

**, CHARACTERIZATION AND ANTIBIOGRAM OF STREPTOCOCCUS  
PNEUMONIAE AMONG PNEUMONEIC PATIENTS IN TWO ISOLATION  
HOSPITALS IN BAUCHI STATE, NIGERIA**

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NIGERIA**

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FACULTY OF LIFE SCIENCES,  
AHMADU BELLO UNIVERSITY, ZARIA  
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## DECLARATION

I declare that the work in this dissertation entitled “Isolation, characterization and antibiogram of *Streptococcus pneumoniae* among pneumoneic patients in two hospitals in Bauchi State, Nigeria” has been performed by me in the Department of Microbiology, Ahmadu Bello University Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this Dissertation was previously presented for another Degree or Diploma at this or any other Institution.

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Ibrahim Abubakar Yusuf

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Date

## CERTIFICATION

This dissertation entitled “ISOLATION, CHARACTERIZATION AND ANTIBIOGRAM OF STREPTOCOCCUS PNEUMONIAE AMONG PNEUMONEIC PATIENTS IN TWO HOSPITALS IN BAUCHI STATE, NIGERIA” by Ibrahim Abubakar YUSUF meets the regulations governing the award of the Degree of Master of Science in Microbiology of the Ahmadu Bello University, and is approved for its’ contribution to knowledge and literary presentation.

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## **DEDICATION**

This Dissertation is dedicated to God Almighty, Who gives me the strength and ability to endure the difficulties encountered in the course of the research.

## ABSTRACT

*Streptococcus pneumoniae* is a Gram positive, alpha-hemolytic, anaerobic organism and a significant human pathogen recognized as a major cause of pneumonia. This study was carried out to isolate, characterize and determine the antibiogram of *Streptococcus pneumoniae* among pneumoneic patients in two hospitals in Bauchi State, Nigeria. Sputum and throat swab samples were collected from a total of 373 patients and inoculated on blood agar for the isolation of pneumococci. Gram staining, sensitivity to optochin as well as bile solubility tests were carried out to identify the alpha-haemolytic streptococcus strains. All isolates biochemically identified as *Streptococcus pneumoniae* were further confirmed using Polymerase Chain Reaction (PCR). The antibiotic susceptibility test of the *Streptococcus pneumoniae* isolated was also determined. Out of the 373 samples analyzed 255 (68.4%) were alpha-hemolytic isolates, of which 14 (3.75%) were biochemically identified as *Streptococcus pneumoniae*. PCR detection of autolysin gene in the isolates revealed that only 9 (2.41%) were molecularly identified as *Streptococcus pneumoniae*. Higher occurrence of 3.8% was observed among the age group of <1-19 years old while the least was 0.86% among the age group of  $\geq 50$  years old but the difference was not statistically significant at  $p > 0.05$ . All the *Streptococcus pneumoniae* isolates were found to be resistant to penicillin, augmentin and tetracycline. However, only one isolate was resistant to clindamycin 1 (11.1%). Penicillin binding protein (Pbp2b) gene was the only resistance gene detected in only one among all the resistant isolates. It was concluded that *Streptococcus pneumoniae* could be a common cause of pneumonia among individuals of <1-19 years in the study area and clindamycin could be used as the antibiotic of choice for treatment of the infection.

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## CHAPTER ONE

### 1.0 Introduction

*Streptococcus pneumoniae* is a Gram-positive, alpha-hemolytic, aero tolerant, anaerobic member of the genus streptococcus (Ryan and Ray, 2004). *Streptococcus pneumoniae* is a significant human pathogenic bacterium recognized as a major cause of pneumonia in the late 19<sup>th</sup> century, and is a subject of many humoral immunity studies.

The organism was termed *Diplococcus pneumoniae* from 1920 (Winslow *et al.*, 1920) because of its appearance in gram-stained sputum. It was renamed *Streptococcus pneumoniae* in 1974 because of its growth in chains in liquid media. *Streptococcus pneumoniae* can be differentiated from *Streptococcus viridans* some of which are also alpha-hemolytic, using optochin test as *Streptococcus pneumoniae* is optochin sensitive. *Streptococcus pneumoniae* can also be distinguished based on its sensitivity to lysis by bile (the so-called bile solubility test) (Far and Mandell, 1994).

The encapsulated gram-positive coccoid bacteria have a distinctive morphology on Gram stain, the so-called “lancet-shaped” diplococci. They have a polysaccharide capsule that acts as a virulence factor for the organism, more than 90 serotypes are known, and these types differ in virulence, prevalence and extent of drug resistance (Musher *et al.*, 1997).

*Streptococcus pneumoniae* has closed circular DNA structure that contains between 2.0 and 2.1 million base pairs, depending on the strain. It has a core set of 1553 genes, plus 154 genes in its virulome, which contribute to virulence, and 176 genes that maintain a non-

invasive phenotype. The genetic information can vary up to 10% between strains (Vander and Opal, 2009).

Despite the name, the organism causes many types of pneumococcal infections other than pneumonia. These pneumococcal diseases include: acute sinusitis, otitis media, meningitis, sepsis, bacteremia, osteomyelitis, septic arthritis, endocarditis, peritonitis, cellulitis, pericarditis and brain abscesses (Musher, 1992).

Transmission of *Streptococcus pneumoniae* occurs as a result of direct person-to-person contact via respiratory droplets and by autoinoculation in person carrying the bacteria in their upper respiratory tract. The pneumococcal serotypes most often responsible for causing infections are those most frequently found in carriers. The spread of the organism within a family or household is influenced by such factors as crowding, season, and the presence of upper respiratory tract infection or pneumococcal disease such as pneumonia or otitis media. The period of communicability is unknown but presumably transmission can occur as long as the organism appears on the respiratory secretions (Nourti and Tsai, 2008).

Pneumonia is an inflammatory condition of the lung – affecting primarily the microscopic air sacs known as alveoli, causing mild to severe illness in all ages (Howell and David, 2009). The air sacs in the lungs become filled with fluid or pus which interferes with the lung's ability to transfer oxygen to the blood.

Pneumococcal pneumonia is the most common clinical presentation of pneumococcal diseases among adults with a short incubation period of about 1 – 3 days (CDC, 2010).

Symptoms generally includes; an abrupt onset of fever and chills. Other common symptoms include; pleuritic chest pain, cough productive of mucopurulent, rusty sputum, dyspnoea (difficulties in breathing), tachypnoea (rapid breathing), hypoxia (poor oxygenation), tachycardia (rapid heart beat) while malaise, weakness, nausea, vomiting and headaches occur less frequently (CDC, 2010).

### **1.1 Statement of the Problem**

Pneumonia remains one of the leading cause of death in the developing countries, although it occurs in all parts of the world affecting all ages, but more common among the young, the elderly and the chronically ill (Ruskanen *et al.*, 2011). According to the same author, pneumonia caused 4 million deaths in all age group (7% of the world yearly total) and affects approximately 156 million children (151 million children in the developing world and 5 million in the developed world). This results in 1.6 million deaths in children under 5years of age of which 95% occur in the developing world.

It has been documented by WHO (2008) and UNICEF (2009 ) that Nigeria ranks second in pneumonia induced death in the world resulting in 6million Nigerian children being infected and 200,000 dying yearly of the disease.

### **1.2 Justification**

Despite the availability of effective chemotherapy pneumococcal pneumonia is still a major health problem in most countries of the world (Nester *et al.*, 1998). This occurs as a result of antibiotic resistance. The organism, *Streptococcus pneumoniae* has a natural transformation system as a mechanism of genetic exchange which underlies the expulsion

of antibiotic resistance in the bacterium over the past many years. For example penicillin resistance is due to altered penicillin binding proteins (Pbps) that exhibit a low affinity for beta lactame antibiotics (Ewig, 1986). *Streptococcus pneumoniae* can also develop antibiotic resistance as a result of therapeutic exposure to antibiotics (Ball *et al.*, 2002), or through enzymatic degradation, alteration of bacteria proteins, efflux of antibiotic, childcare/crowded living conditions and indiscriminate use of antibiotics (Samore *et al.*, 2001; Tenover, 2006).

The incidence of the infection was reported to be higher in children 1 – 5 years of age due to improper development of innate immune system, malnutrition and micronutrient deficiencies (Scott *et al.*, 2000). It may also be due to infection with other diseases such as tuberculosis, viral respiratory disease such as influenza virus and underlying medical condition such as Human Immuno Virus (HIV) infection which are more prevalent in developing world (CDC. 2002). Therefore, Nigeria being among the developing countries and has been documented by WHO (2008) and UNICEF (2009) to rank second in pneumonia induced death in the world, it is therefore important to carry out this research in this part of Nigeria in order to provide base line information for the control and treatment of the disease.

### **1.3 Aim and Objectives**

#### **1.3.1 Aim**

The aim of this study was to isolate, characterize and determine the antibiogram of *Streptococcus pneumoniae* among patients suspected to have pneumonia in two hospitals in Bauchi state, Nigeria.

### **1.3.2 Objectives**

The objectives of the study were:

1. To isolate, characterize biochemically and molecularly *Streptococcus pneumoniae* from pneumoneic patients.
2. To determine the antibiotic resistance pattern of the *Streptococcus pneumoniae* isolates.
3. To determine the presence of antibiotic resistance genes in isolates resistant to some selected antibiotics.

## CHAPTER TWO

### 2.0 Literature Review

#### 2.1 Structure of *Streptococcus pneumoniae*

*Streptococcus pneumoniae* (the pneumococcus) was isolated for the first time by George Sternberg in 1880 and Louis Pasteur in 1881 (Gray, 2000). It was formerly known as *Diplococcus pneumoniae* and belongs to the mitis group within the genus *Streptococcus*. The pneumococcus is a lancet-shaped and mostly encapsulated gram positive bacterium that grows in pairs or short chains in broth culture. On blood agar, pneumococci form small greyish, mostly mucoid or smooth colonies, surrounded by a greenish zone of partial haemolysis (Abdullahi *et al.*, 2012). Noncapsulated pneumococci produce rough colonies. Pneumococci are easily lysed, while the centre of the colony soon becomes depressed because of autolysis.

The pneumococcal cell surface has three layers: the plasma membrane, the cell wall and the capsule composed of polysaccharides. The plasma membrane and the peptidoglycan backbone of the cell wall act as anchors for the cell wall polysaccharides and surface proteins. Two cell wall polysaccharides, teichoic acid and lipoteichoic acid, are common to all pneumococcal strains. Moreover, several surface proteins are conserved across most pneumococcal strains. The best known of these are the lipoprotein pneumococcal surface adhesion A (PsaA), the enzymes immunoglobulin A-protease and neuraminidases and three choline-binding proteins, pneumococcal surface protein A, major pneumococcal autolysin and pneumococcal surface protein C (PspC, also referred to as CbpA or PspA) (Jedrzejewski, 2001). Several new proteins and other surface components involved in the adherence and

virulence mechanisms have been recognised recently (Hammerschmidt, 2006; Mann *et al.*, 2006). A subpopulation of pneumococci produces pilus-like protein structures, able to extend beyond the polysaccharide capsule (Barocchi *et al.*, 2006). One of the most intensively studied intracellular structures is pneumolysin, a toxin common to all clinically relevant pneumococcal strains (Paton, 1996). The capsule is the outermost layer of the cell surface and consists of polysaccharides, which show considerable variation in their chemical composition, amount and antigenic capacity.

## **2.2 Identification of *Streptococcus pneumoniae***

The standard for the clinical diagnosis of pneumococcal infection is growth of viable pneumococci from samples collected from normally sterile body sites, such as blood, cerebrospinal fluid, the pleural cavity or joints. Similarly, the standard method to identify pneumococcal carriage is growth of *S. pneumoniae* from an upper respiratory tract sample such as sputum and throat swab. *S. pneumoniae* grows well on plain blood agar, but the use of selective culture agars that include gentamicin or colistin with nalidixic or oxolinic acid suppresses the growth of other bacteria and thus increases the sensitivity of recovering pneumococci (Dudley *et al.*, 2001; Satzke *et al.*, 2013). The use of culture selective medium is especially important when culturing samples from the upper respiratory tract, a niche for numerous non-pathogenic and potentially pathogenic bacteria (O'Brien and Nohynek, 2003). Pneumococcal growth requires carbondioxide (CO<sub>2</sub>). The optimal pH for pneumococcal growth is around 7.2 and the optimal temperature is between 35°C and 37°C. To increase the yield of pneumococci, specimens are sometimes incubated in an enrichment broth prior to culture like Trypticase Soy Broth. After culture, pneumococci are identified by their characteristic colony morphology and differentiated from other alpha

haemolytic streptococci by their susceptibility to optochin and autolysis in contact with bile salt. The differentiation of atypical pneumococci from other alpha-haemolytic streptococci is sometimes difficult (Hanage *et al.*, 2005). Pneumococci can be identified directly without culture from clinical samples by the immunologic reactions of their antigenic structures with specific antisera or by identifying their genetic material with molecular techniques. These tests may not only detect live bacteria but also killed pneumococci or even fragments of them. These techniques can be used to determine the serotype or to differentiate nonserotypeable pneumococci from other alpha-haemolytic streptococci (Hanage *et al.*, 2005). However, non cultural identification methods offer limited possibilities for further characterisation of pneumococcal properties, since strains cannot be retained for later analysis (Carvalho *et al.*, 2013; Satzke *et al.*, 2013).

