six-week was 9.0% and 10.3% among nine month isolates; and an MIC of 256 was found for three isolates from six-week-old and six isolates from nine-month-old infants. Isolates that were resistant with this MIC level were serotypes 14, 15B, 21 among the pre-vaccination isolates and isolates from nine-month-old infants were only of non-vaccine serotypes 10A, 15A, 15B and 19A. Our finding of the rate of resistance for erythromycin is in agreement with the report from Hawassa (12.9%) of clinical isolates (Daka *et al.,* 2011), but differs from the high rate of resistance (33.2%) reported from Gondar in isolates from carriers (Assefa *et al.,* 2013) and 20.3% from clinical isolates from Bahir-Dar (Anagaw *et al.,* 2013). Our finding is also different from reports from other countries, 24% in Ghana (Mills *et al.,* 2015), 41.7% in Tanzania (Neill *et al.,* 2013), 35.7 % in Italy (Zuccotti *et al.,* 2014), 96% in Taiwan (Kuo *et al.,* 2011), and 29% in Massachusetts,

USA (Lee *et al.,* 2014). The difference in the resistance rate might be due to differences in the study population and variations in the utilization of antibiotics in various settings.

4.1.4 Molecular diversity of the isolates

Characterization of the pneumococcal genetic background may provide the basis for surveillance of replacement disease in the post-vaccine era. PFGE, a genotypic method for the evaluation of total chromosomal DNA, has been considered the 'gold standard' when typing many micro-organisms and has been employed for typing pneumococci though results from this technique cannot be compared with findings of others (Hall 1998, Sa-Leao *et al.,* 2000, Shaaly *et al.,* 2005). In this study, we observed that isolates belonging to the same serotypes produced different finger-print patterns. In contrast, a few isolates of different serotypes produced identical finger-print patterns, showing genetic similarity. This indicated that there was considerable DNA polymorphism within the pneumococcal isolates of the same serotype. Close correlation between serotype and PFGE genotype was observed, although genetic diversity varied by serotype. For instance, we found serotypes 8 and 3 to be mostly clonal, while serotypes 6A and 23F had high genetic diversity and were represented by more than two clones. A similar observation was reported from Israel, a close correlation between serotype and PFGE clone, and the genetic diversity variation by serotype, with some genotypes comprising multiple serotypes (Bar-Meir *et al*., 2015). Clonality among strains with a reduced susceptibility to penicillin was observed for serotype 14 with PFGE genotype F; all the three isolates in the clone had reduced susceptibility to penicillin. Five PFGE genotypes included different serotypes within the clone which is an indication of capsular switching. The overall result of the PFGE analysis in this study showed a high genetic diversity of pneumococcal isolates, which was also reflected in the serotyping.

Multilocus sequence typing (MLST) has greatly advanced our understanding about the population biology of a wide range of bacterial pathogens (Enright and Spratt 1998, Donkor *et al.,* 2013a). In our study, MLST was preformed only for 12 selected isolates from 11 PFGE genotypes, one isolate from each genotype except from one where two

isolates were analyzed. Two isolates of serotypes 1 and 23F within the same PFGE genotype had the same sequence type (ST8930) indicating a likely serotype change due to exchange of the *cps* locus via recombination. A similar observation was reported from Malawi with two unrelated serotypes (19F and 15C) which had the same ST (ST172) (Everett *et al.,* 2012). It was also reported in another study from The Gambia that six STs showed evidence of capsular switching ST172 (23F and 19F), ST802 (23A and 19A), ST847 (23F, 19A), ST1730 (6A and 6B), ST1736 (6A and 6B), and ST1737 (6A 24A)) (Donkor *et al.,* 2011). However, it is necessary to confirm their genetic branch distance with whole genome sequencing to show whether these isolates are closely related or not.

Serotype 14 was reported predominantly (75%) among isolates causing invasive infection in Malawi (Everett *et al.,* 2012), 100% in The Gambian isolates (Antonio *et al.,* 2008), 100% in Ethiopian isolates (Dinku 2013) and isolates were ST63. Though we have done MLST for only one isolate out of three PFGE genotype F isolates, this was identified as ST63. ST63 represents the PMEN 25 clone, which is known to express serotype 15A as reported from Malawi (Everett *et al.,* 2012). Nonetheless, in our study, out of the total of seven serotype 14 isolates, only three were in this PFGE genotype and hence the other four isolates might have different ST types than this strain. We did not identify any new ST among the total of 12 isolates analyzed by MLST. Due to the high cost of MLST, not all our isolates could be analysed.

4.1.5 Other co-colonizers

The upper respiratory tract of children is not a niche for only pneumococci, but is also a residence for other important common respiratory bacterial potential pathogens such as *Staphylococcus aureus*, *Haemophilus influenzae*, and *Moraxella catarrhalis*, which usually behave like commensals, but occasionally cause respiratory or invasive infectious disease. Colonization of this niche is a dynamic process: acquisition and elimination of species, interactions among microbes and between microbes and the host, and interference by environmental factors are suggested to cause a dynamic and complex

microbial interplay (Bosch *et al.,* 2013, Bosch *et al.,* 2016). By reducing nasopharyngeal carriage of VT pneumococci, PCVs may also create ecological niches for colonization of other respiratory pathogens such as *Staphylococcus aureus, Haemophilus influenzae*, and *Moraxella catarrhalis* (Dunne *et al.,* 2013), bacteria that are frequently carried by children and are important causes of disease worldwide (Verduin *et al.,* 2002, Agrawal and Murphy 2011).

In this study, although the primary endpoint was not to analyse impact of the vaccine on other concomitant nasopharyngeal bacteria, we detected *Staphylococcus aureus*, *Haemophilus influenzae*, and *Moraxella catarrhalis* at each stage of our sampling. The highest carriage of *Staphylococcus aureus* (21.7%) was observed in six-week-old infants before the vaccine, and the carriage rate of these bacteria dramatically declined to 4.4% at the age of nine months and again showed slight increment to 7.8% at the age of two years after PCV10 completion. A co-colonisation of *Staphylococcus aureus* with pneumococci was highest (22.4%) at the age of six weeks when compared to other bacteria and at age of nine months and two years. Consistent with our finding, The Gambian and Kenyan studies showed that the prevalence of carriage of *Staphylococcus aureus* was highest in neonates and decreased with age (Kwambana *et al.,* 2011, Hammitt *et al.,* 2014a). The probable reason for lower carriage at the age of nine months and two years in comparison to six weeks is that our samples were collected from the posterior nasopharynx which primarily targeted pneumococci. It has been suggested that the anterior nares is a more appropriate site to fully characterise the effect of the vaccine on *Staphylococcus aureus* carriage (Hammitt *et al.,* 2014a, Feazel *et al.,* 2015).

In several studies, a negative association was reported between NP carriage rate of *Staphylococcus aureus* and pneumococci of particular vaccine types after PCV, showing an increase in the carriage of *Staphylococcus aureus* (Bogaert *et al.,* 2004c, Regev-Yochay *et al.,* 2004b, Madhi *et al.,* 2007, Kuo *et al.,* 2011, van Gils *et al.,* 2011a). Similarly, an increase in carriage rate of pneumococci from six week to nine month and a decline in carriage of *Staphylococcus aureus* in the same children was observed in our study as well, which needs further investigation.

PCV10 (PHiD-CV), a conjugated vaccine with non-typeable *Haemophilus influenzae* protein D, has been showed to reduce the carriage of non-typeable *Haemophilus infulenzae* in under-five children in Kenya and Czech Republic (Hammitt *et al.,* 2014a , Prymula *et al.,* 2011). In another study a higher carriage rate of *Haemophilus influenzae* was observed as an effect of PCV7 (Spijkerman *et al.,* 2012). In contrast to this, several other studies did not find any difference in carriage of these bacteria after PCV (Prymula *et al.,* 2011, van Gils *et al.,* 2011a, van Gils *et al.,* 2011b, Dunne *et al.,* 2012). In our study we detected a very low rate of carriage (3.9%) of these bacteria before the vaccine with a slight increase to 6.3% at the age of nine months which declined again to 4.0% after completion of vaccination at the age of two years. This indicates that it is very difficult to conclude whether the vaccine really impacted on the carriage rate of these bacteria or not. We also did not type the isolates whether they were non-typeable or not and have therefore not undertaken any independent analysis of the impact of the vaccine on non-typeable *Haemophilus influenzae*.

