Rapid identification of *Klebsiella pneumoniae* using PCR based method targeting 16S rRNA gene

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Submitted by

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DECLARATION OF AUTHENTICITY

I hereby humbly declare that this thesis entitled **Rapid identification of *Klebsiella pneumoniae* using PCR based method targeting 16S rRNA gene** submitted by the undersigned has been carried out at Institute for Developing Science and Health Initiatives (ideSHi), Mohakhali, Dhaka under the joint supervision of **Dr. Md. Kaiissar Mannoor** (Scientist and head, Institute for Developing Science and Health Initiatives, ideSHi) as an External Supervisor and **Ms. Namista Islam** (Lecturer, Department of Mathematics and Natural Sciences, BRAC University) as an Internal Supervisor.

The presented dissertation is based on original research work carried out by myself and has not been submitted to any other institution for any degree or diploma. Any reference to work done by any other person or institution or any material obtained from other sources have been accordingly cited and referenced.

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Dedicated to

My Beloved Family

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ABSTRACT

Background of the study: *Klebsiella pneumoniae* (KPN) is a gram negative and one of the most pathogenic bacteria of *Klebsiella* species which accounts for a significant proportion of pneumonia, nosocomial infections, urinary tract infections, intra-abdominal infections, soft tissue infections and septicemia. Current culture-based methods for identifying *Klebsiella pneumoniae* are time-consuming, not highly sensitive and sometimes inconclusive. The aim of the study is to establish a rapid and specific method for identification of *Klebsiella pneumoniae* using the 16S rRNA gene based PCR assay as a possible alternative of traditional phenotypic procedures. **Methods:** A primer set targeting the 16S rRNA gene of *Klebsiella pneumoniae* was designed using CLUSTALW and Primer-BLAST software. Culture on selective media and specific biochemical tests for *Klebsiella pneumoniae*, DNA extraction, PCR, gel electrophoresis and finally nucleotide sequencing was used in this study. Eleven stored clinical *Klebsiella pneumoniae* strains from laboratory stock were examined using the primer set in order to verify the efficiency of the primer. For rapid detection purpose, ten different specimens (wound swab, pus sample) collected in STGG media were examined. DNA was extracted twice from the same specimen; (i) directly from the media- without enrichment and (ii) after 4 hours incubation with enrichment media. **Results**: The designed primer set correctly detected all eleven KPN strains obtained from laboratory stock. Identification of *Klebsiella pneumoniae* in direct swab samples and brief cultivation in enrichment broth required ~4h and ~8h respectively after sample reaches to the laboratory to diagnosis. Similar results were obtained from the KPN strains collected from laboratory stock and the strains that were used for rapid detection (collected from wound, pus swab). All the strains amplified a 657bp product of the 16S rRNA gene of KPN as well as suggesting 99% identity with the 16S r RNA gene of *Klebsiella pneumoniae* by Sanger Sequencing and BLASTn analysis.

Conclusion: The study demonstrates a simple yet useful PCR-based approach for identification of *Klebsiella pneumoniae* that appeared to be more sensitive, specific and resulted in significant time saving in comparison to traditional phenotypic based methods and can serve as a beneficial tool for the rapid detection of *Klebsiella pneumoniae* before the infection can progress to acute phase.

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Abbreviations:

KPN	*Klebsiella pneumoniae*
rRNA	Ribosomal ribonucleic acid
WHO	World Health Organization
CAP	Community acquired pneumonia
HCAP	Health care-associated pneumonia
CDC	Center for Disease Control and Prevention
FBS	Fetal bovine serum
BUHS	Bangladesh University of Health Sciences
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	Ethylene diamine tetraacetic 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. INTRODUCTION:

1.1 Background

1.1.1 Clinical significance of *Klebsiella pneumoniae*

Klebsiella pneumoniae is a common Gram-negative, non-motile, encapsulated, facultative anaerobic bacillus belonging to the family Enterobacteriaceae. *Klebsiella pneumoniae*, also called Friedländer's bacillus, was first described by Friedländer (1882) from the lungs of a patient who had died of pneumonia [1].

