Rapid identification of *Streptococcus pneumoniae* strains using 16S rRNA gene based PCR method.

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DECLARATION OF AUTHENTICITY

I hereby declare that the dissertation "Rapid identification of *Streptococcus pneumoniae* strains using 16S rRNA gene based PCR method" is solely written and submitted by me. The work contained in this dissertation has not been previously submitted to meet requirement for any award or any other examination office or higher education institution. To the best my of knowledge and belief the thesis contains no material previously published on written by another person expect where due reference is made.

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Dedicated To

My   Greatest   Strength

'My Beloved Family'

Acknowledgement

Abstract

Streptococcus pneumoniae strains are responsible for severe diseases, including pneumonia, bronchitis, otitis media, septicemia, meningitis. Early identification of etiologic agent of pneumococcal diseases is essential and usually performed by "Gold Standard" culture based method which is time-consuming and has poor sensitivity. The aim of this study was to develop a PCR based method for rapid diagnosis of pneumococcal diseases by targeting 16S rRNA region of *Streptococcus pneumoniae*. In this study, 16S rRNA based primers were designed using CLUSTALW and Primer-BLAST. Appropriate laboratory tests such as optochin disk test, bile solubility test, gram-staining and catalase test as well as PCR, gel run electrophoresis and nucleotide sequencing was performed to examine the specificity and utility of the primers in *Streptococcus pneumoniae* detection using eighteen stored *Streptococcus pneumoniae* strains. For further validation, the same primers were used against the DNA extracted from six throat swab samples pre-incubated in enrichment broth and DNA extracted directly from same samples without using enrichment broth. It took total 8 hours and 6 hours respectively to achieve the results. After nucleotide sequencing and subsequent BLASTn analysis, it was substantiated; this approach is specific and faster than present diagnostic approach for *Streptococcus pneumoniae* detection.

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Abbreviations:

SPN	*Streptococcus pneumoniae*
rRNA	Ribosomal ribonucleic acid
WHO	World Health Organization
CAP	Community acquired pneumonia
CDC	Center for Disease Control and Prevention
FBS	Fetal bovine serum
STGG media	Skim milk, tryptone, glucose, and glycerin
COPD	Chronic Obstructive Pulmonary Disease
API	Analytical Profile Index
PBS	Phosphate Buffer Saline
BLAST	Basic Local Alignment Search Tool
bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetra acetic acid
μL	Microliter
PCR	Polymerase chain reaction
UV light	Ultraviolet light
$MgCl_2$	Magnesium Chloride
TBE	Tris/Borate/EDTA
RNA	Ribonucleic Acid
BMRC	Bangladesh Medical Research Council
IEDCR	Institute of Epidemiology, Disease Control and Research

Chapter 1:

Introduction

1.1: Background:

Streptococcus pneumoniae is a gram-positive bacterium which is alpha-hemolytic under aerobic condition and beta-hemolytic under anaerobic condition and a member of streptococcus genus [1]. The major cause of pneumococcal bacteremia is pneumonia. The incidence of pneumococcal disease is the highest in children < 2 years of age and in adults > 65 years of age [2]. In developing countries pneumonia is a serious disease in children and it is estimated that more than a million children below the age of 5 each year die from pneumococcal pneumonia [3]. Over ten million new cases of pneumonia in children <5 years are diagnosed annually in Bangladesh [4].

1.2: Character and Morphology:

Classification of *Streptococcus pneumoniae* [1]

Domain: Prokaryotes

Kingdom: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Lactobacillales

Family: Streptococcaceae

Genus: *Streptococcus*

Species: *Streptococcus pneumoniae*

1.3: Habitat

1.3.1: Typical Habitat

Streptococcus pneumoniae are found worldwide within a variety of organisms such as: primates (like the wild Chimpanzee, *Pan troglodytes*), some livestock (like pigs, *Sus scofra domestica*), and felines (*Felis catus*) [5]. They are considered part of the normal flora of upper respiratory bacteria in humans and are found in the highest concentrations among children, the elderly, and those with compromised immune systems [6]. *Streptococcus pneumoniae* can typically be found in the upper respiratory system however, the movement of *Streptococcus pneumoniae* most commonly causes conditions such as pneumonia, conditions such as pneumonia, bacteremia, and meningitis [7].

1.3.2: Conditions Effecting Habitat

Streptococcus pneumoniae are aerotolerant meaning it can exist in an environment with O_2 and features an anaerobic metabolism. Therefore, it does not require O_2. *Streptococcus pneumoniae* however, depends on the organism to provide catalase and peroxidase for its metabolism. As these colonies age, the organism loses its ability to obtain catalyze and peroxidase because of its accumulation of hydrogen peroxide over time [8]. These organisms are typically diplococci which have two cells right next to each other however, longer strands have been observed. They are also non-motile which means they depend on their host organism or other factors for transport [9].
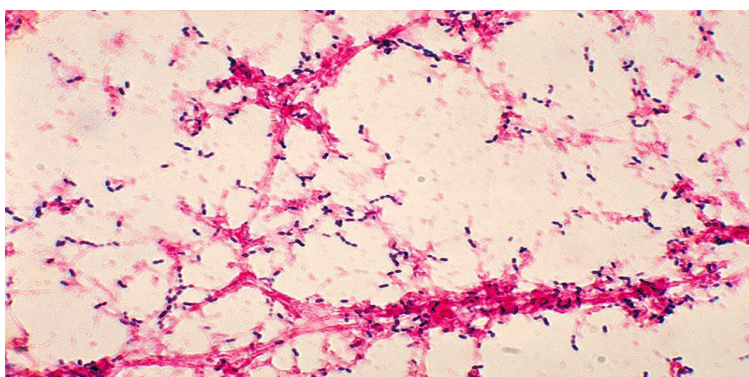
Figure 1.1. *S. pneumoniae* on a blood culture. The small purple dots are dyed purple because *S. pneumoniae* is a gram-positive bacterium. The image also indicates how they usually are found in groups of two. [10]

1.3.3: In Vitro Characteristics

In vitro, *Streptococcus pneumoniae* grow best on blood agar as their metabolism requires blood to function properly. These organisms will grow the best when placed in an environment that offers a 5% CO_2 concentration. [11]. On this media, *Streptococcus pneumoniae* will exhibit α-hemolysis in comparison to β-hemolysis and Ɣ-hemolysis, which have different characteristics. This particular type of hemolysis produces a greenish tint on the media formed from incomplete hemolysis of the blood cells. [12]

1.3.4: Ex Vitro/Pathogenic Characteristics in Respect to Habitat

Ex vitro, in an organism, this bacterium prefers the mucous membranes of the upper respiratory tract. It is estimated that anywhere from 5%-70% of healthy adults have *Streptococcus pneumoniae* living within their upper respiratory tracts [13]. The human body in particular provides a decent environment for this bacterium as it's doubling rate is 20-30 minutes in mixed media containing blood that at 37^0C [14], which is that average body temperature for a human.

1.4: Epidemiology:

Streptococcus pneumoniae is one of the most important human pathogens, and pneumococcal disease is endemic all over the world. In developing countries pneumonia is a serious disease in children and it is estimated that more than a million children below the age of 5 each year die from pneumococcal pneumonia.[15] In the United States the pneumococcus each year probably accounts for 3000 cases of meningitis, 500,000 cases of pneumonia, and 7,000,000 cases of otitis media.[16] The incidence of pneumococcal pneumonia has probably not decreased significantly during the previous century,[17] but the case fatality rate has decreased dramatically with the advent of antibiotics. There has also been a shift toward the disease becoming severe in mostly the elderly and those with underlying diseases. The total yearly incidence of pneumonia in Western populations is around 1% and *S. pneumoniae* is probably responsible for almost half of the cases of community-acquired pneumonia (CAP) [18]. Thus, almost five in 1000 persons each year contract pneumococcal pneumonia, with the incidence being several times higher in the very young and the elderly [19]. The major cause of pneumococcal bacteremia is pneumonia. The total annual incidence of pneumococcal bacteremia in North America and Europe is at least 10 to 20 per 100,000 individuals, but a more correct figure may well be more than 40 per 100,000.[20] The risk of invasive

pneumococcal disease has been found to be more than 20-fold greater in small children if they are attending day care centers.[21,22] Moreover, itis higher still in certain populations such as Alaskan natives.[23] In recent years a substantial increase in the incidence of pneumococcal bacteremia has been noted in several countries, including the United States, Sweden, Norway, and Denmark.[24-27] The incidence of pneumococcal disease is highest during the winter months.[28] One reason for this may be that viral respiratory infections predisposing to pneumococcal disease are more common during winter.[28] Outbreaks of pneumococcal disease may occur if a new strain is introduced in a closed setting, such as schools, military camps, nursing homes, or jails [29].All wild strains of *S. pneumoniae* are provided with a polysaccharide capsule. To date, 90 distinct capsular types have been described [30]. Types that are antigenically related to each other are included in groups (labeled, e.g., 9A,9L, 9N, and 9V), whereas types without close antigenic relationship to other types are given numbers only (e.g. types 1, 2, 3, 4, 5). The capsular polysaccharides are composed of repeating units of oligosaccharides and for most of them the chemical structure is known [31]. Molecular analysis of the genes responsible for the synthesis of some of the capsular substances has shown that they are arranged in cassettes comprising all the genetic material necessary for capsule synthesis [32]. Being naturally transformable, pneumococci may exchange genetic material between different strains. By such processes capsule specificity, in a cassette type-recombination event, can be exchanged in vitro as well as in vivo [33].

1.4.1: Epidemiology in SAARC countries:

The SAARC (The South Asian Association for Regional Cooperation) includes 8 countries: India, Pakistan, Bangladesh, Sri Lanka, Nepal, Bhutan, Maldives, and Afghanistan. The under-five mortality rates (per 1000 live-births) are high in these regions (ranging from 10 for Sri Lanka to 99 for Afghanistan) compared to the western countries (UK=5, and USA=7) as per the 2012 WHO data. The share of under-five deaths due to pneumonia in these regions is as follows: 20.4% in Afghanistan; 15% in India, 14.6% in Pakistan, 13.6% each in Nepal and Bhutan; 11% in Bangladesh, 8.8% in Maldives, and 5.7% in Sri Lanka [34], [35]. The SAARC nations also fall in the zone with high incidence of pneumococcal disease [36], but there is a dearth of studies reporting prevalent serotypes in these regions.

1.5: Distribution of Serotypes of *Streptococcus Pneumoniae* in Children Living in SAARC Countries:

Streptococcus pneumoniae is an encapsulated bacterium with a polysaccharide capsule which is an essential factor in virulence. About 90 distinct pneumococcal serotypes have been identified throughout the world, with a small number of these serotypes accounting for most disease in infants. A study on serotype distribution in SAARC countries was conducted by a group of scientists in India. [37] They divided their study into two parts: population based and hospital based. Participants in their study were children of both sexes and ≤12 years of age (excluding the neonates or young infants <2 months). The combined result from all the SAARC countries they found the common serotypes to be as follows: serotype 1 in Nepal; serotype 14 in Bangladesh and India; serotype 19F in Sri Lanka and Pakistan. They did not find any study from the three SAARC countries: Afghanistan, Maldives, and Bhutan.

(a)

(b)

(c)

(d)

(e)

