CHARACTERIZATION OF *STREPTOCOCCUS PNEUMONIAE* STRAIN ISOLATED FROM PNEUMONIA CASES OF CHILD PATIENTS

A THESIS

BY

MD. NAYEMUL BARI

REGISTRATION NO: 1605456

SEMESTER: JULY- DECEMBER, 2018

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MASTER OF SCIENCE (M.S)

IN

MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJAPUR-5200

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DECEMBER, 2018

Dedicated to

My

Beloved Parents

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The Author
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ABSTRACT

Streptococcus pneumoniae is a leading cause of global morbidity and mortality in children causing pneumonia. The increasing incidence of antibiotic-resistant *S. pneumoniae* strains worldwide poses a threat to the effective treatment of these infections. For this purpose, the present research was conducted during the period of January–November 2018. The samples were collected from three main hospitals of Dinajpur district of Bangladesh. A total of 40 samples were randomly collected & analyzed through different bacteriological, biochemical, moleculer & antibiotic susceptibility testing. Out of 40 samples, positive cases of pneumonia were found to be 37.5% & 15 isolates were isolated. The Frequency of pneumonia in relation to age were 3-5yrs (50%), 6-8yrs (33.33%), 9- 11yrs (25%) & 12-15 (20%). Distribution of organism based on age difference were 3-5 years (7%), 6-8 years (20%), 9-11 years (6.67%) & 12-15 years (30%). The present study reveals that the study area had no significant effect ($P > 0.05$), but age ($P < 0.05$) & socio-economic status ($P < 0.05$) had significant effects on the prevalence of pneumonia in pneumonia patients. In study area, the highest prevalence was found in M Abdur Rahim Medical College Hospital (40%), followed by Arobindo Shishu Hospital (33.33%) & Islami Bank Community Hospital (40%) respectively. Among the age group, the prevalence of pneumonia was highest (50%) in 3-5 years age group, then 33.33% in 12-15 years age group, 25% in 6-8 years age group & 20% in 9-11 years age group. Highest prevalence of pneumonia was found in poor socio-economic status (54.54%), followed by medium (16.66%) & rich (16.66%) socio-economic condition respectively. *Streptococcus pneumoniae* was characterized by 16S rRNA Sequencing & the identified strain was *Streptococcus pneumoniae* NBRC102642. The antibiotic study revealed that all of the isolates of *Streptococcus pneumoniae* were resistant to most of the drugs, but found sensitive to Neomycin, Kanamycin & Streptomycin followed by Erythromycin, Azithromycin & Bacitracin. Avoidence of indiscriminate use of antibiotics & development of nationwide awareness can play an important role for the prevention of pneumonia.

CONTENTS

LIST OF TABLES

LIST OF PLATES

ABBREVIATIONS

DCC	=	Day Care Centre
ED	=	Emergency Department
EUCAST	=	European Committee on Antimicrobial Susceptibility Testing
MDR	=	Multidrug Resistance
MIC	=	Minimal Inhibitory Concentration
PCC	=	Primary Care Centres
PCV	=	Pneumococcal Conjugate Vaccine
PISP	=	Penicillin Intermediate *Streptococcus pneumoniae*
PNSP	=	Penicillin Non-susceptible *Streptococcus pneumoniae*
PRSP	=	Penicillin Resistant *Streptococcus pneumoniae*
PSSP	=	Penicillin Susceptible *Streptococcus pneumoniae*
RTI	=	Upper Respiratory Tract Infection
SPn	=	*Streptococcus pneumoniae*
SPSS	=	Statistical Package for the Social Sciences
WHO	=	World Health Organization
CF	=	Cystic Fibrosis
PCR	=	Polymerase Chain Reaction
FEV1	=	Forced Expiratory Volume in one second
FVC	=	Forced Vital Capacity
CBA	=	Columbia Blood Agar

CHOC	=	Chocolate Agar
DNA	=	Deoxyribonucleic Acid
CFU	=	Colony Forming Unit
TNTC	=	Too Numerous to Count
IQR	=	Interquartile Range
RR	=	Relative Risk
CLSI	=	Clinical and Laboratory Standards Institute
mm	=	Millimeter
NT	=	Non-typable
N/A	=	Not applicable

CHAPTER I
INTRODUCTION

Streptococcus pneumoniae remains a leading cause of serious infections and deaths in young children and the elderly globally (O'Brien *et al.,* 2009). As global efforts to prevent and control pneumococcal disease gain momentum, a good understanding of the population structure of *Streptococcus pneumoniae*, including the circulating pneumococcal serotypes and genotypes would be critical in guiding the development of appropriate interventions. Some studies have reported changes in circulating serotypes causing invasive disease and nasopharyngeal carriage in different parts of the world (Ubukata *et al.,* 2015; Devine *et al.,* 2017). However, most of these studies have been conducted in developed country settings with few in developing countries and there remain large gaps in our understanding of the pneumococcal population structure in developing countries where the burden of disease is disproportionately highest. In addition, changes in recent times, such as the increasingly widespread introduction of routine pneumococcal vaccination and changing patterns of HIV prevalence, could have an impact on the circulating pneumococcal serotypes and genotypes. The estimated incidence of community-acquired pneumonia among children under five years of age in developing countries is approximately 151.8 million new cases per year, 11-20 million of which require hospitalization (Rudan *et al.,* 2004). In developing countries, pneumonia occurs more often and is more severe than in developed nations, and carries higher incidence and mortality rates; pneumonia accounts for one fifth of under-five deaths in the developing world (Bryce *et al.,* 2005).

Regarding penicillin susceptibility of *S. pneumoniae*, minimum inhibitory concentration (MIC) breakpoints for penicillin were first established in the late 1970s, in response to the need for ensuring successful treatment of pneumococcal meningitis. Over the past three decades, increasing MICs to penicillin have emerged in pneumococci, and the percentage of penicillin-intermediate and penicillin resistant strains has risen (Berezin *et al*., 1996; Whitney *et al.,* 2000). In a study conducted by the Regional Vaccine System Project (Projeto de Sistema Regional de Vacinas, SIREVA) between 2000 and 2005, of a total of 8,993 pneumococcal isolates (36.4% of which obtained from patients with pneumonia), 37.8% were penicillin-resistant (Castaned *et al.,* 2009). In light of this growing resistance, penicillins are increasingly being rejected for both empirical and susceptibility-guided

treatment, in favor of more expensive, broad-spectrum antibiotics. However, retrospective and prospective studies in adult and pediatric populations showed that the outcomes of pneumococcal pneumonias caused by purportedly resistant strains but treated with penicillin antibiotics did not differ from those obtained with other antimicrobial agents, suggesting that meningitis breakpoints did not apply to pneumonia. (Palleres *et al.,* 1987; Friedland/Klugman, 1992). Later multicenter studies confirmed these results (Song *et al.,* 2004; Yu *et al.,* 2003). *Streptococcus pneumoniae* is the most common cause of community-acquired respiratory tract infections such as otitis media, sinusitis, and pneumonias (Ishida *et al.* 2008). Globally, pneumococcal diseases account for 1 to 2 million deaths annually in both extremes of age. (Mulholland, 1999). It is supposed to be a very sensitive organism to routine antibiotics especially to penicillins. However, with the isolation of the first clinically significant penicillin-resistant pneumococcus (PRP) in 1967, many studies from different parts of the world have reported an increasing emergence of PRP (Collignon & Bell, 1996). At present, there are not only reports of resistant strains of *S. pneumoniae* to the beta lactam group of antibiotics, but there is also an emergence of multidrug-resistant strains (Lalitha & Manoharan, 2002).

Streptococcus pneumoniae remains a major cause of morbidity and mortality worldwide. Pneumococcus is a commensal of the upper respiratory tract of humans. However, it is also a human pathogen responsible for several respiratory tract infections & serious invasive pneumococcal diseases, such as sepsis and meningitis (O'Brien *et al.,* 2009). Pneumococcal disease has higher incidence among young children, the elderly and the immunocompromised of all ages. Within this latter group, the risk for pneumococcal infection will vary according to the underlying medical conditions but is several folds higher than among healthy people. For example, a study from Canada found that the incidence of IPD among hematopoietic stem cell transplant patients was 30-fold higher than in the general population (Kumar *et al.,* 2008).

As the epidemiology of pneumococcal disease varies with time and place, periodic reassessment with monitoring of prevalent serotypes and patterns of resistance is required for better therapeutic guidance and definition of control strategies. With that in mind, the main objectives of this study were to identify the *S. pneumoniae* serotypes most frequently isolated from children hospitalized for invasive pneumonia; compare these serotypes with those included in conjugate vaccines; and analyze their susceptibility to the antimicrobial agents most often used in pediatric practice. By considering the above-

mentioned justification the current study, were, therefore set with the following objectives.

Research Objectives

- To examine the prevalence and risk factors of carriage of *S. pneumoniae* in the prevaccination era in young children.
- Isolation & identification of *streptococcus* spp. in child patients causing pneumonia.
- Molecular characterization of *streptococcus* spp. by PCR, DNA sequencing & Phylogenetic tree analysis.
- To determine the antibiotic sensitivity pattern of the isolated bacteria.

CHAPTER II
REVIEW OF LITERATURE

Every year in developing countries, some 4.5 million persons, most of them under 5 years of age, die of acute respiratory infections (Berman S, 1991). Most ARI episodes are caused by viral agents and are self-limiting, but the bacterial pneumonias, which occur less frequently, carry a much higher risk of complications and death (Shann, 1986). *Streptococcus pneumoniae* (pneumococcus) and *Haemophilus influenzae* are the two bacterial pathogens most commonly isolated in pediatric pneumonia (Paisley *et al.,* 1984). Since these microorganisms can colonize the upper respiratory tract, aspiration of secretions may lead to pneumonia, especially if an individual's susceptibility to a bacterial infection were increased by malnutrition, viral infection, or other factors that would change the immune response (Musher *et al.,* 1992). Colonization of the upper airways by *S. pneumoniae* can occur during the first few days of life; early colonization is associated with living in a large family and a higher risk of suffering from the disease (Gray *et al.,* 1980).

2.1. Isolation, Molecular characterization & Anti-microbial resistance of *Streptococcus pneumoniae* in child patients

Ebruke *et al.*, 2018. In West Africa, *Streptococcus pneumoniae* remains a leading cause of deaths in young children and serotype 1 strains are particularly important in causing invasive pneumococcal disease despite being rare in nasopharyngeal carriage. The *S. pneumoniae* ST217 clonal complex, consisting of ST217 and the various locus variants has been shown to be the predominant pneumococcal serotype 1 clone in the sub-region. It is unclear how the recent introduction of pneumococcal conjugate vaccine in countries in the sub-region could affect patterns of pneumococcal disease. Improving our understanding of the unique nature of pneumococcal serotype 1 strains within the context of other pneumococcal serotypes circulating in West Africa prior to introduction of PCV would be critical in interpreting any subsequent changes in patterns of disease in this region. Epidemiological studies on *S. pneumoniae* strains in The Gambia revealed that a new dominant clone of serotype 1, ST3081, a single locus variant of ST217, emerged and appeared to have spread across the whole country. ST3081 appeared to have replaced ST618, a triple locus variant of ST217 and the previously dominant pneumococcal serotype 1 lineage circulating in The Gambia for over a decade earlier. This thesis, which

also evaluated antimicrobial resistance patterns, showed that ST3081 isolates were more resistant to co-trimoxazole than ST618 isolates.

Htoon *et al.*, 2018. A cross-sectional, laboratory-based descriptive study was carried out on 231 ARI patients who were aged 1 month to less than 5 years attending YCH from 2013 to 2015. Nasopharyngeal swab specimens were taken after obtaining informed consent from the parents or guardians of the patients. Antimicrobial susceptibility profile was determined by modified Kirby-Bauer method. In addition, molecular serotyping of *S. pneumoniae* was done by Sequential Multiplex Polymerase Chain Reaction. The isolation rate of *S. pneumoniae* was 7.4% (17/231). A total of nine different serotypes were detected. The most common serotype was found to be 6A/6B/6C/6D (52.9%) followed by non-type able serotypes (29.4%), 19F, 23F and 35F/47F serotypes (11.8% each) and 23A, 11A/11D, 34 and 20 serotypes (5.9% each). Carriage of multiple serotypes was observed in 29.4% of *S. pneumonia* isolates. 70.6% of *S. pneumoniae* isolates were found to be penicillin non-susceptible and multi-drug resistance was observed in 41.2% of strains.

Michel *et al.*, 2018. A cross sectional study was carried out from May to October 2014. HIV infected patients suspected of LRTIs attending the Center Medical laboratory and those followed up at the authorized treatment center of Yaounde Military Hospital in Cameroon were enrolled. Sputum was collected from each patient and cultured; identification of microorganisms was performed following standard methods. The disk diffusion method was used for antibacterial susceptibility testing according to the Antibiogram Committee of French Society for Microbiology guidelines. a total of 51 (25.5%) isolates of *S. pneumoniae* were recovered from sputum samples obtained from 200 HIV infected patients aged 19-66 years old (mean age: 36±10.087 years old); 144 (72%) of them were female (sex ratio M/F: 1/3). *S. pneumoniae* carriage was not age dependent (P = 0.384) and was significantly higher in male compared to female (P = 0.008). *S. pneumoniae* isolates were susceptible to amoxicillin-clavulinic acid (100%), pristinamycin (100%), erythromycin (100%) and cefixime (98.04 %). Highest resistance rates were recorded with fusidic acid (100%), fosfomycin (100%) and tetracycline (100%).

Marta *et al.*, 2017. Evaluate the susceptibility profile of serotypes of *Streptococcus pneumoniae* associating them with pneumococcal invasive diseases, as well as antimicrobial therapies. This is a retrospective cross-sectional research involving secondary data from 1998 to 2013, in the northeastern macroregion of Sao Paulo state, Brazil, composed of Araraquara, Barretos, Franca and Ribeirao Preto regions, with 90 municipalities. At Instituto Adolfo Lutz, isolated strains from patients with PID were subjected to identification, serotyping and antimicrobial susceptibility testing. Results: From 796 strains analyzed, 14.8% (*n* = 118) were resistant to penicillin, being 3% (*n* = 24) with intermediate resistance and 11.8% (*n* = 94) with full resistance, especially in patients with meningitis. Moreover, resistance to ceftriaxone was 5.3%: 34 (4.3%) with intermediate resistance and 8 (1%) with full resistance. We point out that the greatest level of resistance profiles was observed against sulfamethoxazole/trimethoprim: 350 (49.4%). On the other hand, antimicrobial susceptibility was described above 90% to chloramphenicol: 99.6% (*n* = 696), erythromycin: 94.7% (*n* = 664), ceftriaxone: 94.7% (*n* = 754) and fully susceptible to vancomycin. Among the 18 most common serotypes, 9V and 14 showed less susceptibility to SMT, to penicillin and ceftriaxone; 19A to SMT and penicillin; 1 to SMT; 12F and 3 to chloramphenicol; 6A to SMT; 6B 23F to erythromycin and penicillin.

Sedigheh *et al.*, 2017. In total, 73 isolates were collected and diagnosed as *S. pneumoniae.* Isolates were susceptible to ofloxacin 95.9%, vancomycin 93%, penicillin 78%, trimethoprim-sulfamethoxazole 61.6%, ceftriaxone 53.5%, meropenem 52%, cefotaxime 46.5%, and erythromycin 8.2%. Of the 15 PRSP isolates, the *pbp2b* gene was identified in 12 (80%). In 1 penicillin-intermediate *S. pneumoniae* isolate the *pbp2b* was detected.

Fouzia *et al.*, 2017. This was a cross-sectional study conducted in the Department of Microbiology, Dow Diagnostic Reference and Research Laboratory, Karachi, Pakistan, during the period of January 2014 to October 2015. Isolation and identification of clinical isolates were performed by using standard and specific methods. Antibiotic susceptibility was performed by using Kirby Bauer method. Maximum numbers of *S. pneumoniae* were recovered from sputum, pus and blood and a higher percentage of the organisms were isolated from females. Among non-invasive S. pneumococcal infections, children, adolescents and adults were found to be highly resistant against Cotrimoxazole, Clindamycin and Ofloxacin, respectively. In invasive S. pneumococcal infections, highest

resistance was observed against Penicillin G, Cotrimoxazole and Macrolides in children, adolescents and adults, respectively.

