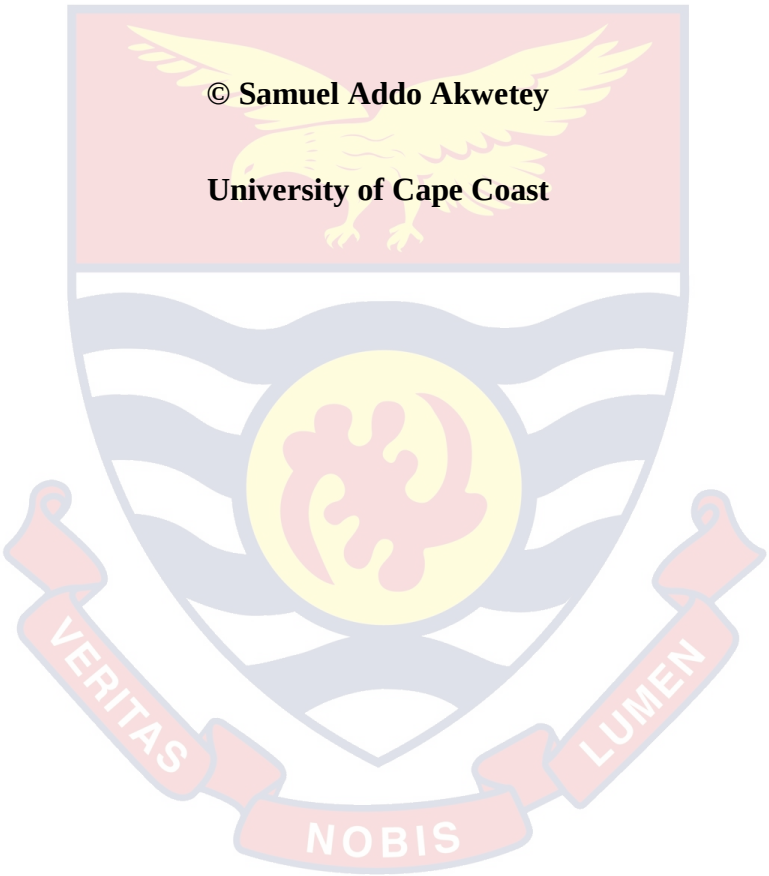
UNIVERSITY OF CAPE COAST

NASOPHARYNGEAL CARRIAGE, ANTIBIOTIC SUSCEPTIBILITY PATTERNS AND SEROTYPE DISTRIBUTION OF *STREPTOCOCCUS PNEUMONIAE* AMONG CHILDREN UNDER FIVE IN CAPE COAST, GHANA

SAMUEL ADDO AKWETEY

2020



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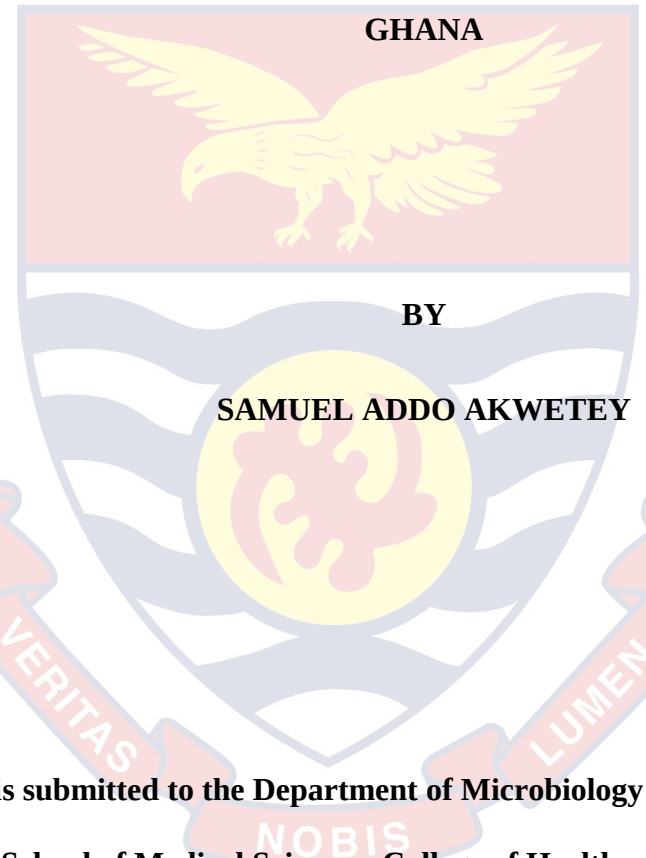

BY

SAMUEL ADDO AKWETEY

Thesis submitted to the Department of Microbiology and Immunology of the School of Medical Sciences, College of Health and Allied Sciences, University of Cape Coast in partial fulfilment of the requirements for the award of Master of Philosophy degree in Infection and Immunity.

MARCH 2020

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature …….. Date……

Name: ……

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature…… Date…….

Name: …….

Co-Supervisor's Signature…… Date……

Name: ……

ABSTRACT

The introduction of the 13 valent pneumococcal conjugate vaccine has been reported to reduce nasopharyngeal carriage of vaccine types of the pneumococcal serotypes and the antibiotic resistant strains of the bacteria. However, studies conducted have revealed serotype replacement of the vaccine types. The goal of this study was to determine the nasopharyngeal carriage and serotype distribution of *S. pneumoniae* among children under five years in the post-vaccination era. A cross-sectional study was conducted where nasopharyngeal swabs were taken from 398 children in the Cape Coast Metropolis. Pneumococcal isolates were identified using optochin sensitivity testing and the presence of *lytA* and *cpsA* genes. Isolates were subjected to antibiotic susceptibility testing against different classes of antibiotics. Multiplex PCR was done on the isolates to serotype and determine the presence of penicillin resistance genes such as *PBP1a*, *PBP2b* and *PBP2x*. The overall carriage prevalence was found to be 23.4%. About 51.6% of the isolates belonged to known serotypes. The most occurring serotypes were 6A/B (10.8%), 23F (8.6%), 14 (7.5%) and 19F (6.5%). All the isolates were susceptible to Levofloxacin whereas 60.2%, 87.1% and 64.5% were non-susceptible to oxacillin, co-Trimoxazole and tetracycline respectively. About 50.5% of the isolates were multidrug resistant. Most of the oxacillin non-susceptible isolates were vaccine serotypes. All the isolates contained at least one of the penicillin resistant genes. In conclusion, antibiotic resistant vaccine type serotypes still persist even though new non-vaccine serotypes were detected. This indicates the presence of newly emerged serotypes.

KEYWORDS

13-valent pneumococcal conjugate vaccine

Antimicrobial resistance

Penicillin resistance genes

Serotypes

Streptococcus pneumoniae

ACKNOWLEDGEMENTS

I would first like to show my profound appreciation to my supervisors, Godwin Kwakye-Nuako, PhD and Desmond Omane Acheampong, PhD, both of the Department of Biomedical Science for their professional guidance, time, patience and advice they offered me during my work. To Ms Rachael Odarkor Mills of Department of Biomedical Science, to you, I say thank you for providing expert advice on the subject matter. I am truly grateful.

I am also grateful to my Head of Department, Samuel Victor Nuvor, PhD and the Postgraduate coordinator, Dorcas Obiri-Yeboah, PhD for approving to support my research financially and to the rest of the faculty of the Department of Microbiology and Immunology for their academic advice.

To Boss Cole of Korle Bu Teaching Hospital and my friends for contributing to the success of my research, I also say thank you.

DEDICATION

In memory of my mother:

Ms Florence Kabutey

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LIST OF ABBREVIATIONS

WHO	World Health Organisation
CDC	Centers for Disease Control and Prevention
lytA	autolysin A
ply	pneumolysin
cpsA	capsular polysaccharide A
DNA	Deoxyribonucleic acid
RT-PCR	real time-Polymerase Chain Reaction
mPCR	multiplex PCR
BA	Blood agar
IPD	Invasive pneumococcal disease
PCV-13	13-valent pneumococcal conjugate vaccine
NT	Non-typable
VT	Vaccine type
NVT	Non-vaccine type
PPV	Pneumococcal polysaccharide vaccine
PNSP	Penicillin non-susceptible pneumococcus
PSSP	Penicillin susceptible pneumococcus
CLSI	Clinical and Laboratory Standards Institute

CHAPTER ONE

INTRODUCTION

The normal flora of the human body are known to provide some benefits to humans. The existence of normal flora in a niche most importantly help prevent the manifestation of disease by preventing colonisation of other pathogens, as colonization often precedes infection. Notwithstanding, normal flora can cause disease and other complications. *Streptococcus pneumoniae* is an example of such flora that resides only in the nasopharynx. It is known to cause localised and systemic diseases, especially in individuals with weak immune system such as children under two years and adults older than 65 years. The organism is the leading cause of bacterial meningitis and community-acquired pneumonia as well as can cause outbreaks. Ghana, as a country, has had its fair share of outbreaks caused by virulent serotypes in 2005 and 2016 with high case fatalities. Even though diseases caused by *S. pneumoniae* are treated with antimicrobial agents, the increase in antimicrobial resistance has made disease treatment of pneumococcal diseases difficult. Given this problem, the pneumococcal conjugate vaccine (PCV-13) was introduced into Ghana's childhood immunisation programme in May 2012. The epidemiology of the organism in this post-vaccination era, is not well understood. Knowledge about this will help fill some gaps and inform policy changes concerning the use of antimicrobials in treating diseases caused by the organism.

Background of the Study

The nasopharynx of children has normal bacterial flora such as *Moraxella catarrhalis*, *Hemophilus influenzae*, *Neisseria meningitidis*,

Staphylococcus aureus and *Streptococcus pneumoniae*, among other *alpha-hemolytic streptococci* that do not usually cause disease (Levinson, 2016). *Streptococcus pneumoniae* is a commensal of the oral cavity and pharynx. It belongs to the *mitis* group based on the phylogenetic grouping (Greenwood, Barer, Slack, & Irving, 2012). *S. pneumoniae* exhibits alpha-hemolysis when cultured on blood agar which is characterised by the presence of a greenish pigment around the colonies. However, on chocolate agar, a greenish-yellow pigment is often seen (CDC, 2011). The organism can further be identified based on colony morphology, oxygen requirements, biochemical profile and by molecular methods such as the detection of autolysin A (*lyt A*), capsular polysaccharide A (*cps A*) and pneumolysin (*ply*) genes, just to mention a few (Sourav et al., 2010). *S. pneumoniae* is one of the major causes of bacterial infections worldwide due to the arsenal of virulence factors it possesses ((Brooks & Mias, 2018).

The colonisation of the nasopharynx by the pneumococcus often precedes invasive disease. The carriage or colonisation of the pneumococcus in the nasopharynx is found in about 5%-90% of healthy people, with the carriage in healthy children being significantly higher than in healthy adults (CDC, 2017). The nasopharyngeal carriage of *S. pneumoniae* is estimated to be 20%-90% in healthy children in Africa (WHO, 2019) whereas in Ghana, it is between 27% and 51%, determined by the limited studies conducted in the country in the pre-vaccination era (Denno et al., 2002; Donkor et al., 2010; Dayie et al., 2013; Mills, Twum-danso, Owusu-agyei, & Donkor, 2015). Protection against the establishment of pneumococcal infections first occurs at the mucosal membrane where the normal flora competes for space, thereby

checking the growth of the pneumococcus (Segura, Calzas, Grenier, & Gottschalk, 2016). This mechanism is also known as colonisation resistance. In some cases, colonisation resistance is followed by the opsonisation-mediated action of the C3b component of the classical pathway of the complement system. This antibody-initiated complement pathway is thought to be the significant immune mechanism, protecting the host against pneumococcal infections. The said mechanism of protection usually involves antibodies and immune cells from the lungs, liver and spleen (Bogaert, Groot, & Hermans, 2004). However, in some cases, when the host-pathogen relationship is disturbed by some factors such as malnutrition, viral infections and immune deficiency, highly pathogenic pneumococcal strains can cause disease. In other words, risk factors that supress the immune system promote the disease. Healthy children, older adults, smokers and individuals with conditions such as sickle-cell disease, asthma, diabetes, human immunodeficiency virus, heart diseases and lymphoma have a high risk of pneumococcal diseases (CDC, 2018).

Moreover, the highly pathogenic pneumococcal strains have several virulence factors such as the pilus, pneumolysin, pneumococcal surface protein A and a polysaccharide capsule that enables the bacteria to colonise, invade and cause disease (Brooks & Mias, 2018). The antigenic polysaccharide capsule as well as the other virulence factors protects the bacterial cell against clearance by the immune system and induce inflammatory response which aids in the transmission of the bacteria (Mayanskiy et al., 2014).

The pneumococcus has been associated with various localised infections such as sinusitis, and acute otitis media caused by contiguous spread and systemic infections such as pneumonia, endocarditis, as well as septicaemia, mostly caused by hematogenous spread (WHO, 2019). These infections can lead to meningitis, peritonitis and osteomyelitis with the long-term sequelae resulting in hearing and neurological deficits in 24.7% of individuals who are lucky to have survived the illness (WHO, 2019).

The World Health Organization global estimates in 2008, showed that 5% of all case-child mortality in children under five years of age were due to pneumococcal infections (WHO, 2012). In 2015, it was estimated that 294,000 out of 5.83 million deaths occurring in children under 5 years was associated with pneumococcal diseases (Wahl et al., 2018). In Ghana, *S. pneumoniae* was the leading cause of bacterial meningitis in 1992 in Kumasi (Mackie, Shears, Frimpong, & Mustafa-Kutana, 1992). It was further implicated in an outbreak in 2016, in the northern belt of the country, with high case fatalities (Kwambana-adams et al., 2017).

The pneumococcus is classified into several serotypes based on the antigenic structure of the capsular polysaccharide, and there are over 98 serotypes of the pneumococci (Dube et al., 2018). Worldwide, about 23 of the over 98 serotypes are responsible for >70% of invasive pneumococcal disease (IPD) in children under five years of age (WHO, 2019). *S. pneumoniae* serotypes 3, 4, 6B, 9V,7F, 14, 18C, 19F, and 23F cause the majority of invasive disease with *S. pneumoniae* serotypes 6B, 7F, 14, 18C, 19F, and 23F usually associated with infections in children, whereas serotypes 3 and 4 predominate in infections in adults (Imo, Rene, Ocklenburg, & Linden, 2010).

S. pneumoniae serotypes vary worldwide based on geographical location, age and with time (Dayie et al., 2013). Diseases caused by these serotypes are treated with antibiotics such as penicillin and macrolides (Erythromycin). However, the public health burden with regards to invasive pneumococcal diseases exacerbated due to the increasing resistance of the causative organism to beta-lactams and the macrolides (Donkor et al., 2010). Resistance to penicillin was first noticed over 50 years ago, and it has been a public health burden ever since, with varying prevalence worldwide (Moujaber, Osman, Rafei, Dabboussi, & Hamze, 2018). Resistance to the fluoroquinolones, such as levofloxacin and chloramphenicol are uncommon but gradually increasing (Cavalieri et al., 2005).

Due to the high incidence of pneumococcal disease among children under five years, the pneumococcal conjugate vaccine-13 (PCV-13) was introduced worldwide and into the national immunisation programme in 2010. However, PCV-13 was introduced concurrently with the rotavirus vaccine, in Ghana in May 2012 (WHO, 2014). This programme aimed to reduce the incidence of invasive pneumococcal disease (IPD) in children as well as reduce the prevalence of drug-resistant serotypes of *S. pneumoniae* (Adetifa et al., 2012). The 13-valent pneumococcal conjugate vaccine is effective against the thirteen most virulent serotypes involved in invasive diseases such as serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F, 3, 6A and 19A (Esposito & Principi, 2015). PCV-13 is most effective in children, particularly those less than two years of age. It increases the immune response to the polysaccharides capsule by conjugating the polysaccharides to a carrier protein (WHO, 2015). The conjugated vaccine effectively induces protection against the

pneumococcal nasopharyngeal carriage, thereby reducing transmission in the community. Moreover, the vaccine is effective against mucosal and systemic infections (WHO, 2015).

Statement of the Problem

The asymptomatic nasopharyngeal carriage of *S. pneumoniae* mostly occurs in children less than five years. This has strongly been associated with the development of associated disease and the spread of the pathogen (Marchisio, Esposito, Schito, & Marchese, 2002). *Streptococcus pneumoniae* is one of the common causes of bacterial meningitis in Ghana. Mackie et al., (1992) reported that over 50% of bacterial meningitis in Kumasi was caused by *S. pneumoniae*, with a mortality rate of 36.4%. The pneumococcus has also been involved in several outbreaks with a high case fatality rate in the northern parts of the country (Leimkugel et al., 2005). In 2016, a large outbreak of meningitis was reported in the Brong Ahafo region of Ghana. The prevalence of *S. pneumoniae* in the epicentre district was 363 suspected cases per 100,000 people (Kwambana-adams et al., 2017). Moreover, there continues to be a high incidence of pneumococcal meningitis in the northern belt of which the vaccine type, serotype 1 is still the main cause (62%) with an incidence of 1.8 cases per 100,000 children under 5 years (Bozio et al., 2018).

Pneumococcal carriage is the main means by which the infection is transmitted from person to person, and the strain of bacteria carried in the nasopharynx of healthy children is the same as the infection-causing strains circulating in the community (Marchisio et al., 2002). In view of this, the more the people that carry the bacteria, the higher the rate of transmission. Meanwhile, acquisition of *S. pneumoniae* is also a precursor for invasive

pneumococci disease (Bogaert et al., 2004). Carriage studies among children conducted in some West African countries such as Nigeria and Gambia among children under five years obtained carriage prevalence of >71% (Adetifa et al., 2012; Usuf et al., 2016). Studies in Ghana have found high pneumococcal carriage among children in Kumasi (51.4%), Accra (34%) and Tamale (31%) (Dayie et al., 2013; Denno et al., 2002). In a separate study, the carriage rate of 48.9% was recorded among children attending a paediatric hospital in Accra (Mills, Twum-danso, Owusu-Agyei, & Donkor, 2015). Unfortunately, nasopharyngeal carriage of antibiotic-resistant *S. pneumoniae* has increased gradually over the last few years (Marchisio et al., 2002), compounding the already alarming situation.

The 13-valent pneumococcal conjugate vaccine, which was introduced to reduce the transmission of the pathogenic and antibiotic-resistant pneumococcal isolates, has been reported to have drastically decreased the incidence of invasive pneumococcal diseases worldwide (Kaplan et al., 2015; Mackenzie et al., 2016). However, there have been recent reports about the emergence of non-vaccine related pneumococcal disease (Mackenzie et al., 2016; Devine et al., 2017).

There are limited studies investigating the antibiotic susceptibility patterns of pneumococcal isolates. Nevertheless, these few studies only estimated the prevalence of antibiotics resistance without further investigating the genetic basis for the resistance of the pneumococcus to these antibiotics. The emergence of penicillin resistance has, however, been determined to some extent in most countries (Mayanskiy et al., 2014; Zettler et al., 2006). These studies, found increasing trend in penicillin resistance and identified the

penicillin-binding protein (*PBP)* genes to be the most frequently occurring gene coding for resistance in the pneumococci. Additionally, the few studies conducted in Ghana recorded high resistance to commonly used antibiotics, especially penicillin and some group of macrolides. Forty-five percent of pneumococcal isolates by Dayie et al., (2013) also showed intermediate resistance to penicillin, while two isolates showed full resistance. Additionally, sixty-three per cent of isolates from Mills et al., (2015) were resistant to penicillin, while 87% of the isolates showed multiple drug resistance.

This study therefore seeks to establish the serotype distribution of the pneumococcus seven years after the introduction of PCV-13 in Ghana. The occurrence of serotype replacement, as well as the reduction in vaccine-type serotypes in circulation and invasive disease, has been documented elsewhere (Kamng'ona et al., 2015; Kwambana-adams et al., 2017; Ladhani et al., 2018). Furthermore, there are geographical variations in pneumococcal serotype distribution globally (Devine et al., 2017). As such, capsular switching and serotype replacement may not be the same in both developed and developing countries. The relation between serotypes in circulation before and after the introduction of the PCV 13 has not been explored extensively in Ghana with only a hand few of the studies conducted in the post-vaccination era (Donkor et al., 2017; Dayie et al., 2018; Dayie et al., 2019; Dayie et al., 2019). Hence this study will provide relevant information to fill important knowledge gaps regarding pneumococcal surveillance in the post-vaccination era in Ghana. This surveillance includes monitoring the non-vaccine types and predicting the types that could cause invasive pneumococcal disease. Also, the study will

provide information on the effectiveness of the PCV 13 vaccine currently being rolled out in Ghana.

Aim

To investigate the carriage prevalence, antibiotic susceptibility patterns and serotype distribution of *Streptococcus pneumoniae* carriage in children less than five years of age in the post-vaccination era.

Objectives

To determine the:

i. prevalence of *S. pneumoniae* carriage in the Cape Coast Metropolis
ii. pneumococcal serotypes and the coverage provided by the 13-valent pneumococcal conjugate vaccine.
iii. antibiotic susceptibility patterns among isolates.
iv. genetic bases of resistance of *S. pneumoniae* to penicillin.

Significance of the Study

Studies of the prevalence of different serotypes and their resistance patterns can provide useful information on the indications for more rational therapeutic, preventive strategies. The resistance of pneumococcus to the various antibiotics varies based on the geographical area, and since current literature has made contradictory reports on the possible increase in antibiotic resistance among the non-vaccine serotypes (Obolski et al., 2018), as it is known that the vaccine mostly carry resistant genes, this study will provide some information on that in the Ghanaian perspective.

The antibiotic susceptibility profile of carriage isolates can be used as an estimate of the incidence of resistance in isolates. Estimating antibiotics

susceptibility patterns could reflect antibiotics use and provide information to guide the use of antibiotics. It could also form the basis for conducting antibiotic resistance surveillance since only a few studies have been done worldwide and only one in Accra, Ghana in this post-vaccination era (Dayie et al., 2019; Emgård et al., 2019; Southern et al., 2018). Also, data from the antibiotic surveillance will contribute to achieving one of the World Health Organisations' strategies for implementation of the action plan on antimicrobial resistance. This study will provide novel data on the genetic basis for penicillin resistance seen in pneumococcal isolates from Ghana. This may be useful information for antibiotic formulators to provide improved medications to combat the pneumococcus.

Delimitations

This study is a cross-sectional study, and it covered both Cape Coast South Constituency and Cape Coast North Constituency in the Cape Coast Metropolis. Only two circuits (Aboom circuit and Ola circuit) out of four circuits were covered. Immunisation centres found in the following areas were selected; Akotokyir, Kwaprow, Ewim, Bakaano, Amamoma and the University of Cape Coast community. Variables included in the study were sex, religion, age and the type of facility whiles variables such as symptoms of respiratory diseases were excluded.

Limitations

In this study, sample and data collection was conducted at selected schools and immunisation centres to cover children aged 59 months to as low as four months. However, the sample collection did not cover every part of the Cape Coast Metropolis, and so the data obtained might not be an exact

representation of the entire population. The study is a cross sectional study and not a longitudinal study. In view of that, the study cannot determine the precise dynamics of carriage, acquisition rates or estimate the carriage duration of the pneumococcus. Moreover, serotyping was done using the sequential multiplex which employs the use of 40 primers for identifying 70 serotypes out of the 98 known serotypes. Penicillin and ceftriaxone resistance are normally determined using the minimum inhibition concentration. However, penicillin resistance was rather determined by detecting *PBP* genes whereas ceftriaxone resistance was determined a different guideline.

Definition of Terms

Serotypes: They are variations within the same species of microorganisms based on their surface antigens.

Antibiotics: These are agents that are used to kill bacteria or inhibit their growth.

Antibiotic resistance: It occurs when infection-causing bacteria do not respond to the action of the antibiotics.

Multiple drug resistance (MDR): It is the resistance of a microbe to three or more classes of antibiotics.

Nasopharyngeal carriage: It is the colonisation of a microbe in the nasopharynx of an individual.

Colonisation: It is the appearance or presence of bacteria in the nasopharynx without causing disease.

Organisation of the Study

This entire thesis is organised in five chapters excluding the abstract, appendix and references:

Chapter one describes the background of the study with a focus on keywords such as Streptococcus pneumoniae, invasive pneumococcal disease, serotypes, antibiotics and antibiotic resistance. Also, the primary purpose of the study is stated together with the specific questions the study seeks to answer. This chapter further looks at issues that the study seeks to address, such as research knowledge gaps, and contradictions and the purpose the data obtained from the study will serve.

In chapter two, similar studies conducted worldwide were reviewed and written following a conceptual framework. Emphasis was made on the gaps left by the reviewed literature to be filled, and the methodologies that were adopted or modified with reasons. This chapter also shows global reports on nasopharyngeal carriage prevalence, circulating serotypes and the prevalence of the resistance isolates of *S. pneumoniae*.

Chapter three looks at the materials used, and methods employed to conduct the study. The study design, study area and target population are mentioned, including their characteristics. The chapter also provides detailed information on how the samples and data were collected and how the obtained data were processed and analysed.

The processed and analysed data were displayed and discussed in chapter four. The results and discussions were made based on the arrangement of the specific objectives.

The final chapter concludes the entire thesis by first summarising the first three chapters of the thesis and finally concluded by stating the findings and making possible explanations for any discrepancies that might have occurred with the results. Recommendations were also made based on the key and inconclusive findings.

Chapter Summary

In summary, this chapter described the background of the study with a focus on keywords such as Streptococcus pneumoniae, invasive pneumococcal disease, serotypes, antibiotics and antibiotic resistance. Also, the primary purpose of the study is stated together with the specific questions the study seeks to answer. This chapter further looks at issues that the study seeks to address, such as research knowledge gaps, and contradictions and the purpose the data obtained from the study will serve.

CHAPTER TWO

LITERATURE REVIEW

Introduction

Streptococcus pneumoniae, also called pneumococcus, is an opportunistic pathogen residing in the nasopharynx. However, the pneumococcus is known to cause diseases, collectively called invasive pneumococcal diseases. Depending on its serotype, the organism can produce in children, elderly and individuals an underlying health problem. This chapter looks at the biology of *Streptococcus pneumoniae* and the global reports on the nasopharyngeal carriage prevalence, circulating serotypes and the prevalence of the resistant isolates of *S. pneumoniae*. Emphasis is made on the gaps left by the reviewed literature to be filled, and the methodologies that were used in the identification of the serotypes, its limitations and advantages. This aims to further understand the organism in relation to the carriage and distribution of its serotypes and antimicrobial resistance to commonly used antimicrobials in healthy children in the post-vaccination era.

Characteristics and Identification of *Streptococcus pneumoniae*

Streptococcus pneumoniae as a prokaryote belongs to the Phylum Firmicutes, Class Bacilli, Order Bacillales and Family Streptococcaceae (Vos et al., 2011). The genus *Streptococcus* comprises of most human pathogenic and commensal microorganisms residing on the mucosal membranes of the upper respiratory tract. They are gram-positive cocci that grow either in chains or in pairs (diplococci). It is a facultative anaerobe, that is, it can survive in both anaerobic and aerobic conditions (Brooks & Mias, 2018). Streptococci are non-spore forming, and non-motile as well as catalase-negative organisms

and can be characterised based on phylogenetic analysis, blood haemolysis properties and by Lancefield grouping method.

The genus is placed into five groups based on their pathogenic properties. The pyogenic group comprises of pathogens that cause the production of pus at the site of infection. The mutans group residing on the human tooth are responsible for dental caries. The anginosus, salivarius and bovis groups form the commensal microbiota. The anginosus and salivarius groups reside in the pharynx and oral cavity while members of the bovis group are in the colon. *Streptococcus pneumoniae* belongs to the mitis group and resides in the nasopharynx. They are known to cause acute otitis media, pneumonia and meningitis. The pneumococcus is grouped together with the viridans streptococcus in the mitis group because of their genotypic and phenotypic relatedness (Haslam & Geme III, 2018). Other members of the mitis group are *S. mitis*, *S. oralis*, *S. pseudopneumoniae*, *S. sanguinis*, *S. australis* (Slotved, Facklam, & Fuursted, 2017).

Based on the haemolytic activity of the genus-group, *Streptococcus pneumoniae* together with the other members of the mitis group and anginosus group exhibit alpha haemolysis (α-haemolysis) on blood agar plates. Alpha-haemolysis is characterised by the presence of a green pigmentation, caused by the action of pneumolysin on haemoglobin, around the colonies (CDC, 2016). Though the pneumococcus shows α-haemolysis, it is not considered a member of the group *viridans streptococci*. Other groups such as the pyogenic groups exhibit beta-haemolysis (β-haemolysis) while the bovis and mutans groups show no haemolysis on blood agar plates (Greenwood et al., 2012).

The Lancefield method of classification, proposed by Rebecca Lancefield, is based on the distinct immunogenicity of the polysaccharide cell wall of the various species. This serological method of classifying Streptococci based on the C carbohydrates in the polysaccharide cell wall was classically used to differentiate β-haemolytic streptococci (Greenwood et al., 2012). Human pathogenic Streptococci such as *S. pyogenes* and *S. agalactiae* belong to group 'A' and 'B' respectively. However, *Streptococcus pneumoniae* and the viridans streptococcus cannot be grouped (Greenwood et al., 2012).