The genus *Klebsiella* was named by Trevisan (1885) to honour the German microbiologist, Theodor Albrecht Edwin Klebs (1834–1913).Pathogenic *Klebsiella* strains belonging to *Klebsiella pneumoniae*subsp. *Pneumonie* are medically most important *Klebsiella* species[2].

K. pneumoniae infections are frequently preceded by gastrointestinal colonization and the gastrointestinal tract is believed to be the most important reservoir for transmission of the bacteria.A number of virulence factors have been suggested in *K. pneumoniae* , including the prominent polysaccharide capsule expressed by the vast majority of clinical isolates as well as different adhesins, lipopolysaccharide (LPS) and iron-scavenging proteins[3, 4]

1.1.1.1 Infectious Syndromes

Klebsielleae organisms cause a wide range of clinical syndromes in humans; including community acquired pneumonia and nosocomial pneumonia (hospital-acquired, ventilator-associated, and health care–associated), urinary tract infection, wound infection, nosocomial infection, abdominal infection, soft tissue infection, surgical site infection, intravascular device infection, bacteraemia and life-threatening septic shock.

Pneumonia

Klebsiella pneumoniae has been known primarily as a pathogen causing community-acquired pneumonia and nosocomial pneumonia (hospital-acquired, ventilator-associated, and healthcare–associated).Community acquired pneumonia (CAP) mainly

affects hosts with underlying conditions (e.g., alcoholism, diabetes, or chronic lung disease). Pulmonary infection is especially common among residents of LTCFs (Long term care facilities) and hospitalized patients because of increased rates of oropharyngeal colonization. Mechanical ventilation is an important risk factor [5].

Urinary tract infection (UTI)

Klebsiella pneumoniae is a common cause of urinary tract infections (UTIs) especially in immunocompromised individuals.

Abdominal infection

Klebsiella pneumoniae causes a spectrum of abdominal infections similar to that caused by *E. coli* but is less frequently isolated from these infections.

Other infections

As an opportunistic pathogen, *Klebsiella* primarily attacks immunocompromised individuals who are hospitalized and have severe underlying diseases. It is estimated that *Klebsiella* species cause 8% of all hospital-acquired infections. *Klebsiella* cellulitis or soft tissue infection most frequently affects devitalized tissue (e.g., decubitus and diabetic ulcers, burn sites) and immunocompromised hosts. *Klebsiella* causes some cases of surgical site infection, hematogenously derived endophthalmitis (especially in association with hepatic abscess), and nosocomial sinusitis[5, 6].

Bacteremia

Klebsiella infection at any site can produce bacteremia. Bloodstream infections caused by this organism may lead to severe sepsis and septic shock that result in high mortality. Patients with intravenous cannulae, temporary pacing wires and those receiving chronic haemodialysis are particularly susceptible[7].

1.1.2Epidemiology

Klebsiella species are broadly prevalent in a variety of environmental sources: such as soil, vegetation, water and colonize on the mucosal surfaces of mammals. In healthy humans, the prevalence of *K. pneumonie* colonization is 5–35% in the colon and 1–5% in the oropharynx; the skin is usually colonized only transiently[8]. It becomes more prevalent in hospital patients, particularly if they have received antibiotics. It is

believed to reach the lungs by aspiration from the oropharynx and certain groups of patients are particularly predisposed. These include alcoholics, diabetics, those who have been endotracheally intubated and those who are elderly or otherwise debilitated by disease[7].