Araceli *et al.*, 2016. A total of 126 pneumococcal isolates were collected. Pneumonia was the most frequent diagnosis (40.5%), followed by meningitis (29.4%), septicemia (16.7%), and other clinical entities, including otitis media and conjunctivitis (13.5%). The most frequent serotypes before the introduction of heptavalent pneumococcal conjugate vaccine (PCV7) were 19F, 23F, 7F, and 35B. Serotypes 3, 6A, 10A, 12F, and 15A/B increased after the introduction of PCV7. Serotype 19A was isolated most frequently in the pneumonia and meningitis cases only after the introduction of PCV7, and it displayed a high resistance to penicillin.

Takafumi *et al.*, 2016. All strains of *S. pneumoniae* were isolated from clinical specimens collected from pediatric patients. The minimun inhibitory concentration was measured and the strains were classified according to the Clinical Laboratory Standards Institute criteria. The isolation rates of penicillin-intermediate resistant *S. pneumoniae* (PISP) and penicillin-resistant *S. pneumoniae* (PRSP) were compared based on seven patient factors. Logistic regression analysis was also performed. The sum of the isolation rates for PISP and PRSP for each period was 64.6%, 67.0%, 56.2%, 76.9% and 49.5%, respectively. Among the patient factors, age category 1 (<3 years, ≥3 years), age category 2 (infant, toddler and preschooler, schoolchild), siblings (absence, presence), and pre-treatment with antimicrobial agents (absence, presence) were associated with significant differences in the isolation rate of PISP + PRSP. An interaction was observed between pre-treatment with antimicrobial agents and schoolchild, and the isolation rate of PISP + PRSP was higher in patients with both pretreatment with antimicrobial agents and schoolchild.

Indre *et al.*, 2016. A total of 900 children under six years of age who presented to primary care centre or a hospital emergency department with acute respiratory tract infection were enrolled in the study. Nasopharyngeal swabs were obtained and cultured for SPn. Positive samples (n=367) were serotyped and tested for antimicrobial susceptibility. Associations of pneumococcal non-susceptibility with study site, season, age, sex, attendance of day care centre and treatment with antimicrobials (between one and six months prior the study) were evaluated. About a half (56.7 %) of SPn strains were susceptible to all the antibiotics tested. Pneumococcal non-susceptibility to penicillin,

erythromycin, clindamycin and trimethoprim–sulphamethoxazole was 15.8, 21.3, 16.9 and 27.3 %, respectively. None of the tested isolates was resistant to norfloxacin or vancomycin. We found a geographical variation of pneumococcal resistance within the cities of the country. Age, sex, the attendance of day care centre and treatment with antimicrobials prior the study was not significantly associated with a carriage of non-susceptible SPn strains. Among non-susceptible SPn serotypes, 67.9 %–82.4 % were present in currently available pneumococcal conjugate vaccines.

Jailton *et al.*, 2016. In 2010, the 10-valent pneumococcal conjugate vaccine (PCV10) was introduced into the Brazilian childhood vaccination programme. Concerns have been raised that non-vaccine serotypes could increase in prevalence and reduce the benefits of vaccination; therefore, we examined non-PCV10 isolates recovered from meningitis during pre- (January 2008–May 2010) and post-vaccine (June 2010–December 2012) periods. Surveillance for pneumococcal meningitis was established at the Reference Hospital of Infectious Diseases in Salvador, Brazil. Serotypes were determined by multiplex PCR and/or Quellung reaction. Antimicrobial susceptibility testing was conducted by E-test and broth microdilution. Genotyping used PFGE and multi-locus sequence typing. A total of 148 cases of meningitis were identified from January 2008 to December 2012, 77 (52 %) of which were due to non-PCV10 isolates, with 50 (52.1 %) from pre-vaccine and 27 (52 %) from post-vaccine periods. In the post-vaccine period, the non-PCV10 serotypes 12F (n=6; 22.2 %), 10A (n=3; 11.1 %), 15B (n=2; 7.4 %) and 18B (n=2; 7.4 %) were the most prevalent. Forty-three isolates (55.8 %) were non-susceptible to one or more antibiotics. Non-susceptibility to penicillin was observed among serotypes 19A (three isolates), 9N (one isolate) and 12F (one isolate). PFGE and multi-locus sequence typing results demonstrated a wide genetic diversity among the isolates. During the early period following PCV10 introduction, no obvious emergence of a particular serotype was evident among non-PCV10 strains. This study underscores the importance of monitoring any changes among non-PCV10 cases after the introduction of PCV10.

Songyin *et al.*, 2015. Fifteen serotypes were identified among the 94 *S. pneumoniae* clinical isolates that were collected. Prevalent serotypes were 19F (42.6 %), 19A (8.5 %), 3 (8.5 %), and 6B (7.4 %). Potential immunization coverage rates for the 7-, 10- and 13-valent pneumococcal polysaccharide conjugate vaccines were 59.6, 62.6, and 79.6 %, respectively. Resistance rates to tetracycline, erythromycin, and trimethoprim/

sulfamethoxazole were 91.2, 80.2 and 63.8 %, respectively. Resistance rates to penicillin, amoxicillin, ceftriaxone, and cefotaxime were 47.3, 34.1, 19.8, and 18.7 %, respectively. In almost all cases, antimicrobial resistance of the *S. pneumoniae* isolates in patients five years or younger was higher than isolates collected from patients aged 51 years or older.

Christina *et al.*, 2015. Only fifteen of 318 adult CF patients (5%) were ever found to have transient Pneumococcus colonization, and none developed persistent infection although length of carriage varied. As all isolates were stored, capsular serotyping could be performed using a multiplex PCR panel. Capsular serotyping revealed a varied distribution of several serotypes within these isolates. Lung function testing at time of incident Pneumococcus isolation was compared with values before and after isolation and showed no significant reduction in spirometry values, nor was there an increased need for rescue antibacterial therapy.

Anis *et al.*, 2015. Fifty-nine *S. pneumoniae* were isolated from infection (n = 31) and colonization (n = 28) sites of patients (children and adults) attending the National Centre of Bone Marrow Transplantation. All isolates were characterized by serotype, antimicrobial resistance pattern and multilocus sequence typing (MLST).The majority (66.1%) of the isolates belonged to five serotypes all included in PCVs: 6B, 9V, 14, 19F and 23F. The potential coverage of the 10-valent and 13-valent PCV was of 71.2% and 76.3% respectively. Resistance rates were very high and 69.5% of the isolates were multidrug resistant: non-susceptibility rates to penicillin, amoxicillin and cefotaxime were 66.1%, 40.7% and 27.1%, respectively; resistance rates to erythromycin, clindamycin, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole, were 69.5%, 61.0%, 37.3%, 22.0% and 67.8%, respectively. The most frequent serotypes had STs characteristic of multidrug resistant international clones known to be highly successful and important causes of pneumococcal infection: Spain 23F-ST81, France 9V/14-ST156, Spain 6B-ST90, 19F-ST320, and Portugal 19F-ST177.

Fen *et al.*, 2015. A total of 284 clinical pneumococcal isolates (270, 5, 4,3, and 2 of which were isolated from sputum, bronchoalveolar lavage fluid, blood, cerebral spinal fluid, and ear secretions, respectively) from children less than 14 years of age who had not been vaccinated with a conjugate vaccine, were collected between January and December in 2013. All isolates were serotyped by multiplex polymerase chain reaction or quellung reactions and antimicrobial susceptibility testing was performed using the broth

microdilution method. The molecular epidemiology of *S. pneumoniae* was analyzed by multilocus sequence typing (MLST). Among the 284 pneumococcal isolates, 19F (33.5%), 19A (14.1%), 23F (12.0%), and 6A (8.8%) were the most common serotypes and the coverage rates of the 7-, 10-, and 13-valent pneumococcal conjugate vaccines (PCV7, PCV10, and PCV13) were 58.6%, 59.4% and 85.1%, respectively. Antimicrobial susceptibility showed that the prevalence rates of *S. pneumoniae* resistance to penicillin were 11.3% (32/284). Approximately 88.0% (250/284) of the isolates exhibited multi-drug resistance. MLST analysis revealed a high level of diversity, with 65 sequence types (STs) among 267 isolates. Specifically, the four predominant STs were ST271 (24.3%, 65/267), ST320 (11.2%, 30/267), ST81 (9.7%, 26/267), and ST3173 (5.2%, 14/267), which were mainly associated with serotypes 19F, 19A, 23F, and 6A, respectively.

Elizeus *et al.*, 2015. In a population-based survey in April 2008, nasopharyngeal specimens were collected from 152 randomly selected healthy children under 5 years of age in the Iganga/Mayuge Health and Demographic Surveillance Site (HDSS). Medical history and prior treatment were recorded. Demographic characteristics and risk factors for carriage of resistant strains were obtained from the HDSS census. Bacteria were isolated and analysed for antibiotic susceptibility using disk diffusion and E test. *Streptococcus pneumoniae* carriage was 58.6%, and, while most (80.9%) isolates had intermediate resistance to penicillin, none was highly resistant. Whereas no isolate was resistant to erythromycin, 98.9% were resistant to trimethoprim-sulphamethoxazole (co-trimoxazole).

Rapee *et al.*, 2015. Antibiotic resistance in *Streptococcus pneumoniae* is an emerging health problem worldwide. The incidence of antimicrobial-resistant *S. pneumoniae* is increasing, and nasal colonization of *S. pneumoniae* in children increases the risk of pneumococcal infection. In this study, the prevalence of *S. pneumoniae* nasal colonization was studied in Thai children from three different districts. *S. pneumoniae* nasal colonization was found in 38 of 237 subjects (16.0%). The carriage rate indicated higher rates in two rural districts (18.2% and 29.8%) than in the urban district (2.8%). The antibiotic susceptibility pattern was determined using the disk diffusion method. Prevalence of multi-drug resistance *S. pneumoniae* (MDR-SP) was 31.6%. Resistance to commonly prescribed antibiotics was found for ampicillin (5.3%), azithromycin (26.3%), cefepime (2.6%), chloramphenicol (18.4%), clindamycin (18.4%), erythromycin (21.1%),

oxacillin (44.7%), trimethoprim/sulfamethoxazole (78.9%) and tetracycline (15.8%). All isolates were sensitive to ceftriaxone. The pulsed-field gel electrophoresis pattern was used to compare genetic diversity of the *S. pneumoniae* isolates. PFGE demonstrated the variation in genotypes of *S. pneumoniae* from different areas. High prevalence of multi-drug resistance *S. pneumoniae* nasal colonization in healthy Thai children was indicated. Effective strategies for appropriate use of antibiotics are therefore needed in the community.

Geng *et al.*, 2014 reported that, a total of 175 pneumococcal isolates were collected and all strains were resistant to erythromycin and clindamycin, about 39.4% strains were non-susceptible to penicillin G. Overall, 174 (99.4%) isolates were resistant to $3 types of antibiotics. Serotypes 19F (28.1%), 6B (19.7%), 19A (18.0%), and 23F (17.4%) were the most common serotypes in all identified strains. The serotypes coverage of PCV7 and PCV13 were 71.9% and 89.9%, respectively. Four international antibiotic-resistant clones, including Taiwan19F-14 (n=79), Spain23F-1 (n=25), Taiwan23F-15 (n=7) and Spain6B-2 (n=7), were identified. The Taiwan19F-14 clones have a higher non-susceptibility rate in b-lactams than other clones and non-clone isolates (p, 0.001). In addition, 98.7% Taiwan19F-14 clones were positive of ermB and mefA/E genes, compare to 33.3% in other clones and non-clone strains.

Monica *et al.*, 2014. We analysed the distribution of vaccine and non-vaccine *Streptococcus pneumoniae* serotypes and the antimicrobial susceptibility of pneumococcal strains isolated from healthy Romanian children. *S. pneumoniae* carriage was detected in 25.25% of the tested children. Carriage increased from 16.7% among infants to 29.4% in 3–5-year-old children ($p < 0.0001$). The proportions of the serotypes included in pneumococcal conjugate vaccines PCV7, PCV10, and PCV13 among our isolates were 39.9%, 40.1%, and 58.7%, respectively. Erythromycin resistance was 72.5%, and it was significantly lower in non-vaccine serotypes compared with PCV13 serotypes: 57.3% versus 83.6% ($p < 10-7$). Penicillin minimum inhibitory concentrations (MICs) >0.064 mg/l were recorded in 71.6%, but the penicillin MIC was >2 mg/l for only 8.4% of tested isolates.

Aleksandra *et al.*, 2013. The prevalence of MRSP increased from 8% in 1998 to 22% in 2008 (P¼0.0001). MRSP were 51% mef (A) positive, 36% erm (B) positive, 8% dual mef (A) and erm (B) positive and 5% mef (A) and erm (B) negative. Dual mef (A)- and erm (B)-positive isolates increased in prevalence from 3% in 1998 to 19% in 2008 (P¼0.001). The prevalence of PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) decreased from 67% in 1998 to 31% in 2008 (P¼0.0072). The prevalence of serotype 19A, a non-PCV7 serotype, increased by 15% from 1998 to 2008; isolates of serotype 19A were MDR, dual mef (A) and erm (B) positive, genetically related by PFGE and associated with the presence of pili virulence factors.

Eric *et al,* 2013. This was a prospective cross-sectional study, and the subjects were children under 12 years old. Nasal swabs were collected from 50 children with HbSS disease and 50 children without HbSS disease. Nasopharyngeal swabs were collected from another group of 92 children with HbSS disease. The nasal and nasopharyngeal swabs were cultured for *S. aureus* and *S. pneumoniae*, respectively. Susceptibility testing was carried out on the *S. aureus* and *S. pneumoniae* isolates for various antibiotics, including penicillin, ampicillin, cefuroxime, erythromycin, cloxacillin, and cotrimoxazole. The carriage rates of *S. aureus* among pediatric subjects with HbSS disease and those without HbSS disease were 48% and 50%, respectively ($P > 0.05$). *S. pneumoniae* carriage among the pediatric subjects with HbSS disease was 10%. Antibiotic resistance patterns of *S. aureus* carried by children with HbSS disease and children without HbSS disease were similar, and the *S. aureus* resistance rates were >40% for the various antibiotics, with the exception of erythromycin and cloxacillin. Low levels of *S. pneumoniae* resistance (0%–11%) were observed for the various antibiotics tested except cotrimoxazole, which showed an extremely high-percentage resistance (100%).

Xiang *et al.*, 2013. A total of 89 strains were isolated and 87 isolates were included. The five prevailing serotypes were 19F (28.7%), 14 (16.1%), 23F (11.5%), 19A (9.2%) and 6B (6.9%). The most common sequence types (ST) were ST271 (21.8%), ST876 (18.4%), ST320 (8.0%) and ST81 (6.9%) which were mainly related to 19F, 14, 19A and 23F, respectively. The potential coverage by 7-, 10-, and 13-valent pneumococcal conjugate vaccine were 77.0%, 77.0%, and 89.7%, respectively. Among the 87 isolates investigated, 11.5% were resistant to penicillin, and for meningitis isolates, the resistance rate was 100%. Multi-drug resistance (MDR) was exhibited by 49 (56.3%) isolates. Eighty-four

isolates were resistance to erythromycin, among which, 56 (66.7%) carried the ermB gene alone and 28 (33.3%) expressed both the ermB and mefA/E genes.

Susan *et al.*, 2013. *Streptococcus pneumoniae* is a major cause of morbidity and mortality worldwide. Rates of carriage are highest in infants and the elderly. The objectives of this study were to determine the rate of nasopharyngeal colonization by *S. pneumoniae*, and to describe the antibiotic resistant patterns and the serotypes of the carried isolates. A cross-sectional study design was used. Nasopharyngeal swabs were collected from 315 children in the months of October and November 2010 and processed to isolate *S. pneumoniae*. The isolates were serotyped by the Quellung reaction and their antibiotic susceptibilities assessed by the disc diffusion method. The overall nasopharyngeal carriage rate for *S. pneumoniae* was 17%. Seventeen serotypes were detected amongst 55 strains analysed: 6A, 23F, 19F, 13, 6B, 14A, 20, 7C, 1, 15B, 35B, 19A, 11A, 34, 5, 3 and 23A. Susceptibility testing revealed that nearly all (98%) were resistant to cotrimoxazole, 9% were resistant to penicillin and 7% to cefotaxime. Resistance to chloramphenicol and erythromycin was 2% and 4%, respectively. All isolates were fully sensitive to tetracycline.