Phenotypic identification of *Streptococcus pneumoniae*

Streptococcus pneumoniae is a fastidious microorganism, requiring an optimum temperature of 35-37^{o}C for growth. This pneumococcus grows well when cultured on media containing 5-7% sheep or horse blood and incubated in 5-10% CO_2 since the bacteria is an anaerobe and haemolyses blood. To increase the yield of the pneumococcus, the culture is supplemented with 5ug/ml of gentamicin. This is because *S. pneumoniae* is intrinsically resistant to low concentrations of gentamicin due to the low intake of the drug (Cattoir, 2016). The identification and differentiation of the pneumococcus is mostly done based on colony morphology, gram reaction and biochemical reactions. Accurate isolation and identification of the pneumococcus are necessary to distinguish it from its close related streptococcus.

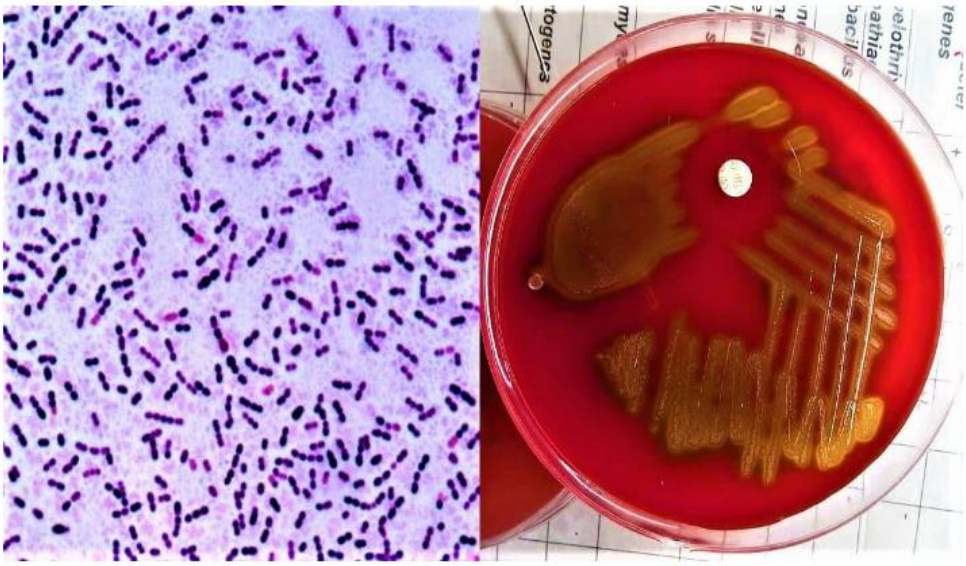


Figure 1. A diagram showing the colony morphology and microscopy of *S. pneumoniae* (Batra, 2018).

Colony morphology

S. pneumoniae grows well on blood agar but can also grow on chocolate agar. On blood agar, the pneumococcus colonies appear grey, raised, small and mucoidal when the isolates are encapsulated or rough when they are non-encapsulated. Colonies of *S. pneumoniae* are relatively similar to viridans streptococci when cultured between 18-24 hours. However, when cultures are incubated for 48 hours, colonies of the pneumococcus become flattened and depressed as compared to the raised colonies of the viridans streptococci. The colonies of the pneumococcus show zones of alpha-haemolysis like the viridans group which distinguishes them from other streptococci groups (Batra, 2018).

Gram reaction

Streptococcus pneumoniae is a gram-positive bacterium when taken through gram staining. The pneumococcus is gram-positive due to the

presence of a thick outer peptidoglycan cell wall which is composed of N-acetylmuramic acid linked with N-acetylglucosamine and a larger amount of teichoic acid (Greenwood et al., 2012). These molecules enable gram-positive bacteria to resist decolouration during staining thereby giving the bacterium its dark purple colouration (Greenwood et al., 2012). When colonies of the pneumococcus have been cultured and incubated for more than 24 hours, they become gram variable, that is neither gram-positive nor gram-negative. This is due to the aging and subsequent dying of the bacterial isolates. The pneumococcus mostly appears as lanceolate and diplococci even though sometimes they may appear in single cocci or in chains.

Biochemical reaction of *Streptococcus pneumoniae*

The various biochemical tests are mostly done to confirm the species the genus Streptococcus. There are three main biochemical tests conducted to identify *S. pneumoniae*; catalase test, optochin test and the bile solubility test.

Catalase test

Microscopically, *Streptococcus* and *Staphylococcus* appear similar with cocci shape, there the catalase test is conducted to differentiate the *Staphylococcus species* from the *Streptococcus species* (CDC, 2016). *Streptococcus species* are catalase-negative because they do not have the catalase enzyme to split hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2).

Optochin test

This test distinguishes *S. pneumoniae* from the other alpha-haemolytic streptococci. The pneumococcus is sensitive to ethylhydrocupreine hydrochloride, also known as optochin. The other species are optochin

resistant even though some pneumococcal strains are resistant as well (CDC, 2016). Isolates with sensitivity zones of at least 14 mm are considered to be pneumococcal strains.

Bile solubility test

The bile solubility test is used as a confirmatory test due to the fact that some of the pneumococci could be optochin resistant. It is therefore known as the 'gold standard' in conventional identification of the pneumococcus (CDC, 2016). The pneumococcus is soluble in bile or 2 % sodium deoxycholate while the other alpha-haemolytic streptococci are resistant (Wessels, Schelfaut, Bernards, & Claas, 2012).

Even though the biochemical tests, especially optochin and bile solubility tests are necessary for distinguishing *S. pneumoniae* from other streptococci, the tests are affected negatively by time and its sensitivity due to the presence of 10% optochin-resistant (Kellogg, Bankert, Elder, Gibbs, & Smith, 2001) and 2% bile insoluble pneumococcus (Richter et al., 2008). It is often important to consider other options such as genotypic identification.

Genotypic Identification of *S. pneumoniae*

Genotypic identification of the pneumococcus through the molecular detection of genes is a more effective method as compared to the traditional method of detection since it does not require viable cultures. Autolysis can sometimes occur, and that could influence the results of the biochemical tests of the bacteria. Molecular detection of the bacteria takes a few hours to be done and it is unaffected if the person is already on antibiotic therapy (Azzari et al., 2010). The molecular detection of the pneumococcus is mostly based on the presence of genes, possessed by all pneumococci, that gives the bacteria its

distinguishable characteristics. Some of these genes are autolysin A (*lytA*), pneumolysin (*ply*), 16S rRNA gene, pneumococcal capsular polysaccharide synthesis (*cps A*) and Xisco gene (Salvà-Serra, Connolly, Moore, & Gonzales-Siles, 2018).

The *lyt A* gene encodes for the enzyme autolysin which is responsible for degrading different bonds in the murein layer leading to the lysis of the cell. The lysis action of the autolysin releases the lipoteichoic and teichoic acids that are mediators of host inflammatory response. This phenomenon often occurs when pneumococcal isolates have been cultured for more than 24 hours or the isolates are exposed to unfavourable conditions. The *ply* gene encodes for pneumolysin, a multifactorial virulence factor in *S. pneumoniae*; with a 471 amino acid toxin that has both host cell lysis, complement activation properties and stimulation of cytokines. *Ply* differs from other gram-positive cholesterol-dependent cytolysins due to the fact that it does not encode for any known motifs significant for cell attachment (Greene, Narciso, Filipe, & Camilli, 2015). This indicates its use for identification of pneumococcus. Sequencing of 16S rRNA genes is another way of differentiating *S. pneumoniae* from its closely related species such as *S. mitis* and *S. pseudopneumoniae.* The *16S rRNA* genes help by identifying the cytosine nucleotide at position 203 of the pneumococcal sequence which is rather substituted for by an adenosine nucleotide in the other members of the mitis group (Salvà-Serra et al., 2018). A novel gene known as the Xisco gene was found to be possessed by all pneumococci and not by any of the members of the mitis group. It is 666bp in length and encodes for a surface protein anchored to the cell wall (Simões et al., 2016).

The antigenic capsule of the bacterium is encoded by the *cps A* gene from which serotyping is done. Despite the high sensitivity of the molecular methods, there are reports regarding the possible misidentification of the pneumococcus (Simões et al., 2016). This was attributed to the fact that there is the transfer of genetic element from the pneumococcus to the other closely related streptococci and vice versa. In the same study, an isolated *S. pneumoniae* displayed an atypical *lytA-BsaAI-RFLP* signature which is known to be exclusively found in its closely related streptococci (Simões et al., 2016). The same study again found atypical *lytA-BsaAI-RFLP* signature in *S. pseudopneumoniae* which is characteristic of *S. pneumoniae* (Simões et al., 2016). With the introduction of the capsular-acting pneumococcal conjugate vaccine worldwide, invasive pneumococcal diseases are now mostly caused by non-vaccine serotypes and emerging non-encapsulated pneumococcus (Keller, Robinson, & Mcdaniel, 2016). The reported cases of non-encapsulated pneumococcus mean that there could be *cps A*-negative pneumococcus.

In view of this, suspected pneumococcal isolates obtained in this study were screened for both *lyt A* and *cps A* gene to minimise misidentification of the bacteria. Isolates that were *lyt A* and *cps A* positive as well as *lyt A* positive and *cps A* negative were all considered as *S. pneumoniae.*

Identification of *S. pneumoniae* Serotypes

The *cps A* gene is one of the genes harboured in the *cps* locus that encode for capsule polysaccharide synthesis of the pneumococcus. The capsule is a major virulence factor responsible for antiphagocytosis and clearance prevention during colonisation of the nasopharynx (Wyres et al., 2013). The pneumococcus was first identified in 1881 by Pasteur and

Sternberg after it was recognized as the pathogen responsible for most of the mortality cases during the influenza pandemic (Geno et al., 2015). Now, *S. pneumoniae* is the cause of both non-invasive and invasive diseases. Before the discovery of antibiotics, immune serum was used to treat patients with pneumonia. This, however, led to the discovery of the serotypes of the pneumococcus when serum taken from convalescent patients showed serologic heterogeneity (Geno et al., 2015). In view of this, serotype-specific antisera were used to treat pneumococcal infections even though it was not always effective and not available for all serotypes. Investigations into the identification of pneumococcal serotypes increased when a Danish Prince died from pneumonia. The serogroup identified at that time was 9 but antisera available was for 9L and 9N. The death of the Danish Prince was actually caused by serotype 9V (Geno et al., 2015).

Currently, about 98 serotypes have been identified with about 96 of them known to be synthesized through the wyz-dependent pathway while the other two synthesized through independent biochemical pathways or synthase-dependent pathway (Wyres et al., 2013). The synthase-dependent mechanism is used in the manufacture of serotype 3 and serotypes 37 capsule where a single enzyme begins the synthesis of the capsules transferring a sugar to a lipid acceptor and subsequently adds more sugars across the cell membrane (Llull, García, & Ló Pez, 2001). The wyz-dependent mechanism involves the addition of nucleotide charged sugars to an undecaprenyl phosphate acceptor preceded by a flippase-mediated transfer across the cell membrane (Geno et al., 2015). Though many serotypes have been identified, about 23 of them are known to be involved in invasive pneumococcal diseases (WHO, 2019). In

other words, different serotypes have different propensities of causing diseases. In view of this, it is necessary for accurate identification of serotypes for surveillance especially during this post-vaccination era in order to detect the emergence of new serotypes and increase of serotypes that seldom found.

Phenotypic identification of pneumococcal serotypes

The phenotypic serotyping of pneumococcus mostly involves the use of antisera to detect serotypes or serogroups based on capsule epitopes. The use of the phenotypic methods is employed worldwide especially in hospital laboratories for diagnosis of infections since it does not require the use of any sophisticated machinery which are costly. This section of the review looks at the commonly used phenotypic methods of serotyping which includes quellung reaction, Latex agglutination and Enzyme-linked immunosorbent assay (ELISA), taking into consideration their strengths and weaknesses.

Quellung reaction

The quellung reaction is the known 'gold standard' for serotyping. It is highly sensitive and specific and, serves as the method to which other methods are compared to (Habib, Porter, & Satzke, 2014). This method involved the use of only 12 reagents since pooled sera identify a serotype of an unknown isolate (Geno et al., 2015). In this method, the antibodies in the rabbit antiserum react with specific capsule epitopes in pneumococcal suspensions. This however, causes a change in the refractive index of light passing through the capsule making the capsule look swollen (Jauneikaite et al., 2015). This approach is used worldwide but it is a slow and laborious process. Again, it is incapable of identifying multiple serotypes in a given sample (Habib et al., 2014).

Latex agglutination method

This phenotypic approach is also commonly used and has some advantages over the quellung. It is more accurate, rapid and less costly since a little amount of antiserum is required (Habib et al., 2014). Also, it requires less expertise to use. The latex agglutination method involves the use of latex beads coated with pooled rabbit antiserum capable of recognising multiple serotypes/serogroups. This test (Pneumotest-Latex) permits the serotyping of pneumococcus by mixing the fresh culture with factor-specific antiserum resulting in a visible clumping on a slide (slide agglutination) (Jauneikaite et al., 2015). The test identifies as much as 91 serotypes out of 98 serotypes.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was developed to detect serotypes of pneumococcus in serum, nasopharyngeal swabs and urine (Leeming, Cartwright, Morris, Martin, & Smith, 2005). Even though this approach has a high sensitivity, it requires high antibody titres to detect pneumococcal antigen. This test is also time-consuming where results are obtained after several days and the reagents used have a limited half-life (Jauneikaite et al., 2015).

Genotypic identification of pneumococcal serotypes

Knowledge of DNA sequences of the *cps* loci of the pneumococcus led to the discovery of many molecular capsular typing approaches. Due to the cost-effectiveness of these approaches, there is a worldwide use of it. Some of the commonly used molecular capsular typing approaches are multiplex PCR (mPCR), real-time PCR (RT-PCR), microarray and whole-genome sequencing (WGS).

Multiplex PCR (mPCR)

This is a type of PCR which was developed to identify 70 serotypes (Pai, Gertz, & Beall, 2006) especially the types that are predominant or known to cause pneumococcal disease including the vaccine serotypes. The 40 PCR primer are grouped into eight reactions/pools with each reaction containing five serotype-specific primers and an identification primer for *cps A*. One of the reactions has a sub-reaction group with primers for identification of serotypes 6C and 6D (Carvalho et al., 2010). This method has high sensitivity (100%) and it is suitable for serotyping pneumococcus in clinical samples (Jauneikaite et al., 2015). The reactions were arranged based on the most predominant serotypes to the least. Though it is rapid, it identifies only a limited number of serotypes. This is due to high sequence homology in the *cps* loci leading to the incomplete characterisation of isolates.

Real-time PCR (RT-PCR)

Another highly sensitive and fast method was developed to directly type pneumococcus directly from culture-negative but *lytA* positive or *cpsA* positive clinical specimen (Azzari et al., 2010). The real-time PCR was designed to have specific probes to detect *ply*, *wgz* and identifies 35 serotypes (Jauneikaite et al., 2015). The sequential triplex RT-PCR type detects the PCV-13 serotypes and 18 other serotypes (Pimenta et al., 2013). The RT-PCR method is less time consuming as compared to the mPCR due to the usage of gel electrophoresis. However, the process requires some level of expertise in optimising primers.

DNA microarray

The microarray was developed with 93 specific probes that detect the serotype-specific *wyz* and *wzx* genes with 12 polymorphisms that identify 22 serotypes and 24 serogroups (Raymond et al., 2013). The method developed by the St. George's medical school involves the preparation of a microarray slide which is scanned with a high-resolution scanner. The output is usually analysed using an empirical Bayesian model to detect pneumococcal serotypes and their relative abundance (Newton, Hinds, & Wernisch, 2011). This method is capable of detecting multiple serotypes in a given sample including carriage of other bacterial species and it is rapid and accurate. It is appropriate for detecting antibiotic resistance genes and novel serotypes (Kamng'ona et al., 2015). The limitations are that an expensive microarray scanner is required and an expert is needed to operate.

Next-Generation Sequencing (NGS) and Whole Genome Sequence (WGS)

Next-generation sequencing was first known to be an expensive approach. However, it is gradually reducing in cost. A method known as the target enriched NGS uses sequencing to identify all pneumococcus including 32 serotypes while the rest are in serogroups (Geno et al., 2015). This approach uses two primer pairs; one that confirms the presence of the pneumococcus species using *lytA* and the other that targets capsular regions of 500 bp in length which are later enriched and sequenced using MiSeq benchtop sequencer (Illumina) (Liyanapathirana et al., 2015). NGS is rapid and has a high throughput. The method can be applied directly to samples and can identify multiple serotypes (Ip et al., 2014). This approach requires bioinformatics expertise.

Whole-genome sequencing analyses of the entire pneumococcus capsular locus using a bioinformatics approach. The reads are mapped to published serotype-specific primer sequences where serotype-specific genes can be confirmed and single nucleotide polymorphisms (SNPs) can be investigated. This helps in distinguishing members of a serogroup especially within serogroup 6 and 11 (Calix et al., 2011). Moreover, it is also appropriate for identifying non-typable pneumococci using the presence of *dexB* and *aliA* primers (Chewapreecha et al., 2014). Whole-genome sequencing requires expertise in bioinformatics, analyses of data and preparation of reagents for sequencing.

Both the phenotypic and genotypic approaches of pneumococcus serotyping have revealed some limitations as shown in Table 1 where they are summarized based on specificity, the number of serotypes detected, level of training required, the time required for serotypes detection and detection of multiple serotypes. A good serotyping method is necessary for a rapid and yet accurate identification of the circulating serotypes. This helps in identifying correctly which serotype is currently causing invasive pneumococcal diseases and so should be included in a vaccine. Moreover, with the increasing discovery of new serotypes which are even more closely related like serogroup 6, an ideal serotyping approach should be able to easily distinguish between closely related serotypes. Even though the Quellung is the gold standard, the latex agglutination test is widely used for the identification of serotype due to the cost of the reagents which are lesser. The reagents used can be prepared in-house and reagents require robust quality control. The phenotypic methods, though widely used, are not rapid and not capable of detecting multiple

serotypes or directly from clinical samples as compared to the genotypic approach. The NGS and WGS methods are rapid and have high sensitivity but require high-level expertise and so are appropriate for research laboratories with such expertise since they are also giving detailed genetic characterisation and information of capsular switching events. This PCR method is highly sensitive, rapid and easy to use as compared to all the methods. It also facilitates the identification of multiple pneumococcal serotypes directly from clinical samples (Azzari et al., 2008). However, mPCR identifies only 40 serotypes including all vaccine types even though more than that are detected by RT-PCR which is more sensitive and so in this study, mPCR was used to serotype the pneumococcal strains.

Table 1: A Summary of the Pneumococcal Serotyping Methods Comparison

Criteria	Phenotypic			Genotypic				
	Quellung reaction	**Latex agglutination**	**ELISA**	**mPCR**	**RT-PCR**	**Microarray**	**NGS**	**WGS**
Specificity	N/A	N/A	98%	100%	98.6%	95.2%	97%	96%
No. of serotypes	91	91	23	70	35	43	38	94
Level of training	2-3	2-3	2	1	2	1-2	1-2	1-3
Time required for results	1-2 days	1 day	1 day	2-3 h	2-3 h	1 day	2-4 day	3-14 day
Detection of multiple serotypes	No	No	Yes	Yes	Yes	Yes	Yes	No

The information was retrieved from Jauneikaite et al. (2015). The specificity was in comparison to conventional methods. Level of training required to use an approach: 1- Basic experience in microbiology and molecular biology, 2-well trained or experienced personnel, 3- specialised knowledge in the use of bioinformatics tools. The time required to identify a serotype is dependent on the experience of the personnel and the number of samples. NGS: Next-Generation Sequencing; WGS: Whole Genome Sequence.

Nontypeable Pneumococci

A few years ago, 90 pneumococcal serotypes were identified and however the number of serotypes has risen to 98 in recent times (Wyres et al., 2013). This shows that novel serotypes are being detected every now and then using highly sensitive methods. This, also, means that there could be pneumococci with novel capsular structures that have not been typed by any antisera or are nontypeable (NT). Designating an isolate as NT is dependent on either of the following factors: Whether the isolates cannot be typed because of the limited number of serotypes a particular serotyping method can detect or the isolate is a non-encapsulated type. The isolate can be misidentified as *S. pneumoniae* or the isolates might have a downregulated capsule (Geno et al., 2015). Based on the genetic make-up of the *cps* loci of NT isolates, a few retain the characteristic *cps* locus composition (Group 1) while most of the NT isolates do not have the machinery that synthesizes the polysaccharide (Group 2) (Park et al., 2012).

In a study to determine the carriage of resistant strains of the pneumococcus, NTs were found to be *cps B*-negative and *aliC/aliD*-positive (Emgård et al., 2019). Group 1 NTs have a *cps* locus with defects mostly found in the *wchA/cpsE* gene. A typical example of such isolate is serotype 8. Group 2 NTs have *PspK/NspA* that compensates for the polysaccharide synthesis machinery by facilitating the epithelial adhesion and subsequent colonisation (Park et al., 2012).

In this study, multiplex PCR was used for serotyping pneumococcal isolates and since this approach can only identify 70 serotypes, there is the

possibility of having a higher number of NTs though they may be *lytA* and *cpsA* positive.

Circulation of Pneumococcus Serotypes among Children after PCV-13 introduction

During the pre-vaccination era, the circulation of pneumococcal serotypes was diverse worldwide (Dube et al., 2018). This was mostly attributed to environmental factors such as the geographical location, climate, time and genetic factors such as the recombination event said to occur at the *cps* locus of the pneumococcus (Donkor, Bishop, Antonio, Wren, & Hanage, 2011). Even though these circulations were diverse, they were dominated by serotypes such as 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F, 3, 6A and 19A which were also the main cause of invasive and non-invasive pneumococcal diseases. The introduction of the pneumococcal vaccines is said to have reduced the incidence of pneumococcal disease especially those by the antibiotic-resistant vaccine serotypes (Balsells, Guillot, Nair, & Kyaw, 2017). The introduction of the pneumococcal vaccines is known to have favoured capsular switching leading to serotype replacement. However, it has been reported that capsular switching has attributed to the recombinational event at the cps locus, is a spontaneous event (Kamng et al., 2015; Wyres et al., 2013). This occurs when the capsular locus is replaced with a locus from another serotype leading to vaccine escape by some resistant serotypes (Donkor et al., 2011). The section of the review looks at the distribution of pneumococcal serotypes among healthy children after the introduction of the 13 valent pneumococcal conjugate vaccines.

In Africa, DR Congo reported the persistence of some vaccine serotypes; 19F, 6, 14 followed by serotypes 11 and 15B/C (Birindwa et al., 2018). Moreover, similar results were reported in Tanzania where serotypes 6, 19F and 23F were 22%, 11% and 8% respectively followed by the non-vaccine serotypes 15B/C and 19B with 11% and 7% respectively (Emgård et al., 2019). Usuf et al. (2016) also confirmed the persistence of serotypes 19F and 19A with 6.8% and 8.5% in the Gambia, 5 years after the introduction of PCV-13. A similar study in Ghana by Dayie et al. (2019) showed a dominance of non-vaccine serotypes such as 23B (11%), 16F (10%), 11A (7%) followed by vaccine serotypes 23F (8%) and 19F (6%). In South Africa, non-vaccine serotypes: 15B/C, 10A, 21, 16F were mostly found among children (Dube et al., 2018).

In Europe, most of the detected circulating serotypes were of the non-vaccine types. In Denmark, Fjeldhøj et al. (2018) found that serotypes 21(13.6%), 23B (12.3%), 35F (11.1%) and 11A (11.1%) were mostly in circulation. Steens, Caugant, Aaberge, & Vestrheim (2015) also reported that in Norway, non-vaccine serotypes15B/C (9%), 21(6%), 22F (5%), 35F (6%), 6C (5%) and 23B (6%) were dominant in the study area. Non-vaccine serotypes 15A (13%), 15B (13%), 23B (9.1%) and 35B (9.1%) were the most dominant circulating in the United Kingdom (UK) (Devine et al., 2017), while in Cyprus and Italy non-vaccine serotypes still dominated (Hadjipanayis et al., 2016; Zuccotti et al., 2014). Detailed information can be found in Table 3.

In America, the serotype occurrences varied probably based on their schedule doses. In Brazil, 6C (12.2%), 11A/D (6.9%), 15B/C (9.2%) and 23A (5.3%) were detected by Neves et al. (2018) while in the USA, Lee et al.

(2014) reported the presence of non-vaccine serotypes in circulation with the exception of 19A even though the scheduled dose is 3p+1. However, in Asia, Chan et al. (2016) reported the use of the 3p+1 schedule dose in Hong Kong and the dominance of non-vaccine serotypes such as 15B/C, 23A, 15A/F and 6C. Despite, the effectiveness of the PCV-13 in Hong Kong, there has been a change in the scheduled dose from 3p+1 to 2p+1 in 2019 (www.info.gov.hk/gia/general/2019/10/P2019041000498).

The persistence of the vaccine serotypes was mostly detected in Africa countries as compared to the rest of the world. Moreover, most of these African countries used 3p+0 schedule dose in the immunisation programmes while most countries in Europe, Asia and America used either 3p+1 or 2p+1 scheduled dose. The difference in the scheduled dose could be one of the reasons for the varying occurrence of vaccine and non-vaccine serotypes.

Table 2: Dominating Serotypes in relation to Schedule Dose after PCV-13 implementation in Africa and Asia

Target population	Dominating serotypes (%)	PCV-13	Schedule dose	Reference
Africa				
Healthy children	19F, 11, 6, 10A, 14, 15B/C	2013	3p + 0	Birindwa et al., 2015
Healthy children	6 (22), 15B/C (11), 19F (11), 23F (8), 19B (7)	2012	3p + 0	Emgard et al., 2019
Healthy children	15B/C, 10A, 21, 16F, 9N, 9V, 19F	2011	2p + 1	Dube et al., 2018
Clinical isolates	19F, 6B, 14	2017	3p + 0	Torimiro et al., 2018
Clinical isolates	6B, 14, 1, 19F	2010	2p + 1	Diawara et al., 2015
Healthy children	23B (11), 16F (10), 11A (7), 23F (8), 19F (6)	2012	3p + 0	Dayie et al., 2019
Healthy children	19A (8.5),19F (6.8), 35B (6), 21(4.8)	2011	3p + 0	Usuf et al., 2018
Asia				
Healthy children	15B/C(16.7),23A (13.1), 15A/F (9.5), 6C (15.5)	2011	3p + 1	Chan et al., 2016
Clinical isolates	19F (33.5), 19A (14.1), 23F (12), 6A (8.8)			Pan et al., 2015
Clinical isolates	19F (42.6), 19A (8.5), 3(8.5), 6B (7.4)			Huang et al., 2015

Table 3: Dominating Serotypes in relation to Schedule Dose after PCV-13 implementation in Europe and America

Target population	Dominating serotypes (%)	PCV-13	Schedule dose	Reference
Europe				
Healthy children	21(13.6), 19F (2.5), 23B (12.3), 35F (11.1), 11A (11.1)	2010	2p + 1	Fjeldhoj et al., 2018
Clinical isolates	15A, 23B, 21, 18A, 35B, 33F, 15B	2010	2p + 1	Navne et al., 2016
Healthy children	15B/C (9), 21(6), 22F (5), 35F (6), 6C (5), 23B (6)	2011	2p + 1	Steens et al., 2015
Healthy children	11A, 23B, 24F, 35F, 16F	2010	2p + 1	Van Hoek et al., 2014
Clinical isolates	15A, 23B, 24F, 12F	2010	2p + 1	Van der Linden et al., 2015
Healthy children	6C (5), 23A (11), 33335F (13), 15A (5), 15B/C (8)	2010	3p + 0	Zuccotti et al., 2014
Healthy children	15B/C, 11A, 23B, 10A	2010	2p + 1	Southern et al., 2018
Clinical isolates	8(10), 12F (14.2), 10A (8.2), 15B/C (7.9), 3(6.9)	2010	2p + 1	Landhani et al., 2018
Healthy children	15A (13), 15B (13), 23B (9.1), 35B (9.1)	2010	2p + 1	Devine et al., 2017
Healthy children	23A (14.3), 15B (5), 15A (8.9), 6C (8.6), 23B (7.3), 19A (5.4)	2010	2p + 1	Hadjipanayis et al., 2016
America				
Healthy children	15B/C (15), 19A (3.5), 6C (2.8), 11A (2.1)	2010	3p + 1	Lee et al., 2014
Clinical isolates	33F,12, 22F, 15B/C,11, 23A	2010	3p + 1	Kaplan et al., 2015
Healthy children	6C (12.2), 11A/D (6.9), 15B/C (9.2), 23A (5.3)	2010	2p + 1	Neves et al., 2018

Pathogenesis of *Streptococcus pneumoniae*

Streptococcus pneumoniae usually is a commensal but under certain circumstances, it turns to be one of the most virulent human pathogens. The World Health Organization has stated that the pneumococcus is the fourth most frequent cause of bacterial infections such as pneumoniae and meningitis (WHO, 2014). The pneumococcus is also the frequent cause of other upper and lower respiratory infections such as otitis media, sinusitis and bronchitis (Infante, McCullers & Orihuela, 2015). The pneumococcus is transmitted to susceptible hosts through horizontal transmission via airborne droplets where it colonises the nasopharynx asymptomatically (carriage). Once the hosts immune system and/or resident microbiome is unable to clear the bacterium, the pneumococcus using the arsenal of virulence factors invades the underlining sterile tissues and organs thereby becoming pathogenic. The virulence factors help the pneumococcus to adhere to the epithelium, escape host immune response and invade tissues (Brooks & Mias, 2018). In summary, the life cycle of the pneumococcus involves transmission, colonisation and invasion, followed by the manifestation of diseases as shown in figure 2.