In our analysis, *Moraxella catarrhalis* was the second highest co-colonizer next to *Staphylococcus aureus*. The carriage rate of this bacterial species was highest at six week before vaccination and dropped down significantly at nine months and showed slight increment at the age two years after completion of vaccination. Different investigators have reported inconsistent associations (no difference, increase and decease of carriage) from randomized controlled trials, and cross sectional studies done to analyse the impact of PCV7 on carriage rate of common commensals of the nasopharynx, in relation to *Moraxella catarrhalis* (van Gils *et al.,* 2011b, Dunne *et al.,* 2012, Spijkerman *et al.,* 2012).

4.2 Limitation of the study

Although at inception, the aim was to conduct this study before the introduction of the vaccine into the routine vaccination programme of the country, due to challenges including time needed for review and approvals, the investigation started one year and

four months after the introduction of the vaccine. Because of this, it could be argued that the data generated at the age of six weeks before the participants took the vaccine, may not be free of impact of vaccine in general (because of possible ongoing impact of vaccination in the community already); and hence, the comparison we did before and after completion of the vaccine may have been compromised. Nevertheless, considering the time it takes for herd immunity to be established in a community, the short time that had elapsed overall can only have very minimal effect.

Furthermore, in our study we did not have a control arm of unvaccinated children in follow ups, the vaccine has already been introduced into the country nationwide; and it was not ethical to have a non-vaccinated group as control. In addition, we did not determine the level of (concentration) antibody in the serum at each stage of the sampling to measure efficacy of the vaccine and rule out effects of non-responsiveness or vaccine failure. We have assumed that all vaccinated children had adequate levels of antibodies to impact on carriage and analyzed the results accordingly. If we had done this, we would have been able to correlate the level of protection to specific serotypes included in the vaccine. In addition, because of the high cost of MLST, we did the analysis for only 12 isolates from 6 week and nine month isolates and we are not sure whether there would be further differences in ST in the study population from already reported ST data sets. The isolates from two-year-old children were not molecularly characterized due to logistic and time constraints. These data will be valuable in further comparisons and analysis of trends in the study population as baseline.

4.3 Conclusions

To our knowledge, this study is the first to see the impact of PCV10 in the country with a larger sample size, and follow-up of the same study participants for two years by analyzing the carriage rate dynamics in children from infancy to two years. It is also the first study to characterize a relatively large number of isolates by serotyping, antibiotic susceptibility testing, and gentotyping using PFGE and MLST. As such, the study fills

critical knowledge gaps and provides a very valuable data set for future monitoring of trends on impact of PCV10 vaccination on pneumococcal diseases in the country.

Pneumococcus remains a major cause of community-acquired pneumonia, meningitis, and sepsis, and non-invasive diseases such AOM, sinusitis etc worldwide, especially in young children. In Ethiopia, according to the FMOH, health and health related indicators of 2008 Ethiopian Fiscal Year; pneumonia was the first among the ten causes of hospital admission and mortality for under-five children (FMOH 2015). Information on pneumococcal carriage in the pre-vaccine period is essential to predict and assess the impact of PCV in poor resource settings like Ethiopia, where disease surveillance is particularly difficult.

This study has confirmed that the acquisition of pneumococci in infants in the study population starts as early as six weeks, before the first dose of the vaccine is given. The overall carriage rate dynamics at the three sampling stages showed a trend of increment at the age of nine months, with a decline again at the age of two years. A total of 59 serotypes were identified from the nasopharynx of infants sampled at six week and nine months and two years, showing circulation of a high diversity of serotypes in the country, with a potential to replace vaccine serotypes and cause non-vaccine type pneumococcal diseases. Nearly 80% of the isolates from the six-week- old children who have not yet started the vaccine were non-vaccine types, which indicate that the currently available vaccine PCV10 does not cover the majority of serotypes carried at this age.

After completion of the vaccine, the carriage rate of vaccine serotypes of pneumococci declined significantly, indicating the benefit of introducing the vaccine. However, the overall carriage rate had a significant increase. Some children were also colonized by serotypes that are included in the vaccine despite completion of vaccination, which shows that PCV10 vaccine may not fully protect from nasopharyngeal colonisation, even for a few of those serotypes it is designed to cover against.

The use of PCV13, currently in use in several high and middle income countries, some African countries has an added value by increasing the coverage to at least 10% more serotypes. It will, in particular, cover against serotypes 6A and 19A. The isolates of these two serotypes were resistant to more than two antibiotics in this study.

A reduced level of susceptibility to penicillin was observed in more than a quarter of the isolates from nine-month-old children. This is an alarming situation and needs to be monitored. We recommend a strict guideline for antimicrobial use and close follow-up of resistance patterns among clinical isolates.

The molecular typing of isolates using PFGE and MLST revealed diverse pneumococci circulating in the study area and the existence of capsular switching events. As the first study upon the initial introduction of the vaccine, the findings of this study will be vital guidance for other future investigations.

4.4 Recommendations

This study was conducted only in Addis Ababa, and does not portray a complete picture of the situation in the country. To gain a complete, country-wide picture, it is essential to scale up the study by including different geographic and socio-demographic features of the country.

Nasopharyngeal carriage rate determination, at one point in time, is not the best primary end-point to analyse the impact of the vaccine. Continued, dynamic surveillance is required to determine vaccine impact and to monitor changes in pneumococcal population biology. Therefore, subsequent investigations have to be conducted both on carriage and on clinical cases to assess the impact of the vaccine and dynamics of the serotypes.

In this study, we investigated nasopharyngeal carriage variation and serotype diversity in infants before and after vaccination. However, we did not address transmission dynamics

within families in our study, and did not determine course of development of herd immunity as a result. It would be necessary to conduct further research to look into these issues further.

The number of day-care centres and kindergartens is increasing in major towns of the country and attendance of day-care centres and/or kindergartens is one major risk factor for dissemination of pneumococci. It is necessary to investigate the prevalence and diversity of pneumococci in day-care centres and kindergartens.

We have observed that there is an indication of multiple serotype carriage. It is, therefore, necessary to determine to what extent multiple carriage is present, and whether this does drive genetic exchange more than single carriage, and whether genetic re-arrangement from non-pneumococcal bacteria occurs in the nasopharynx.

The vaccination schedule for PCV10 in Ethiopia consists of the vaccine being given at the age of six weeks, ten weeks and fourteen weeks without any booster dose. We have observed that some children carried the vaccine types at the age of nine months and two years, despite completion of vaccination as scheduled. This might be due to low concentration of antibodies produced against the specific serotypes to start with, waning of the antibody produced or a combination of both. Therefore, studies should be conducted to determine if waning occurs, and when it happens to determine at what age another booster dose would need to be included if at all. In addition, it is necessary to answer if there are specific characteristics of the vaccine serotype pneumococci carried by children that lead to persistence or recolonization, despite vaccination i.e. how these serotypes manage to protect themselves from vaccine-induced anti-carriage immunity.

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Annexes

Annex 1: Patient information sheets

Annex 1.1 Study participant information sheet for parents/guardians: For children's taking PCV10 vaccine at Health Centre or Hospital (English version)

Title of the project: The Impact of Ten-valent Pneumococcal Conjugate Vaccine (PCV10) on *Streptococcus pneumoniae* (pneumococcus) Nasopharyngeal Carriage Rate: Serotyping, Molecular Characterization and Antibiotic Sensitivity pattern in Ethiopia.

Principal investigator : WondewosenTsegaye Sime (AAU, AHRI)

Co-investigators: Yimtubeznash W/Amanuel MD, MSc PhD, AAU

Abraham Aseffa, MD, PhD, AHRI

Name of institutes: Addis Ababa University and Armauer Hansen Research Institute

We would like to invite your child to participate in a research study. You may decide whether or not you want to allow your child to participate. Please take time to make your decision. Carefully read the following and ask any questions that you may have.