Polymerase chain reaction (PCR) tests are based on amplification of nucleic acids of the pneumococcal genes (van Belkum *et al.*, 1996; Saukkoriipi *et al.*, 2002). PCR tests are increasingly used for research as well as for clinical purposes, although the precise role that they can play has not yet been established (Palmu *et al.*, 2004; Satzke *et al.*, 2013). In general, PCR based methods are highly sensitive in detecting bacteria even in low densities (Chien *et al.*, 2013). PCR methods are also valuable in detecting pneumococci in normally sterile body sites during antimicrobial treatment. The disadvantage of PCR methods is that they may also recognise genetic material that has migrated from the adjacent organs of the host or from non pneumococcal strains that are close relatives to pneumococci, or even airborne contaminants (Carvalho *et al.*, 2013). Thus, PCR may detect viable bacteria that fail to grow in culture, for example, due to small amount or dilution of the sample, or biofilm formation (Palmu *et al.*, 2004; Post *et al.*, 2004 and Hallstoodley *et al.*, 2006).

Trzciński *et al.* (2013) detected viable pneumococci in a remarkable proportion of samples that were initially negative by conventional culturing but positive by PCR when they recultured and carefully examined the original samples.

### **2.3 Classification of *Streptococcus pneumoniae***

*S. pneumoniae* are classified into serotypes and serogroups according to the antigenic properties of the capsular polysaccharide (Konradsen, 2005). The formerly used American nomenclature named serotypes according to their order of discovery. Currently, the Danish nomenclature is used worldwide. It classifies serologically cross reacting serotypes into serogroups. Of the known 46 groups, some comprise a single known serotype, while some represent two or more immunologically related serotypes. More than 90 serotypes have been distinguished. In addition, some strains cannot be serotyped because they are none encapsulated. These strains are also called ‘rough’ strains because of their specific colony morphology. For decades, the standard for serotyping pneumococci after their identification in a culture has been the Neufeld test, also called the Quellung or capsular swelling test as described by Karin O. (2000). It is based on microscopic visualisation of the capsular swelling and agglutination of cells in reaction to adding specific antisera on a pure pneumococcal culture (Konradsen, 2005). The method requires time, resources and a well-trained staff to interpret the results. Alternative serotyping methods such as counterimmunoelectrophoresis and latex agglutination tests (Slotved *et al.*, 2004) have been developed to reduce the work load.

Currently, PCR-based assays to identify serotype specific sequences within pneumococcal capsular genes are increasingly used for pneumococcal serotyping. Novel PCR-based

methods seem precise and effective. Especially simultaneous amplification of multiple sequences in a single reaction—a process referred to as multiplex PCR—is promising in reducing the cost and workload in identifying multiple carriages (Brito *et al.*, 2003). The genetic methods have offered substantially increased possibilities for understanding the characteristics and functions of pneumococcal virulence factors in their interaction with host cells. Molecular characterisation has revealed that pneumococcal strains expressing the same serotype relatively often belong to otherwise diverse clones. Although rare, it is also possible that pneumococci of a single clonal type express different serotypes, a phenomenon known as capsular switching (Jefferies *et al.*, 2004)

#### **2.4 Establishment of Carriage and the Pathogenesis of Pneumococcal Infection**

*S. pneumoniae* gains entry into the human host by adhering to the mucosal epithelium of the respiratory tract. Pneumococci often remain in the nasopharynx as commensals without causing symptoms or any apparent harm to the host. The condition of harbouring pneumococci in the upper respiratory tract is called ‘carriage’ or ‘colonisation’. Even asymptomatic carriage can be considered as infection if it causes an antigen-antibody response. Pneumococci can be inhaled as aerosols or aspirated into the alveoli of the lungs, causing pneumonia. Local infection may progress to systemic infection by transfer of bacteria into the circulation via pulmonary capillaries through the vascular endothelial cells. The pathogenesis of pneumococcal infection depends on the virulence of the bacteria, i.e. their capacity to adhere to and invade the host cells and escape clearance by the host defence mechanisms (Tuomanen *et al.*, 1995).

## 2.5 Adherence of Pneumococci to Human Cells

In the human respiratory tract *Streptococcus pneumoniae* first encounters the mucosa covered by a mucus layer and epithelial cells. A healthy mucosa inhibits bacterial attachment physically by the washing action of the saliva, the cough reflex, and the viscous mucus layer which acts as a physical barrier and transfers the bacteria outwards from the body by aid of beating movements of cilia attached to the mucosal surface. The mucosa also produces antibacterial substances that kill bacteria or inhibit their growth (Coles *et al.*, 1996). These include lysozyme, lactoferrin and antimicrobial surfactant proteins. The ample commensal flora on the respiratory tract protects the mucosa by interfering with growth and invasion of pathogenic bacteria. If pneumococci survive the host defence mechanisms in the mucus, they may adhere to the epithelial cells of the respiratory mucosa. Adherence is mediated by binding between the bacterial surface structures and their target cells throughout the human respiratory tract. The epithelial cells express glycoconjugate receptors bearing specific carbohydrates on their surface, such as sialic acid residues, oligosaccharides and glycosaminoglycans, and pneumococci initially bind loosely to these receptors (Tonnaer *et al.*, 2006; Voss *et al.*, 2012). However, for effective adherence, colonisation and disease development, pneumococci need stronger binding to protein receptors like the polymeric immunoglobulin receptor or keratin 10 (Zhang *et al.*, 2000; Sanchez *et al.*, 2010).

On the bacterium side, several choline-binding proteins, enzymes and other structures of the cell surface are essential in the adhesion of bacterial ligands to their receptors (Khan and Pichichero, 2012). Recent molecular research has remarkably increased our knowledge about the versatility of the pneumococcal surface components and adherence molecules as

well as the host structures involved in the adhesion process (Bergmann and Hammerschmidt, 2006; Tonnaer *et al.*, 2006). It is now known that pneumococci not only directly bind to the host receptors but also have a complex dynamic interplay with several host proteins. This interaction facilitates adherence and subsequent internalisation of pneumococci into the host cells. During the initial contact between pneumococci and the host cells, pneumococcal enzymes reduce the viscosity of the mucus, inhibit the ciliary function, provide nutrients to the bacteria, destroy immunoglobulin activity and inhibit the opsonophagocytosis by human neutrophils. An important mechanism for pneumococci to interact with the host cells is to modify the host structures that contain sialic acid and sugars and expose the target cell receptors for adherence (Bergmann and Hammerschmidt, 2006). For more effective colonisation, pneumococci need adherence molecules, surface proteins and so-called ‘microbial surface components recognising adhesive matrix molecules’. Pneumococci either react directly with the target receptors, or recruit host proteins in the extracellular matrix and serum to act as molecular bridges in attachment of the bacteria to the target cells and in promoting their migration through the cell barriers (Voss *et al.*, 2012). A special mechanism facilitating pneumococcal attachment to the target cells is phase variation, in which bacteria undergo spontaneous and reversible changes in their phenotypic expression between transparent and opaque colony morphologies (Kim and Weiser, 1998).

The transparent variants, expressing less capsule and increased or altered surface proteins and cell-wall carbohydrate-containing structures, show increased adherence to the human epithelial cells (Overweg *et al.*, 2000). A reduction in the amount of capsule may be a necessary step for efficient colonisation, as a thinner capsule allows greater exposure of

pneumococcal cell surface molecules important for interaction with the host cells and adherence. However, experimental research in mouse models has shown that some amount of capsule is required for effective colonisation (Magee and Yother, 2001). Furthermore, the pneumococcal polysaccharide capsule is important for the initial survival of the bacterium on the mucosal membrane, one mechanism being its negative charge (Nelson *et al.*, 2007).

## **2.6 Invasion by Pneumococci and Development of Pneumococcal Infection**

To cause disease, pneumococci must be internalised into the epithelial cells, migrate across the epithelial barrier and survive in an environment with versatile host protective mechanisms. Mucosal diseases, like otitis media and pneumonia, develop when pneumococci multiply and cause cell damage in the mucosal sites of the human body. For development of invasive disease, e.g. bacteraemia and meningitis, Pneumococci need to be translocated through the vascular endothelium and gain access to the blood stream, the cerebrospinal fluid, the lung parenchyma or other normally sterile body sites. In general, pneumococcal adherence appears to be mild until the host cells are activated by inflammatory cytokines (Cundell *et al.*, 1995; Tuomanen, 2000). The host cell activation increases the number of receptors and induces alterations in the cell surface (Henriques-Normark and Tuomanen, 2013). Cell activation also starts a signalling mechanism needed for translocation of bacteria into and through the cells (Iovino *et al.*, 2013). Pneumococcal structures may be involved in the cell activation and the up-regulation of the host cell receptors (Zhang *et al.*, 2000; McCullers, 2006).

Inflammation has a major role in the virulence of pneumococci (Tuomanen, 2000; Jedrzejak, 2001). The inflammatory process includes an influx of neutrophils and destruction of the structure and function of the host cells (Henriques-Normark and Tuomanen, 2013). The pneumococcal cell wall components induce activation of a wide array of cytokines, enzymes and the complement, which leads to strong inflammation (Tomasz and Saukkonen, 1989). Several pneumococcal proteins contribute to the development of inflammation and cell damage, and inhibit the host defence mechanisms (Gillepsie and Balakrishnan, 2000; Bergmann and Hammerschmidt, 2006). Many adhesive surface proteins and enzymes are multifunctional and promote bacterial transport across the host epithelial and endothelial cells (Iovani *et al.*, 2013). An important pneumococcal virulence factor is pneumolysin, an intracellular toxin, which enhances the host inflammatory process and impairs the function and viability of the host cells. Pneumolysin may also cause direct host cell damage (Paton, 1996). Pathogenesis is promoted by pneumococcal autolysins, which cause destruction of pneumococci, thus enhancing the release of cell wall components and internal toxins from dying bacteria and further increasing the inflammation. This process may be facilitated by antimicrobial treatment (Tuomanen *et al.*, 1987). In addition, the pneumococcal pilus-like structures augment the inflammatory process. Although the versatile pneumococcal structures are known to participate in the pathogenesis of pneumococcal disease (Kadioglu *et al.*, 2008), the polysaccharide capsule is still considered the most important virulence factor of *Streptococcus pneumoniae*. The capsular polysaccharides do not elicit inflammatory response and they do not appear to be toxic (Henriques-Noemark and Tuomanen, 2013). The main role of the capsule in the virulence of pneumococci is to protect the bacteria from phagocytosis by polymorphonuclear leucocytes and thus enhance their survival in the

human organism. Pneumococci survive better in the blood and exhibit greater systemic virulence in the opaque phase than in the transparent phase of their morphology, probably because of the thicker capsule (Kim and Weiser, 1998). Pneumococcal virulence depends on both the amount and the chemical composition of the capsule and it varies across different serotypes (Gillepsie, 1989). Differences in the pneumococcal capsule type were found to have significant effects on pneumococcal growth in a mouse model, both in the nasopharynx and in the lungs. However, both the capsule type and the genetic background were important, and their joint influence varied with the site of infection (Kadioglu *et al.* 2002).

The involvement of the platelet-activating factor receptor in the development of pneumococcal invasive disease was recognised in the 1990s (Cundell *et al.*, 1996). The activated receptor mediates adherence to and translocation through human cells, either by direct binding or by initiating a signalling cascade that leads to host inflammation and bacterial invasion (Henriques-Normark Tuomanen, 2013). However, inhibition of platelet-activating factor receptors has been shown to reduce pneumococcal infection only partly, indicating that other receptors are also needed in pneumococcal invasion (Iovani *et al.*, 2013). A well-known receptor capable for the uptake of pneumococci in the respiratory epithelial cells and their transport through the epithelial barrier is the polymeric immunoglobulin receptor, which binds to the pneumococcal protein PspC (Elm *et al.*, 2004). The laminin receptor promotes the initial adhesion of pneumococci to the human microvascular brain cells and may thus initiate the passage through the bloodbrain- barrier (Orihuela *et al.*, 2009).

