DETERMINATION OF ERYTHROMYCIN AND PENICILLIN RESISTANCE GENES IN *STREPTOCOCCUS PNEUMONIAE* FROM PATIENTS ATTENDING SELECTED HOSPITALS IN KADUNA STATE, NIGERIA

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JUNE, 2019

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHYLOSOPHY DEGREE (Ph.D.) IN MEDICAL MICROBIOLOGY

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JUNE, 2019

DECLARATION

I, declare that the work in this Thesis entitled **DETERMINATION OF ERYTHROMYCIN AND PENICILLIN RESISTANCE GENES IN *STREPTOCOCCUS PNEUMONIAE* FROM PATIENTS ATTENDING SELECTED HOSPITALS IN KADUNA STATE, NIGERIA** has been performed by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this Thesis was previously presented for another degree or diploma at this or any other Institution.

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Name of Student	Signature	Date

CERTIFICATION

This Thesis entitled **DETERMINATION OF ERYTHROMYCIN AND PENICILLIN RES ISTANCE GENES IN *STREPTOCOCCUS PNEUMONIAE* FROM PATIENTS ATTEND ING SELECTED HOSPITALS IN KADUNA STATE, NIGERIA** by Haruna Yadock LUSA meets the regulations governing the award of the degree of Doctor of Philosophy (Ph.D.) of Medical Microbiology of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my late parents Mr. and Mrs. Lusa Yadock.

ABSTRACT

Streptococcus pneumoniae (Pneumococcus) is both an aggressive pathogen and a normal flora of the human microbiome with an array of over 90 serotypes.Penicillin-resistant *S. pneumoniae* (PRP) and Erythromycin resistant Pneumococci (ERP) has emerged as a worldwide problem. *S. pneumoniae* develops penicillin resistance through recombination with other PRP strains or closely related streptococcal species that alter its penicillin binding protein (PbP) genes *pbp1a*, *pbp2b*, and *pbp2x*. These altered PbP gene sequences of PRP differ greatly within the PRP subset and from the sequences of penicillin-susceptible *S.pneumoniae* (PSSP),whileresistanceto macrolides in S.pneumoniae arises primarily due to macrolide resistant Genes (MRGs) including; erythromycin ribose methyltransferase encoded by *ermB* or an efflux system leading to selective efflux of 14 and15 membered macrolides encoded by *mef(A)*.Erythromycin ribose methyltransferase (*ermB*) typically confers high level resistance to macrolides, lincosamides and streptogramin B (MLS_B phenotype) whereas *mef A* confers low le vel resistance to macrolides. The study aimed to detect *streptococcus pneumoniae* strains with erythromycin and penicillin resistance genes. A facility based cross sectional study with 450 patients attending selected hospitals in Kaduna State. The enrolled patients were selected by systematic random sampling presenting with clinically suspected respiratory tract infections (RTIs). Two hundred and thirty-seven (52.7%) of the subjects were males and 213(47.3%) were females aged between 10- 70 years with symptoms of respiratory tract infections. Two hundred and seven (46.0%) of the samples yielded positive growth of which 41(9.1%) were *Streptococcus pneumoniae* isolated in 5% blood agar in the presence of 5% CO_2,,morphology,

identified with bile solubility, optochin and antibiotic susceptibilities. MIC determination was done with E-test for penicillin and erythromycin while molecular determination was done for serotypes macrolide and penicillin resistance genes by PCR.

Incidence of 20.5% Penicillin resistant pneumococci (PRP) and 29.4% Erythromycin resistant pneumococci (ERP) were established among the elderly patients age $\geq$ 50 years. Economic status of the patients had a significant relationship to Penicillin and Erythromycin resistance (P=0.0004**) determined by disc diffusion technique. Thirty-one (75.6%) of the Pneumococcal isolates (n=41) were penicillin non-susceptible pneumococci (PNSP)[MICs 1.0-15.5µg/mL; MIC_{50}=8.0µg/mL) and 31(75.6%) were erythromycin non-susceptible pneumococci(ENSP) [MICs 0.12-6.0µg/mL;MIC_{50}=0.90µg/mL]determined by E-Test methodology.

Four pneumococcal serotypes were detected in the study area: 3(33.3%), 13(33.3%), 20(16.7%), 23F (16.7%) by conventional PCR serotyping strategy. PCR assay and Agar-gel electrophoresis for the simultaneous detection of *lyt A,* Penicillin resistance genes (PRGs: *PbP1a*, *2b, 2x*) and Macrolide resistance genes (MRGs: *ermB* and *Mef A*) was carried out. The results show 3 of the pneumococcal strains harbor mutations in *PbP1a* and *PbP 2x*; six of the strains express *ermB* and *mefA* genes. This study shows that pneumococcal drug resistance in the area is predominantly mediated by *ermB* and *mefA* genes with serotypes 3 and 13 having the highest frequency among the isolates suggesting the use of more specific gene targets may in the near future solve the problem of inaccurate identification of pneumococcal infections of the lower respiratory tract. The finding suggests the use of more specific gene targets in the near future may solve the problem of inaccurate identification of pneumococcal infections of the lower respiratory tract. This study also highlights the danger in empirical use of these groups of antibiotics. Our facilities must be enforced to put in place policies that will guarantee protection and safety of patients, their relatives and heath personnel.

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ABBRIVIATIONS

LTCFs	-	Long term Care Facilities
CAP	-	Community- acquired Pneumonia
HIV	-	Human immunodeficiency Virus
TB	-	Tuberculosis
ERM	-	Erythromycin ribosomal methylase gene
MEF	-	Macrolide efflux gene
PRSP	-	Penicillin Resistant *Streptococcus pneumoniae*
PbP	-	Penicillin binding Proteins
BHI	-	Brain Heart Infusion
Lyt A	-	Autolysin A gene
DNA	-	Deoxyribonucleic Acid
SPSS	-	Statistical Package for Social Sciences
PCR	-	Polymerase Chain Reaction
ABUTH	-	Ahmadu Bello University Teaching Hospital
ACHF	-	Acute-care Health Facility
ERP	-	Erythromycin Resistant Pneumococci
PRP	-	Penicillin Resistant Pneumococci
MIC	-	Minimum Inhibitory Concentration
MRGs	-	Macrolide Resistance Genes
PRGs	-	Penicillin Resistance Genes
E-test	-	Epsilometer- Test
ENSP	-	Erythromycin Non susceptible Pneumococci

PVC - Pneumococcal Conjugate Vaccine

PPV - Pneumococcal Polysaccharide Vaccine

ARI - Acute Respiratory Infection

URT- Upper Respiratory Tract

MDROs - Multi-drug Resistant Organisms

CDC - Center for Diseases Control and Prevention

RNA - Ribonucleic Acid

CPS - Capsular Polysaccharide

WHO - World Health Organization

HAP - Hospital Acquired Pneumonia (nosocomial)

HCAP - Hospital Community- Acquired Pneumonia

SYMBOLS

χ^2- chi-square

µg – microgram

Pvalue - probability value

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Antibiotic resistant bacteria that are difficult or impossible to treat are becoming increasinglyco mmon and are causing a global health crisis (Abla *et al.,* 2014). Drug-resistance trends are not well documented in most developing countries due to limited laboratory capacity and acute respiratory infections (ARI) are often treated empirically with antibiotics (Schrang *et al.*, 2001).Erythromycin and penicillin belongs to the group frequently used, clinically important antibiotics for treatment of infections caused by Gram-positive bacteria such as*Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes*. The actionof and resistance to these groups of antibiotics remain cryptic (Marne and Alexander, 2005).*Streptococcus pneumoniae* is the most common cause of bacterialARI and Pneumococcal

resistance is the principal cause of concern regarding treatment failures for ARI (Schrang *et al.*, 2001). Pneumococcus is also the most common cause of community acquired pneumonia (CAP), sporadic bacterial meningitis and bacteremia worldwide (Mohammed *et al.*, 2012). The global emergence of antimicrobial resistance and dissemination of penicillin and macrolde resi stant *Streptococcus pneumoniae* strains has become a serious clinical concern worldwide (Mohammed *et al.*, 2012; Alba *et al.*, 2014). Pneumococcal infection is an important public health concern throughout the world causing disease and death comparable to that of Human immune-deficiency virus (HIV), Malaria and Tuberculosis (Iwalokun *et al.*, 2012). As a major bacterial pathogen, *Streptococcus pneumoniae* infection starts from colonization of the upper respiratory tract (URT), causing respiratory tract diseases such as pneumonia, bronchitis, otitis media and sinusitis.

The bacteria evade host immunity, causing systemic infections such as bacteremia, sepsis and meningitis (Gillespie and BalaKrishnan, 2002; Ortgvist *et al*., 2005).

Pneumococci are normally very sensitive to penicillin, which is lethal at concentrations <0.1 μg/mL.The typical penicillin minimum inhibitory concentration (MICs) for highly resistant strains is 4–8 μg/mL. The threshold MIC at which treatment failure is likely to occur is unclear because of scarce clinical data (Dayie *et al*., 2013). Resistance to macrolides in *S. pneumoniae* arises primarily due to Macrolide resistant Genes (MRGs) including erythromy cin ribosomal methylase encoded by *erm (B*) or an efflux system leading to selective efflux of 14 and 15memberedmacrolides encoded by *mef(A)*.Erythromycin ribosomal methylas e (*ermB*) typically confers high level resistance to Macrolides, lincosamides and streptogramin B(MLS_B phenotype) whereas *mef*(A) confers low level resistance to macrolides (Wierzbowski *et al*.,2007).Erythromycin-susceptible isolates do not possess any of the MRGs (Hasanuzzaman, 2017). In Penicillin resistant *Streptococcus pneumoniae (*PRSP*)*, Beta-lactamase activity has not been detected, which is due exclusively to mutations in their natural target. The penicillin binding proteins (PBP) prevent binding and make them indifferent to $\beta -$lactams and also decreased their binding affinity with these drugs (Ferroni and Berche, 2001).

Highly resistant strains have a reduced capacity to bind to the molecules of antibiotics in at least three of the five existing PBPs; *PbP1a, PbP2x* and *PbP2b* (Maurer *et al*., 2008).

The WHO in 2005, estimated that about 1.6 million people die of pneumococcal disease every year (Bandettini and Melioli, 2012). Nigeria is currently one of the highest pneumococcal disease burdened countries and accounts for 5% of the global burden (Iwalokun *et al*., 2012; Iliyasu *et al*., 2014). The diagnosis of the agent of Pneumonia has an essential role in ensuring the most appropriate and effective therapy for the individual patients and is critical to guiding the development of diagnostic techniques, treatment and prevention strategies.

Older adults admitted into Long- term health care (LTHC) Facilities are at higher risk for invasive pneumococcal disease and death than older adults residing in communities (Kupronis *et al*., 2003). High resistance rates of 56.6% and 66.7% to erythromycin and Penicillin were reported by some workers in Nigeria (Akanbi *et al*., 2012; Iwalokun *et al*., 2012). Findings by Onipede *et al*.(2009) reported 100% of invasive pneumococcal isolates from children to be sensitive to erythromycin which was similar to findings by Adeleye *et al*.(2008) among sputum isolates from HIV-infected pediatrics and adult patients.

1.2 Statement of Research Problem

Information on the molecular epidemiology of *Streptococcus pneumoniae* associated with respiratory tract (RT) infections in Nigeria is scarce. Little is known about the population biology of *Streptococcus pneumoniae* in developing countries, although the majority of pneumococcal infections occur in these settings (Donkor *et al*., 2013).Nigeria accounts for 5% of the global burden of pneumococcal infections. Our Health care facilities do not have policies and procedures in place to protect vulnerable patients from vaccine-preventable illness; specifically pneumococcal disease. It is on record that multi-drug resistant organisms (MDROs) are acquired in hospitals often affecting younger and older people who are very ill(File, 2016;Prina et al.,2015).

Pneumococcal vaccination is not compulsory in Nigeria and hence this vaccine preventable infection(Pneumonia) has continued to be an outlier. The increase in prevalence of antimicrobial resistant bacterial pathogens has severe implications for the future treatment and prevention of infectious diseases in both human and animals.

Human respiratory tract infections cause diseases such as pneumonia, bronchitis, otitis media and sinusitis and under some circumstances; bacteria invade host cells and evade host immunity, causing systemic infections such as bacteremia, sepsis and meningitis (Mohammed *et al*., 2012). The lack of detection and molecular characterization of resistant *Streptococcus* *pneumoniae* associated with RT infections hampers the development of more effective control practice for this infection in Nigeria.

1.3 Justification for the study

Pneumococcal infections are treated with Penicillin as first drug of choice, and erythromycin is frequently used as a substitute for penicillin against Streptococcal and Pneumococcal infections. Penicillin and erythromycin resistance have emerged with alarming high frequencies. Penicillin and macrolide resistant Pneumococci have been reported, especiall y in several Asian countries. Existing data on Pneumococcal carriage and disease in Nigeria have limitations, including small sample sizes, poor yield of pneumococci and very few isolates are serotyped. Consequently, the current prevalence of serotypes in carriage and disease is largely unknown in Nigeria.

In conventional method of identifying etiologic agent of community-acquired pneumonia(CAP), Sputum Gram stains and culture are the first diagnostic step for identifying pneumococcal pneumonia and provide information on antibiotic susceptibility. Prospective studies with high-quality sputum samples showed relatively high sensitivity 82% and specificity 97%. The formulation of conjugate vaccine to cover serotypes in the US and Europe and another to protect against serotypes in the developing world was proposed on the assumption that the same serotypes account for most infections in the two areas of the world.

Long term health care (LTHC) facilities and Acute-care Health facilities are essential components of health care delivery to many patients; unfortunately, these facilities are also recognized as “reservoirs” of antibiotic resistance. This study is therefore imperative to isolate, characterize and detect virulent *Streptococcus pneumoniae* with penicillin and erythromycin resistance determinants from patients attending selected hospitals in Kaduna state, Nigeria so that more effective control measures can be developed for this infection in the State and Nigeria in general.

1.3 Aim and objectives of the Study

Aim

The aim of this study was to determine Erythromycin and Penicillin resistance genes in *Streptococcus pneumoniae* isolated from patients attending selected health facilities in Kaduna State, Nigeria and the objectives are:

1. To isolate and phenotypically characterize *Streptococcus pneumoniae* from sputum samples of patients attending selectedHealth facilities in Kaduna State

2. To determine the susceptibility of the Pneumococcal isolates to eight commonly used antibiotics

3. To identify sociodemographic risk factors associated with Penicillin and Erythromycin resistance using well-structured questionnaire.

4. To determine the MICs of Erythromycin and Penicillin resistant Pneumococci.

5. To serotype Penicillin and Erythromycin resistant pneumococcal isolates in the study area.

6. To detectMacrolide (*ermB* and *mefA*) and Penicillin (*PbP1a, PbP2b*and *PbP2x*) resistance genes in isolates confirmed to be resistant to Erythromycinand Penicillin G.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Streptococcus pneumoniae* (Pneumococcus)

S. pneumoniae was first identified in the late 1800s, and early on was recognized as the most common cause of lobar pneumonia (Musher, 2010).*Streptococcus pneumoniae* has been defined as Gram-positive bacteria in the shape of slightly pointed cocci, usually found in pairs (diplococci), but also singly and in short chains. They are α-hemolytic with individual bacteria between 0.5 and 1.25µm in diameter. *Streptococcus pneumoniae* do not form spores and are non-motile, though they sometimes possess pili used for adherence. They are found normally in upper respiratory tract, including the throat and nasal passages being mesophilic and living optimally at temperatures between 30-35^{o}C(Microbewiki,2011) .*Streptococcus pneumoniae* (Pneumococcus) is a classic example of a highly invasive Gram-positive, extracellular bacterial pathogen that is a major cause of morbidity and mortality globally causing more death than any infectious disease (Birgitta and Elaine, 2013).The medical burden of disease it causes is amongst the greatest in the world in causing disease and death comparable to that of Human immune-deficiency virus (HIV), Malaria and Tuberculosis (TB) (Iwalokun *et al*., 2012). The inflammatory response to pneumococci, one of the potent in medicine has revealed the double-edge sword of clearance of infection with a cost damage to host cells, with highest risk among smallest children and the elderly. CDC. (2008) reported ~1 million children below 5 years of age dying of Pneumococcal diseases. In a study carried out by Iroha *et al*. (2012), 305 sputum samples were collected; 30 (9.8%) of *S. pneumoniae* were isolated from patients within the age range of 20-40 years, 140 (45.9%) from patients within 41-60, 135 (44.2%) from patients within 60 years and above.

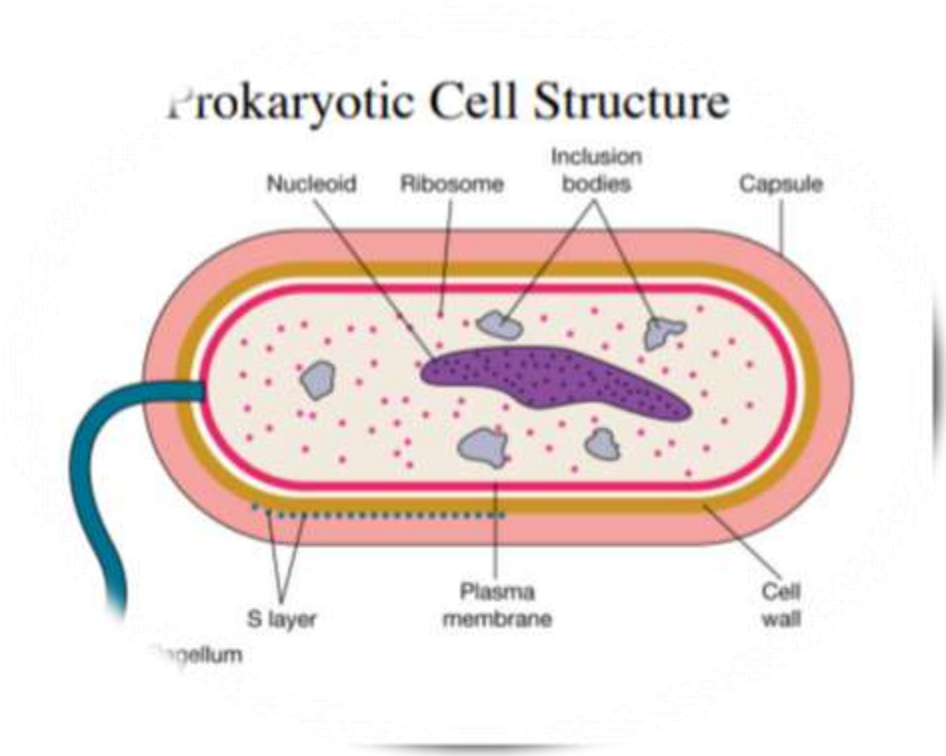


Fig.2.1.1 Structural components of a bacterial cell adopted from wikipedia

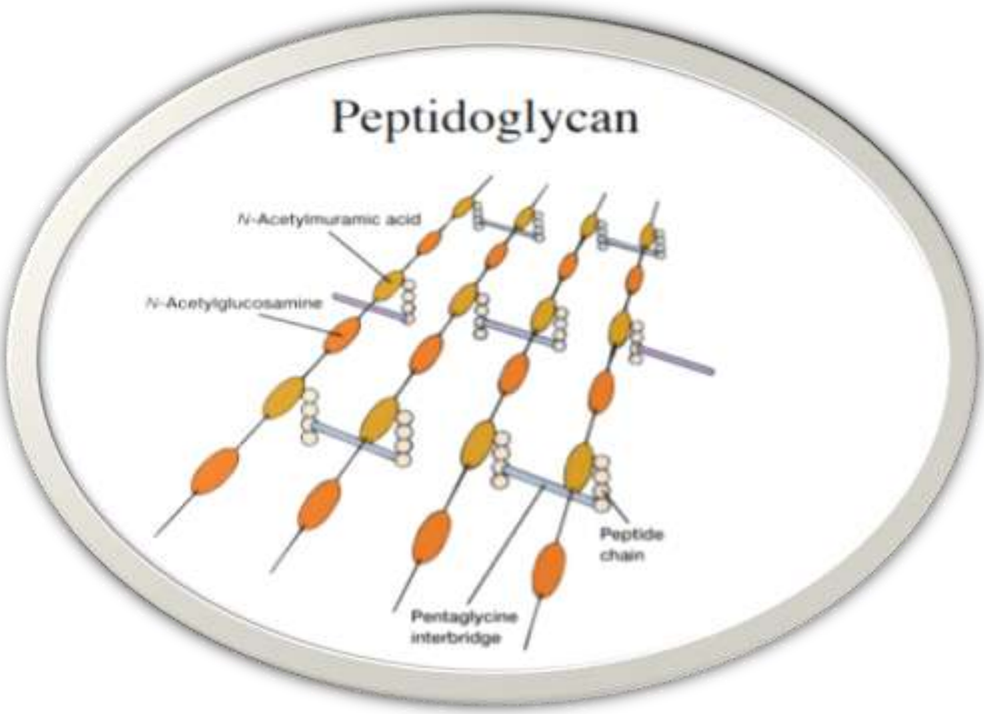


Fig.2.1.2 Structure of Cell wall Peptidoglycan adopted from wikipedia

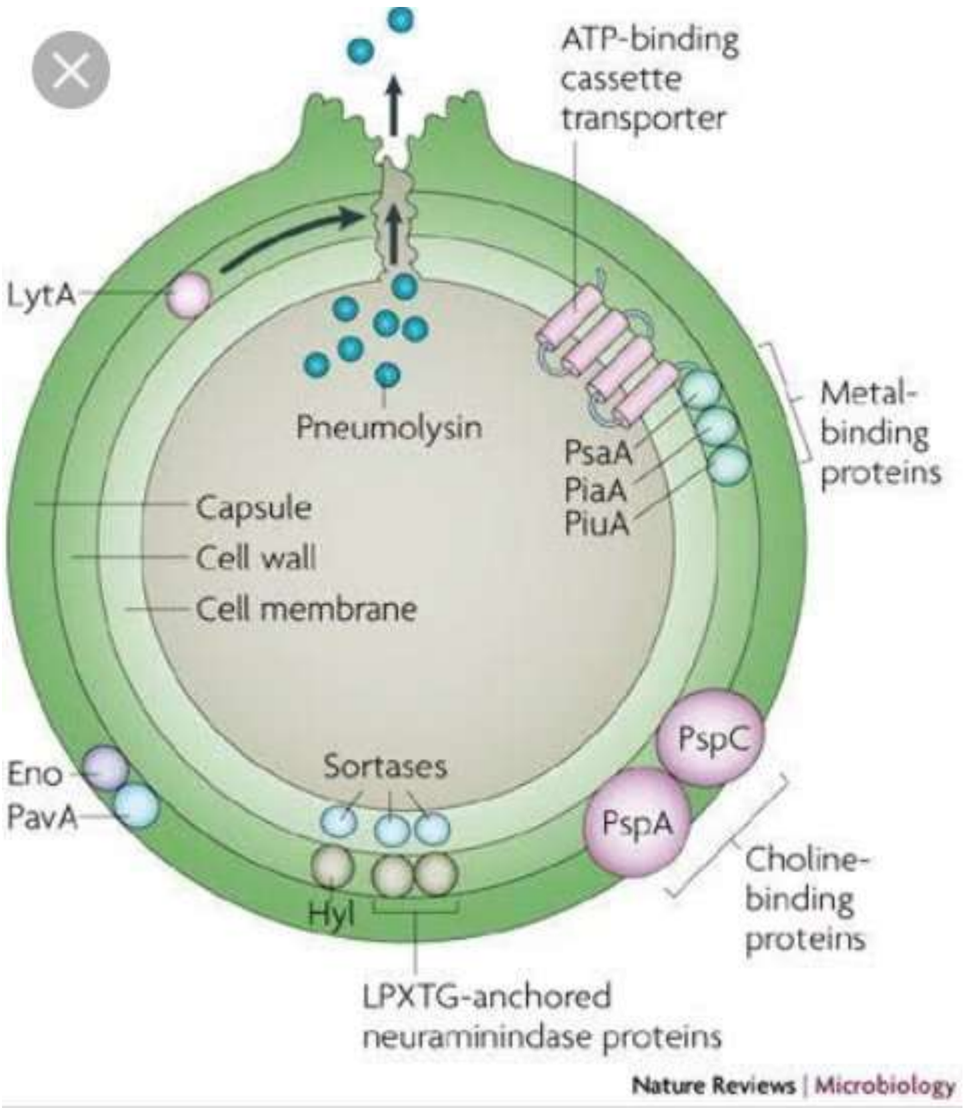


Fig.2.1.3 Pneumococcal virulence factors adopted from Nature Reviews (2008)

2.2 Pneumococcal Pathophysiology

2.2.1 Pneumococcal surface structures

The surface of this bacteria is covered by a polysaccharide capsule overlaying the cell wall comprising of peptidoglycan and teichoic acid. The peptidoglycan has a classical Gram-positive structure of N-acetyl glucosamine, N-acetyl muramic acid, and a Lysine-containing stem peptide; the teichoic acid usually contains a ribitol phosphate backbone covalently attached phosphoryl choline (Pcho) (Birgitta and Elaine, 2013). The impact of the variation aids the bacteria in evading host defenses, such as C-reactive protein(CRP),which target Pcho and leukocytes in blood while promoting attachment to host cells in the nasopharynx and expresses less capsule and less of choline binding protein; the Pneumococcal surface binding protein A(PsPA),but more of the choline binding proteins A(CbPA) and autolysin(LytA),while opaque phase predominates in the blood and characterized by increased capsular polysaccharide and PsPA and less CbPA (Trappetti *et al.*,2011)

2.2.2 Pneumococcal capsule and serotypes

Capsular polysaccharides (CPS) are surface exposed and immunogenic and provide a target for the host immune response. Consequently, there has been selection for mechanisms by which encapsulated bacteria evade the host immune system, and in most encapsulated species, this has been achieved by the generation of antigenic diversity, such that strains of the pathogen may express one of a number of different CPS (Spratt *et al.*, 2004).