1.1.3Incidence

According to the reports from Centers for Disease Control and Prevention (CDC), pneumonia was the eighth-leading cause of death in 2006. About 15 million children worldwide die each year as a consequence of acute respiratory infections, one-third of them from pneumonia, and 96% of these deaths occur in developing countries [7]. In 2006, The World Health Organization (WHO), reported pneumonia as the leading killer of children under the age of five worldwide. Though *K. pneumoniae* causes fewer than 10% of CAPs, but more than 20% of nosocomial pneumonias.Alcohol abuse is the most common underlying condition for community-acquired *K. pneumoniae* pneumonia. Other underlying conditions that predispose to *Klebsiella* infections are diabetes mellitus and COPD (Chronic Obstructive Pulmonary Disease). *Klebsiella* ranks next to *Escherichia coli*, accounting for 8% of endemic hospital infections and 3% of epidemic outbreaks [2]. *K. pneumoniae* accounts for only 1–2% of UTI episodes among otherwise healthy adults but for 5–17% of episodes of complicated UTI, including infections associated with indwelling urinary catheters. *Klebsiella pneumoniae* is the second most common cause of Gram-negative bloodstream infections**.** Infections of the urinary tract, respiratory tract, and abdomen (especially hepatic abscess) each account for 15–30% of episodes of *Klebsiella* bacteremia. Intravascular device–related infections account for another 5–15% of episodes, and surgical site and miscellaneous infections account for the rest [8, 9].

1.2 Clinical Manifestations of *Klebsiella* pneumonia

The invasive nature of *K. pneumoniae* strains appears to correlate with an extreme "stickiness" of these colonies on agar plates: this is known as the hypermucoviscosity phenotype. In *Klebsiella pneumoniae* CAP, a syndrome of pleuritic chest pain, hemoptysis, and bloody sputum (occasionally with a "currant jelly" appearance) is classic but rarely seen. A classic radiographic appearance of *Klebsiella* pneumonia is upper lobe consolidation (especially on the right) with a bulging or bowed fissure.

Klebsiella pneumoniae can also cause lung abscess in patients with HCAP. Pulmonary necrosis, pleural effusion, and empyema can occur with disease progression[7, 9].

1.3 Microbiologic Assay

Conventional methods that allow the detection of *Klebsiella pneumoniae* include microscopic observation, gram staining, traditional culture based techniques, biochemical tests, immunological assays etc.

MICROSCOPY

Microscopic examination remains the most commonly requested microbiological investigation in suspected *Klebsiella pneumoniae* specimens. However, a major limitation of microscopic examination is that it cannot distinguish between infection, colonization, and contamination when the specimen is collected through the oropharynx. Additionally, microscopy lacks sensitivity in specimens with less than 104 CFU per millilitre. Laboratory examination of expectorated sputum is routinely carried out and the adequacy of an expectorated sample may be judged microscopically in terms of the number of buccal epithelial cells present [7, 9].

GRAM'S STAIN

The Gram-stained smear is an essential and necessary part of evaluation of sputum and tracheal aspirates for determining the quality and acceptability of specimens for bacterial culture. The Gram stain may be difficult to interpret as *Klebsiella spp.* due to the presence of oropharyngeal commensals in lower respiratory tract. The application of routine Gram staining to sputum is an area of controversy, some experienced clinicians using it, others not.

CULTURE

Blood culture: It is normal practice to carry out blood culture on all patients admitted to hospital with suspected pneumonia since a positive culture may occur in 10–30% of cases.

Microbiologic culture of respiratory specimens allows definitive identification of the suspected pathogens and permits determination of bacterial susceptibility to antimicrobial agents. Because many pathogens of the lower respiratory tract are also

members of the oropharyngeal flora, culture results must be correlated with the Gram stain findings, including the presence or absence of polymorphonuclear leukocytes[7, 9].

SEROLOGIC TESTING

The cause of *Klebsiella pneumoniae* infections can be suggested by detection and quantitation of humoral (e.g., antibody) responses to bacteria. The serologic methods commonly used in diagnostic laboratories include enzyme immunoassay, immunoprecipitation, immunodiffusion (ID), complement fixation (CF), immunoblotting (including Western blot), agglutination, hemagglutination inhibition, and indirect immunofluorescence assay. The problems often associated with serological detection assays are reduced reproducibility, sensitivity and specificity with high cost.

BIOCHEMICAL TESTS

K. pneumoniae can be identified by Analytical profile index system (API) (BioMérieux, Inc.) or by traditional biochemical tests, and the whole process requires at least 24 to 48 hours.

1.4 Overview of the study

Klebsiella pneumoniae is an important opportunistic pathogen, which has been reported worldwide and the treatments of *Klebsiella pneumoniae* infections are often complicated.