BACKGROUND

Streptococcus pneumoniae (Pneumococcus) accounts among the most important human pathogens, with high morbidity and mortality rates and has one of the largest public health and economic impacts of any bacterial infectious agent in both developing and industrialized countries. Nasopharyngeal (NP) carriage in children plays a major role in its spread within the family, school, and daycare centre / kindergartens.The government of Ethiopia, Ministry of Health has introduced the ten-valent pneumococcal conjugate vaccine recently into routine vaccination programme.

PURPOSE OF THE STUDY

The purpose of this study is to test the impact of the newly introduced pneumococcal vaccine (PCV10) in Ethiopia on carriage rate of the pneumococcus and other bacteria in children less than five years old, and to further characterize the isolates.
The expected duration of the study is 2 years and a total of 785 participants will be enrolled in this study.

STUDY PROCEDURE

The study will involve a multiple nasal swab taking from the child's nasal cavity which will be taken at age of 6 week, when the new borne come for the first vaccine before the vaccination 9 months after completion of the vaccine when the child come to meseals vaccine and 24 months. The swab is a small cotton wool pad which is passed up the nose. The swabs will be transported to the laboratory in AHRI for further analysis. A questionnaire will also be used in this study where you will be asked questions related to clinical history about the child and family members and other questions that are helpful for the analysis of risk factors.

RISKS & DISCOMFORT

Samples will be taken with trained health personnel. Taking sample with the swab might cause slight discomfort and watering of the eyes. You can ask the health personnel to stop taking the swab at any point if your feel that your child is distressed.

EXPECTED BENEFIT

Taking part in this research study may not benefit you child personally, but the findings from this study can help doctors, researchers and people involved in the prevention, control and monitoring of pneumococcal diseases to learn new things that will help others.

COMPENSATION

No compensation will be provided for participating in this study but you will be compensated for your actual travel expenses incurred for making the visit.

CONFIDENTIALITY

The results of the study will be kept confidentially with the exception of the investigators involved in the study, and the samples and the results of the study will be used only for this research purpose. Your name or your child's name will not appear in any report.

VOLUNTARY PARTICIPATION/WITHDRAWAL

The participation of your child in this study is completely voluntary and you have the right to refuse to allow your child to be in this study. You can stop the participation of your child in the study at anytime after giving your consent. This decision will not affect in any way the current or future medical care or any other benefits to which your child is otherwise entitled.

WHOM TO CONTACT

In case of you need any further information about the study please contact:-

- Dr. Wondewosen Tsegaye, Tel. 0911408127
- Dr. Yimtubezinash Woldeamanuel ,Tel. 0911225832
- Dr. Abraham Aseffa , Tell: 0911247525-

INFORMED CONSENT FORM for PARENTS/GUARDIANS: For children attending daycare centre/kindergarten

This statement certifies that I am the parent / guardian of my child _____ have read/ or has been read to me and understood the information about this study which will test the impact of the newly introduced pneumococcal vaccine in Ethiopia on carriage rate of the pneumococcus in children less than five years old. The procedure has been explained to me and I have asked questions for clarification and obtained satisfactory responses in a language that I understood. I have been given enough time to respond. I understand that my child can withdraw from the study at any time and there are no any consequences on the benefits or quality of care concerning my child.

I, on behalf of my child agree to allow that sample to be collected by cotton swab from my child's nose that could be used for this research purpose. I will also provide information to be filled in the questionnaire prepared for this study. By signing this document I consent on behalf of my child to voluntarily participate in the project. A copy of the informed consent will be given to me.

Parent/guardian name: _____

Signature: _____ date: _____

Name of Person Obtaining Consent: _____

Signature: _____ date: _____

Witness name: _____

Signature: _____ date: _____

Annex 1.2 Amharic translation of patient information sheet and informed consent (children's taking PCV10 vaccine at Hospital or Health Centre)

ለጥናቱ ተሳታፊ ልጆች ወላጆች/አሳዳጊዎች የሚሰጥ መረጃ (በሆስፒታልና በጤና ጣቢያ የሳንባ ምች ክትባት ለሚወስዱ ህፃናት)

1. **<u>የጥናቱ ርእስ</u>:-** የሳንባ ምች ክትባት የበሽታው አምጪ ተህዋስ/ባክቴሪያ እና ሌሎች ተዋህስያን ላይ ያለው ተጽእኖና የበሽታው አምጪ ተህዋስን ዝርዝር ህብለ ዘር ጥናት

2. **<u>የጥናቱ ዋና ተመራማሪዎች</u>**
ዶ/ር ወንድወሰን ፀጋዬ (አዲስ አበባ ዩኒቨርስቲና እና አርማወር ሃንሰን የምርምር ተቋም)
ዶ/ር ይምጡበዝናሽ ወልደአማኑኤል (አዲስ አበባ ዩኒቨርስቲ)
ዶ/ር አብርሃም አሰፋ (አርማወር ሃንሰን የምርምር ተቋም)

በዚህ ጥናት ልጅዎ ተሳታፊ እንዲሆን የእርሶን ሙሉ ፈቃደኝነት ያስፈልገናል። በጥናቱ ልጅዎ ተሳታፊ እንዲሆን ወይም እንዳይሳተፍ የእርሶ ውሳኔ ነው። ስለዚህ ጊዜ ወስደው ይህን ስለ ጥናቱ የተሰጠዎትን መረጃ በማንበብ ልጅዎ የጥናቱ ተሳታፊ እንዲሆን ፍቃደኝነቶን እንዲገልጹልን እንጠይቃለን። ስለጥናቱ ማንኛውንም ዓይነት ጥያቄ ሊያነሱልን ይችላሉ።

3. **<u>መግቢያ</u>**
የሳንባ ምች የሚያስከትለው ተዋህስ/ባክቴሪያ ህመም በስከተልና በግደል በዓለም ላይ በተለይ በታዳጊ አገራት እያስከተለ ያለው ጉዳት ከፍተኛ ነው። በአገራችን ኢትዮጵያ ለዚህ በሽታ መከላከያ የሚሆነውን ክትባት በመደበኛው የክትባት ስርዓት ውስጥ ከቅርብ ጊዜ ወዲህ እንዲካተት ተደርጓል።

4. **<u>የምርምሩ ዓላማ</u>**
በኢትዮጵያ በቅርብ የተጀመረውን የሳንባ ምች ክትባት በሽታውን ለመከላከልና ለመቆጣጠር የተጀመረው ክትባት አምጪ ተዋህስ ባክቴሪያና ለሌሎች በሰርን ውስጥ የሚገኙ ተዋህስያን ላይ ያለውን ተፅኖ ጥናት ማድረግና የበሽታውን አምጭ ህብለ ዘር ላይ ጥናት ማካሄድ ነው። ጥናተ የሚወስደው ጊዜ ሁለት ዓመት ሲሆን የጥናቱ ተሳታፊዎች ብዛት 785 ይደርሳል።

5. **<u>የጥናቱ ዘዴዎች</u>**
በዚህ ጥናት በጤና ጣቢያ ወይም ሆስፒታል በገች የሳንባ ምች ክትባት የሚወስዱ በዚህ ጥናት ውስጥ ክትባቱ ከመሰጠቱ አስቀድሞ የህፃኑ/ዋ እድሜ 6 ሳምንት፣ 9 ወር እና 24 ወር ላይ ናሙና ይወሰዳል። ናሙናው የሚወሰደው በናሙናው አወሳሰድ በሰለጠነ ባለሙያ ነው። የተወሰደው ናሙና ወደ አርማው ሃንሰን ላቦራቶሪ ተወስዶ የሳንባ ምች ሊያስከትል የሚችለው ተዋህስ ባክቴሪያ ስርጭት መጠን ምን ያህል እንደሆነ ምርመራ ይካሄዳል። በተጨማሪም የጥናቱ አካል የሆነ መጠይቅ የልጁ ወላጆች ወይም አሳዳጊዎች ምላሽ እንዲሰጡ ይደረጋል።