The polysaccharide capsules are anti-phagocytic and spatially hinder the access of leukocytes to complements fixed on the under laying cell wall. The capsule locus, transcribed as a single operon, showing similar organization in all strains with genes encoding specific capsule type flanked by genes common to all types (Reshmi, 2012).

Hathaway*et al.* (2012) in a study reported that polysaccharide capsule of Streptococcus pneumoniae defines over ninety serotypes, which differ in their carriage prevalence and invasiveness for poorly understood reasons. They also showed there is a link between serotype and growth in-vitro. In the same study Hathaway and her colleagues suggested that expression of CPsA, the first capsule gene correlated inversely with capsule thickness. *Streptococcus pneumoniae* (the pneumococcus) produces 1 of 91 capsular polysaccharides (CPS) that define the serotype (Mavroidi*et al.*, 2015)

An association has been established between the type of polysaccharide capsule surrounding the bacteria and harmless colonization versus invasive disease and capsules that are costly for the bacteria to make are produced in lower quantities; their production limiting the growth of the bacteria in nutrient-restricted conditions (Hathway *et al.*, 2012). Ninety-three capsular serotypes have been identified in *Streptococcus pneumoniae*, each differing in chemical structure and immunogenicity (Calix and Nahm, 2010). Ifedayo *et al.* (2012) identified 42 serotypes of pneumococcus in a study carried out in a Nigerian population. All wild strains of *Streptococcus pneumoniae* are provided with a polysaccharide capsule.

Some serotypes are antigenically related to each other and such related types are included together in groups, for example ,9A,9L,9N,and 9V,while types without a close antigenic relationship to other types are given numbers only, for example 1,2,3,4,and 5.The capsular polysaccharides are composed of repeating units of oligosaccharides and for most of them the exact chemical structure is known(Mats,2017).

Serotyping of pneumococci in different disease conditions in Nigerian adults and children yielded serotypes, 1, 2, 3 and 5(Onyemelukwe and Greenwood, 1982).

Johnson *et al.* (2010) asserted that only a few serotypes tend to be associated with invasive diseases. In west Africa serotypes 1 and 5 are major causes of invasive pneumococcal disease, accounting for more than 30% of all cases in the region (Adegbola *et al.*, 2006).About eight thousand pneumococcal sequence types have been described in the *Streptococcus pneumoniae*database (MLST web) covering a wide range, from different geographical locations, while little data on African isolates have been documented, in contrast to the developed world.

The formulation of one conjugate vaccine to cover serotypes in the US and Europe and another to protect against serotypes causing infections in the developing world was proposed on the assumption that the same serotypes account for most of the infections in these two areas of the world (Jaime *et al*., 2005). The pneumococcal polysaccharide capsule enables the organism to evade host defenses on the mucosal surface during carriage and systemic defenses in invasive disease (Hyams *et al.*, 2010; Morona *et al.*, 2006). Nicholas *et al.* (2013), in a study of penicillin-resistance and serotype distribution of *Streptococcus pneumoniae* in Ghanaian children less than 6 years of age got an overall prevalence of 34% in Accra and 31% in Tamale. The predominant serotypes were 19F, 6B, 23F and 6A with 23% non-typable serotypes in Accra and 12% in Tamale. Two isolates (serotypes 19F and 6B) from Tamale had an MIC>2µg/ml and were classified as penicillin-resistant with 45% of the isolates having intermediate resistance.

DNA-based methods targeting the capsular polysaccharide synthesis (*cps*) locus are being developed (Marcus *et al*., 2012).It has been documented that the first four genes at the 5' end of the *cps* locus are involved in the regulation of capsule production and are conserved in all serotypes (Bentley *et al*., 2006).The middle region of the operon contains serotype-specific genes that have been used as targets of PCR-based serotyping methods (Pai *et al.*, 2006. Marcus *et al*., (2012) described a cheap, efficient, and less complex method of serotyping with high serotype coverage identifying pneumococcal serotypes in one single amplification reaction. Donkor *et al*.,2013 reported seventy-three invasive and carriage pneumococcal isolates from West Africa covered 7 serotypes (1, 3, 5 , 6A, 11, 14, and 23F).A retrospective study carried out by Tarrago *et al*.(2008) in Spain identified 175 pneumococcal isolates representing 35 serotypes(1,2 ,3, 4 ,5 , 6A, 6B, 7F, 7A, 8, 9V, 9N, 9A, 10A, 10F. 11A, 12F, 13, 14, 15B, 15C, 16F, 17F, 18C, 19A, 19F, 20, 22F, 23F, 23A, 23B, 24A, 33F,34, and 37) The pneumococcal population in West Africa is quite divergent, and serotypes that are common in invasive disease (such as serotypes 1 and 5) are more likely to be clonal than serotypes that are common in carriage(Dankor *et al*.,2013).

2.2.3 Pneumococcal pili

The pili in *Streptococcus pneumoniae* are multimeric filamentous surface structures composed of subunit proteins with LPxTG motifs recognized by Sortases and attached to the cell wall. Adhesion involves two pathogenicity islets encoding pili, PI-1 and PI-2. PI-1 encoded by *rlrA* accessory region, influence colonization, virulence, and inflammatory response in mouse challenge models. Pili enable *S. pneumoniae* to attach to epithelial cells in the upper respiratory tract (Barocchi *et al*., 2006).The bacterial pili are considered to participate in bacterial adhesion to a host, a crucial step in bacterial infection (Hilleringmann *et al*.,2008).

2.2.4 Pneumococcal genome

Tettelin *et al*. (2001) reported that the complete annotated 2.16 MbPs genome of

S. pneumoniae encoding 2236 predicted proteins was first published in 2001.In their study, sequence analysis indicated nutritionally fastidious, lactic acid fermenting, facultative anaerobe with a G +C content of 40%.It also showed that with its ability to naturally take up DNA and undergo transformation the genome is exceptionally highly variable with up to 10% of genes showing altered sequences between isolates and 3-5% of the genome showing insertion sequences and truncations. The number of DNA repeat regions is at the top of all available genomes and likely contributing to plasticity .The genomic content of invasive versus noninvasive strains or clonotypes has suggested that swapping of cassettes of “regions of diversity” changes infection dynamics leading to organ specific virulence (Orihuela *et al*., 2004).Romina *et al*.(2011) sequenced the genome of a serotype 11A ,ST62 *S.*

*pneumoniae*invasive isolate(AP200),that was erythromycin-resistant due to presence of erm(TR) determinant, and carried out analysis of the genome organization and comparison with other pneumococcal genome sequence of *S. pneumoniae* AP200 was 2,130,580 bps in length carrying 2216 coding sequences(CDS),56 tRNA, and 12 rRNA genes.

The genome of *S. pneumoniae* D39, the virulent strain used in the revolutionary experiments of Griffiths and McCarthy, consists of a single circular chromosome with about 2 million bps. Its GC content is 39.7%; and it codes for 1914 proteins and 73 RNAs (Lanie *et al*., 2007).

2.3 Pneumonia

Pneumonia is a bacterial or viral infection of the lungs. It is an infection of the air sacs in the lungs. Symptoms can include fever, chills, shortness of breath, coughing that produce phlegm, and chest pain. Those at greater risk are: Babies and toddlers, People who have had a recent viral infection, Smokers,People with suppressed immune systems, People who drink excessive alcohol, Patients in hospitals and People aged 65 years or older (Southern Cross, 2016).CAP is defined as pneumonia acquired outside a hospital or long-term care facility. It occurs within 48 hours of hospital admission or in a patient presenting with pneumonia who does not have any of the characteristics of healthcare-associated pneumonia (i.e., hospitalized in an acute care hospital for 2 or more days within 90 days of infection; resided in a nursing home or long-term care facility; received recent intravenous antibiotic therapy, chemotherapy, or wound care within the past 30 days of the current infection; or attend a hospital or hemodialysis clinic(Fariba and Thomas,2013).Community-acquired pneumonia (CAP) can also be defined as an acute infection of the pulmonary parenchyma in a patient who has acquired the infection in the community, as distinguished from hospital-acquired (nosocomial) pneumonia (HAP). A third category of pneumonia, designated healthcare-associated pneumonia (HCAP), is acquired in other healthcare facilities, such as nursing homes, dialysis centers, and outpatient clinics. The rationale for the separate designation of HCAP was that patients with HCAP were at higher risk for multidrug-resistant (MDR) organisms. However, several studies have shown that many patients defined as having HCAP are not at high risk for MDR pathogens (Chalmers *et al*., 2014; Gross *et al*., 2014; Yap *et al*., 2013).Although interaction with the healthcare system is potentially a risk for MDR pathogens, underlying patient characteristics are also important independent determinants of risk for MDR pathogens (File, 2016).CAP is a common and potentially serious illness. It is associated with considerable morbidity and mortality, particularly in older adult patients and those with significant comorbidities (Prina *et al*., 2015).Community-acquired pneumonia (CAP) can be caused by a variety of pathogens. The predominant bacterial pathogen is *Streptococcus pneumoniae*. Other common pathogens include *Haemophilus influenzae*, the atypical bacteria (*Mycoplasma pneumoniae, Chlamydia pneumoniae*, and *Legionella* spp), oropharyngeal aerobes and anaerobes (in the setting of aspiration), and respiratory viruses. A randomized trial and a retrospective study have suggested that *Staphylococcus aureus* is increasing as a cause of CAP requiring admission to the hospital [some are due to community-associated methicillin-resistant *S. aureus* (MRSA)strains] (File *et al*.,2010; Restrepo *et al*.,2008). Gram-negative bacilli (Enterobacteriaceae and *Pseudomonas aeruginosa*) are the cause of CAP in some patients. The frequency of other causes, such as *Mycobacterium tuberculosis*, *Chlamydiapsittaci* (cause of psittacosis), *C. burnetii* (cause of Q fever), and endemic fungi vary in different epidemiologic settings (File, 2016).Studies utilizing molecular diagnostic methods have reported the rate of detection of a viral etiology in patients with CAP at approximately 30 percent; influenza has been identified in many of these patients (Gadsby *et al*., 2016). The rate of mixed viral-bacterial infection is approximately 20 percent; such mixed infections have been found to be associated with more severe CAP and longer hospitalization than CAP caused by bacteria alone (Johansson,2011).

2.3.1 Pathogenesis

A number of pathogens can give rise to CAP, generally categorized into typical and atypical pathogens. Typical bacterial pathogens that cause CAP include *Streptococcus pneumoniae* (penicillin-sensitive/resistant strains), *Haemophilus influenza* (ampicillin-sensitive/resistant strains), and *Moraxella catarrhalis*(all strains penicillin-resistant) for approximately 85% of CAP cases. CAP is usually acquired via inhalation or aspiration of a pulmonary pathogen into a lung segment or lobe. Less commonly, CAP results from secondary bacteremia from a distant source, such as *Escherichia coli* urinary tract infection and/or bacteremia. Aspiration pneumonia is the only form of CAP caused by multiple pathogens (e.g., aerobic/anaerobic oral organisms). *Klebsiella pneumoniae* CAP occurs primarily in persons with chronic alcoholism and *Staphylococcus aureus* may cause CAP in patients with influenza. *Pseudomonas aeruginosa* is a cause of CAP in patients with bronchiectasis or cystic fibrosis (Fariba and Thomas, 2013). Atypical pathogen CAP manifests a variety of pulmonary and extra pulmonary findings (e.g., CAP plus diarrhea). Atypical CAP can be divided into those caused by either zoonotic or non-zoonotic atypical pathogens. Zoonotic atypical CAP pathogens include *Chlamydophila* (*Chlamydia*), *psittaci* (psittacosis), *Coxiella burnetii* (Q fever), and *Francisella tularensis* (tularemia). Non-zoonotic atypical CAP pathogens include *Mycoplasma pneumoniae*, *Legionella* species, and *Chlamydia pneumoniae*. These organisms account for approximately 15% of all CAP cases (Fariba and Thomas, 2013). Firm adherence of bacteria to the alveolar epithelium and subsequent replication and initiation of host damage responses lead to the classic evolution of lobar pneumonia. The interaction of secreted products and cell surface-anchored bacterial components with the alveolar epithelium and innate immune defenses drives this process. The pore-forming toxin pneumolysin and hydrogen peroxide released in copious amounts by the bacteria disrupt the alveolar epithelium and edema fluid accumulates in the alveolar space (Canvin *et al.*, 1995). Lipoteichoic acids, lipoproteins, and an array of proteins associated with the bacterial surface further amplify interactions with the host. During this early host response, bacteria gain firm adherence to the alveolar epithelium using several protein adhesins, including the histidine triad family of lipoproteins, LPxTG proteins, choline-binding proteins, and pili (Löfling *et al*., 2011). Proteins anchored by the LPxTG motif that bind the extracellular matrix include PavB, PfbA, PclA, and PsrP, an enormous serine rich protein (Jensch *et al*., 2010). Pili, encoded by PI-1 or PI-2, bind bacteria to respiratory epithelial cells (Barocchi *et al*., 2006; Bagnoli *et al*., 2008). The Cbp family of proteins non-covalently attached to the phosphoryl choline determinant on the pneumococcal teichoic acid has a broad range of functions from proteases to cell wall-modifying enzymes to complement interactors to adhesins (Rosenow *et al*., 1997). CbpA in particular is important in binding pneumococci to host cells and PspA interacts with many innate host defense components. Pneumococci have been identified as a pathogen that is both aggressive and normal in human respiratory microbiome (Blaschke, 2016). *Streptococcus pneumoniae* is a crucial pathogen that causes community-acquired pneumonia (CAP). CAP in adults is often treated with a combination of β-lactam antibiotics and macrolides (Mandell *et al*., 2003).

In virtually every aspect of the infectious process, the pneumococcus has set the rules of the Gram-positive pathogenesis game. Pneumococcal diseases range from mild respiratory tract mucosal infections such as otitis media and sinusitis to more severe diseases such as pneumonia, septicemia, and meningitis. The cell wall of pneumococci is a major determinant of the course of disease. The interaction of PCho on the cell wall teichoic acid binds to PAFr and enables bacterial invasion of cells and transmigration across barriers. It also enables the cell wall itself to traffic to all organs. In the tissues, cell wall subcomponents generate intense inflammation by interacting with TLR2 and Nod2. Signaling through these receptors activates NF-κB pathways, cytokine, and chemokine production, leading to intense neutrophil recruitment (Birgitta and Elaine, 2013). Streptococcus pneumoniae secretes a potent cytotoxin, pneumolysin, which is a member of the cholesterol-dependent, pore-forming cytotoxin family. It plays an important role in the respiratory tract, not only by lysing host cells but also by inhibiting the mucociliary beat of respiratory cells and separating epithelial cell tight junctions. It similarly contributes to the pathogenesis of otitis media by cytotoxic effects on the hair cells of the cochlea in the middle ear. Pneumolysin activates the classical complement pathway and may be recognized by TLR4, thereby evoking an inflammatory response (Marlley *et al*., 2003).

2.4 Pneumococcal antibiotic resistance

The beginning of the antibiotic era to the mid-1970s, show pneumococcus remained uniformly susceptible to all classes of antibiotics that had been active against the organism (Iliyasu *et al*., 2015). In 1977 and 1978 outbreaks of penicillin-resistant pneumococci (PRP) were reported in Durban and Johannesburg, South Africa. (Appelbaum *et al*., 1977; Jacobs *et al*., 1978).The PRP appeared to have acquired genetic material that encoded resistance to both penicillin and other commonly used antibiotics. The major mechanism of resistance involves the introduction of mutations in genes encoding penicillin-binding proteins and selective pressure is thought to play an important role and high-level penicillin resistance, macrolide resistance and multidrug-resistance often complicate the management of pneumococcal infection and make choosing empiric antimicrobial therapy for suspected cases of meningitis and pneumonia increasingly difficult. An alarming increase in infections due to penicillin non-susceptible pneumococci (PNSP) has been documented in nearly all countries in the world (Witney *et al*., 2000; Dorn *et al*., 2001). Benbachir *et al.* (2001) reported 30.4% of pneumococci as non-susceptible to penicillin in four African cities (Abidjan, Casablanca, Dakar and Tunisia). In Kano, 93%, 92%, 84%, and 21% of pneumococci were non-susceptible to penicillin, co-trimoxazole, tetracycline and ampicillin, respectively.(Habib *et al*.,2003). PNSP rates of 18% were reported in Sokoto, 29.72% in Jos, and 11.76% in Lagos. (Emele, 2000; Kandakai-Olukemi and Diso, 2009; Oduyebo et *al*.,2006). The rapid emergence of antibiotic resistance is adding to the burden of pneumococcal disease by significantly contributing to the numbers of treatment failures and deaths from this disease (Garba *et al*., 2015). Penicillin-non susceptible isolates are also frequently resistant to other antibiotic classes (Witney *et al*., 2003). This situation according to the investigators justifies a continuous surveillance to provide appropriate recommendations for the treatment of pneumococcal infections. There is a lack of data on antibiotic resistance from West and North Africa (Benbachir *et al.*, 2001). *Streptococcus pneumoniae* has shown a significant increase in antibiotic resistance over the past 20 years;suggesting that this was likely due to its use of a natural transformation system used for genetic exchange, developing resistance to antibiotics through mutation and natural selection. Pneumococcus grows relatively at fast rate reaching great cell densities in infectious environments; a condition which favors natural transformation and natural selection towards antibiotic resistance (Todar, 2003).Antibiotic resistant bacteria that are difficult or impossible to treat are becoming increasingly common and are causing a global health crisis. Antibiotic resistance is encoded by several genes, mainly of which can transfer between bacteria. The mechanism of antibiotic resistance include the prevention of access to drug targets,changes in the structure and protection of antibiotic targets and the direct modification or inactivation of antibiotics(Blair *et al*.,2015). In a study by Benbachir *et al*. (2001) the resistances to eight antibiotics of 375 isolates was carried out by *E*- test. Overall, 30.4% of the isolates were non-susceptible to penicillin G, 25.6% were intermediate and 4.8% were resistant. 96.3% Amoxicillin were susceptible and parenteral third-generation cephalosporin was 92.7% highly active. Resistance to chloramphenicol was detected in 8.6% of the isolates. High levels of resistance were noted for erythromycin 28%, tetracycline 38.3%, and cotrimoxazole 36.4%. Resistance to rifampin was 2.1%. (Benbachir *et al*.,2001). Susceptibility studies by Iroha *et al*. (2012) showed that the highest resistance was 182(59.6%) for penicillin, septrin 156(51.2%), erythromycin 120(39.3%),clavulanic/amoxicillin 118(38.7%), cefotaxime 114(37.4%), clarithromycin 100(32.7%), ciprofloxacin 94(30.8%), gentamycin 75(24.6%), cefuroxime 70(22.9%) and ceftriaxone 69(22.6%). A predictive model was created by JÖrg *et al*., (2016) using 3 baseline variables that were independently associated with high-level penicillin resistance: previous β-lactam antibiotic use, previous stay in a risk area (defined as stay in day care facilities, prisons, homeless shelters, nursing homes, or other long-term care facilities), and previous respiratory tract infection. The model was used to identify patients at low and high risk for high-level penicillin-resistant pneumococcal bacteremia. None of the isolates of patients in the low-risk subgroup had ceftriaxone resistance. Patients in the low-risk subgroup could be empirically treated with fluoroquinolone-sparing regimens (JÖrg *et al*., 2016).

2.5 Macrolide Resistance Genes (MRGs)

2.3.1 Erythromycin ribosomal methylase genes (e*rmB*) and Macrolide efflux genes (*mefA*)

In a study carried out by Fukushima *et al*. (2008) Penicillin and macrolide-resistant genes were detected by Real-time quantitative multiplex Polymerase chain reaction (RQ-mPCR) in purulent sputum samples, all were positive for *lytA* gene, and for 51 samples the detected genes were in complete agreement with the isolated *S.pneumoniae*. For the remaining five samples, the genes were not in complete agreement with the isolated *S. pneumoniae*: two samples were false positives for *erm* (*B*); one sample was false positive for *mef(A*); one sample was false positive for *pbp2x* and *pbp2b*; and one sample was false positive for *mef(A)* and false negative for *pbp1a*. The sensitivities and specificities of this assay for detecting genes directly from sputum samples relative to isolated *S. pneumoniae* were 100 and 93.9% for *erm (B)*,100 and 94.8% for *mef(A*).Exposure of cells to low concentrations of erythromycin stimulated its transposition to conjugative coresident plasmids, making it transferable to plasmid-free recipients of the same species (Cochetti,*2008*). Tn*917* was sequenced in the mid-1980s (Stadler and Teuber,*2002)*; its subsequently updated sequence (5,614 bp) revealed five open reading frames (ORFs), of which *orf2* is *erm*(*B)* and *orf4* and *orf5* are two specific transposition-related genes, *tnpR* (resolvase) and *tnpA* (transposase), respectively. After Tn*917*-like sequences had been demonstrated in erythromycin-resistant *S. pneumoniae* isolates.McGee *et al.,*(2001)described in the same species a new composite structure, designated Tn*3872* (23.6 kb), resulting from the insertion of Tn*917* into *orf 9* of Tn*916*, at base 14525 of its published sequence, with the *erm(B)* gene of Tn*917* thus linked to the *tet*(M) gene of Tn*916*(Cochetti,*2008*). Insertion of the *erm(B)* element alone distinguishes transposon Tn*6002* (20,880 bp), detected in both *S. pyogenes* (Brenciani *et al*.,2004) and *S. pneumoniae* (Del Grosso *et al,.2002)* but originally found in *Streptococcus cristatus* and designated Tn*916*Erm, whose complete sequence (later updated) was available in GenBank since 2005. Intraspecific conjugal transfer of Tn*6002* has been demonstrated in *S. pyogenes* (Brenciani *et al*., 2004), whereas transconjugants were obtained from a *S. pneumoniae* donor only using a *S. pyogenes* recipient (Cochetti *et al*., 2005). As shown by Cochetti *et al*.,(2005),another pneumococcal transposon, Tn6003(25,101bp), is distinguishe d by the further insertion into Tn*6002* of the so called macrolide amino lycoside streptothricin (MAS) element(4,225bp). The MAS element is found within the *erm(B)* element between *orfP0* and *orfP1*, at position 4110 of the Tn*6002* sequence(Cochetti *et al*.,2005). The MAS element appears to be a rearrangement of part of plasmid pRE25 (Schwarz *et al*., 2004) of *E. faecalis* and contains, from upstream to downstream, another *erm(B)* gene (lacking the stop codon) with its leader peptide. Conjugal transfer of Tn*6003* failed to *S. pneumoniae* and *S. pyogenes* recipients, whereas it was obtained to an enterococcal recipient (Cochetti *et al.*, 2005).Since early studies, Tn*1545* was shown to be transferable by conjugation to various gram-positive bacterial genera. In the years after its discovery, the genetic organization of Tn*1545* has essentially been analyzed by restriction mapping; only specific portions have been sequenced, including its ends (Calatayud *et al,.2007)*, the three above-mentioned resistance genes, and transposition-related genes such as *int* (integrase) and *xis* (excisase) (*Puopolo et al., 2007*), which are characteristically associated with the conjugative transposons of the Tn*916* family (Ripa *et al*., 2001). Several studies reported a predominance of *mef(A)* gene in United-States and Canada. In European countries, *erm(B)* determinant is prevalent and *mef(A)*-mediated erythromycin resistance was recently reported in about 10% of strains in Belgium and Italy. Results of these investigations show the predominance of the *erm(B)* gene in erythromycin resistant *S. pneumoniae* isolates. *mef(A)*-mediated resistance is effective in Southern France (3.7%) but this rate is the lowest published from European countries.(Marchandian *et al*., 2001).

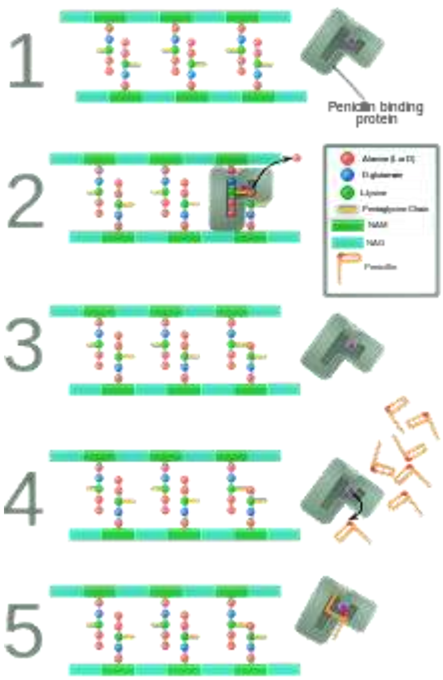
Streptococcus pneumoniae isolates were collected from patients with community-acquired respiratory tract infections during PROTEKT US surveillance study (2000–2003). While the macrolide (erythromycin) resistance rate remained stable at ≈29%, the prevalence of resistant isolates containing both *erm(B)* and *mef(A)* increased from 9.7% in year 1 to 16.4% in year 3, with substantial regional variability. Almost all (99.2%) dual *erm(B)*+*mef(A)* macrolide-resistant isolates exhibited multidrug resistance,(David *et al*.,2005).

The first *mef(A)* carrying structure to be discovered was Tn*1207.1*(7,244bp), a chromosomal element described in *S. pneumoniae* by Santagati *et al.* (Del Grosso,*.2002*) and considered as a defective transposon because the 5′ end of *orf8* appears truncated. It contains eight ORFs, of which *mef(A)* and *msr*(*D)* are the fourth and the fifth; while *orf2* is homologous to site-specific recombinases of gram-positive bacteria, *orf6, orf7*, and *orf8* are homologous to three ORFs of the pneumococcal conjugative transposon Tn*5252*. Tn*1207.1* is integrated at specific site of the pneumococcal chromosome into *celB*, a late competence gene, notably, Tn*1207.1*-carrying pneumococci are unable to become competent(Del Grosso *et al*,**.**2007). Tn*1207.1* has been reported to be transferrable by transformation but not by conjugation **(**Schwarz *et al.*, 2001.), even though variable results have been obtained in mating experiments with different pneumococcal donors (Amezaga *et al*., 2002). Tn*1207.1* is the sole recognized element carrying *mef*(A) in *S. pneumoniae*, in *S. pyogenes* (Mann *et al*.,2012).