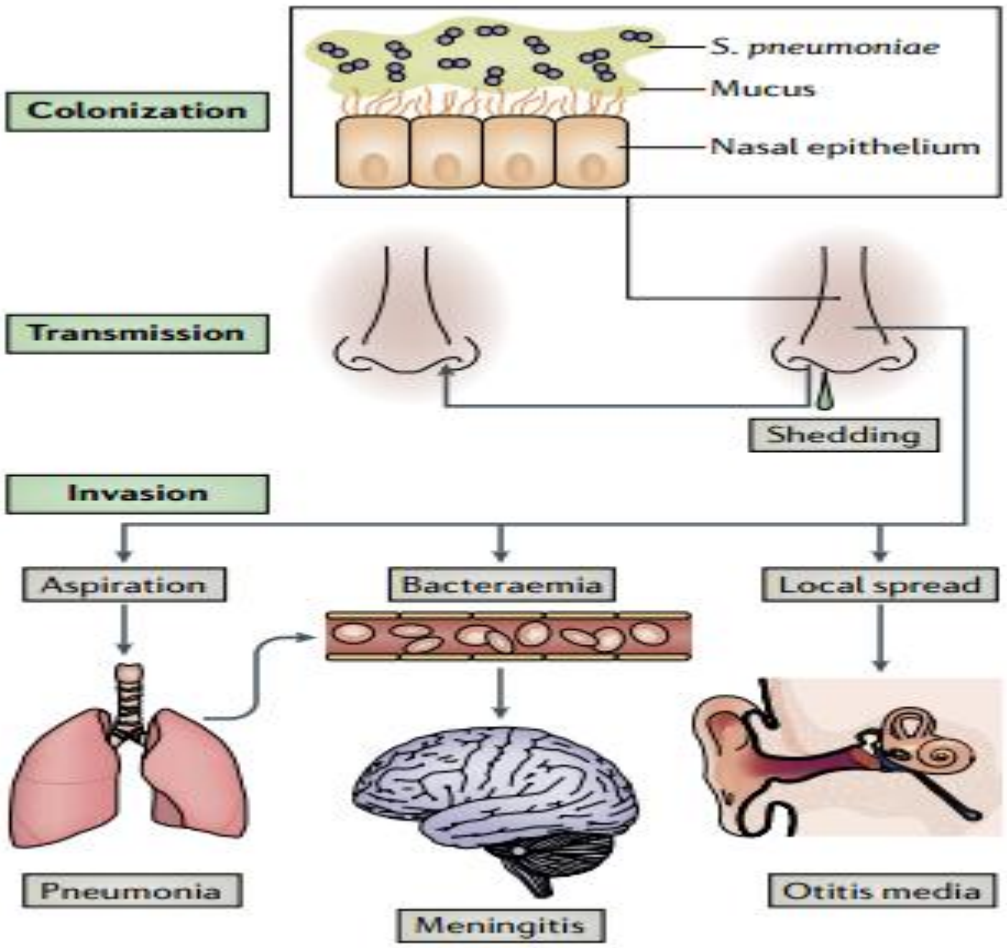


Figure 2. A diagram showing the overview of the life cycle of *S. pneumoniae. Acquisition of S. pneumoniae leads to mucosal colonisation. Colonisation precedes either transmission or invasion into tissues. Transmission to other hosts occurs through shedding of nasal secretions. Migration through the epithelium or endothelium leads to invasive diseases such as pneumonia (Weiser, Ferreira, & Paton, 2018).*

Transmission of *Streptococcus pneumoniae*

The nasopharynx is the main reservoir of respiratory bacteria, including *Streptococcus pneumoniae*. *Streptococcus pneumoniae* is normally transmitted via airborne droplets from carriers, especially children under two years meaning that infants and toddlers are the carriers or human reservoirs of the bacterium (Lipsitch et al., 2012). However, other studies have also suggested that older children are the ones transmitting the pathogen to younger

children and adults (Althouse et al., 2017). Close contact to carriers is not the only means by which the pathogen can be transmitted but indirectly via fomites. When fomites are contaminated with pneumococcus with biofilms, the pathogen can survive for about 4 weeks before drying out and this increases the chance of transmission (Marks, Reddinger, & Hakansson, 2014). The spread from these carriers is intensified during the dry cold months where the nostril secretions are more copious and viral infections increase (Numminen et al., 2015). Pneumococcal carriage is said to increase with increasing viral infections, younger age and crowdedness (Weiser et al., 2018). A study conducted to investigate the transmission of the bacterium showed that more of the pneumococcus was found in the nasal secretions shed by young mice with influenza A virus co-infection (Siegel, Roche & Weiser, 2014). Infection caused by the influenza A virus induce inflammation which in turn stimulates the increased flow of mucus (Siegel, Roche, & Weiser, 2014). Pneumococcus density is also associated with secretion volume as a result of childhood rhinorrhoea. Pneumolysin (*ply*), one of the virulence factors possessed by the pneumococcus, induces inflammation through its pro-inflammatory effects. This facilitates the clearance of the bacterium from the respiratory tract (Zafar et al., 2019). Encapsulated pneumococcus is readily shed and transmitted. This is because the thick capsule enables the escape of the bacteria from the mucus lining the respiratory tract while pneumococcus with no or thinner capsules are poorly shed because they bind effectively to mucins due to the adhering proteins that are poorly shielded by the capsule (Brooks & Mias, 2018).

Streptococcus pneumoniae efficiently transmits and avoids clearance with the use of its virulence factors. The mucosal surfaces of the host's respiratory tract are protected by the immunoglobulin A1 (IgA1) which facilitates the clearance of pathogens. The pneumococcus evades the action of IgA1 by releasing the IgA1 protease or zinc metalloprotease ZmpA which cleaves the hinge region of the IgA1 thereby avoiding the agglutination action of the antibody (Roche, Richard, Rahkola, Janoff, & Weiser, 2015). The cleaving of the mucosal IgA results in the change of the surface charge and increase in the physical proximity of the pneumococcal cell wall (Bogaert et al., 2004).

Colonisation mechanism of *S. pneumoniae*

The colonisation of *S. pneumoniae* is made possible by the cell wall and surface proteins, lying beneath the capsule. The capsule, however, is highly immunogenic and its antigenicity is type-specific. Colonisation involves the adherence of the pneumococcus and evasion of the mucosal immunity of the new host. Before the pneumococcus can attach to the epithelium, it encounters the mucus, which is composed of mucin glycoproteins and also contains antibodies and antimicrobial peptides (Weiser et al., 2018). The mucus layer contains nutrients for the pathogen and yet serves the purpose of preventing the bacterium from attaching itself to the epithelium. Neuraminidase A (NanA) and B-galactosidases (BgaA) enzymes of the pneumococcus also cleaves N-acetylneuraminic acid from mucins, glycoproteins, glycolipids and oligosaccharides on host cell surface thereby decreasing the viscosity of the mucus and exposing the N-acetyl-glycosamine receptors (Limoli, Sladek, Fuller, Singh, & King, 2011). The pneumococcus

binds asymptomatically to the N-acetyl-glycosamine component of the non-inflamed epithelial cells. The adherence of the pathogen is mediated by the pneumococcal surface adhesin A (PsaA), one of the cell-wall-associated surface proteins, by changing the physicochemical characteristics of the pneumococcus (Bogaert et al., 2004). The pneumococcus also has a number of adhesion proteins that aid in the adherence and for virulence. Pneumococcal adherence and virulence proteins A (PavA), and B (PavB) and enolase (Eno) binds to the fibronectin and plasminogen extracellular matrix proteins (Weiser et al., 2018). Moreover, choline-binding protein A (CbpA) binds to vibronectin and protein factor H. Studies are on-going to further understand the colonisation of the pneumococcus by discovering more adhesins as shown in Figure 3. Normally, colonisation of *S. pneumoniae* precedes the manifestation of the disease. The pneumococcal disease involves the release of inflammatory factors such as interleukin 1 (IL 1) and Tumor Necrosis Factor (TNF). Inflamed epithelial and endothelial cells upregulate receptors such as the platelet-activating-factor (PAFR) which have a high affinity for the pneumococcal cell wall choline (Bogaert et al., 2004). The binding of the cell wall choline to the PAFR facilitates the internalisation and transcellular migration of the pneumococcus through the respiratory epithelium and vascular endothelium of the host. Another cell surface protein, choline-binding protein L (CbpL) binds to the cytokine-activated cells via the sialic acid and Lacto-N-neotetraose to facilitate migration of the mucosal barrier (Gutiérrez-Fernández et al., 2016).

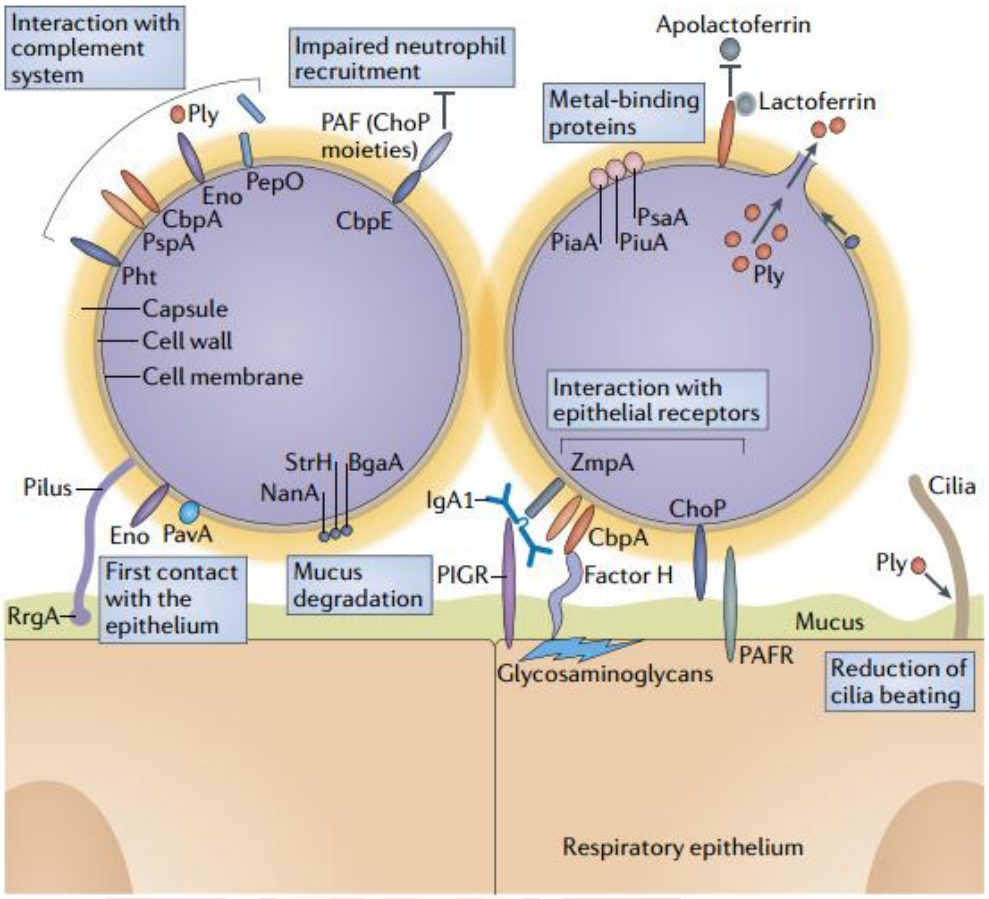


Figure 3. Colonisation mechanisms of *S. pneumoniae* to host epithelium. *Colonisation involves mucus degradation, epithelium attachment and evading the immune response. Enzymes such as neuraminidase A (NanA) and B-galactosidases (BgaA) as well as B-N-acetylglucosaminidase (StrH) degrades the mucus. The autolysin (LytA) causes the release of pneumolysin (Ply) which in turn degrades most of the cilia thereby reducing ciliary beating. Pneumococcal surface protein (PspA) acquires iron for the pathogen and also prevents the antimicrobial action of apolactoferrin. The pili, pneumococcal adherence and virulence proteins A (PavA), and B (PavB) and enolase (Eno) attaches to the epithelium by binding to suitable receptors. The IgA protease or zinc metalloprotease (ZmpA) avoids the action of the humoral immunity by cleaving IgA (Weiser et al., 2018).*

Risk factors for *S. pneumoniae* Colonisation in Healthy children

S. pneumoniae is known to be carried mostly by children, the elderly and individuals with weaker immune systems such as diabetic patients, HIV patients, those with cardiovascular diseases, cancers and smokers (CDC, 2017). Nasopharyngeal carriage of the pneumococcus among the healthy,

varies based on the geographical locations as already shown Table 4, time and age of the children (Bogaert et al., 2004). With regards to age, a cohort study done among children showed that the carriage of the pathogen reached a peak of 55% at age 3 years and dropped afterwards (Bogaert et al., 2004). Birindwa et al. (2018) showed that children two years and older had increased pneumococcal carriage. Previous hospitalisation and antibiotic use were also associated with *S. pneumoniae* carriage (Birindwa et al., 2018; Haile, Gidebo, & Ali, 2019). The pneumococcus is known to thrive during inflammatory conditions in the upper respiratory tracts. The inflammation occurs mostly due to viral infections as *S. pneumoniae* colonisation increases with the presence of Influenza A virus. Moreover, inflammation causes a conformational change at the surface of the host cell where there is the upregulation of receptors that facilitates the adherence of the pneumococcus (Weiser et al., 2018). Inflammatory conditions are normally characterised by running nose or rhinorrhoea of which the pathogen has been found to be associated among children with such signs as well as other respiratory symptoms (Chan et al., 2016; Fjeldhøj et al., 2018; Mills et al., 2015).

Table 4: Nasopharyngeal Carriage of *S. pneumoniae* among Healthy Children

Target population	carriage (%)	PCV-13	Schedule dose	Reference
Africa				
Healthy children	21.0	2013	3p + 0	Birindwa et al., 2015
Healthy children < 24 months	31.0	2012	3p + 0	Emgard et al., 2019 (Tanzania)
Healthy children < 50 weeks	54.0	2011	2p + 1	Dube et al., 2018 (South Africa)
Healthy children under 5 years	54.0	2012	3p + 0	Dayie et al., 2019 (Ghana)
Healthy children	21.5			Assefa et al., 2019 (Ethiopia)
Healthy children < 12 months	37.6	2011	3p + 0	Usuf et al., 2018 (The Gambia)
Asia				
Healthy children < 24 months	5.5	2011	3p + 1	Chan et al., 2016
Europe				
Healthy children < 24 months	26.0	2010	2p + 1	Fjeldhoj et al., 2018 (Denmark)
Healthy children < 60 months	62.0	2011	2p + 1	Steens et al., 2015 (Norway)
Healthy children under 5 years	47.7	2010	2 + 1	Van Hoek et al., 2014
Healthy children under 60 months	26.0	2010	3 + 0	Zuccotti et al., 2014
Healthy children under 5 years	51.9	2010	2 + 1	Southern et al., 2018
Healthy children under 4 years	32.0	2010	2 + 1	Devine et al., 2017
Healthy children	25.3	2010	2 + 1	Hadjipanayis et al., 2016
America				
Healthy children	31.0	2010	3 + 1	Lee et al., 2014

Immune Response to *S. pneumoniae*

The immune system has its mechanism of preventing colonisation of the pathogen and subsequent manifestation of pneumococcal disease. However, pneumococcal diseases do manifest due to age as children under five are known to have a naïve immune system whereas the elderly experience depreciating immune response. Other factors that influence nasopharyngeal carriage may also influence disease manifestation. The two arms of the immune response to the pathogen are discussed here, including how age affects their action.

Innate immune responses

Also known as the first line of defence, the innate immune response is non-specific where it recognises foreign particles using cells and receptors. The respiratory epithelial cells are the first defence line confronted by the pathogen. The epithelial cells prevent the pathogen from invading the underlying organs and tissues (Murphy & Weaver, 2017). The epithelial cells consist of goblet cells and ciliated cells. The goblet cells secrete negatively charged mucus that moisturises the surfaces and traps dust and pathogens whereas the cilia beat together in uniformity to move the trapped pathogens up the throat to the mouth (Whitsett & Alengha, 2015). The simultaneous action of the cilia with the mucus to clear pathogens is called mucociliary clearance (Nelson et al., 2007). The epithelial cells can release cytokines and chemokines when inflamed and also releases defensins, lysozyme and human apolactoferrin that can kill the pneumococcus. Lysozymes acts as a bactericidal agent and lyses cells whereas the apolactoferrin lyses cells and sequester iron (Whitsett & Alengha, 2015). However, the pneumococcus has

the potential to evade these responses. The capsule of the pathogen is negatively charged and so evades the mucus through electrostatic repulsion (Dockrell & Brown, 2015). The D-alanine in teichnoic acids of the cell wall of the pathogen deactivates the antimicrobial peptides (human apolactoferrin, lysozyme and defensins) by increasing the positive charge of their medium, the mucus which is further degraded by the action of the neuraminidase which removes sialic acids (Kovács et al., 2006). The pneumococcus under certain conditions undergoes phase variation where the capsule thickens to evade entrapment in mucus and thins to facilitate adherence to the host receptors (Mook-Kanamori, Geldhoff, van der Poll & van de Beek, 2011). Infants possess immature submucosal glands, goblet cells and also have a lower number of ciliated cells and so are incapable of mucociliary clearance.

Immune cells such as neutrophils and macrophages are utilised during innate immune response. Neutrophils are the most abundant of all white blood cells and usually the first to be recruited to the site of infection (Kolaczkowska & Kubes, 2013). Neutrophils kill pneumococcus either by releasing primary and secondary granules that breaks down the cell wall of pathogens or by phagocytosis. The primary granules contain defensins whereas the secondary granules contain lysozymes for cell lysis (Weiser et al., 2018). The macrophages are known as tissue monocytes. They kill pneumococcus directly through phagocytosis and they can be recruited by other immune cells including neutrophils via cytokine signalling (Arango & Descoteaux, 2014). Macrophages undergo opsonophagocytosis where they engulf pathogens that have been opsonized by the complement system by binding to the Fc portion of immunoglobulins using their receptors called the macrophage receptor with

collagenous structure (MARCO) which also helps in engulfing non-neutralised antigens (Dockrell & Brown, 2015). Macrophages are activated by the toll-like receptors (TLRs) 2 and 4 based on the pattern recognition receptors found on the pneumococcus (Dockrell & Brown, 2015). Infants receive minimum protection by the neutrophils due to reduced cytokine signalling, impaired phagocytic activity and poor bactericidal function (Simon, Hollander & McMichael, 2014). Macrophage function is usually in infants and there is poor phagocytic function as well as low TLR4 function (Simon, Hollander & McMichael, 2014).

The pattern recognition receptors (PRRs) are either secreted or located intracellularly and the recognise pathogen-associated molecular pattern molecules (PAMPs) found on pathogens especially viruses and bacteria. Toll-like receptors (TLRs) and Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are examples of the PRRs that recognise PAMPs (Murphy & Weaver, 2017). TLRs on the cell surface activates TLR signalling pathways that lead to the recruitment of neutrophils and cytokine production. So far, 10 TLRs have been identified but TLR2, TLR4 and TLR9 are known to be involved in pneumococcal cell wall identification (O'Neill, Bryant & Doyle, 2009). TLR2 binds to pneumococcal lipoproteins and peptidoglycan and also plays a role in shedding and transmission since it has been found that mice with deficient TLR2 were responsible for pneumococcal transmission (Tomlinson et al., 2014). TLR4 on the other hand recognises pneumolysin (Srivastava et al., 2005) whereas TLR9 recognises bacterial DNA since it is intracellularly located. TLR9 pathway releases cytokines when activated (Kawai & Akira, 2009). The TLRs activates T cells through the secretion of

co-stimulatory molecules, thus, indirectly activates the adaptive immune response (Kawai & Akira, 2009). In contrast to TLRs, NLRs are intracellular and they stimulate the nuclear factor-kappa B (NF-kB) and controls inflammation (Murray, 2009). NLR recognises bacterial peptidoglycan fragment called muramyl-dipeptide, located in the cytosol. This leads to some cascade of events that lead to the recruitment of macrophages and monocytes (Davis, Nakamura & Weiser, 2011).

Adaptive immune responses

In contrast to innate immune response, the adaptive immune response is slow to act, thus in several days after infection and response to specific antigens (Murphy et al., 2016). The adaptive immunity has two response type; the humoral immune response which is characterised by the action of B cells to specific antigens through the production of antibodies and the cell-mediated immune response characterised by the presence of T cells, including its ability to activate other immune cells that has direct actions on pneumococcal cells (Murphy et al., 2016).

Both B cells and T cells are manufactured in the bone marrow. The B cells mature in the bone marrow into antigen-specific antibodies producing cells called plasma cells (Murphy et al., 2016). Colonisation of the pneumococcus at the mucosal lining is controlled by the immunoglobulin A. The function of the IgA is to opsonise the bacterial cell and promote phagocytosis (Mook-Kanamori, Geldhoff, van der Poll & van de Bee, 2011) However, *S. pneumoniae* evades this attack by releasing IgA proteases that cleave the antibody leaving the Fab portion of the antibody still bound to the bacterial antigens. This reduces the negative charge of the capsule and

facilitate host cell adhesion (Weiser et al., 2003). B cells are capable of class switching by differentiating into memory IgM B cells to facilitate pathogen clearance (Zhang et al., 2015).

Though both B cells and T cells are manufactured in the bone marrow, immature T cells migrate to the thymus where they mature into T helper ($CD4^+$) cells and cytotoxic ($CD8^+$) T cells (Murphy & Weaver, 2017). During pneumococcal infections, $CD4^+$ cells are activated by co-stimulatory molecules secreted via the TLR pathway and by antigen-presenting cells (APCs). APCs present foreign antigens on the major histocompatibility complex (MHC) proteins to T cells. Activation of $CD4^+$ cells leads to the differentiation of the cell into Th1 and Th2 cells (Murphy & Weaver, 2017). Th1 helper cells releases interferon-gamma (IFN-y) thereby stimulating cell-mediated immune response through the activation and recruitment of macrophages (Weiser et al., 2018) whereas Th2 cells release interleukin 4 (IL-4) which activates the humoral immune response (Weiser et al., 2018). Th17 releases the cytokine Interleukin 17 (IL-17) during pneumococcal infection and the IL-17 recruits immune cells such as neutrophils and macrophages to the site of infection to promote clearance. However, the action of the Th17 is regulated by the Tregs (Hoe et al., 2017).

B cells in infants have downregulation of co-receptors and so have limited response to foreign antigens. There is also incomplete immunoglobulin class switching and low somatic hypermutations (Simon, Hollander & McMichael, 2015). Maternal IgG antibodies protect for 4 weeks until they deplete but the infant's humoral immunity begins to develop only after two

years. However, studies have shown the presence of IgM antibodies after pneumococcal infection and carriage (Infante, McCullers & Orihuela, 2015). Infants although having skewed Th2 responses have a different population of T cells called γδ T cells that release IFN-γ thereby eliciting a Th1 type immune response (Simon, Hollander & McMichael, 2015).

Invasive Pneumococcal Diseases caused by *S. pneumoniae*

S. pneumoniae is a facultative anaerobe residing asymptomatically in the upper respiratory tract of 20-50% of children and 5 -20% of adults worldwide (Adegbola et al., 2014). Two fates follow colonisation, thus, either they are transmitted to other susceptible hosts or invading underlying tissues or organs using virulence factors that also enable the pathogen to evade immune responses (Kadioglu, Weiser, Paton & Andrew, 2014). Once the pathogen has evaded the immune cells before and after colonisation, it multiplies, forms biofilms, disrupts the colonisation of the non-pathogen microbiome and migrates to surrounding sterile tissues and organs. The pneumococcus can cause localised infections such as sinusitis and otitis media but can also cause invasive diseases such as bacteraemia, pneumonia and meningitis (CDC, 2015).

Pneumococcal meningitis

Meningitis is the inflammation of the three protective layers of the spinal cord and the brain, called meninges. During inflammation, there is upregulation of PAFR which facilitates the interaction with the Chop moieties thereby helping the pneumococcus to breach the blood-brain barrier (BBB) utilising the PAFR recycling pathway (Isaacman, McIntosh & Reinert, 2010). The pneumococcus can also breach the BBB through the binding of the CbpA

to the laminin receptor found on the brain microvascular endothelium (Donati et al., 2010) or the binding of RrgA to polymeric immunoglobulin receptor (PIGR) and platelet endothelial cell adhesion molecule 1 (PECAM 1) by pneumococcal strains with pilus 1 (Donati et al., 2010).

Pneumococcal meningitis is among more than 50% of all bacterial meningitis in the United States and normally occurs in young children. In Ghana, there have been several reports on the occurrence of pneumococcal meningitis. In 1992, Mackie et al. (1992) reported that over 50% of bacterial meningitis in Kumasi was caused by *S. pneumoniae,* with a mortality rate of 36.4%. A similar case of meningitis was reported in 2005 by Leimkugel et al. (2005). In 2016, a large outbreak of meningitis was reported in the Brong Ahafo region of Ghana. The prevalence of *S. pneumoniae* in the epicentre district was 363 suspected cases per 100,000 people (Kwambana-Adams et al., 2017).

Pneumococcal pneumonia

Pneumococcus residing in the nasopharynx can migrate to the lungs where it causes an infection called pneumonia (Kadioglu, Weiser, Paton & Andrew, 2008). The infection leads to the recruitment of neutrophils and other immune cells as a characteristic of inflammation, causing the air sacs to be filled with fluid leading to difficulty in breathing, increased heart rates and coughing frequently (Wunderink & Waterer, 2014). Pneumonia is a major pneumococcal disease and it is mostly community-acquired (CDC, 2015). It spreads from person-to-person, outside the hospital, by contact in the community through the breathing in of aerosol droplets from a carrier (Steel, Cockeran, Anderson & Feldman, 2013). Community-acquired pneumonia is

still the leading cause of death in children under five and there are approximately 900,000 cases of pneumonia worldwide (World Health Organization, World Pneumonia Day 2017, Available from http://www.who.int/life-course/news/events/2017-world-pneumoniaday/en/). Even though the introduction of the PCV has reduced the incidence of pneumococcal diseases, pneumococcal disease is still a public health burden (Figure 4).

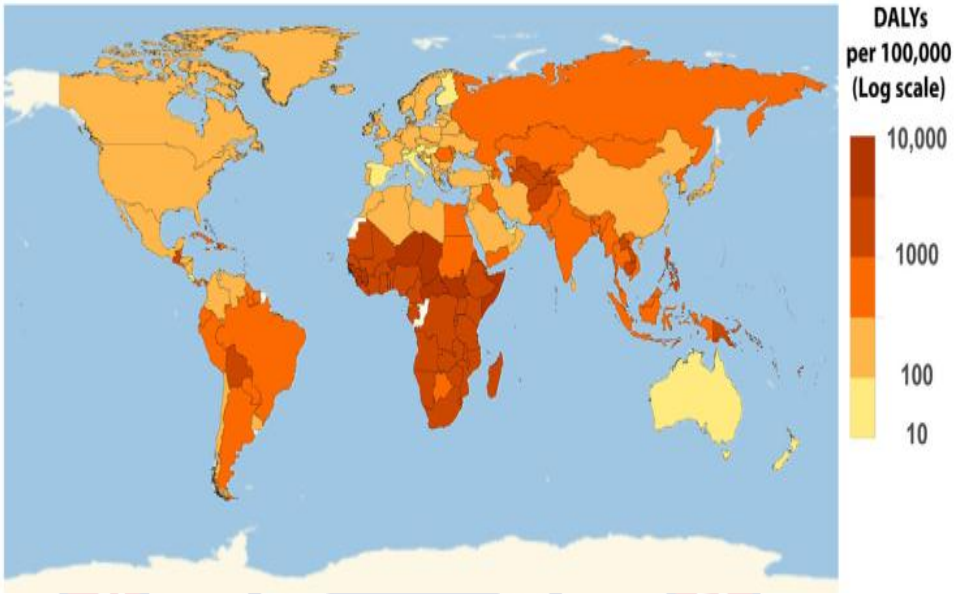


Figure 4. Global disability-adjusted life year (DALY) of pneumococcal pneumonia. Distribution of pneumococcal pneumonia on a log 10 scale in 2016 per 100,000. Information retrieved from Brooks and Mias, (2018).