Laboratory identification of *Klebsiella pnuemoniae* on the basis of gram staining, cultures, microscopic observations and a battery of biochemical tests have their own limitations. These traditional laboratory based methods are time-consuming with low sensitivity, usually requiring several days of incubation. In addition to that, accurate identification at species level of the genus may not be possible in all cases because some of the species share similar biochemical profiles. Although, immunological assays have been proved potentially useful tool for the identification of *Klebsiella* infections, they often show extensive cross reactions with low sensitivity while some organisms can be serologically similar[10, 11].

Therefore, a rapid and sensitive molecular method for the detection of *Klebsiella pneumoniae* is required to control the outbreak of this serious pathogen.

Bacterial identification by Polymerase Chain Reaction (PCR) can provide significant advantages over phenotypic-based methods, including rapid turnaround time, scalability, and high sensitivity. Microbial identification on the basis of 16S rRNA gene sequencing is widely used because the 16S rRNA gene is present in all bacteria. Public databases such as GenBank, the Nucleotide Sequence Database at the European Molecular Biology Laboratory (EMBL-Bank), the DNA Data Bank of Japan (DDBJ) and the Ribosomal Database Project II (RDP II) contain a vast number of bacterial 16S rRNA gene sequences, allowing rapid analysis and providing phylogenetically meaningful information[3, 12, 13].

1.5Objectives of the study

The work presented in this thesis aims to overcome the limitations of conventional laboratory procedures and to establish a specific molecular method. In this study, a simple PCR assay is described with specific primers targeting the 16S rRNA gene for the prompt detection of *K. pneumoniae* from clinical samples. This is the first study at DNA level to specifically detect *Klebsiella pneumoniae* based on 16S rRNA gene in Bangladesh. The core objectives of this research can be summarized as follows:

- To find out a better investigation tool alternative to established phenotypic systems, which can be used to detect *Klebsiella pneumoniae*
- Rapid and specific detection of *Klebsiella pneumoniae* using PCR technique
- Sequencing of 16S rRNA gene to confirm species type.

Chapter 2

Literature Review

2. LITERATURE REVIEW

Klebsiella pneumoniae is one of the important human pathogens in clinical microbiology. Conventional phenotypic-based methods for identifying this organism are time consuming with low sensitivity hence; the need arises for reliable and quick molecular detection methods for the rapid diagnosis of *Klebsiella pneumoniae* infections. Different studies had been carried out to successfully and rapidly detect *Klebsiella neumoniae* based on genotypic methods and in many cases, the researches depended on PCR-mediated amplification of the 16S rRNA gene because the 16S rRNA gene is present in all bacteria. According to [Relman, (1999)], the 16S rRNA gene consists of highly conserved nucleotide sequences, interspersed with variable regions that are genus- or species-specific. PCR primers targeting the conserved regions of rRNA amplify variable sequences of the rRNA gene.

[Kurupati, Prathiba, et al. 2004] stated that the rapid identification of *K. pneumoniae* directly from growth-positive blood can be obtained by real-time PCR aiming the 16s rRNA gene. The experiments, thus demonstrating 100% specificity and 100% sensitivity.

[Jenkins, Ling.,etal.2012] discussed the detection and identification of bacteria in clinical samples amplifying either a single fragment or two fragments of the 16S rRNA gene**.** The PCR results were further compared to culture-based methods where PCR results were found to be more specific. Thus they concluded that, 16S rRNA PCR assays has the potential to make an important contribution to patient management by detecting the presence of bacterial pathogens in culture-negative clinical samples.

[Clifford, Robert J., et al.2012] conducted a study where they detected *Klebsiella pneumoniae*along with other three clinically relevant bacteria pointing the 16S rRNA gene by Real time PCR. Four sets of primers were designed that were capable of positive identification in plate or broth cultures in less than 90 minutes. Therefore, the assay was proved to be rapid, cost-effective and accurate for detecting clinically important bacteria.