Sabrina *et al.*, 2012. This study aimed to determine the magnitude of nasopharyngeal carriage, antimicrobial resistance and serotype distribution of *Streptococcus pneumoniae* in healthy children under 5 years of age in Tanzania. Nasopharyngeal swabs were obtained from 300 healthy children attending a child health clinic at Muhimbili National Hospital in Dar es Salaam, Tanzania. *S. pneumoniae* was isolated and identified using conventional methods. Antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion method. Penicillin MICs and serotypes were determined by an agar gradient diffusion method and the Quellung reaction, respectively. A total of 105 samples (35 .0%) were positive for *S. pneumoniae* and 115 serotypes were detected (ten specimens yielded two serotypes each). Overall, 78 of 115 isolates (67.8 %) were penicillin-nonsusceptible pneumococci (PNSP). The resistance levels of *S. pneumoniae* to trimethoprim– sulfamethoxazole, tetracycline, erythromycin, chloramphenicol and ceftriaxone were 82.6, 10.4, 6.0, 3.5 and 0.0 %, respectively. Multidrug resistance was detected in 19 isolates (16.5 %). The most prevalent serotypes were 19F (n525, 21.7 %), 6B (n515, 13.0 %), 9V (n514, 12.2 %) and 13 (n514, 12.2 %). Of the 64 pneumococcal isolates potentially covered by the seven-valent pneumococcal conjugate vaccine (PCV7),

44 (68.8 %) were PNSP. A high prevalence of PNSP, common pneumococcal serotypes circulating worldwide, was found, and many of the resistant pneumococci strains are covered by the PCV7. These findings indicate that the carriage rate of such resistant strains could be influenced by an appropriate vaccination programme in the study setting and by reinforcing regulations on the rational use of antimicrobial agents.

Cristina *et al.*, 2011. The study included 107 children. The most common serotypes were 14 (36.5%), 1 (16%), 5 (14.6%), 6B (6.3%) and 3 (4.2%). The proportion of identified serotypes contained in the heptavalent, 10-valent and 13‑valent conjugate vaccines was 53.1, 86.5, and 96.9%, respectively. Pneumococcal strains were sensitive to penicillin (minimum inhibitory concentration, MIC $\leq$ 2 μg/mL) in 100 cases (93.5%) and displayed intermediate resistance (MIC= 4 μg/mL) in 7 cases (6.5%). No strains were penicillin-resistant (MIC $\geq$ 8 μg/mL) according to the Clinical and Laboratory Standards Institute 2008 standards. Tested isolates were highly sensitive to vancomycin, rifampicin, ceftriaxone, clindamycin, erythromycin, and chloramphenicol.

Semczuk *et al.*, 2010. *Streptococcus pneumoniae* strains were isolated from children on admission to The Children's Memorial Health Institute, Warsaw, Poland between Jan 1999 and Dec 2007. A total of 784 isolates of *S. pneumoniae* were collected. Susceptibility to antibiotics was analyzed in two periods: 1999-2002 and 2003-2007. All the strains (185) collected in the first period originated from children with pneumococcal diseases. Of the 599 strains obtained in the second period, 234 were isolated from children with *S. pneumoniae* infection and 365 from children with nasopharyngeal carriage.

Lian *et al.*, 2010. A total of 171 *Streptococcus pneumoniae* isolates causing invasive disease were isolated from Chinese children. The serotype distribution and antimicrobial resistance were tested. The results suggested that the 7-valent pneumococcal conjugate vaccine has a preventive effect among children and that there should be long-term surveillance for serotype 19A.

Kiran *et al.*, 2010. There are reports of emergence of resistant strains of *S. pneumoniae* showing resistance to penicillin from all over the world, and now, resistance to multiple drugs (multidrug-resistant strains) has been added to it. However, scanty reports are available so far from India, depicting such resistance. Penicillin resistance was screened

by 1 µg oxacillin disk on Muller-Hinton blood agar followed by Minimum Inhibitory Concentration (MIC) detection by the agar dilution method according to the Clinical Laboratory Standards Institute (CLSI) guidelines. Antibiotic susceptibility for other antibiotics was carried out by the Kirby Bauer disk diffusion method followed by an E-test with HiComb test strips from Hi-media. Out of 50 isolates, 4% (95% Confidence Interval - 1.4, 9.4) showed total resistance to penicillin, whereas, 10% (95% CI; 1.6, 18.3) showed intermediate resistance. These penicillin-resistant pneumococci (4%) were also found to be multidrug-resistant (MDR) strains. Maximum resistance was observed for cotrimoxazole and tetracycline (24% each with 95% CI; 12.2, 35.8) followed by erythromycin and ciprofloxacin (14% each with 95%CI; 4.4, 23.6).

Katherine *et al.*, 2009. We measured the burden of pneumococcal pneumonia by applying the proportion of pneumonia cases caused by *S. pneumoniae* derived from effi cacy estimates from vaccine trials to WHO country-specific c estimates of all-cause pneumonia cases and deaths. We also estimated burden of meningitis and non-pneumonia, non-meningitis invasive disease using disease incidence and case-fatality data from a systematic literature review. In 2000, about 14·5 million episodes of serious pneumococcal disease (uncertainty range 11·1–18·0 million) were estimated to occur. Pneumococcal disease caused about 826 000 deaths (582 000–926 000) in children aged 1–59 months, of which 91 000 (63 000–102 000) were in HIV-positive and 735 000 (519 000–825 000) in HIV-negative children. Of the deaths in HIV-negative children, over 61% (449 000 [316 000–501 000]) occurred in ten African and Asian countries.

Merja *et al.*, 2009. Reported the 1007 pneumococci collected in 2002, 21.5%, 12.1%, and 14.4% were non-susceptible to erythromycin, penicillin and tetracycline, respectively. Multiresistance was detected in 10.5% of the isolates. Only 0.1% of the isolates were non-susceptible to ceftriaxone (non-meningitis breakpoint) and <1.5% to fluoroquinolones. Two isolates were nonsusceptible to linezolide. In 2002-2006, erythromycin resistance increased from 16% (2002) to 28% (2006) (Poisson regression, $p < 0.0001$), penicillin non-susceptibility from 8% to 16% (< 0.0001) and penicillin resistance from 0.8% to 3.7% ($p = 0.03$). Tetracycline resistance remained stable (~10%), as did the proportion of multiresistant isolates (~5%). Levofloxacin and ceftriaxone resistance was rare. Serotypes 14, 9V, 6B, 19F and 19A were the most frequently non-susceptible to erythromycin or penicillin. In both sets of collections of pneumococci, the highest prevalence of erythromycin resistance was among isolates derived from 0- to 2-

year-old children: in 2006, 45.8% of isolates were resistant to erythromycin in this age group. In 2002, disk diffusion testing revealed 26/1007 (2.6%) pneumococcal isolates that produced one to several clearly visible colonies inside the growth inhibition zone, indicating heterogeneous resistance to telithromycin. The telithromycin MIC50 and MIC90 of these isolates were 2 and 4 mg/L (range 0.063 to 8 mg/L), respectively, when measured by the agar dilution method, but with CLSI broth microdilution in a normal atmosphere the MIC50 and MIC90 were 0.125 and 1 mg/L, respectively (range 0.063 – 2 mg/L). The telithromycin MIC50 and MIC90 of the zone isolates (isolated from the colony growing inside of the inhibition zone) was 32 mg/L and 64 mg/L, respectively, according to the agar dilution method in 5% CO2, whilst they were 4 and 8 mg/L with CLSI broth microdilution in ambient air. This type of telithromycin resistance has not previously been described. All such isolates were *erm* (B) positive and two of them also carried *mef* (E), but the exact underlying mechanism of telithromycin resistance remained unresolved. Telithromycin resistant isolates had seven distinct sequence types, of which ST193 was the most frequent (n = 19). Other sequence types were 133, 273, 271, 2248, 2306 and 2307. PFGE results were in accordance with the MLST results. ST193 isolates were all 19A serotype variants of the PMEN clone Greece21-30, while ST273 is a representative of the PMEN global clone Greece6B-22 and ST271 is a single locus variant of a multi-drug-resistant Taiwanese19F ST236 clone.

Elina *et al.*, 2008. Pneumolysin-PCR in pleural fluid significantly improved the microbiologic diagnosis of empyema by increasing the detection rate of pneumococcus almost tenfold to that of pleural fluid culture (75 % vs. 8 %). In whole blood samples, PCR detected pneumococcus in only one child with pneumonia and one child with pneumococcal empyema. Sputum induction provided good-quality sputum specimens with high microbiologic yield. *Streptococcus pneumoniae* (46 %) and rhinovirus (29 %) were the most common microbes detected. The quantification results of the paired sputum and nasopharyngeal aspirate specimens provided support that the majority of the bacteria (79 %) and viruses (55 %) found in sputum originated from the lower airways. Pneumonia was detected in 14 % of children with influenza infection. A history of prolonged duration of fever, tachypnea, and pain on abdominal palpation were found to be independently significant predictors of empyema.

Chinwendu *et al.*, 2008. Human immunodeficiency virus (HIV)-infected patients have an increased rate of pneumococcal infections. Within the HIV-infected population, patients with low CD4 cell counts have a higher rate of pneumococcal infection. The purpose of our study was to determine pneumococcal carriage and to examine the serotypes carried by HIV-infected patients after the introduction of the conjugate vaccine. Nasopharyngeal swabs were obtained from patients during routine clinic visits. Samples were cultured on blood agar plates with gentamicin and screened for alpha-hemolysis, optochin sensitivity, and bile solubility. Capsular serotypes were determined by multiplex PCR, multibead assay, or latex agglutination. Antibiotic susceptibility was determined by the Etest method. Multilocus sequence typing was also performed. Of the 175 patients enrolled, 120 patients had absolute CD4 cell counts above 200/mm3 and 55 had counts below 200/mm3. A total of six (3.4%) patients carried pneumococci. All but one of these patients had received the 23-valent pneumococcal vaccine within the previous 5 years. Five of the isolates were serotypes that are not included in the 7-valent conjugate vaccine. Immunization with the pneumococcal polysaccharide vaccine does not prevent colonization in HIV-infected patients; however, the observation of carriage of serotypes not included in the conjugate vaccine may be due to herd immunity and serotype replacement effects in the general population.

Ataiza *et al.*, 2007. A total of 232 pneumococcal strains were isolated, including 126 (54.31%) strains from male patients. Patients had an age range of 0 to 62 years and were distributed into four age groups: 0 to 5, 6 to 17, 18 to 50, and above 50. From the 36 distinct serotypes identified, eight were more prevalent: 14, 6B, 18C, 5, 19F, 23F, 9V, and 6A. The oxacillin test identified 67 penicillin-resistant strains, out of which 47 were confirmed by the E test as having intermediate level of resistance. None of the strains exhibited high-level resistance.

Nao *et al.*, 2006. It was previously reported that two oligonucleotide primer sets (spn9802 and spn9828) for discriminating *Streptococcus pneumoniae* from pneumococcus-like oral streptococcal isolates using PCR had been developed. In this study, PCR amplification of the lytA, ply, spn9802 and spn9828 genes was used to identify presumptive *S. pneumoniae*. Two genetic groups were identified by analysing sputum samples from 28 patients with community-acquired pneumonia: the lytA-positive, ply-positive, spn9802-positive and spn9828-negative group, and the lytA-positive, ply-positive, spn9802-positive and spn9828-positive group. Isolates of the former group were resistant to

optochin, while those of the latter group showed susceptibility to optochin. The lytA-positive, ply-positive, spn9802-negative and spn9828-negative isolates, and lytA-positive, ply-positive, spn9802-negative and spn9828-positive isolates were not detected in sputum from patients with pneumonia. Subsequently, a total of 92 saliva samples from healthy individuals was screened by PCR using these primer sets. The lytA-positive, ply-positive, spn9802-positive and spn9828-negative group was identified more frequently in saliva from healthy children than in saliva from older healthy individuals and patients with pneumonia. The lytA-positive, ply-positive, spn9802-positive and spn9828-positive group was found frequently in saliva from healthy children, and in saliva and sputum from patients with pneumonia. This study demonstrates a rapid, optimal screening method for the genotypic identification of presumptive *S. pneumoniae* by PCR using four genes highly specific for *S. pneumoniae.*

Jill *et al.*, 2003. Twenty-nine cases of SPIN were identified from a total of 4428 episodes of *S. pneumoniae* infection in children. Sixty-six percent were male, and 55% were white; the mean age was 18.1 day (_8.2). Ninety percent of infants were >38 weeks' gestation. Two mothers had bacterial infections at delivery; 1 had *S. pneumoniae* isolated from both blood and cervix, and 1 had clinical amnionitis. The primary diagnoses in the neonates were bacteremia (8), meningitis (8), bacteremic pneumonia (4), septic arthritis/ osteomyelitis (1), and otitis media (8). Thirty percent of infants with invasive SPIN presented with leukopenia/neutropenia, but this did not predict poor outcome. The infecting pneumococcal serogroups were 19 (32%); 9 (18%); 3 and 18 (11% each); 1, 6, and 14 (7% each); and 5 and 12 (3.5% each). Twenty-six percent of invasive neonatal infections were caused by serogroups 1, 3, 5, and 12, which are not contained in the heptavalent pneumococcal vaccine. In contrast, 6% of invasive nonneonatal disease was caused by these same nonvaccine serogroups. Susceptibility testing demonstrated that 21.4% of isolates were penicillin nonsusceptible and 3.6% were ceftriaxone nonsusceptible. Three (14.3%) neonates with invasive SPIN died; all deaths occurred within 36 hours of presentation. Deaths did not appear to be related to pneumococcal serogroup or susceptibilities.

Jorg *et al.*, 2003. Previous antibiotic exposure is one of the most important predictors for acquisition of penicillin-nonsusceptible *Streptococcus pneumoniae* infection. To determine the impact of duration of exposure to different antibiotic classes, a study of 303 patients with *S. pneumoniae* bacteremia was undertaken. Ninety-eight cases of bacteremia

(32%) were caused by a penicillin-nonsusceptible isolate. Bivariate analysis revealed that use of β-lactams, sulfonamides, and macrolides within the last 1 and 6 months before presentation was associated with PNSP bacteremia (P <.05). Fluoroquinolone consumption was not related to bacteremia due to PNSP (P >.1). Both short- and long-term β-lactam use significantly increased the risk for PNSP infection. Logistic regression analysis revealed that use of β-lactams and macrolides in the 6 months before the first positive blood culture result were independent risk factors (P < .05). Risk for acquiring PNSP infection depends on both the class of antibiotic to which the patient was exposed and the duration of therapy.

Joaquim *et al.*, 2002. Reported the use of new diagnostic methods such as immunological techniques and polymerase chain reaction has proven invaluable for specific diagnosis and epidemiological investigation, showing adequate sensitivity, specificity and promptness of results, with the aim of guiding therapy properly. Review of epidemiological studies of community-acquired pneumonia showed that *Streptococcus pneumoniae* is still one of the most significant etiologic agents in all age groups, in developing and industrialized countries. Resistance of this agent to penicillin and cephalosporins is increasing in all continents and is worrisome. Atypical agents such as *Mycoplasma pneumoniae* and *Chlamydia pneumonia* are common in community-acquired pneumonia, mainly in children older than 4 years, representing one third of the cases in industrial countries. However, their prevalence in developing countries remains to be determined. Respiratory syncytial virus is also a very common etiology of community acquired pneumonia and may cause severe infections, mainly in infants and younger children. The introduction of new conjugated vaccines for *Streptococcus pneumoniae* and *Haemophilus influenzae* type b resulted in significant reduction of morbidity and mortality of pneumonia in children.