6. **<u>የሚደርሰው ጉዳትና ህመም</u>**
የሰርን ውስጥ ናሙና፡ ይህ ናሙና የሚወሰድበት መሳሪያ ህጻኑ/ዋ ላይ ምንም አይነት ጉዳት የማያስከትል ሆኖ የተዘጋጀ ሲሆን በሰርን ውስጥ በጥንቃቄ በማስገባት ለ10 ሰከንድ በሰርን ውስጥ ይቆያል። በተወሰነ ደረጃ ያለመመቸት ሁኔታ የሚፈጥርበት አጋጣሚ አለ። በዚህ ምክንያት ከህጻኑ/ዋ እንባ ሊኖረው ሊኖራት ይችላል። ከልጅዎ ናሙናው በሚወሰድበት ጊዜ የተጨነቀ መስሎ ከተሰማዎት በየትኛው ሰዓት ናሙና እንደተወሰደ ሊያስቆሙ ይችላል።

7. **<u>የተሳታፊ መብት</u>**

ልጅዎት በጥናቱ እዲሳተፍ ማድርግ በፍቃደኝነት ላይ የተመሰረተ ስለሆነ በጥናቱ የማሳተፍና በማንኛውም ሁኔታና በፈለጉበት ጊዜ ልጅዎትን ከጥናቱ የማስለወጣት መብት አለዎት። ስለልጅዎት ጥያቄዎችን አለመመለስና ናሙናውን እንዳይሰጥ ማድርግ መብት አለዎት። የመሳተፍ መብት በሙሉ ፍላጎት ላይ የተመሰረተ ሲሆን ያለመሳተፍም ምንም የመብት ጉድለት ሊያስከትል አይችልም። ነገር ግን ልጅዎት የጥናቱ ተሳታፊ ከሆነ የሳንባ ምች የሚያስከትለው ተዋህስ ላይ የሚደረገውን ጥናት በማገዝና በሽታንነ ለመቆጣጠር ለሚደረገው ጥረት አስተዋጽኦ አለው።

8. ጥቅም

የልጅዎ በጥናቱ መሳተፍ በቀጥታ ለልጅዎም ሆነ ለእርሶ የሚያስገኘው ጥቅም ባይኖረውም ከጥናቱ የሚገኘው ውጤት አገራቱ አሁን ያስገባችው ክትባት ውጤታማነት በመለካትና ወደፊት የሳንባ ምች የሚያስከትለውን ጉዳት ለመከላከልና ለመቆጣጠር ያለው አስተዋጽኦ የጎላ ነው።

9. ማበረታቻና ማካካሻ

በተሳትፎ ምክንያት ማበረታቻ እርስዎም ሆኑ ልጅዎት አታገኙም። ልጅዎትን በጥናቱ ማሳተፍ ነፃ ምርጫዎት ነው። ነገር ግን ከናሙና አወሳሰድ በተያያዘ ለሚያወጡት ተጨማሪ የመጓጓዣ ወጪዎችና ለመጠይቁ መልስ ለመስጠት ለሚያጠፉት ጊዜ 50 ብር ለጥናቱ በመጡ ጊዜ ሁሉ ይሰጦታል።

10. ሚስጥር አጠባበቅ

የሚሰጡት መረጃ እና የምርመራ ውጤት በፍፁም ሚስጥራዊነቱ የተጠበቀ ነው። የእርሶና ልጅዎት ስም በማንኛውም ሪፖርት ላይ አይጠቀስም። ከተጠቀሰው አላማ ውጭ የልጅዎት ናሙናዎች ለሌላ አገልግሎት አይውሉም። ነገር ግን ለሌላ አገልግሎት አስፈላጊ ሆኖ ከተገኘ ፍቃደኝነትዎን እንደገና እንጠይቃለን።

11. በፍቃደኝነት ስለመሳተፍና ጥናቱን ስለማቋረጥ

ልጅዎት በጥናቱ እዲሳተፍ ማድርግ በፍቃደኝነት ላይ የተመሰረተ ስለሆነ በጥናቱ የማሳተፍና በማንኛውም ሁኔታና በፈለጉበት ጊዜ ልጅዎትን ከጥናቱ የማስወጣት መብት አለዎት። ስለልጅዎት ጥያቄዎችን አለመመለስና ናሙናውን እንዳይሰጥ ማድረግ መብት አለዎት። የመሳተፍ መብት በሙሉ ፍላጎት ላይ የተመሰረተ ሲሆን ያለመሳተፍም ምን የመብት ጉድለት ሊያስከትል አይችልም። ነገር ግን ልጅዎት የጥናቱ ተሳታፊ ከሆነ የሳንባ ምች የሚያስከትለው ተዋህስ ላይ የሚደረውን ጥናት በማገዝና በሽታን ለመቆጣጠር ለሚደረገው ጥረት አስተዋፅኦ አለው።

12. ግንኙነት ወይም ምንም አይነት ጥያቄ ካለዎት

ተጨማሪ መረጃዎችን ማግኘት ከፈለጉና ጥያቄ ካለዎት የሚከተሉትን ባለሙያዎች በአካልም ሆነ በስልክ ማግኘት ይችላሉ።

ዶ/ር ወንድወሰን ፀጋዬ (አዲስ አበባ ዩኒቨርስቲና እና አርማወር ሃንሰን የምርምር ተቋም)
ስልክ **0911 408127**
ዶ/ር ይምጡበዝናሽ ወልደአማኑኤል (አዲስ አበባ ዩኒቨርስቲ) ስልክ **0911 225832**
ዶ/ር አብርሃም አሰፋ (አርማወር ሃንሰን የምርምር ተቋም) ስልክ **0911 247525**

ልጅዎ የጥናቱ ተሳታፊ እንዲሆኑ በመፍቀዶ ያለዎትን መብት የሚከተሉትን አካላት መጠየቅ ይችላሉ።
አህሪ አለርት ኤቲክስ ሪበው ኮሚቴ ስልክ ***251-11-3211567***

ለጥናቱ ተሳታፊ ልጆች ወላጆች/አሳዳጊ

የስምምነት ማስረጃ (የሣንባ ምች ክትባት ለሚከተቡ ልጆች)

እኔ ይህ የምሰጠው ማረጋገጫ የ---------- ወላጅ/አሳዳጊ መሆኔንና በአገራቱ የሳንባ ምችን ለመከላከል የተጀመረው ክትባት በበሽታው አምጭ ተዋህስ/ባክቴሪያ እና ሌሎች ተዋህሳን እድሜያቸው አምስት ዓመት ያልሞላቸው ህፃናት ሰርን ውስጥ መኖር ወይም መከሰት ላይ ሊያስከትል የሚችለው የተፅኖ ጥናት ላይ

- የተሰጠኝ መረጃ በቅጡ አንብቤ ተነቦልኝ ተረድቻለሁ፣
- ስለ ናሙና አወሳሰዱ ሁኔታ በበቂ ሁኔታ በሚገኝ ቋንቋ ገለፃ ተደርጎልኝ ያልገባኝ ነገር ካለ እንድጠይ ድል ተሰጥቶኝ ለጠየኳቸውም በቂ ማብራሪያ አግኝቻለሁ፣
- ልጄም በጥናቱ ተሳታፊ እንዲሆን በቂ የማሰቢያ ጊዜ ወስጄ ውሳኔዬን እንድገልፅ እድል ተሰጥቶኛል፣
- ልጄም በማናቸውም ጊዜ ከጥናቱ ማቋረጥ ወይም ያለመሳተፍ ሙሉ መብቱ መሆኑና በጥናቱ ተሳታፊ ባለመሆኑ ወይም በማቋረጡ ማግኘት የሚገባውን ምንም አገልግሎት ሊጓደልበት እንደማይችል ተገልፆልኛል፣

ስለዚህ ልጄን በመወከል የጥናቱ ተሳታፊ እንዲሆንና ከሰርኑ ውስጥ ናሙና እንዲወስድ መሉ ፍቃደኝነቴን በፊርማዬ እያረጋገጥኩ የጥናቱ አካል ለሆነው መጠይቅም አስፈላጊውን ምላሽ ሁሉ ለመስጠት ተስማምቻለሁ፡፡ የስምምነቱም አንድ ግልባጭ እንዲደርሰኝ ተደርጓል፡፡

☐ጥናቱ ተሳታፊ ልጅ ወላጅ/ አሳዳጊ ስም፡ ……
ፊርማ፡……ቀን፡……
ስምምነቱን የስፈረመው ስም ……
ፊርማ፡ ……ቀን፡ ……
የእማኝ ስም፡……..
ፊርማ፡……ቀን፡ ……..