(f)
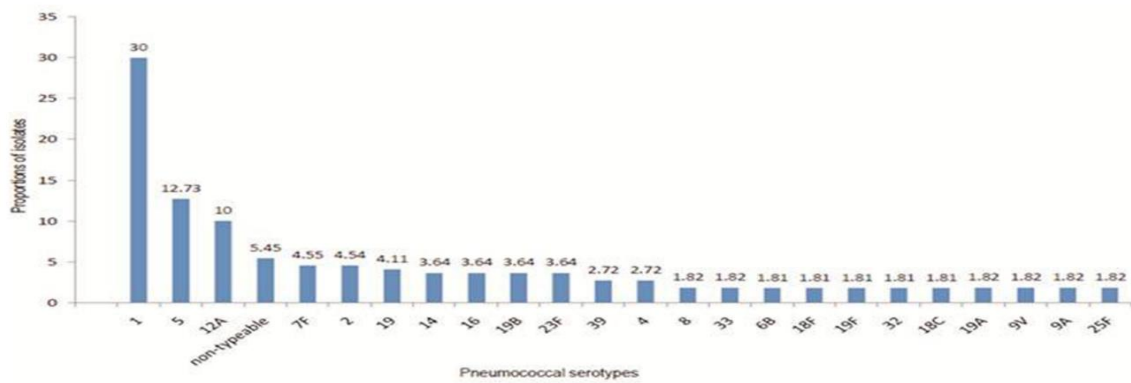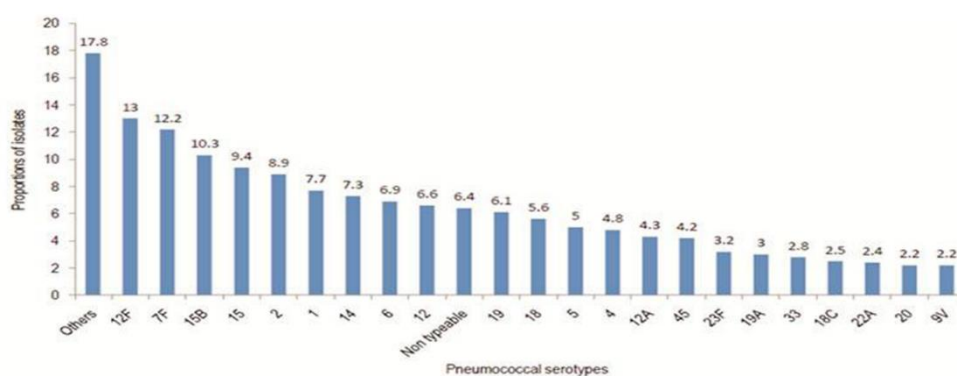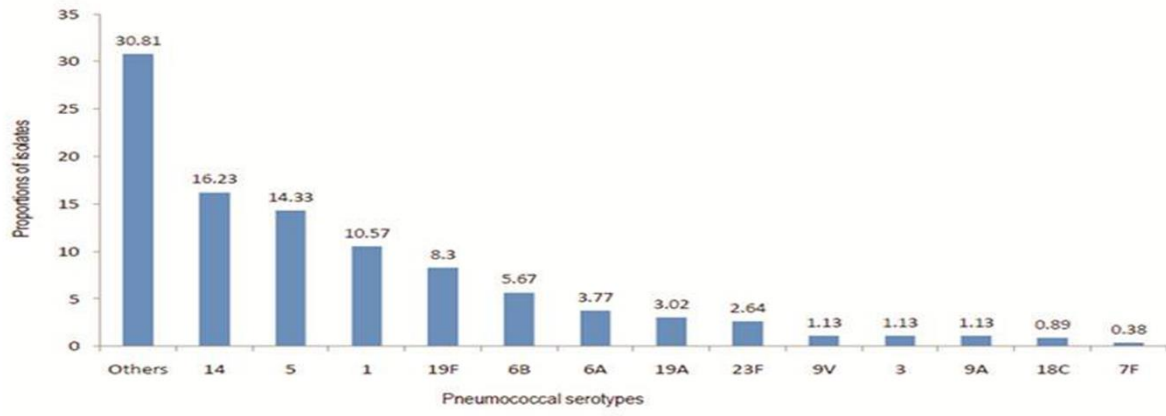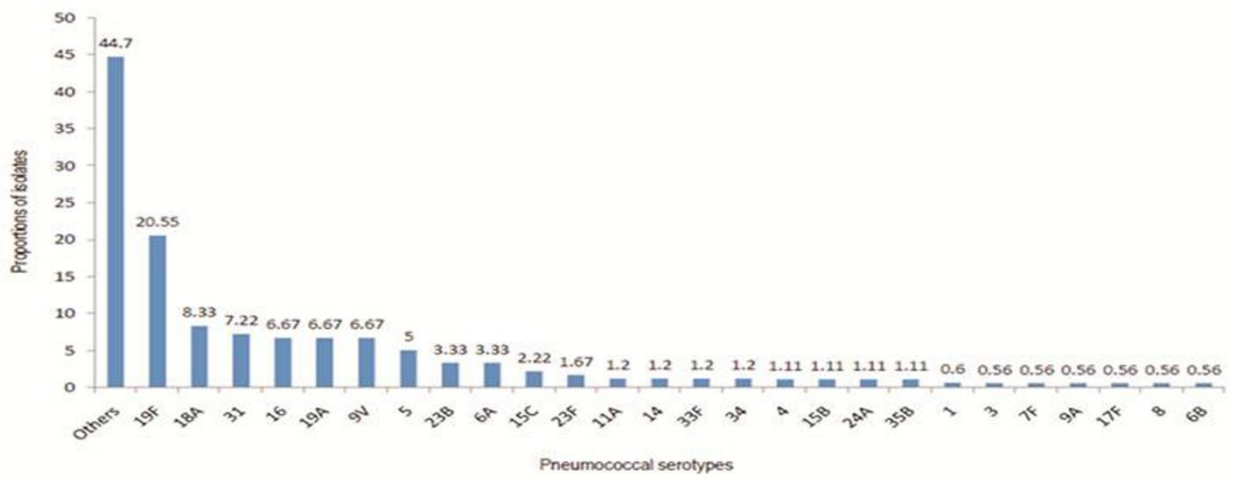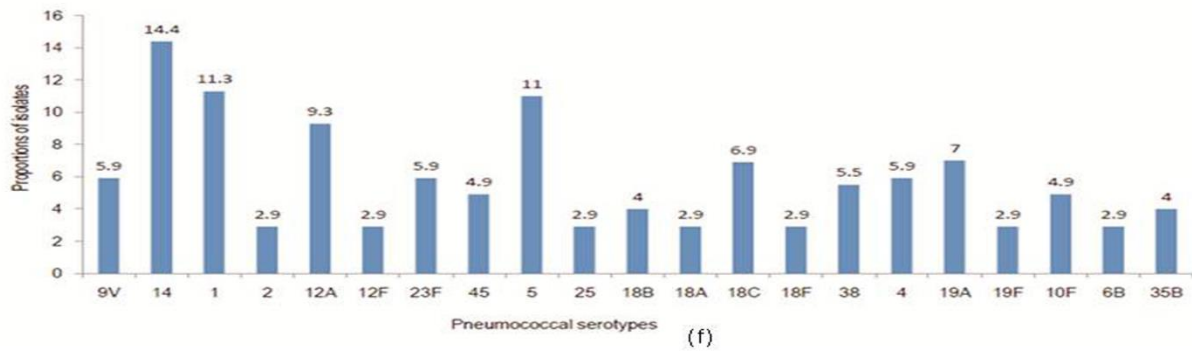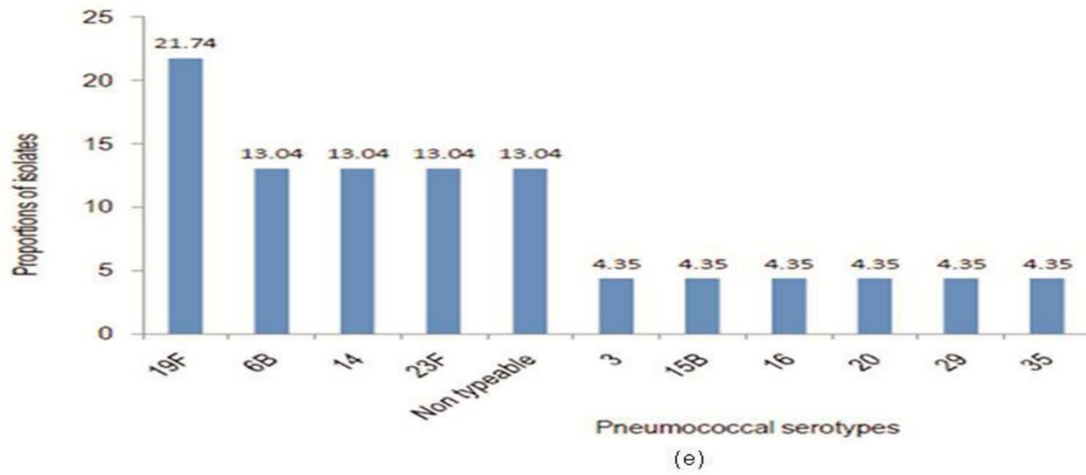
Figure 1.2: Combined serotype data from (a) Nepal; (b)Bangladesh (hospital based); (c): India; (d): Pakistan; (e): Sri Lanka (f): Bangladesh (population based). [38]

1.5.1: Hospital based studies:

The combined data of the 4 studies from Nepal showed serotype 1 was most common followed by 5, and 12A (Figure 3a).

From the data of the 4 Bangladesh studies showed that "others serotype" were most common. Among the identified ones, 12F, 7F, 15B, 15, 2, 1, and 14 were more common (Figure 3b).

The data from 2 Indian studies showed that "others serotypes" were most common. Among the identified ones, 14 was the most common followed by 5, 1, 19F, and 6B (Figure 3c).

The data from 2 Pakistan studies showed that "others serotypes" were most common. Among the identified ones, 19F was the most common followed by 18A, 31, 16, 19A, 9V, and 5 (Figure 3d).

The data from 1 Sri Lankan study showed serotype 19F to be the most common followed by 6B, 14, 23F, and non-typeable (Figure 3e).

1.5.2: Population based studies

There were two population based prospective studies from Bangladesh [38]. The combined data showed serotype 14 to be the most common, followed by 1, 5, 12A, 19A, and 18C (Figure 3f).

1.6: Diagnosis of pneumococcal diseases:

Acute respiratory infections are still among the major causes of childhood mortality in developing countries, including Bangladesh. The classic description of the clinical features of pneumococcal pneumonia is sudden onset of chills and pleuritic chest pain followed by fever and then cough productive of rusty sputum. The laboratory diagnosis of invasive pneumococcal disease (IPD) is mainly done by culture-based methods that have been used for many decades. The most significant recent method have is antigen detection assays, whereas the role of nucleic acid amplification tests has yet to be fully clarified. Despite developments in laboratory diagnostics, a microbiological diagnosis is still not made so specific in most cases of IPD, particularly for pneumococcal pneumonia. The limitations of existing diagnostic tests impact the ability to obtain accurate IPD burden data and to assess the effectiveness of control measures, such as vaccination, in addition to the ability to diagnose IPD in individual patients. There is an urgent need for improved diagnostic tests for pneumococcal disease—especially tests that are suitable for use in under resourced countries like Bangladesh. Isolation of S.

pneumoniae from a normally sterile body site provides conclusive evidence of pneumococcal infection, but this is achieved for only a minority of cases of IPD.Pneumococcal pneumonia, the most common manifestation of IPD, can be particularly difficult to diagnose.The problems appear obtaining high-quality lower respiratory tract samples for testing and with uncertainty regarding the differentiation of infection from colonization. Besides, antibiotic use will also significantly reduce the ability to isolate *S. pneumoniae* from clinical samples. Despite the global importance of pneumococcal disease, there have been few recent developments in laboratory diagnostics.

1.6.1: Microscopy and Culture:

During microscopic evaluation, *S. pneumoniae* appear as lancet-shaped, gram-positive diplococci or chains of cocci (figure 4). However, typical appearances can be altered by antimicrobial therapy, and over-decolorization of the stain can give the false impression that they are gram-negative diplococci. Nevertheless, the quellung reaction is a more specific method for pneumococcal detection from pure cultures or sputum samples [39]. Although the quelling reaction is generally regarded as being highly specific for pneumococcus, cross reactions have been reported with other streptococcal polysaccharides [39], and un-encapsulated strains will produce false-negative results.
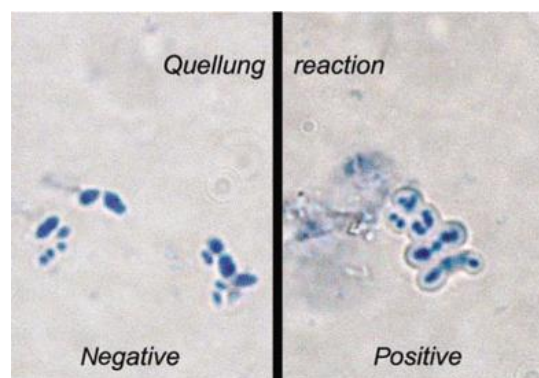
Figure 1.3: Gram stain of a sputum sample showing *Streptococcus pneumoniae* as gram-positive diplococci.
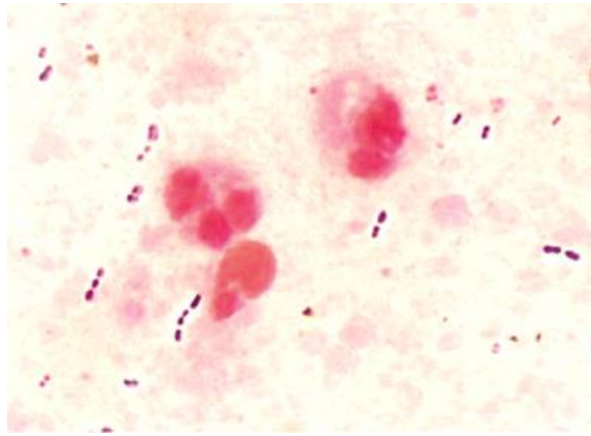
Figure 1.4: Quellung reaction, showing both positive (for *Streptococcus pneumoniae*) and negative results.

After overnight incubation at 37°C with 5% CO_2 on 5% sheep blood agar, *S. pneumoniae* colonies appear to be small, grayish, and mucoid and are surrounded by a greenish zone of α-hemolysis. After 24–48 h of incubation, the colonies become centrally depressed ("draughtsman" colonies) (figure 6). Further identification is important to confirm the identity. Laboratory differentiation between *S. pneumoniae* and other viridans streptococci is usually accomplished by 2 key reactions: optochin susceptibility and bile solubility.*S. pneumoniae* isolates are typically susceptible to optochin and are bile soluble, whereas other viridans streptococci are typically resistant to optochin and are bile insoluble. Although bile solubility is generally regarded as being very sensitive and specific for identification of *S. pneumoniae*, the finding that up to 10% of *S. pneumoniae* isolates can be resistant to optochin has reduced reliance on the latter test [40]. Commercial slide agglutination, coagulation, and DNA probe hybridization tests are alternative methods for rapid identification of *S. pneumoniae* isolates [41, 42]. All of these methods are highly sensitive but occasionally produce positive results with other viridans streptococci (specificity range, 85%–95%) [43,44].

Figure 1.5: *Streptococcus pneumoniae* growing on sheep blood agar as "draughtsman" colonies. The disk contains optochin and is surrounded by a zone of inhibition. [45]

1.6.2: Blood cultures:

The isolation of *S. pneumoniae* from blood culture provides a definite diagnosis of pneumococcal disease.Although *S. pneumoniae* is regarded as the most common cause of community-acquired pneumonia in all age groups, rates of positive blood culture results for adults hospitalized with pneumonia are typically only 3%–8% [46-49] and are lower in children [50,51]. In pneumococcal meningitis, documented bacteremia occurs more frequently than in pneumonia, and reported rates of positive results are often >50% [52,53]. The relatively low documented rates of bacteremia in patients with IPD involve several factors, including prior administration of antimicrobials and the intermittent nature of bloodstream invasion by *S. pneumoniae*. In addition, *S. pneumoniae* releases autolysin during the stationary growth phase, that results in cell death and creates difficulties growing on media. [54].

1.6.3: Cerebrospinal Fluid (CSF) examination:

For the diagnosis of pneumococcal meningitis, the combination of Gram stain and bacterial culture of CSF samples will identify most cases. In one large review, Gram stain smears of CSF samples detected *S. pneumoniae* with a sensitivity of 84% and specificity of 98%, but prior administration of antibiotics significantly reduced the yield for both Gram stain smear and culture [55]. Processing a CSF specimen for culture as soon as possible is vital for optimal culture performance, because bacterial viability decreases over time.

1.6.4: Sputum examination:

In the absence of documented bacteremia, the diagnosis of pneumococcal pneumonia can be challenging, especially in children who may not produce sputum.Under the microscopic, numerous gram-positive diplococci in a sputum sample containing <10 squamous epithelial (SEC) cells and >25 polymorphonuclear (PMN) cells per low-power field (magnification, ×100) [56] or ⩾10 leukocytes for each SEC [57] indicates a patient with pneumonia is strongly suggestive of pneumococcal pneumonia. This is further supported if *S. pneumoniae* is the predominant isolate in cultures of sputum specimens. Poor-quality sputum samples, which contain relatively low numbers of PMN cells and high numbers of SEC cells, should not be processed, because they are likely to represent commensal oropharyngeal flora.Several clinical studies have shown that sputum culture and Gram stain are still useful for the diagnosis of pneumococcal pneumonia, as long as specimens are of high quality and, ideally, were obtained before the administration of antibiotic therapy or up to 24 h after the initiation of therapy. One prospective study revealed that high-quality sputum samples can be obtained from a substantial proportion of adults with community-acquired pneumonia and that the sputum Gram stain had sensitivity of 57% and specificity of 97% for the diagnosis of pneumococcal pneumonia [57]. For bacteremic pneumococcal pneumonia in adults, sputum Gram stain and culture have sensitivities of 80% and 93%, respectively, if an adequate specimen has been produced before therapy [57]. The reason why sputum culture has been shown to have variable sensitivities in different studies is not necessarily the inadequacy of the microbiological tool itself; it can be the result of various factors, such as delayed processing of a sputum sample or processing of an inadequate sample, the patient's failure to produce a sputum sample, and the administration of antimicrobial therapy before obtaining a specimen.

1.6.5: Lung aspirate examination:

Transthoracic needle aspiration has the potential to improve the diagnostic yield of pneumococcal pneumonia, especially in individuals with large peripheral lesions, including in children who may not produce sputum [58–62]. This is a relatively safe procedure if it is performed by experienced staff, but it has yet to be widely adopted because of its invasive nature and concerns about complications. The less compliant lungs of older adults are more prone to pneumothoraxes after the procedure, and patients receiving anticoagulation may bleed.