## **2.7 Interactions between Pneumococci and other Nasopharyngeal Bacteria**

The nasopharynx is a common niche for a range of non-pathogenic species as well as *Streptococcus pneumoniae* and other potential bacterial pathogens such as *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus*. Interactions between different species in the establishment of carriage and development of disease are not well known, and the complex mechanisms of their mutual competition and synergism are currently being intensively studied. Understanding these mechanisms is important for the prevention and treatment of pneumococcal diseases since between-species competition offers an opportunity for other species to replace those eliminated by vaccination or antimicrobial treatment. One species may also benefit if another produces enzymes that reduce antibiotic activity or if the species gains antimicrobial resistance by horizontal gene transfer (Juhász *et al.*, 2014). Non-pneumococcal alpha-haemolytic streptococci inhibit pneumococcal growth in vitro and negative associations between these commensals and pneumococci have been observed in vivo (Santagati *et al.*, 2012; Friedel *et al.*, 2013). A number of studies have shown a negative correlation between the growth of pneumococci and *Staphylococcus aureus* in the nasopharynx (Chien *et al.*, 2013; Shiri *et al.*, 2013). In contrast, there are conflicting results regarding bacterial interactions between *Staphylococcus aureus* and *Haemophilus influenzae*. Both negative and positive associations have been observed between these bacteria in the nasopharynx of children (Abdullahi *et al.*, 2008; Dahlblom *et al.*, 2012; Ruohola *et al.*, 2013). Experimental and in vitro studies provide evidence for both synergistic and competitive interactions between these two species (Ratner *et al.*, 2005). Concurrent colonisation of epithelial surfaces by these species induces synergistic proinflammatory responses (Ratner *et al.*, 2005). Several mechanisms of the mutual competition between *Streptococcus pneumoniae* and

*Haemophilus influenzae* over the ecological niche have been suggested, including competition for nutrients and receptors (King, 2010).

In animal experiments, *Haemophilus influenzae* has been associated with decreased frequency and density of pneumococcal growth (Margolis *et al.*, 2010). *Streptococcus pneumoniae* has been shown to inhibit the in vitro survival of *Haemophilus influenzae* through the production of hydrogen peroxide and neuraminidase (Shakhnovich *et al.*, 2002). *Haemophilus influenzae* for its part activates the complement dependent killing of pneumococci (Lysenko *et al.*, 2005). *Haemophilus influenzae* also enhances carriage-induced antibody production against pneumococcal proteins, at least in children vaccinated with a pneumococcal conjugate vaccine (Xu and Pichichero, 2014).

The complexity of between-species interactions was demonstrated in a study in which colonisation by *Haemophilus influenzae* was negatively associated with *Streptococcus pneumoniae* during upper respiratory infection, but when *Haemophilus influenzae* and *Moraxella catarrhalis* colonised the host simultaneously, both were positively associated with *Streptococcus pneumoniae* (Pettigrew *et al.*, 2008). *Haemophilus influenzae* out-competed a non-virulent strain of *Streptococcus pneumoniae* in experimental co-colonisation in mice, resulting in selection of more virulent pneumococcal strains (Lysenko *et al.*, 2010). Viral upper respiratory infection may also affect the relative occurrence of different nasopharyngeal bacteria (Friedel *et al.*, 2013).

## **2.8 Biofilm Formation**

One possible explanation for the conflicting findings on between-species interactions is biofilm formation. Biofilms are complex three-dimensional structures, comprising bacteria

from one or several species, hyper-adhesively attached to the cell surfaces and encased within a self produced extracellular matrix (Marks *et al.*, 2013). Bacteria organised in biofilms interact with each other using intercellular messenger molecules and form special structures for the delivery of nutrients and removal of metabolic waste products (Post *et al.*, 2004). In biofilms, bacteria elicit weaker inflammatory responses and are more resistant to host defences and other environmental challenges, including desiccation and antimicrobial treatment, as compared to planktonic strains, i.e. strains not organised in a biofilm (Blanchette- Cain *et al.*, 2013).

Bacteria living in biofilms may be undetectable with conventional culture methods but detectable with PCR-based methods (Post *et al.*, 2004) In animal models, pneumococcal biofilm formation has been associated with enhanced adhesive capacity, persistent carriage and decreased virulence (Sanchez *et al.*, 2011; Marks *et al.*, 2012). Pneumococci in biofilms have expressed high proportions of transparent phenotypes, fit for colonisation, along with decreased amount of capsule, enhanced production of adhesins and decreased production of pneumolysin (Sanchez *et al.*, 2011). The presence of *Haemophilus influenzae* has been observed to increase pneumococcal biofilm formation in vitro and in vivo (Weimer *et al.*, 2010), but this may be strain specific and affected by factors associated with the host (Krishnamurthy Kyd, 2014). Although it has been suggested that pneumococcal biofilm formation enhances survival of bacteria and prolongs colonization rather than promotes invasive disease. Biofilms have been observed in association with a variety of infections as well (Tapiainen *et al.*, 2010).

## **2.9 Immunity Related to Pneumococcal Carriage and Infection**

In addition to the physical and biochemical host defence mechanisms of the mucosa, humans have a versatile complex of immunological defence mechanisms to protect themselves against pathogenic microbes. The immune system can be classified into different overlapping and co operating subsystems. The innate (unspecialised) immune system is not pathogen-specific and therefore it is independent of previous contacts with pneumococci. The adaptive (specialised) system is based on development of protection mechanisms after recognition of pneumococcal structures. The innate immunity operates through leucocytes and circulating proteins (the latter mechanism occasionally referred to as ‘humoral innate immunity’). Adaptive immunity has been divided in two components. First, antibody-dependent (humoral) immunity refers to recognition of different polysaccharide or protein molecules (antigens) by the host and production of antigen specific antibodies. The antibodies bind to their target antigens and start a process aiming at destruction of the bacteria and preventing their attachment and invasion. Second, the adaptive cell-mediated immunity operates through leucocytes and does not involve antibodies. In the case of mucosal infections, the immune system first operates at the mucosal level. The systemic immunity operates through the circulation in the whole body (Gregor *et al.*, 2003).

## **2.10 Immunological Responses to Pneumococcal Carriage**

When pneumococci escape the physical and biochemical host defence mechanisms of the upper respiratory tract mucosa, the bacteria invade the tissue beneath the epithelium and stimulate the mucosal innate immune reaction. After the pneumococcal structures have been recognised by the host receptors, the release of cytokines and other chemical mediators recruits leucocytes into the upper respiratory mucosa. This leucocyte migration has an important role in the clearance of pneumococcal carriage (Krone *et al.*, 2013). Inflammation and cell damage further accelerate the process of the innate immune response. In the systemic innate immune reaction, the pneumococcal structures, together with host proteins like the C-reactive protein, trigger complement activation, which results in a cascade of biochemical reactions producing molecules that cover (opsonise) the bacteria. The opsonisation makes the bacteria easier to be embedded (phagocytised) and killed by polymorphonuclear leucocytes and macrophages.

The proteins produced by the complement cascade also enhance recruitment and activation of additional cells and molecules participating in the process. Furthermore, the inflammatory vasodilatation enhances the migration of phagocytic leucocytes from vessels into the infected tissue. Although very quick, the innate immunologic mechanism is relatively ineffective, as pneumococci have many mechanisms to escape clearance by complement activation (Lachmann *et al.*, 1984). As a link between the innate and adaptive immunity, dendritic cells and macrophages process the pneumococcal antigens and present them to leucocytes responsible for the development of adaptive immunity. The adaptive immune system targets pneumococcal infections specifically and is developed after pneumococcal strains have been encountered by the host.

The best known adaptive immune mechanism is production of antibodies against the polysaccharides of the pneumococcal capsule and a number of other pneumococcal antigenic structures. The importance of adaptive cell-mediated immune mechanisms independent of antibodies has been recognized only recently and is less understood (Kadioglu *et al.*, 2004) and (Malley, 2010). At the mucosal level, the antibody production takes place in the so-called ‘mucosa-associated lymphoid tissue’ beneath the epithelium. The pneumococcal polysaccharide and protein antigens induce production of mucosal antibodies, transported back to the mucosal surfaces and secreted. The majority of these secretory antibodies belong to the immunoglobulin A class. Their main function is to prevent bacterial attachment and invasion into the mucous membrane by binding to the bacterial adhesins and blocking their interaction with the receptors in the epithelial cells. Systemic antibodies against pneumococcal antigens circulate in the blood. They are produced in the lymph nodes and in the spleen and opsonise bacteria for phagocytosis and interfere with the function of different pneumococcal structures.

The capsular polysaccharides induce serotype specific antibody production. The production is independent of T-cells, as polysaccharide antigens can bind directly to B-cells and activate them to proliferate and differentiate to antibody-producing plasma cells. The antibodies interfere with the function of their target antigens, for example, by preventing adhesin-mediated binding of pneumococci to the host cells (Khan and Pichichero, 2012) or reducing the cytotoxic effects of pneumolysin (Hirst *et al.*, 2004). Epidemiological studies have shown that even infants are capable of producing antibodies against the pneumococcal polysaccharides and proteins. The antibody concentrations in serum and saliva increase

during the first 2 years of life but remain mostly below the level measured in adults (Turner *et al.*, 2013).

Increases in antibody concentrations have been measured between the acute and convalescent phases of culture-confirmed pneumococcal infection, although this seems to occur only in a minority of events and vary by age, antigen and previous pneumococcal contacts (Bogaert *et al.*, 2006). In adults, significant increases in polysaccharide antibody concentrations against some pneumococcal serotypes were observed, if either the individual or a family member carried the serotype during a 10 month follow-up (Goldblatt *et al.*, 2005). The observed kinetics of the development of antibodies in children has varied remarkably between different serotypes and by different protein antigens (Zhang *et al.*, 2006).

### **2.11 Transmission and Carriage of *Streptococcus pneumoniae***

*Streptococcus pneumoniae* is transmitted between human hosts by respiratory secretions, either as airborne droplets or through contaminated hands or fomites (Marks *et al.*, 2014). If pneumococcal adherence to the mucosa is not prevented by the host defence mechanisms, pneumococci settle on the mucosa of the human nose, nasopharynx or oropharynx. Pneumococcal carriage remains usually asymptomatic, the bacteria staying on the mucosal surface without causing any harm until the bacteria are cleared. Only a few carriage episodes proceed to disease (Ryan and Ray, 2004)

## 2.12 Exposure to Pneumococci and Transmission

The major reservoir of pneumococcal strains in the population is upper respiratory tract carriage. Asymptomatic carriage is the source for the vast majority of transmissions. Transmission occurs most likely when individuals are in close contact with one another. Since young children have the highest frequency of pneumococcal carriage, they form the most important source for pneumococcal transmission. For this reason, most transmission studies have been carried out in families with young children or in day care facilities. The importance of intra-familial spread of pneumococci was first recognized already several decades ago (Gwaltney *et al.*, 1975). Family studies continue to be crucial in learning about the dynamics of pneumococcal transmission and colonisation. In a study among Bangladeshi families, exposure within families to selected target serotypes signified more than a ten-fold increase in the rate of acquisition in other family members (Erasto *et al.*, 2010). Large families, crowded living conditions (Regev-Yochay *et al.*, 2012) and respiratory infections (Tigoi *et al.*, 2012) favour pneumococcal spread within families. Young children are particularly susceptible to acquiring *Streptococcus pneumoniae*. The risk for acquisition is especially high if the young child is exposed by a carrying family member (Verhagen *et al.*, 2013a). However, adults are less susceptible to acquiring *Streptococcus pneumoniae*, even when exposed at home (Shiri *et al.*, 2013). Adults with children in the family tend to carry pneumococci more often than adults living without children (Regev-Yochay., 2004), but the parents still carry much less often than their children (Hussain *et al.*, 2005).

Day care attendees usually show higher prevalence of pneumococcal carriage than unselected healthy children. Thus, day care centres provide an effective environment for

transmission of pneumococci. In a study of pneumococcal transmission among Finnish day care attendees and their family members, about 80% of new serotypes detected in the family were estimated to be attributable to the day care attendee (Horti *et al.*, 2009). In this model, 65% of acquisitions in day care attendees were estimated to originate from fellow day care attendees, 25% from the community, and only 10% from family members. Clustering of strains within day care centres and schools has been demonstrated in several studies (Leino *et al.*, 2008). Adults introduce new strains into the family less often than children because adults carry pneumococci less frequently. However, once colonised, adults may effectively transmit the bacterium to susceptible family members (Shiri *et al.*, 2013).

### **2.13 Prevalence of Pneumococcal Carriage**

The frequency of pneumococcal carriage in a population is usually characterised by the point prevalence of carriage, or sometimes as the proportion of positive findings out of the total number of samples in studies with repeated samples. The prevalence of carriage depends on the acquisition and clearance rates of pneumococcal carriage in the population. The prevalence of pneumococcal carriage increases during the first months of life, as infants become exposed and start acquiring pneumococcal strains after birth (Labout *et al.*, 2008). In several populations the prevalence levels off or peaks already during the first year of life, (Mueller *et al.*, 2012) whereas in others the prevalence continues to increase at least through the second and sometimes the third year of life (Millar *et al.*, 2009). After the peak prevalence, carriage starts to decrease slowly but usually remains relatively prevalent up to about 3 or 7 years of age, after which it decreases more quickly (Reis *et al.*, 2008).