Annex 2. Questionnaire for gathering data to determine the impact of PCV10 vaccine (should be filled at the age of 6 weeks, 9 months and 24 Months)

Date_____

Child Code _____

Part 1. Background Information

1.1.Name Of Health centre_____

1.2.1. City_____

1.2.2 Sub-city_____

1.2.3 Kebele_____

1.2.4. House No._____

1.2.5. Telephone: Mobile _____

House No. _____

1.2. Name of the infant _____

1.3 Address of the parent: 1.3.1 Name of the parent_____

1.3.2 City_____

1.3.3 Sub-city_____

1.3.4 Kebele_____

1.3.5. House No._____

1.3.6. Telephone: Mobile _____

House No. _____

Schedule for next contact Date_____Time_____

Part 2. Possible risk factors for analysis

S.no	Variable	Response and response code (Tick √)
1	Sex of the child	1.Male 01 ☐ 2.Female 02 ☐
2	Age of the child in Months	----------

3	Weight of the child	----------		
4	Height	----------		
5	*Attendance of other Day-care centre kindergarten/ before	1.Yes 2. No	01 02	☐ ☐
6	*If Q5 yes, number of kindergarten/daycare centre attended before	----------		
7	The presence of other siblings attending Day-care centre kindergarten	1.Yes 2. No	01 02	☐ ☐
8	If yes Q6, number of cildren attending Day-care centre /kindergarten	----------		
9	Total size of family members	----------		
10	Number of under five children in the family (number of siblings)	----------		
11	Number of rooms in the house	----------		
12	Sharing of Bedrooms with siblings	1.Yes 2. No	01 02	☐ ☐
13	If Q12 yes, number of children sharing a single room	----------		
14	Sharing of Bed with siblings	1.Yes 2. No	01 02	☐ ☐
15	If Q14 yes , number of children per Bed	----------		
16	History of respiratory tract infection (Sinusitis, bronchitis, pneumonia etc)	1.Yes 2. No	01 02	☐ ☐
17	If Q16 yes, how many times	----------		
18	History of otitis media	1.Yes 2. No	01 02	☐ ☐
19	If yes18, number of times of otitis media	----------		
20	History of hospitalization	1.Yes 2. No	01 02	☐ ☐
21	If yes Q20, number of times hospitalized	----------		
22	*Use of antibiotics within the last six	1.Yes	01	☐

	months (the child)	2. No 02 ☐
23	*If yes Q22, number of times antibiotic usage	----------
24	Mostly used antibiotic used	----------
25	History of antibiotic use by other member of the family in the last six months	1.Yes 01 ☐ 2. No 0 ☐
26	History breast feed	1.Yes 01 ☐ 2. No 02 ☐
27	*If yes Q 26, For how many months	----------
28	Presence separate cooking room	1.Yes 01 ☐ 2. No 02 ☐
29	Energy source for cooking (multiple answer possible	1.Kerosine stove 01 ☐ 2. Electric stove 02 ☐ 3.Charcol 03 ☐ 4. Wood fire 04 ☐ 5. Cylinder Gass stove 05 ☐ 6. Other, specify_____
30	Presence of smoker in the household	1.Yes 01 ☐ 2. No 02 ☐
31	If Q 30 yes, number of smokers	----------
32	*Completion of all vaccination for children according the national guideline	1.Yes 01 ☐ 2. No 02 ☐
33	Education status of the Father	1. Illiterate 01 ☐ 2. Write and read 02 ☐ 3. Completed primary School (up to grade six) 03 ☐ 4. Completed Junior secondary School (up to grade 8) 04 ☐ 5.Completed secondary or Preparatory Schooling

		(completed up to grade 12) 05 ☐ 6.Certificate qualification of any level University graduate (Diploma and above) 06 ☐
34	Educational status of the mother	1. Illiterate 01 ☐ 2. Write and read 02 ☐ 3. Completed primary School 03 ☐ (up to grade six) 4. Completed Junior secondary School (up to grade 8) 04 ☐ 5.Completed secondary or Preparatory Schooling (completed up to grade 12) 05 ☐ 6.Certificate qualification of any level University graduate (Diploma and above) 06 ☐
35	Total monthly average income of the family (Father, mother, other family member lump sum)	----------

Remarks

- * = Not applicable at the age of six weeks
- Record only on changes when they come at 9 months and 24 Months

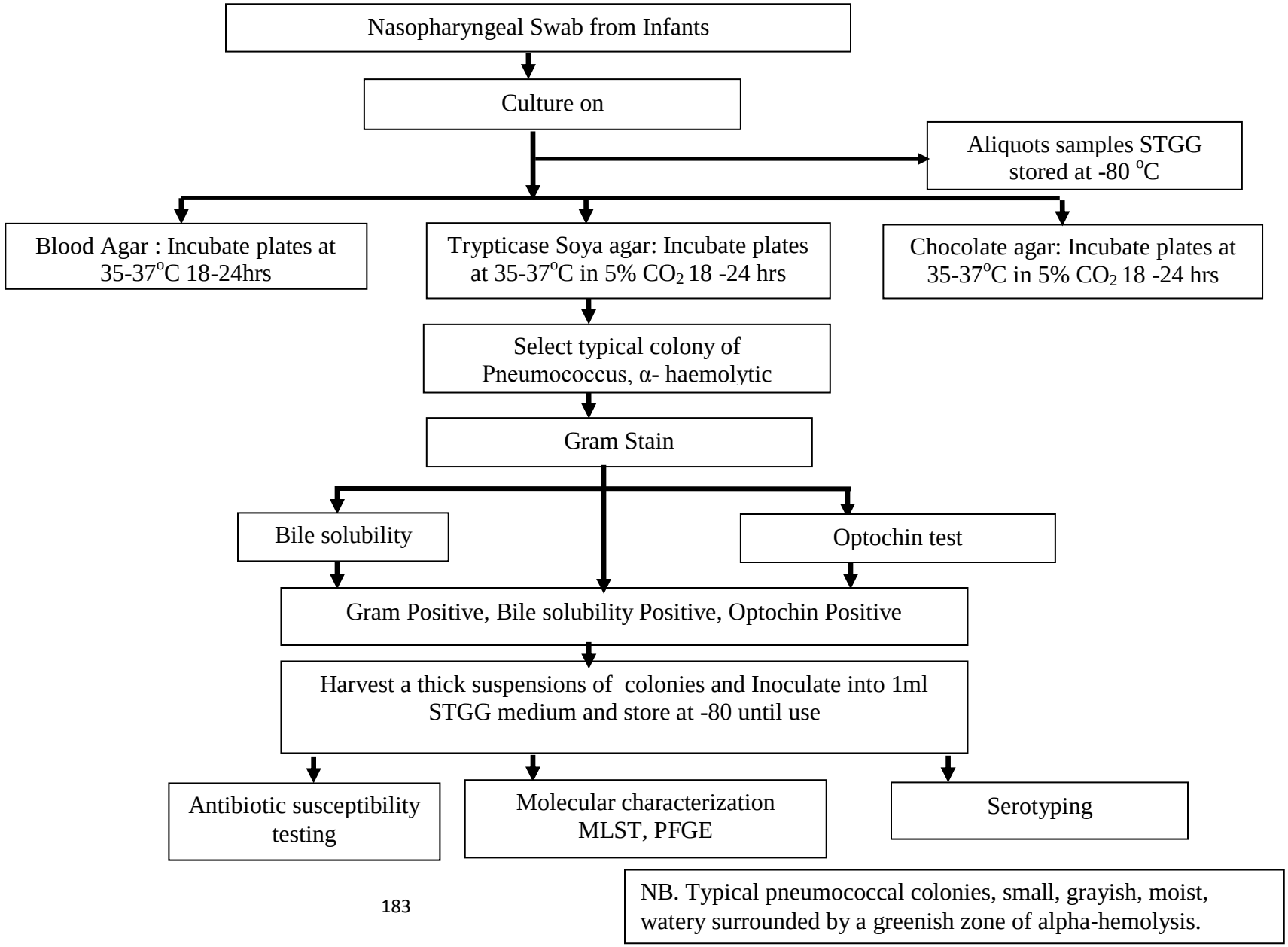
Annex 3. List of Health Institutes and Average Number of Infants Vaccinated in each Sub-city since October 2011at the commencement of the study