Susan *et al.*, 2001. Resistance to penicillin and multiple antimicrobial agents among *Streptococcus pneumoniae* strains is becoming an increasing problem worldwide and in Asia. To determine the prevalence of carriage of *S. pneumoniae* isolates not susceptible to penicillin in young children, we obtained nasopharyngeal swab specimens from 1,978 children (ages, 2 to 6 years) attending 79 day care centers or kindergartens. Three hundred eighty-three strains of *S. pneumoniae* were isolated from these children. Fifty-eight percent of these isolates had reduced susceptibility to penicillin, 123 (32.1%) were intermediate, and 100 (26.1%) were resistant. A very high penicillin MIC (4 g/ml) was

found in 3.3% of the isolates. The isolates also demonstrated high rates of resistance to other antimicrobial agents (51.2% to cefaclor, 50.2% to cefuroxime, 42.8% to cefotaxime, 80.7% to trimethoprimsulfamethoxazole, 77% to erythromycin, 60% to clindamycin, and 33.7% to chloramphenicol). No isolate was resistant to fluoroquinolone. Multidrug resistance (not susceptible to the -lactams and three or more other classes) was found in 39.4% of the isolates. Risk factors for the carriage of *S. pneumoniae* not susceptible to penicillin were multiple physician visits in the preceding 3 months and use of antimicrobial agents by the individual or by household members in the preceding 3 months. In the logistic regression analysis, only the use of antimicrobial agents in the preceding 3 months was an independent risk factor (P 0.004; odds ratio, 2; 95% confidence interval, 1.2 to 3.2). This study demonstrated the high prevalence of antibiotic-resistant *S. pneumoniae* in healthy young children in the community in Hong Kong.

Matthew *et al.*, 2001. In April 1998, surveillance nasopharyngeal cultures were obtained from 368 children aged <8 years in community A and 369 children in community B. The number of antibiotic courses per child within 1 year before culture was higher in community B than A (mean 2.2 vs 1.7). Conversely, oral cephalosporins were more frequently used in community A than B (community A: 22% received cephalosporins within 4 months; community B: 12%). Colonization with *S. pneumoniae* was detected in 24% of children in community A and 14% in community B; 36% of isolates from community A and 28% of isolates from community B were resistant or intermediately susceptible to at least 1 antibiotic tested. Reduced susceptibility was most common to trimethoprim-sulfamethoxazole and cefaclor (28% and 26%, respectively). Pneumococcal carriage (susceptible or resistant) was independently associated with age <5 years (odds ratio [OR]: 2.2), childcare exposure (OR: 2.4), presence of a sibling with a positive culture (OR: 3.3), and residence in community A (OR: 1.7). Among carriers, age <2 years (OR: 2.6), use of cephalosporins within the preceding 4 months (OR: 2.7), and having a sibling colonized with resistant *S. pneumoniae* (OR: 5.5) were independent predictors of reduced susceptibility or resistance. Each pair of resistant isolates from siblings was indistinguishable by pulsed field gel electrophoresis and other molecular typing techniques. Several pneumococcal isolates from these isolated rural areas had the molecular characteristics of international clones of multipledrug– resistant pneumococci that have been associated with worldwide spread.

Nasrin *et al.*, 1999. A total of 461 nasal swabs were collected and *S. pneumoniae* was isolated from 171 (37.1%). Penicillin resistance was found in 12.3% of these isolates, with high-level resistance in 0.6%. Resistance rates were higher for cotrimoxazole (44.4%) and erythromycin (18.1%) than for penicillin. Multidrug resistance was found in 19% of these isolates. There was a significant association between the attendance at a day care centre and carriage of pneumococcal (53% vs 32%, odds ratio (OR) 2.4, 95% confidence interval (CI) 1.5–3.7, P< 0.001). Children who attended day care centres and had received anti-biotics during the 4 months prior to swab collection were three times more likely to carry an antibiotic-resistant isolate than children who had neither attended a day care centre nor received antibiotics (68% vs40%, OR 3.1, 95% CI 1.2–8.4, P= 0.02).

Aura *et al.*, 1997. *Streptococcus pneumoniae* is one of the principal causal agents of acute respiratory infection (ARI) in children, and its resistance to antibiotics has increased worldwide. This study examined the patterns of susceptibility to antibiotics of *S. pneumoniae* that had colonized the upper respiratory tract of 272 children hospitalized for pneumonia in two hospitals in Santafe de Bogota. *S. pneumoniae* was isolated from 114 patients (42%). Diminished susceptibility to penicillin was noted in 19 isolates (17%), with 12 (11%) having an intermediate level of sensitivity and 7 (6%) showing outright resistance. Only 1 of the 19 isolates resistant to penicillin also showed resistance to ceftriaxone. There was diminished sensitivity to erythromycin in 3 isolates (3%), to chloramphenicol in 6 (5%), and to co-trimoxazole (trimethoprim + sulfamethoxazole) in 46 (40%). Resistance to multiple drugs was found in 7 isolates (6%). The most commonly encountered penicillin resistant serotype was 23F (68.4%). An association was observed between age, prior use of antibiotics, and colonization by *S. pneumoniae* with reduced penicillin sensitivity or multiple-drug resistance. This study confirmed the presence of antibiotic-resistant *S. pneumoniae* in Colombia and highlights the importance of the rational use of antibiotics and of the implementation of epidemiologic surveillance for this agent.

Vilhjalmur *et al,* 1996. Total antimicrobial sales for children (6223 prescriptions) among the four areas for which data were available ranged from 9.6 to 23.2 defined daily doses per 1000 children daily (1.1 to 2.6 courses yearly per child). Children under 2 consumed twice as much as 2-6 year olds (20.5 v 10.9 defined daily doses per 1000 children daily). Nasopharyngeal specimens were obtained from 919 children, representing 15-38% of the

peer population groups in the different areas. Pneumococci were carried by 484 (52.7%) of the children, 47 (9.7%) of the isolates being resistant to penicillin or multiresistant. By multivariate analysis age (<2 years), area (highest antimicrobial consumption), and individual use of antimicrobials significantly influenced the odds of carrying penicillin resistant pneumococci. By univariate analysis, recent antimicrobial use (two to seven weeks) and use of co-trimoxazole were also significantly associated with carriage of penicillin resistant pneumococci.

CHAPTER III

MATERIALS AND METHODS

3.1 Study design

A cross-sectional study design was employed. Blood & Nasopharyngeal swab samples of pneumonia patients were collected at different intervals during the study period.

3.2 Study area and period

This study was conducted from January-November 2018 at different hospitals of Dinajpur district of Bangladesh. All microbiological analysis was carried out in microbiology laboratory of Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur.

The samples were collected from three main hospitals of Dinajpur district that includes: M Abdur Rahim Medical College Hospital, Dinajpur, Aurobindo Shishu Hospital, Dinajpur & Islami Bank Community Hospital, Dinajpur.

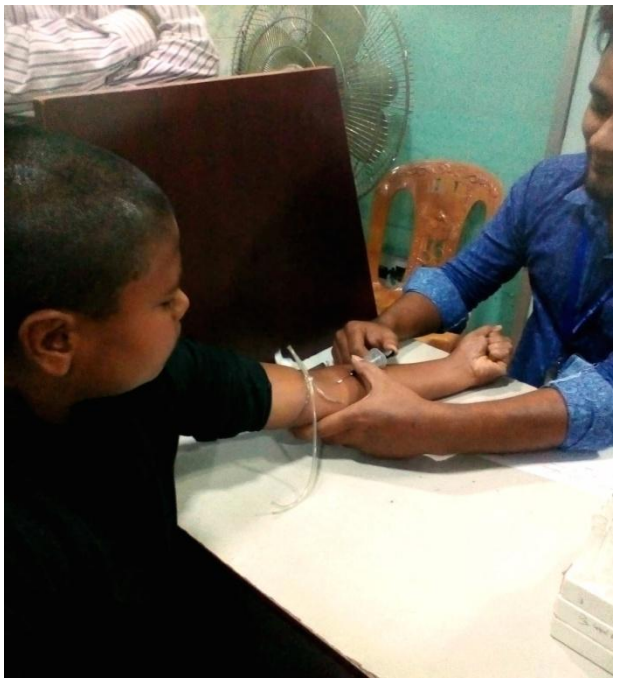

Plate No. 1. Sample Collection

3.3 Collection of Sample

40 samples were collected from three hospitals of Dinajpur district. Out of 40 samples, 20 samples were collected from M. Abdur Rahim Medical College Hospital. 15 samples were collected from Arobindo Shishu Hospital of Dinajpur & the rest of 5 samples were collected from Islami Bank Community Hospital, Dinajpur (IBCH).

3.4 Experimental Layout

The present study was conducted into three phases; in first phase, samples were collected by different age of pneumonia patients & hospital and age of the patients were selected. In the second phase isolation and identification of the organisms from the collected sample using cultural, staining, biochemical & molecular characterization was done. In third phase drug resistant pattern of the selected isolates were determined by using different antibiotic discs available in the market. The experimental layout of the present study is shown in figure:

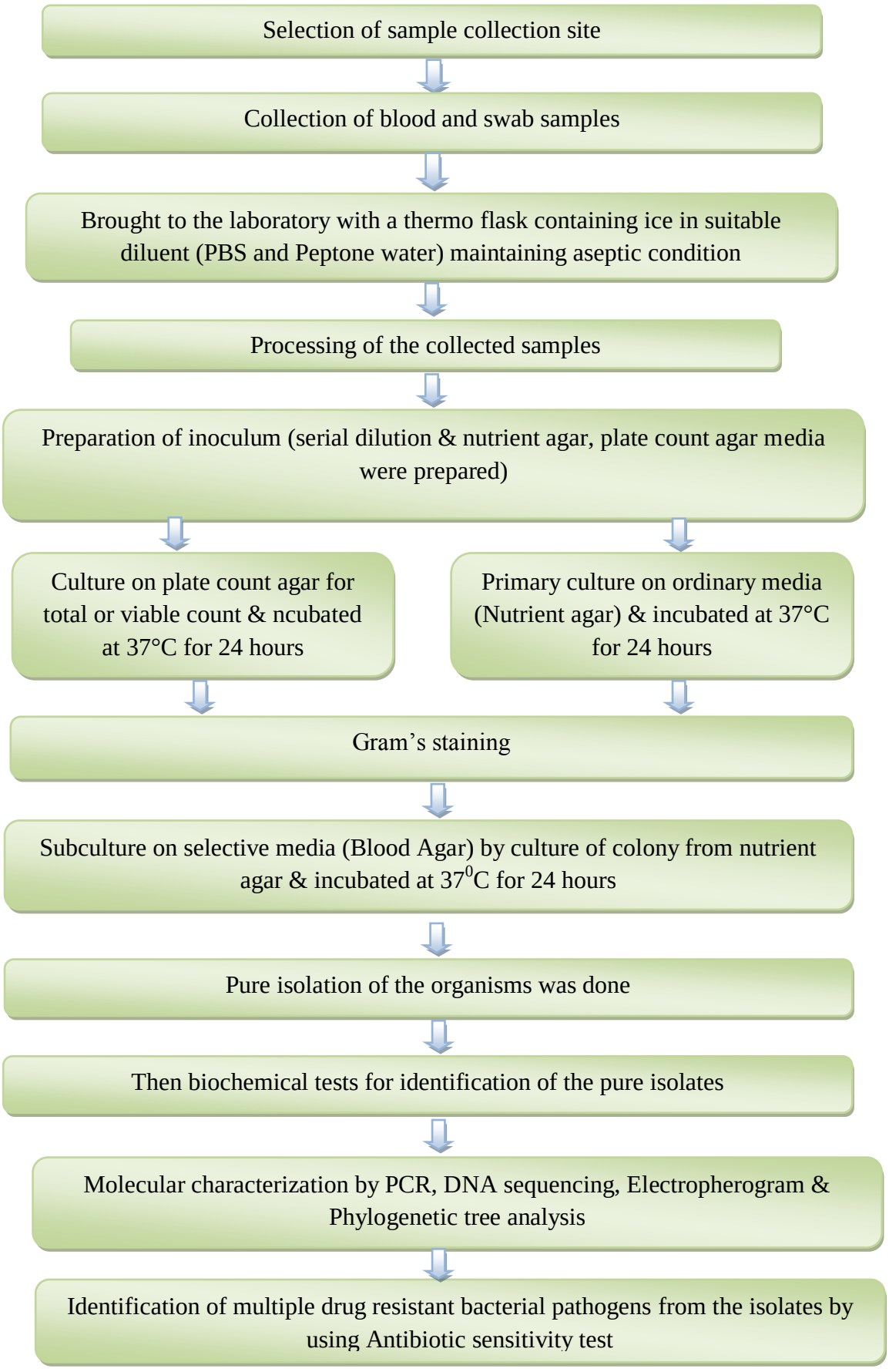

Plate No. 2: The schematic illustration of the experimental layout

3.5 Sample processing, isolation and identification of bacteria

All samples that were collected from different hospitals of Dinajpur district were transported to the microbiological laboratory of department of Microbiology, HSTU, in cool conditions and processed within two hours of collection. To determine the total viable plate count, serial 10-fold dilutions of samples were prepared in physiological saline, and 0.5 mL aliquot was spread plated on plate count agar (PCA). Plates were incubated for 24 hours at 37ºC before bacteriological counts were done.

The number of colonies on each plate having 30–300 colonies was counted by using a digital colony counter. Finally, the bacterial count was reported CFU/mL as follows:

$$CFU/mL = \frac{\text{C}olonies\ counted \times \text{Dilution factor}}{Actual\ volume\ of\ sample\ in\ plate, mL}$$

After that, based on colony morphology representative colonies were picked and sub-cultured on differential media such as blood agar. After obtaining pure colonies and recording important features such as hemolysis on blood agar isolated organisms were identified biochemically in a systematic way following standard methods (Holt JG *et.al.)*

3.6. Bacteriological Media

3.6.1 Media used in the Experiment

3.6.1.1 Solid Media

1. Plate Count Agar

Plate Count Agar (PCA), also called Standard Methods Agar (SMA), is a microbiological growth medium commonly used to assess or to monitor "total" or viable bacterial growth of a sample. PCA is not a selective medium (Cheesbrough, 1985).

2. Nutrient Agar Medium (HI-MEDIA, India)

Nutrient agar is a basic media that is used for cultivating of non-fastidious microorganisms (Cheesbrough, 1985).

3. Blood Agar Medium (HI-MEDIA, India)

For the isolation, cultivation and detection of hemolytic reaction of fastidious microorganisms. Blood Agar Base is suitable to isolate and cultivate a wide range of microorganisms with difficult growth. Upon adding blood, it can be utilized for determining hemolytic reactions *Streptococcus pneumoniae* gives alpha hemolysis. *Streptococcus pyogenes* gives beta hemolysis (Clin. Path 1951).

3.6.1.2 Liquid Media

- Nutrient broth (Difco)
- Methyl Red-Voges Proskauer (MR-VP) broth (Difco)
- 1% Pepton Water (Difco)
- Motility Indole Urease Broth (Difco)
- Buffered peptone water Broth

3.6.2. Reagent

- Crystal violet dye
- Grams iodine
- Alcohol
- Safranin
- Saline
- Iodine solution
- Kovac's reagent
- Methyl- red solution
- 3% H_2O_2
- P –Amino dimethylanilin oxalate
- Phenol red
- Phosphate buffered saline (PBS) solution
- 10% sodium deoxycholate (Bile salt) solution.

3.7 Materials used for bacterial genomic DNA isolation

- TE buffer
- 10% (w/v) Sodium dodecyl sulfate (SDS)
- 20 mg/ml protinase k (stored in small single-use aliquots at -20°C)
- 3 M Sodium Acetate, pH 5.2
- 25:24:1 Phenol/Chloroform/Isoamyl alcohol
- Isopropanol
- 70% Ethanol
- 95% Ethanol
- 1.5 ml Microcentrifuge tubes

3.7.1 Meterials used for Polymerase Chain Reaction

Table 1: PCR Reaction Mixture for 16s rRNA

Buffer	2.5 µl
dNTP	2.5 µl
$MgCl_2$	2.5 µl
Forward Primer (27F)	1.0 µl
Reverse Primer (1492R)	1.0 µl
Nano Pure Water	12.5 µl
DNA	2.0 µl
Taq DNA Polymerase	1.0 µl
Final Volume	25 µl

- Primers used for PCR :

  16S rRNA gene region was amplified with the universal primers.

  - Forward primer- 27F (5' AGAGTTTGATCCTGGCTCAG 3')
  - Reverse primer- 1492R (5' TACCTTGTTACGACTT 3')
- Thermal Cycler (Thermo cycler, ASTEC, Japan)
- 2% Agarose gel
- Gel casting tray with gel comb
- TAE buffer
- Microwave oven
- Conical flask

- Electrophoresis apparatus (Biometra standard power pack P 2T)
- 100 bp DNA size marker
- Bromphenicol blue of loading bufter
- Ethidium bromide (0.5 µl/ml)
- Distilled water
- UV trans-illuminator

3.8 Glassware and Appliances

The different types of important equipment used for this work are listed in following: Distilled water, Sterile bent glass or plastic spreader rods, Micropipette (5-50µl; 10-100µl; 50-500µl; 100-1000µl), Freeze (-20°C),Refrigerator (4°C), Spirit lamp, Vortex Mixture, Labeling tape**,** Experimental test tube, Stopper, Petri dish, Conical flask, Durham's tube, Slide, Microscope, Cotton, Immersion Oil**,** Autoclave**,** Thermometer, Incubator**,** Jar, Beaker, Cylinder, Electric Balance, Filter paper, Bacteriological loop etc.