In high risk populations with a large burden of pneumococcal disease, the turnover and acquisition of new strains is rapid and the prevalence of pneumococcal carriage is high. Extremely high prevalence levels (~90%) have been reported among African children in The Gambia and Ethiopia and among HIV positive children in Tanzania (Anthony *et al.*, 2012). Similarly, very high pneumococcal prevalence has been observed among highland children in Papua New Guinea and Australian indigenous children (Montgomery *et al.*, 1990). The turnover of pneumococcal strains is slower in industrialised countries and middle- and high-income populations, and the average carriage prevalence in healthy children under school age usually settles at a lower level, at about 20–50% (Faden *et al.*, 1990).

In studies conducted among ill children and in day care centres, the prevalence tends to be higher. High point prevalences (70-90%) have been observed occasionally also in industrialized countries in day care centres or orphanages, especially in young children, (Vestrheim *et al.*, 2008). In contrast, low carriage levels (3–20%) have also been reported, but in these studies at least some participating children have been older than five years of age. Adults carry *Streptococcus pneumoniae* less frequently than children. Across a wide range of studies, the prevalence of pneumococcal carriage has been 10% or less (Granat *et al.*, 2007). Higher levels of about 15–20% have been reported in some studies in day care centres and military service (Levine *et al.*, 2012). Among high risk populations even adults carry pneumococci frequently, and the prevalence has been as high as ~50–60% among Gambian villagers (Hill *et al.*, 2006). However, the carriage prevalence still remains clearly lower in adults as compared to children in the same population or family (Mueller *et al.*, 2012). It is not well known how much of the low carriage frequency in adults is attributable

to immunological resistance against acquisition, or is due to low sensitivity to detect carriage of short duration. The prevalence of carriage in older children and adolescents is between those found in children and adults (Hill *et al.*, 2006). There is only sparse knowledge about pneumococcal carriage among the elderly, the other major risk group for invasive pneumococcal disease. However, carriage seems to be rare in this age group (Ridda *et al.*, 2010; Palmu *et al.*, 2012), except among some institutionalized populations (Nuorti *et al.*, 2000).

#### **2.14 Clinical Manifestations of Pneumococcal Pneumonia**

Pneumonia is an acute infection of the lung tissue, filling the alveoli with exudation and interfering with breathing and gas exchange. Bacterial pneumonia develops mostly as a result of aspiration of the bacteria from the nasopharynx or inhalation of the bacteria directly in air-borne droplets (Musher, 1992). The clinical diagnosis of pneumonia is based on the symptoms (cough, fever, pain or difficulty breathing, or gastrointestinal symptoms (Korppi *et al.*, 2008; Cevey-Machere *et al.*, 2009) and clinical signs (crackles or decreased breath sounds detected by auscultation of the lungs and signs of difficult breathing (Puumalainen *et al.*, 2008). A WHO working group has published guidelines for standard interpretation of chest radiographs in verifying the diagnosis of pneumonia in children, especially for purposes of epidemiological studies and vaccine trials (Cherian *et al.*, 2005). Classical pneumococcal pneumonia is considered to present with lobar or segmental consolidation in chest radiography and to respond promptly to penicillin (Toikka *et al.*, 1999; Virkki *et al.*, 2002), but these characteristics are not reliable (Cevey-Machere *et al.*, 2009). If the bacteria spread to the blood and cause bacteraemic pneumonia, they may be detectable with blood culture (Tuerlinckx *et al.*, 2013). Viruses are frequently detected in

association with childhood pneumonia, either as the only causative agents or as counterparts in mixed bacterial-viral infections (Ruskanen *et al.*, 2011). Especially the influenza virus is known to predispose to pneumococcal pneumonia (Sharestha *et al.*, 2013) and influenza-associated pneumococcal pneumonia tends to be especially severe (McCullers, 2006).

### **2.15 Prevention Strategies for Pneumococcal Diseases**

Vaccination is the most effective way to prevent pneumococcal invasive diseases and efforts have been paid to investigate its potential in reducing the burden of pneumococcal infections. A 23-valent polysaccharide vaccine has been available since 1983. It has some effect against bacteraemic disease in adults, but children younger than two years of age show minimal immune responses against most polysaccharide antigens. The efficacy of the polysaccharide vaccine against mucosal infections is poor (Eskola *et al.*, 1999). When the polysaccharide antigens are conjugated to carrier proteins, however, these antigens become immunogenic also in young children and induce immunologic memory. The first conjugate vaccine was licensed in 2000. The vaccine was primarily targeted to prevent invasive diseases, and it included antigens against seven serotypes (4, 6B, 9V, 14, 18C 19F and 23F). This vaccine covered >80% of serotypes causing pneumococcal invasive disease in children in the USA (Johnson *et al.*, 2010). The corresponding coverage was 60–70% in Europe before the vaccination era, with some additional coverage if serotypes cross-reacting with the vaccine serotypes are also considered. (Isaacman *et al.*, 2010), while coverage was only about 50% or less of serotypes causing invasive diseases in children in Asia and Africa, areas with the highest burden of pneumococcal diseases (Hausdorff *et al.*, 2000; Johnson *et al.*, 2010). The next step in the vaccine development was to add serotypes

1 and 5, which are common causes of invasive disease in many developing countries. In studies in South Africa and The Gambia, an experimental, unlicensed 9-Valent vaccine showed 72-83% efficacy against paediatric invasive pneumococcal diseases and 17-37% efficacy against all-cause radiologically confirmed pneumonia (Klugman *et al.*, 2003; Cutts *et al.*, 2005).

A 10-valent vaccine including an additional serotype 7F prevented 100% of vaccine type invasive disease in Finnish children (Palmu *et al.*, 2013). By the end of 2013, pneumococcal conjugate vaccines had been introduced into childhood vaccination programs in 103 countries, and the global coverage of infant immunisation was estimated to be 25% (WHO, 2014). Following vaccine introduction, the incidence of invasive vaccine-type disease has declined remarkably, not only in the vaccine target population but also in other age groups. This indicates herd immunity, i.e. indirect protection through reduced carriage and transmission of the vaccine serotypes by vaccinated children (Whitney *et al.*, 2003; Isaccman *et al.*, 2010). Although the direct effects in the target population for vaccination are clearly beneficial in most countries, the benefit of the indirect effect has been questioned because the other type of indirect effect, i.e. replacement disease in the non target population has been relatively high.

## **2.16 Other Determinants of Pneumococcal Carriage and Pneumococcal Infection**

Studies from different parts of the world document an extraordinary wide range of pneumococcal carriage prevalence (3.5-90%) in children (Cardozo *et al.*, 2006a). This variation is likely due to differences across the study populations with respect of age, current and underlying health status and treatment, genetic background as well as socio-

economic living conditions and cultural habits. In addition, different sampling and laboratory methods may contribute to the differences. Factors affecting the occurrence of upper respiratory infections are mostly the same as those associated with pneumococcal carriage (Gisselsson-Solen *et al.*, 2014) and also pneumococcal invasive diseases share similar risk factors. However, there are also differences, and it is not well known whether these differences are due to shortcomings in the methodology in investigating the associations, or whether there are mechanisms by which some factors specifically affect the progress of carriage to disease.

The incidence of respiratory tract infections starts to increase after protection by maternal antibodies has waned by 6 months of age (Turner *et al.*, 2013), especially in developed populations (Hagerman *et al.*, 2013). Pneumococcal carriage often starts to increase already during the first months of life. The increase of pneumococcal carriage frequency may continue during the second and third years of life, after which it remains at a relatively high level up to school age, whereas the incidence of respiratory infections usually peaks earlier at about one year of age or even earlier (Kaltoft *et al.*, 2000). The maturation of immunological mechanisms, increasing acquired immunity to microbes as well as anatomical changes in the respiratory tract are the likely factors contributing to decreasing susceptibility to the infection by age. Some but not all studies have associated the increased risk specifically with young age of siblings (Nilsson and Laurell, 2001), with their day-care attendance (Aniansson *et al.*, 1992), with their pneumococcal carriage, respiratory symptoms (Millar *et al.*, 2009) or with sharing a sleeping room with siblings (Teele *et al.*, 1989).

Many chronic diseases and conditions impair the host defence mechanisms, predisposing to pneumococcal pneumonia. Anatomical or functional defects in the lungs, liver, kidneys, nervous system and adjacent organs, metabolic and neoplastic diseases or their treatment predispose to these infections (Rose *et al.*, 2014). Globally, the most important of immunological deficiencies predisposing to pneumococcal infections are those caused by human immunodeficiency virus (HIV) infection (Madhi *et al.*, 2000), hereditary sickle cell disease (Ramakrishnan *et al.*, 2010) and various causes of anatomical or functional splenic dysfunction.

The effect of these conditions on pneumococcal carriage is not clear, and the treatment often interferes with interpretation (Cardoso *et al.*, 2006b), but at least immunological disorders are suggested to affect the carriage (Verhagen *et al.*, 2013b). Obvious immunodeficiencies are rarely diagnosed in children suffering from frequent repeated episodes of respiratory infections, but minor or transient deviations in their immunological system may occur (Veenhoven *et al.*, 2004). Severe atopy, allergy, asthma and gastrointestinal reflux probably predispose to respiratory infections (Patria *et al.*, 2013) but their impact on carriage has remained controversial and associations with pneumococcal carriage have been found only occasionally (Borer *et al.*, 2001).

In developed countries, differences in socio-economic conditions are relatively small and the roles of income, education and employment of parents, type of housing, living conditions and other markers of socio-economic status on carriage of pneumococci, respiratory infections in children have remained controversial (Labout *et al.*, 2008). However, crowding has been identified as a risk factor for these infections in several studies also in developed countries (Rapola *et al.*, 2001). Furthermore, when investigated in

detail, a diet rich in added sugar increased the frequency of pneumococcal carriage in Finnish children (Tapiainen *et al.*, 2014). It is likely that the high occurrence of pneumococcal carriage and infectious diseases in developing countries and high risk populations in developed countries is largely attributable to socio-economic factors, malnutrition, vitamin A deficiency (Verhagen *et al.*, 2013b), poor housing or hygiene, smoky firewood, and crowding (Coles *et al.*, 2009). Poor access to health care may worsen these problems (Jain *et al.*, 2005).

Genetic and environmental factors affecting the risk of infection are difficult to differentiate from each other. Indigenous populations with high risk of pneumococcal infections do not share common genetic heritage, although according to a recent theory, their non-European background could refer to a genetic background for their higher susceptibility to pneumococcal infections against which people with a European background could be genetically less susceptible (Laayouni *et al.*, 2014). However, the socio-economic status and cultural lifestyles of indigenous people often differ from those of the non-indigenous populations, and this difference complicates the interpretation of epidemiological data. Furthermore, the declining rate of invasive pneumococcal diseases in Navajo children aged less than 3 years from 1989 to 1996 speaks for environmental factors affecting the risk in this population (O'Brien *et al.*, 2004). The incidence of pneumococcal pneumonia, like many other bacterial infections, follows a distinct winter seasonal patterns (Dowell *et al.*, 2003). These seasonal epidemics probably results from a combination of increased carriage of the bacterium and increased susceptibility to developing disease (Hodges and Macleod, 146). Carriage is considered a prerequisite for disease and the majority of young children carry pneumococcus in the nasopharynx. Once a person is colonized with pneumococcus,

the likelihood that invasive pneumococcal disease (IPD) will develop depends on the virulence of the strain and host's susceptibility (Bogaerti *et al.*, 2004). The host susceptibility is influenced by seasonal factors and environmental conditions like dryness, dust, decreased sunlight etc, which might affect immunity or integrity of respiratory mucosa (Dowell *et al.*, 2003). Genetic factors may also predispose individuals to infectious diseases through inherited anatomical structures (Renko *et al.*, 2007), specific hereditary diseases (Ramakrishnan *et al.*, 2010), or immunological aberrations (Veenhoven *et al.*, 2004).

Tobacco smoke contains chemicals that cause inflammation of the respiratory tract mucosa and harm its function. Adherence of bacteria to human cells has been shown to be more effective in smokers than non-smokers (Ozlu *et al.*, 2008). Active smoking has been identified as a very strong risk factor for invasive pneumococcal disease in adults (Nuorti *et al.*, 2000). In children, the real quantity of exposure to smoke is difficult to measure and it may be associated with socio-economic factors. Thus, the role of passive smoking is clearly recognised in many studies as a risk factor for pneumococcal carriage (Cardozo *et al.*, 2008) and pneumococcal invasive disease (von Mollendorf *et al.*, 2014), but remains undetected in others (Lee *et al.*, 2010). In Alaska, the presence in the household of at least one person chewing tobacco rather than the presence of a smoker was an independent risk factor, indicating that the effect of parental smoking may be at least partly due to increased susceptibility of the smoking parents to infections and subsequent transmission of infection to their children (Greenberg *et al.*, 2006). Indoor air pollution due to combustion of biofuels is regarded as a risk factor for pneumococcal carriage and acute respiratory infections in developing countries.