1.6.6: Antigen Detection Assays:

The detection of pneumococcal antigen in clinical samples, particularly urine samples, dates back at least to 1917 [63]. Over the past few decades, commercial latex agglutination tests targeting capsular polysaccharide antigens of S. pneumoniae have been widely used, although their use has been controversial.In one large study, the sensitivity of a latex agglutination assay was high for detection of *S. pneumoniae* in CSF samples, but all samples that yielded positive results also demonstrated the causative organism on Gram stain [64]. Moreover, false-positive results were common, especially for urine samples, for which most positive results were erroneous. However, latex agglutination tests have still found a role for diagnosing pneumococcal pneumonia and meningitis in communities with limited laboratory facilities [65].The recent development of a rapid immunochromatographic test (ICT) that detects the C polysaccharide cell wall antigen common to all strains of S. pneumoniae (NOW *S. pneumoniae* urinary antigen test; Binax) has renewed interest in antigen detection. This test has quite good sensitivity and specificity. In all studies, a proportion of patients with positive blood or sputum culture results have negative NOW test results. Consequently, the NOW test should be used in conjunction with other testing methods. The test result can remain positive for several weeks [66,67], and pneumococcal vaccination may produce false-positive reactions [68]. The utility of the NOW test for children is still being defined because of the high rate of false-positive results in children, which results from nasopharyngeal colonization with S. pneumoniae [69–70]. Other limitations of the test are its relatively high cost and the inability to provide antimicrobial susceptibility data.

1.6.7: Nucleic Acid Amplification Tests:

Nucleic acid amplification tests, such as PCR isan important diagnostic tool and are now representative of the current microbiological zeitgeist. Its attractiveness in a diagnostic laboratory setting stems from the following attributes: it can detect minute amounts of nucleic acid from potentially all pathogens, it does not depend on the viability of the target microbe, it is probably less affected by prior antimicrobial therapy than culture-based methods, and it provides results within a short time frame.To date, nucleic acid amplification tests have had variable performance for diagnosing IPD. In the setting of pneumonia, PCR has a sensitivity for detecting *S. pneumoniae* in blood samples ranging from 29% to 100% [71], although there is a tendency for the performance to be better in children than in adults.When testing sputum

samples, reported PCR positivity rates have ranged from 68% to 100% for samples from patients with pneumonia [71], although it is unclear how often this reflects colonization of the upper respiratory tract rather than infection [72].Unlike for pneumococcal pneumonia, detection of pneumococcal DNA in CSF specimens can be useful for diagnosis of pneumococcal meningitis. This is perhaps unsurprising, given the high bacterial concentration in CSF in the presence of meningitis and the lesser concerns about contamination with colonizing bacteria. Although they have yet to be extensively evaluated, the sensitivity and specificity of PCR applied to CSF samples is high for diagnosis of pneumococcal meningitis (92%–100% and 100%, respectively), and this finding has been demonstrated in a variety of field settings [73–77]. PCR has also been successfully used with other samples obtained by invasive means, such as pleural fluid [78,79] and lung aspirate [80] specimens.

1.7: Literature review:

The incidence of invasive pneumococcal disease (IPD) varies across countries and is probably largely underestimated since diagnosis of invasive pneumococcal infections, usually performed by culturing from whole blood or cerebrospinal fluid (CSF), requires the presence of viable pathogens in the clinical samples. Although there is still no easy way to establish the diagnosis of pneumococcal disease, some dedicated scientists had studied and done their best to solve the problems. Such group of scientists from the Department of Pediatrics, State University of New York, University of Buffalo, and Children's Hospital of Buffalo, Buffalo, New York, published "New Technique (the NOW Test) for Rapid Detection of *Streptococcus pneumoniae* in the Nasopharynx". [81] Although the NOW test was originally introduced as a rapid pneumococcal antigen detection test for use with urine samples, it was successfully adapted to nasopharyngeal samples. It is an in vitro rapid immunochromatographic assayfor the detection of *S. pneumoniae* cell wall polysaccharide in urine specimens from patients with symptoms of pneumonia. The test kit incorporates rabbit anti-*S. pneumoniae* antibody adsorbed onto a nitrocellulose membrane. If pneumococcal cell wall polysaccharide is in the specimen, an easily discernible pink-to-purple line appears within 15 min on the membrane.However, Howard*et al.,*2002 in their study, adapted this test to detect pneumococcal antigen in the nasopharynxes of patients (children less than 15 years old) colonized with *S. pneumoniae*. The procedure, they conducted according to the directions in the kit. The sensitivity, specificity, positive predictive value, and negative predictive value of the test were 92.2, 97.7, 95.9, and 95.5%, respectively. Another Group of Scientists, developed a 16S rRNA gene based PCR for

the identification of *Streptococcus pneumoniae* and compared with four other species-specific PCR assays as its differentiation from closely related but less pathogenic streptococci are a challenge [82]. In their study they developed PCR-assays and proved, 57°C is specific for *S. pneumoniae*, not amplifying *S. pseudopneumoniae* or any other streptococcal strain or any strains from other upper airway pathogenic species. They found only two among five PCR-assays are accurate, which do not misidentify *S. pseudopneumoniae* as *S. pneumoniae*. Lastly, another study by the scientists from Department of Veterinary Science, University of Camerino, Italy,Department of Technology and Biotechnology of Animal Productions, Infectious Diseases Unit, University of Perugia, Italy, showed rapid identification of *Streptococcus equisimilis* in equine nasal swab samples [83].DNA for PCR, they extracted both from the colonies grown on agar media and from enrichment broth aliquots after incubation with nasal swab samples. They identified *S. equisimilis*by bacteriological isolation in 23 out of 99 swab samples, and PCR assays on these colonies were fully concordant with bacteriological identification (kappa statistic = 1.00). In addition, PCR of the enrichment broth aliquots confirmed the bacteriological results and detected *S. equisimilis* in 6 samples more than the bacteriological examination (kappa statistic = 0.84).They prepared 4ml T14 enrichment broth for the 6 hours incubation of the nasal swab samples which consisted of phosphate buffered saline pH 7.2, 2% fetal calf serum (Celbio, Italy), 0.0005% amphotericin B (Bristol-Myers Squibb, Italy) and then spread on agar plates containing 5% sheep blood and *Streptococcus* selective supplement (Oxoid, Italy). For DNA extraction both from colonies on the agar plate and from enrichment broth, they used digestion buffer (Tris-HCl 10 mmol/L, EDTA 1 mmol/L, pH 8.0 containing 5 U/μL lysozyme; Sigma, Italy), proteinase K20 μg/μL (Eurobio, France) andafter incubation, boiling and several centrifugation steps they did PCR and found the desired result which was absolutely specific.

Chapter 2:

Method and Materials

2.1: Working place:

All project tasks were executed in the laboratory of institute for developing Science and Health initiatives (ideSHi), Institute of Public Health Building, 2nd Floor, Mohakhali Bazar Rd, Dhaka 1212, Bangladesh, from January, 2017 to August, 2017. Here bio-safety level 2 (BSL-2) is maintained and all the laboratory tasks were done inside biological safety cabinet.

2.2: Bacterial isolates:

I worked with 18 strains of *Streptococcus pneumoniae* which were obtained from the preserved bacterial stock of collected nasal swab samples from AREI study in ideSHi Laboratory. A universal primer was designed to detect any strain of *Streptococcus pneumoniae* from nasal swab sample. Besides, 6 throat swab samples were collected from the lab members in order to prove rapid diagnosis of pneumonia patient within 6 hours.

2.3: Processing of the samples:

Nasal Swab Samples, which were preserved at -70°C freezer, were thawed and sub-cultured on the blood agar (BD Difco™) (trypticase soya agar enriched with 5% sheep blood) using streak plate method that encourages the growth of *Streptococcus pneumoniae.* After 24-48 hours of incubation in an anaerobic condition at 37°C, plates were observed very carefully and strains were stocked again in cryotube with glycerol for long term preservation. Then DNA isolation was performed from these samples. On the other hand, for the rapid diagnosis purpose, throat swab samples were collected in the cryotube containing STGG media and DNA was extracted using both with an enrichment and without an enrichment media.

2.4: Preparation of STGG (Skim Milk Tryptone Soya Broth Glucose Glycerin) medium:

Field studies of Streptococcus pneumoniae (pneumococci) nasopharyngeal (NP) colonization are hampered by the need to directly plate specimens in order to ensure isolate viability. A medium containing skim milk, tryptone, glucose, and glycerin (STGG) is used to transport and store NP materials.

The solution was dispensed in 1.0-ml amounts into cryotubes. These were autoclaved at 15 lb/in^2 and 121°C for 10 min. After the tubes were cooled, they were screwed on tightly and stored at −20°C or refrigerated at 4°C until used. All tubes were used within 6 months of preparation. The medium was tested for sterility by plating the entire volume of one tube from each lot onto Trypticase soy agar with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) and incubating the plate at 37°C for 48 h. If the growth of any organism was observed, the lot was discarded. STGG tubes were vortexed for approximately 20 s immediately before being inoculated with an NP swab specimen to draw into suspension the precipitate which develops during storage.

2.5: Preparation of the bacterial plating:

2.5.1: Blood Agar:

Blood Agar is an enriched medium often used to grow fastidious organisms and to differentiate bacteria based on their hemolytic properties. It contains general nutrients and 5% sheep blood. It is useful for cultivating fastidious organisms and for determining the hemolytic capabilities of an organism. Some bacteria produce exoenzymes that lyse red blood cells and degrade hemoglobin; these are called hemolysins. Bacteria can produce different types of hemolysins. Beta-hemolysin breaks down the red blood cells and hemoglobin completely. This leaves a clear zone around the bacterial growth. Such results are referred to as β-hemolysis (beta hemolysis). Alpha-hemolysin partially breaks down the red blood cells and leaves a greenish color behind. This is referred to as α-hemolysis (alpha hemolysis). The greenish color is caused by the presence of biliverdin, which is a by-product of the breakdown of hemoglobin. If the organism does not produce hemolysins and does not break down the blood cells, no clearing will occur. This is called γ-hemolysis (gamma hemolysis). The hemolysins produced by streptococci perform better in an anaerobic environment. Because of this, it is standard procedure to streak a blood plate and then stab the loop into the agar to provide an area of lower oxygen concentration where the streptolysins can more effectively break down the blood cells. *Streptococcus pneumoniae* does alpha haemolysis on blood agar.

Figure 2.1: Types of hemolysis on Blood agar [84]

Procedure:

- Samples were inoculated into 20ml of Blood agar taken on a medium sized Petri plate and incubated at $37C^0$ for 24 hours.
- After the 24 hours of incubation, the morphology and cultural characteristics of the colonies on the media was observed to identify and confirm the presence of *S. pneumoniae.*
- Presence of a partial clear zone around the colonies that is, alpha hemolysis was taken to be positive result for the presence of *S. pneumoniae*
- Identification tests were performed next for further confirmation of *S. pneumoniae.*

2.5.2: Identification of the *S. pneumoniae*:

The strains were further confirmed by several tests. These tests include:

- ✓ Optochin disk test
- ✓ Bile soluble test
- ✓ Catalase test
- ✓ Gram Staining

2.5.2.1: Optochin disk test:

Optochin (ethyl hydrocupreine hydrochloride) sensitivity test is used for the presumptive identification of alpha-hemolytic streptococci as *Streptococcus pneumoniae*. Optochin is used to differentiate *Streptococcus pneumoniae* from other alpha-hemolytic streptococci. *Streptococcus pneumoniae* strains are sensitive to Optochin although some strains are optochin-resistant and other alpha-hemolytic streptococcal species are Optochin-resistant. The Optochin test is performed on a blood-agar medium using a disk diffusion principle. Optochin sensitive *Streptococcus pneumoniae* surrounding the disk impregnated with optochin are lysed, due to changes in surface tension, creating a clear zone of inhibition.

2.5.2.1.1: Procedure:

- A few isolated colonies of the alpha- hemolytic organism was taken and streaked onto a blood-agar plate.
- An Optochin disk was placed, in the streaked area and incubated it at $37C^0$.
- The plates were examined after 18 – 24 hours of incubation and measured the zone of inhibition if applicable.

2.5.2.1.2: Reading the optochin test results:

Using a 6 mm, 5 µg disk, a zone of inhibition of 14 mm or greater indicates sensitivity and allows for presumptive identification of pneumococci. Zone of inhibition should be measured in millimeters, including diameter of disk. In the case of an isolate completely resistant to optochin, the diameter of the disk (6 mm) should be recorded. A smaller zone of inhibition (< 14 mm) or no zone of inhibition indicates that the bile solubility test is required. It is important to remember that pneumococci are sometimes optochin-resistant.

2.5.2.2: 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.

2.5.2.2.1: Preparation of Bile solubility reagent:

Preparation of 2% sodium deoxycholate (bile salt) solution: 2g of sodium desoxycholate was dissolved into 100 ml sterile distilled water.

2.5.2.2.2: Procedure:

- A milky suspension from overnight culture on blood agar was prepared in 1ml of 0.5% saline.
- Turbidity was adjusted to that of McFarland No. 1 standard.
- Two 1ml gram vials were taken and the suspension was divided into two vials (test and control) of 0.5ml.
- In one vial, 0.5ml, 2% sodium desoxycholate (bile salts) was added and in another vial, 0.5ml normal saline was added, that was used as control. (include preparation of 2% sodium desoxycholate in sterile distilled water or equivalent- Sandra)
- The vials were vortexed and incubated CO_2 incubator or candle jar (35-37)C^o for up to 2 hours.
- *S. pneumoniae* vial will be completely transparent without turbidity (comparing with control vial), while any other alpha hemolytic streptococci vial will remain turbid after the 2 hours of incubation.
- The test should not be performed on old cultures as the active enzyme might be lost.

2.5.2.3: Catalase Test:

Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; H_2O_2. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme. Catalase mediates the breakdown of hydrogen peroxide

H_2O_2 into oxygen and water. To find out if a particular bacterial isolate is able to produce catalase enzyme, small inoculum of bacterial isolate is mixed into hydrogen peroxide solution (3%) and is observed for the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. Catalase-positive bacteria include strict aerobes as well as facultative anaerobes. They all have the ability to respire using oxygen as a terminal electron acceptor. Catalase-negative bacteria may be anaerobes, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor (i.e. Streptococci).

2.5.2.3.1: Percentage of H_2O_2 used on catalase test:

For routine testing of aerobes, 3% hydrogen peroxide is used and 15% H_2O_2 solution is used for the identification of anaerobic bacteria. Catalase test is used to differentiate aerotolerant strains of Clostridium (catalase negative) from Bacillus species (catalase positive). The superoxol catalase test used for the presumptive speciation of certain Neisseria organisms requires a different concentration of H_2O_2 .

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \text{ (gas bubbles)}$$
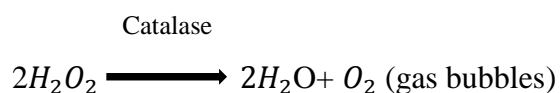
2.5.2.3.2: Procedure:

A drop of the catalase reagent (30% hydrogen peroxide) was taken on autoclaved glass slides. The glass slides were labeled according to the sample being tested. A colony for each of the bacterial isolates was taken from a blood agar plate and placed on the reagent drops on the glass slides. An immediate bubble formation indicated a positive result no bubble formation indicates negative result.

2.5.2.4: Gram Staining:

Gram staining is a used to differentiate two large groups of bacteria on the basis of their cell wall Gram positive and Gram negative. Gram positive bacteria are stained violet as they contain a thick layer of peptidoglycan in their cell walls. This thick layer of peptidoglycan layer can retain the crystal violet which is used for staining purpose. On the other hand, Gram negative bacteria are stained reddish pink due to their thinner peptidoglycan wall, which does not retain the crystal violet during the decoloring process. *S. pneumoniae* is a gram-positive organism

and thus appears violet on staining. A colony of the bacterial isolate was touched with a burnt loop and placed on a sterile glass slide. A drop of saline was taken by the loop and then the bacterial colony was smeared on the glass slide. The glass slide was heat fixed and the smear was allowed to dry. A drop of crystal violet, the primary stain was added to the smear and after one minute, the crystal violet was carefully washed off the glass slide. A drop of the mordant, iodine was next added and then after one minute the grams iodine was carefully washed off the slide. A drop of 70% ethanol was added as the decolorizing agent and washed off after 45 seconds. Safranin, the counter stain was added and after 60 seconds it was washed off the glass slide. The slide was allowed to dry off completely, after which it was observed under the microscope.