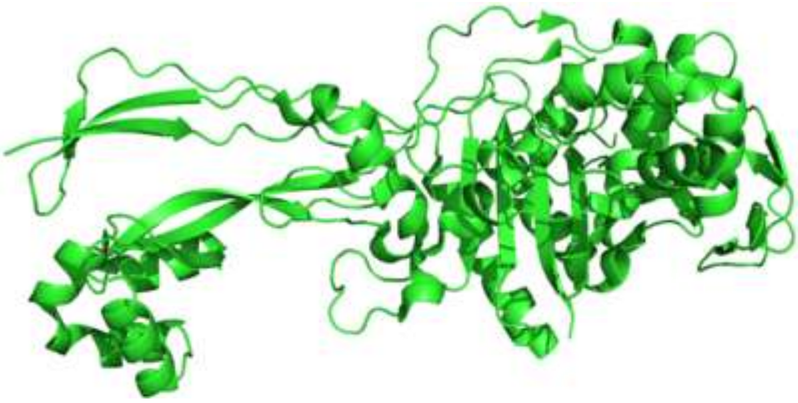
2.6 Penicillin Resistance Genes (PRGs)

2.6.1 Penicillin binding proteins (PbPs)

A number of ways and means have evolved to provide resistance to eubacteria challenged by β-lactams. Some pathogens resist by expressing low-affinity targets for these antibiotics, the penicillin-binding proteins (PBPs). Even within this narrow focus, a great variety of strategies have been uncovered such as the acquisition of an additional low-affinity PBP, the overexpression of an endogenous low-affinity PBP, the alteration of endogenous PBPs by point mutations or homologous recombination or a combination of the above (Jacques,2008).



Penicillin binding proteins catalyze the cross-linking of bacterial cell wall



Ribbon representation

Fig.2.2: Penicillin binding Proteins; Adopted from wiki.png-photos

thr	**gln**	**ala**	**tyr**	**gly**	**ser**	**phe**
ACT	**CAG**	**GCT**	**TAC**	**GGT**	**TCA**	**TTC**
CCT	**GCC**	**TTT**	**TCT**	**AGA**	**CCA**	**ATG**
pro	**ala**	**phe**	**ser**	**arg**	**pro**	**met**

Extensive nucleotide and amino acid residue changes for same coding region of PBP in susceptible strains of *S.pneumoniae*

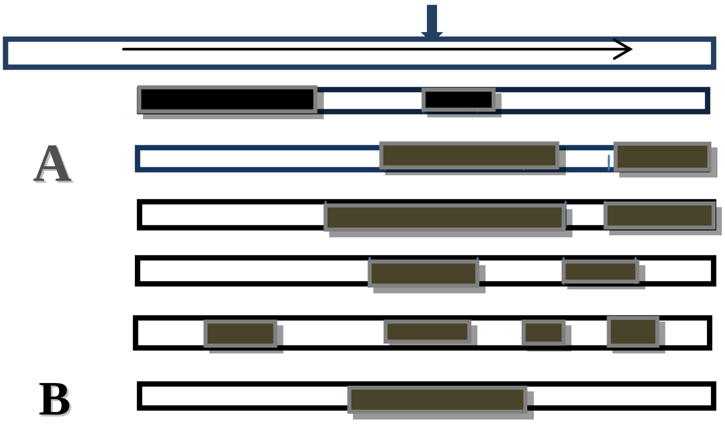


Fig.2.3: Comparison of mosaic PBP2b genes from 2 types (A and B) of PRP to wild type PBP2b sequence top.
Key: Vertical arrow indicates location of SXXK motifHorizontal arrow indicates Transpe ptidase domain proteinOpen regions indicate wildtype sequence Shaded boxes indicate regions containing sequences altered from wild type but derived from related donor

Penicillin binding Proteins are membrane-bound D, D-peptidases that have evolved from serine proteases, to which their biochemical activity is mechanistically similar.(Ghuysen,1994) These enzymes catalyze the transpeptidation reaction that cross-links the peptidoglycan of the bacterial cell wall. *B-lactam* antibiotics, which are substrate analogues, covalently bind to the PBP active site serine and inactivate PBPs at concentrations that are about the same as the MICs. The high-molecular-weight PBPs in *Streptococcus pneumoniae* are PBP 1 (which can be resolved into PBPs la and 1b, which appear functionally interchangeable) and PBP 2 (actually three proteins, 2a, 2b, and 2x). These are essential and have high penicillin-binding affinity. PBP 3 is the low-molecular-weight PBP in pneumococci (Henry, 1999). The expression of β-lactamases or additional low-affinity PBPs has never been reported in pneumococcus. Instead, β-lactam-resistant strains of *Streptococcus pneumoniae* always harbor modified versions of their own class A and class B PBPs that are poorly acylated by β-lactams (Hakenbeck *et al.*, 1980). Five PBPs from *Streptococcus pneumoniae*, PBP1a, 1b, PBP2b, PBP2x and sometimes PBP2a were found to be altered in resistant clinical isolates. Mosaicity is the product of recombination events between different alleles within a species or between orthologous genes of related species. Mosaic sequences of *pbp* genes are very difficult to classify and organize. Comparison of nucleotide sequences originating from susceptible strains show that they exhibit the same level of polymorphism as other loci, with one or two amino acid substitutions over the protein length (Laible *et al.*, 1991) as shown in fig.2.3. In contrast, mosaic *pbp* genes show blocks of sequences that differ from non-mosaic alleles by about 14–23%. The diverging blocks span various lengths of the transpeptidase domain or even most of the extracellular domain. The degree of difference suggested that the diverging sequence blocks originate from other streptococcal species, and that multiple sources had been tapped by pneumococcal strains to survive antibiotic selection. *S. pneumoniae* develops penicillin resistance through recombination with other penicillin-resistant *S. pneumoniae* (PRSP) strains or closely related streptococcal species that alter its penicillin binding protein (pbp) genes *pbp1a*, *pbp2b*, and *pbp2x* . These altered pbp gene sequences of PNSP differ greatly within the PNSP subset and from the sequences of penicillin-susceptible *S. pneumoniae* (PSSP) (Ronald *et al*., 2010). In contrast, mosaic *pbp* genes show blocks of sequences that differ from non-mosaic alleles by about 14–23%. The diverging blocks span various lengths of the transpeptidase domain or even most of the extracellular domain. The degree of difference suggested that the diverging sequence blocks originate from other streptococcal species, and that multiple sources had been tapped by pneumococcal strains to survive antibiotic selection. *S. pneumoniae* develops penicillin resistance through recombination with other penicillin-resistant *S. pneumoniae* (PRSP) strains or closely related streptococcal species that alter its penicillin binding protein (pbp) genes *pbp1a*, *pbp2b*, and *pbp2x*. The altered pbp gene sequences of PNSP differ greatly within the subset and from the sequences of penicillin-susceptible *S. pneumoniae* (PSSP) (Ronald *et al.*, 2010).

2.6 Penicillin resistant Pneumococci (PRP)

Penicillin-resistant *S. pneumoniae* has emerged as a worldwide problem in the past decade. Penicillin-non susceptible *Streptococcus pneumoniae* (PNSP) remains a major global health threat because *S. pneumoniae* is the predominant bacterial pathogen of community-acquired pneumonia and a major cause of meningitis and of otitis media in children. In the United States, penicillin resistance in *S. pneumoniae* has been observed in up to 34% of all isolates. This is very similar to the prevalence of penicillin resistance in a study by Doern *et al.*(2001) who reported 33%. One-third (33 of 98) of these resistant isolates had high-level penicillin-non susceptibility. Multiple logistic regression analysis showed that previous β-lactam use, previous stay in a risk area, and previous respiratory infection were independently associated with PNSP bacteremia (*P*< .01) (Vernet *et al.*, 2008).Penicillin-resistant *S. pneumoniae* has emerged as a worldwide problem in the past decade. Penicillin-non susceptible *Streptococcus pneumoniae* (PNSP) remains a major global health threat because *S. pneumoniae* is the predominant bacterial pathogen of community-acquired pneumonia and a major cause of meningitis and of otitis media in children. In the United States, penicillin resistance in *S. pneumoniae* has been observed in up to 34% of all isolates. This is very similar to the prevalence of penicillin resistance in a study by Doern *et al.*(2001) who reported 33%. One-third (33 of 98) of these resistant isolates had high-level penicillin-non susceptibility. Multiple logistic regression analysis showed that previous β-lactam use, previous stay in a risk area, and previous respiratory infection were independently associated with PNSP bacteremia (*P*< .01) (Vernet *et al.*, 2008). Pneumococci are normally exquisitely sensitive to penicillin, which is lethal at concentrations <0.1 μg/mL. Typical penicillin MICs for highly resistant strains is 4–8 μg/mL. The threshold MIC at which treatment failures is likely to occur is unclear because of a paucity of clinical data. Serum concentrations averaging 10–15 μg/mL are readily achievable with high-dose intravenous penicillin regimens, which should provide a comfortable margin above the MIC for all but the most resistant strains. However, at lower doses or with less active β-lactam, the margins would be less, increasing the likelihood of treatment failure. Treatment failure can and does occur with meningitis caused by a penicillin-resistant strain, because achievable concentrations of penicillin and other β-lactam antibiotics in cerebrospinal fluid average only a fraction, 5%–15%, of those in serum. On the basis of animal models, anecdotal cases, and pharmacokinetic considerations, MICs >0.25 μg/mL are likely to be associated with penicillin treatment failure. Since ~25% of *S. pneumoniae* isolates in the United States exhibit intermediate (0.1 μg/mL $\geqslant$ MIC $\geqslant$ 1 μg/mL) or high-level (MIC > 1 μg/mL) penicillin resistance (Doern *et al*., 2001). Pneumococcal isolates were screened for penicillin resistance by use of the oxacillin disk test and classified as penicillin susceptible (MIC, $\leqslant$0.06 µg/mL), intermediate-level penicillin non susceptible (MIC, 0.12–1 µg/mL).A high-level penicillin non susceptible (MIC, $\geqslant$2 µg/mL) was recorded by use of the E-test methodology (AB Bio disk). All penicillin-susceptible and intermediate-level PNSP (MIC, $\leqslant$1 µg/mL) were classified as "penicillin-susceptible/intermediate-level and penicillin-non susceptible *S. pneumoniae*" (PSISP). The outcome of the study was high-level PNSP bacteremia (MIC, $\geqslant$2 µg/mL). Isolates were also tested for ceftriaxone susceptibility and classified on the basis of the new interpretive criteria for nonmeningeal isolates: drug MICs of $\leqslant$1, 2, and $\geqslant$4 µg/mL for susceptible, intermediate, and resistant isolates, respectively (NCCLS, 2002). Penicillin resistance in pneumococci is the result of the acquisition of series of stepwise mutations in PBP genes. Mutations that reduce the amount of drug binding are selected for by β-lactam antibiotics. In the laboratory, very low-level resistance to penicillin can result from a point mutation leading to amino acid substitution within the transpeptidase domain of the PBP. Mutations described in the PBP 2x gene of penicillin-resistant mutants of *S. pneumoniae* R6 are instructive. Mutations occurred within the penicillin-binding domain of the PBP 2x molecule, often in regions near one of the three penicillin-binding motifs, SXXK (which contains the active site serine), S(Y) XN, and K (H) T(S) G. One-point mutation resulted in a modest increase in MIC from 0.02 - 0.16 μ/mL. A second mutation doubled that MIC to 0.32. Additional mutations in PBP 2x were associated with higher levels of resistance, with MICs of 1.28 μg/mL. However, trans formants with a PBP 2x double, triple, or quadruple mutant all had MICs of 0.32 μg/mL. The more extensively PBPs are altered, both in structure and in number of distinct protein species, the higher the MIC. Penicillin MICs are <0.1 μg/mL in strains expressing wild type PBPs 1 and 2, which have high-affinity penicillin binding. Strains having intermediate resistance MICs show low-affinity binding to some, but not all PBPs. Such a strain, for example, might have PBP 2a and 2x showing a modest reduction in the normal binding without alterations of PBPs 1 or 2b. Strains with MICs >1 μg/mL typically have more marked reductions in binding affinity affecting all PBPs. The low-molecular-weight PBP 3 has been thought to play no role in resistance, because its affinity is unchanged in highly resistant strains. However, reduced expression of PBP 3 lowers resistance, suggesting that it may play a supportive function. The reason for the widely differing MICs among the various β-lactam antibiotics is that specific compounds will differ in the degree to which binding is affected by a structural change or changes in one or more PBPs. (Henry, 1999).

2.7 Prevalence of Erythromycin and Penicillin Resistant Pneumococci in Nigeria

The prevalence of the PRP and ERP strainshave continued to increase in many parts of the world which is much higher than the reported values in Kaduna State. High resistance rates of 56.6% and 66.7% to erythromycin were reported by some workers in Nigeria (Akanbi *et al.*, 2012; Iwalokun *et al.*, 2012). Contrary to findings by Onipede *et al.*, (2009) who reported 100% of invasive pneumococcal isolates from children to be sensitive to erythromycin which is also similar to findings by Adeleye *et al.*, (2008) among sputum isolates from HIV-infected pediatrics and adult patients. It has been reported that pneumococci resistant to penicillin are more likely to become resistant to other antibiotic than penicillin-susceptible strains (Doern *et al*., 1996). This was shown in a study where the resistance percentages among the cefuroxime, erythromycin, chloramphenicol, amoxicillin–clavulanic acid and azithromycin were consistently higher among penicillin-resistant strains than among penicillin-susceptible isolates (Iliyasu *et al*., 2015). Kandakai-Olukemi and Diso,(2009) also reported similar findings. The sensitivities and specificities of the molecular method for detecting genes from sputum samples relative to isolated *streptococcus pneumoniae* were 100 and 93.9% for *erm(B)* ,100 and 94.8% for *mef(A),*94.4 and 97.5% for *PbP1a,*100 and 94.1% for *PbP2x,* and 95.6% for *PbP2b.*Multidrug-resistant clones of *S. pneumoniae* were detected by Farell *et al.*(2008) expressing both *erm(B)* and *mef(A)* with the strains increasing in prevalence from 9.7% to 16.4% of macrolide-resistant isolates in 2000-2003.The heavy burden of community-acquired infectious diseases in most developing countries dictates a heavy requirement for antibiotics, often empirically. Chloramphenicol and TPM/SMX are commonly used as first-line drugs in the integrated management of childhood illness (IMCI) scheme (Obaro *et al*., 2011). In addition to TPM/SMX, penicillin and erythromycin are also widely use in sickle cell anemia and HIV/AIDS patient to prevent opportunistic infections (Grange *et al*.,2003). These coupled with wide spread antibiotic self-medication,(Iwalokun *et al*.,2012; Arikpo *et al*.,2011) may be promoting rapid emergence of an tibiotic resistance in Nigeria. Iliyasu *et al*., (2015) isolated 132 Pneumococci out of which twenty-two (16.7%) of the isolates were fully sensitive to penicillin while 73 (55.3%) and 37 (28.0%) were intermediately and fully resistant, respectively. One hundred and twenty-seven (96.2%) of the isolates were fully resistant to trimethoprim–sulphamethoxazole. Eleven (8.5%) were fully resistant to amoxicillin and 104 (78.8%) and 17 (12.9%) were intermediately resistant and fully susceptible. One hundred and six (80.3%) of the isolates were fully susceptible to chloramphenicol, while resistance to penicillin was shown to infer resistance to other antibiotics.

2.8 Epidemiology of Pneumococcal infections

Pneumococcal infections are thought to spread from person to person via droplets/aerosols and nasopharyngeal colonization as a prerequisite for pneumococcal disease. The carriage rate peaks around 2–3 years of age and diminishes thereafter to <10% in the adult population. However, adults with small children at home may have a higher carriage rate. The bacteria enter the nasal cavity and attach to the nasopharyngeal epithelial cells and may then either stay as a colonizer or spread further to other organs, such as the ears, sinuses, or via bronchi down to the lungs and then potentially penetrate the mucosal barrier to enter the blood stream and/or cross the blood–brain barrier to cause meningitis (Birgitta and Elaine, 2013).Spread by aerosol, pneumococci are harbored in the nasopharynx. This interaction most commonly leads to clearance and serotype-specific immunity. Progression to otitis media is very common in children. Invasive disease involves spread to the lungs and bloodstream. The most serious development is meningitis. This series of invasive steps is a pattern shared by the three major bacterial pathogens of children (pneumococcus, *Haemophilus*, and meningococcus) and is driven by a very basic interplay between innate invasion and innate immunity (Birgitta and Elaine, 2013).

Nicholas *et al.*, (2013) posited that *Streptococcus pneumoniae* (Pneumococcus) is an important cause of morbidity and Mortality among children less than 5 years of age, patients with debilitating diseases, the elderly (≥65 years) and immuno-compromised individuals. The Pneumococcus is a classic example of a highly invasive, Gram-positive, extracellular bacterial pathogen and is an important public health concern throughout the World with global burden in causing disease and death comparable to that of HIV, Malaria and TB, with Nigeria currently accounting for about 5% of the global burden as reported by Iwalokun *et al*., (2012) and Iliyasu *et al*., (2014).

An in-vitro antimicrobial resistance profile of 11 Penicillin-resistant pneumococcal strains isolated from nasopharyngeal samples was carried out in Jos, Nigeria by Kadakai-Olukemi and Dido,(2009) in which they obtained resistance to Ampicillin 6(54.54%),Erythromycin 4(36.36%),Tetracycline 3(27.27%), Chloramphenicol 3(27.27%), and Ciprofloxacin 1(9.09%). In a similar study in University of Ibadan Teaching Hospital, Nigeria, Akanbi *et al.*, (2004) obtained a resistance profile of 83% Penicillin G, 73.8% Ampicillin, 56.6% Erythromycin, 22. 8% Ofloxacin,20.0% Ciprofloxacin, 28.0% Ceftriaxone,18.0% Cefuroxime and 17.0% Ceftazidime. Eighty-three (83) serotypes were determined by agglutination by latex particles and/or by the Neufeld test using monovalent antisera (Statens Serum Institute). Among the 100 isolates, 57% were non-susceptible to penicillin (PNSP), 46% were intermediate and 11% were resistant (MIC range 2-4 μg/ml). Resistance rates to other antibiotics were as follow: erythromycin (22%), tetracycline (20%), and cotrimoxazol (51%). All the strains were suscepti ble to chlor-amphenicol, vancomycin and levofloxacin. The predominant serotypes were 14, 19F, 23F, and 6B accounting for 50.6% of tested strains. Non-penicillin susceptibility was associated with serotype 14 (88.23%), 6B (80.00%), 19F (61. 53%), and 23F (57.14%). In children ≤ 5 years of age, the rate of this serotypes were 14 (23.33%), 19F (13.33%), 23F (13.33%) and 6B (10%). Pneumococcal vaccination is not compulsory in Algeria. *Streptococcus pneumoniae* isolates (N = 31,001) were collected from patients with community-acquired respiratory tract infections during the Prospective Resistant Organism Tracking and Epidemiology for Ketolide Telithromycin (PROTEKT) US surveillance study (2000–2003). While the macrolide (erythromycin) resistance rate remained stable at ≈29%, the prevalence of resistant isolates containing both *erm(B)* and *mef(A)* increased from 9.7% in year 1 to 16.4% in year 3, with substantial regional variability. Almost all (99.2%) dual *erm* (*B*) +*mef* (*A*) macrolide-resistant isolates exhibited multidrug resistance,(David *et al*.,2005) whereas 98.6% and 99.0% were levofloxacin- and telithromycin-susceptible, respectively. These strains were most commonly isolated from the ear or middle-ear fluid of children. One hundred and fifty-two representative *erm(B*) +*mef* (*A*) isolates, >90% were clonally related to the multidrug-resistant international Taiwan^{19F}-14 clonal complex 271 (CC271). Three hundred and sixty-six erm (*B*) +*mef* (*A*) isolates from the PROTEKT global study (1999–2003): show 83.3% were CC271, with the highest prevalence seen in South Africa, South Korea, and the United States. The study confirms the increasing global emergence and rapidly increasing US prevalence of this multidrug-resistant pneumococcal clone.Several studies reported a predominance of *mef (A)* gene in United-States and Canada. In European countries, *erm (B)* determinant is prevalent and *mef (A)*-mediated erythromycin resistance was recently reported in about 10% of strains in Belgium and Italy. Results of these investigations show the predominance of the *erm (B)* gene in erythromycin resistant *S. pneumoniae* isolates. Macrolide efflux (*mefA)*-mediated resistance is effective in Southern France (3.7%) but this rate is the lowest published from European countries (Marchandian *et al*., 2001).

2.9 Diagnosis of Pneumococcal Pneumonia

The diagnosis of *S. pneumoniae* infection is frequently problematic. The clinical signs and symptoms of pneumococcal infections cannot be differentiated reliably from disease of alternative etiology. The “gold standard” diagnostic method is still culture. Cultures are not infrequently negative in infections considered likely on clinical grounds to be of pneumococcal origin, particularly after administration of antibiotics (Mursher *et al*., 2004).

New sensitive diagnostic methods are valuable, not only in determining the etiologic agent but also in monitoring the epidemiology of IPD within the general population and vaccine recipients. PCR for the diagnosis of IPD has proven to be limited in sensitivity when applied to blood and urine (Sheppard *et al*., 2004).Antigen detection assays have also been described with several of this methods targeting C-polysaccharide in urine (Smith *et al*., 2003). The increasing use of Pneumococcal vaccine in the developing world,calls for the need to appropriately diagnose and understand the etiology of pneumonia, so as to define the burden of pneumococcal disease and monitor vaccine efficacy and effectiveness(Vernet *et al*.,2011). Conventional serotyping by Quellung reaction is costly, time- inefficient and error prone.

A meeting on the diagnosis, detection, and serotyping of pneumococcal disease organized by PATH and Foundations Mèrieux, 2009; recommended Chest X-ray, antigen detection in urine,*S. pneumoniae* quantitative PCR in nasopharyngeal aspirates and sputum and C-reactive protein or procalcitonin measurement in blood .It was further recommended proteomics to identify pneumococcus-specific antigens in urine or host markers in blood expressed during pneumonia and new technologies based on bead-based assays, microarrays, or deep sequencing developed to determine in a single test capsular serotype, resistance profile and genotype(Vernet *et al*., 2011).

E-test has been shown to be a good alternative for testing pneumococcal MIC when compared with the standard agar dilution method (Iliyasu *et al*., 2015). A cross-sectional study was conducted in Ebonyi State University Teaching Hospital (EBSUTH) Abakaliki to assess the prevalence of drug resistant strains of *Streptococcus pneumoniae* isolated from sputum samples of patients with pneumococcal infections attending the outpatient clinics. A total of 305 sputum samples of patients with clinically diagnosed pneumonia were collected and inoculated on 5% sheep-blood agar, incubated at 35°C for 24 h in 5-10% CO_2. Colonies were Gram-stained; alpha-hemolysis colonies were tested with a 6mm optochin disk followed by bile solubility test. Susceptibility testing panels of the following antibiotics: penicillin, ciprofloxacin, clavulanic acid/amoxicillin, septrin, erythromycin, gentamycin, clarithromycin, cefotaxime and cefuroxime were tested against isolated strains of *Streptococcus pneumoniae* using disc diffusion method (Iroha *et al*., 2012).

Molecular techniques have been under development for the last 30 years, but progress throughout the last decade has been particularly rapid. In hybridization, the DNA in a sample is rendered single stranded and allowed to combine with a single-stranded probe. Other variations include the binding of a capture probe to a solid support. After binding of the target, the probe can hybridize. Probes can be labeled with a variety of reporters, including radioactive isotopes, antigenic substrates, enzymes or chemiluminescent compounds. One important development is molecular beacons. Two years after its first description by Mullis, and thus only 15 years ago, the first diagnostic application of PCR was published by Saiki *et al.*(1988). The technique became broadly used after the introduction of a thermostable DNA polymerase from *Thermus aquaticus* (Taq DNA polymerase) and the development of automated oligonucleotide synthesis and thermocyclers. PCR involves cycles of heating the sample for denaturing, annealing of the primers, and elongation of the primers by a thermostable DNA polymerase. In theory, each round of amplification gives a doubling of the number of DNA target molecules, but the process is seldom 100% efficient because of the presence of inhibitors, and in later rounds of amplification DNA polymerase may become limited(Fluit *et al.*,2001). However, during the last few years, new developments in labeling technology have expanded the applicability of PCR. One such development was the use of 5′-fluorescence-labeled oligonucleotides that were blocked at their 3′ ends, thereby preventing elongation by DNA polymerase. Besides this special oligonucleotide, PCR has two traditional oligonucleotides which function as primers and are chosen in regions flanking the special oligonucleotide. The special oligonucleotide hybridizes with the target and is removed by the 5′→3′ exonuclease activity of Taq DNA polymerase during primer extension, resulting in enhanced fluorescence that can easily be detected(Blaschke,2016).