There are also factors that decrease the susceptibility of children to pneumococcal carriage and infection. Human milk has properties that protect against bacterial attachment in the human respiratory epithelial cells (Aniansson *et al.*, 1990). The milk secretory immunoglobulin A antibodies inhibit microbes on the mucosa, while antimicrobial molecules such as lactoferrin and oligosaccharides may resemble the receptors for pneumococcal attachment (Hanson *et al.*, 2002). It has also been suggested that breast milk has a unique capacity to stimulate the immune system of the infant after which it might respond better to infections (Hanson, 1998). Breast-feeding is often associated with other potential factors affecting bacterial carriage or disease, e.g. age, nutritional and hygienic quality of alternative food, time spent in day care, education of the parents or other socioeconomic factors. Hence, it appears plausible that such confounding could explain some of the controversial findings about the protective effect of breast-feeding against pneumococcal carriage (Duffy *et al.*, 1997), and invasive pneumococcal diseases (Hanson *et al.*, 2002).

Xylitol inhibits the growth of *Streptococcus pneumoniae* (Kontiokari *et al.*, 1995). It has been found to reduce pneumococcal adherence to human epithelial cells in vitro in a Finnish study (Kontiokari *et al.*, 1998), but not in Spanish study (Ruiz *et al.*, 2011). Xylitol decreased the occurrence of pneumococcal infection in Finnish day care children when used regularly five times a day (Uhairi *et al.*, 2000). In animal studies, xylitol did not decrease nasopharyngeal carriage of pneumococci in rats (Kontiokari *et al.*, 1998) but activated neutrophils and increased the survival time of rats during experimental pneumococcal sepsis (Renko *et al.*, 2008). Also probiotics decrease pneumococcal adhesion to human epithelial cells in vitro (Wong *et al.*, 2013). The ingestion of probiotics

reduces pneumococcal carriage in adults (Gluck and Gebbers, 2003), and a beneficial effect in preventing respiratory infections in children has been suggested (John *et al.*, 2013), although in a randomised, controlled trial no effect was seen against either pneumococcal pneumonia or pneumococcal carriage (Hatakka *et al.*, 2007).

The use of antimicrobial agents to treat pneumococcal pneumonia also affects pneumococcal carriage. With very few exceptions, lower carriage rates have been reported in studies that compare children with recent antimicrobial treatment in the last month to children without treatment (Dagan *et al.*, 1998; Ghaffar *et al.*, 2000). There appears to be an association between clearance of the carried strain and its susceptibility to the antimicrobial agent. Carriage of susceptible strains is reduced whereas carriage of non-susceptible strains remains at the same level (Dagan *et al.*, 2000), resulting in an increase in the proportion of non-susceptible strains. Consequently, antimicrobial treatment selects resistant strains, and recent or frequent antimicrobial use has proven to be a risk factor for carriage of non-susceptible strains both at the individual and population levels (Goossens *et al.*, 2005; Guillemot *et al.*, 1998). In an Israeli study, a resistant strain in the nasopharynx replaced the initially susceptible strain in the respiratory tract within a few days after the start of antimicrobial treatment of Pneumococcal pneumonia (Dagan *et al.*, 2001).

### **2.17 Pneumococcal Antibiotic Resistance**

The pneumococcus (*Streptococcus pneumoniae*) continues to be a common cause of serious and life threatening infections, including pneumonia, bacteraemia and meningitis. It is also a frequent cause of respiratory tract infections, such as otitis media and sinusitis (Klugman, 1990; Collignon and Bell 1996). During the first half of the 20th century *S. pneumoniae*

was susceptible to penicillin and most other antimicrobial agents. However, in recent years *S. pneumoniae* has exhibited increased resistance to standard agents including penicillin, erythromycin, chloramphenicol, and extended spectrum cephalosporins. Since antibiotics first became widely used in the World War II era, they have saved countless lives and blunted serious complication of many feared infectious diseases, however many antimicrobials are not as effective as they used to be. Widespread use of antibiotics is thought to have spurred evolutionary adaptations that enable bacteria to survive these powerful drugs (Ball *et al.*, 2002).

For many years *S. pneumoniae* was uniformly susceptible to penicillin G with MICs of <0.1 µg/ml, allowing most physicians to treat persons who had severe infections with penicillin alone without testing for resistance. Since the 1960s, however, resistance to penicillin and other antimicrobial agents has spread rapidly and was first reported in Australia in 1967, in New Guinea in 1969, in South Africa in 1977, and in many other countries throughout Africa, Asia, and Europe (Jacobs *et al.*, 1978).

Many penicillin resistant strains are also resistant to other drugs such as chloramphenicol, erythromycin, tetracycline and trimethoprim-sulfamethoxazole (Collignon and Bell, 1996). As multi-drug resistant strains become increasingly prevalent, treatment options became limited. The clinical impact of antimicrobial resistances on the outcomes of invasive and non-invasive Drug Resistant *Streptococcus pneumoniae* (DRSP) infections remains largely unknown. To make appropriate empiric antimicrobial choices clinicians need a reliable and current assessment of the level of antimicrobial resistance in the community (Kentuck, 1994). Surveillance data collected at the center for disease control and prevention (CDC) have shown that high level resistance to penicillin increased more than 60-fold from 0.02%

for 1979 to 1987 to 1-3% in 1992 for pneumococcal isolates from invasive infections (Breiman *et al.*, 1994). In some communities, at least 30% of isolates are non-susceptible to penicillin (Whitney *et al.*, 2000). However, Levine *et al.* (1995) considered clindamycin as an alternative drug for the treatment of Nursing home acquired pneumonia.

## CHAPTER THREE

### 3.0 Materials and Methods

#### 3.1 Study Area

Bauchi State is located on latitude  $10.637^{\circ}\text{N}$  and longitude  $10.0807^{\circ}\text{E}$  in the North-Eastern region of Nigeria. It occupies a total landmass of  $49,119\text{km}^2$ . It has 20 Local Government Areas with a population of 4,653,066 (NPC, 2006).



Figure 3.1: Map extract of Bauchi State showing the sampling locations

KEY:  General Hospital Toro,  Abubakar Tafawa Balewa University Teaching Hospital, Bauchi

### 3.2 Study Population

The study population included patients of all ages who were attending Abubakar Tafawa Balewa University Teaching, Bauchi and General Hospital Toro, Bauchi State and were suspected to have pneumonia during the study period and consented to the study.

### 3.3 Ethical Approval

The ethical approval was obtained from Bauchi State Health Research Ethics Committee and Abubakar Tafawa Balewa University Teaching Hospital Research Ethics Committee

### 3.4 Sample Size

The sample size was calculated using 25.6% prevalence rate recorded in Nigeria by Taura

*et al.* (2013) in Kano using the formula;  $n = \frac{Z^2 P(1 - P)}{d^2}$

n = Sample size

P = Prevalence rate (25.6%) = 0.256

Z = Standard Normal Distribution at 95% confidence limit = 1.96

d = Absolute desired precision at 5% = 0.05

Hence,

$n = 1.96^2 \times 0.256(1-0.256)$

\_\_\_\_\_ = 292.67 which was rounded up to 373.

0.05<sup>2</sup>

### **3.5 Sample Collection**

Sputum were collected from 249 and 124 patients in Abubakar Tafawa Balewa University Teaching Hospital (ATBUTH) and General Hospital Toro (GHT) respectively into a clean, sterile, dry, wide-necked, leak proof universal container while in children who could not cough out sputum, throat swab samples were collected from March, 2014 to October, 2015. The samples were transported immediately to the ATBUTH's Microbiology Laboratory for processing (Cheesebrough, 2006).

### **3.6 Media Preparation**

All media used during this research were of analytical grade and were prepared according to manufacturer's instructions. Blood agar (Oxoid), trypticase soy agar (Oxoid) and Mueller-Hinton agar (Oxoid) were used in this work.

### **3.7 Sample Processing and Isolation of *Streptococcus pneumoniae***

The purulent part of the sputum was homogenized gently with 2ml sterile normal saline and streaked on a freshly prepared blood agar using a sterile wire loop and then incubated at 37°C for 18 hours in a candle jar (Cheesebrough, 2006). Typical isolates that were alpha-haemolytic, Grampositive Streptococci were subcultured on fresh blood agar medium for purification. After purification, the isolates were inoculated on Trypticase Soy agar slants for subsequent microscopic and biochemical identification.

### **3.8 Microscopic Examination of the Isolates**

The colonies that showed  $\alpha$ - hemolysis on blood agar were Gram stained. Those that appeared Gram positive Streptococci were subjected to biochemical characterization (Kellogg *et al.*, 2001).

### **3.9 Biochemical Characterization of the Isolotes**

The following biochemical tests were carried out.

#### **3.9.1 Optochin sensitivity test**

Colonies of the alpha-hemolytic, Gram-positive isolates were inoculated on blood agar plates. The optochin disc was applied on the inoculated blood agar plates and incubated at 37°C for 24 hours in a candle jar. The zone of inhibition around the optochin disc was recorded and interpreted. A zone size  $\geq 14$ mm indicates susceptibility which is diagnostic of *Streptococcus pneumoniae* while a zone size  $< 14$ mm indicates optochin resistance and suggests that bile solubility test should be carried out to identify *Streptococcus pneumoniae* (Tankeshwar, 2013).

#### **3.9.2 Bile solubility test**

One millilitre of saline suspension of 16 hours old alpha-hemolytic, Gram-positive colonies from the blood agar plates was prepared and the turbidity adjusted to 0.5 Mcfarland standards. Half millilitre of the suspension was dispensed in two tubes. Half millilitre of 2% sodium deoxycholate was added to one tube marked as test and equal amount of saline was added to the second tube marked as control and incubated at 37°C for 3hrs. Clearance or loss of turbidity in the tube marked test indicates that the organism is lysed by bile salt

which biochemically confirmed the isolates as *Streptococcus pneumoniae* while persistent turbidity indicates that the organism is not *Streptococcus pneumoniae* (Tankeshwar, 2013).

### **3.10 Molecular Characterization of the Isolates**

This was carried out using the Polymerase Chain Reaction (PCR) technique.

#### **3.10.1 DNA Extraction**

The DNA of the organism was extracted as described by Iwolakun *et al.* (2012) as follows; Colonies from fresh culture of bile soluble isolates were picked and inoculated in to Leuria Bertani (LB) medium and incubated at 37°C for 24 hours. One millilitre of the overnight culture was added into 1.5ml Eppendorff tube and centrifuged at 10,000g for 1minute and the supernatant was discarded. Five hundred microlitre of lysis buffer was added, vortexed and incubated at room temperature for 30minutes. The mixture was transferred to spins columns and centrifuged at 10,000g for 1minute, the filtrate and collection tube were discarded.

The spin column was put into fresh collection column and 300µl of wash buffer I was added and centrifuged at 10,000g for 1minute and the filtrate was discarded. Three hundred microlitre of wash buffer II was added and centrifuged at 1000g for 1minute and the filtrate discarded. The spin column was then centrifuged at 10,000g for 2minutes to remove residual alcohol and salts. The collection was discarded and the spin column placed in 1.5ml Eppendorff tube. Fifty microlitre elusion buffer was added and incubated at room temperature for 2 minutes. The column then centrifuged at 10,000g for 2 minutes, the spin

column was then discarded and the DNA remained in the Eppendorf tube for subsequent use.

### **3.10.2 PCR Detection of Autolysin (LytA) Gene**

One hundred and seventy five microlitre of the master mix was put in an Eppendorff tube followed by addition of 14 $\mu$ l each of the forward and reverse primer of autolysin gene (LytA) (957bp) (Nagai *et al.*, 2001). Seven microlitres of 25M MgCl<sub>2</sub> was then added to the mixture followed by 42 $\mu$ l of nuclease free water. Eighteen microlitres of the reaction mixture was dispensed into PCR tubes and to each tube, 7 $\mu$ l of the DNA template was added. The mixture was centrifuged at 10,000g for few seconds and loaded into the thermocycler.

The amplification of the LytA gene was done at 35 cycles under the following conditions; DNA denaturation at 95°C for 5 minutes, 94°C for 30 seconds, primer annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 7 minutes (Iwolakun *et al.*, 2012).

### **3.10.3 Agarose Gel Electrophoresis**

Two grams of 2% powdered agarose was placed in 200ml conical flask followed by addition of 100ml of 1 $\times$ TBE (Tris Borate EDTA) and the mixture was microwaved. It was allowed to cool to 45<sup>0</sup>C followed by addition of 5 $\mu$ l ethidium bromide (10mg/ml) and the mixture poured into the already assembled casting tray with combs positioned and allowed to gel. After gelling, the combs were removed and the tray was placed in the tank containing running buffer.

Fifteen microlitre of each sample was mixed with 5µl of gel loading dye (Bromothymol blue) and loaded into each well on gel. The preparation was connected to power pack and ran at 80v for 30 minutes. The gel was then photographed with Polaroid film under transmitted UV light to see the bands of the target genes (Iwolakun *et al.*, 2012).