Sub-City	Health Institute Providing the Vaccine		Average number of Vaccinated Infants per month
	Public (Health Centre)	Private	
Yeka	Yeka	Dinberwa MCH	1,563
	Kotebe		
	Entoto No. 1		
Nifas Silk Lafto	Nifas Silk Woreda 1	Biruk Higher Clinic	2,469
	Nifas Silk Woreda 2	Tsion Higher Clinic	
	Nifas Silk Woreda 6	St. Marry Higher Clinic	
	Nifas Silk Woreda 9		
	Nifas Silk Woreda 12		
	Nifas Silk Woreda 05		
Lideta	Teklehaimanot	Betel Hospital	1,164
	Lideta		
	Beletshachew		
Kolfe Keranio	Kolfe	Aynalem Higher Clinic	3,104
	Alem Bank	Betel Hospital	
	Woreda 24	Sipara Higher Clinic	
	Philipos	SOS Higher Clinic	
	Awolya		
Kirkos	Kazachies	Betezata Hospital	1,432
	Kirkos	BGM Hospital	
	Meshualekia	Landmark Hospital	
Gulele	Shiromeda		1,454
	Selam		
	Free Methodist		
Bole	Woreda 17	Kadisco Hospital	3,987

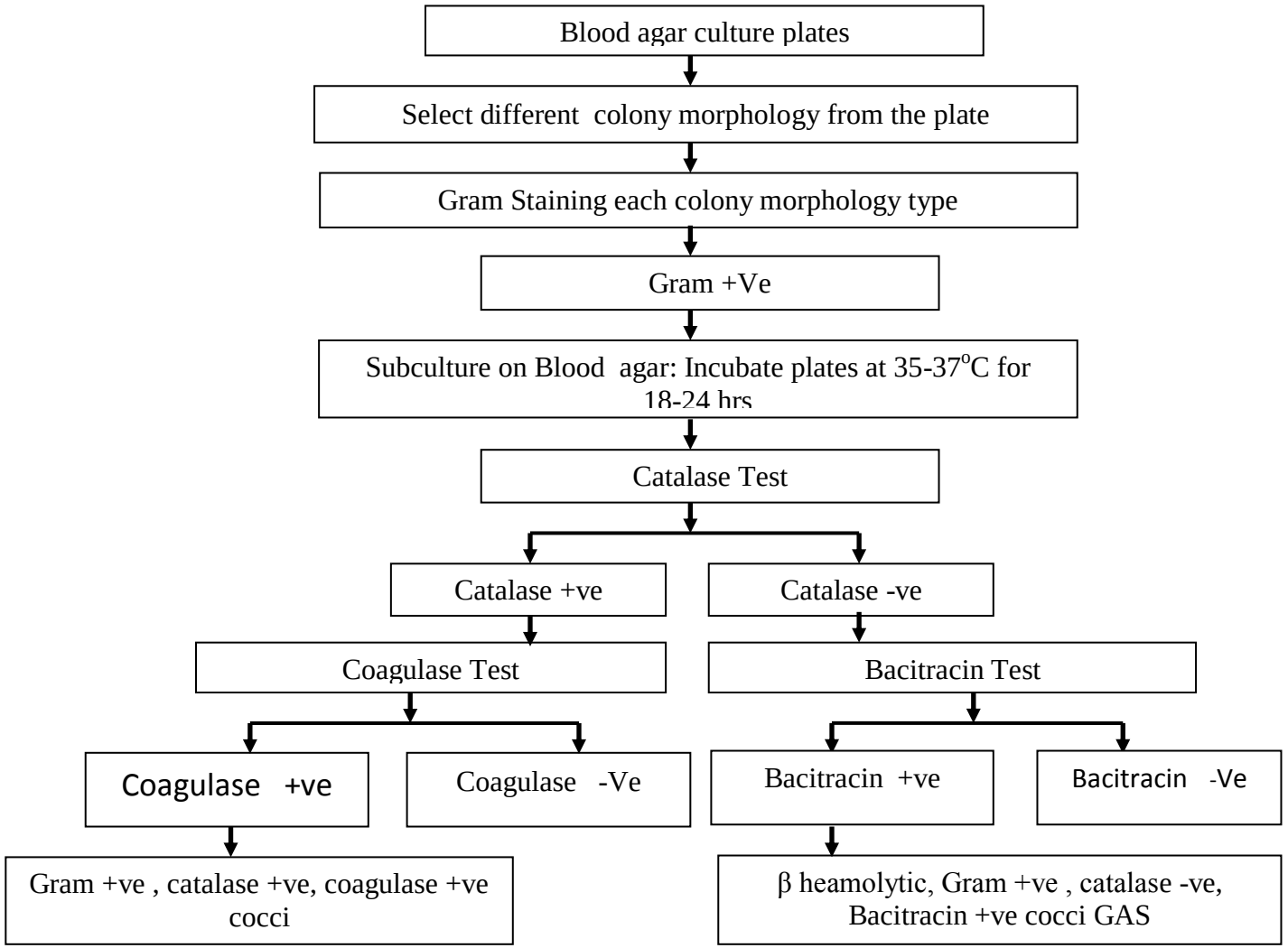
	Bole 17/20	MCM Hospital	
	Dile Fre	Betsega Hospital	
	Bulbula	Addis Howot Hospital	
		Hayat Hospital	
		Choice Higher Clinic	
		Brase Hospital	
Arada	Arada	Aynalem Higher Clinic	2,115
	Gulele	Abebech Gobena Higher Clinic	
	Kebena	Meskerem Higher Clinic	
		Tibebu Hospital	
		Ethio Tebib Hospital	
		Hemen Hospital	
		3 Children Higher Clinic	
		Ethiopia Higher Clinic	
Addis Ketema	Addis Ketema	Girum Hospital	1,471
	Woreda 7	Al-Afia Higher Clinic	
	Woreda 18		
Akaki Kality	Akaki	Zembaba Hospital	1,732
	Kality		
	Saris		
Total	**36**	**30**	**20491**