Bacteraemia

Bacteraemia is simply defined as the presence of bacteria in the blood (CDC, 2015). Bacteraemia precedes a number of pneumococcal diseases such as meningitis and septicaemia and it is responsible for 12,000 cases per year (CDC, 2015). The pneumococcus is able to traverse the endothelium through the interaction between ChoP and PAFR or CbpA and PIGR leading to

hematogenous spread. Once in the blood, the capsule thickens and there is upregulation of surface proteins such as PspA (Weiser et a., 2018).

Bacteraemia is not the only means by which the pneumococcus can attach the BBB and traverse it. However, localised infections such as sinusitis can precede meningitis and such a non-haematogenous spread is facilitated by exogenous sialic acid (Weiser et al., 2018). Sinusitis occurs as a consequent of the presence of the pneumococcus in the fluid trapped in the sinuses (CDC, 2015). *S. pneumoniae* can also migrate to the middle ear to cause an infection known as otitis media. Otitis media normally occurs in young children and Centers for Disease Control (CDC) has reported that about 60% of children have at one point in the life had an ear infection (CDC, 2015).

Common Antibiotics Used for the Treatment of *S. pneumoniae* Infections and their Resistance Mechanisms

Pneumococcal diseases were on the rise until they were managed with antibiotics such as penicillin in 1967. Afterwards, other classes of antimicrobials such as macrolides and quinolones were mostly used in the treatment of pneumococcal diseases. However, resistance to these antimicrobials increased which compelled doctors to use particular agents for specific diseases. This review focuses on the mechanism of action of the commonly used antibiotics, the mechanism of resistance of the pneumococcus to these drugs and its epidemiology.

Beta-lactam antibiotics- Penicillins and Cephalosporins

Beta-lactam agents such as penicillin and cephalosporins are characterised by their core structure which is the beta-lactam nucleus (Rantala, 2009). Penicillin was the first beta-lactam to be discovered and used for the

treatment of bacterial infections including those caused by pneumococci. The original penicillin, penicillin G, was improved to provide a broader spectrum of activity and have increased activity against beta-lactamases (Greenwood et al., 2012). Cephalosporins, even though are beta-lactam agents, have a beta-lactam ring attached to six-membered dihydrothiazine ring instead of a five-membered thiazolidine ring as seen in penicillins and they also exhibit a broad-spectrum activity than almost all the penicillins (Greenwood et al., 2012).

Mechanism of action of beta-lactams

Beta-lactams are used for the treatment of all pneumococci infections, non-meningitis, meningitis and pneumonia. In Ghana, penicillins are still the first-line treatment for pneumococcal infections such as pneumonia in hospitalised children (Ministry of Health, 2010). Second-generation cephalosporins such as cefuroxime are recommended as a second-line treatment for pneumococcal infections whereas Ceftriaxone which is a third-generation cephalosporin is used for the treatment of meningitis (Ministry of Health, 2010).

Beta-lactams are bactericidal agents and they kill bacteria cells by inhibiting cell wall synthesis. The cell wall of the pneumococcus is known as the peptidoglycan which is composed of N-acetylmuramic acid and N-acetylglucosamine residues that are crosslinked (Rang, Ritter, Flower & Henderson, 2015). The penicillins and cephalosporins interfere with the synthesis by inhibiting the transpeptidation by covalently binding to penicillin-binding proteins (PBPs) therefore leading to the prevention of the formation of

the crosslinks. This is due to the fact that the PBPs have transpeptidase and carboxypeptidase activities (Rang et al., 2015).

Mechanism of resistance to beta-lactams

The susceptibility of pneumococcal isolates to beta-lactams such as penicillin and cephalosporins is usually tested using the disk diffusion method. For penicillin, oxacillin disk (1ug) is used to identify penicillin non-susceptible pneumococci where isolate with a zone of inhibition of less than 20 was considered to be non-susceptible (CLSI, 2017) whereas ceftriaxone non-susceptible pneumococci are reported when the zone of inhibition is less than 26mm (Jorgensen et al., 1994). However, the use of the disk diffusion might be inappropriate in determining whether an isolate is resistant or not and do not correlate with minimum inhibition concentration (MIC) results. In view of this, MIC testing should be done for non-susceptible isolates (Cavalieri et al., 2005).

S. pneumoniae confers resistance to beta-lactams such as penicillin and cephalosporins through the alterations in three out of six PBPs: PBP1a, PBP2b and PBP2x. The altered genes can be obtained from other closely related members through transformation followed by recombinational events. Alterations in the PBP genes reduces the affinity of the beta-lactams to the target site. An intermediate resistance is caused by alterations in either PBP2b or PBP2x whereas full-blown resistance is caused alternations in all three PBP genes (Moujaber et al., 2018; Nagai et al., 2001). Alterations in PBP1a and PBP2x confers resistance to cephalosporins such as cefotaxime and ceftriaxone (Cavalieri et al., 2005). Moreover, the PBP genes are not the only ones that can confer resistance to penicillin and cephalosporins. The MurMN

operon encodes for the proteins, MurM and MurN, which replaces linear muropeptides with branched ones thereby causing abnormalities in the peptidoglycan synthesis (Smith & Klugman, 2001).

Glycopeptides - Vancomycin

Vancomycin is a tricyclic glycopeptide antimicrobial. It is produced from a nocardioform actinomycete called *Amycolatopsis orientalis* (Rantala, 2009). Vancomycin has a large molecule and so are unable to penetrate the outer membrane of Gram-negative bacteria (Greenwood et al., 2012). Another glycopeptide is teicoplanin which is long-lasting as compared to vancomycin (Rang et al., 2015).

Mechanism of action

Vancomycin is restrictively used to treat infections caused by Gram-positive bacteria and the last resort for multidrug-resistant pneumococcus where it is combined with gentamycin to increase the intracellular concentration of gentamicin (Moujaber et al., 2018).

Even though vancomycin exerts its activity by inhibiting cell wall synthesis just like penicillin and cephalosporin, its mechanism is different. Thus, vancomycin inhibits cell wall synthesis by inhibiting the release of the synthesis building blocks thereby preventing the growing chain of the peptidoglycan (Rang et al., 2015).

Mechanism of resistance

Currently, there are no reported cases of Vancomycin-resistant pneumococci isolates and so any detected resistant isolates may be associated with identification errors or antibiotic susceptibility testing errors (Cavalieri et

al., 2005). In view of this, there are no resistant breakpoints for isolates to vancomycin (CLSI, 2017). However, vancomycin-tolerant pneumococci have been reported in Korea (Sung et al., 2006). Vancomycin-tolerant pneumococci were discovered to have a mutation in the histidine kinase gene, vncS and this mutation leads to suppression of autolytic activity (Moujaber et al., 2018).

Amphenicols - Chloramphenicol

Chloramphenicol is a bacteriostatic antibiotic with a broad-spectrum activity. It is a nitrobenzene-containing moiety, produced naturally from *Streptomyces venezuelae*. However, it is not frequently used due to the possible occurrence of a side effect such as aplastic anaemia although rare (Greenwood et al., 2012).

Mechanism of action

Chloramphenicol inhibits protein synthesis. Protein synthesis in prokaryotes is such that the 30S subunit of the ribosome binds to the messenger RNA after which the 30S subunit attaches to the 50S subunit to form a 70S subunit. The 70S subunit moves along the mRNA from one codon to the other and from the A position to the P position on the ribosome. Chloramphenicol inhibits transpeptidation by reversibly binding to the 50S subunit and prevents the binding of the amino acid-containing tRNA to the acceptor site thereby preventing peptide bond formation (Rang et al., 2015)

Mechanism of resistance

The determination of resistant isolates can be done using the disk diffusion method where resistant isolates show a zone of inhibition of less than 21mm whereas a MIC breakpoint of less than 9ug/ml (CLSI, 2017).

Chloramphenicol-resistant pneumococci isolates are rarely detected worldwide.

Pneumococci isolates become resistant to chloramphenicol through the acquisition of the plasmid chloramphenicol acetyltransferase (*cat*) which is encoded by the cat gene, a gene that reduces the ability of the drug to attach to the ribosome. This occurs by modifying the enzymes associated with chloramphenicol acetylation leading to the generation of O-acetoxy chloramphenicol products (Moujaber et al., 2018).

Tetracyclines – Tetracycline

Tetracyclines are also bacteriostatic antibiotic with a broad-spectrum activity. They were first discovered in the late 1940s. Tetracyclines are composed of four linear six-membered hydrocarbons which are fused (Rantala et al., 2009). Examples of tetracyclines are doxycycline, oxytetracycline, chlortetracycline, tetracycline, minocycline and tigecycline.

Mechanism of action

Tetracyclines are active against both Gram-positive and Gram-negative bacteria, some atypical bacteria and protozoan parasites such as *plasmodium* spp. They are also used to treat respiratory tract infections such as chronic bronchitis and community-acquired pneumonia (Rang et al., 2015). Tetracyclines also inhibit protein synthesis. During protein synthesis, a tRNA with an amino acid binds to the A site in the ribosome by complementary base-pairing which later moves to the P site. Tetracycline enters the bacterial cell by passive diffusion through porins and competes with the amino-acyl

tRNA for the A site on the mRNA-ribosomal complex (Greenwood et al., 2012).

Mechanism of resistance

Pneumococci isolates are considered to be susceptible when the zone of inhibition is above 27mm and a MIC breakpoint of 1ug/ml or less (CLSI, 2017). Due to the increase of tetracycline-resistant pneumococci, tetracycline is rarely used in the treatment of pneumococcal diseases (Rang et al., 2015).

Pneumococci isolate exhibit resistance to tetracycline using one of the two mechanisms: acquisition of *tet* genes such as *tetA*, *tetB*, *tetC* and *tet31* which encodes for efflux pumps or *tetT*, *tetW*, *tetM* and *tetO* which encodes for proteins that protect the ribosome (Moujaber et al., 2018). Another genetic element, formerly called omega *cat-tet* but now called Tn5253, has been discovered that contains two resistance genes that confer resistance to both tetracycline (*tet*) and chloramphenicol (*cat*) (Moujaber et al., 2018).

Macrolides, Lincosamides and Streptogramin B (MLS_B)

Macrolides are antimicrobials with many-membered macrocyclic lactone rings which are attached to one or many deoxy sugars. The 14-membered macrolide includes Erythromycin, which is naturally obtained from an actinomycete called *Saccharopolyspora erythraea.* The lactone ring of erythromycin is attached to L-cladinose and D-desoamine (Rantala et al., 2009). There are 15-membered macrolides such as Azithromycin, a semisynthetic derivative of erythromycin, and an azalide and 16-membered macrolides such as Spiramycin (Greenwood et al., 2012).

The lincosamide, Clindamycin superseded lincomycin due to it remarkable activity against Gram-positive bacteria such as streptococci, staphylococci and anaerobic bacteria whereas Streptogramins such as quinupristin and dalfopristin have synergistic effects (Greenwood et al., 2012).

Mechanism of action

Erythromycin has a spectrum of activity similar to penicillin; it is effective against many Gram-positive bacteria but not Gram-negative rods. The macrolides have bactericidal activity by preventing protein synthesis. During protein synthesis, the transfer RNA is translocated from the A site to the P site and then to the E site, in relation to the messenger RNA. Macrolides bind to the 50S ribosomal subunit of the rRNA, specifically the domain V of the 23S rRNA within a tunnel that serves as a channel for growing the peptide chain thereby preventing the translocation of the tRNA. This leads to the premature release of the peptide chains (Rang et al., 2015).

Clindamycin is also effective against many penicillin-resistant staphylococci. It has a similar mechanism of action as the macrolides and chloramphenicol (Rang et al., 2015). Streptogramins such as quinupristin and dalfopristin have poor activity when used alone but when used together, they produce a synergistic effect methicillin-resistant staphylococcus (Rang et al., 2015). Streptogramins also act on the 50S rRNA as macrolides. Dalfopristin acts on the 50S rRNA, blocking the peptidyl transferase centre and causing a conformational change. This leads to an increase in the concentration of quinupristin by 100-fold, interfering with polypeptide chain formation (Rantala et al., 2009).

Mechanism of resistance

S. pneumoniae exhibits two major mechanisms against macrolides: methylation of the ribosome encoded by the *ermB* gene and macrolide efflux pumps encoded by the *mefA* gene. The phenotypic expression of *ermB* and *mefA* or *mefE* is known as MLS_B and M phenotypes respectively. The third mechanism of resistance occurs but it is rare. It involves mutations in the 23S rRNA or protein L4 or L22 of the ribosome (Mosleh, Gharibi, Alikhani, Saidijam, & Vakhshiteh, 2014).

The *ermB* gene confers resistance to macrolides (MIC > 1ug/ml) and cross-resistance to lincosamides (MIC > 1ug/ml) and streptogramins (MIC > 4ug/ml) (CLSI, 2017) by methylating adenine a position 2058 (A2058) leading to a reduction in the binding affinity to domain V 23S rRNA (El Ashkar et al., 2017). Some isolates express MLS_B resistance constitutively ($cMLS_B$) whiles others are not normally induced to express the gene ($iMLS_B$). Pneumococcal isolates that have the inducible *ermB* gene are normally resistant to the 14- and 15-ring member macrolides and susceptible to the 16-ring member macrolides, lincosamides and streptogramin. The D-test is mostly done to detect $iMLS_B$ (Rantala et al., 2009). The presence of the *mefA* gene encodes for the macrolide efflux pumps which causes resistance to 14- and 15-ring member macrolides only (El Ashkar et al., 2016).

The *mef* genes are examples of the major facilitator superfamily (MFS) where there is the extrusion of drugs coupled with ion exchange. Even though *mefA* and *mefE* have 90% similarity at the DNA level, they are found at different locations on the pneumococcal chromosome elements: *mefA* is found

on a chromosomal element while *mefE* is carried on the macrolide element genetic assembly (Rantala et al., 2009).

Fluoroquinolones

Quinolones such as nalidixic acid have a narrow spectrum of activity and so was used for the treatment of urinary tract infections caused by Gram-negative bacteria before it was superseded by broad-spectrum antimicrobials such as ofloxacin, levofloxacin and moxifloxacin (Rang et al., 2015).

Mechanism of action

Fluoroquinolones are effective against Gram-negative and Gram-positive bacterial infections including penicillin and cephalosporin-resistant bacteria and members of Enterobacteriaceae (Greenwood et al., 2012). Fluoroquinolones exert their bactericidal activity by inhibiting the action of DNA gyrase, also known as topoisomerase II in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria which is involved in the negative supercoiling in DNA leading to the inhibition of transcription and replication (Rang et al., 2015).

Mechanism of resistance

Resistance to fluoroquinolones is mostly mediated by mutations in genes such as *gyrA*, *gyrB*, *parC* and *parE*, collectively called quinone-resistance-determining regions (QRDRs) (Moujaber al., 2018). Mutations in only *parC* leading to amino acid substitution confer low-level resistance in *S. pneumoniae* but the addition of mutations in *gyrA* confers high-level resistance. Mutations in *parC* can be either Ser79Phe, Ser79Tyr, Asp83Tyr or Asp83Gly whereas mutations in *gyrA* can be Ser84Phe, Ser84Tyr or Glu85Lys

(Bast et al., 2000). Mutations in *gyrB* and *parE* also confer resistance to fluoroquinolones but with minimal effects.

Sulfonamides - Trimethoprim and Sulfamethoxazole (co-Trimoxazole)

Sulfonamides were first discovered before the discovery of penicillin in the 1960s (Rantala et al., 2009). Sulfonamides were used in the treatment of infectious diseases in a dye known as protonsil dye. The structural formula of sulfonamides is the structural analogues of para-aminobenzoic acid (PABA), a molecule needed for the manufacture of folic acid in bacteria (Rang et al., 2015).

Mechanism of action

Due to the increasing resistance to sulfonamides, sulfamethoxazole was administered in combination with trimethoprim. Both drugs act to inhibit folate synthesis in bacterial cells by competing with PABA and they both broad spectrum of activity (Cattoir, 2016).

Sulfonamides inhibit the folate synthesis pathway by competing with PABA for the dihydropteroate synthetase thereby inhibiting the action of the enzyme, that is, the conversion of PABA to folate. The folate is the precursor for the synthesis of thymidylate. Trimethoprim also interferes with a step in the folic acid synthesis by inhibiting the action of dihydrofolate reductase. This is possible due to its high affinity to the enzyme because of the structural similarities with folate (Rang et al., 2015).

Mechanism of resistance

The first co-Trimoxazole-resistant pneumococcus was identified in 1972 and since then there have been increasing resistance reported worldwide.

Co-Trimoxazole-resistant isolates (zone diameter ≤ 15mm or MIC ≥ 4/76µg/mL) (CLSI, 2017) are known to have point chromosomal mutations in *folA* and *folP*. *folA* and *folP* encode for the dihydrofolate reductase and dihydropteroate synthase respectively (Moujaber et al., 2018). Resistance to Trimethoprim is characterised by single amino acid substitution (Ile100Leu) in the *folA*-encoding enzyme, while resistance to sulfamethoxazole is characterised by the insertion of mostly two amino acids in the *folP*-encoding enzyme sulphonamide-binding site (Cornick et al., 2018).

Pneumococcal Vaccines

Pneumococcal vaccines are introduced to provide serotype-specific protection against invasive pneumococcal disease-causing serotypes and prevent the spread of antibiotic resistance. The first case of vaccination was recorded in 1911 where a group of South African gold miners were vaccinated with a whole-cell pneumococcus vaccine which yielded 25% to 50% effectiveness and also led to the discovery of serotype-specific pneumococcus (Geno et al., 2015). In this section of the literature review, emphasis will be on the types of pneumococcal vaccines, their mechanisms and limitations.

Polysaccharide vaccine (PS vaccine)

The first pneumococcal polysaccharide vaccine (PPV) was introduced in the 1940s and even though it was effective, it was still withdrawn due to the success rates of chemotherapy at that time. In view of that, doctors preferred to treat pneumococcal infections with antimicrobials (Geno et al., 2015). The PPV-14 was reintroduced in the 1970s due to the increase in case fatalities as a result of treatment failures associated with antibiotic resistance. PPV-14 was licensed in 1977 and later used in the US in 1983 (Goonewardene et al., 2019).

The current PS vaccine, PPV-23, contains an appreciable amount of CWPS, 25µg polysaccharide per each contained in the vaccine and has wider coverage than the currently used conjugate vaccine (Table 5) (Geno et al., 2015). PS vaccines stimulate an immune response by directly stimulating B cell response without depending on the T cells (Figure 5). This, however, leads to poor immunogenicity in children below 2 years and produces no B cell memory (Song, Moseley, Burton & Nahm, 2013). Other shortcomings that come with the use of this vaccine is that it is not effective against community-acquired pneumonia mostly found in young children and IPD in immunocompromised individuals (Moberley, Holden, Tatham & Andrews, 2013). PS vaccines are also not effective in preventing nasopharyngeal colonisation and exhausts memory B cell concentrations (Geno et al., 2015).

Pneumococcal conjugate vaccine (PCV)

The pneumococcal conjugate vaccine was introduced to provide increased protection among children under 2 years since about 80% of IPDs occur in those children. To increase the immunogenicity, PCVs are made such that the polysaccharides are conjugated to proteins (Goonewardene et al., 2019). However, the immunogenicity depends on the method of conjugation, the amount of the polysaccharide and the type of carrier protein. PCV-7 was the first PCV vaccine to be discovered and it was licensed in the US in 2000 (CDC, 2010). This was later followed by PCV-10 and PCV-13 to provide a wider serotype coverage. PCV-10 was first introduced in Canada in 2008 whereas PCV-13 was first approved in Chile in 2009 (Geno et al., 2015). The licensed PCVs are summarised in Table 5.

Table 5: Comparisons of Pneumococcal Vaccines

Vaccine	Year licensed	Carrier Protein	Serotypes	Conjugation method
PPV-23	1983	None	1, 2, 3, 4, 5, 6B, 7F, 8, 9V, 9N, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F	None
PCV-7	2000	CRM_{197}	4, 6B, 9V, 14, 18C, 19F, 23F	Reductive amination
PCV-10	2008	NTHi protein D; tetanus toxoid; diphtheria toxoid	PCV7 + 1, 5, 7F	Bifunctional spacer
PCV-13	2009	CRM_{197}	PCV10 + 3, 6A, 19A	Reductive amination

Adapted from Geno et al. (2015).

PCVs act by indirectly stimulating B cell immune response via T cell activation. This leads to the production of antibodies for a longer period and availability of memory cells (Figure 5) (Goonewardene et al., 2019).

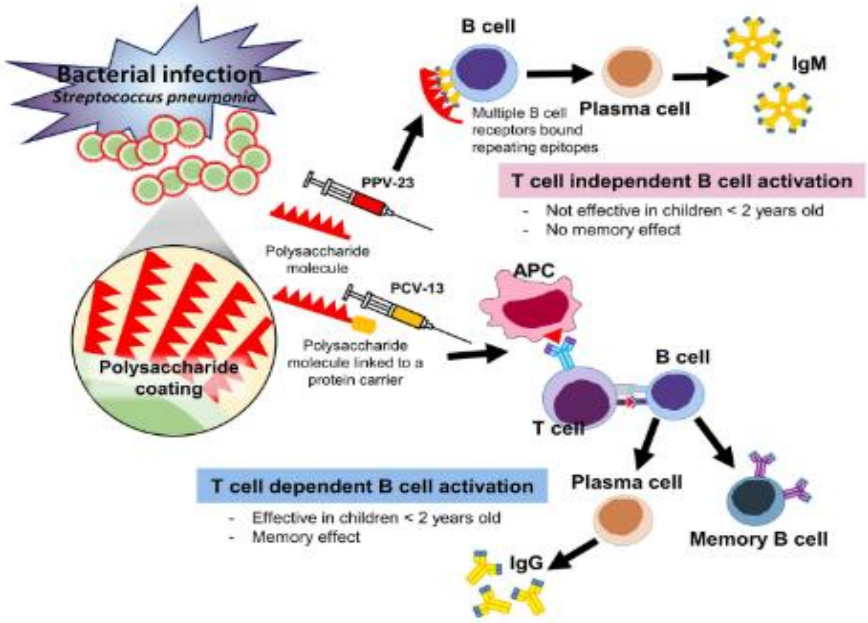


Figure 5. Comparison of mechanisms of pneumococcal vaccines. *Polysaccharides in PPV-23 stimulates B cell directly leading to its differentiation in plasma cells. Plasma cells produce antibodies. Polysaccharides in PCVs stimulate B cells via T cell activation which leads to the differentiation into both plasma cells and memory B cell.*

Recommendations for pneumococcal vaccinations in healthy children

In 2018, it was documented that 142 countries had included pneumococcal vaccine into their national immunisation programme (NIP) (IVAC, 2018). WHO recommends that, for PCV administration, either the 3p+0 schedule (3 primary doses without booster) or the 2p+1 schedule (2 primary doses with a booster) would be suitable. For the 3p+0 schedule, the first should be administered at age 6 weeks whereas the last is given within 9 months. The interval between doses should be between 4-8 weeks and depending on countries national programme, the doses can be administered at 6, 10 and 14 weeks or 2, 4 and 6 months (Goonewardene et al., 2019). For the 2p+1 schedule, doses are given at 8 weeks intervals for infants. The first dose is administered at 6 weeks whereas the booster is usually between 9 and 15

months (Goonewardene et al., 2019). Developed countries normally apply the 3p+1 (3 primary doses without booster) where the doses are given at an interval of 2 months starting from 2 months old infants. The booster is given between 12 and 15 months.

Chapter Summary

This chapter in summary, reviews literature on similar carriage studies including identification and serotyping methods, the weakness and strengths and the types pneumococcal vaccines available. This chapter highlights the various identification methods and serotyping methods that were employed in this study and the reasons for that.

CHAPTER THREE

METHODOLOGY

Introduction

This study investigated the serotype and antimicrobial resistance occurrence and distribution of *Streptococcus pneumoniae* among healthy children in a selected area. Pneumococcal carriage prevalence, serotype distribution and resistance patterns of some selected antimicrobials were established. This chapter covers the materials and methods used to achieve the various objectives of this study. It explains the research design, describes the study area and sampling procedure, data collection and laboratory procedures, and data analysis.

Study Area and Population

The study was conducted in the Cape Coast Metropolis, which is one of the twenty-two (22) Metropolitan, Municipal and District Assemblies (MMDAs) in the Central region of Ghana. Cape Coast Metropolis is the smallest metropolis in the country, but a very important part of Ghana as tourist hub, located on $1^{o}15'$W longitude and $5^{o}06'$N latitude. It occupies an area of approximately 122 square kilometres (km^2), with a population density of 1502/ km^2 as of 2018 and a growth rate of 1% per year. The metropolis is bounded on the south, west, east and north by the Gulf of Guinea, Komenda Edina Eguafo/Abrem Municipal, Abura Asebu Kwamankese District and Twifo Hemang Lower Denkyira District respectively. According to the 2010 population and housing census, the population of the metropolis was

approximately 170,000 with males and females representing 48.7% and 51.3% respectively. The population was projected to be 183,937 in September 2018.

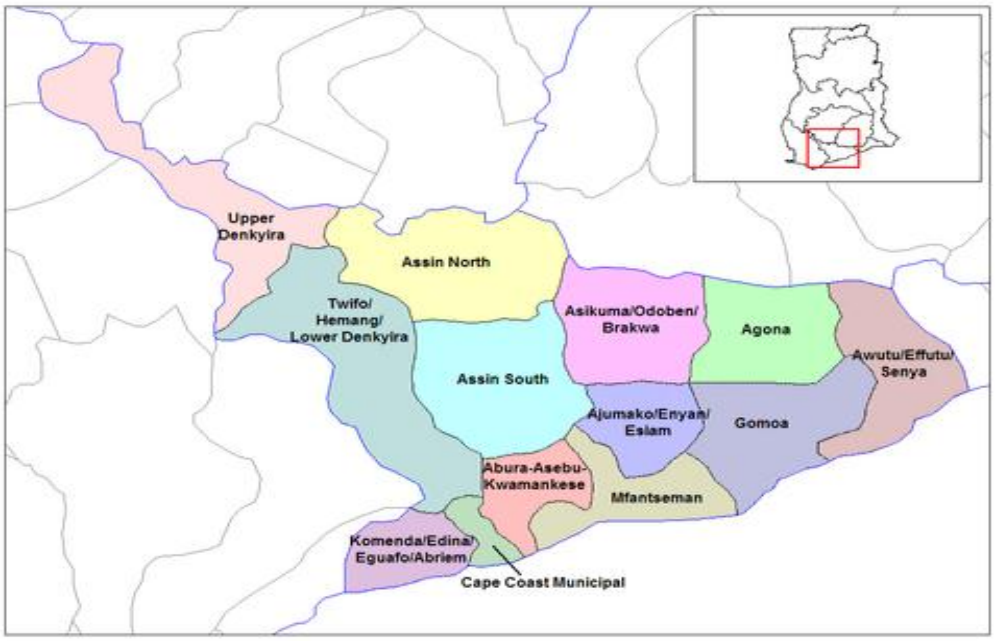


Figure 6. A map showing the study area (Ghana districts.com)

Study Design and Sampling Procedure

The study was a cross-sectional study, where data of a target population with varying characteristics and demographics, is taken at a point in time to prove and disprove assumptions. This study design is cost-effective and appropriate for prevalence studies since the study was mainly concerned with the determination of nasopharyngeal carriage and distribution among the various demographics. This study was conducted at selected immunisation centres and nursery or kindergartens in the Cape Coast Metropolis. A list of the schools was obtained from the Ghana Education Service regional office in Cape Coast from which the schools were randomly selected. All children attending the immunisation centres were conveniently sampled following parental consent. Both centres were chosen due to the age ranges of children found in these centres. The immunisation centres help with the selection of

children between 4 months and 24 months while the preschools promote the selection of children 12 months to 59 months.

Inclusion and Exclusion Criteria

All healthy children under the age of 5 years of consenting parents were eligible for the study at the immunisation centres and selected pre-schools. Children above five years attending the immunisation centres or pre-schools were not included in the study. Those with symptoms of upper and lower respiratory infection as at the time of sample collection were also excluded from the study.

Sample Size Determination

The sample size for this study was determined using the one sample, dichotomous outcome formula. The minimum number of participants included in the study was determined using the formula below;

$$N = \frac{Z^2 (P) (1-P)}{d^2}$$

Where Z = 1.96 is the standard score for the confidence interval of 95%

P = 0.5 is the prevalence for the largest possible sample size (Mills et al., 2015)

d = allowable error (d) of 5%

The calculated sample size = 385

Specimen and Data Collection

Health-care personnel were trained on how to obtain nasopharyngeal swabs for cultures from the study participants. Samples from the nasopharynx were obtained using a Copan plastic mini tip flocked swab, and the WHO-

recommended guidelines were followed (Satzke et al., 2014). The flexible minitip flocked swab stick (Copan Diagnostics Inc) was inserted into the posterior nasopharynx, rotated gently, removed and placed in a labelled vial containing 1ml of sterilised skimmed milk-tryptone-glucose-glycerine (STGG) transport medium (Satzke et al., 2014). The collected specimen was immediately transported on ice to the laboratory (Microbiology Laboratory, Department of Biomedical Science, UCC) within eight hours in accordance to the World Health Organization protocol for evaluation of pneumococcal carriage, vortexed for 10-20s to disperse the microbes from the swab and stored at -80^{o}C until ready for further characterisation.