2.5.2.5: Enrichment Medium:

An enrichment media was used to support the growth of *Streptococcus pneumoniae* from the throat swab samples. The samples were being enriched in that media for about 4 hours in the shaker incubator at $37C^0$ in an anaerobic condition at 250 rpm (rotation per minute). Then DNA isolation was done. Consequently, polymerase chain reaction and agarose gel electrophoresis were performed to get the desired results. However, DNA isolation was also done from throat swab samples without using any enrichment medium, so that it was easy and reliable to compare the two results.

2.5.2.5.1: Preparation of the Enrichment medium:

For the preparation of enrichment medium 800µl of Todd Hewitt Broth and 200 µl of Fetal Bovine Serum was used. Then 40µl of sample was added in it.

- ### Todd Hewitt Broth

Todd Hewitt Broth is used for the cultivation of streptococci and other fastidious microorganisms in a laboratory setting. The nitrogen source is provided by Heart Infusion in Todd Hewitt Broth. Yeast Enriched Peptone supplies vitamins and essential minerals. Dextrose is a carbon source and a stimulant for hemolysin production [85]. Sodium Chloride maintains the osmotic balance of the medium. Disodium Phosphate and Sodium Carbonate act as buffers to aid in neutralizing acid production from dextrose fermentation and protect hemolysin from inactivation [85].

- **Fetal Bovine Serum:**

Fetal bovine serum (FBS) is a ubiquitously used essential supplement in cell culture media. However, it was used in our study for specific bacterium's growth. Serum provides different growth factors and hormones, binding and transport proteins, attachment and spreading factors, additional amino acids, vitamins and trace elements, fatty acids and lipids, protease-inhibitors, "detoxification" (due to binding and inactivation), (colloid)osmotic pressure, reduction of shear stress etc.

2.5.2.6: Quality control:

It is important to maintain the quality while doing any laboratory experiments. The procedures for quality control primarily monitor the accuracy of the work by checking the bias of data with the help of (certified) reference samples and control samples and the precision by means of replicate analyses of test samples as well as of reference and/or control samples.

2.5.2.6.1: Quality control for confirmation tests of the organisms:

A negative control was set up for all the four types of confirmation tests that were performed. By doing this, we can check whether any contamination has occurred during the experiment or any external source is affecting the experiment or not.

2.5.2.7: DNA Isolation by boiling method: (For PCR optimization)

DNA, the building block of life is the genetic material for all living organisms and it contains information that is crucial for heredity. DNA isolation is necessary for genetic analysis including scientific, medical, or forensic purposes. Presence of lipids, proteins, polysaccharides and some other impurities in the DNA preparation can interfere with DNA analysis methods by reducing the quality of DNA, which leads to its shorter storage life. DNA can be isolated from any living or dead organism. Commonly used sources for DNA isolation are whole blood, hair, sperm, bones, nails, tissues, saliva, epithelial cells, urine, bacteria, animal tissues, and plants. The traditional methods for the isolation of DNA are more time consuming and the reagents used are costly. Therefore, using boiling method, DNA can be extracted very easily. This method is not time consuming and can be done rapidly. Obtaining DNA template by this method, template can be used for polymerase chain reaction (PCR).

2.5.2.7.1: Procedure:

1. At first an Eppendorf was taken and 1ml of 0.9% normal saline was added in it.

2. About 4/5 colonies from over-night culture were taken by sterile loop and mixed them in the saline.

3. After that, homogenize mixture was done by applying a light vortex.

4. Then the Eppendorf was kept in the boiling water for about 10 minutes.

5. After 10 minutes had passed, the Eppendorf was immediately kept in ice for 1 minute.

6. Lastly, centrifugation was done at 12000 rpm for 10 minutes and supernatant was collected in another Eppendorf.

7. The supernatant contains the desired DNA and the Eppendorf was stored at -20 degrees Celsius refrigerator.

2.5.2.7.2: DNA Isolation procedure by boiling method (After enrichment of Nasal Swab and Throat Swab Samples):

1. At first all the samples were transferred in the Eppendorf from the cryotubes after 4 hours of enrichment.

2. Then Centrifugation was done for about 10 minutes at 13000 rpm.

3. After that pellet was collected and dissolve it in 500 µl of 0.9% normal saline by applying vortex.

4. Next, centrifugation was done for about 1 minute at 200000 g.

5. Then again pellet was collected and dissolve it in 500 µl of 1% autoclaved PBS.

6. The Eppendorf was kept in boiling water for 10 minutes.

7. After 10 minutes had passed, the Eppendorf was kept in ice for 1 minute immediately.

8. Lastly, centrifugation was done at 13000 rpm for 10 minutes.

9. Now, supernatant was collected which contains desired DNA and stored at -20 degrees Celsius.

2.5.2.7.3 Measurement of DNA concentration and purity:

DNA concentration was measured with EON spectrophotometer (NanoDrop™, USA) using Take 3 plate. Approximately 2µl Nuclease Free water was used as blank and 2µl of DNA sample was loaded on the Take 3 plate and OD (optical density) was measured spectrophotometrically. The concentration was measured in ng/µl. The purity was checked using OD ratio at 260 nm/280 nm reading. The result was evaluated with Nano drop Software.

2.5.2.8 Genotypic detection of definite base pair (415bp) of *Streptococcus pneumoniae*:

In order to confirm the presence of definite base pair (415bp) among 16srRNA region of *Streptococcus pneumoniae*, molecular techniques such as PCR and sequencing were done.

2.5.2.8.1: Designing a PCR primer:

The DNA sequences of 16srRNA genes were retrieved from the nucleotide database of National center for Biotechnology Information (NCBI). The retrieved FASTA sequence was then used to design specific primers using Primer-BLAST.

2.5.2.8.2: Software:

Primer-3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/)

Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)

Primer-Blast is a widely used program for designing PCR primers. PCR is an essential and ubiquitous tool in genetics and molecular biology. Primer-Blast can also design hybridization probes and sequencing primers. PCR is used for many different goals. Consequently, primer blast has many different input parameters that can be controlled and that tell exactly what characteristics make good primers for certain goals.

Method:

- ✓ http://www.ncbi.nlm.nih.gov/tools/primer-blast/ is browsed.
- ✓ Sequences are pasted.

✓ Parameters are changed if necessary.

✓ Get primers is clicked on and few minutes are required to get the result.

Figure 7: Uploading query sequence into Primer-BLAST

The sequence of the primers specific to the 16rRNA gene of *Streptococcus pneumoniae* along with their melting temperature (Tm), and guanine-cytosine (GC %) content is given below:

Table 2.1: Primers specific to 16s rRNA gene of *Streptococcus pneumoniae* and their melting temperature, size and GC content:

Primer name	Primer sequence (5'-> 3')	GC content	Product length	Tm [°C]
NM_2 (F)	GGAGCTTGCTCCTCTGGATG	60%	20	60.18
NM_2 (R)	ACCGTCACAGTGTGAACTTTCC	50%	22	61.00

Product size:415bp

2.5.2.9: Polymerase Chain Reaction:

Polymerase chain reaction (PCR) is a common laboratory technique used to make millions or billions copies a particular region of DNA. This DNA region can be anything which is desired by the researcher. Typically, the goal of PCR is to make enough of the target DNA region so that it can be analyzed or used in some other way. For instance, DNA amplified by PCR may be sent for sequencing, visualized by gel electrophoresis, or cloned into a plasmid for further experiments. PCR is used in many areas of biology and medicine, including molecular biology research, medical diagnostics, and even some branches of ecology. Here, in this study PCR was done to amplify DNA so that the desired band can be observed by gel electrophoresis.
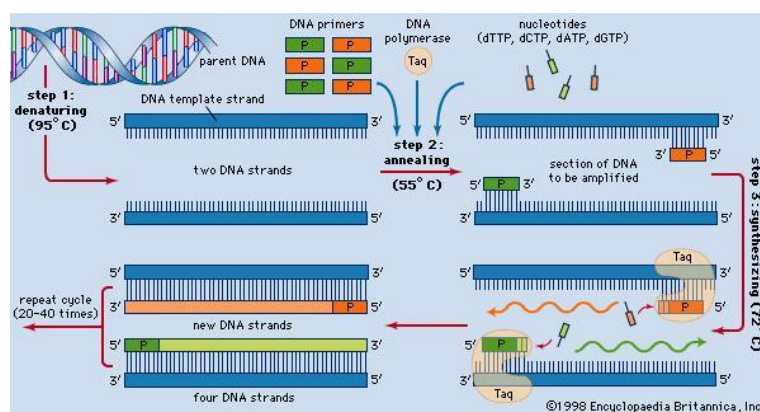
Figure 8: Principle of polymerase chain reaction [86]

2.5.2.9.1: Taq polymerase

Like DNA replication in an organism, PCR requires a DNA polymerase enzyme that makes new strands of DNA, using existing strands as templates. The DNA polymerase typically used in PCR is called Taq polymerase, after the heat-tolerant bacterium from which it was isolated (*Thermus aquaticus*).*T. aquaticus* lives in hot springs and hydrothermal vents. Its DNA polymerase is very heat-stable and is most active around 70°C (a temperature at which a human or E. coli DNA polymerase would be nonfunctional). This heat-stability makes Taq polymerase ideal for PCR.

2.5.2.9.2: Conventional PCR:

PCR was performed using a T100 ™ thermal cycler (Bio-Rad, USA). The final reaction volume was 10µl which contains-

- 1 µl 10X PCR buffer with $MgCl^{2+}$ (Clontech, code no: R001A),

- 1µl of dNTPs mixture (2.5 mM),

- 0.5µl forward and reverse primers,

- 0.1µl of Taq polymerase (Clontech, code no: R001A)

- 3.9 µl Nuclease Free Water

- 3 µl of template DNA**.**

Table 2.2: Thermal cycling profile PCR master mix

Component	Amount
2.5 mM dNTPs	1µl
10X buffer(Mg^{2+})	1µl
Forward primer	0.5µl
Reverse primer	0.5µl
Taq polymerase	0.1µl
Nuclease free water	5.9µl
Template DNA	1µl
Total volume	**10 µl**

⇨ The amount of template DNA was changed when it was necessary. Therefore, the amount of nuclease free water was also changed according to that.

⇨ Taq Polymerase was added right before loading the sample in the PCR machine (Infinigen, USA). All the steps were performed in the Eppendorf PCR cooler rack.

Table 2.3: Thermal cycling profile

PCR condition	Temperature	Time	
Initial denaturation	94°C	3 minutes	
Denaturation	94°C	30 seconds	
Annealing	58°C	30 seconds	35 Cycles
Extension	72°C	60 seconds	
Final extension	72°C	10 minutes	
	10°C	∞	

2.5.2.9.3: The steps of PCR

The key ingredients of a PCR reaction are Taq polymerase, primers, template DNA, and nucleotides (DNA building blocks). The ingredients are assembled in a tube, along with cofactors needed by the enzyme, and are put through repeated cycles of heating and cooling that allow DNA to be synthesized.

The basic steps are:

Denaturation (94°C): This step heats the reaction strongly to separate, or denature, the DNA strands. This provides single-stranded template for the next step.

Annealing (58°C): This step cools the reaction so the primers can bind to their complementary sequences on the single-stranded template DNA.

Extension (72°C): This step raises the reaction temperatures so Taq polymerase extends the primers, synthesizing new strands of DNA.

Cycle repeated 35 times in this PCR reaction, which generally took maximum 2 hours.

2.5.2.10: Gel Electrophoresis:

The results of a PCR reaction are usually visualized using gel electrophoresis. Gel electrophoresis is a technique in which fragments of DNA are pulled through a gel matrix by an electric current, and it separates DNA fragments according to size. Amplified DNA is loaded in wells in the gel close to the negative electrode. In the presence of an electrical field, negatively charged DNA moves toward the positive pole through the small holes that make up the gel matrix. These holes allow the shorter fragments of DNA to migrate faster than their longer counterparts. Once the reaction is complete, the length of the amplified DNA can be accurately determined by comparing with a DNA ladder. Amplified PCR products were analyzed by electrophoresis on 1%, 1.5% and 2% agarose gel and definite amount of agarose powder (Ultrapure, Invitrogen, USA) was dissolved in definite amount of 1X TBE buffer. Then it was heated to dissolve in a microwave oven for about 1-2 minutes. The mixture was then allowed to cool down at room temperature. To the cooled agarose gel, 2µl of Gel red (Biotium, Cat no: 41003, USA) was added. The gel was then poured on the gel casting tray previously set with the comb and allowed to solidify. While pouring the melted gel mix solution into the gel tray, care was taken so that no bubbles were formed.

2.5.2.10.1: Detection of PCR product by Agarose Gel Electrophoresis:

4/5µl of the PCR product was mixed with 2µl of loading dye and was loaded into the individual wells of the gel. A ladder of size 1Kb plus (Invitrogen, USA) was used to ensure amplification of the desired gene and measure the exact product size which was estimated to be within 1,500bp. Amplified PCR products were electrophoresed at 130 volts for 1 hour. The separated DNA bands were observed on a Gel documentation system (Bio-Rad, USA) under Ultraviolet light.

2.5.2.10.2: PCR Product purification:

MinElute PCR Purification Kit Protocol (using a microcentrifuge)

This protocol is designed to purify double-stranded DNA fragments from PCR reactions resulting in high end-concentrations of DNA. Fragments ranging from 70bp to 4 kb are purified from primers, nucleotides, polymerases, and salts using MinElute spin columns in a microcentrifuge.

Important points before starting

■ Ethanol (96–100%) to Buffer PE before use, should be added.

■ All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.

■ pH indicator I volume of 1:250 to Buffer PB (i.e.,120 µl pH indicator I to 30 ml Buffer PB or 600 µl pH indicator I to 150 ml Buffer PB) should be added. The yellow color of Buffer PB with pH indicator I indicates a pH of 7.5.

■ pH indicator I to entire buffer contents should be added. pH indicator I to buffer aliquots should not be added.

■ If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

2.5.2.10.3: Procedure

1. Buffer PB volume of 5 should be added to 1 volume of the PCR reaction and should be mixed. It is not necessary to remove mineral oil or kerosene.

For example, 250 µl of Buffer PB should be added to 50 µl PCR reaction (not including oil).

2. When pH indicator I has been added to Buffer PB, color should be checked that the mixture is yellow. If the color of the mixture is orange or violet, then 10 μl of 3 M sodium acetate (pH 5.0) should be added and should be mixed. Then color of the mixture will turn to yellow.

3. A MinElute column in a provided 2 ml collection tube in a suitable rack should be placed.

4. To bind DNA, the sample to the MinElute column should be applied and centrifugation for 1 min should be done.

For maximum recovery, all traces of sample to the column should be transferred.

5. Flow-through should be discarded. The MinElute column back into the same tube should be placed.

6. To wash, 750 μl Buffer PE was added to the MinElute column and centrifuged for 1 min.

7. Flow-through was discarded and placed the MinElute column back in the same tube. Then the column was centrifuged for an additional 1 min at maximum speed.