### **3.11 Determination of Antibiogram of the *S. pneumoniae* Isolates**

Antibiotic susceptibility testing was performed using selected therapeutically relevant groups of antibiotics which includes; Beta-lactame agents (penicillin, augmentin), Macrolides (erythromycin), Tetracyclines (tetracycline), Glycopeptides (vancomycin), Chloramphenicol (chloramphenicol) and Lincosamides (clindamycin) as described by Kirby and Bauer (1966) and interpreted according to CLSI standard (2014) on plates of Mueller-Hinton agar supplemented with blood.

#### **3.11.1 Preparation of the Inoculum**

Four isolated colonies of the test organism were picked using a sterile inoculating loop and suspended in 2ml sterile saline. The saline tube was vortexed to create a smooth suspension. The turbidity of the suspension was adjusted to 0.5 McFarland ( $1.5 \times 10^8$  CFU/ml) standard by adding more of the organism when the suspension was too light or diluting with sterile saline when the suspension was too heavy (Cheesebrough, 2006).

### **3.11.2 Antibiotic Susceptibility Assay**

A sterile swab was used to swab 1ml of the inoculum over the entire surface of the Mueller-Hinton agar plates and allowed to stand at room temperature for 5 min. The appropriate antibiotic discs were placed on the surface of the inoculated agar plates using a sterile forcep and incubated at 37°C for 24 hours in a candle jar. After the incubation period, the zone sizes of growth inhibition were measured to the nearest millimeter using a metre rule and interpreted according to the CLSI (2014) criteria.

### **3.12 Molecular Detection of Antibiotic Resistance Genes of the Isolates**

PCR assay was used to detect the antibiotic resistance genes which includes; penicillin resistance gene (Pbp2b), tetracycline resistance gene (TetM), chloramphenicol resistance gene (CatS) and erythromycin resistance gene (MefA).

### **3.13 Statistical Analysis of Data**

Data obtained were entered and analysed using SPSS 11.0 Statistical software.

Comparison was done using Chi-Square (Iwolakun *et al.*, 2012).

**Table 3.1: Primers Sequence for the Genes Screened in the Study**

<b>Primer code</b>	<b>Sequence 5' – 3'</b>	<b>Bp</b>	<b>Reference</b>
<b>LytA</b>	CAACCGTACAGAATGAAGCGG	957	(Nagai <i>et al.</i> , 2001)
	TTATTCGTGCAATACTCGTGCG		
<b>Pbp2b</b>	CCTATATGGTCCAAACAGCCT	147	(Nagai <i>et al.</i> , 2001)
	GGTCAATTCCTGTGCGCAGTA		
<b>MefA</b>	CTGTATGGAGCTACCTGTCTGG	402	(Nagai <i>et al.</i> , 2001)
	CCCAGCTTAGGTATACGTAC		
<b>TetM</b>	GAAGTGTATCCTAATGTGTG	377	(Warsa <i>et al.</i> , 1996)
	GATACTCTAACCGAATCTCG		
<b>CatS</b>	GGACTGAGTGTAAGTCTGAC	30kb	(Trieu-Cuot <i>et al.</i> , 1993)
	CCATACCGTTGCGTATCAC		

## CHAPTER FOUR

### 4.0 Results

#### 4.1 Microscopic Examination of the Isolates

In this study, 373 samples; 249 from Abubakar Tafawa Balewa University Teaching Hospital (ATBUTH) and 124 from General Hospital Toro (GHT) were collected and analysed, of which 255 (68.4%) revealed alpha-hemolytic, Gram positive streptococci isolates.

#### 4.2 Biochemical Characterization of the Isolates

The biochemical characterization of the isolates revealed that 7 (2.81%) isolates from ATBUTH were optochin sensitive while 10 (4.01%) were bile soluble. On the other hand, 2 (1.61%) isolates from GHT were optochin sensitive while 4 (3.22%) were bile soluble. This gave a total of 14 (3.75%) biochemically identified pneumococcal isolates.

#### 4.3 Molecular Detection of *Streptococcus pneumoniae* Isolates and Age Distribution

After PCR amplification of the organism's specific gene called autolysin (LytA) using the LytA specific primer, 9 of the biochemically identified isolates were molecularly identified as *Streptococcus pneumoniae* with the amplicon size of 957bp as shown in plate II. The other isolates that were not identified as *Streptococcus pneumoniae* are shown in plate III. Seven isolates from ATBUTH were molecularly confirmed as *Streptococcus pneumoniae* as shown in Table 4.3, of which 6 (75%) were obtained from the age group of < 1-19 years old. None of the isolates obtained from the age group of 20-49 years was positive of *Streptococcus pneumoniae*. While only 1(50%) *Streptococcus pneumoniae* was obtained

from the age group of >50 years old. Therefore, the total occurrence of *Streptococcus pneumoniae* isolated in ATBUTH was 70%.

Two isolates from GHT were molecularly confirmed as *Streptococcus pneumoniae* as shown in Table 4.4 of which 2 (66.6%) were obtained from the age group of < 1-19 years old. None of the isolates obtained from the age group of 20-49 and that of >50 years old was *Streptococcus pneumoniae*. The total *Streptococcus pneumoniae* isolated in GHT was 50%.

#### **4.4 Seasonal Distribution of the *S. pneumoniae* Isolates**

During the dry season, 87 samples were examined from ATBUTH and 3 (3.4%) *S. pneumoniae* were isolated while 36 sample were examined from GHT and 1(2.8%) *S. pneumoniae* was isolated. This makes a total of 4 (3.3%) *S. pneumoniae* isolated during the winter period of the study. Similarly, during the rainy season, 162 samples were examined from ATBUTH and 4 (2.5%) *S. pneumoniae* were isolated while 88 samples were examined from GHT and 1 (1.1%) *S. pneumoniae* was isolated. This gives a total of 5 (2.0%) *S. pneumoniae* isolated during the summer period of the study.

#### **4.5 Occurrence of *S. pneumoniae* Isolates in the Two Hospitals Studied**

A total of 212 samples were obtained from the age group of < 1-19 in the two hospitals studied. Eight (3.8%) samples were positive for *Streptococcus pneumoniae*. Forty-five (45) samples were obtained from the age group of 20-49 in the two Hospitals studied and none of them was positive for *Streptococcus pneumoniae*. One hundred and sixteen (116) samples were obtained from the age group >50 years old in the two Hospitals studied and 1

(0.9%)) sample was positive for *Streptococcus pneumoniae*. The overall occurrence of *Streptococcus pneumoniae* in the two Hospitals studied was 2.41%. However, the P-Value was 0.137761 which is greater than 0.05 (i.e  $P > 0.05$ ), indicating that there is no statistically significant difference between the age distribution and the occurrence of *Streptococcus pneumoniae*.

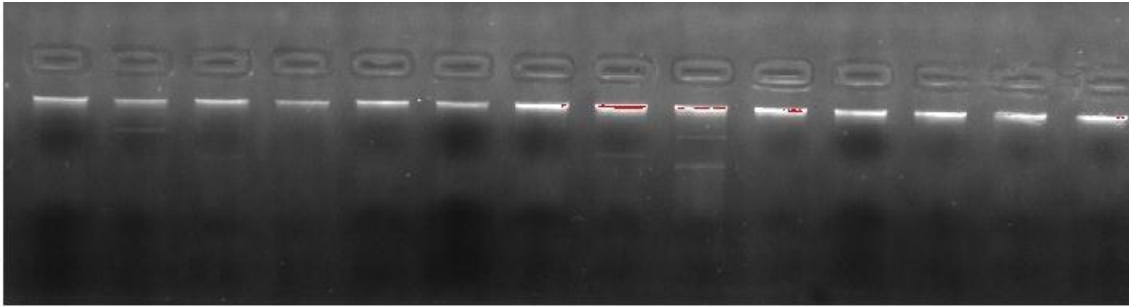
**Table 4.2: Microscopic Characterization of the *Streptococcus pneumoniae* Isolates**

Sampling location	No. of Samples collected	No. of $\alpha$ -haemolytic Isolates (%)	No. of Gram-Positive Streptococci isolates (%)
ATBUTH	249	187(75.1)	187(75.1)
GHT	124	68(54.8)	68(54.8)
Total	373	255(68.4)	255(68.4)

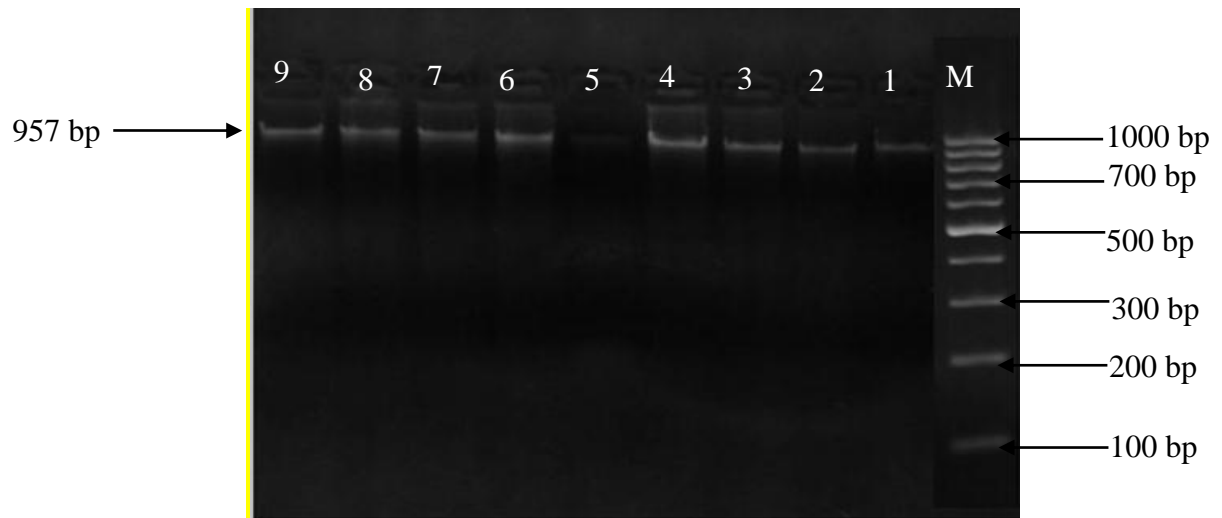
**Key:** ATBUTH= Abubakar Tafawa Balewa University Teaching Hospital, GHT= General Hospital Toro

**Table 4.3: Biochemical Characterization of the *Streptococcus pneumoniae* Isolates**

Sampling Location	No. of samples collected	No. of optochin sensitive isolates (%)	No. of bile soluble isolates (%)	No. of biochemically identified isolates (%)
ATBUTH	249	07(2.8)	10(4.0)	10(4.0)
GHT	124	02(1.6)	04(3.2)	04(3.2)
Total	373	09(2.4)	14(3.8)	14(3.8)

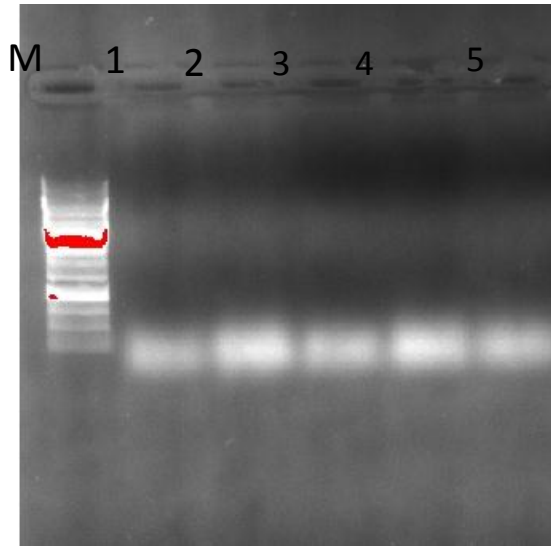


**Plate I:** Agarose Gel Photo of Genomic DNA of the *S. pneumoniae* Isolates



**KEY:** Lane M: 100 bp DNA ladder, Lanes 1-9: PCR amplicons of LytA

**Plate II:** PCR Products of Autolysin (LytA) Gene for *Streptococcus pneumoniae* Isolates



**Key:** Lane M: 100 bp DNA ladder and Lanes 1-5: PCR amplicons of LytA

**Plate III:** Negative PCR Products of Autolysin (LytA) Gene for *Streptococcus pneumoniae* Isolates

**Table 4.4: Occurrence of Confirmed *Streptococcus pneumoniae* Isolates Among Pneumoneic Patients at Abubakar Tafawa Balewa University Teaching Hospital Based on Age Group**

Age group (yrs)	No. of isolates screened	No. positive for <i>S. pneumoniae</i> (%)
< 1-19	08	6(75)
20-49	0	0(0.0)
>50	02	1(50)
Total	10	7(70)

**Table 4.5: Occurrence of Confirmed *Streptococcus pneumoniae* Isolates Among Pneumoneic Patients at General Hospital Toro Based on Age Group**

Age group (yrs)	No. of isolates screened	No. positive for <i>S. pneumoniae</i> (%)
< 1-19	3	2(66.6)
20-49	0	0(0.00)
>50	1	0(0.00)
Total	4	2(50.0)

**Table 4.6: Seasonal Distribution of the *S. pneumoniae* isolates**

Sampling location	Dry season		Rainy season	
	No. of samples Collected	No. of <i>S. pneumoniae</i> isolated (%)	No. of samples Collected	No. of <i>S. pneumoniae</i> isolated (%)
ATBUTH	87	3(3.4)	162	4(2.5)
GHT	36	1(2.8)	88	1(1.1)
Total	123	4(3.3)	250	5(2.0)

**Table 4.7: Occurrence of *S. pneumoniae* Isolates in the Two Hospitals Studied**

Sampling subjects(yrs)	Number of samples screened	Number of <i>Streptococcus pneumoniae</i> isolated (%)
< 1-19	212	8(3.77)
20-49	45	0(0.00)
>50	116	1(0.86)
Total	373	9(2.41)

( $P > 0.05$ : 0.137761)

#### **4.6 Antibiogram of the *Streptococcus pneumoniae* Isolates**

The diameter of the zone of inhibition of each antibiotic tested was measured in millimeters and recorded for each *Streptococcus pneumoniae* isolate as shown in Table 4.6. The zone diameters were interpreted according to the CLSI (2014) criteria shown in appendix I to determine the resistant isolates. All the nine (100%) isolates were resistant to penicillin, augmentin and tetracycline. Five (55.55%) isolates were resistant to chloramphenicol; three isolates (33.33%) were resistant to vancomycin as well erythromycin, while only one (11.11%) isolate was resistant to Clindamycin.