3.9 Morphological Characterization of Bacteria by Gram's Staining Method

The most widely used staining procedure in microbiology is the gram stain ,discovered by the Danish scientist and physician Hans Christian Joachim Gram in 1884, Gram staining is a differential staining technique that differentiates bacteria into two groups: gram-positives and gram-negatives. The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. Gram-negative bacteria are decolorized by the alcohol, losing the color of the primary stain, purple. Gram-positive bacteria are not decolorized by alcohol and will remain as purple. After decolorization step and a counterstain is used to impart a pink color to the decolorized gram- negative organisms.

3.10 Preparation of Gram's staining solution

The four solutions:

- Crystal violet
- Gram's iodine
- 95% Alcohol
- Safranin (Counterstain).

3.10.1 Gram staining procedure

1. Clean glass slides were taken.
2. A sterile technique was used, a smears of each of the organisms was prepared. Smear made of a drop of water on the slide was placed then each organism separately to the drop water with a sterile was transferred. A circular motion of the inoculating loop of organism was mixed and spreads.
3. Air–dry the smears were allowed then heat-fixed in the usual manner.
4. Smears were flooded with crystal violet and let stood for 1 min then slides were washed with tap water.
5. Smears were flooded with grams iodine mordant and let stood for 1 min. Slides were washed with tap water.
6. Smears were decolorized with 95% ethyl alcohol. Slides were washed with tap water.
7. Smears were counter stain with safranin for 30 sec. Slides were washed with tap water.
8. Slides were examined under oil immersion. (James G. Cuppuccion, Natalie Sherman, 1996).

3.11 Biochemical Tests

Isolated organism with supporting growth characteristics of suspect identified by biochemical tests. Several types of biochemical tests were performed in this study to confirm the specific bacteria that are as follows:

1. Catalase Test
2. Indole Test
3. Methyl Red (MR) Test
4. Voges Proskauer (VP) Test
5. Lactose Fermentation Test
6. Triple Sugar Iron (TSI) Test
7. Bile Solubility Test

3.11.1 Catalase Test

This test was used to differentiate bacteria which produce the enzyme catalase. To perform this test, a small colony of good growth pure culture of test organism was smeared on a slide. Then one drop of catalase reagent (3% H_2O_2) was added on the smear. The slide was observed for bubbles formation. Formation of bubble within few seconds was the indication of positive test while the absence of bubble formation indicated negative result (Cheesbrough, 1985).

3.11.2 Indole Test

Two milliliter of peptone water was inoculated with the 5 ml of bacterial culture and incubated at 37°c for 48 hours. Kovac's reagents (0.5ml) were added, shake well and examined after 1 minute. A red color in the reagent layer indicated indole test positive. In negative case there is no development of red color (Cheesbrough, 1985).

3.11.3 Methyl Red Test (MR)

Sterile MR-VP broth was inoculated with the test organism and following incubation at 37°c for 24 hours. if the organism would ferment glucose via the mixed acid fermentation pathway like lactic, acetic, which decreases the PH , hence upon the addition of the indicator methyl red the broth becomes red in color and yellow color indicated a negative result (Cheesbrough, 1985).

3.11.4 Voges-Proskauer Test (VP)

Voges Proskauer Test – If the organism would ferment glucose via the butylenes glycol pathway, an intermediate product, acetyl methyl carbinol or acetone which is neutral is converted to diacetyl upon the addition of the VP – Reagent –B (40% KOH with 0.3% creatine) in the presence of VP – Reagent – A (5% alpha- naphthol in abs. methyl alcohol). Diacetyl is red in color. Negative is yellow color (Cheesbrough, 1985).

3.11.5 Triple Sugar Iron (TSI) Test

This tube medium is used to identify Gram-negative enteric bacilli based on the following biochemical characteristics (Cheesbrough, 1985)

- Glucose fermentation – indicated by yellow butt
- Lactose fermentation – indicated by yellow slant

- Hydrogen sulfide production – indicated by blackening of the medium
- Gas production – indicated by presence of a crack, bubble or gas space
- pH indicator – phenol red
- Hydrogen sulfide indicator – ferric ammonium citrate with sodium thiosulfate.

3.11.6 Bile Solubility Test

Bile Solubility Test is the test, which differentiate *Streptococcus pneumoniae* (positive-soluble) from alpha-hemolytic streptococci (negative- insoluble). *Streptococcus pneumoniae* is bile soluble whereas all other alpha-hemolytic streptococci are bile resistant. *S. pneumoniae* has an autolytic enzyme, which can be demonstrated by allowing a broth culture to age in the incubator; at 24 hours, the broth is turbid; after a few days, the medium will become clear. Bile or a solution of a bile salt (e.g., sodium desoxycholate) rapidly lyses pneumococcal colonies. Lysis depends on the presence of an intracellular autolytic enzyme, amidase. Bile salts lower the surface tension between the bacterial cell membrane and the medium, thus accelerating the organism's natural autolytic process. Bile salts activate the autolytic enzyme, which induces clearing of the culture (Cheesbrough, 1985).

3.11.6.1 Reagents for Bile Solubility Test

10% sodium deoxycholate (bile salt) solution

- Dissolve 10 gram of sodium deoxycholate into 100 ml sterile distilled water.

3.12 Antibiotic susceptibility testing

Once the bacteria is isolated and identified from each sample collected, the standard Kirby-Bauer disk diffusion method was used to determine the antimicrobial susceptibility profiles of the isolates according to the recommendations of National Committee for Clinical Laboratory Standards (CLSI 2013).

Bacterial inoculum was prepared by suspending the freshly grown bacteria in 4–5 ml sterile nutrient broth and the turbidity was adjusted to that of a 0.5 McFarland standard.

The antimicrobial susceptibility testing was performed using Mueller-Hinton medium, Antibiotic disks were applied using a sterile forceps. Agar plates were incubated at 37°C for 18 hours.

After overnight incubation at 37 °C, the diameter in millimeters of the zones of inhibition around each of the antimicrobial discs was recorded and categorized as resistant or sensitive in accordance with company recommendations. All isolates were tested for sensitivities to 10 of routine and practical antibiotics.

3.12.1 Antimicrobial agents with their disc concentration

Sl. No	Antimicrobial agents	Symbol	Disc concentration (µg/disc)	Diameter of zone of inhibition (ZOI)		
				Resistant	Intermediate	Susceptible
1	Penicillin	P	10	≤ 11	12-21	≥ 22
2	Ampicillin	AMP	25	≤ 13	14-16	≥ 17
3	Amoxicillin	AM	30	≤ 13	14-17	≥ 18
4	Amikacin	AK	30	≤ 14	15-16	≥ 17
5	Cephalexin	CN	30	≤ 14	-	≥ 14
6	Ciprofloxacin	CIP	5	≤ 20	21-30	≥ 31
7	Gentamycin	GEN	10	≤ 12	13-14	≥ 15
8	Kanamycin	K	30	≤ 13	14-17	≥ 18
9	Erythromycin	E	15	≤ 13	14-17	≥ 18
10	Azithromycin	AZM	10	≤ 13	14-17	≥ 18
11	Neomycin	N	30	≤ 12	13-16	≥ 17
12	Vancomycin	VA	30	≤ 9	10-11	≥ 12
13	Streptomycin	S	10	≤ 11	12-14	≤ 15
14	Bacitracin	B	10	≤ 8	9-12	≤ 13

Source: CLSI- 2013 [Note: S=Sensitive, R=Resistant, I=Intermediate]

3.13 Maintenance of stock culture by sterile buffered glycerin method

After the experiment it was necessary to preserve the isolated organisms for longer periods. For this purpose, pure culture of at least one organism per isolates were stored in sterilized 80% glycerin and used as stock culture. The equal volume of 80% glycerin and bacterial culture were mixed and sealed with paraffin wax and stored at 37° c. The isolated organisms were given code name for convenience. (Buxton and Fraser, 1977).

3.14 Laboratory Preparation

All items of glassware including test tubes, pipettes, cylinders, flasks conical flasks, glass plates, slides, vials and other necessary instruments cleaned by detergents powders. The glassware then cleaned by brushing, washed thoroughly and finally sterilized by autoclaving for 15 minutes at 121°c under 15 lbs. pressure per square inch. Autoclaved items were kept in oven at 50°C for further use.

3.15 Preparation of Culture Media

3.15.1 Solid Media

3.15.1.1 Plate count agar (PCA)

17.5g added to 1 litre of distilled water. Dissolved by bringing to the boil with frequent stirring, then mixed & distributed into final containers. Sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, the medium was poured into sterile petri dishes & allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37°C overnight to check their sterility & used for cultural characterization or stored at 4°C for further use (Cater, 1979).

3.15.1.2 Nutrient agar Media

28 grams of nutrient agar was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely (necessary calculation was done for required number of plates).The medium was then sterilized by autoclaving sterilized by autoclaving for 15 minutes at 121°c under 15 lbs. pressure per square inch. After autoclaving, the medium was poured into each sterilized petri dishes and allowed to solidify. After solidification all petri dishes was incubated at 37°c for overnight to check their sterility and used for cultural characterization (Cater, 1979).

3.15.1.3 TSI Slant

65 grams TSI agar base powder was mixed in 1000 ml of cold distilled water in a flask and mixed thoroughly, then heated to boiling for dissolving the medium completely (necessary calculation was done for required number of test tubes).The medium was then sterilized by autoclaving for 15 minutes at 121°c maintaining a pressure of 1.2 kg/.Then 20/10 ml of medium was poured into each sterilized test tubes and allowed to cool and to

solidify (kept in horizontal position). After solidification test tube were used for biochemical characterization and incubated at 37°c for 24 hours (Cater, 1979).

3.15.1.4 Blood Agar

40 gams of Blood agar base (HI-MEDIA, India) powder was dissolved in 1000ml of distilled water. The medium was sterilized by autoclaving at 1.2 kg/cm^2 Pressure & 121°C for 15 minutes & 45°C. Then 5-10% sterile defibrinated blood was added to the medium and distributed to sterile Petridishes and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characteriation or stored at 4°C refrigerator for future use. (Cater, 1979).

3.15.2 Liquid Media

3.15.2.1 Nutrient Broth

13 grams of nutrient broth was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely(necessary calculation was done for required number of plates). The solution is then distributed in test tubes, stoppered with cotton plugs and sterilized in the autoclave machine at 121°c and 15 pounds pressure per square inch for 15 minutes. The sterility of the medium was checked by incubating overnight at 37°c and used for general cultural medium (Cater, 1979).

3.15.2.2 Methyl red- Voges Proskauer (MR-VP) Broth

17 grams of MR-VP medium was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely(necessary calculation was done for required number of plates). The solution is then distributed in test tubes, stoppered with cotton plugs and sterilized in the autoclave machine at 121°c and 15 pounds pressure per square inch for 15 minutes. The sterility of the medium was checked by incubating overnight at 37°c and used for biochemical characterization (Cater, 1979).

3.16 Preparation of Reagents

3.16.1 Methyl red solution

The indicator MR solution was prepared by dissolving 0.05 gram of Bacto methyl-red in 28ml of 95% alcohol and diluted to 22 ml with addition of distilled water (Merechant and Packer, 1967).

3.16.2 Alpha- Naphthol solution

Alpha- Naphthol solution was prepared by dissolving 5 gram of 1-Naphthol in 100 ml of 95% ethyl alcohol (Merechant and Packer, 1967).

3.16.3 Kovac's Reagent

The solution was prepared by mixing 25 ml of concentrated Hydrochloric Acid in 75 ml of Amyl alcohol and to this mixture 5 grams of paradimetyl –aminobenzyldehide crystals were added. This was then kept in a flask equipped with rubber cork for future use (Merechant and Packer, 1967).

3.16.4 Potassium hydroxide solution

To prepare 40% KOH solutions add 4gm KOH solids with 100 ml distilled water (Merechant and Packer, 1967).

3.16.5 Phosphate buffered Saline Solution

For preparation of phosphate (PBS) solution, 8 grams of sodium chloride, 2.89 gram of disodium hydrogen phosphate disodium hydrogen phosphate, 0.2 gram of potassium chloride and 0.2 gram of potassium hydrogen phosphate were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely. The solution was then sterilized by autoclave at 121°cmaintainig a pressure of 15 pounds per square inch for 15 minutes and stored at refrigerator until use. The p^H of the solution was measured by a p^H meter and maintained at 7.0-7.2 p^H (Cheesbrough 1994).

3.17 PCR Amplification, Sequencing of 165 rRNA Genes with Universal Primers and Phylogenetic Analysis of *Streptococcus pneumonae*

3.17.1 Basic protocol of bacterial genomic DNA isolation

Bacteria from saturated liquid culture are lysed and proteins are removed by digestion with protinase-k. Cell wall debris, polysacharides and remaining proteins are removed by Phenol-chloroform extraction and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation.

Procedure

- A 25 ml of liquid culture was inoculated with *Streptococcus pneumonae*.
- 1.0 ml of the overnight culture was allowed to spin in a microcentrifuge tube for 5 minutes at 10000 rpm.
- The supernatant was discarded.
- This step was repeated.
- The pellet was re-suspended in 467 µl TE bufer by repeated pipetting. 30 µl of 10% SDS & 3 µl of 20 mg/ml Proteinase-k to give a final concentration of 100 µl/mg Proteinase-k in 0.5% SDS. Thoroughly mixed & incubated 30 min for 1 hr at 37°C.
- Approximately equal volume (500 µl) of Phenol/Chloroform/Isoamyl alcohol was added. Mixed thoroughly but very gently to avoid shearing the DNA, by inverting the tube until the phase are completely mixed.
- Then the tubes were centrifuged at 12000 rpm for 10 minutes.
- The aqueous & viscous supernatant (~400 µl) were removed to a fresh microcentrifuge tube, leaving the interface behind. An equal volume of Phenol/ Chloroform/ Isoamyl alcohol extract were added thoroughly mixed & allowed to spin in a microcentrifuge at 10000 rpm for 5 min.
- The supernatant was transfered to a fresh tube.
- 1/10th volume of 3M sodium acetate was added & mixed well.
- 0.6 volume of isopropanol was added to precipitate the nucleic acids & kept on ice for 10 minutes.
- Centrifuged at 13500 rpm for 15 minutes.
- The supernatant was discarded.

- The obtained pellet was washed with 1 ml of 95% ethanol for 5 minutes. Then centrifuged at 12000 rpm for 10 minutes.
- The supernatant was discarded.
- The pellets was dried as there is no alcohol.
- The pellet was re-suspended 50 µl of TE & then 7.5 µl of RNase. The DNA was stored at 4°C for short term and at -20°C for long term.

3.17.2 PCR amplification & sequencing of 16S rRNA

Condition of PCR:

Table 2: Condition of PCR

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	5 min	01
Denaturation	95°C	30 sec	35
Annealing	56 °C	30 sec	
Extension	72 °C	1.5 mn	
Final extension	72 °C	10 min	01
Holding	4 °C	Hold	-

3.17.3 Electrophoresiss

Process of Electrophoresis:

- Preparation of gel.
- Sample application in the gel.
- Adjustment of voltage or current (gel-electrophoresis about 70-100 volts).
- Set up run time about 30-60 minute
- When DNA migrated sufficiently, as judged from the migration of bromphenicol blue of loading buffer, the power supply was switched off.
- The gel stained in ethidium bromide (0.5 µl/ml) for 10 minutes, in a dark place.
- The gel was distained in distilled water for 10 minutes. The distained gel was placed on the imaging system in the dark chamber of the image documentation system.
- The UV light of the system was switched on; the image was viewe on the monitor, focused, acquired and saved in an USB flash drive.

3.17.4 Nucleotide seqence accession number and BLAST analysis

The nucleotide sequence 16S rRNA gene region date was submitted to NCBI nucleotide sequence database. Using BLAST tool, phylogenic tree, primer pairs were designed from NCBI database search tool.