8. The MinElute column was placed in a clean 1.5 ml microcentrifuge tube.

9. To elute DNA, 10 μl Buffer EB (10 mM Tris·Cl, pH 8.5) or water was added to the center of the membrane, then the column was kept stand for 1 min, and then centrifuge was done for 1 min.

10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA.

2.5.2.11: DNA sequencing (Sanger sequencing method)

DNA sequencing is a laboratory method used to determine the sequence of a DNA molecule. It is also called Sanger Sequencing as the method was developed by Frederick Sanger. In Sanger sequencing, the DNA to be sequenced serves as a template for DNA synthesis. A DNA primer is designed to be a starting point for DNA synthesis on the strand of DNA to be sequenced. Four individual DNA synthesis reactions are performed. The four reactions include normal A, G, C, and T deoxynucleotide triphosphates (dNTPs), and each contains a low level of one of four dideoxynucleotide triphosphates (ddNTPs): ddATP, ddGTP, ddCTP, or ddTTP. The four reactions can be named A, G, C and T, according to which of the four ddNTPs was included. When a ddNTP is incorporated into a chain of nucleotides, synthesis terminates. This is because the ddNTP molecule lacks a 3' hydroxyl group, which is required to form a link with

the next nucleotide in the chain. Since the ddNTPs are randomly incorporated, synthesis terminates at many different positions for each reaction.

Following synthesis, the products of the A, G, C, and T reactions are individually loaded into four lanes of a single gel and separated using gel electrophoresis, a method that separates DNA fragments by their sizes. The bands of the gel are detected, and then the sequence is read from the bottom of the gel to the top, including bands in all four lanes. For instance, if the lowest band across all four lanes appears in the A reaction lane, then the first nucleotide in the sequence is A. Then if the next band from bottom to top appears in the T lane, the second nucleotide in the sequence is T, and so on. Due to the use of dideoxynucleotides in the reactions, Sanger sequencing is also called "dideoxy" sequencing.

The DNA samples were sent at IEDCR (Institute of Epidemiology, Disease Control and Research) for sequencing. The calculation of cycle sequencing was obtained according to the measured template concentration.
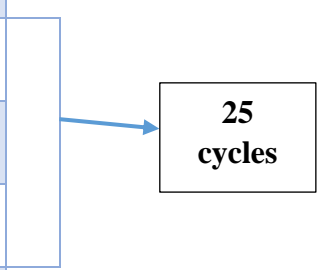
Table 2.4: Master cycle components with amount

Component	Amount
5X PCR sequencing buffer	2.0μl
Big dye(2.5X)	0.50μl
Primers	0.2 μl
Template	1-10 ng
Water	up to 10 µL

The tubes containing the template were spun, and 10-20 ng/µL (depending on the concentration) of each of the purified PCR products were added to the 8-tube PCR strip. Then nuclease free water was added to the mixture to make the total volume 10 µL. The PCR tubes were centrifuged at 4000 rpm for 3 minutes. Then, the PCR strip was placed in the Mastercycler® gradient (Cat. No. 4095-0015, USA Scientific) Thermal Cycler and subjected to following thermal cycling profile: pre-denaturation at 95°C for 10 minutes; 25 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 5 seconds and extension at 72°C for 4 minutes; and a final extension at 72°C for 6 minutes.

Table 2.5: Thermal cycling profile for cycle sequencing

PCR condition	Temperature	Duration	
Initial denaturation	95°C	10 minutes	
Denaturation	95 °C	10 seconds	
Annealing	55 °C	5 seconds	**25 cycles**
Extension	72 °C	4 minutes	
Final extension	72 °C	6 minutes	
	10 °C	∞	

After completion of cycle sequencing, the reaction plate was centrifuged at 4100 rpm for 2 minutes. Then, 45µl of SAM solution and 10 µl of X-terminator (Applied Biosystems, USA) were added per 10µl volume. Both of the solution aid in removal of impurities by desalting as salt interferes with electro-kinetic injection and elimination of remaining labeled ddNTPs, thus, minimizing background noise produced by dye blobs in the sequencing results. Before addition, the X-terminator solution was vortexed properly at maximum speed for at least 30 seconds, until it became homogenous. As it was difficult to pipette the highly dense X-terminator solution out from the bottom of its container, wide bore micropipette tips were used. Later, the reaction plate was sealed and vortexed for half an hour. The mixture was then centrifuged at 4100g for 2 minutes and the supernatant collected for capillary electrophoresis. 10 µl of supernatant was transferred to a fresh sequencing tube. Before placing the sequencing tubes into the capillary electrophoresis instrument, it was covered with Septa mat. Rest of the supernatant was stored at +4°C for later use.

2.5.2.11.1 Sequence analysis:

Sequencing data were analyzed by Chromas Lite 2.4 software to identify the sequence alignments for showing identity and detecting mutations. The obtained sequence was subjected to further analysis using Basic Local Alignment Search Tool (BLAST) for finding sequence similarity with sequences already reported in online databases.

Basic Local Alignment Search Tool (BLAST): https://blast.ncbi.nlm.nih.gov/Blast.cgi

2.5.2.11.2: BLAST (Basic Local Alignment Search Tool)

BLAST is an algorithm that can compare and align a query nucleotide or protein sequence with a number of sequences contained in its database. It finds regions of local similarity between the sequences by calculating the statistical significance of matches. It is both rapid and sensitive and hence is used by millions of biologists. It is available online at the National Center for Biotechnology Information (NCBI) website (Lobo, 2008).

2.5.2.11.3: Method:

☐   https://blast.ncbi.nlm.nih.gov/Blast.cgi is browsed

☐   Sequences are pasted

☐   Parameters are changed if necessary

☐   BLAST is clicked on and few minutes are required to get the result.

Chapter 3: Results

3.1 Confirmation of *Streptococcus pneumoniae*:

The strains were confirmed using appropriate laboratory tests. The test results were observed and recorded. The stored strains were confirmed by culturing them on blood agar plate through overnight incubation at anaerobic condition. Colonies showing typical morphological characteristics of *Streptococcus* were further taken for confirmation tests. A series of confirmation tests- Optochin disk test, Bile soluble test, Catalase test, Gram staining investigated the presence of *S. pneumoniae*. Through these tests, eighteen *S. pneumoniae* isolates were finally confirmed.

3.1.1 Colony morphology of *Streptococcus pneumoniae* strains:

After 24-48 hours incubation of *Streptococcus pneumoniae* isolates in anaerobic condition on Blood agar plate, the colony appearance and morphology were observed for the confirmation of bacterial strains.
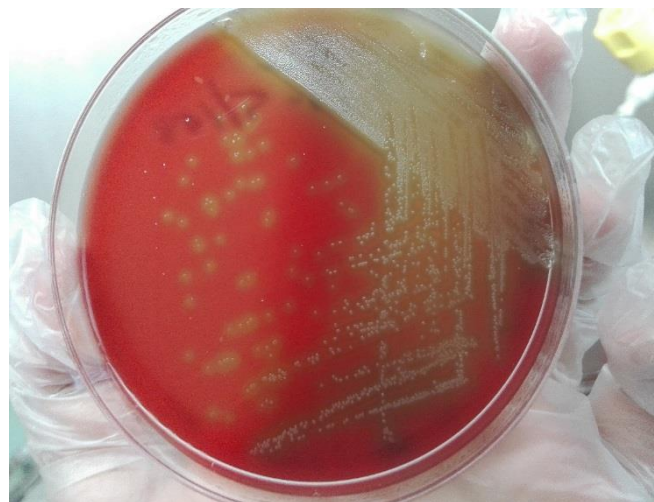
Figure 3.1: Colony appearance of *Streptococcus pneumoniae* isolates on Blood agar plate from nasal swab sample.
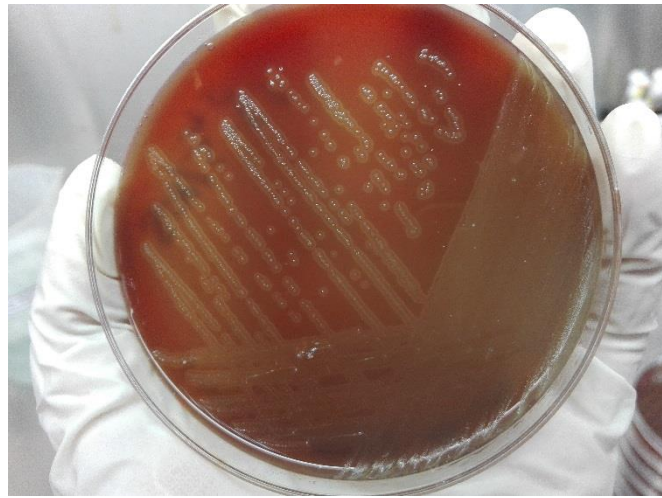
Figure 3.2: Subculture of *Streptococcus pneumoniae* isolates on Blood agar from throat swab sample.

Table 3.1: Colony characteristics of the isolates on Blood agar medium

Bacterial isolate	Colony Characteristics					
	Size	Margin	Elevation	Pigment	Form	Consistency
Streptococcus pneumoniae	Small	Entire	Raised center	Greenish	Circular	Smooth

3.1.2: Optochin Disk Test:

The growth on the surface of the plate was observed, especially in the vicinity of the antibiotic disk. If there is a visible zone of inhibition surrounding the disk, the microbe is susceptible to the antibiotic. A presumptive identification for *S. pneumonia* was made to check whether the alpha-hemolytic colony produces a zone of inhibition of 14mm or greater around the disk.
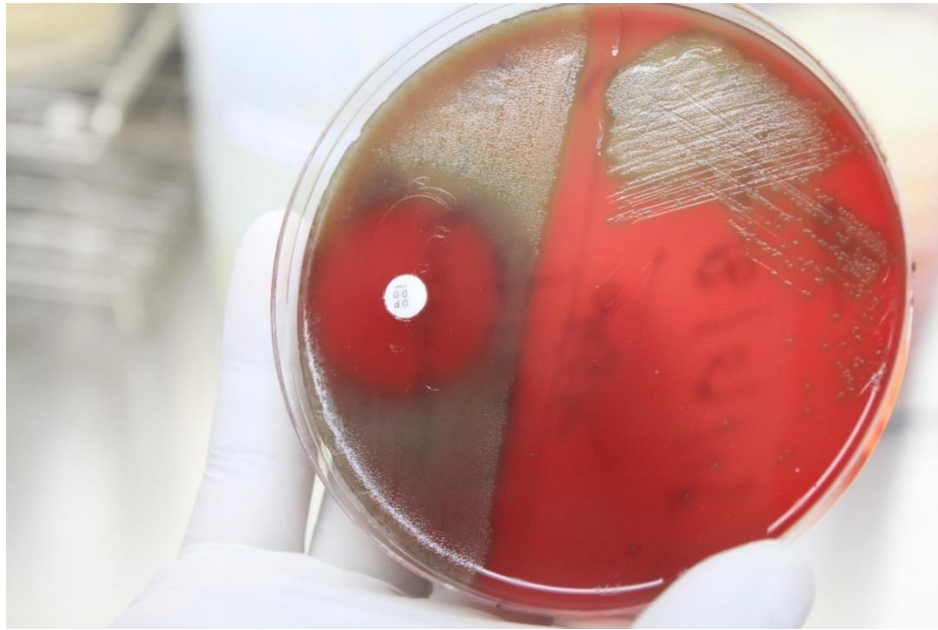
Figure 3.3: Optochin-sensitive (zone > 14mm) *Streptococcus pneumoniae* growing around an Optochin Differentiation Disk on Blood Agar incubated in CO_2 for 24 hours at 37ºC.

3.1.3: Bile solubility test:

Organisms producing smaller zone sizes (≤14mm) should be tested for bile solubility. Organisms with questionable zone sizes (6-14mm) around the disk should be presumptively identified as pneumococci only if it is bile soluble. In this experiment, there was no optochin resistant strain but still bile solubility test was applied for all the eighteen strains for further confirmation of the bacterial strain. All of the eighteen strains were bile soluble confirming that the strains were *S. pneumoniae*.
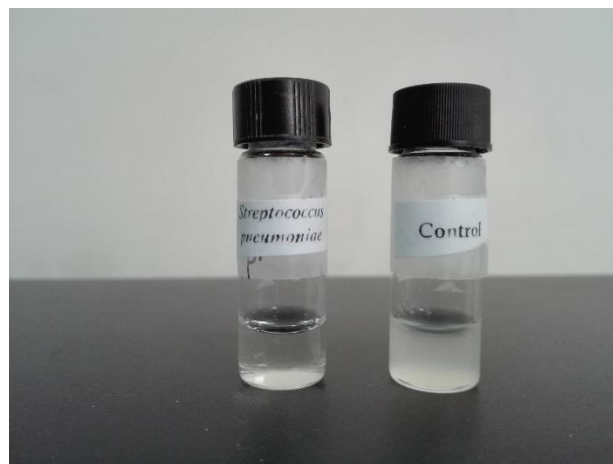
Figure: 3.4 A clearing of the turbidity in the bile tube (*Streptococcus pneumoniae*) but not in the saline control tube indicates a positive test. Experiment was done from nasal swab samples.

3.1.3.1: Bile solubility test: (From six throat swab samples)

Bile solubility test was also performed for six throat swab samples which were collected from the lab members. All of the six samples were bile soluble confirming that the strains were *S. pneumoniae*.
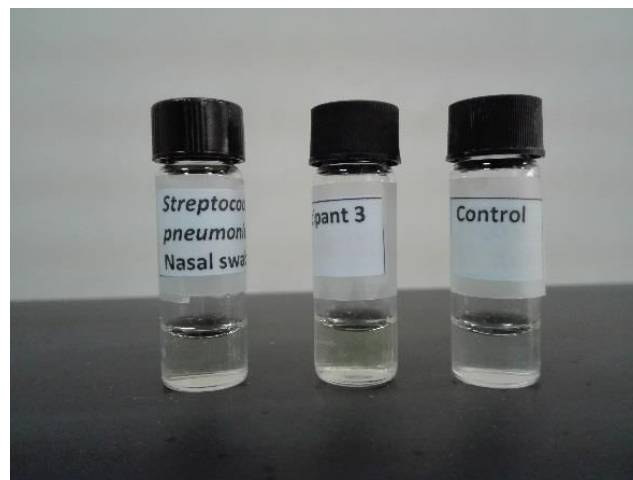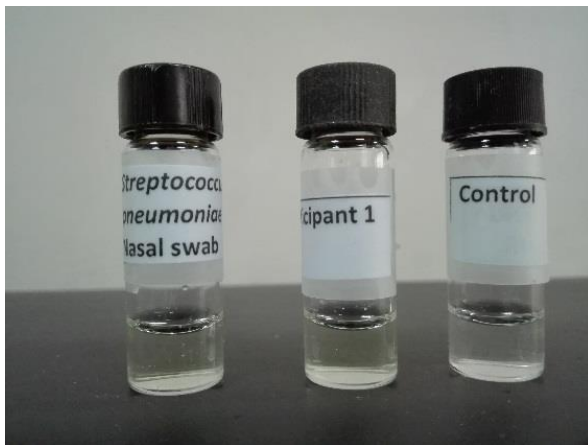
Figure: 3.5 A clearing of the turbidity in the bile tube for participant 1, participant 2 and pertcipant 3 after applying bile solubility test reagent. Comparing with a postive control (*Streptococcus pneumoniae* from nasal swab) indicates a positive test whereas, "control" tube is a negative control contianing only bacterial suspension.
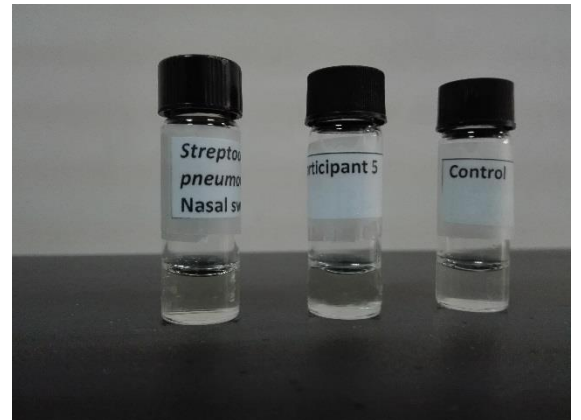
Figure: 3.6 A clearing of the turbidity in the bile tube for participant 4, participant 5 and perticipant 6 after applying bile solubility test reagent. Comparing with a postive control (*Streptococcus pneumoniae* from nasal swab) indicates a positive test whereas, "control" tube is a negative control contianing only bacterial suspension.