All the *Streptococcus pneumoniae* isolates exhibited multiple antibiotics resistance as observed in table 4.8. Three isolates were resistant to three of the seven antibiotics with MAR index of 0.42. Four isolates were resistant to five out of the seven antibiotics with MAR index of 0.71. Only one isolate was resistant to six antibiotics with MAR index of 0.85.

#### **4.7 Agarose Gel Electrophoresis of Antibiotic Resistance Genes Amplified by PCR**

Penicillin binding protein (Pbp2b) with the amplicon size of 147bp which codes for penicillin resistance was the only resistant gene detected among others screened as shown in plate IV, lane 1.

**Table 4.8: Antibiotic Susceptibility of the *Streptococcus pneumoniae* Isolates**

Isolate's Number	Zone of inhibition(mm)						
	VAN(30µg)	AUG(30µg)	E(15µg)	CHL(30µg)	CLIN(2µg)	P(10µg)	TET(30µg)
<b>1</b>	17 (S)	10 (R)	14 (R)	19 (R)	23 (S)	07(R)	0(R)
<b>2</b>	18 (S)	12 (R)	16 (I)	20 (R)	24 (S)	08(R)	0(R)
<b>3</b>	19 (S)	12 (R)	17 (I)	21 (S)	26 (S)	06(R)	0(R)
<b>4</b>	22 (S)	10 (R)	11 (R)	19 (R)	22 (S)	0(R)	0(R)
<b>7</b>	20 (S)	09 (R)	22 (S)	31 (S)	36 (S)	08(R)	0(R)
<b>9</b>	16 (R)	08 (R)	0 (R)	25 (S)	0 (R)	0(R)	0(R)
<b>10</b>	15 (R)	13 (R)	21 (S)	20 (R)	18 (I)	07(R)	0(R)
<b>12</b>	17 (S)	10 (R)	22 (S)	22 (S)	21 (S)	07(R)	0(R)
<b>14</b>	15 (R)	14 (R)	21 (S)	18 (R)	19 (S)	0(R)	0(R)
<b>%R</b>	3 (33.3)	9(100)	3 (33.3)	5 (55.5)	1 (11.1)	9 (100)	9 (100)

**KEY:** VAN= Vancomycin, AUG= Augmentin, E= Erythromycin, CHL= Chloramphenicol,  
CLIN= Clindamycin, P= Penicillin, TET= Tetracycline, mm= Millimeter,  $\mu\text{g}$  = microgram

MARI: Multiple Antibiotics Resistance Index, %R= Percentage Resistance

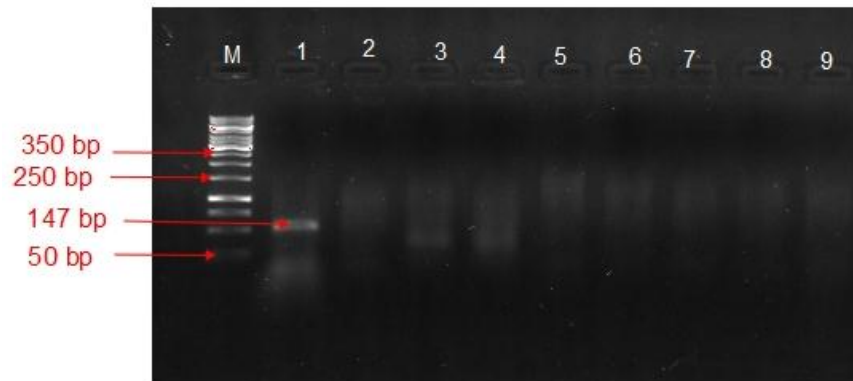
S= Sensitive

I= Intermediate

R= Resistant

**Table 4.9: Multiple Antibiotic Resistance of the *Streptococcus pneumoniae* Isolates**

Isolate	Antibiotic resisted	MARI
1	AUG, E, CHL, P, TET	0.71
2	AUG, CHL, P, TET	0.57
3	AUG, P, TET	0.42
4	AUG, E, CHL, P, TET	0.71
7	AUG, P, TET	0.42
9	VAN, AUG, E, CLIN, P, TET	0.85
10	VAN, AUG, CHL, P, TET	0.71
12	AUG, P, TET	0.42
14	VAN, AUG, CHL, P, TET	0.71



**KEY:** Lane M: 50 bp DNA ladder Lane 1: PCR Amplicon of pbp2b

Plate IV: PCR Products of Penicillin Resistance Gene (Pbp2b) of *Streptococcus pneumoniae* Isolates

## CHAPTER FIVE

### 5.0 Discussion

From the cultural plates, the alpha-haemolytic isolates were presumed to be *Streptococcus pneumoniae*. This is because pneumococci produce an enzyme called pneumolysin which breaks down hemoglobin into a green pigment that can be observed as a green zone surrounding the colonies of the organism on blood agar plates (Musher *et al.*, 2000).

Of the 14 (3.75%) biochemically identified *Streptococcus pneumoniae* from the 373 sputum and throat swab samples examined in this study, 9 (2.41%) were molecularly confirmed as *Streptococcus pneumoniae*. This finding could be due to the fact that *Streptococcus pneumoniae* shares  $\geq 99\%$  similarities with *Streptococcus pseudopneumoniae* and differs only in possession of autolysin gene by *Streptococcus pneumoniae* as reported by Marcus *et al.*, (2012) or some inconsistencies in the PCR amplification procedures.

Occurrence of the pathogen varies with sampling location, where it was found to be higher (70%) in Abubakar Tafawa Balewa University Teaching Hospital, located in the urban setting compared to (50%) found in General Hospital Toro which is located in the rural setting. This could be attributed to more exposure to risk factors such as crowded living conditions and day care attendance of children in the urban setting compared to rural communities which favor pneumococcal spread within a population. This is also similar to the findings of Ahmed *et al.* (2015) in Northern Nigeria who reported higher occurrence (86%) of the pathogen among urban settlers compared to 12% observed among the rural settlers. Similarly, higher occurrence of 3.8% for *Streptococcus pneumoniae* was observed among the age group of <1-19 years old while the least was 0.86% among the age group of

$\geq 50$  years old but the difference was not statistically significant at  $p > 0.05$ . The highest occurrence of the pathogen in the age group of <1-19 years old in this study could probably be attributed to risk factors such as poor hygiene, crowded living conditions, poor socio-economic status, malnutrition, immature immune system, as well as immune compromised conditions due to HIV, other respiratory tract infections and poor breast-feeding. Hsieh *et al.* (2012) also attributed it to cohabitating with young children carrying the bacterium in their upper respiratory tract or day care attendance. The 3.8% occurrence of the pathogen in the age group of <1- 19 is lower than the 15.8% reported by Wroe *et al.* (2012) in Ghana. This variation could be due to differences in identification procedures carried out in this study which includes extension of the identification to the molecular level which is more sensitive and specific over the conventional cultural and biochemical methods.

The maturation of immunological mechanisms, increasing acquired immunity to microbes, anatomical changes in the respiratory tract as well as widespread use of antibiotics before presentation to hospital are the likely factors that could account for the absence of the pathogen in the age group of 20-49 years old. This finding agrees with that of Lipsitch *et al.* (2012) who stated that there is a decreased incidence of *Streptococcus pneumoniae* in adults compared to small children because as the age increases, so does immune mediated clearance of pneumococcal serotypes.

Occurrence of *Streptococcus pneumoniae* in the age group of  $\geq 50$  years old in this study could probably be related to their weak immune system due to old age as reported by Hill *et al.* (2006). Nuorti *et al.* (2000) also partly attributed this to their behavioural pattern like smoking and alcoholism. This result agrees with the report of Iwolakun *et al.* (2012) who reported higher (53.3%) occurrence of *S. pneumoniae* in children compared to 46.7%

observed in adults. Ruskanen *et al.* (2011) also reported that even though pneumococcal pneumonia affects all ages but is more common among the young, the old and the chronically ill.

There was higher occurrence of *Streptococcus pneumoniae* (3.3%) during the dry season compared to 2.0% occurrence in the rainy season. This could be associated with exposure to environmental factors like dryness and dust during the dry season, which might affect immunity or integrity of the mucosa thereby, increasing susceptibility of the host to the pathogen. This agrees with the reports of Weinberger *et al.*, (2014) who reported 48.5% occurrence of *Streptococcus pneumoniae* in dry season compared 29.7% in the rainy season.

The overall occurrence of 2.41% for *Streptococcus pneumoniae* recorded in this study is much lower than that obtained by Taura *et al.* (2013) in Kano who reported 25.6% occurrence of *Streptococcus pneumoniae* among patients with respiratory tract infection. This difference might be due to the fact that PCR (a more specific tool) was used in the detection of the organism in this study as opposed to the only conventional cultural and biochemical tests used in their study.

There was a 100% resistance to penicillin. The high penicillin resistance observed is not surprising as penicillins are the oldest and most widely used antibiotics. This result agrees with the findings of Iwalokun *et al.* (2012) who reported 100% resistance to penicillin in South-Western Nigeria. Klugman (1990) reported long ago that the greater the quantity and the longer the duration an antibiotic has been in use, the more likely resistant strains emerge to that antibiotic. Similarly, there was a 100% tetracycline resistance rate observed

in this study. This could be due to its high level of consumption because of its easy accessibility by the public especially in countries with limited healthcare budgets as reported by Hryniewicz (2003) and Jacobs *et al.* (2003). The 33.33% resistance rate to erythromycin in this study is higher than the 25.0 % rate of resistance to azithromycin, which is also a macrolide as reported by Garba *et al.* (2015). The macrolides have long been important in treating community acquired pneumonia because of their excellent activity against pneumococci. However, this activity has been eroded by the proliferation of macrolides resistant strains due to excessive use of the antibiotic by public and continues to increase in many parts of the world, leading to an increasing number of treatment failures in infections caused by macrolide-resistant pneumococcal isolates (Daneman *et al.*, 2006). The rate of resistance to chloramphenicol was found to be 55.55% in this study, which is higher than the 27.7% resistance rate reported in Jos by Kandakai-Olukemi *et al.* (2009) possibly because of the persistent use of the antibiotic over time. It could also be attributed to low cost and ease of accessibility of the antibiotic to the public as reported by Hryniewicz (2003).

In this study, vancomycin resistance was found to be 33.33% which is higher than the 0.3% reported in Asia by Hoban *et al.* (2001). This might be due to wide spread use of extended-spectrum cephalosporins which has been reported to promote emergence of vancomycin resistant pneumococci strains and extended beta-lactamase producing organisms (Patterson *et al.*, 2001). Some Pneumococcal isolates have been reported by Charpentier *et al.* (2000) to exhibit tolerance to vancomycin probably as a result of mutations in several genetic loci. Clindamycin resistance in this study was found to be 11.1%, which is lower than the 15.4% and 20.0% reported by Hoban *et al.* (2001) in Europe and Asia respectively. The low

resistance rate to this antibiotic could be attributed to its low usage due to cost and non-affordability by the public in the study area.

The possible explanation for the observed resistance can be attributed to the facts that the organism has a natural transformation system as a mechanism of genetic exchange which underlies antibiotics resistance over the past many years as reported by Ewig (1986) or through enzymatic degradation, alteration of bacterial proteins and efflux of antibiotics as reported by Samore *et al.* (2001) and Tenover. (2006). It could also be as a result of therapeutic exposure to antibiotics as observed by Ball *et al.* (2002).