2.9.1 Detection ofPneumococcal Resistance genes

Various methods have been reported to detect point mutations in target genes, including sequence-specific oligonucleotide probe hybridization,sequencing of the target genes, RFLP, radio isotopic or non-radio isotopic SSCP analysis, mismatch amplification mutation assay PCR, and allele-specific PCR in combination with RFLP. Probes were developed to detect mutations in staphylococcal gyr*A* genes associated with resistance, but the probes have proved difficult to use, and not all resistant strains demonstrate differential binding of the probes. Most of the studies have employed DNA sequencing of PCR-amplified DNA for the detection of alterations in the target enzymes. The primers, however, are mostly species specific. In Studies of 36 *S. aureus* isolates by non-radio isotopic SSCP (nRI-SSCP) for the presence of point mutations in the gyrA gene, direct DNA-sequencing analysis of the PCR-amplified DNA fragments confirmed the results obtained by nRI-SSCP. These authors identified seven mutational types which were separated from the wild type in a single electrophoretic step within 3 h after PCR amplification. The authors concluded that the use of the nRI-SSCP method allows relatively rapid analysis of DNA from a large number of strains. Since the electrophoretic pattern is theoretically specific for each mutation, identification of a specific mutation is possible by comparison of the mobility of the sample DNAs with that of control DNA carrying known mutations. Thus, nRI-SSCP analysis is a simple and useful method not only for the detection of point mutations associated with quinolone resistance but also for the investigation of epidemiologic markers. In an extended study, mutations in the grl*A* and gyr*A* genes of 344 clinical strains of *S. aureus* were analyzed by combinations of nRI-SSCP, restriction fragment length analysis, and direct sequencing. Five types of single-point mutations and four types of double mutations were observed in the grl*A* genes, while four types of single-point mutations and four types of double mutations were found in the gyr*A* genes. Although the power of direct sequencing was greater for the detection of gyr*A* mutations, the authors concluded that SSCP analysis is a rapid, simple, and effective method for detection of point mutations in both the grl*A* gene and the gyr*A* gene of S. aureus strains. Meanwhile, several investigators have applied SSCP analysis to the detection of mutations, mainly in gyr*A*, in several bacterial species. By SSCP analysis, 18 band patterns could be differentiated, with each pattern corresponding to a distinct mutation. The band patterns were reproducible and distinct from each other. Since SSCP analysis is simple and rapid, it might also be suitable for epidemiological surveillance of organisms involved in outbreaks or for epidemiological resistance studies (Blaschke, 2016).

2.10 Description and Pharmacokinetics of Erythromycin and Penicillin

2.10.1 Erythromycin

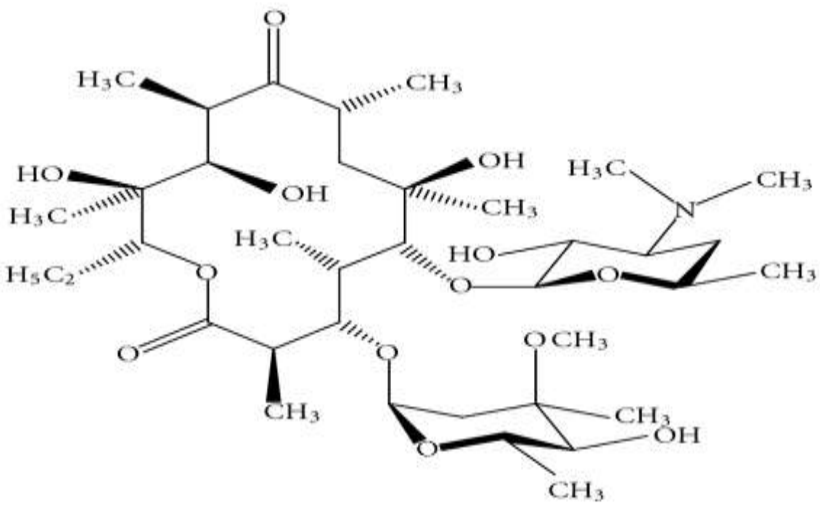


Fig. 2.4: Molecular structure of Erythromycin-the-14-membered prototype macrolide: Adopted from Wikipedia contributors (25 June, 2019)

Erythromycin is produced by a strain of *Saccharopolyspora erythrea* (formally *Streptomyces erythraeus)*and belongs to the macrolide group of antibiotics. It is basic and readily forms salts with acids.Erythromycin ethyl succinate is an ester of erythromycin suitable for oral administration (Pill identifier, 2013)

Generic Name: erythromycin

Chemical Data: $C_{37}H_{67}NO_{13}$

Mol. Mass: 733.93 g/mol

Brand Names: Eryc, Ery-Tab, E.E.S, Eryped, PCE, [E-Mycin(Pharmacia&Upjohn), Ilosone(Eli Lilly), and Pediazole(Abbot) are discontinued brands)(Pill identifier, 2013; BNF49, 2005).

Adverse Reaction and Side effects

The most frequent side effects of oral erythromycin are gastrointestinal and are dose- related. They include nausea, vomiting, abdominal discomfort, diarrhea and anorexia. Symptoms of hepatitis, hepatic dysfunction, and or abnormal liver function test result may occur. Allergic reactions with rash eosinophilia may occur rarely. Ototoxicity may occur.Erythromycin, sold under the brand names Ery-Tab, Akne-Mycin, E.E.S. Eryc, and Pediamycin. The drug is prescribed for infections like pneumonia, whooping cough (pertussis), sexually transmitted diseases, Legionnaires' disease, urinary tract infections (UTIs), and acne. Erythromycin belongs to group of drugs known as macrolide antibiotics, which work by stopping bacteria from making proteins that they need to survive and function properly. There are no specific warnings about drinking alcohol while taking erythromycin, but there's some evidence that alcohol might result in delayed absorption of the antibiotic. Erythromycin is available in many different dosage forms including cream, gel, tablet, and solution for injection into the veins. Depending on the condition, doses of erythromycin tend to range from the following: 250 milligrams (mg) to 500 mg, every six to 12 hours; 500 mg every 12 hours; 333 mg every eight hours (Wiley, 2015) .In the first-line treatment of severe invasive pneumococcal infection in our environment the use of ceftriaxone is recommended; however, in a resource-constrained settings chloramphenicol, amoxicillin or amoxicillin–clavulanic acid may be an alternative, with high prevalence of penicillin resistance in, the use of penicillin for empiric treatment of suspected pneumococcal infection is no longer recommended (Iliyasu *et al*., 2015).

Mechanism of action of Erythromycin

Erythromycin displays bacteriostatic activity or inhibits the growth of bacteria, especially at higher concentrations.By binding to the 50s subunit of the bacterial rRNA complex protein synthesis and subsequent structure and function processes critical for life or replication are inhibited [Katzung, (nd)].

2.10.2 Penicillin

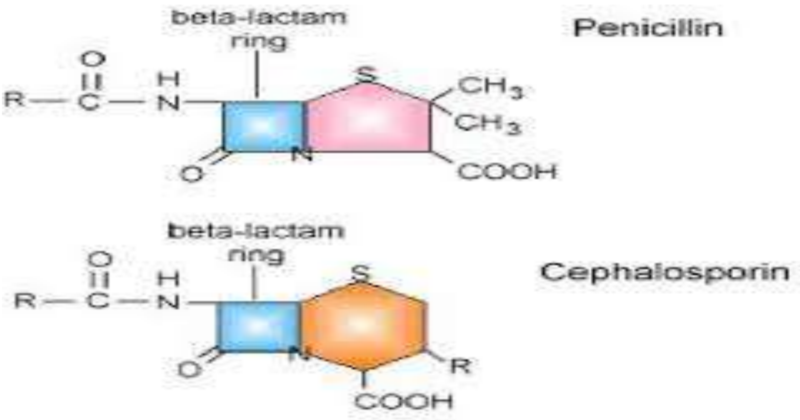


Fig.2.5: Molecular structure and β-lactam ring: Adopted from Wikipedia contributors (25 June, 2019)

Penicillin is one of the first and still one of the most widely used antibiotic agents derived from the Penicillium mold (*Penicillium notatum*) (Jorge, 2016), It is a group of antibiotics which include Penicillin G(intravenous use),Penicillin V (oral use) and Procaine Penicillin and benzathine Penicillin(intramuscular).The term “Penicillin” is often generally used to refer to benzyl penicillin(Penicillin G, the original penicillin found in 1928). Penicillin has been the drug of choice worldwide for the treatment of pneumococcal infections. However, penicillin resistant strains have emerged, resulting in a shift to the use of other drugs, e.g. cefotaxime, chloramphenicol and erythromycin (Dankor *et al*., 2010).

Adverse effect: ≥ 1% of people associated with use of penicillin includes:

Diarrhea, hypersensitivity, nausea, rash, neurotoxicity, urticarial and superinfection including candidiasis; infrequent adverse effect include fever, vomiting, erythema, dermatitis, angioedema, seizures especially in people with epilepsy and pseudomembranous colitis. Other effects include serum sickness.(type III hypersensitivity) (Rossi,2007).

Mechanism of Action of Penicillin

Penicillin and other ß-Lactam antibiotics act by inhibiting PBPs, which normally catalyze cross-linking of bacterial cell wall.ß-lactam antibiotics inhibit the formation of peptidoglycan cross-linking in the bacterial cell wall, achieved through binding of the four membered ß-lactam ring of penicillin to the enzyme DD-trans peptidase, as a result the DD-trans peptidase cannot catalyze the formation of cross-links and an imbalance between cell wall production and degradation develops bacteriostatic action causing the cell to rapidly die (Antunez *et al.*,2006).

2.11 Host Immunity against Pneumococcus

The majority of respiratory pathogens have phosphoryl choline (PCho) on their surfaces. PCho is on the cell wall teichoic acid of pneumococci. PCho, by mimicking the host chemokine PAF, binds to the host cell platelet-activating factor receptor (PAFr) and its scaffold of β arrestins, leading to the uptake of bacteria into a vesicle and transmigration across the epithelial or endothelial barrier. This simple invasion step is countered by innate immune elements. C-reactive protein binds to PCho on the bacteria;Surfactant contains abundant PCho, which inhibit bacterial contact with the host cell.In a study carried out by Reshmi *et al.*(2012), theyobserved that pneumococcal surface proteins A(PsPA); a major virulence factor of pneumococci, effectively inhibits C3 deposition and that PsPA's terminus has a choline-binding domain that anchors PsPA to the phosphocholine (PC) moieties on the pneumococcal surface and CRP, another important host defense molecule, also binds to PC. The binding of CRP to pneumococci enhances complement C3 deposition through the classical pathway.Control of bacterial multiplication in the lung requires innate immune recognition of the pneumococcal pathogen-associated molecular patterns (PAMPs) that initiate recruitment of neutrophils and the accumulation of hemorrhagic debris as the hallmark of pulmonary consolidation. Pneumococcal peptidoglycan components are known to induce signaling by TLR2 and LPS binding protein (Weber *et al*., 2003).The **Nuclear Factor** kappa-light-chain-enhancer of activated B cell (NF-κB) signaling leads to the release of cytokines, particularly IL-6 and IL-1ß, which then induces neutrophil recruitment and macrophage activation. The lung is unique in enabling neutrophil recruitment specifically in response to pneumococcus in both a CD-11/CD-18 dependent and independent manner (Doerschuk *et al*., 2000). Although required for clearance of bacteria, the intense inflammation induced by neutrophils contributes to lung damage; neutropenia (although not a complete absence of neutrophils) may attenuate damage and improve outcome (Marks *et al*., 2007). Many respiratory pathogens have converged on a common strategy to advance mucosal disease to bacteremia. This process is termed "innate invasion" and is counteracted by the very early participants induced in innate immunity (Thornton *et al*., 2010). The key host receptor for innate invasion is the platelet-activating factor receptor (PAFr). Many reports have shown that respiratory pathogens decorate their surfaces with phosphoryl choline (PCho), an example of molecular mimicry as PCho is the ligand critical to the activity of the chemokine (PAF). Binding of PCho to PAFr initiates uptake of the bacteria into a vacuole that is then trafficked across the host cell where bacteria can be released to seed the bloodstream (Brigitta and Elaine, 2013). Capsular polysaccharides (CPS) are surface exposed and immunogenic and provide a target for the host immune response. Consequently, there has been selection for mechanisms by which encapsulated bacteria evade the host immune system, and in most encapsulated species, this has been achieved by the generation of antigenic diversity, such that strains of the pathogen may express one of a number of different CPS (Spratt *et al*., 2004).Pneumolysin activates the classical complement pathway and may be recognized by TLR4, thereby evoking an inflammatory response (Marlley *et al*., 2003). Pneumolysin has been shown to influence bacterial uptake into human dendritic cells, to inhibit cellular activation and cytokine secretion, and to induce a caspase-dependent apoptosis (Braun *et al*., 2002). Nonfunctional pneumolysin, such a serotype 1 influences inflammation induced by the interaction of bacteria and dendritic cells and might affect the development of diseases such as empyema (Littmann *et al.*, 2009).

2.12 Prevention and Control of pneumococcal infections

Patients are often treated with antibiotics for longer than necessary. Antimicrobial stewardship programs can help to shorten the duration of antibiotics and narrow the spectrum of antibiotics (Avdic *et al*., 2012).Antibiotic resistant bacteria that are difficult or impossible to treat are becoming increasingly common and are causing a global health crisis. Antibiotic resistance is encoded by several genes, mainly of which can transfer between bacteria. The mechanism of antibiotic resistance include the prevention of access to drug targets, changes in the structure and protection of antibiotic targets and the direct modification or inactivation of antibiotics(Alba *et al.*,2015).Streptococcus pneumoniae is a major cause of sepsis, meningitis and respiratory disease Worldwide. Pneumococcal conjugate vaccines (PCVs) have now been implemented in many countries worldwide, including Singapore (Elita *et al*., 2014).

2.12.1 Therapeutic treatment of invasive Pneumococcal pneumonia

Erythromycin is an antibiotic in the class of antibiotics known as macrolide antibiotics which also includes azithromycin (Zithromax, Zmax) and clarithromycin (Biaxin). It is used to treat several types of infections including upper/lower respiratory tract infections, skin infections, acute pelvic inflammatory disease, erythrasma,(erythema) etc. caused by bacteria such as S*treptococcus pyogenes, Streptococcus pneumoniae, Mycoplasma pneumoniae, Staphylococcus aureus, Neisseria gonorrhoeae*, and many others. Erythromycin, like all macrolide antibiotics, prevents bacterial cells from growing and multiplying by interfering with their ability to make proteins while not affecting human cells. Determination of whether a patient with community-acquired pneumonia (CAP) can be treated safely as an outpatient or requires hospitalization with or without intensive care unit (ICU) admission is essential before selecting an antibiotic regimen(Review, 2014).The two most commonly used prediction rules are the Pneumonia Severity Index (PSI) and CURB-65(Fine *et al.*, 1997). PSI risk class correlates directly with mortality rate; risk class I is associated with a 0.1 percent mortality rate, compared with 0.6 percent for class II, 0.9 to 2.8 percent for class III, 8.2 to 9.3 percent for class IV, and 27.0 to 29.2 percent for class V (Mandell *et al.*, 2007). On the basis of these mortality rates, risk class I and II patients can generally be treated as outpatients, risk class III patients are to be treated in an observation unit or with a short hospitalization and risk class IV and V patients require hospitalization (Metlay and Fine, 2003). CURB 65 uses five prognostic variables (Lim *et al.*,2003): Confusion (based upon a specific mental test or disorientation to person, place, or time),Urea (blood urea nitrogen in the United States) >7 mmol/L (20 mg/dL),Respiratory rate ≥30 breaths/minute, Blood pressure (BP) (systolic <90 mmHg or diastolic ≤60 mmHg), Age ≥65 years.The choice of initial therapy is complicated by the emergence of antibiotic resistance amongst*S.pneumoniae*,the most common bacterium responsible

for CAP, as well as concern for MRSA in severe CAP. Empiric therapy and pathogen directed therapy-antibiotic therapy is typically began on an empiric basis, since the causative organism is not identified in an appreciable proportion of patients (Prina *et al*., 2015). Antibiotics should be started as soon as possible once the diagnosis of CAP is considered likely. The clinical features and chest radiographic findings are not sufficiently specific to determine etiology and influence treatment decisions. The Gram stain of respiratory secretions can be useful for directing the choice of initial therapy if performed on a good quality sputum sample and interpreted by skilled examiners using appropriate criteria (Mandell *et al.*, 2007). Pathogen-directed treatment (PDT) and empiric broad-spectrum antibiotic treatment (EAT): PDT wasbased upon microbiologic studies (rapid diagnostic tests) or clinical presentation; EAT pati ents received a beta lactam beta-lactamase inhibitor plus erythromycin or, if admitted to the intensive care unit (ICU), ceftazidime and erythromycin. Adverse events were more frequent in the EAT group but were primarily related to the specific antimicrobial choice i.e., erythromycin (Van der Eerden *et al.*, 2005).The selection of antimicrobial regimens for empiric therapy is based upon a number of factors, including: The most likely pathogen(s), Clinical trials proving efficacy, Risk factors for antimicrobial resistance. The choice of empiric therapy must take into account the emergence of antibiotic resistance among *S. pneumoniae*, the most common cause of CAP in adults who require hospitalization, Medical comorbidities, which may influence the likelihood of a specific pathogen and may be a risk factor for treatment failure and epidemiologic factors such as travel and concurrent epidemics (e.g. Middle East respiratory syndrome corona virus, avian influenza).Additional factors that may affect the choice of antimicrobial regimen include the potential for inducing antimicrobial resistance, pharmacokinetic and pharmacodynamics properties, safety profile, and cost (File *et al.*, 2004).Risk factors for CAP due to gram-negative bacilli include previous antibiotic therapy, recent hospitalization, immunosuppression, pulmonary comorbidity (e.g., cystic fibrosis, bronchiectasis, or repeated exacerbations of chronic obstructive pulmonary disease that require frequent glucocorticoid and/or antibiotic use), probable aspiration, and multiple medical comorbidities (e.g., diabetes mellitus, alcoholism) as reported by Shindo *et al.*,2013.

Summarized briefly, risk factors for drug-resistant *S. pneumoniae* in adults include: Age >65 years, Beta-lactam, macrolide, or fluoroquinolone therapy within the past three to six months, Alcoholism, Medical comorbidities, Immunosuppressive illness or therapy and Exposure to a child in a daycare center. Another risk factor is prior exposure to the healthcare setting such as from prior hospitalization or from residence in a long-term care facility.Recent therapy or repeated courses of therapy with beta-lactams and macrolides, or fluoroquinolones are risk factors for pneumococcal resistance to the same class of antibiotic (Kuster *et al.*, 2014). Optimal doses of the beta-lactams (ceftriaxone, cefotaxime, ampicillin-sulbactam) have not been studied adequately, the higher doses, at least initially, until the minimum inhibitory concentrations (MICs) against possible isolates (e.g., *Streptococcus pneumoniae*) are known. In patients (particularly those with bronchiectasis or chronic obstructive pulmonary disease [COPD] and frequent antimicrobial or glucocorticoid use) who may be infected with *Pseudomonas aeruginosa* or other resistant pathogens, therapy should include agents effective against the pneumococcus, *P. aeruginosa*, and *Legionella* spp. Acceptable regimens include combination therapy with a beta-lactam antibiotic and a fluoroquinolone, such as the following regimens: Piperacillin-tazobactam (4.5 g every six hours) **or** Imipenem (500 mg IV every six hours) **or** Meropenem (1 g every eight hours) **or** Cefepime (2 g every eight hours) **or** Ceftazidime (2 g every eight hours) PLUS Ciprofloxacin (400 mg every eight hours) **or** Levofloxacin (750 mg daily).For penicillin-allergic patients, the type and severity of reaction should be assessed. The great majority of patients who are allergic to penicillin by skin testing can still receive cephalosporin (especially third-generation cephalosporins) or carbapenems. If there is a history of a mild reaction to penicillin (**not** an IgE-mediated reaction, Stevens Johnson syndrome, or toxic epidermal necrolysis), it is reasonable to administer a cephalosporin or carbapenem using a simple graded challenge (e.g., give 1/10 of dose, observe closely for one hour, then give remaining 9/10 of dose, observe closely for one hour). Skin testing is indicated in some situations. For penicillin-allergic patients, if a skin test is positive or if there is significant concern to warrant avoidance of a cephalosporin or carbapenem, options include aztreonam (2 g IV every six to eight hours) plus levofloxacin (750 mg daily) or aztreonam plus moxifloxacin plus an aminoglycoside.

Macrolide-containing regimens — observational studies have suggested that combination regimens containing a macrolide plus a beta-lactam result in better clinical outcomes in patients with severe CAP, especially bacteremic pneumococcal pneumonia; this is likely due to the immunomodulatory effects of macrolides (Martin-Loeches *et al.*,2010).

If a sputum culture reveals methicillin-susceptible *Staphylococcus aureus* (MSSA), therapy should be changed to nafcillin (2 g IV every four hours) or oxacillin (2 g IV every four hours).In patients who are treated with the combination of an intravenous beta-lactam and a macrolide who have risk factors for drug-resistant *S. pneumoniae* (DRSP), replace the intravenous beta-lactam with high-dose amoxicillin (1 g orally three times daily) to complete the course of therapy. When DRSP is not a concern, amoxicillin can be given at a dose of 500 mg orally three times daily or 875 mg orally twice daily. In patients who have already received 1.5 g of azithromycin who do not have *Legionella pneumonia*, do not continue atypical coverage. Conversely, in patients who have not received 1.5 g of azithromycin, give amoxicillin in combination with a macrolide or doxycycline. An alternative for patients without risk factors for DRSP is to give a macrolide or doxycycline alone to complete the course of therapy. The dosing for macrolides and doxycycline is: Azithromycin-500 mg once daily; Clarithromycin-500 mg twice daily; Clarithromycin XL – Two 500 mg tablets (1000 mg) once daily; Doxycycline– 100 mg twice daily.

2.12.2 Pneumococcal Conjugate Vaccine (PCV) and Pneumococcal Polysaccharide Vaccine (PPV)

Efforts to control infection have led to the deployment of polysaccharide vaccines and an understanding of antibiotic resistance. The inflammatory response to pneumococci, one of the most potent in medicine, has revealed the double-edged sword of clearance of infection but at a cost of damage to host cells. In virtually every aspect of the infectious process, the pneumococcus has set the rules of the Gram-positive pathogenesis game. Pneumococcal diseases range from mild respiratory tract mucosal infections such as otitis media and sinusitis to more severe diseases such as pneumonia, septicemia, and meningitis.(Birgitta and

Elaine,2013). Medically, the three major bacterial pathogens of children have historically been the pneumococcus, *Haemophilus*, and meningococcus. All three are naturally transformable and undergo autolysis in the stationary phase. They share the same progression of disease from the nasopharynx to blood and brain and use phosphoryl choline on their surfaces to bind to the same series of receptors in the pathway of innate invasion (Thornton et al., 2010). Whereas vaccination has succeeded in dramatically reducing disease caused by *Haemophilus* and meningococcus, pneumococcus remains a poorly controlled outlier. The huge number of pneumococcal serotypes (compared to the small number for the other two pathogens) remains the main challenge in developing strategies to control this global pathogen (Birgitta and Elaine, 2013).Introduction of pneumococcal vaccines in Nigeria is a priority as part of the Accelerated Vaccine Introduction Initiative (AVI) of the Global Alliance for Vaccines and Immunization (GAVI). However, country data on the burden of pneumococcal disease (IPD) is limited and coverage by available conjugate vaccines is unknown (Ifedayo et al., 2012). Since vaccination is the most effective public health tool to prevent IPD, pneumococcal conjugate vaccines (PCVs) containing 7, 10 and 13 serotypes have been developed although PCV-7 is being replaced by PCV-10 and -13 because it contains limited serotypes. Unlike the 23 serotype-containing polysaccharide vaccine, PCVs are more immunogenic in young children, induce immunological memory and provide serotype-specific protection against pneumococcal carriage. Although they contain serotypes most associated with disease and antimicrobial resistance worldwide, variation of serotypes geographically and antimicrobial resistant strains prevent universal protection. In addition, replacement in carriage and invasive disease by non-vaccine containing and drug resistant serotypes of pneumococci occurs with use of PCVs (Farrell *et al.*, 2007). In many countries needing PCV the most, data on the burden of pneumococcal carriage and disease; as well as the prevalent serotypes and coverage by PCVs is limited or unknown. Nigeria is one of the 10 countries where two-thirds of all deaths due to pneumonia in children <5 years occur (Akanbi *et al*., 2009; WHO, 2007). It is also one of the 15 countries with the highest estimated number of cases of clinical pneumonia(Rudan *et al*.,2008), pneumococci serotypes 1, 2, 3 and 5 were the serotypes associated with IPD in children and adults in North East, Nigeria(Onyemelukwe and Greenwood,2007) . More recently, a small study in Eastern Nigeria found 69% pneumococci carriage rates in children <5years attending routine paediatric clinics (Nwachukwu and Orji, 2008).These problems call for urgent detection and surveillance of pneumococcal serotypes in Kaduna State and Nigeria in general as indicated in section 1.2 of this study to provide better treatment for Pneumonia. Falade *et al*., 2009 in a hospital based surveillance of invasive bacterial infections found pneumococcus was implicated in the majority of pneumonia, meningitis and bacteremia in the children studied. Existing data on pneumococcal carriage and disease in Nigeria have limitations including small sample sizes, poor yield of pneumococci and/or that very few isolates are serotyped and results from earlier studies are out of date. Consequently, the current prevalence of serotypes in carriage and disease is largely unknown in Nigeria. Baseline pneumococcal carriage and invasive rates data are crucial for assessing the impact of vaccination and for monitoring serotype changes and antimicrobial resistance patterns. Our serotyping results indicate that PCV-13 covers approximately half of the serotypes carried by healthy children ≤6 years of age in our study (48% in Accra and 51% in Tamale), while PPV-23 provides coverage for 55% of all isolates in our sample. In total, PCV-13 covers 55% of the isolates with intermediate penicillin resistance, compared with PPV-23 which covers 61%. The coverage rates were similar in both Accra and Tamale. The two isolates that were fully penicillin resistant were serotypes 19A and 6B, both of which were covered by PCV-13 and PPV-23. The majority of serotypes (80%) seen in children under 24 months of age were covered by PCV-13. A higher proportion of isolates from children aged 3-6 years were not covered by PCV-13 (Nicholas *et al*., 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

Kaduna State is geographically located at the Centre of Northern Nigeria, in the Northwest geopolitical zone. The state lies between latitude 90 and 140^0 N of the equator with a time of 1 hour ahead of the GMT. It has two distinct seasons; the wet (rainy) season which lasts from April to October and the dry season which lasts from November to March. The state shares boundaries with Niger state to the west, Zamfara, Katsina and Kano states to the North, Bauchi and Plateau to the east and FCT Abuja and Nasarawa state to the south. It has a population of 6.63 million based on the 2006 census projections. The state is divided into three senatorial Zones as follows: Northern (Zone 1), Central (Zone2), and Southern (Zone3) Zones. Zone 1 comprises of Zaria, Soba, Sabon Gari, Makarfi, Kudan, Ikara, Kubau and Lere LGAs; Zone 2: Birnin Gwari, Giwa, Chikun, and Kaduna south, Kaduna North, Kajuru and Igabi LGAs andZone 3: Zangon Kataf, Jama'a, Kauru, Kaura, Kachia, Sanga, Jaba and Kagarko LGAs. Kaduna State has 739 Primary health facilities, 29 secondary care facilities and five tertiary hospitals.Kaduna state is administratively divided into 23 local government areas (LGAs). There are several districts and wards in each LGA (Abubakar *et al.*, 2013).