Data was also collected from consenting parents or guardians of participating children at the immunization centres with the aid of a pretested questionnaire. The questionnaire explored areas of socio-demographic information such as age, sex, religion, type of facility and vaccination status.

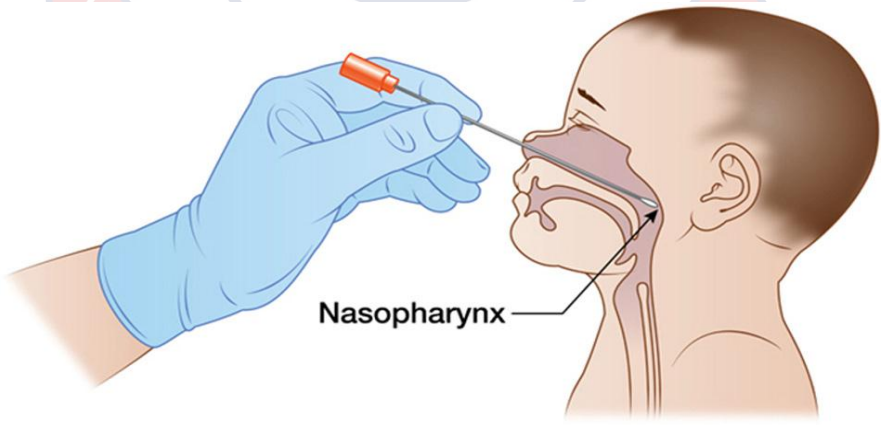


Figure 7: A diagram showing the collection of a nasopharyngeal swab
Source: Satzke *et al*., 2014

Laboratory Methods

Isolation and identification of *Streptococcus pneumoniae*

The frozen specimen was first sub-cultured onto 5 % sheep blood agar plate supplemented with 5µg/ml gentamycin. The blood agar plates were incubated at 37^{o}C in 5-10 % CO_2 using a canister jar for 18-24 hours. Bacteria colonies that exhibited alpha haemolysis were taken through further identification processes such as microscopic morphology. Gram-positive microbes and diplococci shaped, and catalase-negative were taken through Optochin testing. This was done by sub-culturing a colony of the suspected organisms on 5% sheep blood agar plate and an optochin disc (6mm, 5µg) placed carefully on the agar using sterile forceps. The plates were incubated again at 37^{o}C in 5–10 % CO_2 for 18-24 hours. Samples susceptible to Optochin by exhibiting inhibition zone of greater than or equal to 14mm were considered to be *Streptococcus pneumoniae.* Pneumococcal isolates were stored on STGG media at -80^{o}C until serotyping and molecular screening of resistance genes. Molecular identification was conducted by screening for *lytA* gene which encodes for the enzyme autolysin. The isolation and identification procedure were adopted from O'Brien et al., 2003 with little modification whereby the *lytA* gene was detected. *S. pneumoniae* ATCC 49169 served as a positive control throughout the study.

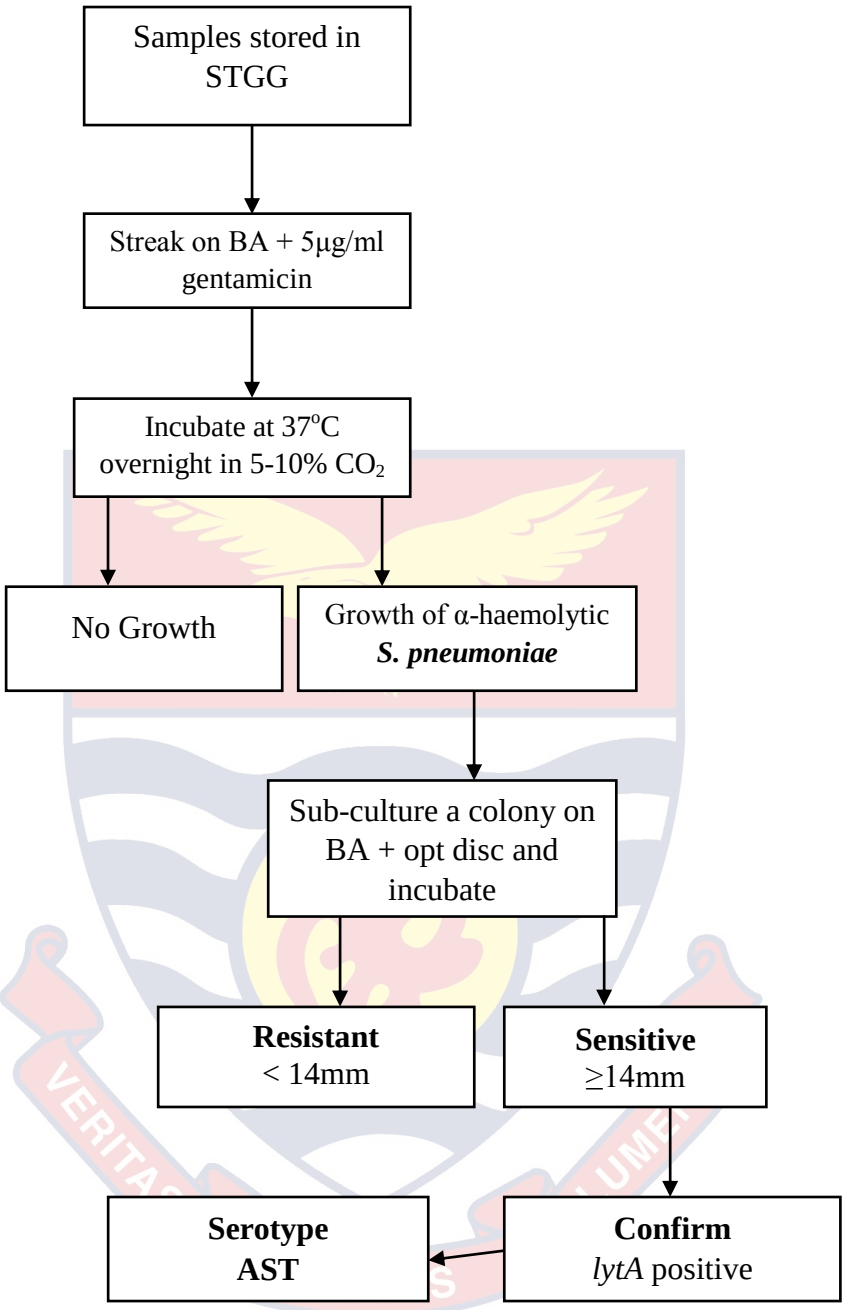


Figure 8: A flowchart showing the workflow for the isolation of *S. pneumoniae* STGG: skimmed milk-tryptone-glucose-glycerine; BA: Blood agar; AST: Antibiotic susceptibility testing

Serotyping of *Streptococcus pneumoniae*

Isolates identified as *S. pneumoniae* were further serotyped by conventional sequential multiplex PCR using the CDC reaction mix set up for Africa(https://www.cdc.gov/streplab/downloads/pcr-africa-clinical-

specimens.pdf). The multiplex PCR used to detect a total of 70 serotypes, was performed using eight sequential reactions. Forty-three primer pairs were used to detect serotypes 1, 2, 3, 4, 5, 6A/B/C/D, 6C/D, 7C/B/40,7F/A, 8, 9N/L, 9V/A, 10A, 10F/C/33C, 11A/D, 12F/A/B/44/46, 13, 14, 15A/F, 15B/C, 16F, 17F, 18C/F/B/A, 19A, 19F, 19Fvar, 20, 21, 22F/A, 23A, 23B, 23F, 24F/A/B, 31, 33F/A/37, 34, 35A/C/42, 35B, 35F/47F, 38/25F/25A and 39. A primer pair to amplify the polysaccharide capsule (*cps A*) was added to serve as an internal control by targeting all pneumococcal isolates. The primer pairs, sequences and product sizes are listed in the Appendix B.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was done to determine the susceptibility patterns of the isolated bacteria to some selected antibiotics using Kirby Bauer's disk diffusion method following the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2017). A bacterial suspension was prepared by picking single colonies of the isolates and mixing with normal saline until homogeny is established. The suspension was adjusted to 0.5 McFarland to ensure that the quantity of bacterial cells is approximately $1.05x10^8$ CFU/mL. The standard inoculum was swabbed onto Muller-Hinton agar with 5% sheep blood. Antibiotic discs were carefully dispensed on the agar using sterilised forceps and incubated at 37℃ for 16-24 hours. After incubation, the zones of growth inhibition were determined and then reported as sensitive (S), intermediate (I) and resistant (R). These were compared to the standard zones of inhibition reported by the Clinical Laboratory Standard Institute and Jorgensen et al (CLSI, 2017; Jorgensen et al., 1994). The standard zones of inhibition are summarised in the Table 6

below. The antibiotic discs used were co-Trimoxazole (CT, 1.25/23.75µg), Tetracycline (TE, 30µg), Chloramphenicol (CHL, 30µg), Ceftriaxone (CRO, 30µg), Levofloxacin (LEV, 5µg), Vancomycin (VAN, 30µg), Oxacillin (OX, 1µg), Erythromycin (ERY, 15µg) and Clindamycin (DA, 2µg). The inducible clindamycin resistance was determined by disk diffusion using D-test (CLSI, 2017). Minimum inhibition concentrations (MICs) of some randomly selected isolates (n=15) were determined using Penicillin G Etest strips (Liofilchem). Breakpoints at ≤0.06µg/ml, 0.12 - 1µg/ml and ≥2µg/ml were defined as susceptible, intermediate and resistant respectively according to the CLSI guidelines. The positive isolated strains that were resistant to three or more classes of antibiotics were regarded as multidrug-resistant (Bogaert et al., 2004). The oxacillin and ceftriaxone disks were used to screen for beta-lactam resistance.

Table 6: Inhibition Zones and their Corresponding Susceptibility Patterns

Antimicrobial Agent	**Susceptible (S)**	**Intermediate (I)**	**Resistance (R)**
*Ceftriaxone, *30µg*	≥ 29	26 – 28	≤ 25
Levofloxacin, 5*µg*	≥ 17	14 – 16	≤ 13
Vancomycin, 30*µg*	≥ 17	---------	--------
Erythromycin, 15*µg*	≥ 21	16 – 20	≤ 15
Clindamycin, 2*µg*	≥ 19	16 – 18	≤ 15
Oxacillin, 1*µg*	≥ 20	---------	---------
Tetracycline, *30µg*	≥ 28	25 – 27	≤ 24
Chloramphenicol, *30µg*	≥ 21	---------	≤ 20
co-Trimoxazole, *1.25/23.75µg*	≥ 19	16 – 18	≤ 15

Source: Clinical and Laboratory Standards Institute (2017) and *Jorgensen et al. (1994)

Detection of Serotypes and Antibiotic Resistance Genes

DNA extraction

The stored pneumococcal isolates were taken from the -80^{o}C freezer and sub-cultured on blood agar supplemented with 5 % sterile sheep blood. The cultured isolates were incubated at 37^{o}C for 18-24 hours in 5-10 % CO_2. Bacterial colonies were suspended in 200 µL of sterile distilled water and adjusted to 1M McFarland standard. The suspension was immediately heated at 100^{o}C for 8-10 minutes, and immediately frozen at -20^{o}C for 1 minute and centrifuged for 5 minutes (Espinosa, Báez, Percedo, & Martínez, 2013). The supernatants that contain the extracted DNA were stored at -20^{o}C until further use.

Molecular identification of *Streptococcus pneumoniae*

Molecular identification of the pneumococcus was done using the *lytA* primer gene. The primer sequence and amplicon size is shown in Table 7 below. Amplifications of *lytA* gene was performed under the following conditions: Pre-denaturation at 94^{o}C for 3 minutes followed by 30 amplification cycles of 94^{o}C for 30 seconds as denaturation, annealing at 55^{o}C for 90 seconds, extension at 72^{o}C for 30 seconds, and 72^{o}C for 10 minutes as the final extension.

Molecular capsular typing

The 41 primer pairs were grouped together in eight sequential reactions. Each reaction included five primer pairs which targeted serotype-specific regions of five different serotypes and included a control that targets all the positive pneumococcal isolates by detecting the *cps* A gene. The first reaction consisted of *cps* A, 14, 1, 5, 4 and 18C/F/B/A while reaction 2

consisted of *cps* A, 16A/B/C/D, 19F, 23F, 25F/A/38, 9V/A. The reactions are summarised in the Appendix C.

Polymerase Chain Reaction for serotyping

The Polymerase Chain Reactions were performed in 25-ul volumes of a mixture containing 12.5 µL of 1X Master Mix (Taq polymerase, dNTPs, 1.5mmol/L of $MgCl_2$), 3.5 µL of PCR H_2O, 5 µL of crude DNA template and varying volumes of primers of concentration of 0.3 µM. Amplification of genes was performed using a thermocycler (Biometra UNOII) under the following conditions: Pre-denaturation at 95^{o}C for 15 minutes followed by 35 amplification cycles of 94^{o}C for 30 seconds as denaturation, annealing at 54^{o}C for 90 seconds, extension at 72^{o}C for 60 seconds, and 72^{o}C for 10 minutes as the final extension and finally at 4^{o}C until ready for further use. Specific single serotype primers were used to confirm isolates that showed a band size corresponding to a particular serotype.

Detection of pneumococcal penicillin resistance genes

The resistance genes conferring resistance to the beta-lactams were determined using *PBP 1a, PBP 2x* and *PBP 2b* specific primers. The primers and their sequences have been summarised in Table 3.2.

Table 7: List of Primers used for Identification and Resistance Genes Screening

Primer name	Primer sequence 5'-3'	Product size (bp)	Reference
PBP 2x-F	GTCATGCTGGAGCCTAAATT	277	Zettler et al., 2006
PBP 2x-R	AACCCGACTAGATAACCACC		
PBP 2b-F	ACTCAGGCTTACGGTTCATT	359	Zettler et al., 2006
PBP 2b-R	ACGAGGAGCCACACGAACAC		
PBP la-F	AGGTCGGTCCTAGATAGAGCT	423	Zettler et al., 2006
PBP la-R	GAGCTACATAGCCAGTGTC		
Lyt A-F	GAAATTAATGTGAGTAAATTAAGA ACAG	900	Dayie et al., 2019
Lyt A-R	TTTTACTGTAATCAAGCCATCTGGC		

Polymerase chain reaction for resistance genes

The Polymerase Chain Reactions were performed in 25-µL volumes of a mixture containing 12.5 µL of 1X Master Mix (Taq polymerase, dNTPs, 25 mmol/L of $MgCl_2$), 10uL of PCR H_2O, 0.5 µL of DNA template and 1uL each of primers (0.4 µM). Amplifications of *PBP2b, PBP2x* and *PBP1a* genes were performed using a thermocycler (Biometra UNO II) under the following conditions: Pre-denaturation at 94^{o}C for 3 minutes followed by 35 amplification cycles of 94^{o}C for 30 seconds as denaturation, annealing at 58^{o}C for 30 seconds, extension at 72^{o}C for 2 minutes, and 72^{o}C for 5 minutes as the final extension.

Polymerase chain reaction product detection

The PCR products were mixed with 6X loading dye (Thermo scientific) in a 5:1 ratio and 5 µL of the mixture was loaded onto 2 % agarose gel made from 1X TAE (Tris-HCL Acetic acid EDTA) and stained with 4S

Red Plus Nucleic Acid stain (Life Sciences Biotechnology, China, cat. No: A606695), along with a 5µL of 100 bp Gene rule (Life Sciences Biotechnology, China). The amplicons were subjected to electrophoresis at 100 V for 1 hour 30 minutes. The gel images were visualised using a UV transilluminator (Cleaver Scientific Ltd), and the sizes of the amplicons were determined by comparing them to the gene ruler (Thermo scientific).

Data analysis

Data were entered into a database and IBM SPSS Statistics version 21 (IBM Corp., Armonk). Statistically, significant differences and associations between categorical data were analysed using the chi-square tests or fisher's exact test where necessary for quantitative data. A p-value of < 0.05 was considered statistically significant. The presence of resistant genes and serotypes was analysed descriptively and expressed as proportions or using bar charts.

Ethical considerations

The study was carried out in accordance with institutional ethical guidelines. Ethical clearance was obtained from the Institutional Review Board (UCCIRB/EXT/2017/21) of the University of Cape Coast, Ghana. Written informed consent to participate in the study was obtained from parents/guardians before inclusion in the study.

Chapter Summary

In this study, Cape Coast Metropolis was selected as the study area with children under five years being the target population from which the sample was taken. However, the sample collection did not cover every part of

the Cape Coast Metropolis, and so the data obtained might not be an exact representation of the entire population. The study is a cross sectional study and not a longitudinal study. In view of that, the study cannot determine the precise dynamics of carriage, acquisition rates or estimate the carriage duration of the pneumococcus. Moreover, serotyping was done using the sequential multiplex which employs the use of 40 primers for identifying 70 serotypes out of the 98 known serotypes.

CHAPTER FOUR

RESULTS AND DISCUSSION

Results

This study explored the carriage of *Streptococcus pneumoniae* among healthy children under 5 years of age residing in the Cape Coast Metropolis, Ghana, 5 years after the introduction of the pneumococcal conjugate vaccine-13. The study was a cross-sectional study which is appropriate for the determination of carriage prevalence, and included serotyping using Sequential Multiplex PCR (SM-PCR) and screening for resistance genes using conventional PCR. The statistical comparisons were done using the Chi-square test or Fisher's exact where necessary. Data were mainly expressed using charts and tables.

Characteristics of the Study

Nasopharyngeal samples were collected from 398 healthy children of consenting parents between January and March 2018. Six (53%) health care centres and 18 (47%) randomly selected preparatory schools in the Metropolis were used. Of the 398 samples obtained, 200 (50.3%) and 198 (49.7%) were from males and females respectively. The median age was 24.5 months with an interquartile range of 11 to 50 months; 199 (50%) were ≤ 24months and 199 (50%) were > 24 months. The sampling covered 10 communities with most samples (18.6%) being obtained from Aboom wells and the least (2.3%) from Bakano as shown in Table 8.

Nasopharyngeal Carriage of *S. pneumoniae* in association to Study Characteristics

The overall pneumococcal carriage in the Cape Coast Metropolis was 23.4% (93/398) as shown in Table 9. The pneumococci isolates were identified conventionally by optochin susceptibility and molecular confirmation was achieved by screening for the *lyt A* gene. With regards to the carriage among sexes, males had a higher carriage (49/200; 24.5%) as compared to females (44/198; 22.2%), but there was no association (p=0.591). The carriage among healthy children in the different age groups was similar; 23.1% and 23.6% respectively. No association was found between age and carriage (p=0.906). Similarly, there was no association between nasopharyngeal colonisation and the type of facility, location of facility, ethnic groups and religion (Table 9). There was an association between the nasopharyngeal colonisation and the type of preparatory school (p=0.013) as the carriage in private schools (36.2%) were higher than in public schools (18.6%) as shown in Table 10.

Table 8: Characteristics of Healthy Children enrolled in the Study

Variables	n (%) or median (IQR)
Sex	
Male	200 (50.3)
Female	198 (49.7)
Age (months)	**24.50 (11-50)**
≤ 24	199 (50)
> 24	199 (50)
Facility	
School	187 (47.0)
Hospital	211 (53.0)
Location of facility	
Amamoma	69 (17.3)
Kwaprow	35 (8.7)
Akotokyir	11 (2.8)
UCC	45 (11.3)
Aboom wells	74 (18.6)
Pedu	10 (2.5)
Abura	15 (3.8)
Bakano	9 (2.3)
Ewim	42 (10.6)
Kakumdo	22 (5.5)
Ethnic group	
Fante	213 (53.5)
Other Akans	121 (30.4)
Ga-Dangme	14 (3.5)
Northern tribes	13 (3.3)
Ewe	31 (7.8)
Foreign tribe	6 (1.5)
Religion	
Christian	364 (91.5)
Muslim	34 (8.5)
Total	398 (100.0)

IQR: Interquartile range; UCC: University of Cape Coast

Table 9: Nasopharyngeal Carriage Prevalence and Distribution among Demographic Characteristics

Variables	Total	Carriage N (%)	X^2 value	P-value
Sex			0.288	0.591
Male	200 (50.3)	49 (24.5)		
Female	198 (49.7)	44 (22.2)		
Age (months)			0.014	0.906
≤ 24	199 (50)	46 (23.1)		
> 24	199 (50)	47 23.6)		
< 11	110 (27.6)	26 (23.6)	3.472	0.482
12 – 23	82 (20.6)	17 (20.7)		
24 – 35	44 (11.1)	14 (31.8)		
36 – 47	42 (10.6)	12 (28.6)		
> 48	120 (30.2)	24 (20.0)		
Facility			0.027	0.869
School	187 (47.0)	43 (23.0)		
Hospital	211 (53.0)	50 (23.7)		
Location of facility			9.203	0.419
Amamoma	100 (25.1)	28 (28.0)		
Kwaprow	55 (13.8)	8 (14.5)		
Akotokyir	24 (6.0)	5 (20.8)		
UCC	47 (11.8)	9 (19.1)		
Aboom wells	74 (18.6)	14 (18.9)		
Pedu	10 (2.5)	4 (40.0)		
Abura	15 (3.8)	6 (40.0)		
Bakano	9 (2.3)	2 (22.2)		
Ewim	42 (10.6)	11 (26.2)		
Kukuado	22 (5.5)	6 (27.3)		
Ethnic group			3.217	0.667
Fante	213 (53.5)	46 (21.6)		
Other Akans	121 (30.4)	33 (28.3)		
Ga-Dangme	14 (3.5)	2 (14.3)		
Northern tribes	13 (3.3)	2 (15.4)		
Ewe	31 (7.8)	9 (29.0)		
Foreign tribe	6 (1.5)	1 (16.7)		
Religion			0.160	0.689
Christian	364 (91.5)	86 (23.6)		
Muslim	34 (8.5)	7 (20.6)		
Total	398 (100.0)	93 (23.4)		

Table 10: Prevalence of Carriage within Selected Facilities

Variables	Total	Carriage N (%)	X^2 value	P-value
Circuits			1.149	0.284
OLA	113 (60.4)	29 (25.7)		
ABOOM	74 (39.6)	14 (18.9)		
Type of school			6.155	**0.013***
Private	47 (25.1)	17 (36.2)		
Public	140 (74.9)	26 (18.6)		
Health facility			7.583	0.181
Amamoma CHPS	69 (32.7)	23 (33.3)		
Kwaprow CHPS	35 (16.6)	5 (14.3)		
Akotokyir CHPS	11 (5.2)	1 (9.1)		
UCC RCH	45 (21.3)	8 (17.8)		
Bakano RCH	9 (4.3)	2 (22.2)		
Ewim RCH	42 (19.9)	11 (26.2)		

*P<0.05=Significant association between independent variables and carriage

Serotype, Distribution and Pneumococcal Vaccine Coverage

Serotyping of the 93 pneumococcal isolates was done using a sequential multiplex PCR out of which only 48 (51.6%) were typed with the remaining 45 (48.4%) being non-typeable (NT) by multiplex PCR. Out of the 48 identified serotypes, 5 were included in the PCV-13 vaccine. They are; serotypes 6A/B (n=10, 10.8%), 14 (n=7, 7.5%), 19F (n=6, 6.5%), 23F (n=8, 8.6%) and 19A (n=2, 2.2%). The non-vaccine types include; 10A (n=1, 1.1%), 11A (n=2, 2.2%), 12F (n=2, 2.2%), 15A (n=3, 3.2%), 15B/C (n=1, 1.1%), 16F (n=2, 2.2%), 34 (n=1, 1.1%) and 23B (n=4, 4.3%) as shown in Figure 9. The most occurring serotypes were 6A/B (10.8%), 23F (8.6%), 14 (7.5%) and 19F (6.5%) which are all vaccine types, forming (35.5%) of the serotyped isolates. However, the non-vaccine serotypes (64.5%) were (including non-typable) more than the vaccine types.

The distribution of serotypes were similar among the age groups where the serotypes, 10A, 15BC and 16F were found among children ≤24 months and the rest found among children >24 months (Table 11). However, among the vaccine types, 19F (50%) and 19A (50%) were evenly distributed among both age groups while 6A/B (60%) and 23F (62.5%) were predominant among children >24 months. Serotype 14 was mostly found among those ≤24 months. With regards to sex, 19A and 12F were only isolated from males whiles 11A and 10A were from females. Seventy per cent (70%) of 6A/B isolates were found among females whiles 14 and 19F isolates were found among males were 57.1% and 83.3% respectively. Moreover, the vaccine types (18.2%) mostly exhibited multidrug resistance as compared to the non-vaccine types (10.0%). The immunization coverage of PCV-13 was found to be 35.5% but varied among the age groups and sex.

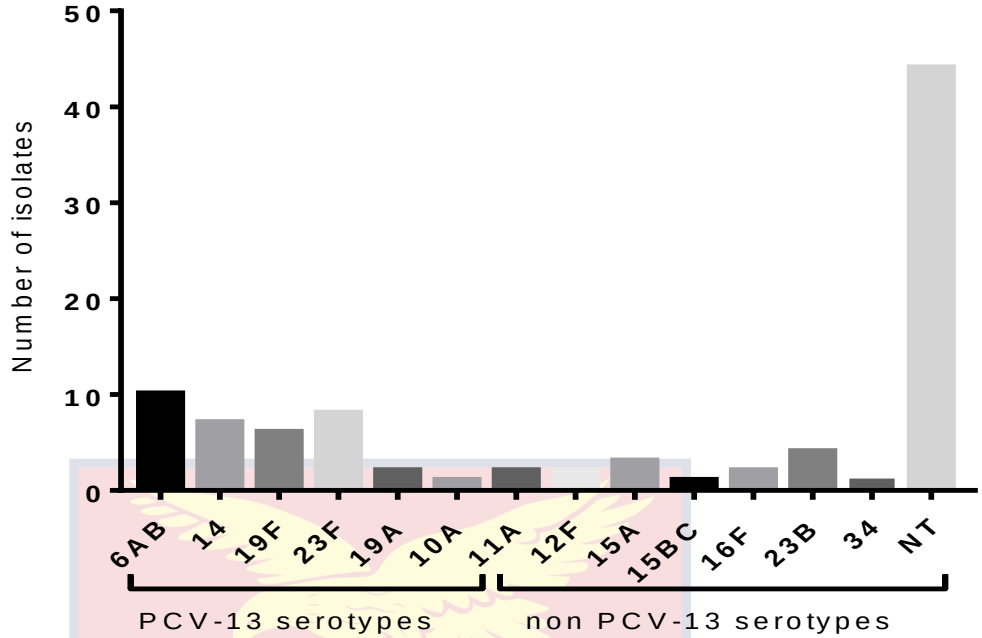


Figure 9: Overall Serotype Distribution and PCV-13 Vaccination coverage among S. pneumoniae isolates

Table 11: Serotype Distribution by Age Group, Sex and Multidrug Resistance (MDR)

Serotype	Total	Age groups (%)		Sex (%)		MDR (%)
	N	≤ 24 months	>24 months	Male	Female	
PCV-13	**33**	**42.4**	**57.6**	**57.6**	**42.4**	**18.2**
6AB	10	40.0	60.0	30.0	70.0	50.0
14	7	57.1	42.9	57.1	42.9	14.3
19F	6	50.0	50.0	83.3	16.7	33.3
23F	8	37.5	62.5	50.0	50.0	50.0
19A	2	50.0	50.0	100.0	0.0	0.0
Non-PCV 13	**60**	**53.3**	**46.7**	**50.0**	**50.0**	**10.0**
10A	1	100.0	0.0	0.0	100.0	0.0
11A	2	0.0	100.0	0.0	100.0	0.0
12F	2	50.0	50.0	100.0	0.0	0.0
15A	3	33.3	66.7	66.7	33.3	33.3
15BC	1	100.0	0.0	0.0	100.0	0.0
16F	2	100.0	0.0	50.0	50.0	0.0
23B	4	75.0	25.0	75.0	25.0	0.0
34	1	100.0	0.0	0.0	100.0	0.0
NT	44	48.9	51.1	51.1	48.9	9.1

PCV-13: Pneumococcal conjugate vaccine; non-PCV 13: non-pneumococcal conjugate vaccine; MDR: Multidrug resistance

Distribution of Antimicrobial Resistance

The isolates demonstrated high resistance to co-Trimoxazole, tetracycline and non-susceptibility to oxacillin with 87.1%, 64.5% and 60.2% respectively (Table 12). Minimum inhibitory concentration test results of fifteen (15) randomly selected oxacillin isolates showed intermediate resistance to Penicillin G (0.06-1.5µg/ml) since resistance to oxacillin is not considered resistance to penicillin, rather non-susceptible to penicillin (CLSI, 2017). The least percentage resistance was observed among isolates that conferred non-susceptibility to ceftriaxone (3/93; 3.2%), resistance to vancomycin (2/93; 2.2%) and clindamycin (4/93; 4.3%) (Table 12). All the isolates were susceptible to levofloxacin. Isolates with multidrug resistance were 12 (12.9%). The dual resistance between penicillin and any other antibiotics such as ceftriaxone, vancomycin, clindamycin and chloramphenicol were 100% while that of penicillin and tetracycline was 71.7% (p=0.036). All the isolates showing multidrug resistance were also non-susceptible to penicillin (Table 13). Most of the serotypes identified showed non-susceptibility to penicillin with a majority of them being vaccine types; 6A/B, 14, 19F and 19A showing 60%, 71.4%, 83.3% and 100% respectively Figure 10.