3.1.4: Catalase Test:

Most cytochrome containing organisms produce a catalase enzyme which breaks down hydrogen peroxide into oxygen and water. When a small amount of a catalase producing organism is introduced into hydrogen peroxide, bubbles of oxygen form as a result of the enzyme's activity. Streptococcus pneumoniae is catalase negative as no bubble formed when hydrogen peroxide was introduced on the slides containing the organism. *Klebsiella pneumoniae* was taken as the positive control.
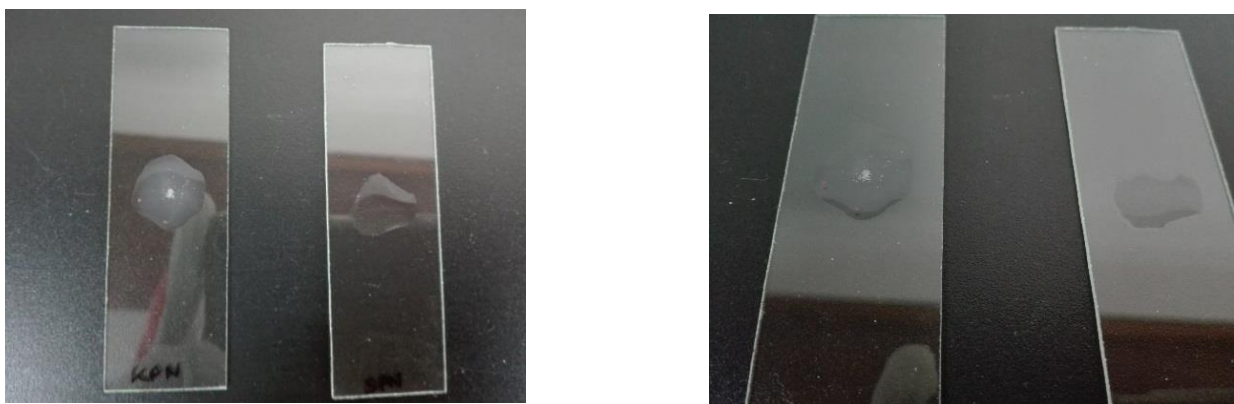
Figure 3.7: Figure: Catalase test result of *Streptococcus pneumoniae* which is negative and is indicated by the right-side slide and *Klebsiella pneumoniae* was taken as positive control that is indicated by the left side slide in both images. In left side image, colony was taken from nasal swab sample plate and in right side image; colony was taken from throat swab sample plate.

Table 3.2: Catalase test result of *Streptococcus pneumoniae* and *Klebsiella pneumoniae* positive control

Organisms	Catalase test results
Streptococcus pneumoniae	Negative
Klebsiella pneumoniae **(Positive control)**	Positive

3.1.5: Gram Staining:

Gram staining result of the strains were observed under microscopic oil emersion lens for the confirmation of cellular morphology and arrangement. All the colonies were purple in color and spherical, remained in chain or pairs denoting that they were gram positive cocci.
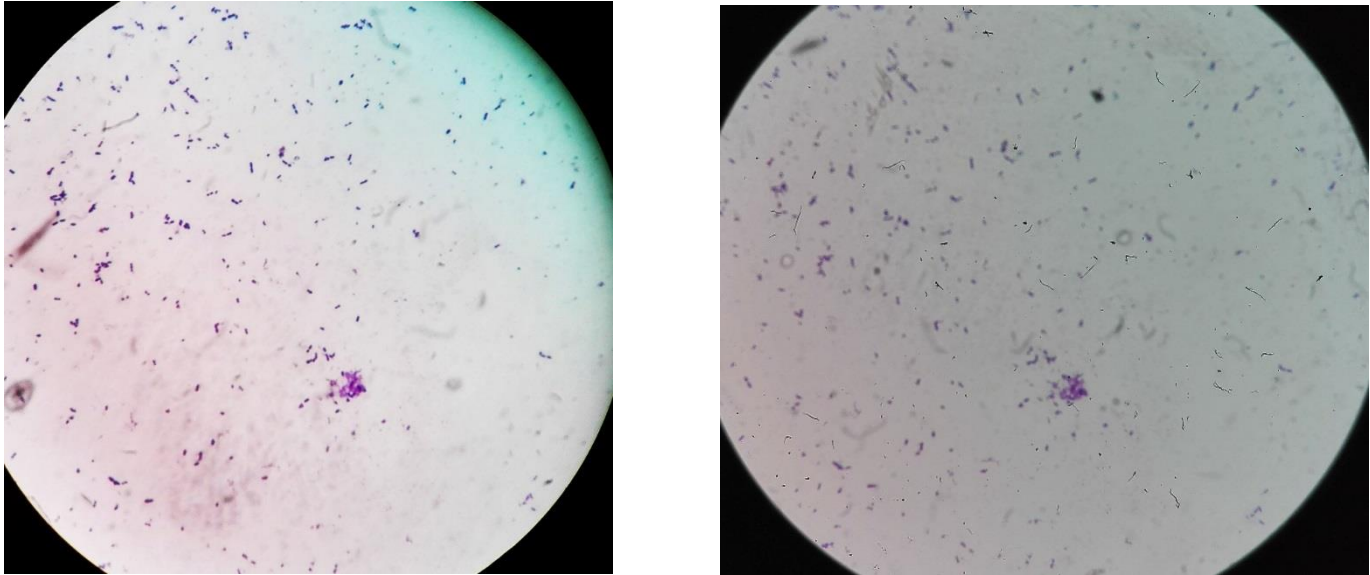
Figure 3.8: Microscopic characteristics of *Streptococcus pneumoniae* after Gram staining. Image at the left side was the result from nasal swab samples and the right side image indicates the results from throat swab samples.

3.2: Genotypic detection:

The 16SrRNA specific primers were used for PCR based detection purpose. The amplified DNA was run in 1.5% agarose gel in order to observe the desired band. To detect the 415bp desired product, the GeneRuler 1Kb Plus DNA Ladder (Invitrogen) was used which is a solution of DNA molecules of different known lengths. After done with gel run, the desired 415bp band was easily compared with the bands of the ladder. Gel run image of clinical samples (Nasal Swab Samples-18 strains) and Throat Swab Samples from the lab members are given below:

3.2.1: Gel run figure of Clinical samples (Nasal Swab Samples)
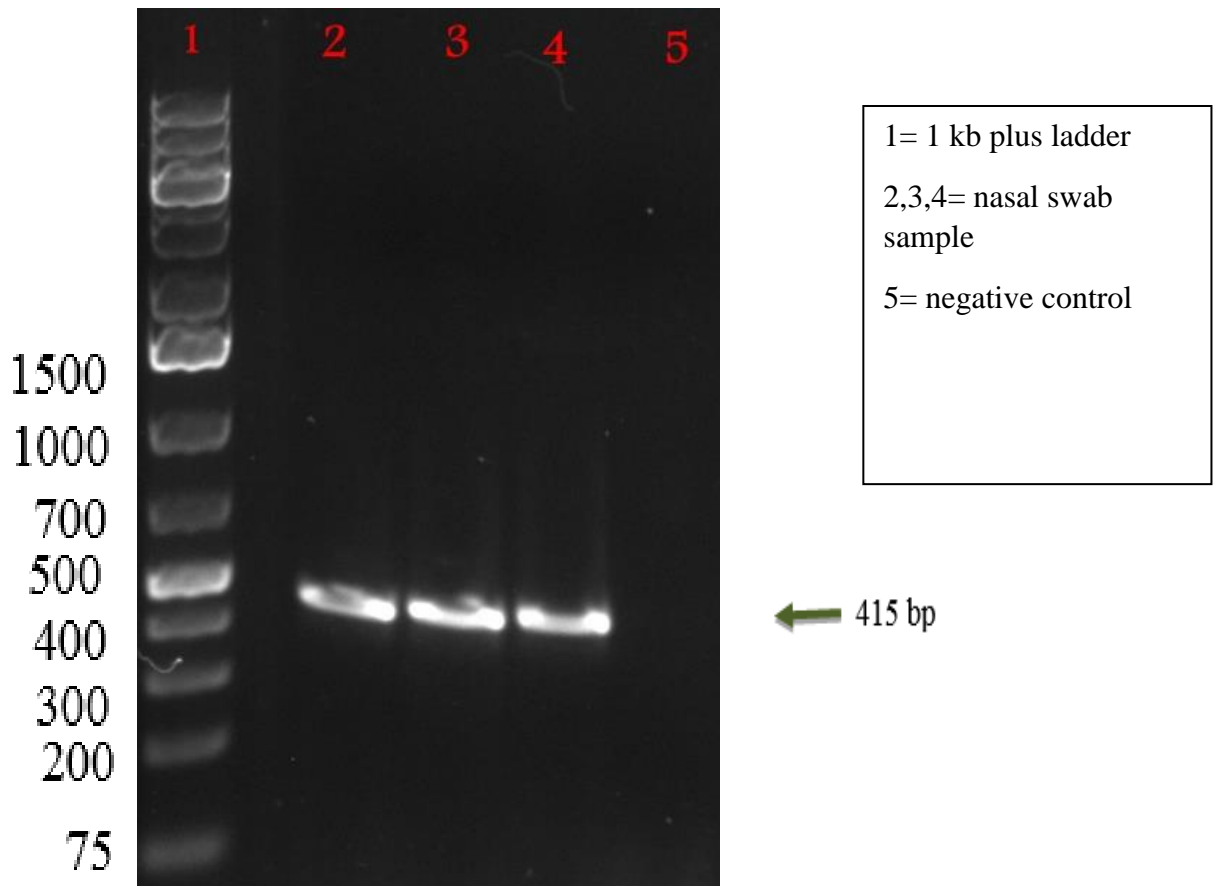
Figure 3.9: Gel electrophoresis result of three nasal swab samples

As the desired band (415bp) appeared after gel electrophoresis, it was confirmed, designed primer pair was appropriate and specific for the detection of *S. pneumoniae*.
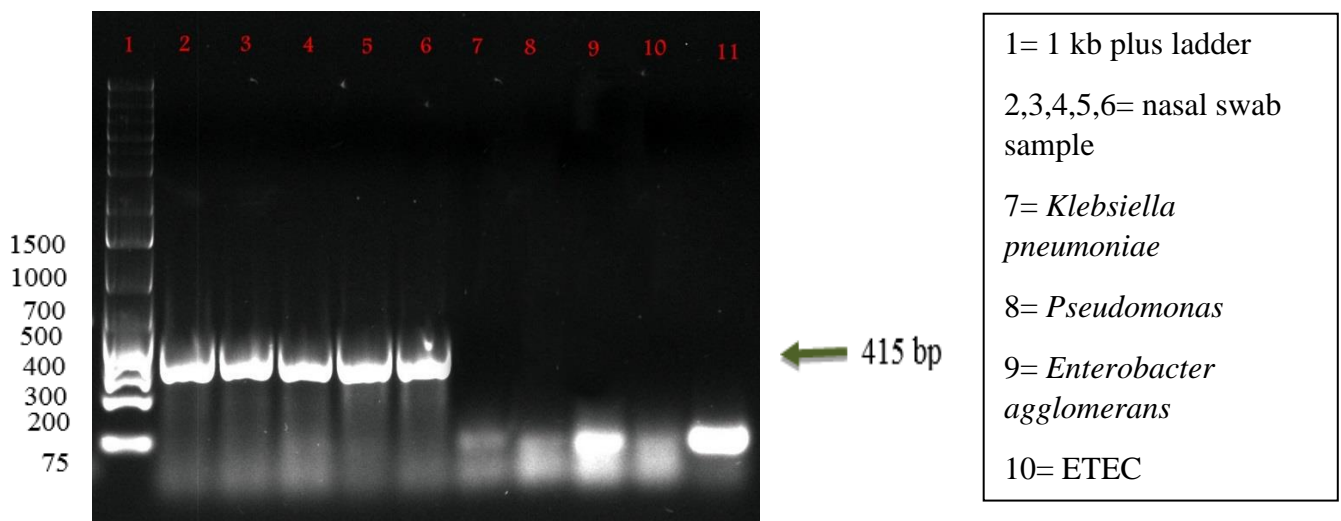
Figure 3.10: Gel electrophoresis result of five nasal swab samples and other five strains except *S. pneumoniae*

In this figure, gel electrophoresis result of five nasal swab samples and other five strains which includes *Klebsiella pneumoniae, Pseudomonas, Enterobacter agglomerans*, ETEC indicates designed primer-pairs could only detect *S. pneumoniae* from the nasal swab samples as appropriate band (415bp) appeared.