CHAPTER IV
RESULTS

The present research was designed to determine the isolation, identification and antibiogram study of bacterial pathogens isolated from blood and nasopharyngeal swab samples of pneumonia patients from different hospitals of Dinajpur district of Bangladesh. The collected samples were subjected to various bacteriological examination such as cultural, biochemical techniques and antibiotic sensitivity pattern in the laboratory of the department of microbiology, HSTU, Dinajpur, for isolation and identification of bacteria. A total number of 40 samples were collected from pneumonia patients (3-15 years) for this study. Out of 40 samples, 15 were found to be positive for pneumonia and total 15 isolates were isolated. Result of morphological, staining, cultural, biochemical, antibiotic sensitivity pattern and percentage of incidence of isolated bacteria are presented in different tables.

4.1. Results of microbial assessment of collected blood and nasopharyngeal swab samples by total viable counts

Serial dilution was performed to reduce the number of bacteria and also to obtained total viable counts. Samples (50µl) were spread on PCA plate up to dilution 10^{-7}. For the growth of bacteria after 24 hours of incubation at 37^0C aerobically and were indicated by the growth of circular, smooth, convex, and gray white or yellowish colonies. Then the number of colonies were observed and recorded. The numbers of total viable count are shown in table 3. In case of PCA, the highest total viable count 8.6×10^9cfu/ml was found in 10^{-7} dilution and lowest 1.6×10^9cfu/ml in 10^{-6} dilution.

Table 3: Results of total viable counts in PCA agar

Dilution	Number of colony	Result
10^{-1}	More than 300	TNTC
10^{-2}	More than 300	TNTC
10^{-3}	More than 300	TNTC
10^{-4}	280	5.6×10^7 cfu/ml
10^{-5}	115	2.3×10^8 cfu/ml
10^{-6}	80	1.6×10^9 cfu/ml
10^{-7}	43	8.6×10^9cfu/ml

Legends: PCA=Plate count agar, TNTC= Too numerous to count, CFU=Colony forming unit

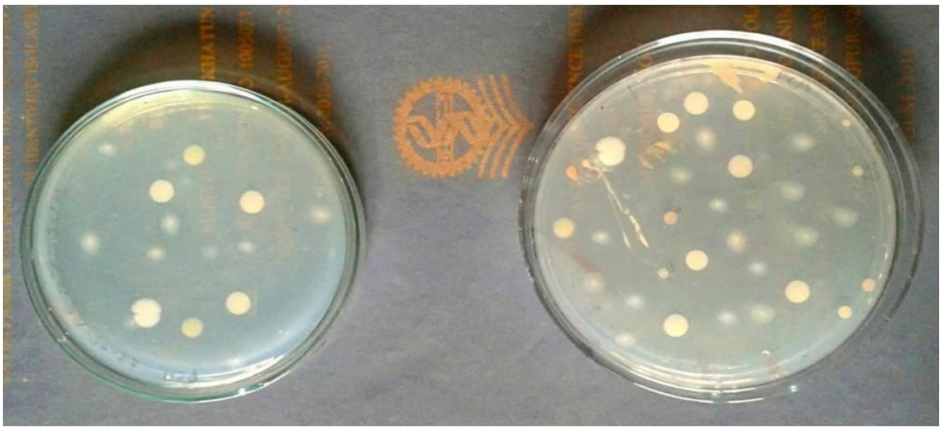

Plate No. 3: Colony count on Plate Count Agar plate

4.2. Isolation and identification of bacteria by different bacteriological Methods:

4.2.1 Results of Cultural Examination

The cultural characteristics of *Streptococcus pneumoniae* on various media are presented in Table 4.

Table 4. The results of cultural characteristics of the bacteria which were isolated from blood & nasopharyngeal swab samples of pneumonia patients

Name of Bacteria	Samples	Staining Characteristic	Name of Media	Colony Characteristics
Streptococcus pneumoniae	Nasopharyngeal swab	Short chain forming Gram positive spherical cells, violet in colour.	Nutrient broth	Uniform turbidity
			Nutrient agar	Convex, smooth & gray white colonies
			Blood agar	Alpha hemolytic colony
	Blood	Short chain forming Gram positive spherical cells, violet in colour.	Nutrient broth	Uniform turbidity
			Nutrient agar	Smooth & gray white colonies
			Blood agar	Alpha hemolytic colony

4.2.2 Results of nasopharyngeal swab sample inoculation on Nutrient broth, Nutrient agar & Blood agar

After inoculation of nasopharyngeal swab sample on Nutrient agar and Nutrient broth, it was observed that mat growth was found on Nutrient agar characterised by convex, circular, white colony & turbidity was found in Nutrient broth. Alpha hemolytic colonies were found on blood agar media.

Plate No. 4: Fresh Nutrient broth (Right), Turbidity on Nutrient broth (Left).

Plate No. 5: Fresh Nutrient agar plate (Right), Organisms on Nutrient agar showing convex, circular, white colony (Left).

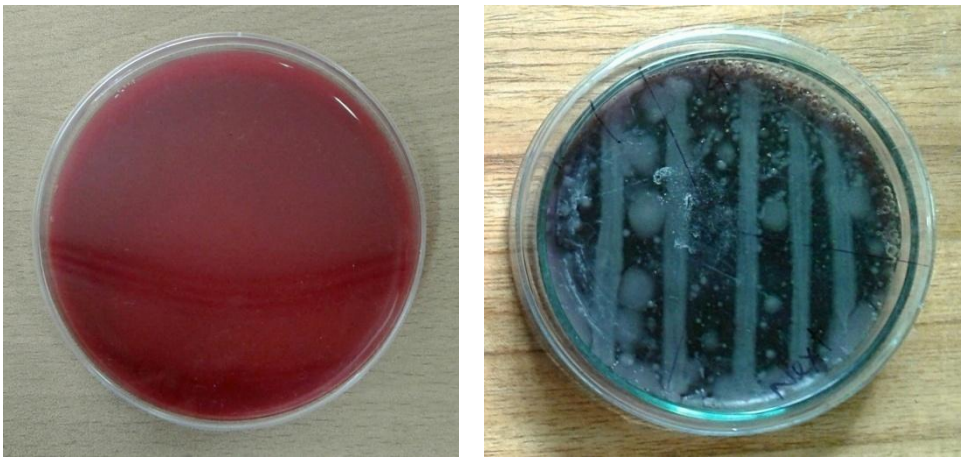

Plate No. 6: Fresh blood agar plate (Right), Alpha hemolytic colonies on blood agar (Left).

4.2.3 Results of Blood sample inoculation on Nutrient broth, Nutrient agar & Blood agar

4.2.3.1 Nutrient broth

Uniform turbidity was found on overnight culture of blood sample in Nutrient broth incubated at 37°C which indicates bacterial growth in the media.

Plate No. 7: Uniform turbidity

4.2.3.2 Nutrient Agar

Nutrient agar plates are spread with the blood sample & revealed the growth of bacteria after 24 hours of incubation at 37^{o}C aerobically which were indicated by the growth of smooth & gray white or yellowish colonies.

Plate No. 8: Culture on Nutrient Agar plate with smooth & gray white colonies

4.2.3.3 Blood Agar

Blood sample of pneumonia patients are taken as inoculum & cultured on nutrient agar plates. Then colonies of the organism are taken from nutrient agar & spread on blood agar plates by sterile platinum wire loops (Subculture). After 24 hours of incubation at 37^{o}C anaerobically (5% CO_2 in anaerobic jar), the growth of bacteria were indicated by characteristic alpha hemolytic colonies.

Plate No. 9: Alpha hemolytic colonies on blood agar

4.2.4 Results of Microscopic Examination

Microscopic observation was performed to observe the morphology of the isolates. Gram positive isolates were found under microscope by oil emmersion lens. Gram positive isolates were spherical chain shape cocci cells & showed purple coloured colonies under 100X microscopy.

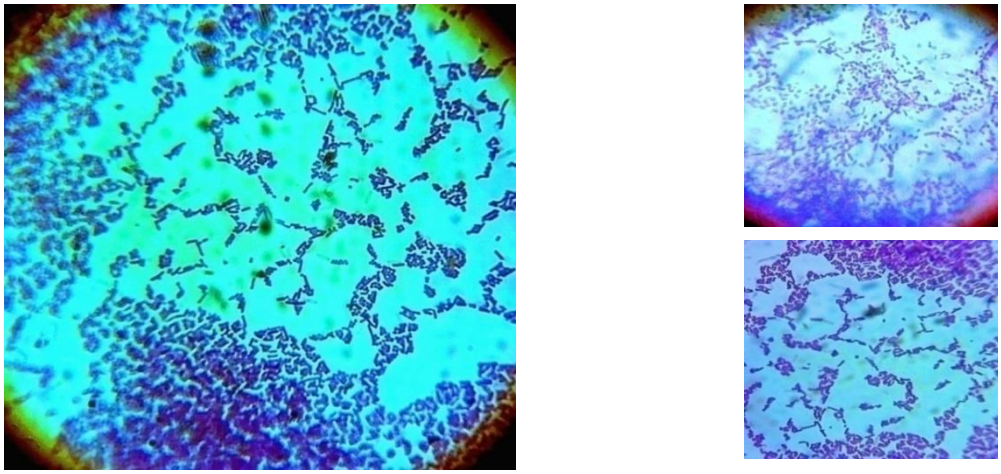

Plate No. 10: Gram positive spherical chain shape cocci were found on Gram's staining.

4.3 Results of Biochemical Tests

Biochemical test was performed to confirm the bacteria that cause pneumonia in pneumonia patients. After observing the colony characters on different media and also gram staining, I have selected biochemical tests to identify bacteria. The following table shows the biochemical test results:

Table 5: Results of Biochemical Tests

Name of the isolate	Catalase	Oxidase	MR	VP	Indole	Lactose Fermentation	TSI	Bile Solubility
Streptococcus pneumoniae	-ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve

Legends: +ve = positive, -ve = negative, MR= Methyl-Red, VP= Voges-Proskauer, TSI= triple sugar iron.

4.3.1 Catalase Test

Streptococcus pneumoniae gives a negative result for Catalase test indicated by absence of bubble formation resulting from production of oxygen gas.

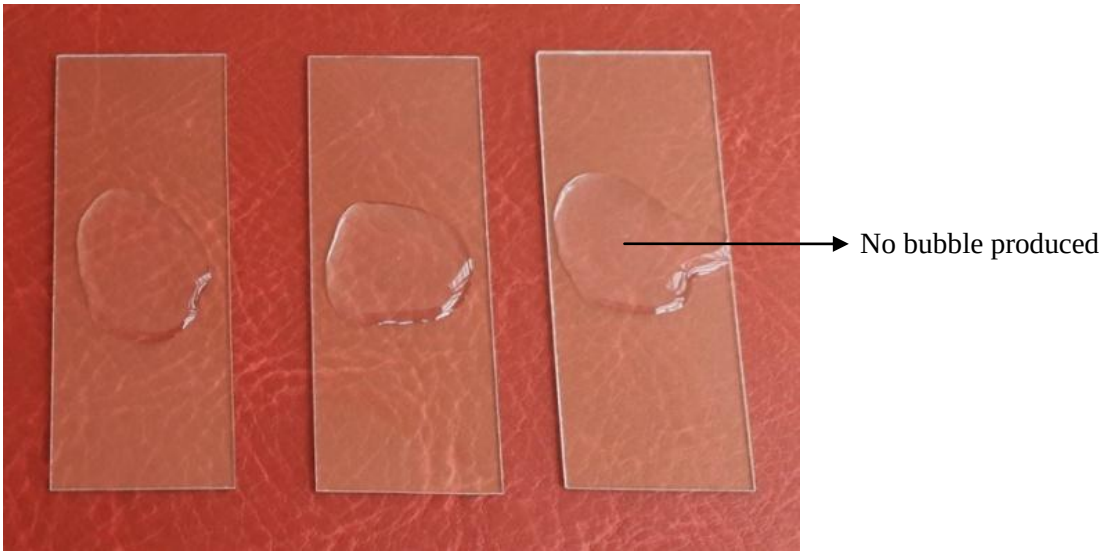

Plate No. 11: No bubble production indicates negative result in Catalase test.

4.3.2 Oxidase Test

Streptococcus pneumoniae gives a negative result in oxidase test indicated by no development of purple colour when the colonies of the organism came in contact with the filter paper soaked with oxidase reagent.

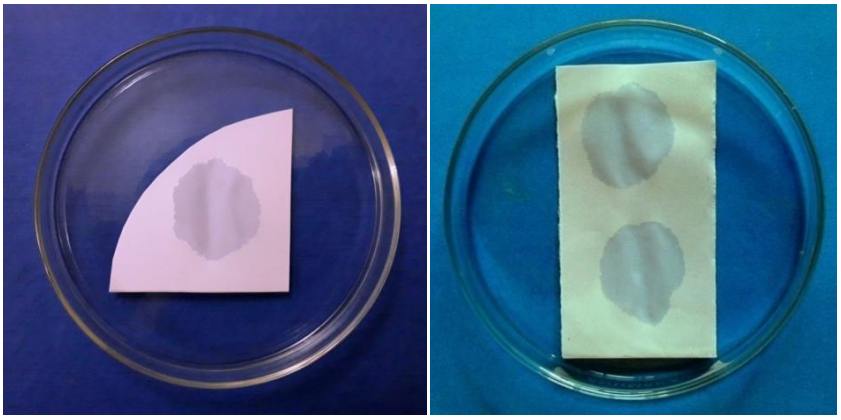

Plate No. 12: No purple colour development indicates a negetive test & absence of oxydase enzyme.

4.3.3 Methyl-Red Test

Streptococcus pneumoniae gives a positive reaction for Methyl-Red test indicated by a bright red coloured ring formation in the reagent layer on the top of the medium.

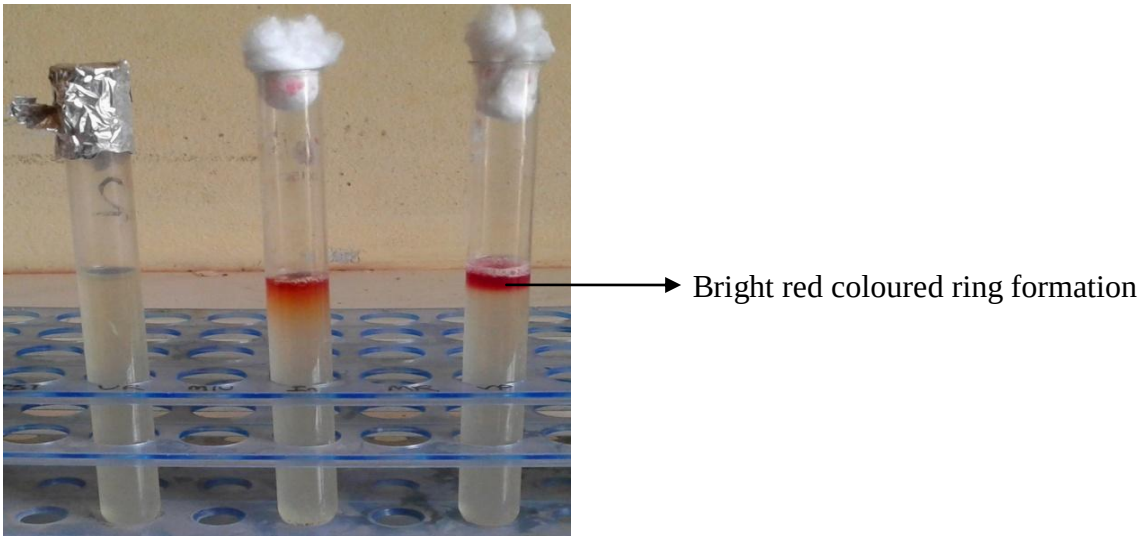

Plate No. 13: Methyl-Red test result was positive indicated by colour change of the medium to bright red ring formation (right) & the left is control.

4.3.4 Voges-Proskaur Test

Streptococcus pneumoniae shows a negative result for Voges-Proskaur test indicated by lack of rose red colour change of the medium.