Source: Addis Ababa city Administration Health Bureau

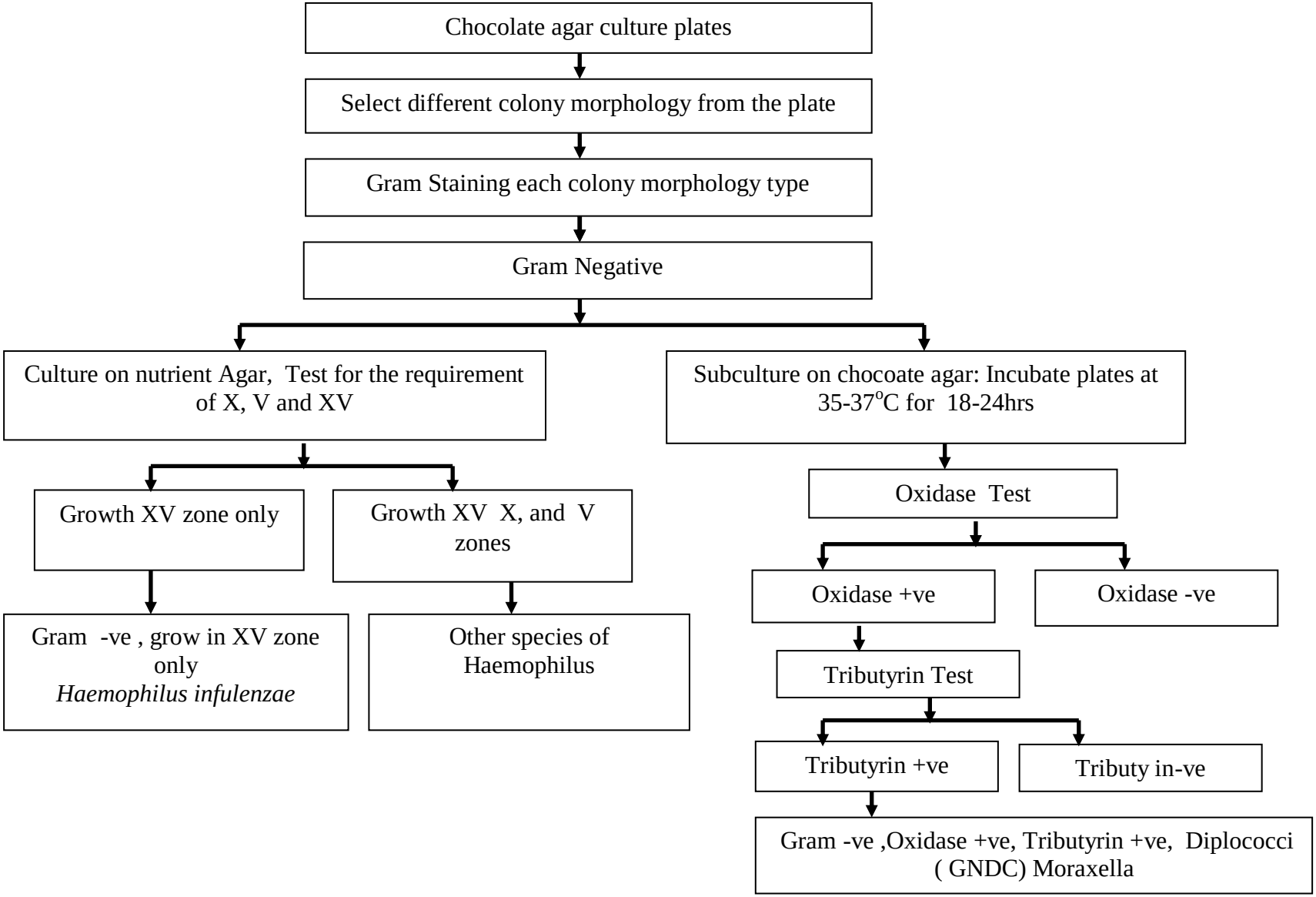
Annex 4. Flowchart: Sequence of Tests for the carriage of *Streptococcus pneumoniae* (Pneumococcus) and impact of PCV10 Study



Annex 5. Flow chart for Identification of *S.aureus* and *Streptococcus pyogenes*



Annex 6. Flow chart for Identificaation of *Haemophilus* and *Moraxella* Species



Annex 7. Pneumococcal Serotyping Protocol by Gel diffusion Method

Materials

- Dextros bullion 1%, pH 7,5
- Agar-Noble – DIFCO 214230
- Horse sera (Fetal claf serum), inactivated
- Distilled H2O
- PBS buffer
- Pneumococcal cultures, from swabs with pure culture, from growth on blood agar plates or from frozen isolates.
- CO_2 thermostat, 37 °C
- Vortex (for mixing/ dissolving pellet)
- Heating plate with magnet stirrer (for preparation/boiling of gel)
- Hole puncher, coupled to "vacuum drive" (to make holes in the gel)
- Sterile glass, centrifuge tubes 10ml, with cork/lids (or other tubes that fits the centrifuge)
- Centrifuge, 3000 rpm (for pelleting bacteria)
- Glass plate 20.5 x 11 cm
- Microscope glass slides 7.6 x 2.6 cm
- Moist chamber for large plates and also for slides
- Desk lamp
- Loupe, if needed

Preparation of DS-tubes:

1 % Dextrose bullion with 10 % inactivated horse Sera: add 100 mL horse sera to 900 mL dextrose bullion. Aliquot about 5ml to each glass tube, as sterile as possible (flame not necessary, change pipettes (30ml) when necessary). Control the sterility by incubating the tubes at 36 °C over night. Store the tubes in fridge before use.

Analyse procedure Serotyping

(for gel typing, excluded SMIs normal procedure for incoming pneumococcal isolates)
The pneumococci are first typed to which type or group they belong (ie non factor types/groups). Isolates belonging to a group are then typed further to their specific factor type. Our primary typing method was gel diffusion where most types and factor types are identified. Remaining types was identified by Quellung reactions. We used the Latex-kit when the isolate do not react with any of the antisera in gel diffusion or Quellung reactions, but the isolate are positive in "Bile solibility test"

Day 1

1. Inoculate the bacteria to the tubes with DS buillion (we grow the bacteria in parallel on blood plates, for propagation before freezing and to have fresh cultures possibly needed for Quellung reaction at later stages of the typing)
2. Incubate the DS tubes (and plates) in CO_2 thermostat over night (18-24 h).
   At Fridays: the DS-tubes can be kept in the thermostat over the weekend (but plates should be removed on Saturday and kept at room temperature, to be re-grown Monday -> Tuesday before freezing).

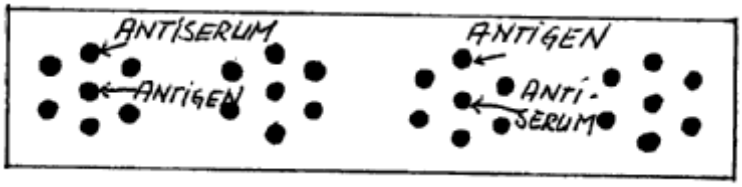
Dag 2

3. Preparation of antigen for the gel diffusion: centrifuge the DS-tube at about 3 000 rpm for 8-10 minutes. Pour off the supernatant. Add about 400µL dH_20 and dissolve the pellet/mix the content of the tube on vortex.
4. Make a protocol for the samples to be serotyped. For analyses of many samples use the form "SMI1536 Geldiffusion II" where the layout of a glass plate (for 30 ml gel) are pre-printed. If there are a few samples to be serotyped, small glass slides (microscope slides) may be used (3ml gel on each slide). Then plan and draw a protocol for the small glass slides.
5. Gel preparation
   - Make 1% gel, exemplified by 50 ml: Mix 0.5g Agar-Noble DIFCO powder+ 50ml PBS buffer in a flask

- Heat to boiling while stirring (magnet) to dissolve all agar powder
- Pour or pipette the gel onto the glass/ glasses; 50 ml on a large glass plate or 3ml each on small glas slides. (Preferably the glass have been placed on a levelled horizontal Table.)
- Let the gel(s) set (about 5 min).
- Place the gels in moist chambers, move to fridge to set further (15 minutes). Small gels can be stored in this way up to a week before use.
- Punch out desired holes in the gel according to the protocol for the samples to be typed, using the vacuum coupled punching devise. Use a pre-printed protocol as a template; placed under the glass plate/slides when punching.
- Fill about 6.5 µL antisera in the center of each "flower", see example 2 below. (Pooled antisera in the first steps, n=12 -> 12 "flowers", as suggested in the big pre-printed template)
- Fill about 6.5 µL antigen in the holes around each "flower" with antisera in the center. 6 samples (antigens) can be tested in each "flower". The same 6 sampes are tested at the same position in the 12 different antisera "flowers"

  When you have one sample only: the antigen are placed in the middle with atisera around, 2 flowers are needed to fit the 12 antisera (example 1 below).
  More than 1, less than 6 samples to be tested: punch our as many holes around the center that you need.

Example 1 Example 2



<u>Interpretation of results, day 3-4</u>

6. Before reading the gel, whip the clean from condensation with a Kleenex or similar paper tissue (on the back side of the glass ie "under" the gel). Hold the glass in an angel with a desk lamp behind to easier read the precipitations.

7. Read the precipitations and make notes on your gel template (paper map of the gel) with a blue pen day1 and a red pen day2. Unspecific results may occur, but are commonly less intense/dense.
8. Use the typing schedule for the pooled antisera to interpret the results. Write the sample number and the results for each sample tested on the paper protocol, next to the map for the gel. Precipitations in two pools are specific for one type (example: precipitates in pool D and R gives serotype 9).