All the pneumococcal isolates found in this study exhibited multiple antibiotic resistances as indicated by their Multiple Antibiotic Resistance Index (MARI), ranging from 0.42-0.85. This indicates high risk spread of resistant pneumococcal isolates because MARI value > 0.2 is considered to originate from high risk sources where antibiotics are often used as reported by Riaz *et al.* (2011).

Among the resistance gene screened, only *pbp2b* was detected with an amplicon size of 147bp as shown in plate IV lane 1. Other genes not detected could be due to the fact that the pneumococcal antibiotic resistance is encoded by different genes, which probably were not targeted in this study, or they may be plasmid based and not chromosomal based.

## CHAPTER SIX

### 6.0 CONCLUSION AND RECOMMENDATION

#### CONCLUSION

1. The study concludes that *Streptococcus pneumoniae* could be a common cause of pneumococcal pneumonia and patients of age group <1-19 years old are more susceptible to the pathogen.
2. PCR amplification of autolysin is a better approach for the identification of *Streptococcus pneumoniae*.
3. Clindamycin can be considered as the most effective therapeutic agent in the treatment of pneumococcal pneumonia.
4. Only one isolate expressed penicillin binding protein (Pbp2b) as a mechanism of penicillin resistance.

#### RECOMMENDATIONS

1. Due to the cost of running PCR, It is recommended that at least the bile solubility test should be routinely performed in cases suspected of pneumococcal etiology even if the isolates are optochin resistant, for accurate identification of *Streptococcus pneumoniae* and adequate therapy of patients.
2. Government in partnership with the non-governmental organizations should put in place well equipped laboratories especially in the rural areas for proper diagnosis of pneumonia before initiating therapy.

3. Government should also support local and national campaigns to promote appropriate antibiotics prescription in order to exert a downward pressure on resistance rates.
4. Every health care facility should have a current data on local antimicrobial resistance patterns of *Streptococcus pneumoniae* to help guide their therapeutic recommendations.

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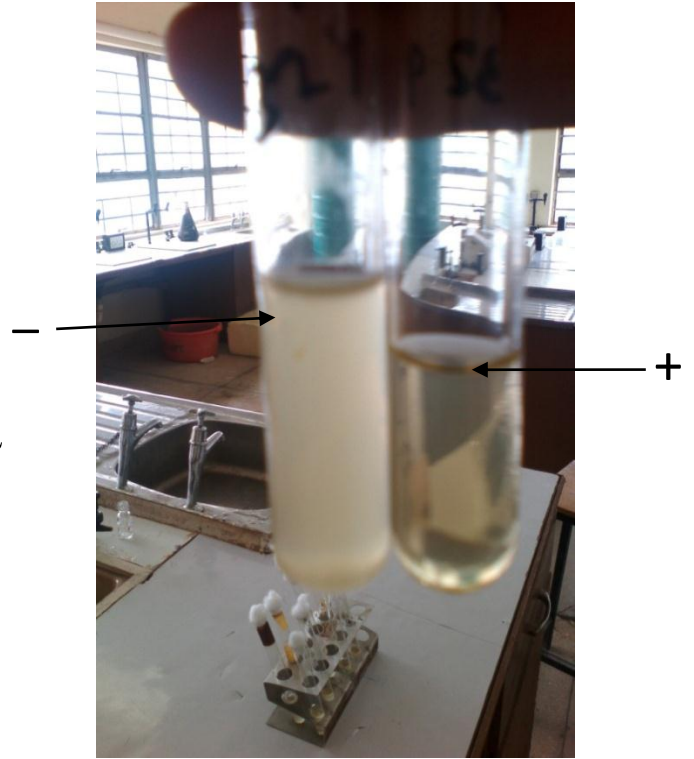
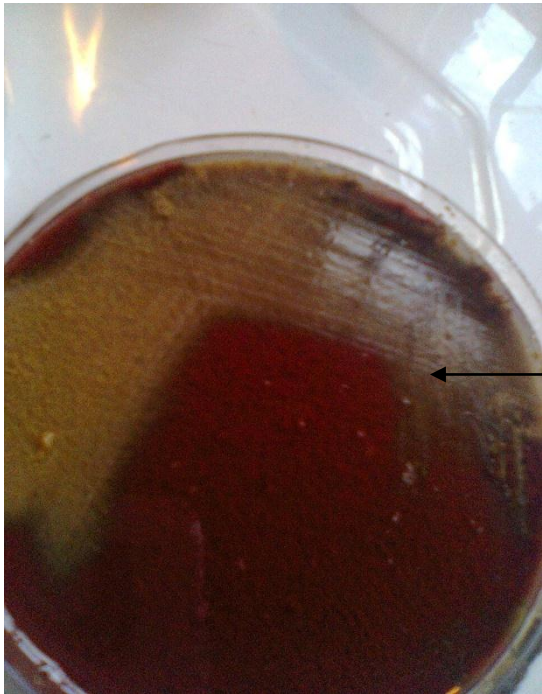
## APPENDICES

Antibiotics( $\mu$ g)	Zone diameter interpretative criteria(mm)		
	Sensitive	Intermediate	Resistance
Penicillin(10)	$\geq 20$	-	-
Erythromycin(15)	$\geq 21$	16-20	$\leq 15$
Tetracycline(30)	$\geq 28$	25-27	$\leq 24$
Chloramphenicol(30)	$\geq 21$	-	$\leq 20$
Clindamycin(2)	$\geq 19$	16-18	$\leq 15$
Vancomycin(30)	$\geq 17$	-	-

KEY: CLSI: Clinical Laboratory Standard Institute

mm: millimeter

Appendix I: CLSI (2014) Standard Interpretation of Zone of Inhibition Sizes for *S. pneumoniae*



Alpha-hemolysis by *S.*

*S. pneumoniae* Isolates

Key: + = bile soluble

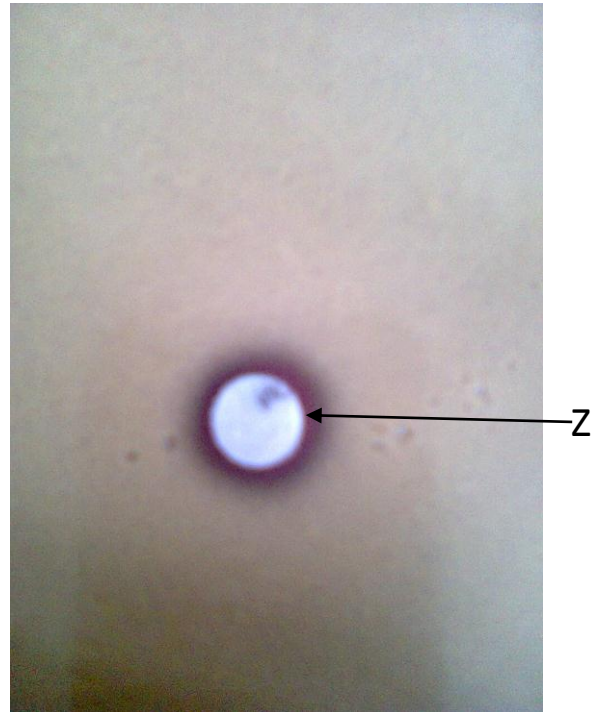
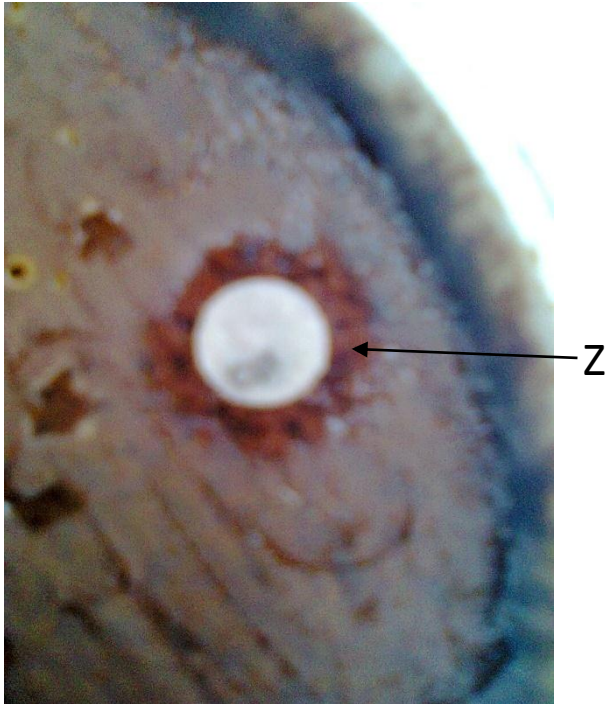
- = bile insoluble

$\alpha$ =alpha-haemolysis

Bile Solubility Test Results of

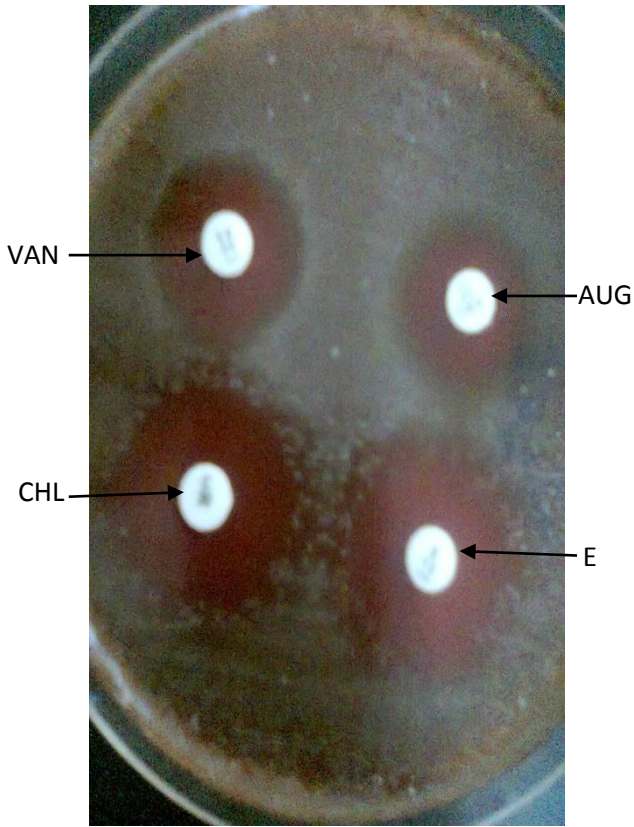
*S. pneumoniae* Isolates

Appendix II: Cultural and Biochemical identification of *S. pneumoniae*.

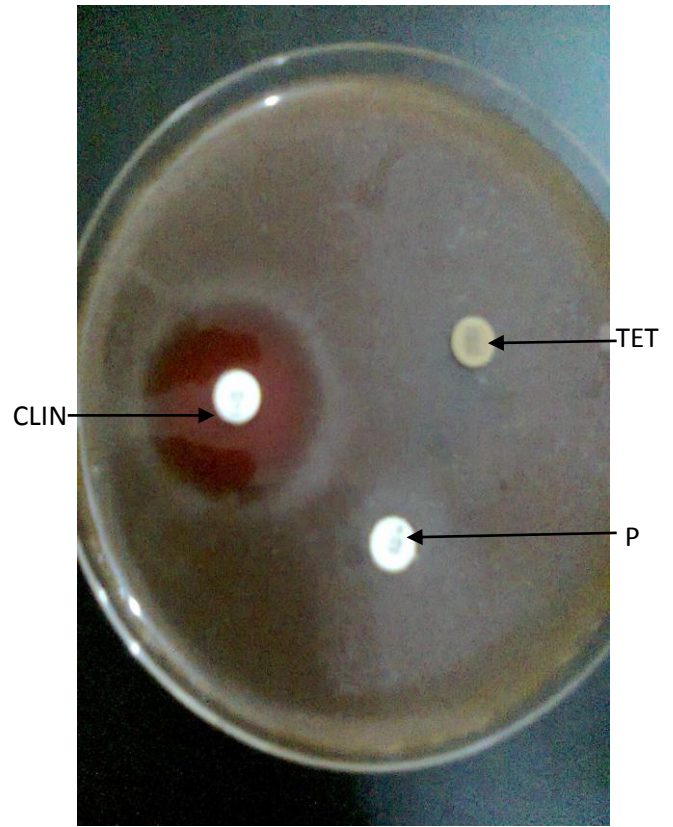


Appendix III: Optochin Sensitivity of *S.pneumoniae* Isolates

Key: Z= zone of inhibition of optochin



**a**



**b**

Appendix IV: Antibiotic Zones of Inhibition of *S. pneumoniae* Isolates

a= Zones of inhibition by VAN (Vancomycin), AUG (Augmentin), E (Erythromycin) and CHL (Chloramphenicol)

b= Zones of inhibition by CLIN (Clindamycin), TET (Tetracycline) and P (Penicillin)