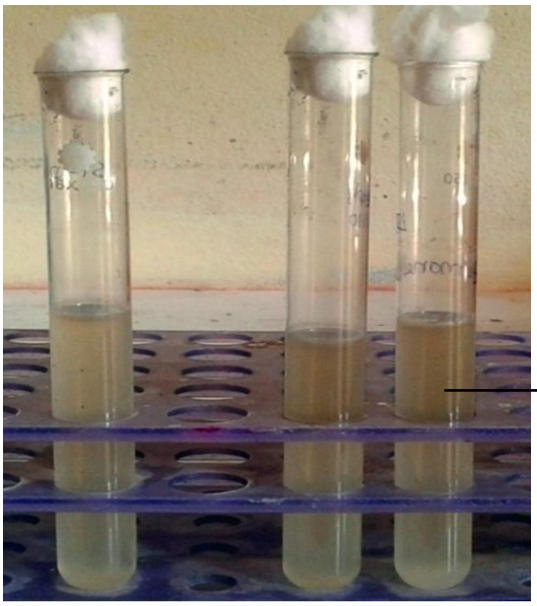

Plate No. 14: The result of Voges-Proskauer test was negative indicated by no red color change of the medium (right) & control (left).

4.3.5 Indole Test

Streptococcus pneumoniae shows a negative result for indole test indicated by no appearance of a cherry red colored ring at the interface of the medium.

Plate No. 15: No cherry red color ring formation indicates negative indole test (right) & control (left).

4.3.6 Lactose Fermentation Test

Streptococcus pneumoniae shows a positive result for Lactose fermentation test indicated by colour change of the medium from red to orange or yellow.

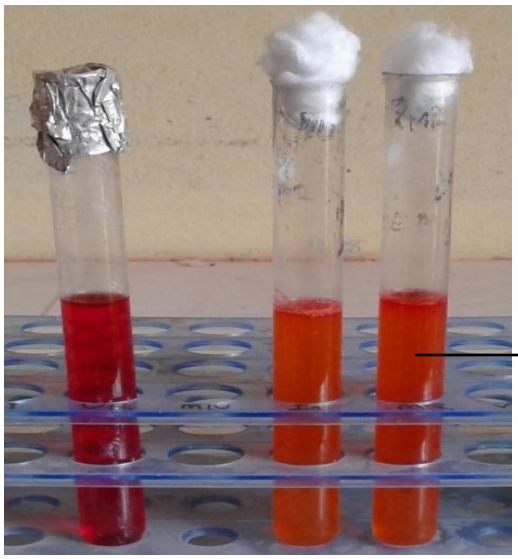

Plate No. 16: Colour change of the medium from red to orange or yellow indicates positive result (right) & control (left).

4.3.7 TSI (Triple Sugar Iron) Test

Streptococcus pneumoniae shows a positive result for Triple Sugar Iron test indicated by yellowing of the medium (Acidic reaction). Acid slant or acid butt indicates that dextrose & sucrose were fermented or dextrose & lactose were fermented or all the three sugars, dextrose, lactose and sucrose were fermented. H_2S was not produced; beacause there was no blackening of the medium.

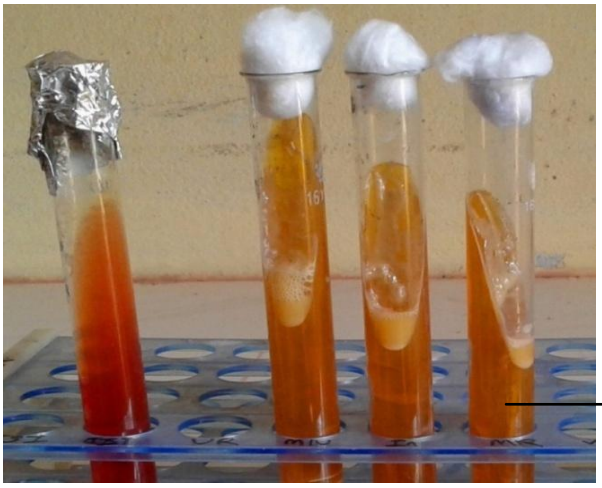

Plate No. 17: Triple Sugar Iron test showing control (left) & positive (right).

4.3.8 Bile Solubility Test

Streptococcus pneumoniae is bile soluble & gives a positive result in bile solubility test indicated by clearing of turbidity in test suspension.

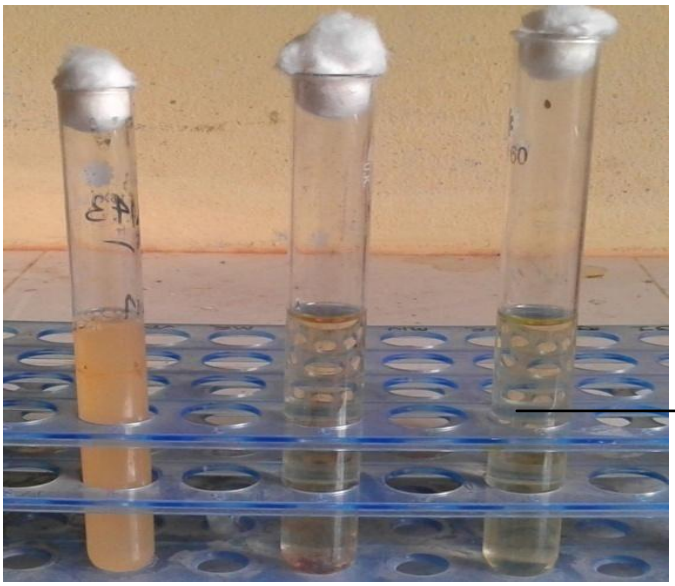

Plate No. 18: Clearing of turbidity in test suspension indicates positive result (right) & the left is control.

4.4 Frequency of pneumonia among pneumonia patients based on different catagories

Out of 40 pneumonia patients, positive cases of pneumonia were found to be 37.5% & 15 isolates were isolated. Frequencies of pneumonia in pneumonia patients based on different catagories were shown in table 6, 7, 8 & 9. Total 37.5% positive cases of pneumonia patients were shown in table 6.

Table 6. Frequency of pneumonia among pneumonia patients

Total number of cases	No. of positive Cases	Percentage
40	15	37.5%

Highest frequency of pneumonia was seen in pneumonia patients of age 3-5 yrs and lowest frequency was noticed in age13-15 yrs of age group which were shown in Table 7.

Table 7. Frequency of pneumonia in pneumonia patients in relation to age

Age	No. Examined	No. Positive	Percentage
3-5yrs	12	6	50%
6-8yrs	10	4	33.33%
9-11yrs	8	3	25%
12-15	10	2	20%
Total	40	15	

Table 8. Distribution of pneumonia patients according to sample collection and types of bacteria

Name of Hospital	No. of Sample	No. of positive case (%)
M Abdur Rahim Medical College Hospital	20	8 (54.5)
Arobindo Shishu Hospital	15	5 (36.36)
Islami Bank Community Hospital	5	2 (9.09)
Total	40	15

Highest number of *Streptococcus pneumoniae* found in 3-5 years of age which were shown in table 9.

Table 9: Distribution of organism based on age difference

Age	Organisms
	Streptococcus pneumoniae
3-5years	6 (7%)
6-8years	4 (20%)
9-11years	3 (6.67%)
12-15	2 (30%)
Total	15 (63.67%)

4.5 Prevalence of pneumonia among pneumonia patients

The table 10 represents the prevalence of pneumonia based on study area, age & socio-economic status. The present results reveales that the study area had no significant (P > 0.05) effect but age (P < 0.05) & socio-economic status (P < 0.05) had significant effects on the prevalence of pneumonia in pneumonia patients. In study area, the highest prevalence was found in M Abdur Rahim Medical College Hospital (40%), followed by Arobindo Shishu Hospital (33.33%) & Islami Bank Community Hospital (40%) respectively. Among the age group, the prevalence of pneumonia was highest (50%) in 3-5 years age group, then 33.33% in 12-15 years age group, 25% in 6-8 years age group & 20% in 9-11 years age group. Highest prevalence of pneumonia was found in poor socio-economic status (54.54%), followed by medium (16.66%) & rich (16.66%) socio-economic condition respectively.

Table 10: Prevalence of pneumonia based on study area, age & socio- economic status

Parameter		No. examined	No. Positive	Prevalence (%)	P value
Study area	M Abdur Rahim Medical College Hospital	20	8	40	0.061
	Arobindu Shishu Hospital	15	5	33.33	
	Islami Bank Community Hospital	5	2	40	
Age	3-5 years	20	10	50	0.018
	6-8 years	12	3	25	
	9-11years	5	1	20	
	12-15 years	3	1	33.33	
Socio-economic status	Poor	22	12	54.54	0.023
	Medium	12	2	16.66	
	Rich	6	1	16.66	

Note: P > 0.05 means not significant,

P < 0.05 means statistically significant.

4.6 Result of PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogenetic Analysis of *Streptococcus pneumoniae*

16S rRNA gene region was amplified with the universal primers, Forward primer- 27F (5'AGAGTTTGATCCTGGCTCAG 3') Reverse primer- 1492R (5' TACCTTGTTACG ACTT 3'). PCR Amplification band was found at 1466 bp.

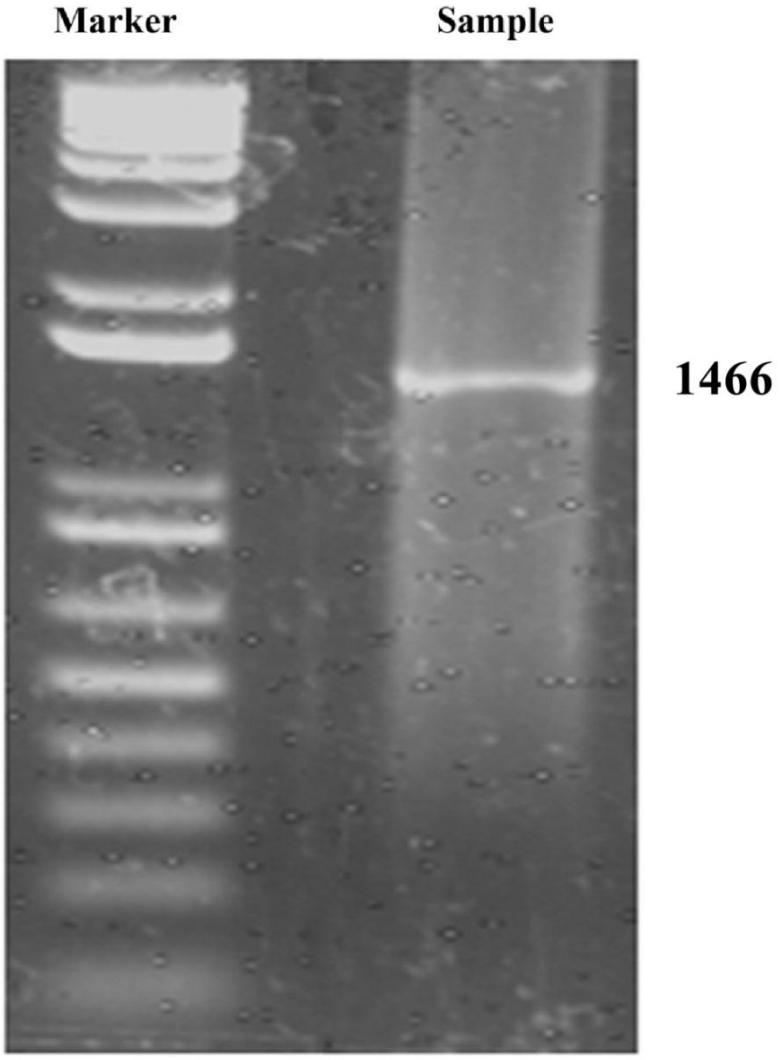

Plate No.19: Result of amplification of 16S rRNA gene region of *Streptococcus pneumoniae* PCR. Note: PCR= Polymerase Chain Reaction, kb= kilo base.

4.6.1 Contig Sequence of *Streptococcus pneumoniae*

Blast: 92%

Identified starin: Streptococcus_pneumoniae_strain _NBRC102642.

>NR_114172.1_Streptococcus_pneumoniae_strain _NBRC102642

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ACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAA
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CGGTTACTTAAGCAGGATGTGAAATCCCCGGGCTCAACCCGGGAACTGCGTT
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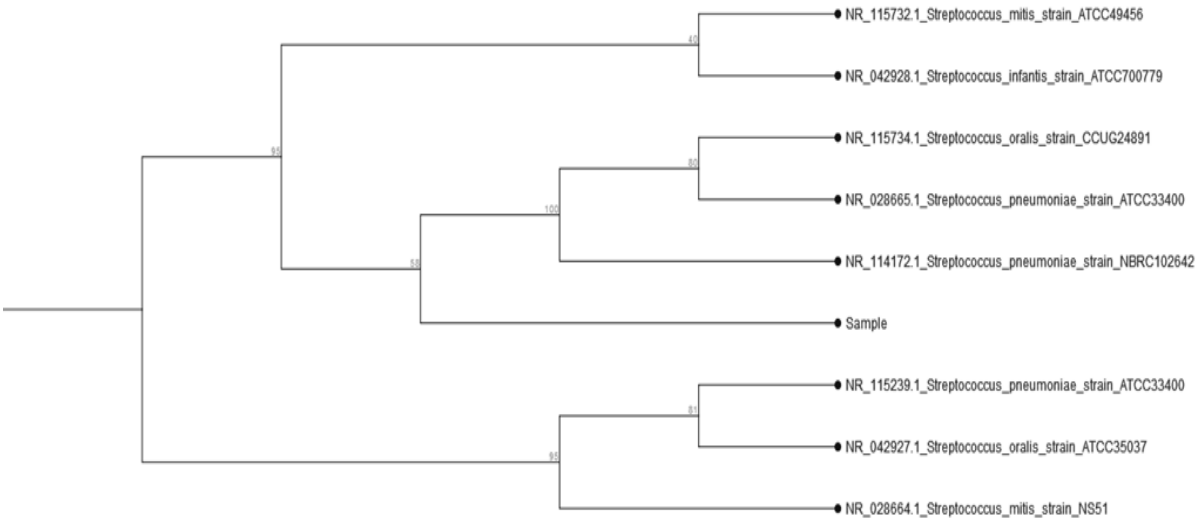
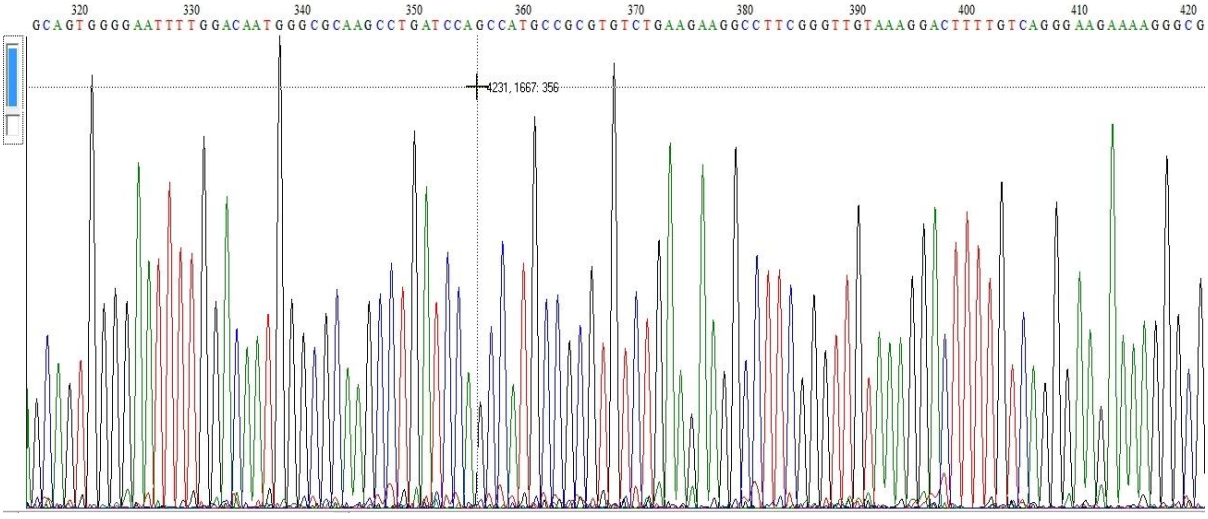
4.6.2 Phylogenetic tree analysis of *Streptococcus pneumoniae*



4.6.3 Result of Electropherogram



4.7 Results of Antibiotic Sensitivity Tests

Several commercially available antibiotics were used for *Streptococcus pneumoniae* are given in Table 11.