- If there is only one precipitate in one pool, test the sample for all serotypes included in that pool to obtain the specific type/group result.
- If there are no precipitations, test the sample in pool G and I. If there then are precipitations in either G or I, test the sample for the types included in the specific pool.
- If there still are no precipitations for a specific sample, prepare a new antigen as described above, and test in A-T+G+I and also with omni-antisera.
- If the isolate persistent negative, use the Latex-kit
- If the isolate is negative to all antisera tested included in the latex kit antisera, optochine sensitive, positive in bile -solubility test and have very small colony morphology on the blood agar plate, the sample is interpreted as non type able (NT).

9. Samples typed to a specific type (as example 1, 3 or 14) are now completed, but those belonging to a group consisting of several factor types (exemplified by group 9 with factor types 9A, L, N and V) are further typed with factor sera which also can be done on a gel for most factor types. By experience, some batches of antisera will not work in gel diffusion, but this may vary between batches of the same factor sera.

Annex 8. PFGE- Pulsed field Gel Electrophoresis Protocol

Bacterial growth

Day1

1. Streak out isolates from -70°C freezer to half of a blood agar plate using a sterile disposable loop. Grow them at 37°C in 5% CO2 over night for a minimum of 12h.

Day2

1. Set the heat block to **37°C and 60°C**. Prepare 1ml PBS in sterile 1.5 ml eppendorf tubes.

2. Suspend 2 white loopes in PBS, wortex.

3. Measure OD at 600nm, use PBS as blank. OD should be around 1.2

4. Transfer 180µl to a new eppendorf tube.

5. Centrifuge 5min at 6000rpm

6. Take away the supernatant and save the pellet

7. Resuspend the pellet in 100 µl PetIV-buffer

8. Put the tube into the heating block at 37°C.

9. Prepare Low melting agarose, for **30 samples: 0.06g agaros + 4ml PetIV**

10. Prepare mould with scotch-tape, and mark wells.

11. Prepare 1:10 lysozyme in water and put it on ice

12. Add 5 µl lyzosyme to the tube, and mix it with 100 µl agarose.

13. Put the mould with agarose in the fridge

14. Mark and prepare 2ml eppendorf tubes with 400 µl EC-buffert and 20µl 1:10 lysozyme. Take out the mould from the fridge and put the "plugs" in the tube, 2 plugs/tube. Place them in water bath, 37°C for at least 3h. Lyses the cell wall

15. Prepare ESP-buffer **30 samples: 7600 µl ES-buffer + 400 µl ProteinasK**

16. Take away EC-buffer. Add 250µl ESP-buffer. Water bath at 56°C, over night. Degrade proteins and gives a cleaner DNA.

Day 3

1. Add 1xTE buffer into Falcon tubes. Transfer plugs to Falcon tubes.

2. Wash 2 times, 30 min.

3. Mark 2ml eppendorf tubes with isolate name.

4. Prepare 2ml eppendorf tubes with 0.1xTE. Dived plugs into 1/3 and transfer it to the tube with 0.1xTE. Leave for 20-30 min.

2. **Prepare MIX1** 1X 32X

- 10xRE-buffer (BufferA) 10µl * X 320 µl
- BSA 1µl *X 32 µl
- Milli Q 89µl *X 2848 µl
- Add 100 µl MIX1 and leave for 30 min in fridge.

3. **Prepare MIX2** 1X 32X

- 10xRE-buffer (BufferA) 15µl * X 480 µl
- BSA 1.5µl *X 48 µl
- Milli Q 131µl *X 4192 µl
- ApaI 2.5µl *X 80 µl

4. Change buffer to MIX2, 150 µl, at 37°C for minimum 2h.

5. Wash them with 200 µl 0.5xTBE in 20min.

6. Prepare the machine with 0.5xTBE and start the pump and then the cooling system.

4. Gel visualization and imaging

Annex 9. MLST Protocol

DNA preparation with QIAGEN, DNeasy Blood & Tissue Kit

1. Grow bacteria on a blood agar plate overnight (37 º CO2).
2. Dissolve bacteria from one fourth of the plate into 180µl lysozymbuffert.
3. Add 20µl lyzosym. Vortex.
4. Incubate at 37 º for at least **30 min**.
5. Add 200µl AL-buffert (Al-buffer first) and 25µl proteinas K. Vortex.
6. Incubate at 70 º for **30 min**.
7. Add 200µl 99,5% EtOH. Vortex.
8. Transfer the mix to a DNeasy-minifilter.
9. Centrifuge 1min 10 000rpm.
10. Place the filter in a new collection tube, add 500µl AW1-buffert.
11. Centrifuge 1min 10 000rpm.
12. Place the filter in a new collection tube, add 500µl AW2-buffert
13. Centrifuge 3min 13 000rpm.
14. Transfer the filter to an eppendorftube, add 100µl MQ H2O.
15. Incubate for 1 min.
16. Centrifuge 1min 10 000rpm. Discard the filter.
17. Store the DNA at -70 º.

PCR Matermix	For each well
MQ H2O	19.75µl
10x PCR-buffer	2.5µl
dNTP mix (10mM)	0.5µl
Forward and reverse primer (20pmol)	1µl
Taq-polymerase	0.25µl

1. Calculate the amount of mastermix to be made depending on the number of samples tested.
2. Mix the mastemix according to volumes above.

3. Add 1µl DNA to each well (7 wells /isolate, 7 different primer pairs)

4. Add 24µl of mastermix to each well (make sure the correct primers are added to the correct wells)

5. Seal the plate and vortex quickly.

6. Start the PCR.

PCR programme. (MLST) Primers used for MLST

Lid 100º C

1. 95ºC 4 min

2. 95ºC 30sec

3. 55ºC 30sec

4. 72ºC 1min

Rep No. 2 - 4, 29 times

5. 72ºC 10min

6. Hold 4ºC

Clean up of PCR products

1. Add 30µl of 20%PEG/2.5M NaCl, vortex.

2. Incubate at room temp for 30 min

3. Centrifuge at 3200rpm 4ºC for 1 hour.

4. Invert plate and spin up to 500rpm on a "celltork"

5. Add 75µl of 70% Etanol

6. Centrifuge at 3200rpm 4ºC for 20 min.

7. Invert plate and spin up to 500rpm on a "celltork"

8. Add 75µl of 70% Etanol

9. Centrifuge at 3200rpm 4ºC for 20 min.

10. Invert plate and spin up to 500rpm on a "celltork"

11. Dry on the bench for 10 min

12. Add 5µl MQ H2O to each well, vortex (spin down)

13. Put in fridge for 10 minVortex and use.

Sequencing Matermix	For each well
Forward or reverse primer (1pmol)	1µl
Purifed PCR product	2µl
Taq-polymerase (Big dye v 3.1)	2µl

1. Calculate the amount of mastermix to be made depending on the number of samples tested.
2. Mix the mastemix according to volumes above.
3. Add PCR product to each well
4. Add 5µl of mastermix to each well (make sure the correct primers are added to the correct wells.
5. Seal the plate and vortex quickly.
6. Start the PCR

PCR programme.(JVSEQ)

Lid 100º C

1. 96ºC 1 min
2. 96ºC 10 sec
3. 50ºC 5 sec
4. 60ºC 4 min

Rep No. 2 - 4, 24 times

5. 60ºC 10 min
6. Hold 4ºC

Precipitation of sequencing products.

1. Add 65µl of 70% EtOH to each well
2. Incubate at -20 º C for 1 hour.
3. Centrifuge at 3500rpm, 4 º C for 1 hour.
4. Invert plate and spin up to 500rpm on a "celltork"
5. Add 150µl 70% EtOH to each well
6. Centrifuge at 3500rpm, 4 º C for 30 min.
7. plate and spin up to 500rpm on a "celltork"
8. Dry on the bench for 10 min
9. Add 20 µl HiDi to each well and leave up to the sequencing room.

Declaration

This thesis is my original work and has not been presented for a degree in any other University, and that all sources of material used for the thesis have been duly acknowledged

Candidate Name: Wondewosen Tsegaye Sime

Signatute _____

Date_____

This Thesis has been submitted for examination with my approval as University advisors:

1. Dr. Yimtubezinash Weldeamanuel (MD, MSc, PhD)

Signature_____ Date _____

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May, 2017