The percentage resistance of the pneumococcal isolates varied in relation to age groups, sex and serotype. Resistant isolates collected from children ≤ 24 months were higher than > 24 months children. Similar results were observed among females and vaccine types. With regards to individual antibiotics, tetracycline resistance was significantly higher in children ≤ 24 months than children > 24 months (p= 0.008) and significantly higher in males

than females (p= 0.001). Chloramphenicol resistance was significantly higher in the vaccine serotypes than the non-vaccine serotypes (p= 0.020).

Table 12: Antibiotic Susceptibility Patterns of Pneumococcal Isolates (n = 93)

Antimicrobial agents	Susceptible (S)	Intermediate (I)	Resistant (R)
	N (%)	N (%)	N (%)
Ceftriaxone	89 (95.7)	1 (1.1)	**3 (3.2)**
Levofloxacin	93 (100)	0	**0**
Vancomycin	91 (97.8)		**2 (2.2)**
Erythromycin	84 (90.3)	3 (3.2)	**6 (6.5)**
Clindamycin	89 (95.7)	0	**4 (4.3)**
Oxacillin	37 (39.8)		**56 (60.2)**
Tetracycline	28 (30.1)	5 (5.4)	**60 (64.5)**
Chloramphenicol	87 (93.5)		**6 (6.5)**
Co-Trimoxazole	7 (7.5)	5 (5.4)	**81 (87.1)**
D-test	91 (97.8)		**2 (2.2)**
MDR			**12 (12.9)**

Table 13: Antibiotic Susceptibility Testing of isolates (n= 93) categorised by Penicillin Susceptibility

	PENICILLIN		
Antimicrobial agents	**PNSP**	**PSP**	**P-value**
Ceftriaxone	4 (100.0)	37 (41.6)	0.148
Levofloxacin	0	37 (39.8)	------
Vancomycin	2 (100.0)	37 (40.7)	0.516
Erythromycin	8 (88.8)	36 (42.9)	0.081
Clindamycin	4 (100)	37 (41)	0.148
Tetracycline	**43 (71.7)**	**17 (60.7)**	**0.036**
Chloramphenicol	6 (100.0)	37 (42.5)	0.078
Co-Trimoxazole	51 (62.9)	2 (28.6)	0.698
MDR	**47 (100.0)**	**37 (80.4)**	**0.0001**

MDR: Multidrug resistance; PNSP: Penicillin Non-Susceptible Pneumococcus; PSP: Penicillin Susceptible Pneumococcus

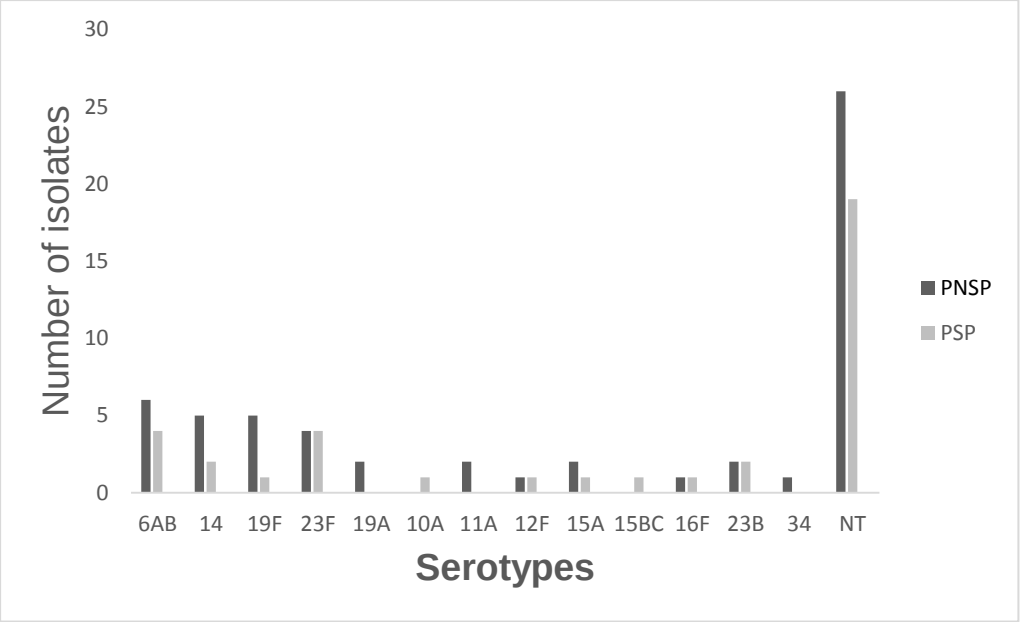


Figure 10: Distribution of Penicillin Susceptibility among Pneumococcal Serotypes

Table 14: Distribution of Antibiotic Resistance of Pneumococcus Isolates to some Selected Antimicrobials among Age groups, Sex and Serotypes

Antibiotics	Resistance of age groups (%)			Resistance of sex (%)			Resistance of serotypes (%)		
	≤ 24 months (n=46)	>24 months (n=47)	p-value	Male (n=49)	Female (n=44)	p-value	Non-vaccine (n=60)	Vaccine (n=33)	p-value
CRO	6.5	2.1	0.361	2.0	6.8	0.256	5.0	3.0	0.618
VA	2.2	4.3	0.570	2.0	4.5	0.601	3.3	3.0	0.937
ERY	10.8	8.5	0.700	6.1	13.6	0.221	8.3	3.0	0.299
OX	56.5	63.8	0.472	59.2	61.4	0.830	55.0	69.7	0.166
TET	**82.6**	**57.4**	**0.008****	**73.5**	**65.9**	**0.001*****	68.3	72.7	0.427
CHL	4.3	8.5	0.677	8.2	4.5	0.478	**1.7**	**15.2**	**0.020***
COT	97.8	87.2	0.111	93.8	90.9	0.588	90.0	97.0	0.414
CLIN	2.2	6.4	0.617	2.0	6.8	0.257	5.0	3.0	0.654
MDR	13.0	12.8	0.755	12.2	13.6	0.607	10.0	18.2	0.061
LEV	0.0	0.0	------	0.0	0.0	------	0.0	0.0	------

CRO: Ceftriaxone; VA: Vancomycin; ERY: Erythromycin; OX: Oxacillin; TET: Tetracycline; CHL: Chloramphenicol; COT: Co-Trimoxazole; CLIN: Clindamycin; LEV: Levofloxacin; MDR: multidrug resistance; *P<0.05; **P<0.01; ***P<0.001= Significant association between independent and dependent variables.

Distribution of Penicillin Resistance Genes among Phenotypic Penicillin Susceptible and non-susceptible isolates, Age groups and Serotype groups

All the isolated strains were screened for the presence of the penicillin binding protein genes. All the isolates contained at least one of the selected genes under study. Penicillin non-susceptible pneumococci as classified using the 1ug oxacillin disk had *PBP2b* (10.7%), *PBP2x* (3.6%), *PBP2b* + *PBP2x* (80.4%) and *PBP2b* + *PBP2x* + *PBP1a* (5.4%). Penicillin susceptible pneumococci had *PBP2b* + *PBP2x* (80.4%) and *PBP2b* + *PBP2x* + *PBP1a* (2.7%). For isolates obtained from children 24 months and below, *PBP2b* was 6.5%, *PBP2x* was 4.3%, *PBP2b* + *PBP2x* was 82.6% and *PBP2b* + *PBP2x* + *PBP1a* was 6.5% whereas for those isolated from children above 24 months, *PBP2b* was 6.4%, *PBP2b* + *PBP2x* was 91.5% and *PBP2b* + *PBP2x* + *PBP1a* was 2.1%. Vaccine serotypes contained 6.1% *PBP2b*, 91.5% *PBP2b* + *PBP2x* and 6.1% *PBP2b* + *PBP2x* + *PBP1a* whereas the non-vaccine serotypes had 6.7% *PBP2b*, 3.3% *PBP2x*, 86.7% *PBP2b* + *PBP2x* and 3.3% *PBP2b* + *PBP2x* + *PBP1a*.

Table 15: Occurrence and Distribution of Penicillin Resistance Genes among selected Demographics

	Penicillin Susceptibility		Age in months N (%)		Serotypes N (%)	
Resistance genes	**PNSP**	**PSP**	**≤ 24**	**> 24**	**VT**	**NVT**
	(N=56)	**(N=37)**	**(N=47)**	**(N=46)**	**(N=33)**	**(N=60)**
PBP2b only	6(10.7)	0(0.0)	3(6.5)	3(6.4)	2(6.1)	4(6.7)
PBP2x only	2(3.6)	0(0.0)	2(4.3)	0(0.0)	0(0.0)	2(3.3)
PBP2b + *PBP2x*	45 (80.4)	36 (97.3)	38 (82.6)	43 (91.5)	29 (87.9)	52 (86.7)
PBP2b + *PBP2x* + *PBP1a*	3(5.4)	1(2.7)	3(6.5)	1(2.1)	2(6.1)	2(3.3)

PBP: penicillin binding protein; VT: vaccine-types; NVT: non-vaccine types; PSP: penicillin susceptible pneumococcus; PNSP: penicillin non-susceptible pneumococcus

Discussion

Streptococcus pneumoniae is one of the opportunistic pathogens residing in the nasopharynx of most people. Nonetheless, it has been the focus of most studies due to its immense contribution to morbidity and mortality in children, the elderly and immunocompromised individuals. Given this public health concern, vaccines have been introduced to help prevent the manifestation of pneumococcal diseases. However, there are studies conducted both in the pre and post-vaccination era worldwide to determine the impact of these vaccinations among populations that are most at risk of the infection. Epidemiological studies are mostly performed from time to time to update knowledge on the dynamics of a population with regards to antibiotic resistance and serotype distribution (Emgård et al., 2019). Such studies are also done among healthy individuals to ascertain the true representation of the pneumococcal population due to the difference in the propensity of different

serotypes to cause disease (Donkor et al., 2011). In Ghana, there have been limited studies on the pneumococcal carriage in the pre-vaccination period and post-vaccination (Mills et al., 2013; Dayie et al., 2013; Dayie et al., 2019). This current study, therefore, explored the carriage, antibiotic resistance distribution and the serotype distribution of the pneumococcus among healthy children under 5 years after the introduction of the PCV-13 into Ghana's immunisation programme.

The collection of nasopharyngeal swabs is known to be the 'gold standard' for the isolation of respiratory pathogens (Satzke et al., 2014). Based on positive culture and *lyt A* gene confirmation, the carriage rate of the pneumococcus was found to be 23.4%. The sample collection was carried out between early January and early March signifying that the study was conducted in the dry season (www.meteo.gov.gh/website) and so the carriage rate of the pneumococcus could have been influenced by the weather conditions. Carriage tends to increase during the cool but dry season (Numminen et al., 2015). The study conducted by Numminen and colleagues showed that colonization prevalence of *S. pneumoniae* was highest in infants between February and April in Kilifi, Kenya. Although there is no data on pre-vaccination pneumococcal carriage prevalence for the Cape Coast Metropolis, our data can be compared to carriage prevalence in other parts of Ghana; Two pre-vaccination carriage studies were carried out in Accra (along the coastal belt similar to the Cape Coast Metropolis) and one in Tamale in 2011 (Dayie et al., 2013). The carriage of *S. pneumoniae* (23.4%) in this study was lower compared to 48.9 % among children attending a paediatric hospital in Accra (Mills et al., 2015). Another study by Dayie and colleagues among nursery

and kindergarten children in Accra and Tamale showed a carriage rate of 34% and 31% respectively (Dayie et al., 2013). However, the carriage rate from this study was lower compared to the few post-vaccination studies that have been conducted in Ghana. They include; two among HIV-infected children and adults (Donkor et al., 2017; Dayie et al., 2019) and one among healthy children in Accra (Dayie et al., 2019). The carriage rate found among healthy children under 5 years was high (54%) in the study done by Dayie and colleagues (2019) as compared to the prevalence in this current study. The differences in the carriage rate could be due to the geographical location and the target population. It is known that, the distribution of the pneumococcus varies with respect to geographical locations and time (Haile et al., 2019).

Similar studies done in some countries in Africa showed carriage rates of 21% (Congo) and 21.5% (Ethiopia) (Birindwa et al., 2018; Haile et al., 2019) whereas in Tanzania, South Africa and the Gambia, the carriage rate was high ranging between 31%-37.6% (Dube et al., 2018; Emgård et al., 2019; Usuf et al., 2018). There was varying prevalence among countries outside Africa; Italy (26%), Norway (62%), UK (32%), England (51.9%), Denmark (26%), USA (31%) (Devine et al., 2017; Fjeldhøj et al., 2018; Lee et al., 2014; Southern et al., 2018; Steens et al., 2015; Zuccotti et al., 2014). Even though, the carriage rates in these countries were higher than that found in the current study, a similar study done by Chan and her colleagues in Hong Kong showed a colonisation prevalence of 5.5% (Chan et al., 2016). Several factors, such as the geographical location might have contributed to this but it could also be that the scheduled dose (3p+1) used at that time could be the major

contributing factor despite the fact that children were less than 2 years and are most at risk colonisation.

This study recorded no association between most of the socio-demographic factors and the carriage prevalence of *S. pneumoniae* (Table 9). There was a slightly high carriage prevalence among males as compared to females as reported in other studies (Emgård et al. 2019; Usuf et al., 2018). Studies have reported that children under the age of two years are more frequently colonised by pneumococci than older children and adults (Kamngóna et al., 2015; Steens et al., 2015; Dube et al., 2018). However, in this study, children older than 2 years had higher carriage prevalence as compared to those two years and below although not significant, this concurs with the findings from Choe et al. (2016). A further look at the detailed breakdown of the age groups showed a prevalence of 23.6% among children 11 months and below which decreased slightly to 20.7% (12-23 months) and peaked around 24-35 months at 31.8% and decreased again from 28.6% to 20% around (36 - 47 months) and ages above 48 months respectively. The rise and fall of the carriage could be attributed to the effect of the PCV-13 vaccination. Ghana's scheduled dose for pneumococcal vaccination is at 6, 10 and 14 weeks and so it is expected that there will be increased effectiveness of the vaccine in the first few months after vaccination. The further decline in the carriage after the rise could be attributed to the maturity of the mucosal immunity associated with increasing age. A similar increase in the carriage has been reported in other studies (Dayie et al., 2019; Usuf et al., 2018). This study, therefore, showed that attending a private school was associated with a high rate of pneumococcal carriage (Table 10). This could have been

occasioned by the age disparity among the schools since it was observed that most of the children in the public schools were mostly older than the required class age and so were mostly at the age category with the least prevalence. This indicates that they may have matured immunity as compared to those in private schools.

Serotyping was done using the sequential multiplex PCR (SM-PCR) as described by the CDC (CDC, 2014). This method involves the molecular detection of serotypes by amplifying selected genes within the *cps* locus. Multiplex PCR detects 70 serotypes out of the 98 known serotypes. This accounts for the larger number (48.4%) of non-typable (NT) in this study even though they tested positive for the *cps A* gene. Among the remaining isolates that could be serotyped, 33 were covered by the PCV-13 while 15 were not. Since the 45 of the *cps A* positive isolates could not be identified probably due to the limiting number of serotypes this method can identify, the non-vaccine serotypes were considered to be 60 (64.5%) whilst the PCV-13 coverage or vaccine types were 33 (35.5%). The increase in the non-vaccine serotypes is suggestive that the PCV-13 might have reduced prevalence of the vaccine types allowing for the increase in the non-vaccine types as reported in other studies (Fjeldhoj et al., 2018; Usuf et al., 2018). The PCV-13 coverage corroborates the results reported by Dayie et al. (2019).

The overall serotype prevalence showed that vaccine types 6A/B (10.8%), 23F (8.6%), 14 (7.5%) and 19F (6.5%) persisted which is similar to that reported by Emgård et al. (2019). The high prevalence of the vaccine type could be because not all the vaccination status of the children was confirmed by checking the immunisation cards especially among most of the school

children and not all the children had received all three doses. The persistence of serotypes/serogroups 6, 19F, 23F and 14 are considered as colonisers and tend to be carried in the nasopharynx for a prolonged period (Dube et al., 2018). Similar studies have reported the persistence of some vaccine-types where Birindwa et al. (2018) reported circulating 19F, the most abundant serotype, in the eastern part of DR Congo. Serotype 19F has also been the major serotype among clinical isolates in Nigeria, Tunisia and China (Huang et al., 2015; Ktari et al., 2017; Torimiro, Fabunmi, & Ajayi, 2018) while Dayie et al. (2019) also reported the persistence of 19F and 23F in their study. Moreover, vaccine-type 19A has been implicated in otitis media patients (Kaplan et al., 2015). A study conducted to compare the incidence rates of the invasive pneumococcal disease in Morocco reported that even though the incidence of IPDs reduced drastically, vaccine types 19F, 1, 6B and 14 were the major cause of IPD in children under 5 years (Diawara et al., 2015). Serotype 1, the major cause of disease outbreaks such as meningitis in the Northern belt of this country was not detected in this study (Kwambana-adams et al., 2017; Leimkugel et al., 2005).

Among the detected non-vaccine types, serotype 23B (4/15) was the most predominant serotype, followed by 15A (3/15). This corroborates the results from Dayie et al., (2019). Comparing this finding to the other pre-vaccination studies in the country shows that serotypes 23B, 15A and 15B/C could be emerging serotypes; since they were not detected or reported in the pre-vaccination studies in Ghana. Serotypes 23B, 15A and 15B/C have been reported to be on the rise and are the major cause of IPDs in Germany, England, South Africa and UK (Devine et al., 2017; Dube et al., 2018;

Southern et al., 2018; Van Der Linden, Falkenhorst, Perniciaro, & Imöhl, 2015). This implies that attention should be given to these serotypes since they might have a high propensity to cause pneumococcal diseases.

Serotype distribution varied among age groups and sex (Table 11). Collectively, vaccine types were mostly not found among children under 2 years which is contrary to what was reported by other studies (Kamng'ona et al., 2015). The decrease of vaccine types among the children under 2 years might be attributed to the effectiveness of the vaccine at the early years which were replaced by the non-vaccine types and therefore explains the similar carriage prevalence among both children 2 years and below and those above years. Concerning the collective distribution of serotypes among sex, non-vaccine types were equally distributed among both sexes even though 15A and 23B were dominant among males. Vaccine types were mostly found among males with 14 and 19F dominating.

Nasopharyngeal carriage precedes pneumococcal disease manifestation, especially pneumonia and meningitis. Pneumococcal diseases have been estimated to cause 11% of all mortality in children less than 5 years of which a large proportion of these deaths occur in low-and-middle-income countries (Birindwa et al., 2018). In 2017, Ghana updated its Standard Treatment Guidelines (STGs) recommended for clinicians to treat infections including paediatric infections (Ministry of Health, 2017). However, there have been reports on the increase of antibiotics resistance among both vaccine and non-vaccine pneumococcus (Chan et al., 2016; Ktari et al., 2017; Obolski et al., 2018).

In this study, most of the isolates were non-susceptible to Penicillin (60.2%) and resistant to Tetracycline (64.5%) and co-Trimoxazole (87.1%) whilst none of the isolates were resistant to Levofloxacin. The results of the current study corroborate the findings of a study by Dayie et al. (2019) where all the isolates were susceptible to Levofloxacin and 63% were resistant to Tetracycline. Erythromycin resistant isolates were 11% as compared to 6.5% in our study whereas Penicillin and co-Trimoxazole resistance was high showing 60.5% and 87.5% respectively. In comparison to the carriage study done by Mills et al. (2015) in Accra, the resistance to co-Trimoxazole, Tetracycline and Erythromycin was low with the exception of penicillin non-susceptible. This indicates the possible impact of PCV-13 on antibiotic resistance.

Penicillin non-susceptible pneumococcus (PNSP) were detected using oxacillin (1ug) disk (≤19mm) which is appropriate for testing for susceptibility in non-meningitis isolates (Cavalieri et al., 2005). The PNSP isolates detected in the current study (60.2%) as compared to penicillin resistance in Cyprus (27.8%), Tanzania (46%), Hong Kong (7.3%), Italy (30.9%) and Brazil (38.9%) (Camilli et al., 2013; Chan et al., 2016; Emgård et al., 2019; Hadjipanayis et al., 2016; Neves et al., 2018) was high but low as compared to Congo (62%) and Nigeria (100%) (Birindwa et al., 2018; Torimiro et al., 2018). The minimum inhibition concentration (MIC) was determined on 15 randomly selected isolates which showed intermediate resistance ($0.12 \leq I \leq 2$ µg/ml) (CLSI, 2017) which suggests that possibly, all the PNSP could be intermediate resistant. In this study, most of the PNSP (80.4%) and PSSP (97.3%) were found to contain both *PBP2x* and *PBP2b* genes. According to

Jalal et al. (1997), the presence of both genes is suggestive that the isolates are PSSP since in their study, the use of primers that identifies both genes has high sensitivity (93%) for penicillin susceptible pneumococcus. Moreover, Jalal and colleagues (1997) also reported that their methods, even though sensitive to detect PSSP and PRSP, was not useful in detecting PISP. According to Zettler et al. (2006), the presence of all three genes was indicative of resistance whereas the presence of one gene (*PBP2b* or *PBP2x*) was indicative of intermediate resistance. This explains why all the isolates with one of the *PBP* genes was found among the PNSP isolates and three out of four PSNP contained all three genes as shown in Table 15. However, three of the isolates containing all three *PBP* genes were PNSP whereas only one was found to be PSSP. This could mean that the three PNSP were expressing resistance whereas the one PSSP was not expressing its resistance.

The distribution of PNSP was assessed among serotypes (Figure 9). Most of the detected vaccine serotypes were non-susceptible to penicillin and among the predominant non-vaccine serotypes (15A and 23B), most of the 15A isolates were resistant (2/3, 66.7%) whiles PNSP were evenly distributed among 23B isolates. However, 11A isolates were all non-susceptible to penicillin.

According to the 2017, Ghana Standard Treatment Guidelines, penicillin is recommended for the treatment of paediatric infections. The high resistance to penicillin might be associated with the easy accessibility to penicillin, low adherence to the proper use of drug and lack of knowledge on the use of the drugs. Treatment of pneumococcal diseases with penicillin might not be suitable even though the resistance were intermediate which

could suggest that administration of penicillin should be done frequently (four or more times daily) to achieve the optimum results (Jacobs, 1999). However, this could exert selective pressure on the pneumococcus leading to a full-blown resistance in the pneumococcus. Also, administration of penicillin with cephalosporins might further exert selective pressure on the pneumococcus since all the isolates that were resistant to the third-generation cephalosporin, ceftriaxone, were also resistant to penicillin (Table 13). This might be due to the similar mode of action the two drugs possess.

The high resistance of the pneumococcal isolates to tetracycline (64.5%) is similar to that reported by Dayie et al. (2019) and even though higher than that reported in Ethiopia (42.3%), it was still lower than that in Congo (78%) and Hong Kong (71.2%) (Birindwa et al., 2018; Chan et al., 2016; Haile et al., 2019). Resistance to tetracycline was reported to be conferred mostly by the *tetM* gene in Accra, Ghana (Dayie et al., 2019) which is involved in ribosomal protection. The dual resistance between penicillin and tetracycline indicates that the proportion of isolates that have resistance to both penicillin and tetracycline (71.7%) are significantly different from the isolates that are sensitive to both drugs ($p=0.036$). This suggests that there is a high probability that isolates resistant to tetracycline were also resistant to penicillin.

High resistance to co-Trimoxazole has also been reported in countries such as Tanzania (97%), Nigeria (81.3%), Congo (94%) and Ethiopia (64%) which concurs with the findings in this study (87%) (Birindwa et al., 2018; Emgård et al., 2019; Haile et al., 2019; Torimiro et al., 2018). A pre-vaccination carriage study in Ghana showed 100% resistance to co-

Trimoxazole (Mills et al., 2015) indicating a slight decrease in resistance. co-Trimoxazole is an over-the-counter drug in Ghana just like penicillin and as such it is easily accessible. It is a commonly used drug due to its synergistic effect against bacterial, protozoan and fungal infections, and also a recommended prophylactic treatment for children with HIV infection (WHO, 2006). Moreover, due to the spread of chloroquine-resistance throughout Africa, there have been a switch to the use of sulphadoxine-pyrimethamine (SP) as the first-line treatment of malaria in children in some countries, for intermittent preventive therapy (IPT) in pregnant women and seasonal malaria chemopreventive in Ghana (Chatio, Ansah, Awuni, Oduro, & Ansah, 2019). This, however, contributes co-Trimoxazole resistance since both co-Trimoxazole and SP have a similar mechanism of action; thus, inhibiting enzymes involved in the metabolic synthesis of folic acid possibly allowing cross-resistance mechanism between them (Feikin et al., 2000). Resistance to co-Trimoxazole might have occurred due to the selective pressure exerted on the pneumococcus microbe during the treatment of malaria in the children and also through vertical transmission from the mothers to their newly born children. Resistance to co-Trimoxazole is conferred by mutations in *fol A* and *fol P*, the genes encoding the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes.

This study revealed a low level of tolerance to vancomycin (2.2%) and resistance to erythromycin (6.5%), clindamycin (4.3%) and chloramphenicol (6.5%). However, 2 (2.2%) isolates were found to be Inducible clindamycin (D) test positive which indicates that these isolates exhibit resistance to clindamycin only in the presence erythromycin. The presence of erythromycin

induces the production of rRNA methylases which are encoded for by the *erm* gene.

The distribution of antibiotic resistance varied among age, sex and serotypes (Table 14). Isolates from children 2 years and below had high resistance (32.8%) than children older than 2 years (29.7%). More specifically, the percentage of tetracycline resistance was significantly higher in children 2 years and below than in those older than 2 years (p=0.008). Moreover, tetracycline-resistant isolates were mostly found in males as compared to females (p=0.001) even though collectively, resistant isolates were residing in the nasopharynx of females than males (30.8%). Penicillin non-susceptible pneumococcus (PNSP) were mostly found among children above 2 years (63.8%) and females (61.4%) although not significant. Emgard and colleagues (2019) reported that there is a strong association between females and carriage of PNSP. The proportion of non-vaccine serotypes that were resistant to chloramphenicol were lower than those of vaccine serotypes (p=0.024) as such PNSP were mostly found in vaccine serotypes were more resistant as compared to non-vaccine serotypes.

Multidrug-resistant (MDR) isolates, as defined by resistance to three or more classes of antibiotics, with the exception of ceftriaxone but including isolates containing the three *PBP* genes, were found to be 12.9% which is much lower as compared to the findings (20%) reported by Dayie et al. (2019). However, the PCV-13 coverage (63%) is similar as compared to that (60%) reported by Dayie et al. (2019). The proportion of MDR isolates were mostly serotype 23F and NT (33.3%). Moreover, MDR isolates were predominant among children 2 years and below (52.2%) and in males (53.1%)

(Table 14). This, however, shows that vaccine serotypes are the cause of multidrug resistance as this is also supported by the findings by Chan et al. (2016) which reported that even though only 8 (9.8%) isolates were MDR isolates, 5 isolates were of vaccine types.

As shown in Table 15, almost all of the isolates found among children older than 24 months were PSSP (91.3%) as classified by the presence of *PBP* genes indicating penicillin susceptibility. This is suggestive that the presence of PNSP isolates reduced as age increased and could be related to the effectiveness of the PCV-13 and maturity of the mucosal immunity of the children as they age. In relation to the serotype groups, vaccine types (87.9%) were more susceptible to penicillin as compared to the non-vaccine types (86.7%) as classified by Jalal et al. (1997). This finding, therefore agrees with the findings by Obolski et al. (2017) which indicated that non-vaccine types are resistant to penicillin.

Chapter Summary

The study revealed that 23.4% carriage prevalence among children under five with those attending private schools having a higher risk of carrying the pathogen (0.013). The current pneumococcal vaccine (PCV-13) in use, covers 34.5% of the serotypes detected, indicating that most of the serotypes were the non-vaccine types. However, the vaccine types persisted since the predominant serotypes were 6A/B, 14, 19F and 23F.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATIONS

With the introduction of the 13 valent pneumococcal conjugate vaccine into national immunisation programmes worldwide, there have been reports on the drastic reduction in the incidence of invasive pneumococcal disease and antibiotic resistance caused by vaccine serotypes. A few years afterwards, studies have reported on the non-reducing carriage rates of the pneumococcus despite the reduction of the vaccine serotypes and the emergence of antibiotic resistant non-vaccine serotypes which have the propensity to cause pneumococcal diseases. Meanwhile, there haven't been enough studies in Ghana to verify the claims made concerning the serotype and antibiotic susceptibility distribution of *Streptococcus pneumoniae* among high-risk populations such as children under 5 years. In view of this, this study sought to investigate the pneumococcus serotype distribution by determining the carriage prevalence, the serotypes present in the detected microbes and determining their susceptibility to commonly used antimicrobials including the genetic bases of penicillin resistance. This was achieved by taking nasopharyngeal swabs from children of consenting parents and placing the swabs in storage media for transportation and further storage. Using the appropriate culture methods and confirmation by the molecular detection of the *lyt A* gene, the pneumococcus was detected. The pneumococcus was later taken through antibiotic susceptibility testing using disk diffusion method where the penicillin non-susceptible ones were further tested for the presence of resistance genes. Serotyping of the pneumococcus was done using

multiplex PCR which was used to determine the PCV-13 coverage. The results obtained in this study was compiled and presented in tables and figures.