3.2 Sampling Site and Subjects

Three tertiary health facilities were covered during the study. The Facilities were: Ahmadu Bello University Teaching Hospital, Zaria (Northern Senatorial Zone); Kaduna StateUniversity Teaching Hospital, Kaduna (Central senatorial Zone) and Sir Patrick Ibrahim Yakowa Memorial General Hospital, Kafanchan in the Southern Senatorial Zone (Fig.3.1). One hundred and fifty (**150**) subjects were recruited from each hospital without bias by a purposive random sampling technique.

Fig.3.1: Map of Nigeria showing Senatorial Zones and sampling sites.

3.3 Ethical Approval

Ethical approval was obtained from the Ethical committees of Kaduna State Ministry of Health, Ahmadu Bello University Teaching Hospital Shika, Zaria and Kaduna State University Teaching Hospital (Appendices I-III) attached.

3.4 Study Design

The study was facility-based survey, cross sectional study to probe the LRT infections andthe virulent determinants from the Pneumococcal isolates among the patients in the targetedhospitals.

3.5 Inclusion Criteria

Patients of both sexes, of age ≥ 10 years with symptoms of respiratory tract infections RTIAtte nding the targeted facilities,which consented to participate in the study were included.

3.6 Exclusion Criteria

Patients without symptoms of respiratory tract (RT) infections and those age ≤ 10 years and those who did not consent were excluded.

3.7 Sample Size

The sample size was determined using the following equation (Araoye, 2004)

$$\boldsymbol{n = \frac{z^2pq}{l^2}}$$

Where: $\boldsymbol{n}$ =Sample Size

$z =$ Score for a given confidence interval set at 1.96 for 95% confidence limit.

$p =$ Prevalence value of previous study for Penicillin and Erythromycin resistance was 48 % (Mohammad*et al.*, 2012).

$\boldsymbol{l} =$ Permissible error of estimation, taken as 0.05 (5%)

$$\boldsymbol{q = 1 - p}$$

$$n = \frac{(1.96)^2 X\ 0.48\ X\ 0.52}{(0.05)^2} = 384$$

Three hundred and eighty-four (**384**) was the calculated minimum sample size. Four hundred and fifty (**450**) samples were collected to cover for 10% attrition in the study area.

3.8 Collection of Samples

Sputum samples from patients, age 10-70 years having symptoms of respiratory tract infections attending the selected facilities were collected in wide mouth leak-proof containers. Patients were instructed to collect samples in the morning soon after patient wakes before any mouth wash. Patients were instructed to hold their breath a few seconds-then cough directly into the specimen container. The patients were educated about the difference between phlegm/mucus and oral secretions. The sputum samples were transported to the laboratory as soon as possible andgood-quality sputum samples were used (Fukushima *et al*., 2008). Prior to collection of samples, patients were interviewed using structured questionnaire designed to obtain basic demographic data, history of illness and clinical information concerning the patient (CDC; Tests online, 2014).Patients' consent was also sort before recruiting them for the study.

3.9 Isolation of Lower Respiratory Tract Bacterial Pathogens

Purulent part of the sputum sample was washed in about 5ml of sterile physiological saline to reduce the number of commensals. The washed sputum samples were inoculated by streaking method on plates of 5% Blood agar and incubated immediately in presence of 5% CO_2(provided conventionally) overnight at 37^oC (Mohammed,2015).After 24hrs incubation discrete colonies, mucoid, raised or having draughtsman appearance showing α- hemolysis (greenish zones of inhibition around disc)on blood agar weresuspected as *S. pneumoniae*were sub cultured on fresh media (BA) to purify them. Subsequently they were transferred unto Chocolate Agar slants for further identification and Characterization.

3.10 Characterization of Isolates

The bacterial isolates were identified based on morphological characters, Gram reaction, catalase test, coagulase test, bile solubility and susceptibility to Optochin (Iliyasu *et al.*, 2015).

3.10.1 Gram staining

The culture growing on blood agar plate was used. A loop full of tap water was placed on a slide. Using a sterile cool loop a small sample of the colony was transferred to the drop, and emulsified. The film was then allowed to air dry and was heat fixed. The slide was flooded with crystal violet solution for one minute, washed off briefly with tap water then drained. The slide was thereafter flooded with Gram's Iodine solution, and allowed to act (as a mordant) for one minute, washed off with tap water and drained. Excess water from slide was removed and blotted and it was flooded with 95% alcohol for 10 seconds then washed off with tap water. The slide then was drained and the smear flooded with safranin solution and allowed to counterstain for 30 seconds, washed off with tap water and finally drained and blotted dry. All slides were examined under the oil immersion lens. *S. pneumoniae* appeared as Gram positive elongated (lanceolate) diplococci, or formed short chains(Bacteriology-test, 2014).

3.10.2 Catalase test (Slide Method)

A clean glass slide was divided into two sections with grease pencil. One was labeled as “test” and the other as “control”. A small drop of normal saline was placed on each area. With a sterilized and cooled inoculating loop one colony of the culture from a nutrient agar slant was picked and emulsified on each drop to make a smooth suspension. With a Pasteur pipette, one drop of 3% hydrogen peroxide was placed over the test smear. Nothing was added in the other drop that served as control. Observation was made of the fluid over the smears for the appearance of gas bubbles. The slide was discarded in a jar of disinfectant.

Appearance of gas bubbles indicated positive catalase test (Bacteriology-test, 2014).

3.10.3 Bile Solubility test (Broth solubility)

A heavy suspension of pure culture was prepared in 1.0ml of 0.85% saline and the suspension divided between two tubes (one test and one control), 0.5ml of 10% sodium deoxycholate was added to the test suspension and 0.5ml of 0.85% saline to the control. The suspension was mixed gently and incubated at 37^{o}C for up to 15min, and evidence of clearing of turbidity in the tube marked test was examined and compared with the saline control. Suspension clearing in tube labelled test was considered positive result while suspension remaining turbid in both tubes was considered negative (WHO/CDC, 2003).

3.10.4 Optochin Susceptibility test

An inoculum from a pure culture was transferred aseptically to a sterile plate of nutrient agar, typically by use of cotton-tipped applicator (swab); the inoculum was spread over the plate to distribute the bacteria as evenly and thoroughly as possible over the entire agar surface. A chemically impregnated disk containing Optochin was then aseptically placed in the center of the agar surface. The inoculated plate was incubated at 37^{o}C for 24 hours and the results determined. Growth of the lawn up to the margin of the disk indicates the bacterium is resistant to ethylhydrocupreine hydrochloride (optochin). An obvious clear zone around the disk (≥14mm in diameter) indicates susceptibility (WHO/CDC, 2003).

3.10.5 Antimicrobial susceptibility test

The susceptibility of *S. pneumoniae* was determined by the Kirby-bauer disc diffusion method and interpreted following CLSI guidelines. Breaking points for non-susceptibility and resistance was used to indicate susceptibility of the pneumococci to the antibiotics (Wayne, 2006). The following antimicrobial drugs were included in the tests: Ampicillin (10µg), erythromycin (15µg), penicillin (10U), Cefuroxime (10µg), Azithromycin (10µg), Ceftriaxone (10µg), Chloramphenicol (30µg) and Tetracycline (30µg).Minimum Inhibitory Concentrations of strains displaying resistance to erythromycin and penicillin from the disk diffusion method were determined by E-test methodology (Liofilchem, Italy). MIC strips were: Erythromycin (0.016-256µg/mL) and Penicillin G (0.016-256µg/mL). Resistance to the antibiotics was interpreted according to MIC Test Strip Technical Sheet *Streptococcus pneumoniae* (MIC Guide, 2015).

3.11 Determination of Minimum Inhibitory Concentration (MIC)

MIC Strips were applied to an inoculated Mueller-Hinton agar plate using sterile forceps and incubated at 37°C for 18-24hrs in the presence of 5% CO_2. After incubation, the ellipse intersecting the reading scale on the strip was considered as the MIC (in µg/mL), where the concentration of the antibiotic tested inhibits the microorganism's growth (Liofilchem) (Appendix VI).

3.12 PCR for Confirmation and Detectionof MRGs and PRGs Genes

3.12.1 Identification of *S. Pneumoniae* genesand amplification of the Resistance genes

The autolysin (lytA) gene was targeted to identify *S. pneumoniae* which is the pneumococcal species specific gene. Genes amplified for detection of penicillin resistance were *pbp1a, PbP2b and PbP2x* genes and genes targeting the macrolides genes were *erm (B)* and *mef (A)*. This amplification was done by conventional polymerase chain reaction (PCR).The *lytA* gene encodes the main Pneumococcal Autolysin (*lytA*) used as target DNA to confirm *S. pneumoniae* strains.

Table 3.1: Primers used for *lytA*, *PbP1a, PbP2b, PbP2x and erm (B), mef(A)* resistance genes.

GENE	PRIMER	AMPLICON SIZES
lytA	5′-CAA CCG TAC AGA ATG AAG CGG-3′	295
	3' – TTA TTC GTC CAA TAC TCG TGCG- 5′	
PbP1a	5′-AGT ATA TCA AGA ACA CTG GCT ACG- 3′	195
	3' – GCT TGG AGT GGT TGA GCTA - 5′	
PbP2b	5′- AAA TTG GCA TAT GGA TCTTTTC - 3′	203
	3' - TATTCATCTCTGTCGGTTGC-5′	
PbP2x	5′- AAG TAA CTA TGAACCAGGATCAG - 3′	147
	3' - CGAAGCATTTGTGTTTGT GT- 5′	
erm (B)	5′- GAAAAGCTACT CAAC CAAATA- 3′	224
	3' - AGTATCATTAATCACTAGTGC- 5′	
mef(A)	5′- AGTATCATTAATCA CTAGTGC - 3′	294
	3' - TTCTTCTGGTACTAAAAGTGG - 5′	

(Mohammad *et al*., 2012)

3.12.2 DNA extraction

DNA extraction was carried out by transferring *S. pneumoniae* colonies from culture medium; suspending them in 300µl of phosphate- buffer saline and then centrifuging at 3000 rpm for 15min.The supernatant was set aside and the sediment was used for DNA extraction. The sediments were re-suspended in 50µl of 1x Tris-EDTA (TE) buffer at pH 7.4 and incubated for 10 min at 37^{o}C and incubation at 100^{o}C for 3 minutes. Samples werestored at -20^{o}C until use 1-3 days (Fukushima *et al*., 2008).

3.12.3 PCR amplification of Resistance genes by conventional (mPCR)

PCR amplification was carried out with the cycling parameters in light cycler. After an initial denaturation step at 95^{o}C for 5 min, 30 cycles of amplification was performed as follows: denaturation at 94^{o}C for 30 s, annealing temperature at 58^{o}C for 30 s and extension temperature at 72^{o}C for 30 s (Mohammad et *al*., 2012).

3.13.4 Detection of PCR products

The sizes of PCR products (genes) were detected and analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide (0.5ml). The gel was prepared by mixing 1.5g of agarose into 2ml of Tris-Acetate EDTA (TAE) and 98ml of distilled water and then heating the mixture at 55^{O}C for 2 minutes in a microwave. Fifteen microliter (15µl) of Ethidium bromide was added to the heated mixture and poured into a gel tray fitted in a gel tank. Combs were inserted into the molten gel and it was allowed to solidify at room temperature. After solidification of the agarose gel, 300ml of TAE Buffer (6ml TAE + 294ml distilled water was poured into the gel tank and the combs gently removed. Ten microliter (10µl) of DNA ladder was loaded into the first well of the gel while 10µl of each PCR product was loaded into the remaining wells accordingly. Electrophoretic machine was set at 60V for 45 minutes. The gel was then viewed under the UV light box for documentation.

3.13 Serotyping (PCR Strategy)

3.13.1 PCR amplification of *cpsB* for Sequetyping

Genomic DNA was extracted from a fresh overnight culture of pneumococcal cells. The sequencing primers used were: cps1 ,5'-GCA ATG CCA GAC AGT AAC CTC TAT-3',and cps 2, 5'-CCT GCC TGC AAG TCT TGA TT-3'.To match annealing temperature of the reverse primer, the forward primer was extended in the 5' end by three nucleotides compared to the *in silico* primer sequence. The reaction mixture contained 2µl of genomic DNA,0.3µl Taq polymerase(5U/µl)(Invitrogen),1xPCR buffer(10X),1.5 Mm $MgCl_2$,0.6 Mm deoxynucleoside triphosphates(dNTPs),and 0.4µM each primer, made up to a final volume of 50µl with DNase/RNase-free distilled water(Gibco).The reaction Cycle consisted of an initial denaturation step at 95^{o}C for 30 s ,65^{o}C for 30s and 72^{o}C for 1 minute 30s.Amplicons from PCR were analyzed with 1.5% agarose gel electrophoresis (Marcus *et al*., 2012).

3.13.2 Nucleotide sequencing

The amplicons with expected *cps* band sizes were purified using the PCR purification kit (Qiagen) according to the manufacturer’s instructions. Purified amplicons were cycle sequenced in both directions with the same primers as those used in the initial PCR. Cycle sequencing was performed with BigDye sequence Terminator v.3.1 (Applied Biosystems).

Sequences were analyzed on Genetic Analyzer 3130(Applied Biosystems) and viewed with Bionumerics software version 5(Applied Maths) (Marcus *et al.*, 2012).

3.13.3 *in Silico* Identification of Nucleotides Sequences

The amplicon nucleotide sequences were used to interrogate the GenBank database, and if the highest BLAST bit score gave the serotype.Identification was then considered as a ‘’match’’.Where the query results did not correlate or were not consistent with conventional serotyping methods, the strain/serotype wasconsidered as ‘’misidentified’’ (Marcus *et al*., 2012)

3.14 Data Analysis

Results obtained and data from questionnaires were reduced to percentages and presented in tables and charts. Microsoft excel, (2016) was used to analyze the data obtained and statistical analysis was done using SPSS version 20.0 software programme. Association and relationship between variables was determined using Pearson Chi-square and correlation analysis at 95% confidence interval. P-values were obtained all P values ≤ 0.05 was considered statistically significant association between the variables.

CHAPTER FOUR

4.0 RESULTS

Out of the total of 450 subjects sampled for the study, from the selected hospitals in three senatorial zones of Kaduna State, 237 (52.7%) are males and 213 (47.3%) are females. Bacteri al pathogens of the lower respiratory tract were identified in 207(46.0%) of the sputa.Fig.4.1 shows the distribution of bacterial isolates from sputum samples collected from patients attending selected hospitals in Kaduna State based on locations(n=450), 41 were positive for *Streptococcus pneumonia* giving a prevalence of 9.1%. Other bacterial pathogens of the lower r espiratory tract were isolated as follows: *Streptococcus viridians Group* 56(12.4%), *Staphyloco ccus aureus* 52(11.6%), *Klebsiella pneumoniae* 30(6.7%), Streptococcus *pyogenes* 20(4.4%), *H aemophilus influenzae* 5(1.1%), *Enterobacter aerogenes* 2(0.4%) and *Moraxella catarrhalis* 1(0.2%).Statistical analysis showed a significantassociation (χ^2=13.16; P=0.019; df =2) with respect to frequency of isolates and location. The Zonal distribution of the isolates, show that the highest Pneumococcal isolates were recorded in Northern and Southern Senatorial Zones(34.2% each),48% of *S. aureus* were from Central Senatorial Zone,100% of *M. catarrhalis* were from the Southern Zone (Table 4.1).Fig.4.2 is the proportion of *S. pneumoniae* isolates from the three Senatorial Zones of Kaduna State. The results show that Northern Senatorial Zone and Central Senatorial Zones recorded the highest number of isolates 34.0 % each, while the Southern Zone had 32.0%. The difference is statistically not significant (χ^2 = 0.73; 0.6219;d f=2). Table 4.2 shows antibiotic susceptibility of Pneumococcal isolates by senatorial zones of Kaduna State. The highest resistance to Penicillin was recorded in the Southern senatorial zone (56.1%) followed by Northern senatorial zone (50.0%), while the highest resistance to Erythromycin was recorded in the Central senatorial zone (46.2%) followed by Southern senatorial zone (42.9%). The highest antibiotic resistance recorded in Kaduna State in this study was recorded in the Southern senatorial zone (100%) against cefuroxime. High resistance was also recorded against Chloramphenicol (92.9%) in the NSZ, Ceftriaxone (85.8%) in NSZ and Tetracycline (78.6%) in SSZ. High susceptibility was recorded in NSZ against Azithromycin (78.6%).

Fig.4.3 shows Pneumococcal antibiotic resistance in Kaduna State. The highest resistance was recorded against Cefuroxime (75.8%) followed by Ceftriaxone (65.4%) and Ampicillin (65.9%).There was also high resistance to Penicillin (48.2%) and Erythromycin (41.6%) across the Acute-care health facilities in the state.

Fig.4.4 shows Pneumococcal susceptibility to Penicillin and Erythromycin in Kaduna State. There is higher resistance to Penicillin (48.1%) than Erythromycin (41.6%).

Table 4.3 shows pneumococcal resistance to Penicillin and Erythromycin (PRP and ERP) among patients based on three demographic factors. Subjects in age groups 50-59 and 20-29 had the highest prevalence for penicillin resistant pneumococci(PRP) with 22.6% each, followed by age groups(≥60, 40-49 and,10-19yrs) having 16.1% each. The highest resistance to erythromycin (ERP) was recorded among year group ≥60years with 27.0%, followed by 50-59 and 20-29 with 18.9% each; 40-49 had 13.5%, while 10-19 years had 10.8% respectively. The prevalence for PRP was higher in females (52.0%), while ERP was higher in males (54.2%). Higher prevalence of 70.8% for PRP and 73.9% for ERP was recorded among those who do not know their genotype followed by 29.2% PRP and 26.1% ERP for those with AA genotype. No resistant strains were recorded amongst those with AS and SS genotypes. In this study there was no significant association recorded in PRP and ERP withage but there was a significant association in genotypes (P=0.0134).

With respect to use of Antibiotics, those who have been on antibiotics (last 3 to 6 months) had higher prevalence of PRP (56.0%) and ERP (65.2%), this difference was not statistically significant. In relation to Alcohol consumption, those who did not consume Alcohol had 100%

prevalence of PRP and ERP. Statistically the difference was none estimable (**ne**).For cigarette smoking; those who do not smoke had higher prevalence, for PRP (83.3%) and ERP (86.4%).The difference was not statistically significant. Rate of pneumococcal resistance based on some predisposing risk factors. Those with lower economic status had higher PRP (52.2%) and ERP (54.5%) infections rate; statistically the difference was significant (P=0.0004), while those residing in semi-urban communities had higher resistant infection rates for PRP (44.0%) and ERP (41.7%); the difference not being statistically significant. In respect to marital status, the married participants had higher prevalence for PRP (62.5%) and ERP (65.2%).The Singles had 37.5% for PRP and 34.8% for ERP while the divorced, widowed and separated participants had no recorded cases of resistant pneumococci to Penicillin and Erythromycin, statistically there was no significant association(Table 4.3)

The rate of occurrence of resistant pneumococci to penicillin and erythromycin with reference to some underlying medical conditions as shown in Table 4.5in this study show Human immunodeficiency virus (HIV) negative individuals had the highest rates for PRP (64.0%) and ERP (70.8%); the difference was not statistically significant.Participants who did not know their Acid-fast bacilli (AFB) status had higher prevalence of 45.8% for PRP, while those who were not having Tuberculosis (TB negative) had higher prevalence for ERP (4 2.9%); though the difference was not statistically significant. Diabetic patients had higher prev alence for both PRP (94.4%) and ERP (88.9%);though the difference was not statistically Signi ficant Table 4.4

Out of the 41(9.11%) isolates of *Streptococcus pneumoniae* 31(88.6%) of them were Penicillin non susceptible (PNSP) [MIC1.0-15.5μg/mL; MIC_{50}=8.0μg/mL] and 31(86.1%) were Erythro mycin non susceptible (ENSP)[MIC 0.12-6.0μg/mL;MIC_{50}=0.90μg/mL]determined by E-test methodology (MIC test strips,LIOFILCHEM Products) in the study area as in Tab.4.5Figure 4.5 is showing MICs of Penicillin G and Erythromycin against resistant pneumococci in NSZ with MICs range 2.5-12.6 μg/ml based on CLSI-AST Break points (2015); 7(50.0%) were highly resistant to penicillin (MIC ≥8.0≤12.6 μg/ml) and 5(35.7%) were highly resistant to erythromycin (MIC ≥2.0≤6.0 μg/ml).

Fig.4.6 is showing MICs of Penicillin G and Erythromycin against resistant pneumococci in CSZ based on CLSI-AST Break points (2015); 6(46.2%) were highly resistant to penicillin (MIC ≥8.5≤15.5 μg/ml) and 6(46.2%) were highly resistant against erythromycin (MIC ≥1.2≤4.0 μg/ml).

Fig.4.7 is showing MICs of Penicillin G and Erythromycin against resistant pneumococci in SSZ based on CLSI-AST Break points (2015); 6(46.2%) were highly resistant to penicillin (MIC ≥8.0≤12.5 μg/ml) and 6(46.2%) were highly resistant to erythromycin (MIC ≥1.0≤4.0 μg/ml).

Fig.4.8 is showing Serotype distribution of six pneumococcal serotypes in the study area. Of the twelve resistant pneumococcal isolates (MIC 1.5-15.5μg/mL), 8(66.7%) were serotyped by PCR Serotyping; yielding 6(75.0%) different serotypes and 2(25.0%) non-type able isolates. The most common serotypes were: 3(33.3%), 20(16.7%), 13(33.3%), and 23F (16.7%) as in Table 4.6; showing the distribution of Pneumococcal serotypes with respect to MIC of Penicillin and Erythromycin.Highest Penicillin MIC (15.5μg/mL) was recorded against serotype 3 and MIC(6.0 μg/mL) for serotype 20.

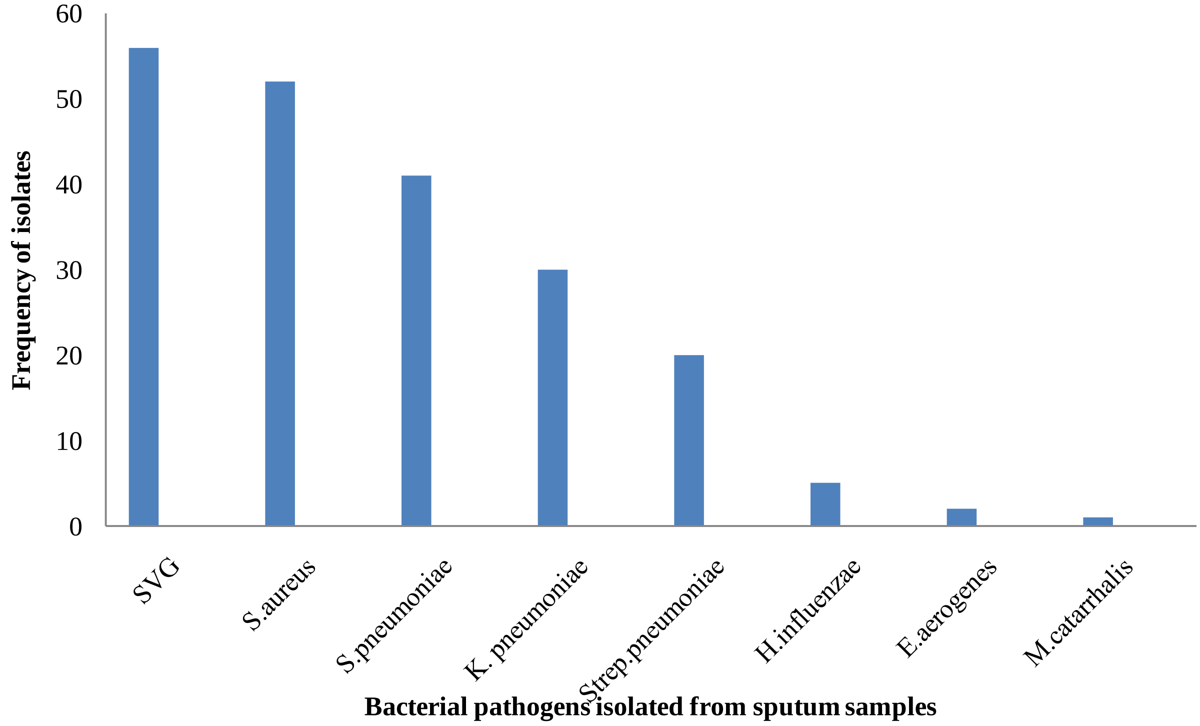


Fig. 4.1: Frequency of bacterial isolates from sputum samples in Kaduna State.

Table 4.1: Distribution of bacterial isolates from sputum samples collected from patients attending selected hospitals base on locations.

Isolates	freq.	NSZ n(%)	CSZ n(%)	SSZ n(%)
S.pneumoniae	41	14(34.2)	13(31.6)	14(34.2)
SVG	56	17(30.4)	22(39.3)	17(30.4)
S.pyogenes	20	5(25.0)	8(40.0)	7(35.0)
S.aureus	52	12(24.0)	24(48.0)	16(32.0)
K.pneumoniae	30	8(26.7)	13(43.3)	9(30.0)
H.influenzae	5	2(40.0)	0(0)	3(60.0)
E.aerogenes	2	2(100.0)	0(0)	0(0)
M. cattarhalis	1	0(0.0)	0(0)	1(100.0)

$\chi 2$=0.019; 13.16; df=2

Key: freq.-number of isolates; NSZ-Northern Senatorial Zone; CSZ-Central Senatorial Zone; SSZ-Southern Senatorial Zone; SVG-Streptococcus viridians' Group.