[Wang, Min, et al. 2008] performed a different study in which they analysed the 16S–23S rRNA Gene Internal Transcribed Spacer Region in *Klebsiella* species.The phylogenetic analysis based on ITS regions revealed the relationships among *Klebsiella* species similarly to that based on 16S rRNA genes.

[Dong, Derong, et al.2015] established a loop mediated isothermal amplification (LAMP) method for the rapid detection of *Klebsiella pneumoniae* targeting the rcsA gene from clinical samples. This method could rapidly identify *Klebsiella pneumoniae* that could be useful tool in clinical screening.

[Hartman, Laurie J., et al. 2009] examined the *rmpA* and *magA* gene of *Klebsiella pneumonia* that are associated with Hypermucoviscosity Phenotype by establishing a rapid Real-time PCR technique.

[Lu, Jang-Jih. 2000] et al designed a universal PCR capable of amplifying a portion of the 16S rRNA gene of *Klebsiella pneumoniae* and other related organisms. They identified the bacteria based on the size of the amplified PCR products as well as the restriction endonuclease digestion patterns.The results of their investigation, suggested that universal PCR coupled with restriction enzyme analysis can be used to detect and identify bacterial pathogens in clinical specimens.

Chapter 3

Methodology

CHAPTER 3: METHODOLOGY

3.1 Place of study

This study was conducted at Institute for developing Science and Health initiatives (ideSHi). Ethical approval for the study was obtained from Bangladesh Medical Research Council (BMRC).

3.2 Period of study

This study was carried out from January 2017 to September 2017.

3.3 Microbiological culture

A total 23 clinical samples were examined in this study.

Eleven clinical strains that were known to be positive controls for *Klebsiella pneumoniae* preserved in -70°C freezer as glycerol stock were used. These nasal swab samples were collected randomly from the paediatric ward of Dhaka Medical College Hospital (DMCH) and Shaheed Suhrawardy Medical College Hospital (SSMCH) for AERI (Acute Respiratory and Enteric Infections) study of ideSHi laboratory.

Additionally, another twelve human clinical isolates were collected from diverse sources including wound swab, throat swab and pus samples from BUHS (Bangladesh University of Health Sciences, Mirpur, Dhaka) were used for direct DNA extraction and DNA extraction from enrichment broth for the rapid detection of *Klebsiella pneumoiae.*

3.4 Sample collection and storage

After performing hand hygiene and wearing clean gloves, samples were collected using autoclaved, sterile cotton swabs. During wound and pus sample collection, the deepest part of the wound should be sampled, avoiding the superficial microflora. Specimens should be obtained before antimicrobial therapy where possible. The swabs were carefully placed in sterile test tubes for transport and stored in STGG media at -70°C storage.