3.2.2: Gel run figure of Throat Swab Samples (collected from Lab members):
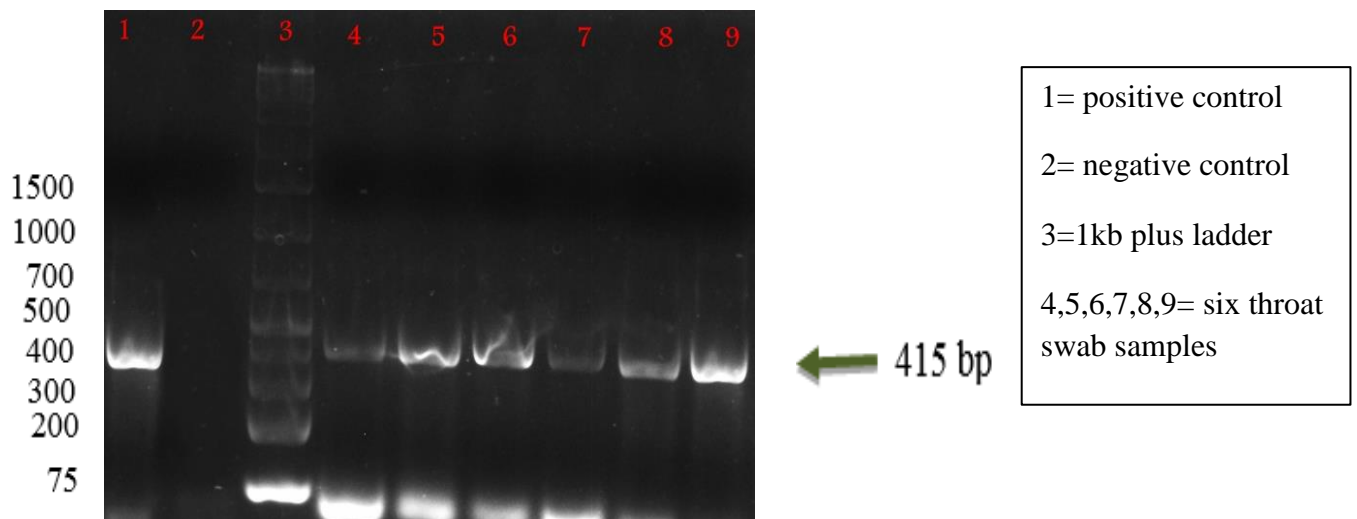
Figure 3.11: Gel run of the throat swab samples collected from six lab members after incubation in enrichment broth.
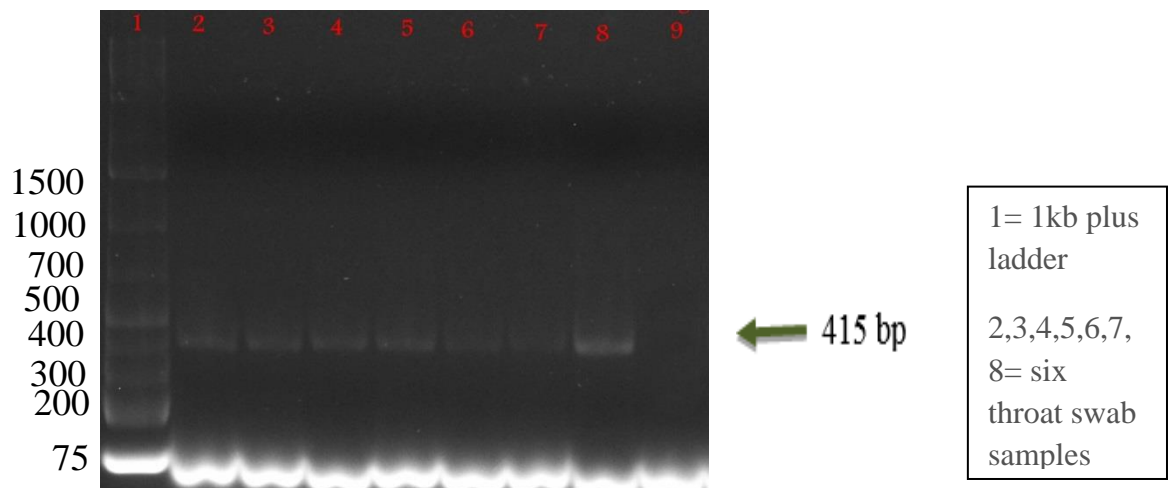
Figure: Gel run of the throat swab samples collected from six lab members without incubation in enrichment broth.

3.3 Purity and amount of purified PCR product: (From culture plate)

The purity and concentration of the DNA sample was measured spectrophotometrically. The ratio of OD at 260 nm and 280 nm of the extracted DNA and the concentration of the PCR product was measured and recorded.

Organisms	Nucleic Acid concentration	Purity (260/280 nm)
Streptococcus pneumoniae 1	51.3	1.80
Streptococcus pneumoniae 2	46.2	1.79

Table 3.3: Purity and concentration of the PCR products

3.4 Purity and amount of purified PCR product: (From throat swab sample without enrichment)

The purity and concentration of the DNA sample was measured spectrophotometrically. The ratio of OD at 260 nm and 280 nm of the extracted DNA and the concentration of the PCR product was measured and recorded.

Table 3.4: Purity and concentration of the PCR products

Participant	Nucleic Acid concentration	Purity (260/280 nm)
Participant 1	33.2	1.72

3.5 Template preparation from culture plate (Sequencing of PCR product)

For sequencing purpose, two templates were prepared from two different strains. After done with PCR and PCR purification, they were analyzed by sequencing. The sequencing data were analyzed by Chromas Lite 2.1 software. This tool generates a four colors chromatogram showing the result of sequencing run. Different bases are represented in different colors that are defined below:

1.Adenosine=Green

2.Guanine=Black

3.Cytosine=Blue

4. Thymine=Red
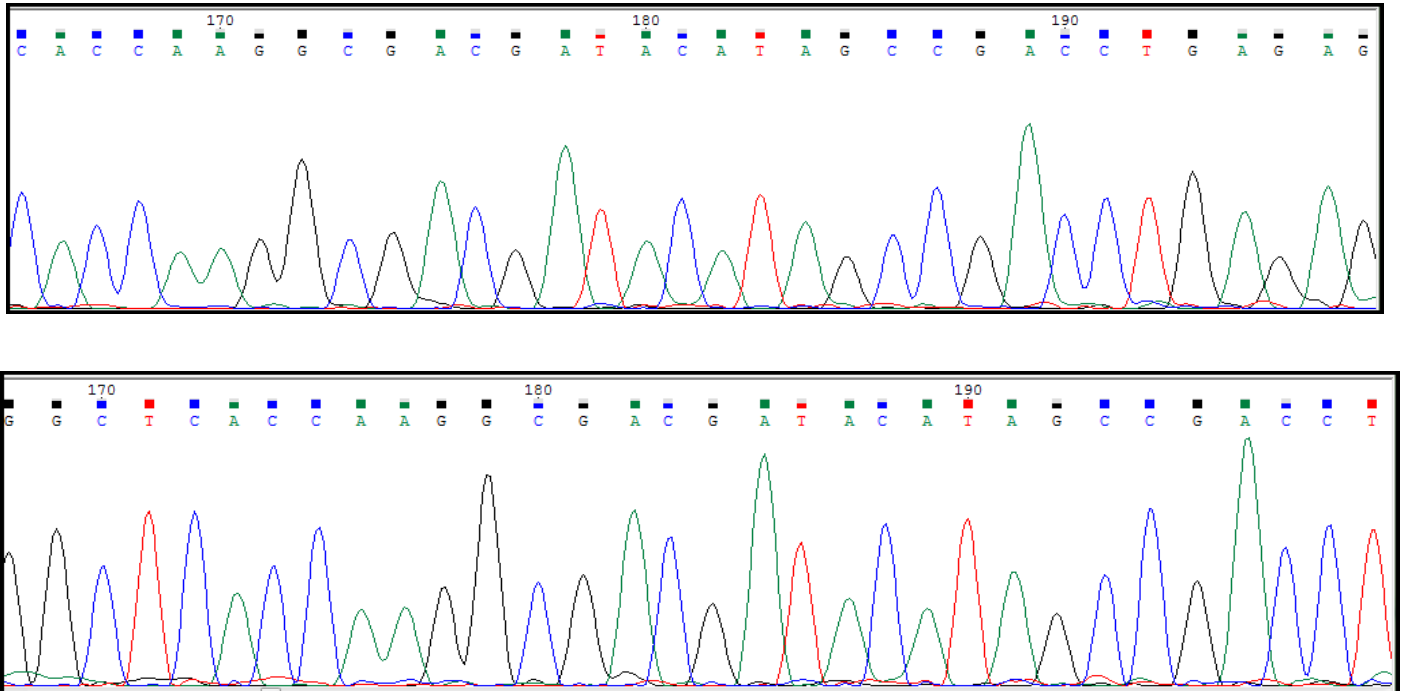
Figure 3.12: Diagrammatic representation of a part of the chromatogram of sequence of two strains. Here, the peaks are well-formed which are shown by different color. There is no background messiness, which indicates, the refined sequence had proper concentration of template and primer. In the chromatogram, the area under the peak is considered as the measure of component concentration.

>*Streptococcus pneumoinae*_16srRNA_1

TGATGARTACGCGTAGGTACCTGCCTGGTAGCGGGGGATAACTATTGGAAACGATA
GCTAATACCGCATAAGAGTAGATGTTGCATGACATTTGCTTAAAAGGTGCACTTGCA
TCACTACCAGATGGACCTGCGTTGTATTAGCTAGTTGGTGGGGTAACGGCTCACCAA
GGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGGAAGTC
TGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTA
AGAGAAGAACGAGTGTGAGAGTGGAAAKACC

>*Streptococcus pneumoinae*_16srRNA_2

CWWSGACGGGTGAGTACGCGTAGGTACCTGCCTGGTAGCGGGGGATAACTATTGGAAACG
ATAGCTAATACCGCATAAGAGTAGATGTTGCATGACATTTGCTTAAAAGGTGCACTTGCATC
ACTACCAGATGGACCTGCGTTGTATTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGCGACG
ATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGGAAGTCTGACCGAGCAACGCCGCGT
GAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGAGAAGAACGAGTGTGAGAGTGG
AAACCCCCSKKGKGGGGGGRRRKAKATAAA

Figure 3.13: Sequencing data obtained using specific forward primer

3.5.1 Nucleotide Analysis:

3.5.2 BLAST results of the nucleotide sequence: The gene sequence was analyzed with NCBI Nucleotide Basic local alignment search tool (BLAST).

Job title: Streptococcus pneumoinae_16srRNA

RID XRK03SRR015 (Expires on 10-11 03:20 am)
Query ID lcl|Query_158685
Description Streptococcus pneumoinae_16srRNA
Molecule type nucleic acid
Query Length 400

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.7.0+ ▷ Citation

Other reports: ▷ Search Summary [Taxonomy reports] [Distance tree of results] [MSA viewer]

Job title: Streptococcus pneumoinae_16srRNA

RID XREYSN3W014 (Expires on 10-11 02:11 am)
Query ID lcl|Query_117865
Description Streptococcus pneumoinae_16srRNA
Molecule type nucleic acid
Query Length 370

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.7.0+ ▷ Citation

Other reports: ▷ Search Summary [Taxonomy reports] [Distance tree of results] [MSA viewer]

Graphic Summary

Figure: 3.14: Results of two strains of *S. pneumoniae*. Here Molecule type is nucleic acid and query length of two sequences was 400 and 370 respectively.
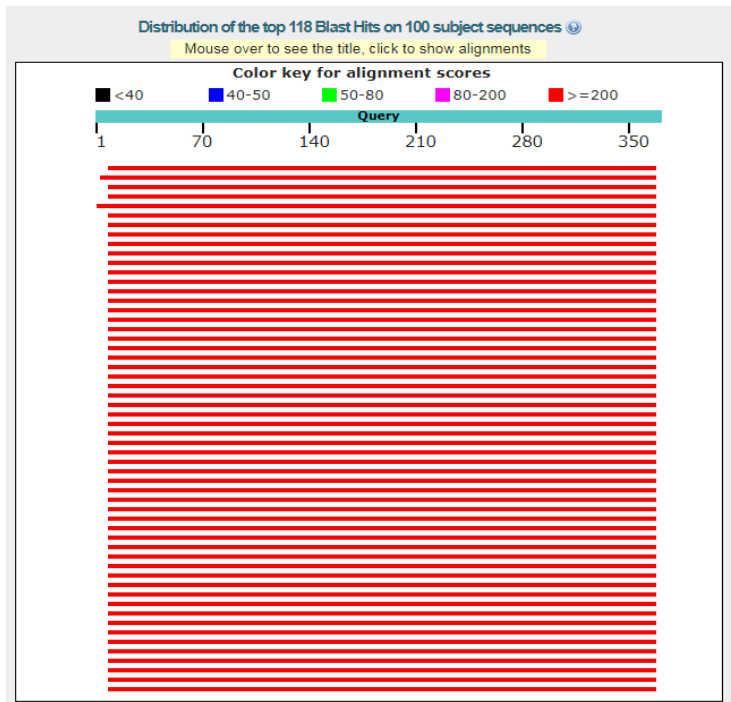
Figure 3.15: Graphic Summary of BLASTn result using the sequence of PCR product. Red straight lines indicating the highest sequence similarities.
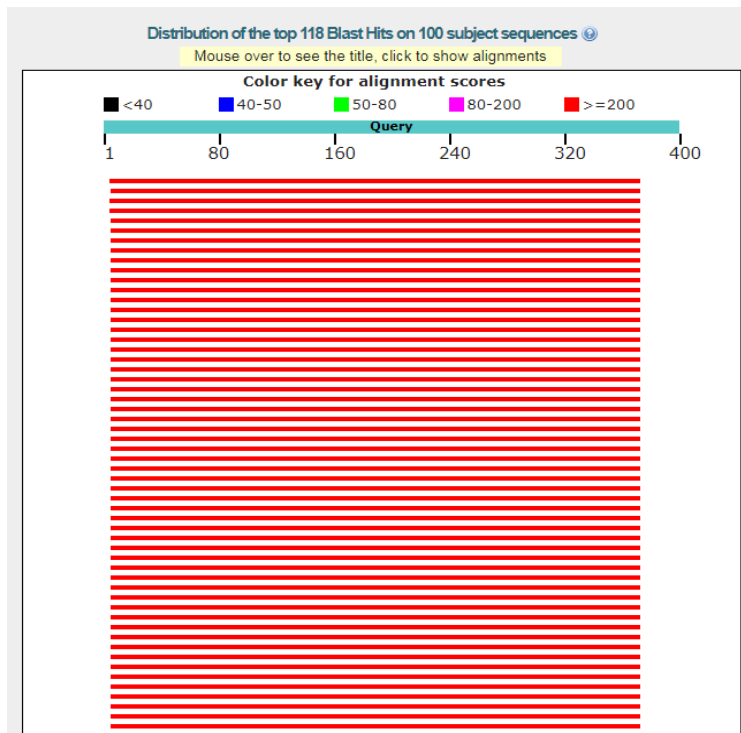
Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus pneumoniae DNA, nearly complete genome, strain: KK0381	656	2621	96%	0.0	99%	AP018043.1
Streptococcus pneumoniae strain PUS085 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661223.1
Streptococcus pneumoniae strain PUS083 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661221.1
Streptococcus pneumoniae strain PUS081 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661219.1
Streptococcus pneumoniae strain PUS076 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661214.1
Streptococcus pneumoniae strain PUS069 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661207.1
Streptococcus pneumoniae strain PUS064 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661202.1
Streptococcus pneumoniae strain PUS063 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661201.1
Streptococcus pneumoniae strain PUS060 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661198.1
Streptococcus pneumoniae strain PUS059 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661197.1
Streptococcus pneumoniae strain PUS053 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661191.1
Streptococcus pneumoniae strain PUS050 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661188.1
Streptococcus pneumoniae strain PUS046 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661184.1
Streptococcus pneumoniae strain PUS043 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661181.1
Streptococcus pneumoniae strain PUS037 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661175.1
Streptococcus pneumoniae strain PUS036 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661174.1
Streptococcus pneumoniae strain PUS035 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661173.1
Streptococcus pneumoniae strain PUS034 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661172.1

Alignments ⬇Download ⌄ GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus pneumoniae DNA, nearly complete genome, strain: KK0381	656	2621	96%	0.0	99%	AP018043.1
Uncultured Streptococcus sp. partial 16S rRNA gene, isolate 42T_22589	656	656	96%	0.0	99%	LT697171.1
Streptococcus pneumoniae strain PUS085 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661223.1
Streptococcus pneumoniae strain PUS083 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661221.1
Streptococcus pneumoniae strain PUS081 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661219.1
Streptococcus pneumoniae strain PUS076 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661214.1
Streptococcus pneumoniae strain PUS069 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661207.1
Streptococcus pneumoniae strain PUS064 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661202.1
Streptococcus pneumoniae strain PUS063 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661201.1
Streptococcus pneumoniae strain PUS060 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661198.1
Streptococcus pneumoniae strain PUS059 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661197.1
Streptococcus pneumoniae strain PUS053 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661191.1
Streptococcus pneumoniae strain PUS050 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661188.1
Streptococcus pneumoniae strain PUS046 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661184.1
Streptococcus pneumoniae strain PUS043 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661181.1
Streptococcus pneumoniae strain PUS037 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661175.1
Streptococcus pneumoniae strain PUS036 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661174.1
Streptococcus pneumoniae strain PUS035 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661173.1
Streptococcus pneumoniae strain PUS034 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661172.1
Streptococcus pneumoniae strain PUS030 16S ribosomal RNA gene, partial sequence	656	656	96%	0.0	99%	KX661168.1

Figure 3.16 Significant alignments with query sequence.