Summary

The results obtained from this cross-sectional study showed a 23.4% carriage prevalence in the overall population with male children being more colonised (24.5%) than female children. Moreover, children older than 24 months had high carriage prevalence while children attending private schools were highly at risk of nasopharynx colonisation (36.2%) by the pneumococcus (p=0.013). Serotyping using multiplex PCR showed the persistence of vaccine serogroups/serotypes such as 6A/B (10.8%), 14 (7.5%), 19F (6.5%) and 23F (8.6%) even though the overall vaccine serotypes were 35.5% and 64.5% for non-vaccine serotypes since all the non-typeables were all *cpsA* positive. This, therefore, indicates the PCV-13 coverage of 35.5%. The antibiotic susceptibility of the pneumococcus to commonly used antibiotics showed that most of the isolates were non-susceptible to oxacillin (60.2%), tetracycline (64.5%) and co-Trimoxazole (87.1%) and none was resistant to levofloxacin. Moreover, the determination of MIC on randomly selected penicillin-resistant isolates showed that all the selected isolates were intermediate resistant. The proportion of isolates resistant to both penicillin and tetracycline were different from those sensitive to both drugs (p=0.036). Tetracycline resistance was significant among children 2 years and below (82.6%), and males (73.5%) while chloramphenicol resistance was mostly exhibited by vaccine serotypes (15.2%). Multidrug resistant isolates were also found to 12.9%.

Conclusion

This study was performed on healthy children below 5 years in the Cape Coast Metropolis, Ghana, six years after the introduction of PCV-13. This study, being the first in this location, showed that 23.4% of the healthy children were colonised by *Streptococcus pneumoniae*. This carriage rate was low in comparison to other similar studies done in the pre- and post-vaccination era in the coastal belt like as this study. The overall serotype prevalence was dominated by vaccine serotype/serogroup 6A/B, 14, 19F and 23F, all of which are included in the 13 valent pneumococcal conjugate vaccine. The persistence of these vaccine serotypes was also found in other countries but not in other parts of this country. This study has however revealed the presence of emerging serotypes, 15A, 15B/C and 23B which were previously not detected by previous studies done in Ghana. Most importantly, based on the molecular detection of the *PBP* genes, low resistance to penicillin and high resistance to tetracycline and co-Trimoxazole was determined, including its distribution among age groups, sex and serotypes that could influence vaccination strategies and policies.

Recommendations

This study is the first of its kind in the central region of Ghana and so serving as a baseline for future studies. It is therefore necessary for continuous surveillance of the serotypes and antibiotic resistance to strengthen knowledge and evidence as stated as one of the policy objectives in "Policy on Antimicrobial Use and Resistance in Ghana". Serotypes 15A, 15B/C and 23B should be monitored since they are known to be the major cause of invasive pneumococcal disease in other countries. In view of the persistence of the

vaccine serotypes, it is suggestive that the Ghana's schedule dose of 3p+0 should be reviewed and possibly consider adding a booster 3p+1 or 2p+1. With regards to the high antibiotic resistance in the pneumococcus, it is recommended that there should be strict adherence to the national plan for antimicrobial use and resistance.

Suggestions for further research

Further research should be a longitudinal study such as cohort study in order to give precise look at the dynamics of carriage, acquisitions rates and duration of carriage of serotypes. Concerning the distribution of carriage and resistance, it will be appropriate for future studies to assess the risk factors associated with them. Moreover, subsequent research should detect non-typeables molecularly using *cpsB, aliC*/*aliD* genes. The molecular bases of resistance to tetracycline and co-Trimoxazole should be looked at, especially with regards with co-Trimoxazole resistance which requires sequencing of *folA* and *folP* gene. Finally, further research should be done to determine the molecular characteristics of circulating genotypes of the pneumococcus using whole genome sequencing.

REFERENCE

Adegbola, R. A., DeAntonio, R., Hill, P. C., Roca, A., Usuf, E., & Hoet, B. (2014). Carriage of *Streptococcus pneumoniae* and other respiratory bacterial pathogens in low and lower-middle income countries: a systematic review and meta-analysis. *PLoS One*, 9(8),103293. doi:10.1371/journal.pone.0103293

Adetifa, I. M. O., Antonio, M., Okoromah, C. A. N., Ebruke, C., Inem, V., Bojang, A., & Adegbola, R. A. (2012). Pre-Vaccination Nasopharyngeal Pneumococcal Carriage in a Nigerian Population : Epidemiology and Population Biology, *7*(1). https://doi.org/10.1371/journal.pone.0030548

Althouse, B. M., Hammitt, L. L., Grant, L., Wagner, B. G., Reid, R., Larzelere-Hinton, F., … Hu, H. (2017). Identifying transmission routes of Streptococcus pneumoniae and sources of acquisitions in high transmission communities. *Epidemiology and Infection*, *145*(13), 2750–2758. https://doi.org/10.1017/S095026881700125X

Azzari, C., Moriondo, M., Indolfi, G., Cortimiglia, M., Canessa, C., Becciolini, L., … Resti, M. (2010). Realtime PCR is more sensitive than multiplex PCR for diagnosis and serotyping in children with culture negative pneumococcal invasive disease. *PLoS ONE*, *5*(2). https://doi.org/10.1371/journal.pone.0009282

Balsells, E., Guillot, L., Nair, H., & Kyaw, M. H. (2017). Serotype distribution of Streptococcus pneumoniae causing invasive disease in children in the post-PCV era: A systematic review and meta-analysis. *PLoS ONE*, *12*(5). https://doi.org/10.1371/journal.pone.0177113

Birindwa, A. M., Emgård, M., Nordén, R., Samuelsson, E., Geravandi, S., Gonzales-siles, L., … Lindh, M. (2018). High rate of antibiotic resistance among pneumococci carried by healthy children in the eastern part of the Democratic Republic of the Congo, 1–12.

Bogaert, D., Groot, R. De, & Hermans, P. W. M. (2004). Streptococcus pneumoniae colonisation Streptococcus pneumoniae colonisation : the

key to pneumococcal disease, *4*(March), 144–154.

Bozio, C. H., Abdul-karim, A., Abenyeri, J., Abubakari, B., Ofosu, W., Zoya, J., … Lessa, F. C. (2018). Continued occurrence of serotype 1 pneumococcal meningitis in two regions located in the meningitis belt in Ghana five years after introduction of 13-valent pneumococcal conjugate vaccine, *137*, 1–10. https://doi.org/10.1371/journal.pone.0203205

Brooks, L. R. K., & Mias, G. I. (2018). Streptococcus pneumoniae's virulence and host immunity: Aging, diagnostics, and prevention. *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2018.01366

Camilli, R., Daprai, L., Cavrini, F., Lombardo, D., D'Ambrosio, F., Del Grosso, M., … Pantosti, A. (2013). Pneumococcal Carriage in Young Children One Year after Introduction of the 13-Valent Conjugate Vaccine in Italy. *PLoS ONE*, *8*(10), 1–10. https://doi.org/10.1371/journal.pone.0076309

Carvalho, M. D. G., Pimenta, F. C., Jackson, D., Roundtree, A., Ahmad, Y., Millar, E. V., … Beall, B. W. (2010). Revisiting pneumococcal carriage by use of broth enrichment and PCR techniques for enhanced detection of carriage and serotypes. *Journal of Clinical Microbiology*, *48*(5), 1611–1618. https://doi.org/10.1128/JCM.02243-09

Cattoir, V. (2016). Mechanisms of antibiotic resistance. *Frontiers in Microbiology*, *6*(FEB), 1–45. https://doi.org/10.3389/fmicb.2015.00034

CDC. (2011). Laboratory Methods for the Diagnosis of Meningitis Caused by Neisseria meningitidis, Streptococcus pneumoniae, and Haemophilus influenzae. CHAPTER 8 Identification and Characterization of Streptococcus pneumoniae. *CDC- Center for Disease Control and Prevention*, *2*, 1–14. Retrieved from http://www.cdc.gov/meningitis/lab-manual/chpt08-id-characterization-streppneumo.pdf

Chan, C. C. K., Subramanian, R., Chong, P., Nelson, E. A. S., Simon, H., Martin, A., & Ip, M. (2016). Pneumococcal carriage in young children after introduction of PCV13 in Hong Kong. *Vaccine*. https://doi.org/10.1016/j.vaccine.2016.05.047

Chan, K. C. C., Subramanian, R., Chong, P., Nelson, E. A. S., Lam, H. S., Li, A. M., & Ip, M. (2016). Pneumococcal carriage in young children after introduction of PCV13 in Hong Kong. *Vaccine*, *34*(33), 3867–3874. https://doi.org/10.1016/j.vaccine.2016.05.047

Chatio, S., Ansah, N. A., Awuni, D. A., Oduro, A., & Ansah, P. O. (2019). Community acceptability of Seasonal Malaria Chemoprevention of morbidity and mortality in young children: A qualitative study in the Upper West Region of Ghana. *PLoS ONE*, *14*(5), 1–12. https://doi.org/10.1371/journal.pone.0216486

Chewapreecha, C., Harris, S. R., Croucher, N. J., Turner, C., Marttinen, P., Cheng, L., … Bentley, S. D. (2014). Dense genomic sampling identifies highways of pneumococcal recombination. *Nature Genetics*, *46*(3), 305–309. https://doi.org/10.1038/ng.2895

Choe, Y. J., Lee, H. J., Lee, H., Oh, C. E., Cho, E. Y., Choi, J. H., … Choi, E. H. (2016). Emergence of antibiotic-resistant non-vaccine serotype pneumococci in nasopharyngeal carriage in children after the use of extended-valency pneumococcal conjugate vaccines in Korea. *Vaccine*, *34*(40), 4771–4776. https://doi.org/10.1016/j.vaccine.2016.08.030

CLSI. (2017). *Performance Standards for Antimicrobial Susceptibility Testing* (27th ed, Vol. 37). Wayne, PA: Clinical and Laboratory Standards Institute.

Cornick, J. E., Harris, S. R., Parry, C. M., Moore, M. J., Jassi, C., Kamng, A., … Everett, D. B. (2018). Genomic identification of a novel co-Trimoxazole resistance genotype and its prevalence amongst Streptococcus pneumoniae in Malawi, (September 2013), 368–374. https://doi.org/10.1093/jac/dkt384

Dayie, N. T. K. D., Arhin, R. E., Newman, M. J., Dalsgaard, A., Bisgaard, M., Frimodt-møller, N., & Slotved, H. (2013). Penicillin resistance and serotype distribution of Streptococcus pneumoniae in Ghanaian children less than six years of age. *BMC Infectious Diseases*, *13*(490), 1–7.

Dayie, N. T. K. D., Baffuor-asare, M., Labi, A., Obeng-nkrumah, N., Olayemi,

E., Lartey, M., … Donkor, E. S. (2019). Epidemiology of Pneumococcal Carriage among HIV-Infected Individuals in the Conjugate Vaccine Era : A Study in Southern Ghana, *2019*.

Dayie, N. T. K. D., Tetteh-Ocloo, G., Labi, A. K., Olayemi, E., Slotved, H. C., Lartey, M., & Donkor, E. S. (2018). Pneumococcal carriage among sickle cell disease patients in Accra, Ghana: Risk factors, serotypes and antibiotic resistance. *PLoS ONE*, *13*(11), 1–14. https://doi.org/10.1371/journal.pone.0206728

Dayie, N. T. K. D., Tettey, E. Y., Newman, M. J., Bannerman, E., Donkor, E. S., Labi, A., & Slotved, H. (2019). Pneumococcal carriage among children under five in Accra , Ghana , five years after the introduction of pneumococcal conjugate vaccine, 1–11.

Denno, D. M., Frimpong, E., Gregory, M., & Steele, R. W. (2002). Nasopharyngeal carriage and susceptibility patterns of Streptococcus pneumoniae in Kumasi, Ghana. *West African Journal of Medicine*, *21*(3), 233–236. https://doi.org/10.4314/wajm.v21i3.28038

Devine, V. T., Cleary, D. W., Jefferies, J. M. C., Anderson, R., Morris, D. E., Tuck, A. C., … Clarke, S. C. (2017). The rise and fall of pneumococcal serotypes carried in the PCV era. *Vaccine*, *35*(9), 1293–1298. https://doi.org/10.1016/j.vaccine.2017.01.035

Diawara, I., Zerouali, K., Katfy, K., Zaki, B., Belabbes, H., Najib, J., & Elmdaghri, N. (2015). Invasive pneumococcal disease among children younger than 5 years of age before and after introduction of pneumococcal conjugate vaccine in Casablanca, Morocco. *International Journal of Infectious Diseases*, *40*, 95–101. https://doi.org/10.1016/j.ijid.2015.09.019

Donkor, E.S., Bishop, C. J., Antonio, M., Wren, B., & Hanage, W. P. (2011). High Levels of Recombination among Streptococcus pneumoniae. *MBio*, *2*(3), 1–8. https://doi.org/10.1128/mBio.00040-11.Editor

Donkor, Eric S., Annan, J. A., Badoe, E. V., Dayie, N. T. K. D., Labi, A. K., & Slotved, H. C. (2017). Pneumococcal carriage among HIV infected

children in Accra, Ghana. *BMC Infectious Diseases*, *17*(1), 1–7. https://doi.org/10.1186/s12879-017-2224-0

Donkor, Eric S., Newman, M. J., Oliver-Commey, J., Bannerman, E., Dayie, N. T. K. D., & Badoe, E. V. (2010). Invasive disease and paediatric carriage of Streptococcus pneumoniae in Ghana. *Scandinavian Journal of Infectious Diseases*, *42*(4), 254–259. https://doi.org/10.3109/00365540903490000

Dube, F. S., Ramjith, J., Gardner-Lubbe, S., Nduru, P., Robberts, F. J. L., Wolter, N., … Nicol, M. P. (2018). Longitudinal characterization of nasopharyngeal colonization with Streptococcus pneumoniae in a South African birth cohort post 13-valent pneumococcal conjugate vaccine implementation. *Scientific Reports*, *8*(1), 1–9. https://doi.org/10.1038/s41598-018-30345-5

El Ashkar, S., Osman, M., Rafei, R., Mallat, H., Achkar, M., Dabboussi, F., & Hamze, M. (2017). Molecular detection of genes responsible for macrolide resistance among Streptococcus pneumoniae isolated in North Lebanon. *Journal of Infection and Public Health*, *10*(6), 745–748. https://doi.org/10.1016/j.jiph.2016.11.014

Emgård, M., Msuya, S. E., Nyombi, B. M., Mosha, D., Gonzales-siles, L., Nordén, R., … Andersson, R. (2019). International Journal of Infectious Diseases Carriage of penicillin-non-susceptible pneumococci among children in northern Tanzania in the 13-valent pneumococcal vaccine era, *81*, 156–166. https://doi.org/10.1016/j.ijid.2019.01.035

Espinosa, I., Báez, M., Percedo, M. I., & Martínez, S. (2013). Evaluación de métodos simples de extracción de ADN para la tipificación de S . suis Evaluation of simplified DNA extraction methods for Streptococcus suis typing. *Rev. Salud Anim*, *35*(1), 59–63.

Esposito, S., & Principi, N. (2015). Impacts of the 13-Valent Pneumococcal Conjugate Vaccine in Children. *Journal of Immunology Research*, *2015*, 1–6. https://doi.org/10.1155/2015/591580

Feikin, D. R., Dowell, S. F., Nwanyanwu, O. C., Klugman, K. P., Kazembe, P.

N., Barat, L. M., … Huebner, R. E. (2000). Increased Carriage of Trimethoprim / Sulfamethoxazole-Resistant Streptococcus pneumoniae in Malawian Children after Treatment for Malaria with Sulfadoxine / Pyrimethamine. *Journal of Infectious Diseases*, *181*, 1501–1505.

Fjeldhøj, S., Laursen, R. P., Larnkjær, A., Mølgaard, C., Fuursted, K., Krogfelt, K. A., & Slotved, H. C. (2018). Probiotics and carriage of Streptococcus pneumoniae serotypes in Danish children, a double-blind randomized controlled trial. *Scientific Reports*, *8*(1), 1–9. https://doi.org/10.1038/s41598-018-33583-9

Geno, K. A., Gilbert, G. L., Song, J. Y., Skovsted, I. C., Klugman, K. P., Jones, C., … Nahm, M. H. (2015). Pneumococcal capsules and their types: Past, present, and future. *Clinical Microbiology Reviews*, *28*(3), 871–899. https://doi.org/10.1128/CMR.00024-15

Goonewardene, S. T., Tang, C., Tan, L. T.-H., Chan, K.-G., Lingham, P., Lee, L.-H., … Pusparajah, P. (2019). Safety and Efficacy of Pneumococcal Vaccination in Pediatric Nephrotic Syndrome. *Frontiers in Pediatrics*, *7*(339), 1–20. https://doi.org/10.3389/fped.2019.00339

Greene, N. G., Narciso, A. R., Filipe, S. R., & Camilli, A. (2015). Peptidoglycan Branched Stem Peptides Contribute to Streptococcus pneumoniae Virulence by Inhibiting Pneumolysin Release. *PLoS Pathogens*, *11*(6), 1–24. https://doi.org/10.1371/journal.ppat.1004996

Greenwood, D., Barer, M., Slack, R., & Irving, W. (2012). *Medical Microbiology. A guide to Miicrobial Infections: Pathogenesis, immunity, laboratory investigation and control* (18 edition). China: Churchill Livingstone.

Gutiérrez-Fernández, J., Saleh, M., Alcorlo, M., Gómez-Mejóa, A., Pantoja-Uceda, D., Treviño, M. A., … Hermoso, J. A. (2016). Modular Architecture and Unique Teichoic Acid Recognition Features of Choline-Binding Protein L (CbpL) Contributing to Pneumococcal Pathogenesis. *Scientific Reports*, *6*, 38094. https://doi.org/10.1038/srep38094

Habib, M., Porter, B. D., & Satzke, C. (2014). Capsular Serotyping of

Streptococcus pneumoniae Using the Quellung Reaction. *J. Vis. Exp.*, *84*, 1–4. https://doi.org/10.3791/51208

Hadjipanayis, A., Efstathiou, E., Alexandrou, M., Panayiotou, L., Zachariadou, C., Petrou, P., & Papaevangelou, V. (2016). Nasopharyngeal Pneumococcal Carriage among Healthy Children in Cyprus Post Widespread Simultaneous Implementation of PCV10 and PCV13 Vaccines, 1–15. https://doi.org/10.1371/journal.pone.0163269

Haile, A. A., Gidebo, D. D., & Ali, M. M. (2019). Colonization rate of Streptococcus pneumoniae, its associated factors and antimicrobial susceptibility pattern among children attending kindergarten school in Hawassa, southern Ethiopia. *BMC Research Notes*, *12*(1), 1–7. https://doi.org/10.1186/s13104-019-4376-z

Huang, S., Liu, X., Lao, W., Zeng, S., Liang, H., Zhong, R., … Wu, X. (2015). Serotype distribution and antibiotic resistance of Streptococcus pneumoniae isolates collected at a Chinese hospital from 2011 to 2013, 1–10. https://doi.org/10.1186/s12879-015-1042-5

Imo, M., Rene, R., Ocklenburg, C., & Linden, M. Van Der. (2010). Association of Serotypes of Streptococcus pneumoniae with Age in Invasive Pneumococcal Disease , *48*(4), 1291–1296. https://doi.org/10.1128/JCM.01937-09

Ip, M., Liyanapathirana, V., Ang, I., Fung, K. S. C., Ng, T. K., Zhou, H., & Tsang, D. N. C. (2014). Direct detection and prediction of all pneumococcal serogroups by target enrichment-based next-generation sequencing. *Journal of Clinical Microbiology*, *52*(12), 4244–4252. https://doi.org/10.1128/JCM.02397-14

Jalal, H., Organji, S., Reynolds, J., Bennett, D., Jnr, E. O. M., Millar, M. R., & Royal, B. (1997). Determination of penicillin susceptibility of Streptococcus pneumoniae using the polymerase chain reaction. *J Clin Pathol: Mol Pathol*, *50*, 45–50.

Jauneikaite, E., Tocheva, A. S., Jefferies, J. M. C., Gladstone, R. A., Faust, S. N., Myron, C., … Clarke, S. C. (2015). Current methods for capsular

typing of Streptococcus pneumoniae. *Journal of Microbiological Methods*, 1–9. https://doi.org/10.1016/j.mimet.2015.03.006

Jorgensen, J. H., Swenson, J. M., Tenover, F. C., Barry, A., Ferraro, M. J., Murray, P. R., & Reller, L. B. (1994). Development of interpretive criteria and quality control limits for macrolide and clindamycin susceptibility testing of Streptococcus pneumoniae. *Journal of Clinical Microbiology*, *34*(11), 2679–2684.

Kamng'ona, A. W., Hinds, J., Bar-Zeev, N., Gould, K. A., Chaguza, C., Msefula, C., … Everett, D. B. (2015). High multiple carriage and emergence of Streptococcus pneumoniae vaccine serotype variants in Malawian children. *BMC Infectious Diseases*, *15*(1), 1–11. https://doi.org/10.1186/s12879-015-0980-2

Kamng, A. W., Hinds, J., Bar-zeev, N., Gould, K. A., Chaguza, C., Msefula, C., … Everett, D. B. (2015). High multiple carriage and emergence of Streptococcus pneumoniae vaccine serotype variants in Malawian children. *BMC Infectious Diseases*, 1–11. https://doi.org/10.1186/s12879-015-0980-2

Kaplan, S. L., Center, K. J., Barson, W. J., Ling-Lin, P., Romero, J. R., Bradley, J. S., … Mason, E. O. (2015). Multicenter surveillance of streptococcus pneumoniae isolates from middle ear and mastoid cultures in the 13-valent pneumococcal conjugate vaccine era. *Clinical Infectious Diseases*, *60*(9), 1339–1345. https://doi.org/10.1093/cid/civ067

Keller, L. E., Robinson, D. A., & Mcdaniel, L. S. (2016). Nonencapsulated Streptococcus pneumoniae : Emergence and, *7*(2), 1–12. https://doi.org/10.1128/mBio.01792-15.Invited

Kellogg, J. A., Bankert, D. A., Elder, C. J., Gibbs, J. L., & Smith, M. C. (2001). Identification of Streptococcus pneumoniae revisited. *Journal of Clinical Microbiology*, *39*(9), 3373–3375. https://doi.org/10.1128/JCM.39.9.3373-3375.2001

Ktari, S., Jmal, I., Mroua, M., Maalej, S., Elhouda, N., Ayed, B., … Hammami, A. (2017). International Journal of Infectious Diseases

Serotype distribution and antibiotic susceptibility of Streptococcus pneumoniae strains in the south of Tunisia : A fi ve-year study (2012 – 2016) of pediatric and adult populations. *International Journal of Infectious Diseases*, *65*, 110–115. https://doi.org/10.1016/j.ijid.2017.10.015

Kwambana-adams, B., Hanson, B., Worwui, A., Agbla, S., Foster-nyarko, E., Ceesay, F., … Antonio, M. (2017). Rapid replacement by non- vaccine pneumococcal serotypes may mitigate the impact of the pneumococcal conjugate vaccine on nasopharyngeal bacterial ecology. *Scientific Reports*, (April), 1–11. https://doi.org/10.1038/s41598-017-08717-0

Ladhani, S. N., Collins, S., Djennad, A., Sheppard, C. L., Borrow, R., Fry, N. K., … Ramsay, M. E. (2018). Rapid increase in non-vaccine serotypes causing invasive pneumococcal disease in England and Wales, 2000–17: a prospective national observational cohort study. *The Lancet Infectious Diseases*, *18*(4), 441–451. https://doi.org/10.1016/S1473-3099(18)30052-5

Lee, G. M., Kleinman, K., Pelton, S. I., Hanage, W., Huang, S. S., Lakoma, M., … Finkelstein, J. A. (2014). Impact of 13-valent pneumococcal conjugate vaccination on Streptococcus pneumoniae carriage in young children in Massachusetts. *Journal of the Pediatric Infectious Diseases Society*, *3*(1), 23–32. https://doi.org/10.1093/jpids/pit057

Leeming, J. P., Cartwright, K., Morris, R., Martin, S. A., & Smith, M. D. (2005). Diagnosis of invasive pneumococcal infection by serotype-specific urinary antigen detection. *Journal of Clinical Microbiology*, *43*(10), 4972–4976. https://doi.org/10.1128/JCM.43.10.4972-4976.2005

Leimkugel, J., Adams Forgor, A., Gagneux, S., Pflüger, V., Flierl, C., Awine, E., … Pluschke, G. (2005). An Outbreak of Serotype 1 *Streptococcus pneumoniae* Meningitis in Northern Ghana with Features That Are Characteristic of *Neisseria meningitidis* Meningitis Epidemics. *The Journal of Infectious Diseases*, *192*(2), 192–199. https://doi.org/10.1086/431151

Levinson, W. (2016). *Medical Microbiology and Immunology*. (M. Weitz & B. Kearns, Eds.) (14 ed). New York: McGraw Hill Education.

Limoli, D. H., Sladek, J. A., Fuller, L. A., Singh, A. K., & King, S. J. (2011). BgaA acts as an adhesin to mediate attachment of some pneumococcal strains to human epithelial cells. *Microbiology*, *157*(8), 2369–2381. https://doi.org/10.1099/mic.0.045609-0

Lipsitch, M., Abdullahi, O., D'Amour, A., Xie, W., Weinberger, D. M., Tchetgen Tchetgen, E., & Scott, J. A. G. (2012). Estimating rates of carriage acquisition and clearance and competitive ability for pneumococcal serotypes in kenya with a markov transition model. *Epidemiology*, *23*(4), 510–519. https://doi.org/10.1097/EDE.0b013e31824f2f32

Liyanapathirana, V., Nelson, E. A. S., Ang, I., Subramanian, R., Ma, H., & Ip, M. (2015). Emergence of serogroup 15 Streptococcus pneumoniae of diverse genetic backgrounds following the introduction of pneumococcal conjugate vaccines in Hong Kong. *Diagnostic Microbiology and Infectious Disease*, *81*(1), 66–70. https://doi.org/10.1016/j.diagmicrobio.2014.09.028

Llull, D., García, E., & Ló Pez, R. (2001). Tts, a Processive-Glucosyltransferase of Streptococcus pneumoniae, Directs the Synthesis of the Branched Type 37 Capsular Polysaccharide in Pneumococcus and Other Gram-positive Species*. *ASBMB*. https://doi.org/10.1074/jbc.M010287200

Mackenzie, G. A., Hill, P. C., Jeffries, D. J., Hossain, I., Uchendu, U., Ameh, D., … Corrah, T. (2016). Effect of the introduction of pneumococcal conjugate vaccination on invasive pneumococcal disease in The Gambia: a population-based surveillance study. *The Lancet Infectious Diseases*, *16*(6), 703–711. https://doi.org/10.1016/S1473-3099(16)00054-2

Mackenzie, G. A., Leach, A. J., Carapetis, J. R., Fisher, J., & Morris, P. S. (2010). Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults : cross-sectional surveys in a population

with high rates of pneumococcal disease. https://doi.org/10.1186/1471-2334-10-304

Mackie, E. J., Shears, P., Frimpong, E., & Mustafa-Kutana, S. N. (1992). A study of bacterial meningitis in Kumasi, Ghana. *Annals of Tropical Paediatrics*, *12*(2), 143–148. https://doi.org/10.1080/02724936.1992.11747559

Marchisio, P., Esposito, S., Schito, G. C., & Marchese, A. (2002). Nasopharyngeal Carriage of Streptococcus pneumoniae in Healthy Children : Implications for the Use of Heptavalent Pneumococcal Conjugate Vaccine, *8*(5), 479–484.

Marks, L. R., Reddinger, R. M., & Hakansson, A. P. (2014). Biofilm formation enhances fomite survival of Streptococcus pneumoniae and Streptococcus pyogenes. *Infection and Immunity*, *82*(3), 1141–1146. https://doi.org/10.1128/IAI.01310-13

Mayanskiy, N., Alyabieva, N., Ponomarenko, O., Lazareva, A., Katosova, L., Ivanenko, A., … Baranov, A. (2014). Serotypes and antibiotic resistance of non-invasive Streptococcus pneumoniae circulating in pediatric hospitals in Moscow, Russia. *International Journal of Infectious Diseases*, *20*(1), 58–62. https://doi.org/10.1016/j.ijid.2013.11.005

Mills, R. O., Twum-danso, K., Owusu-agyei, S., & Donkor, E. S. (2015). Epidemiology of pneumococcal carriage in children under fi ve years of age in Accra , Ghana, (November 2014), 1–6. https://doi.org/10.3109/00365548.2014.994185

Ministry of Health. (2010). *Standard Treatment Guideline* (10th editi). Accra: Ghana National Drugs Programme.