3.5 Flow diagram of the study design

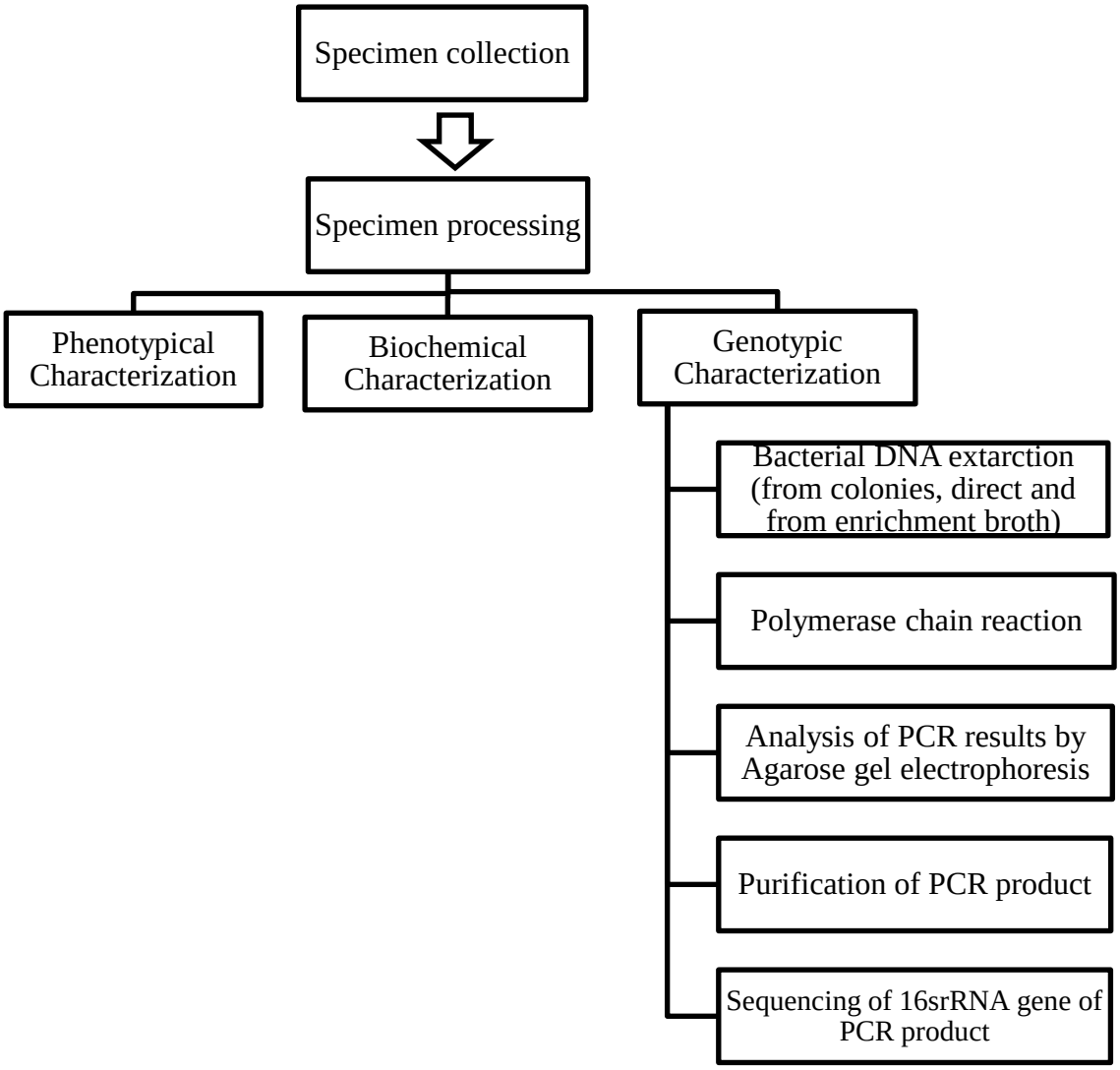


Figure 3.1: Flow chart of study plan

3.6 Conventional microbiological methods

Isolates obtained from ideSHi repository and BUHS had undergone culture on relevant selective media, had been further sub-cultured, and were identified by routine biochemical tests and an API kit based on Clinical and Laboratory Standards Institute guidelines.

3.6.1 Gram stain

Gram staining is a procedure which is used to differentiate between gram positive and gram negative organisms; hence it is differential stain. Gram positive and gram negative cells can be easily differentiated on the basis of the structure of cell wall. In Gram staining procedure, as a result of having thick peptidoglycan layers, Gram positive bacteria retain the crystal violet dye, while Gram negative bacteria shows a pink/ red colour of safranin, a counterstain added after the crystal violet.

Using sterile technique, a drop of saline was placed on the slide and a small amount of the bacteria were then transferred to the drop of saline with a sterile cooled inoculating loop. A smear was then prepared by mixing and spreading the bacteria by means of a circular motion of the loop. The smear was then allowed to air dry followed by heat fixation. The fixed smear was flooded with crystal violet solution and allowed to remain for 1 minute. The crystal violet was rinsed off with distilled water. The slide was flooded with grams iodine solution mainly mordant and allowed to remain for one minute. After that, the smear was decolorized with 95% ethyl alcohol and gently washed with distilled water or tap water. Finally, it was counterstained with safranin for 45 seconds and gently washed with distilled water or tap water. The slide was then blot dried with bibulous paper and examined under oil immersion [14].