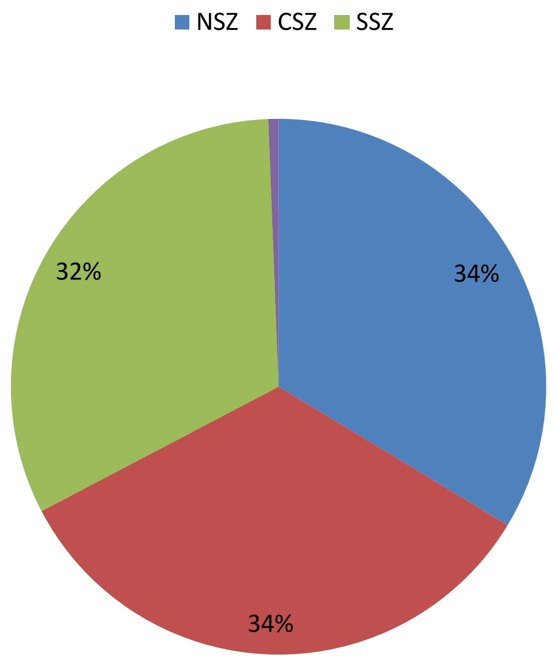
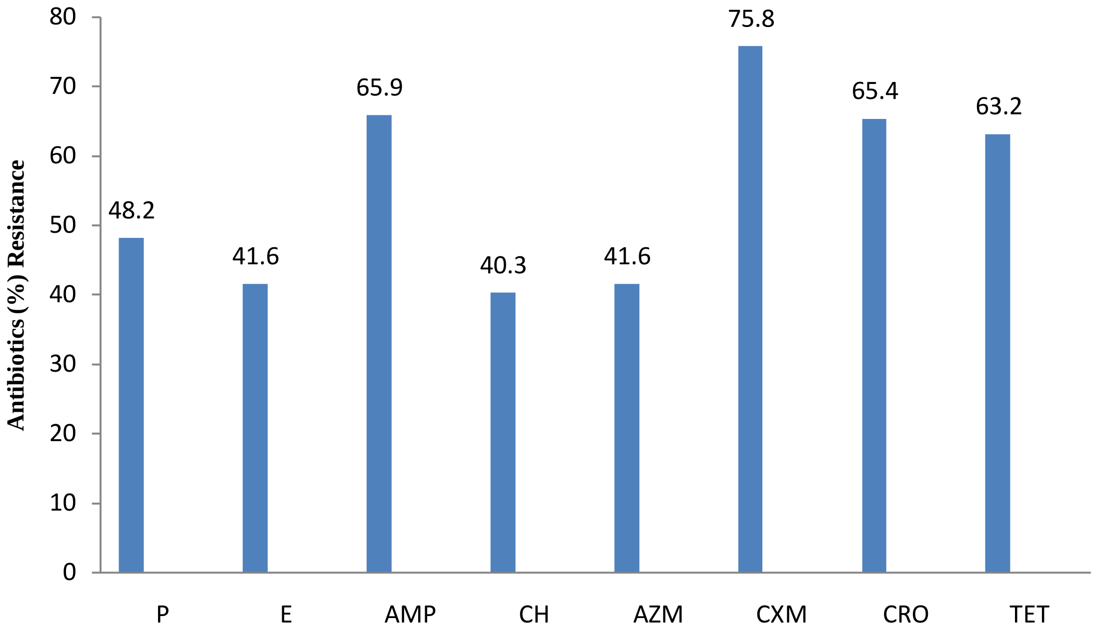


Fig. 4.2: Proportion of *S. pneumoniae* isolates by Senatorial Zones of Kaduna State.
Key: NSZ-Northern senatorial zone; CSZ-Central senatorial zone; SSZ-Southern senatorial zone

Table 4.2: Antibiotic Susceptibility of Pneumococcal isolates(n=41) to some antibiotics by Senatorial Zones of Kaduna State.

Antibiotics	NSZ (%)			CSZ (%)			SSZ (%)		
	S	I	R	S	I	R	S	I	R
P	7.1	42.9	50.0	15.4	46.2	38.5	21.3	28.6	56.1
E	7.1	57.1	35.8	15.4	38.4	46.2	14.2	42.9	42.9
AMP	0.0	28.6	71.4	7.7	23.1	69.2	21.4	21.4	57.2
CH	7.1	0.0	92.9	38.4	30.8	30.8	50.0	42.9	7.1
AZN	78.6	7.1	14.3	46.1	7.8	46.1	14.3	21.4	64.3
CXM	42.9	14.2	42.9	15.4	0.0	84.6	0.0	0.0	100.0
CRO	7.1	7.1	85.8	30.8	23.0	46.2	28.6	7.1	64.3
TET	21.4	21.4	57.2	38.5	7.7	53.8	7.1	14.2	78.6

Key: NSZ-Northern senatorial zone; CSZ-Central senatorial zone; SSZ-Southern senatorial zone; P-Penicillin; E-Erythromycin; AMP-Ampicillin; CH-Chloramphenicol; AZN-Azithromycin; CXM-Cefuroxime; CRO-Ceftriaxone; TET-Tetracycline



Antibiotics

Fig.4.3: Antibiotic Resistance (%) of Pneumococcal isolates from sputum samples in Kaduna State.

Key: P-Penicillin G; E-Erythromycin; AMP-Ampicillin; CH-Chloramphenicol; AZN-Azithromycin; CXM-Cefuroxime; CRO-Ceftriaxone; TET-Tetracycline

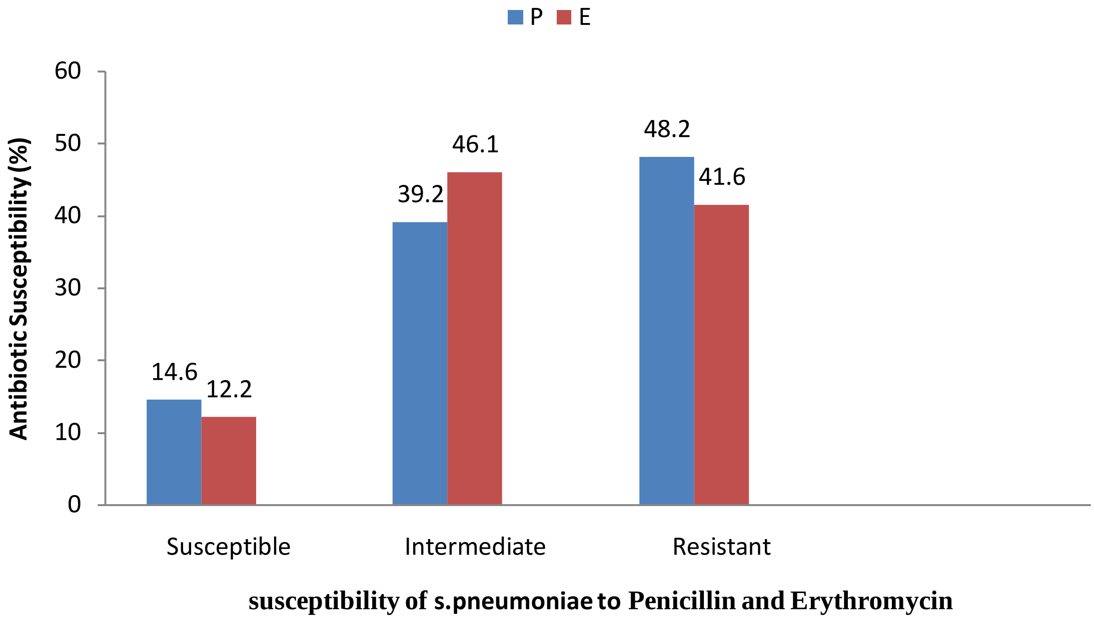


Fig.4.4: Distribution of *S. pneumoniae* susceptibility to Erythromycin and Penicillin
Key: P -Penicillin, E-Erythromycin

Table 4.3 Prevalence of Penicillin and Erythromycin resistant pneumococci according to some demographic factors associated with patients in the three senatorial zones.

Characteristics	PRP	ERP	χ^2-Value	P value
Age (yrs)	n=31	n=37	1.960	0.8547
10-19	5(16.1)	4(10.8)		
20-29	7(22.6)	7(18.9)		
30-39	2(6.5)	4(10.8)		
40-49	5(16.1)	5(13.5)		
50-59	7(22.6)	7(18.9)		
≥60	5(16.1)	10(27.0)		
Sex	n=25	n=24		
Male	12(48.0)	13(54.2)	0.186	0.6659
Female	13(52.0)	11(45.8)		
Genotype	n=24	n=23		
AA	7(29.2)	6(26.1)		
AS	0(0)	0(0)		
SS	0(0)	0(0)		
Don't know	17(70.8)	17(73.9)	10.056	0.0134

Key: PRP-Penicillin resistant Pneumococci; ERP-Erythromycin resistant Pneumococci; χ2-Chi-square; P-Probability value

Table 4.4: Acquiring Pneumococcal infections with some risk factors among patients attending selected hospitals in Kaduna State

Characteristics	PRP	ERP	χ^2-Value	P value
Antibiotics use	n=25	n=23		
Yes	14(56.0)	15(62.2)	0.427	0.5136
No	11(44.0)	8(34.8)		
Alcohol consumption	n=24	n=22		
Yes	0(0)	0(0)ne		
No	24(100)	22(100)		
Cigarette smoking	n=24	n=22		
Yes	4(16.7)	3(13.6)	0.082	0.7746
No	20(83.3)	19(86.4)		
Economic status	n=23	n=22		
High	0(0)	0(0)	18.025	0.0004
Moderate	11(47.8)	10(45.5)		
Low	12(52.2)	12(54.5)		
Residential location	n=25	n=24		
Urban	6(24.0)	6(25.0)		
Semi-urban	11(44.0)	10(41.7)		
Rural	8(32.0)	8(33.3)	0.027	0.9865
Marital status	n=24	n=23		
Married	15(62.5)	15(65.2)	0.038	0.8463
Single	9(37.5)	8(34.8)		
Divorced	0(0)	0(0)		
Widowed	0(0)	0(0)		
Separated	0(0)	0(0)		

Key: PRP-Penicillin resistant Pneumococci; ERP-Erythromycin resistant pneumococci; χ^2-Chi-square; P-Probability value; ne- none estimable

Table 4.5: Acquiring Penicillin and Erythromycin resistant pneumococcal infections based on some under laying medical conditions among patients attending selected hospitals in Kaduna State

Characteristics	PRP	ERP	χ^2-Value	P value
HIV status	n=25	n=24		
Positive	3(12.0)	2(8.4)	0.302	0.8597
Negative	16(64.0)	17(70.8)		
Don't know	6(24.0)	5(20.8)		
AFB Status	n=24	n=21		
Positive	4(16.7)	4(19.0)	0.276	0.8713
Negative	9(37.5)	9(42.9)		
Don't know	11(45.8)	8(38.1)		
Diabetes	n=18	n=18		
Diabetic	1(5.6)	2(11.1)		
Non-diabetic	17(94.4)	16(88.9)	0.370	0.5429

Key: PRP-Penicillin resistant Pneumococci; ERP-Erythromycin resistant pneumococci; χ^2-Chi-square;P-Probability value; HIV-Human immunodeficiency Virus; AFB-Acid Fast Bacilli;

Table 4.6: Antibacterial susceptibility of Penicillin G and Erythromycin (E-Test) method

Antibiotics	n	% Susceptibility			MIC(µg/mL)	
		S	I	R	range	MIC_{50}
PEN G	35	11.4	45.7	42.9	1.0-15.5	8.0
ERY	36	13.9	52.8	33.3	0.12-6.0	0.90

Key: PEN G-Penicillin G, ERY-Erythromycin, MIC_{50}-MIC at 50%, S-susceptible, I-intermediate, R-resistant.

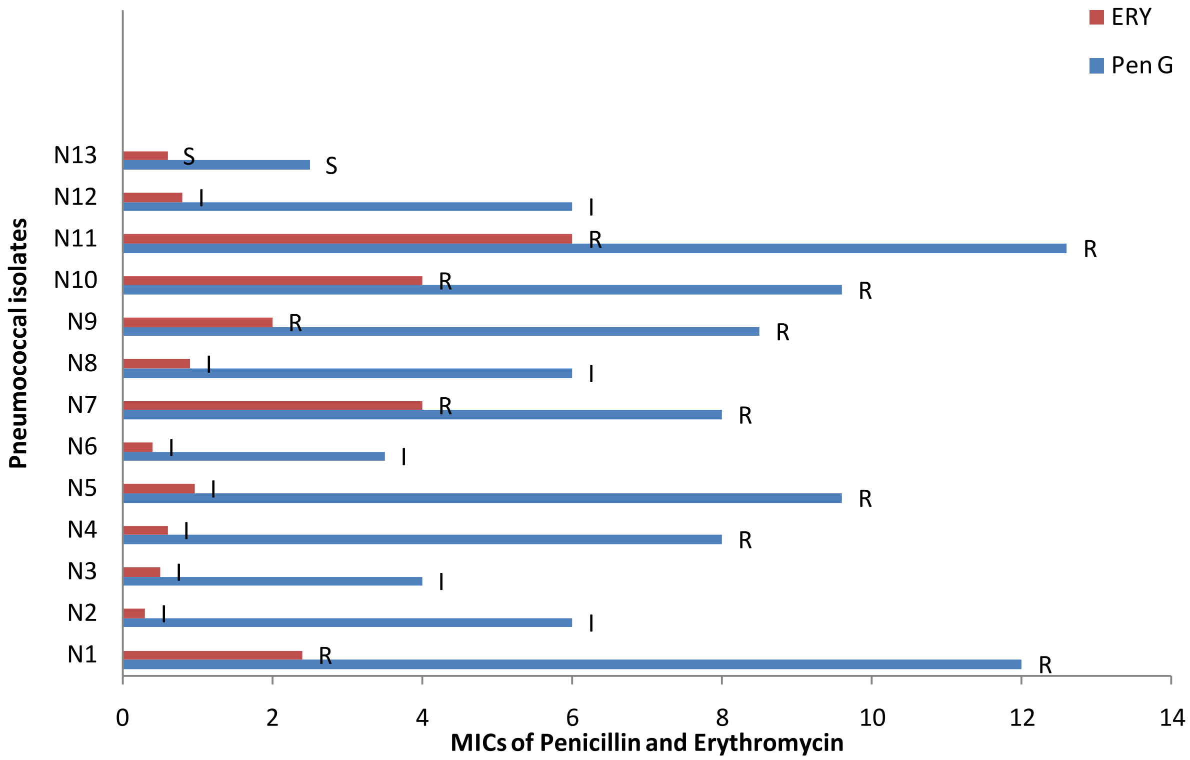


FIG4.5: Minimum Inhibitory Concentrations (µg/mL) of Erythromycin and Penicillin against resistant *S. pneumoniae* in NSZ with CLSI –AST Break points.

Key: S-susceptible, I-intermediate, R-resistant; Key: S-susceptible, I-intermediate, R-resistant; CLSI-Clinical and Laboratory Standards Institute; N-sample number from Northern Senatorial Zone.

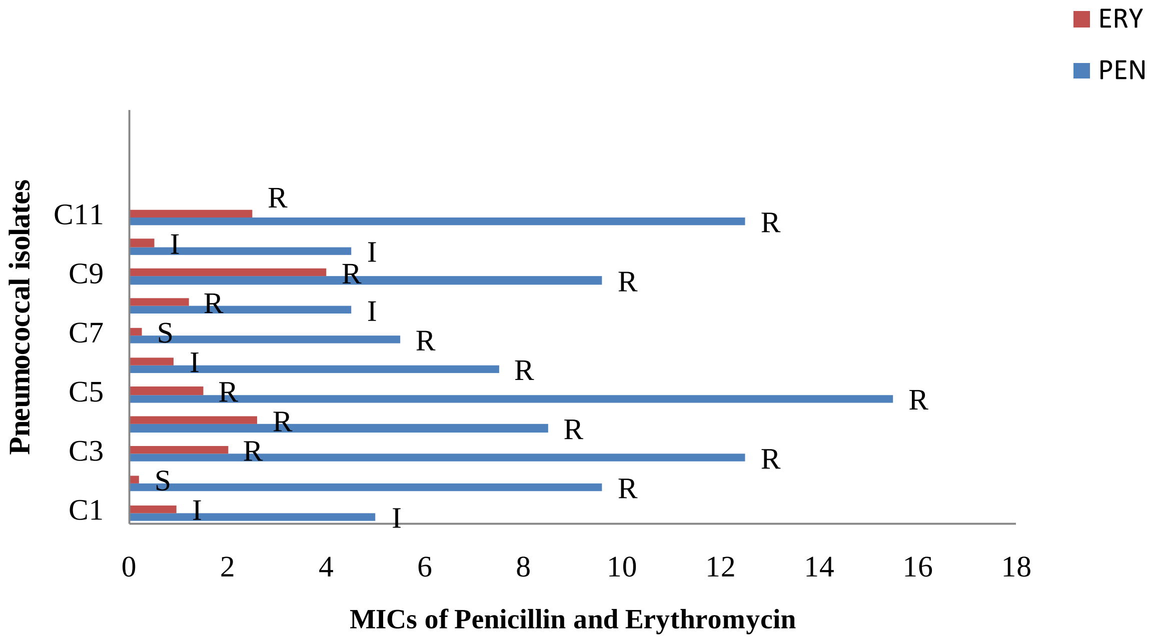


FIG.4.6:Minimum Inhibitory Concentrations (μg/mL) of Erythromycin and Penicillin against resistant *S. pneumoniae* in CSZ with CLSI –AST Break points.

Key: S-susceptible, I-intermediate, R-resistant; Key: S-susceptible, I-intermediate, R-resistant; CLSI-Clinical and Laboratory Standards Institute.

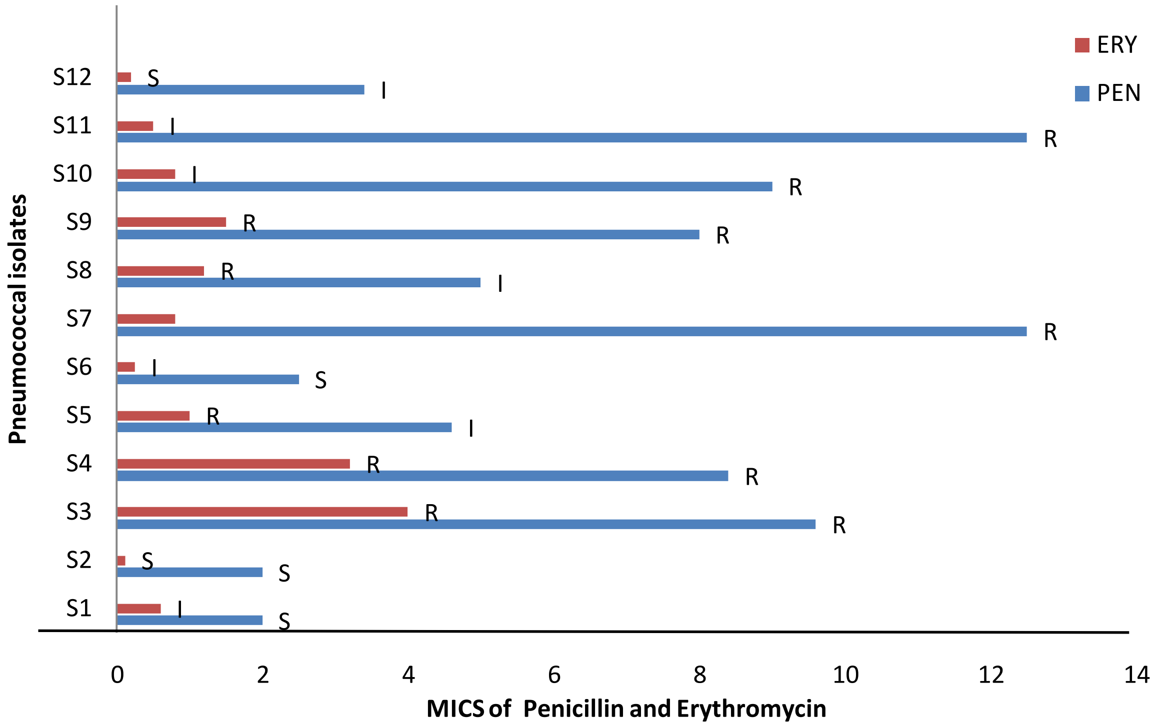


FIG.4.7: Minimum Inhibitory Concentrations (µg/mL) of Erythromycin and Penicillin against *Streptococcus pneumoniae* in SSZ with CLSI- AST - Breakpoints.

Key: S-susceptible, I-intermediate, R-resistant; CLSI-Clinical and Laboratory Standards Institute.

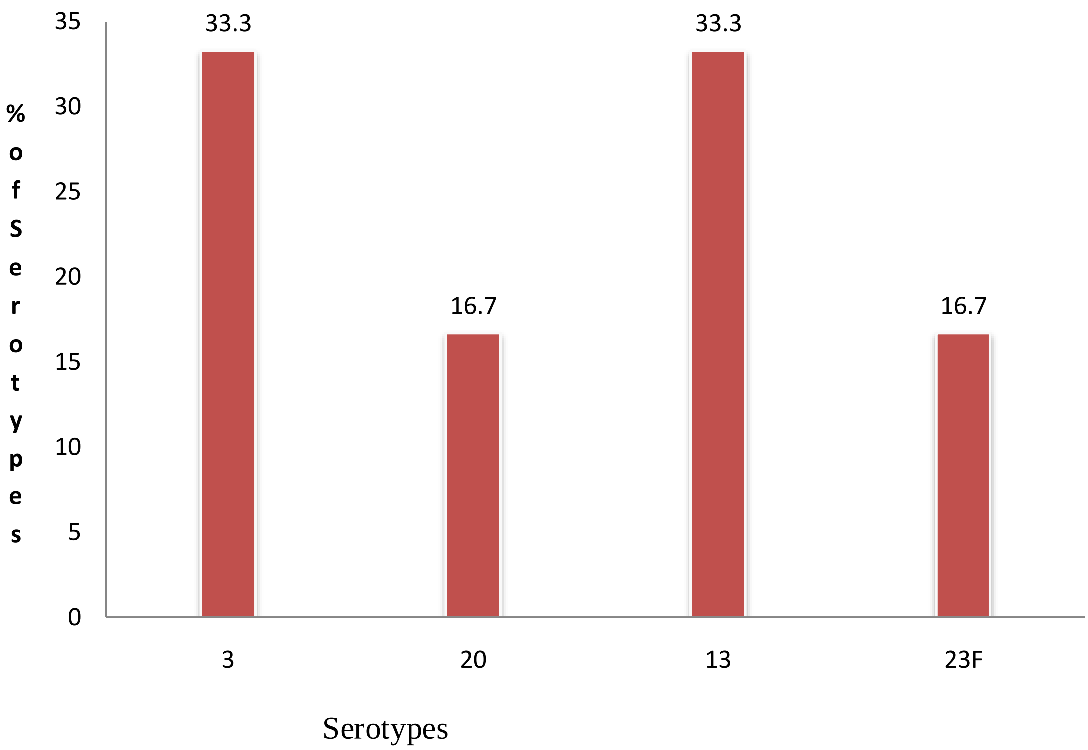


FIG.4.8: Pneumococcal Serotypes distribution in Kaduna State

Table 4.7 is distribution of resistance genes detected based on MIC range (µg/mL).

PCR assay for simultaneous detection of *lytA,* Penicillin resistance genes (altered *PbP1a*, *PbP2x* and *PbP2b*) and Macrolide resistance genes [*erm (B)* and *mef (A)*] was carried out. PCR results in this study showed *lytA* detected in the samples. Gene determination carried out in this study detected 1(11.1%) strains with *PbP1a* gene; 2(22.2%) strains with *PbP2x* gene and in none of the strains was *PbP2b* gene detected, while 3(33.3%) strains had *erm (B)* and 3(33.3%) strains had *mef (A)* gene. None of the strains had both *erm (B)* and *mef (A)* genes and 3(33.3%) strains had neither *erm (B*) nor *mef (A)(*Table 4.8). Agarose –gel Electrographs of the PCR products are shown in plate's I-.VI.

Table 4.7: Distribution of Pneumococcal serotypes with Minimum inhibitory concentrations (MICs) of Penicillin and Erythromycin.