Ministry of Health (GNDP). (2017). *STANDARD TREATMENT GUIDELINES* (7th ed.). Accra: Yamens Press Limited.

Moujaber, G. El, Osman, M., Rafei, R., Dabboussi, F., & Hamze, M. (2018). Molecular mechanisms and epidemiology of resistance in Streptococcus pneumoniae in the Middle East region, *66*, 847–858.

https://doi.org/10.1099/jmm.0.000503

Nagai, K., Shibasaki, Y., Hasegawa, K., Davies, T. A., Jacobs, M. R., Ubukata, K., & Appelbaum, P. C. (2001). JAC Evaluation of PCR primers to screen for Streptococcus pneumoniae isolates and -lactam resistance , and to detect common macrolide resistance determinants, 915–918.

Najafi Mosleh, M., Gharibi, M., Alikhani, M. Y., Saidijam, M., & Vakhshiteh, F. (2014). Antimicrobial susceptibility and analysis of macrolide resistance genes in Streptococcus pneumoniae isolated in Hamadan. *Iranian Journal of Basic Medical Sciences*, *17*(8).

Neves, F. P. G., Cardoso, N. T., Souza, A. R. V., Snyder, R. E., Marlow, M. M., Pinto, T. C. A., … Riley, L. W. (2018). Population structure of Streptococcus pneumoniae colonizing children before and after universal use of pneumococcal conjugate vaccines in Brazil: Emergence and expansion of the MDR serotype 6C-CC386 lineage. *Journal of Antimicrobial Chemotherapy*, *73*(5), 1206–1212. https://doi.org/10.1093/jac/dky001

Newton, R., Hinds, J., & Wernisch, L. (2011). Empirical Bayesian models for analysing molecular serotyping microarrays. *BMC Bioinformatics*, *12*(1), 88. https://doi.org/10.1186/1471-2105-12-88

Numminen, E., Chewapreecha, C., Turner, C., Goldblatt, D., Nosten, F., Bentley, S. D., … Corander, J. (2015). Climate induces seasonality in pneumococcal transmission. *Scientific Reports*, *5*, 1–14. https://doi.org/10.1038/srep11344

Obolski, U., Lourenço, J., Thompson, C., Thompson, R., Gori, A., & Gupta, S. (2018). Vaccination can drive an increase in frequencies of antibiotic resistance among nonvaccine serotypes of Streptococcus pneumoniae, *115*(12), 3102–3107. https://doi.org/10.1073/pnas.1718712115

Pai, R., Gertz, R. E., & Beall, B. (2006). Sequential Multiplex PCR Approach for Determining Capsular Serotypes of Streptococcus pneumoniae Isolates, *44*(1), 124–131. https://doi.org/10.1128/JCM.44.1.124

Park, I. H., Kim, K. H., Andrade, A. L., Briles, D. E., Mcdaniel, L. S., & Nahma, M. H. (2012). Nontypeable pneumococci can be divided into multiple cps types, including one type expressing the novel gene pspK. *MBio*, *3*(3). https://doi.org/10.1128/mBio.00035-12

Rantala, M. (2009). *Antimicrobial Resistance in Streptococcus Pneumoniae in Finland with Special Reference to Macrolides and Telithromycin*. University of Helsinki. Retrieved from http://www.thl.fi

Richter, S. S., Heilmann, K. P., Dohrn, C. L., Riahi, F., Beekmann, S. E., & Doern, G. V. (2008). Accuracy of phenotypic methods for identification of Streptococcus pneumoniae isolates included in surveillance programs. *Journal of Clinical Microbiology*, *46*(7), 2184–2188. https://doi.org/10.1128/JCM.00461-08

Roche, A. M., Richard, A. L., Rahkola, J. T., Janoff, E. N., & Weiser, J. N. (2015). Antibody blocks acquisition of bacterial colonization through agglutination. *Mucosal Immunology*, *8*(1), 176–185. https://doi.org/10.1038/mi.2014.55

Salvà-Serra, F., Connolly, G., Moore, E. R. B., & Gonzales-Siles, L. (2018). Detection of "Xisco" gene for identification of Streptococcus pneumoniae isolates. *Diagnostic Microbiology and Infectious Disease*, *90*(4), 248–250. https://doi.org/10.1016/j.diagmicrobio.2017.12.003

Satzke, C., Turner, P., Virolainen-julkunen, A., Adrian, P. V, Antonio, M., Hare, K. M., … Pneumococcal, W. H. O. (2014). Standard method for detecting upper respiratory carriage of Streptococcus pneumoniae : Updated recommendations from the World Health Organization Pneumococcal Carriage Working Group Standard method for detecting upper respiratory carriage of Streptococcus. *Vaccine*, *32*(2014), 165–179. https://doi.org/10.1016/j.vaccine.2013.08.062

Segura, M., Calzas, C., Grenier, D., & Gottschalk, M. (2016). Initial steps of the pathogenesis of the infection caused by *Streptococcus suis* : fighting against nonspecific defenses. *FEBS Letters*, *590*(21), 3772–3799. https://doi.org/10.1002/1873-3468.12364

Siegel, S. J., Roche, A. M., & Weiser, J. N. (2014). Influenza promotes pneumococcal growth during coinfection by providing host sialylated substrates as a nutrient source. *Cell Host and Microbe*, *16*(1), 55–67. https://doi.org/10.1016/j.chom.2014.06.005

Simões, A. S., Tavares, D. A., Rolo, D., Ardanuy, C., Goossens, H., Henriques-Normark, B., … Sá-Leão, R. (2016). LytA-based identification methods can misidentify Streptococcus pneumoniae. *Diagnostic Microbiology and Infectious Disease*, *85*(2), 141–148. https://doi.org/10.1016/j.diagmicrobio.2016.03.018

Slotved, H. C., Facklam, R. R., & Fuursted, K. (2017). Assessment of a novel bile solubility test and MALDI-TOF for the differentiation of Streptococcus pneumoniae from other mitis group streptococci. *Scientific Reports*, *7*(1), 1–8. https://doi.org/10.1038/s41598-017-07772-x

Sourav, S., Patricia, A., Sharma, S., … R. K.-I. journal of medical, & 2010, undefined. (n.d.). Detection of pneumolysin and autolysin genes among antibiotic resistant Streptococcus pneumoniae in invasive infections. *Ijmm.Org*. Retrieved from http://www.ijmm.org/article.asp?issn=0255-0857;year=2010;volume=28;issue=1;spage=34;epage=39;aulast=Sourav

Southern, J., Andrews, N., Sandu, P., Sheppard, C. L., Waight, P. A., Fry, N. K., … Miller, E. (2018). Pneumococcal carriage in children and their household contacts six years after introduction of the 13-valent pneumococcal conjugate vaccine in England, *16*, 1–14.

Steens, A., Caugant, D. A., Aaberge, I. S., & Vestrheim, D. F. (2015). Decreased carriage and genetic shifts in the streptococcus pneumoniae population after changing the seven-valent to the thirteen-valent pneumococcal vaccine in Norway. *Pediatric Infectious Disease Journal*, *34*(8), 875–883. https://doi.org/10.1097/INF.0000000000000751

Torimiro, N., Fabunmi, A. O., & Ajayi, A. (2018). Serotypes and antibiotic susceptibility profile of encapsulated Streptococcus pneumoniae strains isolated from under five years old children, *12*(April), 990–998.

Usuf, E., Bojang, A., Camara, B., Jagne, I., Oluwalana, C., Bottomley, C., …

Roca, A. (2018). Maternal pneumococcal nasopharyngeal carriage and risk factors for neonatal carriage after the introduction of pneumococcal conjugate vaccines in The Gambia. *Clinical Microbiology and Infection*, *24*(4), 389–395. https://doi.org/10.1016/j.cmi.2017.07.018

Usuf, E., Bojang, A., Hill, P. C., Bottomley, C., Greenwood, B., & Roca, A. (2016). Nasopharyngeal colonization of Gambian infants by Staphylococcus aureus and Streptococcus pneumoniae before the introduction of pneumococcal conjugate vaccines. *New Microbes and New Infections*, *10*, 13–18. https://doi.org/10.1016/j.nmni.2015.12.002

Van Der Linden, M., Falkenhorst, G., Perniciaro, S., & Imöhl, M. (2015). Effects of infant pneumococcal conjugate vaccination on serotype distribution in invasive pneumococcal disease among children and adults in germany. *PLoS ONE*, *10*(7), 1–17. https://doi.org/10.1371/journal.pone.0131494

Wahl, B., Brien, K. L. O., Greenbaum, A., Majumder, A., Liu, L., Chu, Y., … Knoll, M. D. (2018). Articles Burden of Streptococcus pneumoniae and Haemophilus influenzae type b disease in children in the era of conjugate vaccines : global , regional , and national estimates for. *The Lancet Global Health*, *6*(7), e744–e757. https://doi.org/10.1016/S2214-109X(18)30247-X

Weiser, J. N., Ferreira, D. M., & Paton, J. C. (2018). Streptococcus pneumoniae: transmission, colonization and invasion. *Nature Reviews Microbiology*. https://doi.org/10.1038/s41579-018-0001-8

Wessels, E., Schelfaut, J. J. G., Bernards, A. T., & Claas, E. C. J. (2012). Evaluation of Several Biochemical and Molecular Techniques for Identification of Streptococcus pneumoniae and Streptococcus pseudopneumoniae and Their Detection in Respiratory Samples. *Am Soc Microbiol*, *50*(4), 1171–1177. https://doi.org/10.1128/JCM.06609-11

WHO. (2014). *CONCURRENT INTRODUCTION OF TWO VACCINES*. Retrieved from https://www.who.int/immunization/diseases/poliomyelitis/inactivated_pol

io_vaccine/case_study_ghana_en.pdf?ua=1

WHO. (2019). Pneumococcal conjugate vaccines in infants and children under 5 years of age: WHO position paper – February 2019. *Weekly Epidemiological Record*, *94*(8), 85–104. Retrieved from http://orton.catie.ac.cr/cgi-bin/wxis.exe/?IsisScript=KARDEX.xis&method=post&formato=2&cantidad=1&expresion=mfn=003687

Wyres, K. L., Lambertsen, L. M., Croucher, N. J., McGee, L., Von Gottberg, A., Liñares, J., … Brueggemann, A. B. (2013). Pneumococcal capsular switching: A historical perspective. *Journal of Infectious Diseases*, *207*(3), 439–449. https://doi.org/10.1093/infdis/jis703

Zafar, M. A., Hammond, A. J., Hamaguchi, S., Wu, W., Kono, M., Zhao, L., & Weiser, J. N. (2019). Identification of pneumococcal factors affecting pneumococcal shedding shows that the dlt locus promotes inflammation and transmission. *MBio*, *10*(3). https://doi.org/10.1128/mBio.01032-19

Zettler, E. W., Scheibe, R. M., Dias, C. A. G., Santafé, P., Santos, D. S., Moreira, J. D. S., & Fritscher, C. C. (2006). Determination of penicillin resistance in Streptococcus pneumoniae isolates from southern Brazil by PCR. *International Journal of Infectious Diseases*, *10*(2), 110–115. https://doi.org/10.1016/j.ijid.2005.04.005

Zuccotti, G., Mameli, C., Daprai, L., Garlaschi, M. L., Dilillo, D., Bedogni, G., … Chiara, V. (2014). Serotype distribution and antimicrobial susceptibilities of nasopharyngeal isolates of Streptococcus pneumoniae from healthy children in the 13-valent pneumococcal conjugate vaccine era. *Vaccine*, *32*(5), 527–534. https://doi.org/10.1016/j.vaccine.2013.12.003

APPENDIX

Appendix A: Ethical Clearance

UNIVERSITY OF CAPE COAST
INSTITUTIONAL REVIEW BOARD SECRETARIAT
TEL:
E-MAIL:
C/O Directorate of Research, Innovation and Consultancy

OUR REF: UCCIRB/A/2016/194
YOUR REF:
OMB NO: 0990-0279
IORG #: IORG0009096

9TH JANUARY, 2018

Ms. Richael Odarkor Mills
Department of Biomedical Science
University of Cape Coast

Dear Ms. Mills,

ETHICAL CLEARANCE –ID: (UCCIRB/EXT/2017/21)

The University of Cape Coast Institutional Review Board (UCCIRB) has granted **Provisional Approval** for the implementation of your research protocol titled **'Post–vaccination Pneumococcal Carriage in Children Less than Five Years of Age in Cape Coast, Ghana'.** This approval requires that you submit periodic review of the protocol to the Board and a final full review to the UCCIRB on completion of the research. The UCCIRB may observe or cause to be observed procedures and records of the research during and after implementation.

Please note that any modification of the project must be submitted to the UCCIRB for review and approval before its implementation.

You are also required to report all serious adverse events related to this study to the UCCIRB within seven days verbally and fourteen days in writing.

Always quote the protocol identification number in all future correspondence with us in relation to this protocol.

Yours faithfully,

Dr. Samuel Asiedu Owusu
UCCIRB Administrator

ADMINISTRATOR
INSTITUTIONAL REVIEW BOARD
UNIVERSITY OF CAPE COAST
Date: 09/01/18

Appendix B: List Showing Primer Sequences used for Serotyping

Primer name	Nucleotide sequence	Product size (bp)	References
1-F	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA	280	Pai et al., 2006
1-R	CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C		
2-F	TAT CCC AGT TCA ATA TTT CTC CAC TAC ACC	290	Da Gloria Carvalho et al., 2010
2-R	ACA CAA AAT ATA GGC AGA GAG AGA CTA CT		
3-F	ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G	371	Pai et al., 2006
3-R	CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G		
4-F	CTG TTA CTT GTT CTG GAC TCT CGA TAA TTG G	430	Pai et al., 2006
4-R	GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G		
5-F	ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG	362	Pai et al., 2006
5-R	GCT CGA TAA ACA TAA TCA ATA TTT GAA AAA GTA TG		
6A/B/C/D-F	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	250	Pai et al., 2006
6A/B/C/D-R	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA		
6C/D-F	CAT TTT AGT GAA GTT GGC GGT GGA GTT	727	Da Gloria Carvalho et al., 2010
6C/D-R	AGC TTC GAA GCC CAT ACT CTT CAA TTA		
7C/B/40-F	CTA TCT CAG TCA TCT ATT GTT AAA GTT TAC GAC GGG A	260	Pai et al., 2006
7C/B/40-R	GAA CAT AGA TGT TGA GAC ATC TTT TGT AAT TTC		
7F/A-F	TCC AAA CTA TTA CAG TGG GAA TTA CGG	599	Da Gloria Carvalho et al., 2010
7F/A-R	ATA GGA ATT GAG ATT		

	GCC AAA GCG AC		
8-F	GAA GAA ACG AAA CTG TCA GAG CAT TTA CAT	201	Da Gloria Carvalho et al., 2010
8-R	CTA TAG ATA CTA GTA GAG CTG TTC TAG TCT		
9N/L-F	GAA CTG AAT AAG TCA GAT TTA ATC AGC	516	Dias et al., 2007
9N/L-R	ACC AAG ATC TGA CGG GCT AAT CAA T		
9V/A-F	GGG TTC AAA G TC AGA CAG TG A ATC TTA A	816	Da Gloria Carvalho et al., 2010
9V/A-R	CCA TGA ATG A AA TCA ACA TT G TCA GTA GC		
10A-F	GGT GTA GAT TTA CCA TTA GTG TCG GCA GAC	628	Pai et al., 2006
10A-R	GAA TTT CTT CTT TAA GAT TCG GAT ATT TCT C		
10F/C/33C-F	GGA GTT TAT CGG TAG TGC TCA TTT TAG CA	248	Da Gloria Carvalho et al., 2010
10F/C/33C-R	CTA ACA AAT TCG CAA CAC GAG GCA ACA		
11A/D-F	GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G	463	Pai et al., 2006
11A/D-R	GAT TAT GAG TGT AAT TTA TTC CAA CTT CTC CC		
12F/A/B/44/46-F	GCA ACA AAC GGC GTG AAA GTA GTT G	376	Pai et al., 2006
12F/A/B/44/46-R	CAA GAT GAA TAT CAC TAC CAA TAA CAA AAC		
13-F	TAC TAA GGT AAT CTC TGG AAA TCG AAA GG	655	Da Gloria Carvalho et al., 2010
13-R	CTC ATG CAT TTT ATT AAC CG C TTT TTG TTC		
14-F	GAA ATG TTA CTT GGC GCA GGT GTC AGA ATT	189	Dias et al., 2007
14-R	GCC AAT ACT TCT TAG TCT CTC AGA TGA AT		
15A/F-F	ATT AGT ACA GCT GCT GGA ATA TCT CTT C	434	Pai et al., 2006
15A/F-R	GAT CTA GTG AAC GTA CTA TTC CAA AC		
15B/C-F	TTG GAA TTT TTT AAT TAG TGG CTT ACC TA	496	Pai et al., 2006

15B/C-R	CAT CCG CTT ATT AAT TGA AGT AAT CTG AAC C		
16F-F	GAA TTT TTC AGG CGT GGG TGT TAA AAG	717	Da Gloria Carvalho et al., 2010
16F-R	CAG CAT ATA GCA CCG CTA AGC AAA TA		
17F-F	TTC GTG ATG ATA ATT CCA ATG ATC AAA CAA GAG	693	Pai et al., 2006
17F-R	GAT GTA ACA AAT TTG TAG CGA CTA AGG TCT GC		
18C/F/B/A-F	CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC	573	Pai et al., 2006
18C/F/B/A-R	TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC		
19A-F	GAG AGA TTC ATA ATC TTG CAC TTA GCC A	566	Pimenta et al., 2009
19A-R	CAT AAT AGC TAC AAA TGA CTC ATC GCC		
19F-F	GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C	304	Pai et al., 2006
19F-R	GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG		
19Fvar-F	GAC AAT TCT GGT TGA CTT GTT GAT TTT G	585	Menezes et al., 2013
19Fvar-R	CTA CCA AAT ACC TCA CCA GCT TCC		
20-F	GAG CAA GAG TTT TTC ACC TGA CAG CGA GAA G	514	Pai et al., 2006
20-R	CTA AAT TCC TGT AAT TTA GCT AAA ACT CTT ATC		
21-F	CTA TGG TTA TTT CAA CTC AAT CGT CAC C	192	Da Gloria Carvalho et al., 2010
21-R	GGC AAA CTC AGA CAT AGT ATA GCA TAG		
22F/A-F	GAG TAT AGC CAG ATT ATG GCA GTT TTA TTG TC	643	Pai et al., 2006
22F/A-R	CTC CAG CAC TTG CGC TGG AAA CAA CAG ACA AC		
23A-F	TAT TCT AGC AAG TGA	722	Da Gloria

	CGA AGA TGC G		Carvalho et al., 2010
23A-R	CCA ACA TGC TTA AAA ACG CTG CTT TAC		
23B-F	CCA CAA TTA G CG CTA TAT TCA TTC AAT CG	199	Da Gloria Carvalho et al., 2010
23B-R	GTC CAC GCT GAA TAA AAT GAA GCT CCG		
23F-F	GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC	384	Pai et al., 2006
23F-R	CAC AAC ACC TAA CAC TCG ATG GCT ATA TGA TTC		
24F/A/B-F	GCT CCC TGC TAT TGT AAT CTT TAA AGA G	99	Da Gloria Carvalho et al., 2010
24F/A/B-R	GTG TCT TTT ATT GAC TTT ATC ATA GGT CGG		
31-F	GGA AGT TTT CAA GGA TAT GAT AGT GGT GGT GC	701	Pai et al., 2006
31-R	CCG AAT AAT ATA TTC AAT ATA TTC CTA CTC		
33F/A/37-F	GAA GGC AAT CAA TGT GAT TGT GTC GCG	338	Pai et al., 2006
33F/A/37-R	CTT CAA AAT GAA GAT TAT AGT ACC CTT CTA C		
34-F	GCT TTT GTA AGA GGA GAT TAT TTT CAC CCA AC	408	Pai et al., 2006
34-R	CAA TCC GAC TAA GTC TTC AGT AAA AAA CTT TAC		
35A/C/42-F	ATT ACG ACT CCT TAT GTG ACG CGC ATA	280	Da Gloria Carvalho et al., 2010
35A/C/42-R	CCA ATC CCA AGA TAT ATG CAA CTA GGT T		
35B-F	GAT AAG TCT GTT GTG GAG ACT TAA AAA GAA TG	677	Pai et al., 2006
35B-R	CTT TCC AGA TAA TTA CAG GTA TTC CTG AAG CAA G		
35F/47F-F	GAT AAG TCT GTT GTG GAG ACT TAA AAA GAA TG	517	Pai et al., 2006
35F/47F-R	CTT TCC AGA TAA TTA CAG GTA TTC CTG AAG		

	CAA G		
38/25F/25A-F	CGT TCT TTT ATC TCA CTG TAT AGT ATC TTT ATG	574	Pai et al., 2006
38/25F/25A-R	ATG TTT GAA TTA AAG CTA ACG TAA CAA TCC		
39-F	TCA TTG TAT TAA CCC TAT GCT TTA TTG GTG	98	Da Gloria Carvalho et al., 2010
39-R	GAG TAT CTC CAT TGT ATT GAA ATC TAC CAA		
CPS A-F	GCA GTA CAG CAG TTT GTT GGA CTG ACC	160	Pai et al., 2006
CPS A-R	GAA TAT TTT CAT TAT CAG TCC CAG TC		

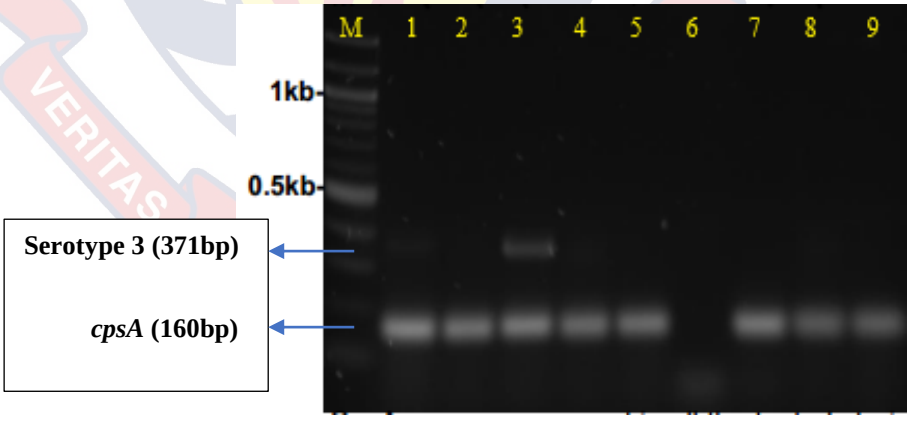
Appendix C: Primer Concentrations and Approach for Molecular Capsular Typing

Reaction number	**Primer pairs used**	**Concentration per reaction (uM)**
1	14-F, 14-R	0.3
	1-F, 1-R	0.3
	5-F, 5-R	0.3
	4-F, 4-R	0.3
	18C/F/B/A-F, 18C/F/B/A-R	0.3
2	6A/B/C/D-F, 6A/B/C/D-R	0.3
	19F-F, 19F-R	0.5
	23F-F, 23F-R	0.5
	25F/25A/38-F, 25F/25A/38-R	0.3
	9V/9A-F, 9V/9A-R	0.5
2.1	6A/B/C/D-F, 6A/B/C/D-R	0.3
	19var-F, 19var-R	0.2
	6C/D-F, 6C/D-R	0.5
3	7C/B/40-F, 7C/B/40-R	0.3
	3-F, 3-R	0.3
	15B/C-F, 15B/C-R	0.3
	7F/A-F, 7F/A-R	0.4
	17F-F, 17R-R	0.5
4	8-F, 8-R	0.2
	12F/A/44/46-F, 12F/A/44/46-R	0.5
	9N/L-F, 9N/L-R	0.5
	22F/A-F, 22F/A-R	0.5
	23-F, 23-R	0.5
5	24F/A/B-F, 24F/A/B-R	0.2

	2-F, 2-R	0.3
	11A/D-F, 11A/D-R	0.3
	19A-F, 19A-R	0.3
	16F-F, 16F-R	0.5
6	21-F, 21-R	0.2
	33F/A/37-F, 33F/A/37-R	0.3
	15A/F-F, 15A/F-R	0.3
	35F/47-F, 35F/47-R	0.3
	13-F, 13-R	0.4
7	39-F, 39-R	0.2
	23B-F, 23B-R	0.2
	35A/C/42-F, 35A/C/42-R	0.3
	20-F, 20-R	0.3
	35B-F, 35B-R	0.5
8	10F/C/33-F, 10F/C/33-R	0.3
	34-F, 34-R	0.3
	10A-F, 10A-R	0.5
	31-F, 31-R	0.5

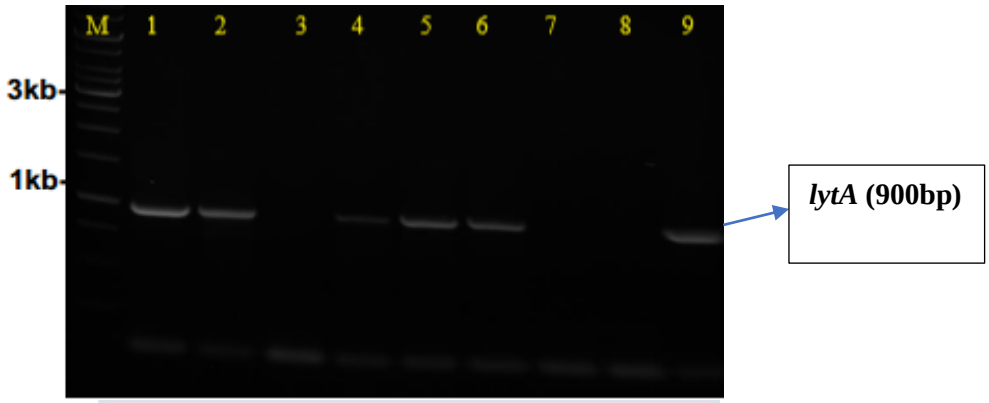
* *cps A* gene primer was included in each reaction at 0.5 uM

Appendix D: Image of Agarose gel showing Serotype Amplicons



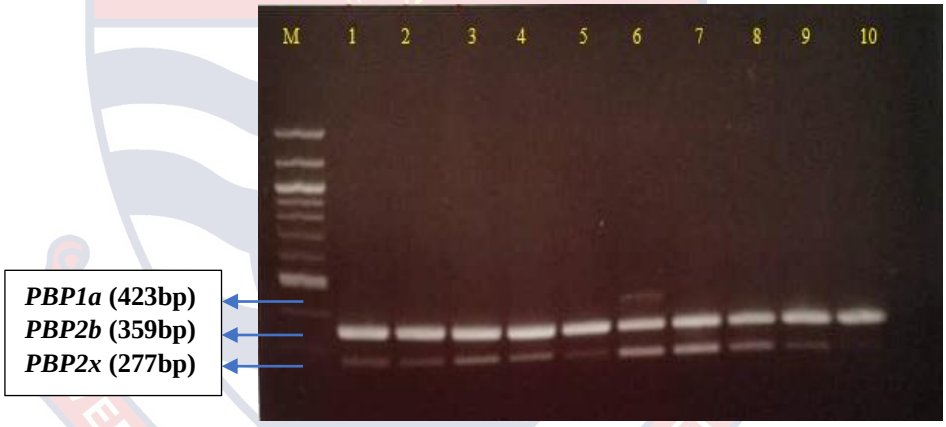
A 4S plus nucleic acid stained-2% agarose gel showing amplicons of two genes after mPCR was done: Serotype 3 (371 bp) and *cpsA* (160 bp) genes. M- 100bp ladder; lanes 1, 2, 3 4, 5, 7, 8 and 9 are positive for *cpsA* genes; Lane 3 is positive for serotype 3 (371bp) whereas the other lanes are negative for other genes.

Appendix E: Image of Agarose gel showing *lytA* amplicons



A 4S plus nucleic acid stained-2% agarose gel showing PCR products of an amplified *lytA* gene (900bp). M-1kbp ladder; lanes 1, 2, 4, 5, 6 and 9 are positives for the *lytA* gene

Appendix F: Image of Agarose gel showing amplicons of *PBP* genes



A 4S plus nucleic acid stained-2% agarose gel showing PCR products of an amplified PBP genes: *PBP1a* (423bp), *PBP2b* (359bp) and *PBP2x* (277bp). M- 100bp ladder; lanes 1, 2, 3, 4, 5, 7, 8, 9 are *PBP2b* and *PBP2x* positive; Lane 6 is *PBP1a*, *PBP2b* and *PBP2x* positive; Lane 10 is *PBP2b* positive