Table 11: Result of antibiotic sensitivity test for *Streptococcus pneumoniae*

Name of antibiotic with disc concentration	Zone of Inhibition (mm)	Interpretation
Penicillin (10)	-	R
Amoxicillin (30)	-	R
Ampicillin (25)	-	R
Methicillin (5)	-	R
Cloxacillin (1)	-	R
Clindamycin (2)	-	R
Ciprofloxacin (5)	-	R
Vancomycin (30)	-	R
Amikacin (30)	-	R
Cefradin (25)	-	R
Cefixime (5)	-	R
Cefaclor (30)	-	R
Cephalexin (30)	-	R
Cefepime (30)	-	R
Co-trimoxazole (25)	-	R
Neomycin (30)	**19**	**S**
Kanamycin (30)	**21**	**S**
Streptomycin (10)	**29**	**S**
Erythromycin (15)	**20**	**S**
Azithromycin (30)	**24**	**S**
Bacitracin (10)	**18**	**S**

Legends: S= Sensitive, R= Resistant & I= Intermidiate

4.7.1 Antibiotic Sensitivity pattern of *Streptococcus pneumoniae*

Antibiotic sensitivity test was carried out using disc diffusion technique on Mueller Hinton agar for all the bacterial isolates to the most common antibiotic agents. The antibiotic agents that were used in this study are given in the Table 13. The antibiotic study revealed that all of the isolates of *Streptococcus pneumoniae* were sensitive to Neomycin, Kanamycin & Streptomycin followed by Erythromycin, Azithromycin & Bacitracin. The isolates were resistant to Penicillin, Amoxicillin, Ampicillin, Methicillin, Cloxacillin, Clindamycin, Ciprofloxacin, Vancomycin, Amikacin & Co-trimoxazole followed by Cefradin, Cefixime, Cefaclor, Cephalexin & Cefepime.

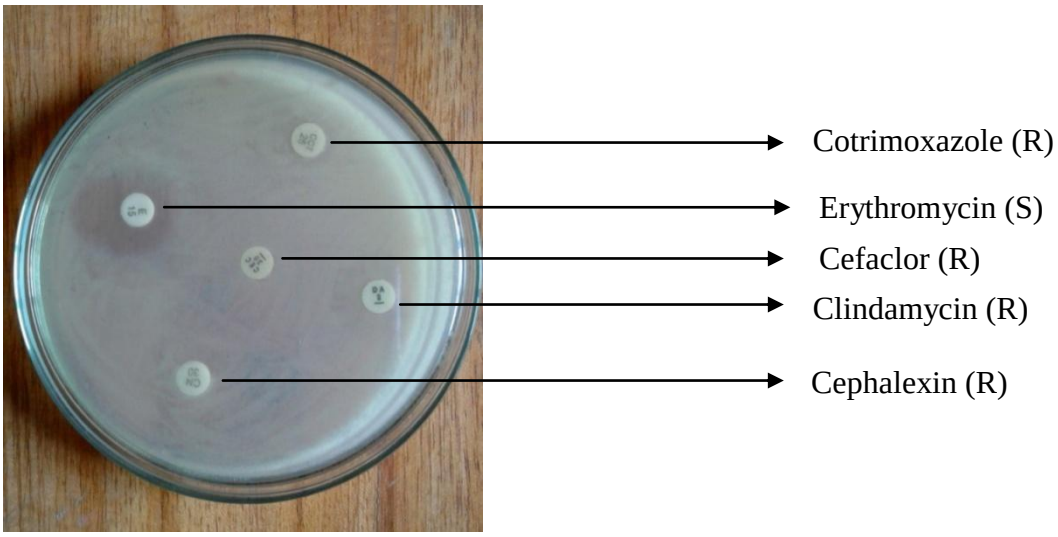


Plate No.20: Antibiotic sensitivity test for *Streptococcus pneumoniae*

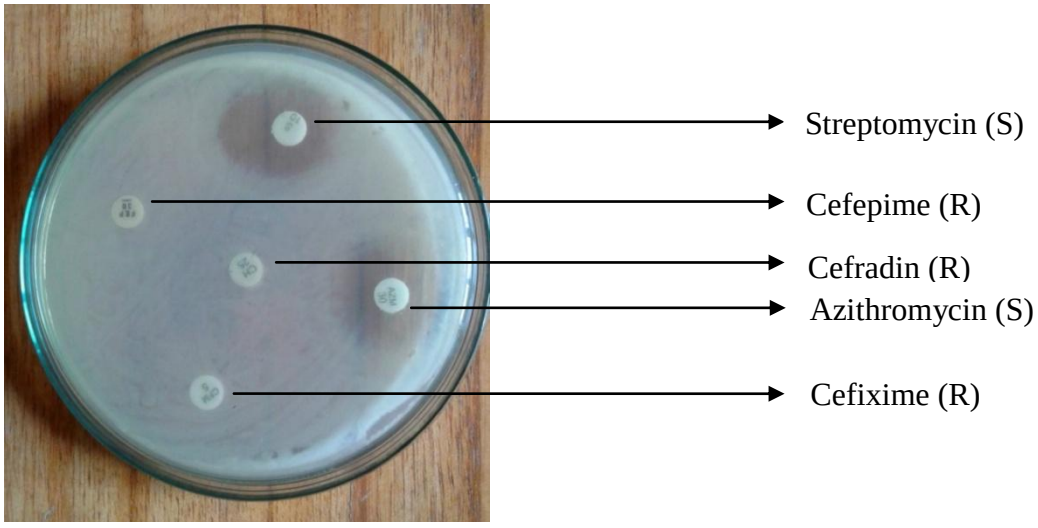


Plate No. 21: Antibiotic sensitivity test for *Streptococcus pneumoniae*

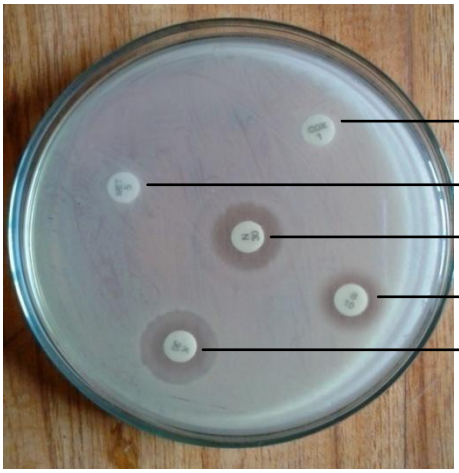


Plate No. 22: Antibiotic sensitivity test for *Streptococcus pneumoniae*

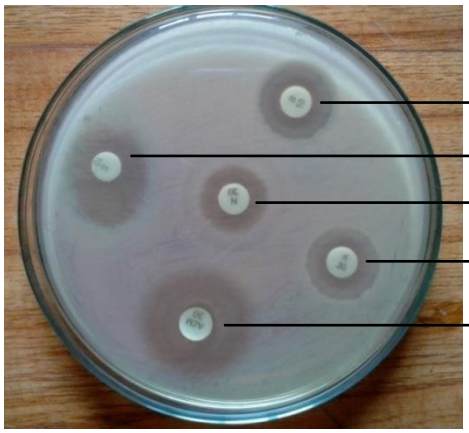


Plate No. 23: Antibiotic sensitivity test for *Streptococcus pneumoniae*

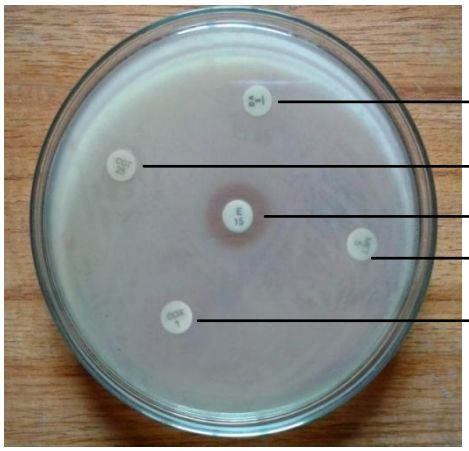


Plate No. 24: Antibiotic sensitivity test for *Streptococcus pneumoniae*

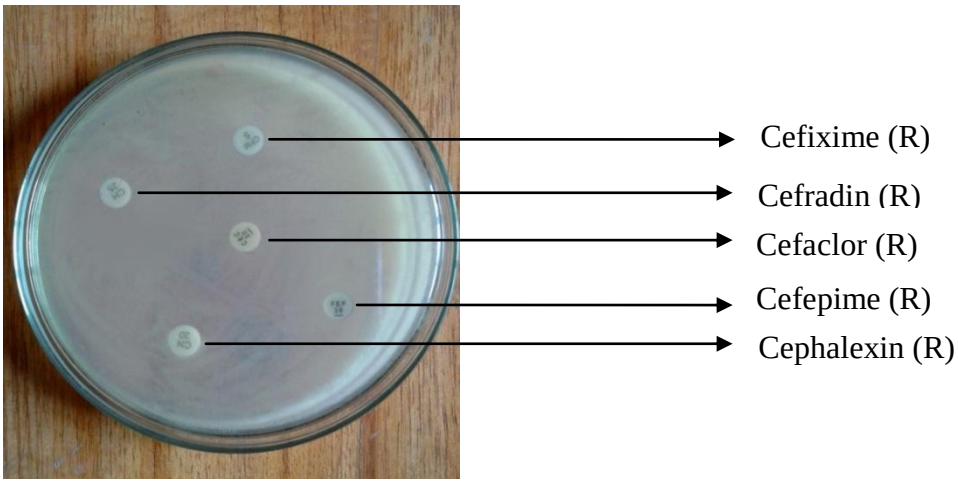


Plate No. 25: Antibiotic sensitivity test for *Streptococcus pneumoniae*

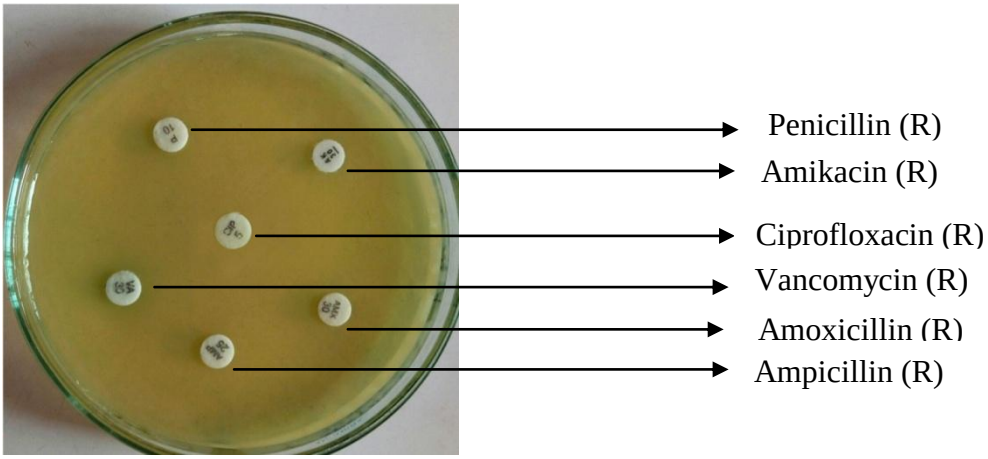


Plate No. 26: Antibiotic sensitivity test for *Streptococcus pneumoniae*

CHAPTER V
DISCUSSION

This study was conducted from January-November 2018 at different hospitals of Dinajpur district of Bangladesh. All microbiological analysis was carried out in microbiology laboratory of Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur. The samples were collected from three main hospitals of Dinajpur district that includes: M Abdur Rahim Medical College Hospital, Dinajpur, Aurobindo Shishu Hospital, Dinajpur & Islami Bank Community Hospital, Dinajpur.

Out of 40 pneumonia patients, positive cases of pneumonia were found to be 37.5% & 15 isolates were isolated. The Frequency of pneumonia in relation to age were 3-5yrs (50%), 6-8yrs (33.33%), 9- 11yrs (25%) & 12-15 (20%). Distribution of organism based on age difference *Streptococcus pneumoniae* of 3-5 years (7%), 6-8 years (20%), 9-11 years (6.67%) & 12-15 years (30%). The present study reveales that the study area had no significant ($P > 0.05$) effect but age ($P < 0.05$) & socio-economic status ($P < 0.05$) had significant effects on the prevalence of pneumonia in pneumonia patients. In study area, the highest prevalence was found in M Abdur Rahim Medical College Hospital (40%), followed by Arobindo Shishu Hospital (33.33%) & Islami Bank Community Hospital (40%) respectively. Among the age group, the prevalence of pneumonia was highest (50%) in 3-5 years age group, then 33.33% in 12-15 years age group, 25% in 6-8 years age group & 20% in 9-11 years age group. Highest prevalence of pneumonia was found in poor socio-economic status (54.54%), followed by medium (16.66%) & rich (16.66%) socio-economic condition respectively.

Antibiotic sensitivity test was carried out using disc diffusion technique on Mueller Hinton agar for all the bacterial isolates to the most common antibiotic agents. The antibiotic study revealed that all of the isolates of *Streptococcus pneumoniae* were sensitive to Neomycin, Kanamycin & Streptomycin followed by Erythromycin, Azithromycin & Bacitracin. The isolates were found resistant to Penicillin, Amoxicillin, Ampicillin, Methicillin, Cloxacillin, Clindamycin, Ciprofloxacin, Vancomycin, Amikacin & Co-trimoxazole followed by Cefradin, Cefixime, Cefaclor, Cephalexin & Cefepime.

In Rio de Janeiro, Barroso *et al*., (2012) isolated, from patients with pneumonia, penicillin-resistant *S. pneumoniae* (clone Spain9V-ST156) linked to serotype. Several

authors reported rates of penicillin resistance of *S. pneumoniae*, isolated from patients with pneumonia & meningitis, ranging from 21.4% to 27.1%, compatible with the 25.5% rate found here. On the other hand, lower values were observed in the state of Parana (15%) and in Salvador (13%-19%), and higher than 50% in Cuba, El Salvador, Honduras and Mexico. For the other non-meningeal pneumococcal infections, our research detected 3% of strains with intermediate resistance and 0.1% with full resistance to penicillin. According to Negrini (2010), in the municipality of Ribeirao Preto, children younger than 5 years presented 3.5% of *S. pneumoniae* with intermediate resistance, and none presented full resistance to penicillin. In 2012, in Brazil, full resistance was not observed to penicillin either, and intermediate resistance was 7.5% and 3.9% for children younger and older than 5 years, respectively.

In Argentina, for children younger than 5 years, 100% of the strains were susceptible to penicillin. In this study, among the pneumococci isolated from patients with pneumonia, just 0.1% was totally resistant to penicillin and 3% of the isolates from non-meningeal infections presented intermediate resistance to penicillin. This piece of data reinforces the consensus of some researches that concluded that patients with pneumonia treated with penicillin presented similar clinical evolution.

In Guatemala and Mexico, a superior proportion of *S. pneumoniae* resistant to ceftriaxone (16.7% and 16.5%, respectively) for non-meningeal infections was verified. In Brazil, in nonmeningeal infections, a rate of 3% of intermediate resistance to ceftriaxone was reported, with no isolation of the pneumococcus with full resistance, a value similar to the one of the region researched in this work. In general, the resistance profile of *S. pneumoniae* to ceftriaxone is low, yet, the importance of monitoring resistance levels is stressed due to the excellence of this antimicrobial for treatment of bacterial meningitis. Strains of *S. pneumoniae* resistant to penicillin are frequently associated with resistance to other classes of antimicrobials. In Porto Alegre, among the 18 penicillin-resistant pneumococcus strains, seven were resistant to at least two other drugs. In this research, 0.9% of multi-resistant *S. pneumoniae* was reported, with simultaneous resistance to penicillin & erythromycin. In the USA, it commonly relates to serotype 19A after the introduction of pneumococcal conjugate vaccine. Besides, *S. pneumoniae* with full and simultaneous resistance to penicillin and ceftriaxone were observed in eight (1%) of the patients.

CHAPTER VI
CONCLUSION

Streptococcus pneumoniae is a leading cause of bacterial pneumonia in children worldwide. In the present study, it was observed that pneumonia was most commonly found in 3-5 years age group & in poor socio-economic condition. The antibiotic study revealed that all of the isolates of *Streptococcus pneumoniae* were resistant to most of the drugs, but found sensitive to Neomycin, Kanamycin & Streptomycin followed by Erythromycin, Azithromycin & Bacitracin. The increasing incidence of antibiotic-resistant *S. pneumoniae* strains are becoming a great problem to treat & control this bacterial species. Use of antibiotics without the recommendation of registered physicians is one of the most important causes of this problem.

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