Serotype	MIC(µg/l)	
	PEN G	ERY
13	12.0	2.4
ND	9.6	4.0
20	12.6	6.0
3	15.5	1.5
3	9.6	4.0
ND	9.6	3.5
23F	9.0	4.0
13	8.4	3.2

Key: ND-Not detected, PEN G-Penicillin G

Table 4.8: Minimum inhibitory concentrations (MICs) of Erythromycin and Penicillin according to detected resistance genes in *Streptococcus pneumoniae* from Kaduna State

PCR Results	MIC range (µg/ml)				
	≥0.001	≥0.01	≥ 0.1	≥1.0	≥10.0
mef(A)	0	0	0	2	1
erm(B)	0	1	1	1	0
mef(A) +*erm(B)*	0	0	0	0	0
PbP1a	0	0	1	0	0
PbP2b	0	0	0	0	0
PbP2x	0	0	0	2	0

KEY: MRGs-Macrolide resistance genes; mef(A)-Macrolide efflux gene A; erm(B)-Erythromycin Ribose methyltransferase gene B; PbP(1a,2b,2x)-Penicillin binding Proteins

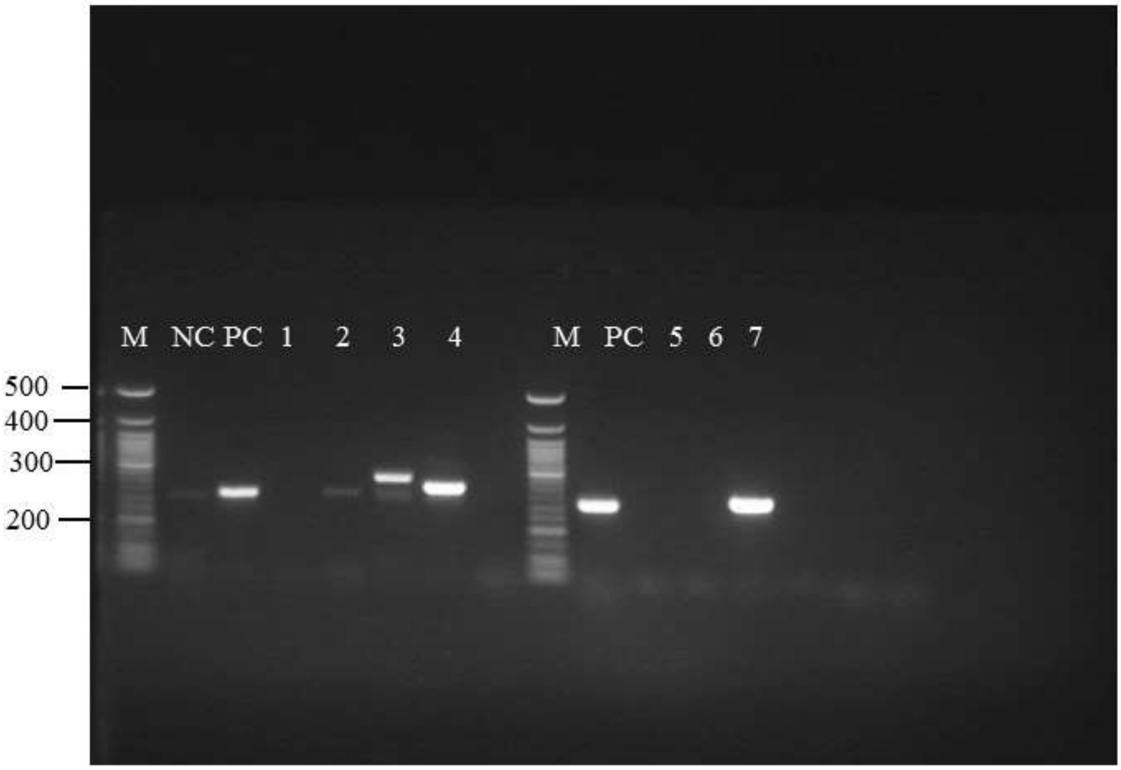


Plate 1: Agarose-gel electrograph of PCR products generated with distinct bands at Lane 7 (*ermB*- 224bp);Lanes 4(*mefA*- 294bp) fragments amplified; Lanes 1,2,5 and 6 are samples showing no amplicons; Lane 3 is a distinct amplicon of pneumococcal *lyt A*(295bp).

Key: M-Marker (100bp); Negative control (NC); Positive control (PC).

NC: Water + all PCR Components; **PC:**DNA template of *S.pneumoniae* ATCC 49619 + all PCR Components.

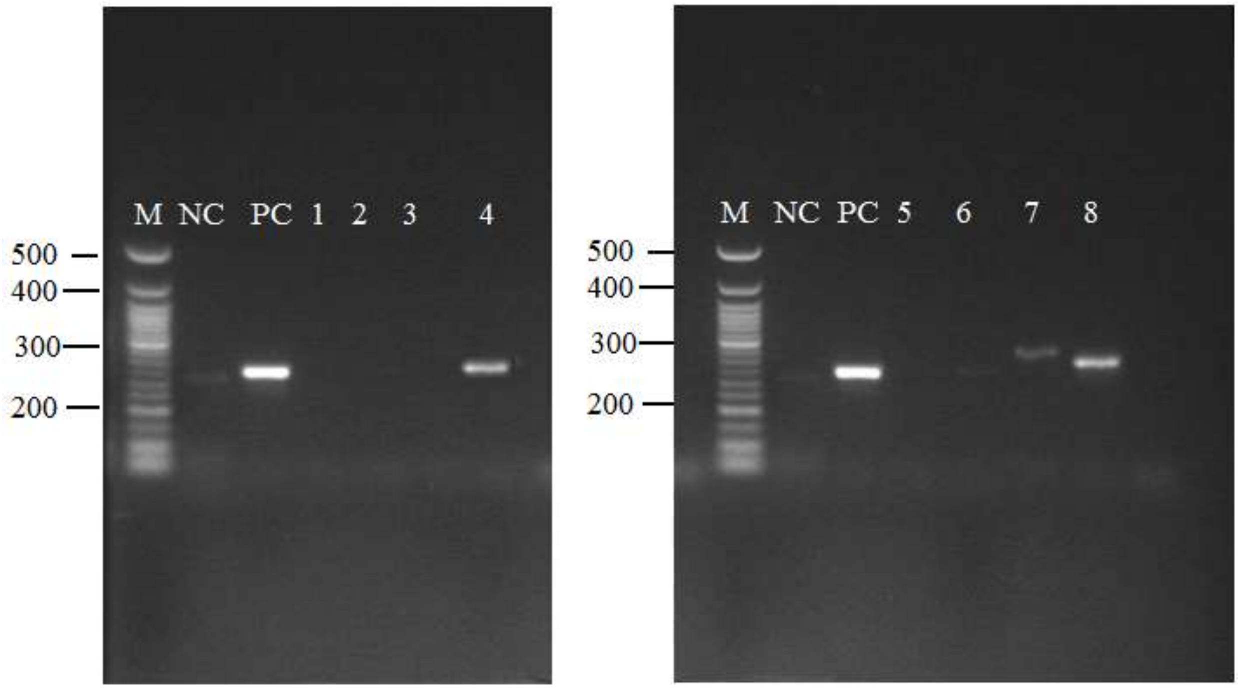


Plate 1I and III: Agarose-gel electrograph of PCR products generated with distinct bands of Lane 4 (*ermB*- 224bp);Lane 8 (*mefA*- 294bp); Lane 7 is a distinct amplicon of *lytA*(295-bp) fragments amplified; Lanes1,2,3,5 and 6 are samples showing no amplicons.
Key: Marker (M); Negative control (NC); Positive control (PC).

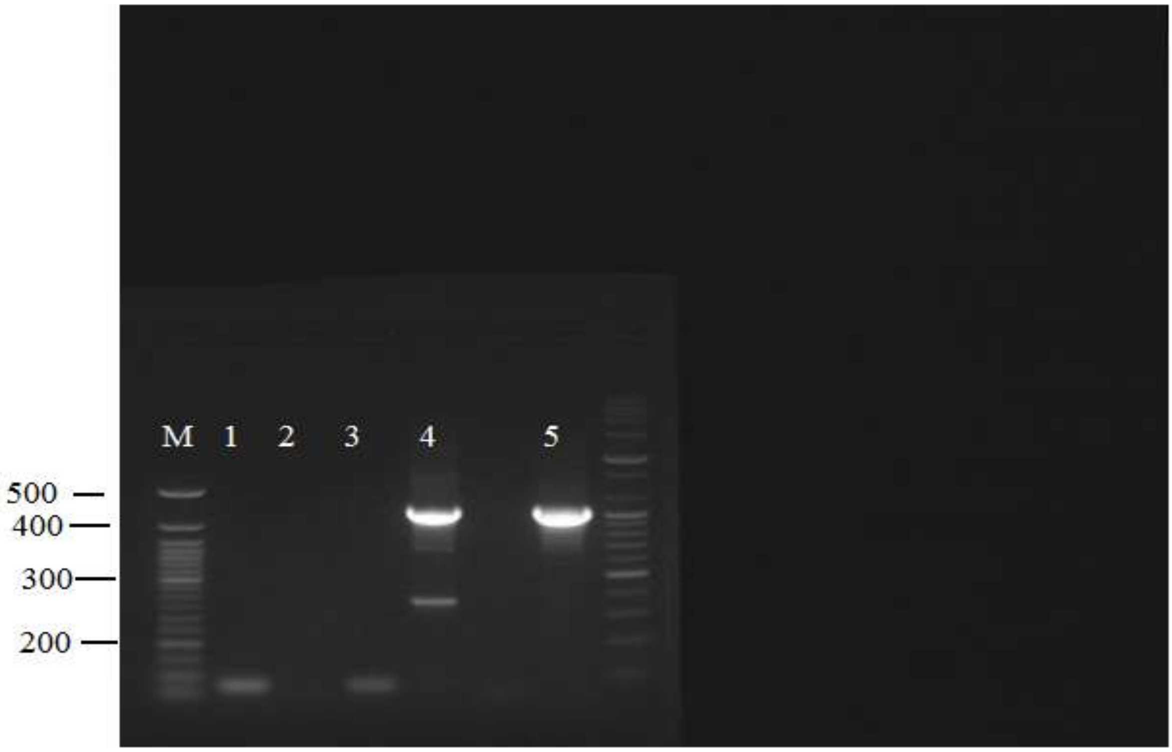
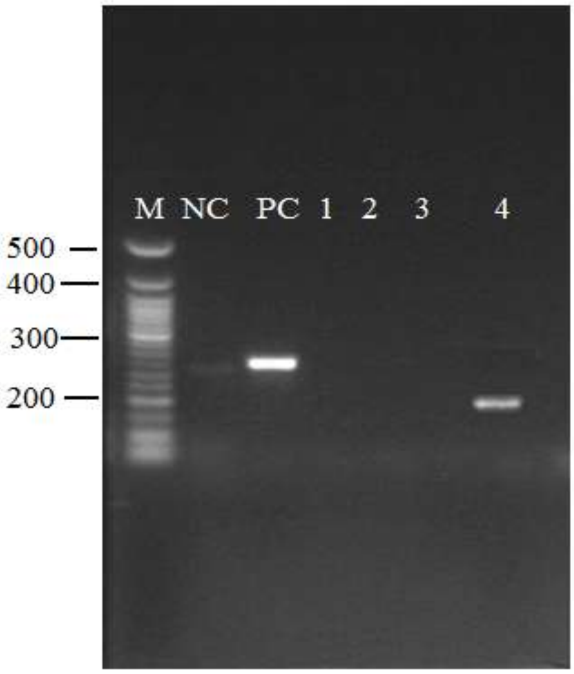
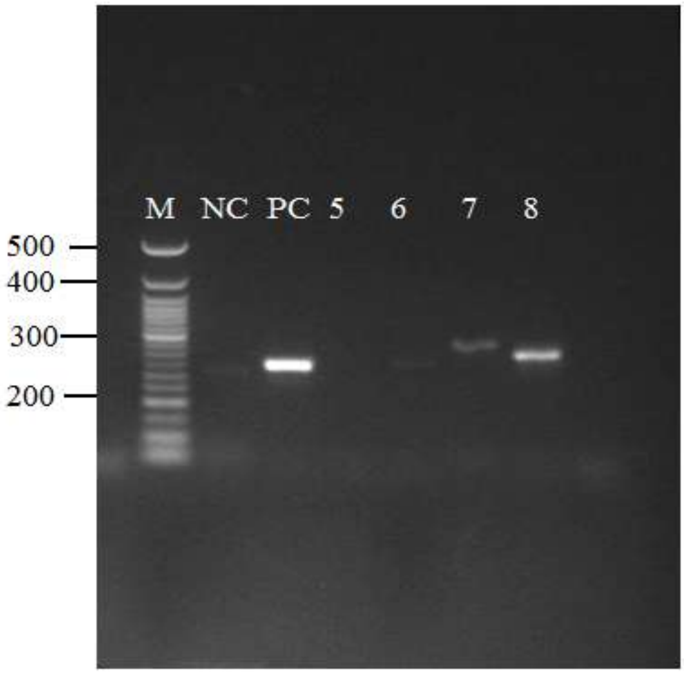


Plate IV : Agarose-gel electrograph of PCR products generated with distinct bands of Lanes 1 and 3(PbP2x- 147bp); Lane 4 (mefA- 294bp) are fragments amplified; Lanes 2 and 5 is sample showing no amplicon.

Key: Marker (M).





PlatesV and VI: Agarose-gel electrograph of PCR products generated with distinct bands of Lane 4(PbP1a- 195bp);Lane 8 (mefA- 294bp); Lane 8(ermB-224bp) fragments amplified;Lanes1,2,3,5 and 6 are samples showing no amplicons.

Key: M-Marker; NC-Negative control; PC-Positive control.

Table 4.9: Sequences producing significant alignment of detected serotypes with isolates deposited at NCBI GenBank

Sample code	Description (Strain)	Serotype	Query (%)	Identity (%)	E-value	Accession No
Z5942	34365	20	100	95	0.0	CR9316791
Z307	34357	13	100	95	0.0	CR9316611
Z569	52402	3	100	95	0.0	Z472101
Z025	OXC141	23F	100	95	0.0	HE9835241

CHAPTER FIVE

DISCUSSION

Streptococcus pneumoniae is both an aggressive pathogen and a normal part of the human microbiome (Blaschke, 2016). *Streptococcus pneumoniae* infections are a leading cause of illness in young children, the elderly and persons with debilitating medical conditions (Schrang *et al*., 2001).

This research documented a Pneumococcal prevalence of 9.1 % which did not agree with other researchers (Mohammad et al., 2011,Iliyasu *et al*., 2014 and Nicholas *et al*., 2013) who reported higher rates of 21.5%, 60% and‘=71% respectively. This possibly may be due to the exclusion of children (≤9years of age) who have higher Pneumococcal carriage (Nicholas *et al*., 2013).Viridian’s Streptococci bacteria are generally disregarded as the etiologic agent of pneumonia even when isolated from cultures of sterile material. The isolation of Streptococcus viridian’s Group in this study is less compared to findings by Freitas *et al.*, (2006).This may be due to chance, or the exclusion of younger patients(≤9years of age) in this study.

Higher resistance to erythromycin and other macrolides used in this study as alternatives to ß-lactams for treatment of Respiratory tract infections(RTI) was recorded as compared to works of some researchers (Abla *et al*., 2013; David *et al.*, 2015), but lower than that recorded by Mohammad *et al*.,2011. Erythromycin resistance in some Countries was found to be higher than those recorded in this study (Song *et al*.,2004;Reinert,2005;Carmargos,2006; Jenkings,2008).This difference might be related to the shift in use of other antibiotics in these areas(Dankor *et al.*,2010). Although the Penicillins had been the drug of choice worldwide for the treatment of pneumococcal infections, a high pneumococcal resistance was recorded for this group of antibiotics as reported by some researchers in Iran and Japan (Sahm *et al.*, 2000 and Pallares *et al*., 2005).

In this study higher prevalence of Penicillin resistant pneumococci (PRP) 20.6% and Erythromycin resistant pneumococci (ERP) 27.0% was obtained among the elderly of age ≥50years which is in agreement with findings by Nicholas *et al.* (2013), the reason probably, they are immunocompromised. Year group 20-29 had higher prevalence for penicillin resistance, which is similar to findings by Mohammed, (2013) probably due to nature of exposure to more risk factors such as drug abuse, HIV and TB that could lower the immunity. The males in this study had higher rates of PRP, similar to findings by Schrang *et al.,* (2001), probably due to more exposure to risk factors associated with antibiotic resistance. David *et al.* (2005) reported higher prevalence of PRP and ERP among patients with AA genotype but this could not be established in this study as those who did not know their genotypes had higher rates. This was surprising since patients with Sickle cell disease (SCD) are at higher risk of fatal sepsis with encapsulated pneumococci because of the inherent auto splenectomy that occurs with SCD (Navalkelle, *et al.*, 2017).

Understandably, Antibiotics previous use, age and Hospitalization had been identified as important risk factors to resistance (WHO, 2001).Higher resistance rates were obtained for those who had previous use of antibiotics; Penicillin 56.0% and Erythromycin 62.2% in this research.This is consistent with works carried out by some researchers (Kuster *et al.*, 2014 and Song *et al.*, 2004).

HIV infection and other underlying medical conditions associated with immune deficiency greatly increase the likelihood of contracting pneumococcal disease (WHO, 2007).The prevalence of PRP and ERP among HIV positive Participants and other comorbidities was low in this work compared to findings by Jordano *et al*. (2004), who recorded more than 60 times higher risk of invasive pneumococcal disease among HIV-infected patients than in population without known HIV-infection.

There were not many variations in ecologic distribution of PRP and ERP in the different locations, in this research. This finding agrees with that reported by Nicholas *et al*. (2013) in a study carried out in Ghana.

Penicillin has been the drug of choice worldwide for the treatment of pneumococcal infections. However, penicillin resistant strains have emerged resulting in a shift to the use of other drugs e.g. cefotaxime, chloramphenicol and erythromycin (Dankor *et al*., 2010).

Investigations from some parts of Nigeria and other countries have shown high prevalence of resistance to Penicillin and other agents. Penicillin resistant pneumococci (PRP) and Erythromycin resistant pneumococci (ERP) had incidenceof20.5% and 29.4% respectively, in this study .The prevalence of PRP closely agrees with findings by Akpede *et al*. (1994) but way below findings' by Habib *et al*. (2003), and Kadankai *et al*. (2009) who recorded prevalence of 85.4% and 93.0% respectively, however rates in this work were higher than that reported by some researchers (Emele, 2000; Oduyebo *et al*., 2006; Abla *et al*., 2013) likely due to chance or differences in the predisposing factors peculiar to the study locations.

Those who were never hospitalized in the last 6-months had higher prevalence rates of PRP and ERP in this study. Patients with Health Care Associated Pneumonia HCAP are at higher risk for multidrug-resistant (MDR) organisms. However, several studies have shown that many patients defined as having HCAP are not at high risk for MDR pathogens (Chalmers *et al*.,2014;Gross *et al*.,2014).Although interaction with the healthcare system is potentially a risk for MDR pathogens, underlying patient characteristics are also important independent determinants of risk for MDR pathogens(File,2016).

Higher resistance rates were obtained for those who had previous use of antibiotics.This is consistent with works carried out by some researchers (Kuster *et al*., 2014 and Song *et al*., 2004).

There are more than 90 types of pneumococcal bacteria. PCV13 protects against 13 types. PCV13 also provides protection against pneumonia (lung infection) (WHO, 2007).

WHO in 2007 recommended the use of pneumococcal conjugate vaccines (PCV) in all countries, urging that the highest priority for introduction be given to countries with high under-five mortality due to Pneumococcal infections. In this study 100% resistance was recorded among recruits to this research who had not received any PCV in their life time. Understandably, Nigeria only joined other African countries to launch pneumococcal conjugate vaccine (PCV10) as part of its Routine immunization schedule on 22nd December, 2014(WHO, 2015).

There were not many variations in ecologic distribution of PRP and ERP in the different locations in this research. This finding agrees with that reported by Nicholas *et al*. (2013) in a study carried out in Ghana.

Pneumococci are normally exquisitely sensitive to penicillin, which is lethal at concentrations <0.1 μg/mL. Typical penicillin MICs for highly resistant strains is 4–8 μg/mL. The rates of penicillin resistance (MICs ≥ 2 mg/liter) ranged from 0% (India and Philippines) to 71.4% (Vietnam), and the rates of resistance to non-beta-lactam agents varied in a similar manner. In this study high-level (MIC ≥2µg/ml) PRP and ERP has been associated with bacterial pneumonia which is similar to findings by Metlay *et al*., (2000);Fukushima *et al*.,(2008) and Abla *et al*.,(2013).

Accurate serotyping is essential for epidemiologic study of *S. pneumoniae* [CDC, (nd)]

The introduction of Pneumococcal conjugate vaccines necessitates continued monitoring of circulating strains to assess vaccine efficacy and replacement serotypes (Marcus *et al*., 2012).Whereas vaccination has succeeded in dramatically reducing diseases but pneumococcus remains a poorly controlled outlier. The huge number of pneumococcal serotypes (compared to the other pathogens) remains the main challenge in developing strategies to control this global pathogen (Birgitta and Elaine, 2013).There are over 90 different capsular serotypes of *Streptococcus pneumoniae*. The serotypes Sequenced and identified in this study were serotypes 3, 13, 20 and 23. Serotype 3 is included in the 10-valent Pneumococcal Conjugate Vaccine (PCV), the 23F serotype is included in the heptavalent conjugate vaccine. The finding is not in complete agreement with findings of Onyemelukwe and Greenwood,1998 who identified serotypes 1,2 3, and 5 in Zaria and Abla *et al*., 2013 in Algeria, probably because their subjects comprises both children and adults. Serotypes 13 and 20 are non-vaccine types. Serotypes 3 and 23 are also among the dominant serotypes as reported by WHO, 2007.The findings are also comparable to that obtained by Tarrago *et al*., 2008; Dankor *et al*., 2014 but completely differs from that reported by Falade *et al*., 2009.

The common resistance genes detected by PCR in this work were *PbP1a*, *PbP2x*, *erm (B)* and *mef (A)*.Frequency of genes detected did not agree with that of Song *et al*., 2004; Zettler *et al*., 2004 and Mohammad *et al*., 2011,but was consistent with findings of Fukushima *et al*., 2008; the differences may possibly be attributed to differences in geographical locations and exclusion of children in this study.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Four hundred and fifty (450) subjects were sampled in this study comprising of 237(52.7%) males and 213(47.3) females. Two hundred and seven (46%) pathogens of the LRT infections were isolated across the Senatorial Zones of Kaduna State, Nigeria. A Pneumococcal prevalence of 9.1% was recorded. The highest antibiotic resistance was against Cefuroxime(75.8%) and the least was against Azithromycin(40.3%).Pneumococcal resistance to erythromycin was 41.6% and penicillin(48.2%).The highest PRP(22.6%) and ERP(27.0%) was recorded among patients' ≥ 50 years of age. An MIC_{50}=8.0µg/mLfor penicillin and $MIC_{50==}$0.9 µg/mLfor erythromycin were established in the study area. Four serotypes were detected in this study including serotypes 3,13,20 and 23F,while resistance genes determined were *PbP 1a*,*PbP2b*, *ermB* and *mefA*.

6.2Conclusion

The highest pneumococcal resistance to penicillin and erythromycin was obtained among patients' ≥50 years of age. Economic status of the patients has a significant relationship to Penicillin and Erythromycin resistance probably due to antibiotic abuse and empiric treatment with antibiotics. A prevalence rate of 19.8% pneumococcal infection was established in this study. The study shows that the isolates did not only show resistance to penicillin but also to other alternative antibiotics such as Erythromycin, Azithromycin, Tetracycline, and cefuroxime and should be used carefully in the future. The indiscriminate use of antibiotics at the community level might be probable cause of resistance obtained in this work. It is also pertinent to note that the resistance pattern shown by Penicillin and Erythromycin discourages a common practice in some of our Health care institutions where sensitivity is done on one antibiotic and another is used for treatment due to availability or cost implication. The implication of this study is a high potential for treatment failure with most antibacterial drugs recommended to empirically treat lower respiratory tract infections. Higher prevalence of *Streptococcus viridians Group (SVG)* recorded in this study than *Streptococcus pneumoniae* implies that the diagnosis of pneumococci can be problematic and if not chanced occurrence; only the use of specific gene targets may solve the problem of inaccurate identification of pneumococci. Serotype 3 and 13 are the most prevalent serotypes causing disease in this study.The study of erythromycin and penicillin resistance genes is imperative for diagnostic purposes, especially for pneumococci that carry *erm(B)* and *mef(A)* genes and harbor PBPs; since *erm(B)* confers high level resistance and *mef(A)* low-level resistance to macrolides while pneumococci harboring PBPs prevent binding and making them indifferent to ß-lactams and also decrease their binding affinity with the drugs. Pneumococcal Macrolide and Penicillin resistance in the Study area is predominantly mediated by *erm(B)* and *mef(A)* gene which encodes for high-level erythromycin methylase and low-level efflux mediated resistance.

6.3 Recommendations

1. Screening for pneumococcal vaccination status should be authorized in patients' ≥ 65 or older. One dose of PCV13 is recommended for all adults' ≥65 years of age.
2. Smoking cessation should be a goal for hospitalized patients with community-acquired pneumonia (CAP) who smoke.
3. Despite the use of empiric therapy, testing for a microbial diagnosis is important in clinical or epidemiologic settings.
4. The practice where sensitivity is done for one antibiotic and another is used for treatment must be discouraged as it is likely to fast track emergence of resistant pneumococci.
5. Monitoring of other kinds of resistance mechanisms is highly recommended.

6. Events that reduce selective pressures deriving antimicrobial resistance must be encouraged such as: More appropriate use of antimicrobials, Vaccination must be enforced (PCV and PPSV) .
7. Our ACHC facilities must put in place policies that will guarantee protection and safety of patients, their relatives and health personnel.
8. Indiscreet use of antibiotics should be restrained to limit the surfacing of resistant strains.
9. Monitoring of sensitivity patterns, emergence of resistant strains as well as MDR strains of *Streptococcus pneumoniae* must be done continuously at the local as well as globally.
10. Antibiotic regimens must be started as soon as diagnosis of CAP is considered likely.
11. Penicillin as the best empirical choice for treatment of infections with *S. pneumoniae* needs to be reconsidered. The use of penicillin as the best choice for treatment of infections with *S. pneumoniae* may need reconsideration because of the emerging trends in the antibiotic resistance.
12. Further study is required to fully determine:

(i) Presence of Non-encapsulated Streptococcus pneumoniae(NESP) in the country

(ii) Presence of Pneumococci in culture negative samples

(iii) Surface protein unique to NESP enhancing colonization and virulence despite their lack of capsules.

6.4 Contributions of the Findings

1. There was a 9.11% incidence rate of *Streptococcus pneumoniae* and serotypes 3, 13, 20,23F are common among patients presenting sputum samples at Acute-care Health facilities in Kaduna state, Nigeria.
2. The occurrence rate of Penicillin resistance among the isolates was 20.5% while it was 29.4% for Erythromycin in the area (Lower than 56.0% those of other reports elsewhere).

3. Mutations among three Pneumococcal strains are seen in *PbP1a* and *PbP2x* while six strains expressed ermB and mefA genes as mediators of drug resistance with Serotypes 3 and 13 showing highest frequency.

4. Finding suggests the use of more specific gene targets in the near future to solve the problem of inaccurate identification of Pneumococcal infections of URT.