The parameters are maximum score, total score, query cover, E value and accession number. Score is given according to the alignment number depending on the match, mismatch and gaps. When query sequence and target sequence is identical then the maximum score is got. Here query cover indicates how many the sequence length is covered by this search. On the other hand, the Expect value (E) is a parameter that indicates the number of hits one can "expect" to see by chance when searching a database of a particular size. It decreases exponentially as the Score (S) of the match increases. Essentially, the E value describes the random background messiness. Therefore, here, when E value is low the identity is high.

Streptococcus pneumoniae 1

Streptococcus pneumoniae 2

Figure 3.17: One of the matched sequences with 16SrRNA gene of two strains of *Streptococcus pneumoniae*.

Here score is 652 and 656. E value is 0.0 for both cases. Only 1 and 2 gaps were present in the aligned sequences. Lastly, identities indicate, among 370 nucleic acids sequence 368 nucleic acids sequences are matched (strain1) and among 359 nucleic acids sequence 358 nucleic acids sequences are matched (strain2) with *S. pneumonia* reference.

3.5.3: Sequencing results (template preparation from throat swab without enrichment)

For sequencing among 6 throat swab samples, 1 random sample was taken and results are given below:
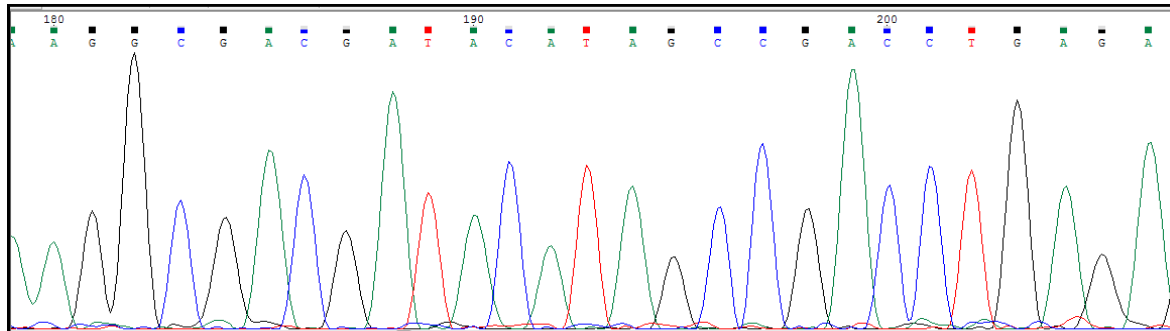
Figure 3.18: Diagrammatic representation of a part of the chromatogram of sequence of one random sample from the throat swab

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>*Streptococcus pneumoniae*_16SrRNA

TCCCYACTCKGCTGATYSTTYSSYYAGKKSCKGYYWGRTMGCGRGGGATAACTA
TTGGAAACGATAGCTAATACCGCATAAYAKTRGWTGTTGCATGAYATYTGYTTR
AAAGGTGCAATTGCAYCACTACCAGATGGACCTGCGTTGTATTAGCTAGTTGGTG
RGGTAACGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT
CTTCGGCAATGGACGGAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTT
TTCGGATCGTAAAGCTCTGTTGTAAGAGAAGAACGAGTGTGAGAGTGGAAAGTT
CCMCYTGTGACGGTAAA

Figure 3.19 FASTA sequence of DNA

3.5.4 Nucleotide Analysis:

3.5.5 BLAST results of the nucleotide sequence: The gene sequence was analyzed with NCBI Nucleotide Basic local alignment search tool (BLAST).

Streptococcus pneumoniae_16SrRNA

Figure: 3.20: Graphic Summary of BLASTn result using the sequence of PCR product. Red straight lines are indicating the highest sequence similarities.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus pneumoniae strain PUS054 16S ribosomal RNA gene, partial sequence	599	599	89%	2e-167	96%	KX661192.1
Streptococcus pneumoniae strain PUS002 16S ribosomal RNA gene, partial sequence	599	599	89%	2e-167	96%	KX661140.1
Streptococcus mitis strain 2.2 16S ribosomal RNA gene, partial sequence	599	599	89%	2e-167	96%	KX453968.1
Streptococcus sp. A12, complete genome	599	2386	89%	2e-167	96%	CP013651.1
Streptococcus mitis strain SVGS_061, complete genome	599	2397	89%	2e-167	96%	CP014326.1
Streptococcus mitis strain KCOM 1350 (= ChDC B183), complete genome	599	2006	89%	2e-167	96%	CP012646.1
Streptococcus sp. C2 16S ribosomal RNA gene, partial sequence	599	599	89%	2e-167	96%	KM225749.1
Streptococcus sp. B2 16S ribosomal RNA gene, partial sequence	599	599	89%	2e-167	96%	KM225741.1
Streptococcus sp. DP15 16S ribosomal RNA gene, partial sequence	599	599	89%	2e-167	96%	KJ575556.1
Streptococcus oralis strain ChDC B680 16S ribosomal RNA gene, partial sequence	599	599	89%	2e-167	96%	KC589364.1
Streptococcus thermophilus partial 16S rRNA gene, isolate 19-5-E	599	599	89%	2e-167	96%	HE974957.1

Figure 3.21: Significant alignments with query sequence

When query sequence and target sequence is identical then the maximum score is got. Here query cover for *Streptococcus pneumoniae* is 89% which indicates 89 out of 100 sequence length is covered by this search. Identity is 96%. On the other hand, the Expect value (E) for this organism is 2e^167, meaning the value is nearer to zero which indicates that there was less random background messiness

Chapter: 4

Discussion

Streptococcus pneumoniae is the main etiologic agent of pneumonia worldwide. The diagnosis of pneumococcal diseases is still not overtly established and is probably largely underestimated since diagnosis of pneumococcal infections, usually performed by traditional culture based method which is considered as "Gold Standard" but have a poor sensitivity. It also requires the presence of viable pathogens in the clinical samples and often have the risk of contamination due to long and multiple steps [87-89]. Moreover, the results are not available before 2-3 days. On the other hand, PCR-based assays for direct detection of pneumococci from clinical samples do not require viable bacteria and have a very high sensitivity, representing an important tool in the diagnosis of pneumococcal infections [90].

According to a study by Musher et al. [91], sensitivity increased in inverse proportion to the duration of prior antibiotic therapy. For example, direct microscopic examination of Gram-stained specimens has some limitations in clinical practice; specifically, inadequate sputum collection and antimicrobial therapy before obtaining sputum specimens lead to low diagnostic yields. In this case, sputum studies found diverse sensitivity (15-100%) [92]. Molecular methods substantially revolutionized microbiological diagnosis. These techniques are more convenient than conventional methods as they are generally faster, more specific and precise. Moreover, they apply more stable genotypic characteristics for identification than traditional techniques using phenotypic characteristics [89]. Developing countries like Bangladesh is particularly at great risk as the methods of diagnosis here is not precise and reliable even then the mortality rate of child and elderly people due to pneumococcal diseases is out of grip.

In this study, species-specific primer pair only detected any strain of *S. pneumoniae*. Furthermore, for rapid diagnosis purpose, PCR was able to detect *S. pneumoniae* both from enrichment broth aliquots after incubation with six throat swab samples and directly from those samples without using enrichment broth. According to published studies regarding diagnosis of pneumococcal diseases, such study in Italy [83], they detected *S. equisimilis* directly from equine nasopharyngeal swab samples. They prepared an enrichment broth called "T14" [phosphate buffered saline pH 7.2, 2% fetal calfserum (Celbio, Italy), 0.0005% amphotericin B (Bristol-Myers Squibb, Italy)] and in total the method took 10 to12 hours including six hours of enrichment to achieve the ultimate results.

In our study, the similar method was followed but in a modified form. The collected throat swab samples were enriched for about four hours instead of six hours and it took only 8 hours in total to perform the whole process. Moreover, the enrichment broth media consisted of Todd Hewitt broth and fetal bovine serum. Todd Hewitt broth was originally developed for the production of antigenic streptococcal hemolysin [93]. On the other hand, the semi solid STGG medium where the samples were stored, was used so that it can enhance recovery of pneumococci even at low organism concentrations [94].

Furthermore, when DNA extraction was performed directly from the samples without using any enrichment broth, it took only 6 hours to get the results. Bile soluble test was performed and results were positive for all throat swab samples. After nucleotide sequencing and subsequent BLASTn analysis, it was confirmed that designed primer pair could randomly detect *Streptococcus pneumoniae*. Though, the organism shares over 99% of 16S rRNA gene homology with other viridans group streptococci, such as *Streptococcus pseudopneumoniae*, *Streptococcus mitis*, and *Streptococcus oralis* [95], the query coverage, maximum score, identity and E-value appeared as best for *S. pneumoniae*.

According to the other published reports regarding the diagnosis of pneumococcal diseases, a specific gene of the any species of Streptococci was targeted and a primer-pair was designed according to that gene in order to detect certain species. Such study in Korea, they developed a quantitative real-time PCR assay targeting the capsular polysaccharide biosynthesis gene (cpsA) as species-specific detection tool for *S. pneumoniae* [96]. But neither they used any enrichment broth nor directly extracted DNA from the samples rather conventional culture based method was performed. Moreover, as they targeted a specific gene instead of 16S rRNA region, there is a possibility of high mutation rate of that gene but 16S rRNA genes are universally present in all bacteria, they have conserved, essential function, so they evolve slowly and number of random mutations can be correlated with the evolutionary distance between species [97]. Another study in Italy, they used real-time PCR and multiplex sequential PCR but bacterial genomic DNA was extracted from biological samples using the QIAmpDNeasy Blood & Tissue kit to improve the ability to diagnose IPD [98] which is time consuming.

However, this study gives us a ray of hope about the rapid diagnosis of pneumococcal diseases as the primer-pair showed highest specificity towards *Streptococcus pneumoniae*. Besides the whole procedure of the diagnosis can be performed within six to eight hours which is undoubtedly the most rapid way of diagnosis.

Despite having some strong points, this study has some limitations too. The number of the throat swab samples should be more. On the other hand, the primer pair should only detect *Streptococcus pneumoniae* and should not misidentify other species of Streptococci. In this case it would better if multiplex PCR was used. By this method, two or more unique target sequences can be amplified simultaneously so that one set of primers can able to amplify an internal control to verify the integrity of the PCR while the second set of primer is targeted to DNA sequence of interest.

Chapter: 5

Conclusion

Conventional culture method of diagnosis is an inexpensive but time-consuming though is considered as "Gold Standard" and there are certain limitations associated with it. Molecular methods have been increasingly incorporated in laboratories, particularly for the detection and characterization of isolates and for the diagnosis of diseases due to fastidious, slow growing, nonviable or non-cultivable organisms which cannot be detected by conventional culture techniques. The introduction of these techniques and their automation enables to provide appropriate treatment for the patient. These tests provide timely results which are useful for high-quality patient care at a reasonable cost and are associated with improvement in patients care. Moreover, molecular methods are rapid and highly sensitive and specific.

However, this study describes the specificity of designed primer pairs on 18 strains of *S. pneumoniae*. A rapid PCR protocol is developed for the detection and identification of *S. pneumoniae* both directly from the throat swab samples and from the same samples, pre-incubated in enrichment broth by amplification of a 16S rRNA region specific for the species. Best result appeared for the *S. pneumoniae* among other streptococci group organisms when nucleotide blastn was performed. Therefore, this method could be useful for rapid diagnosis and epidemiological purposes for the detection of *S. pneumoniae* from the samples.

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Appendices:

Appendix-I

Media composition

All the media was autoclaved at 121°C for 15 minutes. The composition of the media used in the present study has been given below:

Recipe for the preparation of STGG medium:

Name of the ingredients	Measurement
1. Skim milk powder (Difco, Detroit, Mich.)	2g
2. Oxoid tryptone soy broth	3g
3. Glucose	0.5g
4. Glycerol	10ml
5. Distilled water	100ml

Todd Hewitt broth (Sigma-Aldrich)

Ingredients	Amount (g/L)
Beef Heart Infusion	500.00
Peptic Digest of Animal Tissue	20.00
Dextrose	2.00
Sodium Chloride	2.00
Sodium Carbonate	2.50
Sodium Phosphate	0.40

Luria-Bertani Broth:

Ingredients	Amount (g/L)
Peptone (Himedia,India)	5.0
Yeast Extract (Himedia,India)	2.5
NaCl (Sigma, Germany)	5.0

Mueller Hinton Agar (Oxoid, England):

Ingredients	Amount (g/L)
Beef, dehydrated infusion form	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar (Himedia, India)	15.0

Appendix-ΙΙ: Buffers and Reagents

Tris Boric Acid EDTA (TBE) Buffer (500 ml):

To 500 ml distilled water, 5.4 g Tris HCL powder, 2.75 g boric acid and 0.5M EDTA of 2 mlwere dissolved. The pH of the buffer was adjusted to 8, autoclaved and stored at room temperature.

Phosphate Buffer Saline (PBS):

The composition along with amount is mentioned below:

1X PBS; 10 mMPhosphate (pH 7.2-7.4)

Reagents	For 100 ml
Sodium chloride(NaCl) (0.136M)	0.80
Di-sodium hydrogen Orthophosphate dodecahydrate $Na_2HPO_4.12H_2O$	0.137
Potassium phosphate monobasic (KH_2PO_4) (2mM)	0.0275
Potassium Chloride (KCl) (2.68mM)	0.02

Appendix-III

Instruments:

The equipment used throughout the study are listed below:

Instruments	Manufacturer
Autoclave	WiseClave
Refrigerator	Electra, Samsung (+4°C)- to store bacteria; Vestfrost (+4°C)- to store bacterial medium
Freeze	Vestfrost (-20°C) to store stock antibiotics; ESCO (-80°C) to store stock bacteria.
Incubator	Memmert
Shaking Incubator	WiseCube
Oven	WiseVen
Water bath	WiseBath
Micropipette	(2-20µl)- Gilson and Costar® (20-200µl)- Gilson and Costar® (200-1000µl)- Gilson
Bio-Safety Cabinet	ESCO Class-II Type-A2 Labculture® Biological Safety Cabinet
Vortex Mixture Machine	WiseMix
Weighing Machine	OHAUS®
Weighing Paper	Fisherbrand®
Spectrophotometer	Eon™ BioTek®
96-Well Plate	Nunc™ 96F Microwell Plate
Centrifuge Machine	Thermo SCIENTIFIC
Light Microscope	OLYMPUS CX41
T 100 ™ thermal cycler	Infinigen
Gel documentation machine	Bio-Rad
Take 3 plate	Bio-Tek
Antibiotic disks	Oxoid