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APPENDICES

APPENDIX I

KADUNA STATE ETHICAL APPROVAL

MINISTRY OF HEALTH, KADUNA STATE

All Communication to be addressed to:
THE HON. COMMISSIONER
Quoting Reference and Date
Telephone: 234-248048
Website: ttp://www/moh.kd.gov.ng.
Email: info@moh.kd.gov.ng.

Independence Way,
P.M/;B 2014
Kaduna.
Kaduna State, Nigeria.

MOH/ADM/744/VOL.I/265

15th April, 2015

Health Research Ethical Committee Kaduna State
Ministry of Health.

To: ....

Ministry of Health Research Ethical Clearance

Re:- Determination of Erythromycin and Penicillin Resistance Genes in *Streptococcus pneumoniae* from Patients Attending Long-term Health Care Facilities (LTHCFs) in Kaduna State.

This is to inform you that the research described in the submitted protocol, the consent form and other participant information materials have been reviewed and given approval by the Health Research Ethics Committee.

Name of Principal Investigator:	Lusa Haruna Yadock
Date of Receipt of Application:	23rd February, 2015
Date of Ethical Approval:	14th April, 2015
Research Period:	3 years

You are kindly requested to give researchers maximum cooperation during the period of research.

However researchers are kindly requested to submit a copy of their findings to the State Ministry of Health please.

F.A. KURAH
Secretary Research Ethical Committee

APPENDIX II

ABUTH ETHICAL APPROVAL

HEALTH RESEARCH ETHICS COMMITTEE

AHMADU BELLO UNIVERSITY TEACHING HOSPITAL

SHIKA - ZARIA, NIGERIA.

E-mail: abuthshika@yahoo.com

website: www.abuth.org

Chairman of Board: Chief. Shuaib Oyedokun Afolabi
Chief Medical Director: Prof. Lawal Khalid, MBBS, FMCS, FWACS, FRCS(ED)
Chairman, Medical Advisory Committee: Prof. Abdullahi Mohammed, MBBS, FWACP, FICS
Director of Administration: Barr. Ishak Bello, LL.B, BL, LL.M, PGDM, ANAN, FCAI

ABUTH/HREC/CL/05

Our Ref: ____________

8th June, 2017

Your Ref: ____________

Date: ____________

ABUTH HREC FULL ETHICAL CLEARANCE CERTIFICATE

Determination of the presence of Eryhromycin and Penicillin Resistance Genes in Strepto Coccus Pneumoniae from Patients attending Acute-Care Health Care(ACHC) Facilities in Kaduna State.

ABUTH Ethics Committee assigned number:	-	ABUTHZ/HREC/S2/2015
National Health Research Ethics Comm. NO.	-	NHREC/10/12/2015
Name of the principal Investigator:	-	Mr. Lusa Haruna Yadock
Address of the Principal Investigator:	-	Dept. of Microbiology, ABUTH, Zaria
Date of receipt of valid application:	-	14th March, 2017
Date of meeting when final determination On ethical approval was made:	-	11th April, 2017

This is to inform you that the research described in the submitted protocol, the consent forms and other participant information materials have been reviewed and ***given full approval by the Health Research Ethics Committee.***

Please note: this approval dates from * 24th May, 2017 - 24th May, 2018

No participant recruitment into this research may be conducted outside these dates.

All informed consent forms in this study must carry the ABUTH HREC number assigned to this research and the duration of ABUTH HREC approval of the study.

This HREC expects that you submit your application as well as an annual report for ethical clearance renewal 3 months prior to expiration of study dates. This is to enable you obtain renewal of your approval and avoid interruption of your research.

If there is delay in starting the research, please inform the ABUTH HREC so that starting dates can be adjusted accordingly.

No changes are permitted in the research without prior approval by ABUTH HREC, except in circumstances outlined in national code for Health Research Ethics: http://www.nhrec.net.

ABUTH HREC reserves the right to conduct compliance assessment visits to your research site without prior notification.

12/6/17

Prof. Aisha. I. Mamman MBBS, FMCPath
Chairperson, ABUTH HREC

APPENDIX III
KADUNA STATE UNIVERSITY TEACHING HOSPITAL APPROVAL

BARAU DIKKO SPECIALIST HOSPITAL,
KADUNA, NIGERIA

HOSPITAL MANAGEMENT BOARD
MINISTRY OF HEALTH, KADUNA STATE

BDSH/KD/SUB.322/III

14/12/2015

The HOD Laboratory,
The Head GOPD,
Barau Dikko Teaching Hospital,
Kaduna.

<u>**LETTER OF INTRODUCTION: LUSA HARUNA YADOCK**</u>

I am directed to write and introduce the above named Researcher to carry out research work on **"Determination of Erythromycin and Penicillin Resistance Genes in Streptococcus Pneumoniae from Patients Attending Long-term Health Care Facilities (LTHCFs) in Kaduna State".**

Kindly give him the necessary assistance.

Thank you.

Suleiman Mustapha
Hosp. Secretary
For: Medical Director

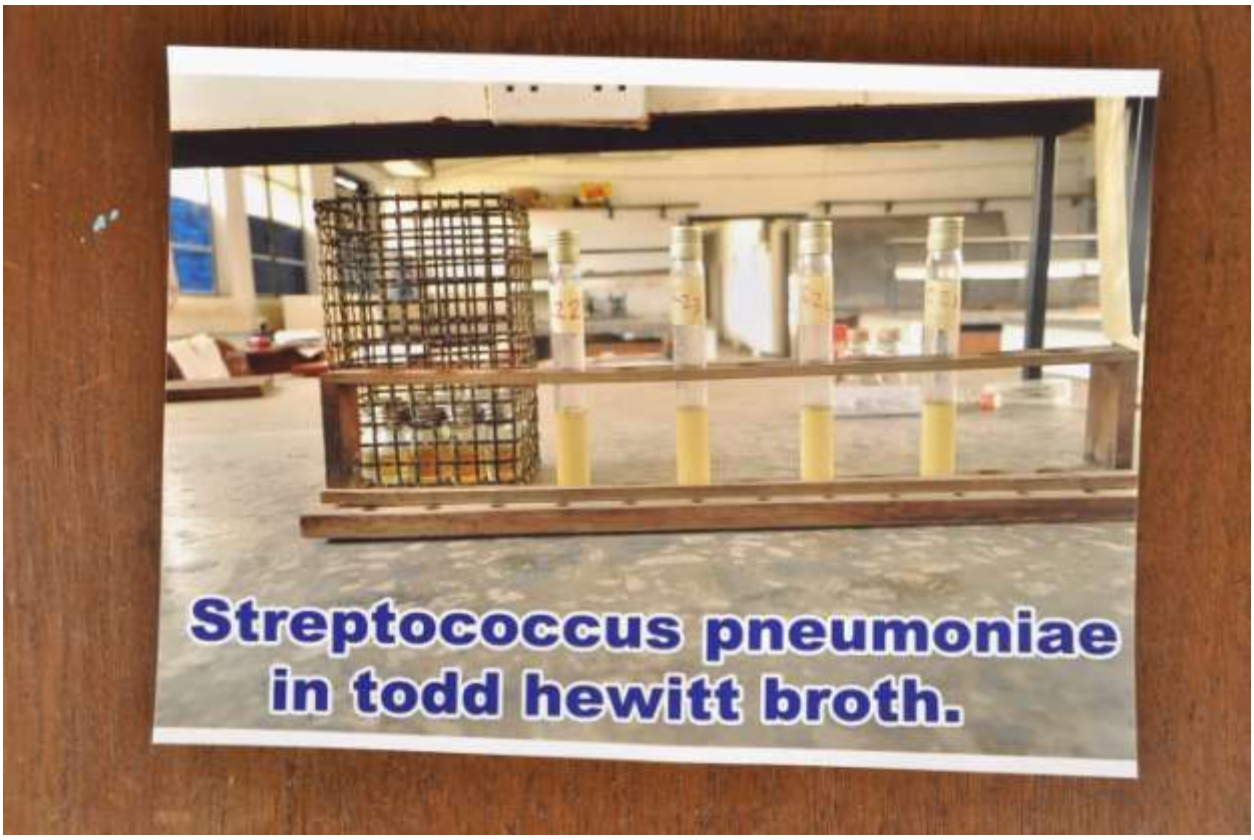
Lafia Road, P.O.Box 9727, Kaduna, Nigeria. Board Chairman: , Medical Director: 08033146100
Website: baraudikkohospital.com, email: enquiries@baraudikkohospital.com

APPENDIX IV

MICs OF PENICILLIN AND ERYTHROMYCIN

APPENDIX V

INOCULATED TODD HEWITT BROTH



APPENDIX VI
DETERMING MIC BY E-TEST METHODOLOGY

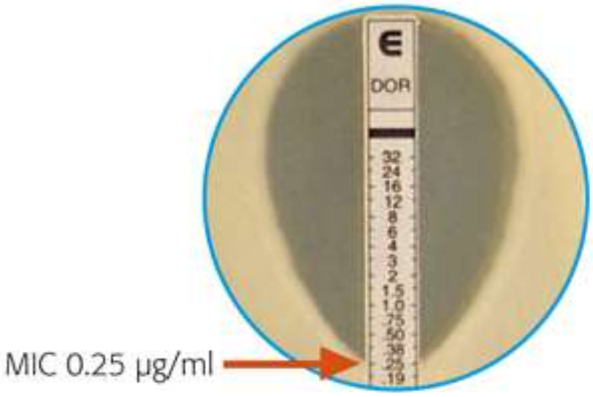


Fig.2: E-test reading scale for determining MIC (Liofilchem,Guide)

APPENDIX VII
SEQUENCE OF ISOLAT ES

CZ4

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SSZ5
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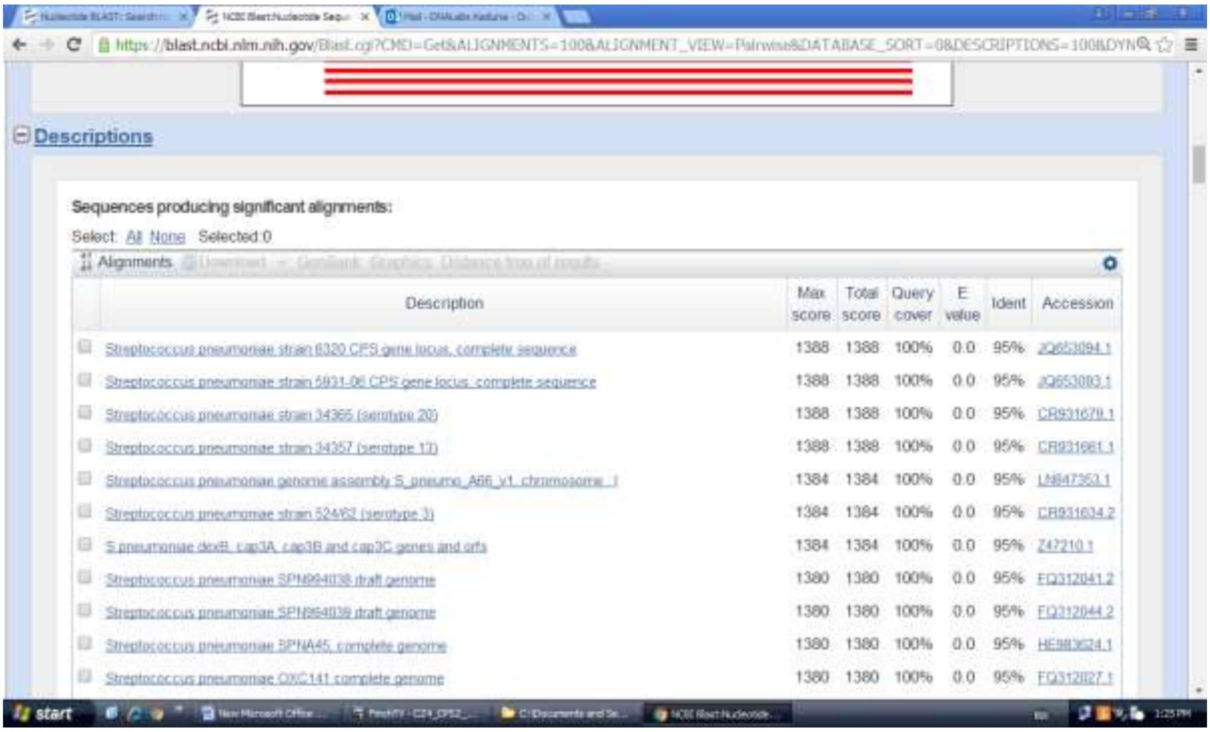
APPENDIX VIII

SEQUENCE PROCESS

Cz4

APPENDIXIX

SEQUENCES SHOWING SIGNIFICANT ALLIGNMENTS 1



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Descriptions

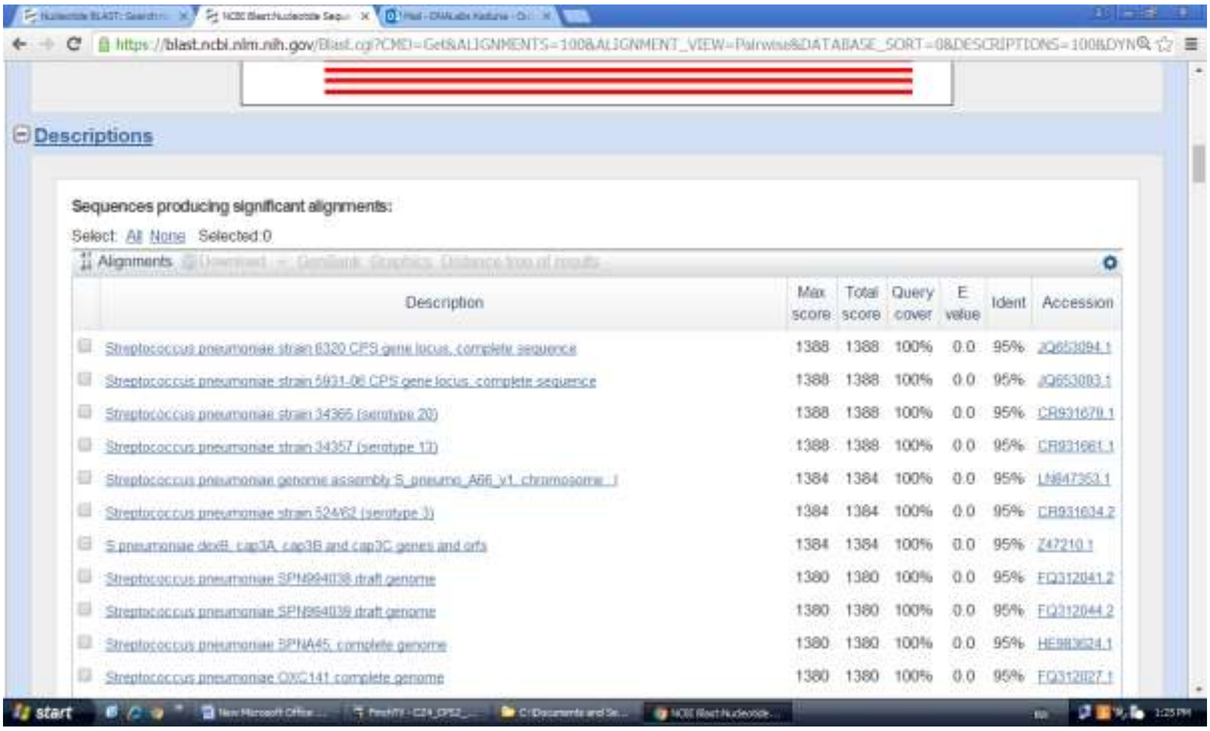
Sequences producing significant alignments:

Select: All None Selected:0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus pneumoniae strain 6320 CPS gene locus, complete sequence	1388	1388	100%	0.0	95%	JQ653094.1
Streptococcus pneumoniae strain 5931-06 CPS gene locus, complete sequence	1388	1388	100%	0.0	95%	JQ653093.1
Streptococcus pneumoniae strain 34365 (serotype 20)	1388	1388	100%	0.0	95%	CR931679.1
Streptococcus pneumoniae strain 34357 (serotype 13)	1388	1388	100%	0.0	95%	CR931661.1
Streptococcus pneumoniae genome assembly S_pneumo_A66_v1, chromosome : I	1384	1384	100%	0.0	95%	LN847353.1
Streptococcus pneumoniae strain 524/62 (serotype 3)	1384	1384	100%	0.0	95%	CR931634.2
S.pneumoniae dexB, cap3A, cap3B and cap3C genes and orfs	1384	1384	100%	0.0	95%	Z47210.1
Streptococcus pneumoniae SPN994038 draft genome	1380	1380	100%	0.0	95%	FQ312041.2
Streptococcus pneumoniae SPN994039 draft genome	1380	1380	100%	0.0	95%	FQ312044.2
Streptococcus pneumoniae SPNA45, complete genome	1380	1380	100%	0.0	95%	HE983624.1
Streptococcus pneumoniae OXC141 complete genome	1380	1380	100%	0.0	95%	FQ312027.1

SEQUENCES SHOWING SIGNIFICANT ALLIGNMENTS 2



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Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus pneumoniae strain 6320 CPS gene locus, complete sequence	1388	1388	100%	0.0	95%	JQ653094.1
Streptococcus pneumoniae strain 5931-06 CPS gene locus, complete sequence	1388	1388	100%	0.0	95%	JQ653093.1
Streptococcus pneumoniae strain 34365 (serotype 20)	1388	1388	100%	0.0	95%	CR931679.1
Streptococcus pneumoniae strain 34357 (serotype 13)	1388	1388	100%	0.0	95%	CR931661.1
Streptococcus pneumoniae genome assembly S_pneumo_A66_v1, chromosome : I	1384	1384	100%	0.0	95%	LN847353.1
Streptococcus pneumoniae strain 524/62 (serotype 3)	1384	1384	100%	0.0	95%	CR931634.2
S.pneumoniae dexB, cap3A, cap3B and cap3C genes and orfs	1384	1384	100%	0.0	95%	Z47210.1
Streptococcus pneumoniae SPN994038 draft genome	1380	1380	100%	0.0	95%	FQ312041.2
Streptococcus pneumoniae SPN994039 draft genome	1380	1380	100%	0.0	95%	FQ312044.2
Streptococcus pneumoniae SPNA45, complete genome	1380	1380	100%	0.0	95%	HE983624.1
Streptococcus pneumoniae OXC141 complete genome	1380	1380	100%	0.0	95%	FQ312027.1

APPENDIX X

CONSENT FORM

CONSENT FORM FOR RESEARCH ON "DETRMINATION OF ERYTHROMYCIN AND PENICILLIN RESITANCE GENES IN STREPTOCOCCUS PNEUMONIAE FROM PATIENTS ATTENDING LONG-TERM HEALTH CARE FACILITIES (LTCFs) IN KADUNA STATE''.

A research on Erythromycin and Penicillin resistant pneumococci in respiratory tract (RT) infections among patients attending Long-term care facilities is here by being conducted by Mr. Lusa Haruna Yadock for the award of a Ph.D. Medical Microbiology.

The purpose of this research is to determine the frequency, Serotypes and molecular pattern of resistant pneumococci in patients with RT infections. The result will help in the formulation of therapeutic regimens for patients with such resistant bacterial infections and to improve physicians' ability to treat the infections to reduce morbidity and mortality rates from pneumococci resistant to the most common antibiotics used for the treatment of respiratory tract infections in Nigeria. You are expected to produce 2ml of sputum sample, as will be instructed by the researcher assisted by well trained Technicians. In the course of giving your sample, you will not be subjected to any painful procedures to collect the phlegm/mucus.

If your sample yields resistant pneumococci, you will be given prescription to treat the bacterial infection.

If you agree to participate in this research please sign below.

Name and address of participant Signature/thumb print------------
--- Tel--------------------------------
Date-------------------------------

Name of researcher Signature/------------------------
----------------------------------- Date-------------------------------

Name and address of witness
…………………………………….. Signature/------------------------
Tel----------------------------------
Date---------------------------------------

APPENDIX XI

RESEARCH QUESTIONNAIRE

RESEARCH QUESTIONNAIRE

This questionnaire is aimed at getting socio-demographic and clinical data from you. Your cooperation and responses would add value to this research. Any information you provide will be treated with a high sense of confidentiality. The research work titled "Determination of erythromycin and penicillin resistance genes in Streptococcus pneumoniae from patients attending long-term health care facilities in Kaduna state" would guide physicians /clinicians in delivering effective control for pneumococcal diseases in the state.

Please tick as appropriate and provide answers where necessary

Name/Number of patient (optional)…………………………………………………

Study NO……………………………………..

Age…………………………………………………

Sex: Male [ ] Female [ ]

What is your Marital status: single [ ] married [ ] divorced [ ] windowed [ ] separated [ ]

Place of residence: urban [ ] semi-urban [ ] rural [ ]

Which of the following symptoms below are you having?

Fever[ ] headache[ ] chest pain[ ] cough[ ] chills[ ] shortness of breath[ ]

Nausea [ ] confusion

Are you on antibiotics? Yes [ ] no [ ]

What is your AFB Status: Negative [ ] positive [ ] don't know [ ]

What is your HIV Status? Negative [ ] positive [ ] don't know [ ]

Hospitalization in last 6 month: Yes [ ] No [ ]

Have you been immunized against Pneumococcal infections (PCV)? Yes [ ] No [ ]

Socio-economic status: High [ ] Moderate [ ] Low [ ]

Genotype: AA [ ] AS [ ] SS [ ] Others………………………………..

Do you take Alcohol? Yes [ ] No [ ]

Do you Smoke? Yes [ ] No [ ]

APPENDIX XII

MINIMUM INHIBITORY CONCENTRATIONS BREAK POINTS

MIC Test Strip Technical Sheet ***Streptococcus pneumoniae***

Specimen
Blood, CSF and respiratory sites (sputum, tracheal aspirate, middle ear fluid, nasopharynx).

Medium	Mueller Hinton II Agar (Sheep blood 5%), Ref. 10131 or Mueller Hinton Fastidious Agar (Horse blood 5% + 20 mg/L β-NAD), Ref. 10132.
Inoculum	Suspension in Mueller Hinton Broth (Ref. 24107) to 0.5 McFarland (Ref. 80400). 1 McFarland (Ref. 80401) for mucoid.
Incubation	35 ± 2°C/ 5% CO_2/ 20-24 hours.
Interpretation of results	Bactericidal drugs: interpret the M.I.C. at complete inhibition of growth including microcolonies, hazes and isolated colonies. Bacteriostatic drugs: interpret the M.I.C. at 80% inhibition when trailing is seen.

		Quality Control (M.I.C. µg/mL)	CLSI INTERPRETATION M.I.C. Criteria (µg/mL)			EUCAST INTERPRETATION M.I.C. Criteria (µg/mL)		Examples of ANTIBIOGRAM	
		S. pneumoniae ATCC® 49619	S	I	R	S	R	140 mm petri dish	90 mm petri dish
AZM	AZITHROMYCIN (-CO_2)	0.06-0.25	≤0.5	1	≥2	≤0.25	>0.5		
AZM	AZITHROMYCIN (+CO_2)	0.5-2							
P	PENICILLIN G Parenteral (non-meningitis) Parenteral (meningitis) Oral	0.25-1	 ≤2 ≤0.06 ≤0.06	 4 - 0.12-1	 ≥8 ≥0.12 ≥2	 ≤0.06 ≤0.06	 >2 >0.06	✓ 0.002 - 32	✓ 0.002 - 32
CTX	CEFOTAXIME meningitis non-meningitis	0.03-0.12	 ≤0.5 ≤1	 1 2	 ≥2 ≥4	≤0.5	>2	✓ (or CRO)	✓ (or CRO)
CRO	CEFTRIAXONE meningitis non-meningitis	0.03-0.12	 ≤0.5 ≤1	 1 2	 ≥2 ≥4	≤0.5	>2	✓ (or CTX)	✓ (or CTX)
C	CHLORAMPHENICOL	2-8	≤4	-	≥8	≤8	>8		
CLR	CLARITHROMYCIN (-CO_2)	0.03-0.12	≤0.25	0.5	≥1	≤0.25	>0.5		
CLR	CLARITHROMYCIN (+CO_2)	0.06-0.25							
CD	CLINDAMYCIN (-CO_2)	0.03-0.12	≤0.25	0.5	≥1	≤0.5	>0.5		
CD	CLINDAMYCIN (+CO_2)	0.06-0.25							
ETP	ERTAPENEM	0.03-0.25	≤1	2	≥4	≤0.5	>0.5		
E	ERYTHROMYCIN (-CO_2)	0.03-0.12	≤0.25	0.5	≥1	≤0.25	>0.5	✓	
E	ERYTHROMYCIN (+CO_2)	0.06-0.25							
IMI	IMIPENEM	0.03-0.12	≤0.12	0.25-0.5	≥1	≤2	>2		
LEV	LEVOFLOXACIN	0.5-2	≤2	4	≥8	≤2	>2		
LNZ	LINEZOLID	0.25-2	≤2	-	-	≤2	>4		
MRP	MEROPENEM meningitis non-meningitis	0.06-0.25	≤0.25	0.5	≥1	 ≤0.25 ≤2	 >1 >2	✓	
OFX	OFLOXACIN	1-4	≤2	4	≥8	≤0.12	>4		
TE	TETRACYCLINE	0.06-0.5	≤1	2	≥4	≤1	>2		
SXT	TRIMETHOPRIM-SULFAMETHOXAZOLE 1/19 [1]	0.12-1	≤0.5	1-2	≥4	≤1	>2	✓	
VA	VANCOMYCIN	0.12-0.5	≤1	-	-	≤2	>2	✓	

Notes
1. Values on the MIC scale refer to the first component of the combination.

References

- CLSI M100-S25, 2015. Performance Standards for Antimicrobial Susceptibility Testing.
- CLSI M07-A10, 2015. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically.
- EUCAST. Breakpoint tables for interpretation of MICs and zone diameters. Version 5.0, 2015.

MIC Test Strip, Patent No. 1395483

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