3.6.2 Selective Media:

Much of the study of microorganisms depends on its ability to grow in the laboratory, and this is possible only if suitable culture media are available for the growth of microorganism. A culture medium is defined as a solid or liquid preparation used for the growth, transport, and storage of microorganisms. The effective culture medium must contain all the nutrients required for the growth of the microorganism. Selective media allows the growth of certain type of organisms, while inhibiting the growth of

other organisms. These media contain antimicrobials, dyes, or alcohol to inhibit the growth of the organisms not targeted for study.

For the identification and confirmation of *Klebsiella pneumoniae* from the collected samples, two selective media were used- MacConkey Agar (MAC) and Eosin Methylene Blue (EMB) Agar. The colony morphology and the cultural characteristics of the inoculated samples on these two media ensured the confirmation of *Klebsiella pneumoniae.*

3.6.2.1MacConkey Agar (MAC)

MacConkey Agar is a selective and differential culture media commonly used for the isolation of enteric Gram-negative bacteria. It is based on the bile salt-neutral red-lactose agar of MacConkey. Crystal violet and bile salts in incorporated in MacConkey Agar to prevent the growth of gram-positive bacteria and fastidious gram-negative bacteria, such as *Neisseria* and *Pasteurella*. Gram-negative enteric bacteria can tolerate to bile salt because of their bile-resistant outer membrane. MacConkey Agar is selective for Gram negative organisms, and helps to differentiate lactose fermenting gram negative rods from Non lactose fermenting gram negative rods. It is primarily used for detection and isolation of members of family Enterobacteriaceae and *Pseudomonas* spp. On MAC, *Klebsiella* spp produce pink mucoid colonies.

3.6.2.2 Eosin Methylene Blue (EMB) Agar

EMB agar is a both selective and differential culture medium. It is selective culture medium for gram-negative bacteria (selects against gram positive bacteria) and is commonly used for the isolation and differentiation of coliforms and fecal coliforms. EMB media assists in visual distinction Enterobacteriaceae, other non-pathogenic lactose-fermenting enteric gram-negative rods, and the *Salmonella* and *Shigella* genera. The combination of the two dyes eosin and methylene blue inhibits most Gram positive bacteria but allows many Gram negative organisms to grow. Gram negative bacteria that ferment the lactose produce acid which turns the colonies dark purple as the acid acts upon the dyes. In addition, certain lactose-fermenting bacteria produce flat, dark colonies with a green metallic sheen. Other lactose fermenters produce larger, mucoid colonies, often purple only in their centre. On EMB agar, *Klebsiella* spp. produces large, mucoid pink to purple colonies. *E.coli* colonies have a

characteristic green sheen. Lactose non-fermenters are either colourless or light lavender.

3.6.3Biochemical characterization of the bacteria

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology laboratory manual instructed by (Cappuccino and Sherman 2005). The biochemical tests performed were; Triple sugar iron agar test (TSI), IMViC test (Indole production test, Methyl red test, Voges-Proskauer test, Citrate utilization test), Urease test, Nitrate reduction test, Catalase test, Oxidase test and Motility test.

3.6.3.1 Triple Sugar Iron Agar test

Triple sugar iron (TSI) agar is a tubed differential medium used in determining carbohydrate fermentation and H2S production. Gas from carbohydrate metabolism can also be detected. Bacteria can metabolize carbohydrates aerobically (with oxygen) or fementatively (without oxygen). TSI differentiates bacteria based on their fermentation of lactose, glucose and sucrose and on the production of hydrogen sulphide.

Triple sugar iron slants were prepared in the test tubes and autoclaved at 15 psi 121◦C. The inoculating needle was sterilized in the blue flame of the Bunsen burner till it turned into red hot and then allowed to cool. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes by means of a stab and streak inoculation method. The tubes were incubated for 24 hours at 37◦C [14].

3.6.3.2Indole Production test

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. It is used as part of the IMViC procedures, a battery of tests designed to distinguish among members of the family Enterobacteriaceae. The presence of indole when a microbe is grown in a medium rich in tryptophan demonstrates that an organism has the capacity to degrade tryptophan. Detection of indole, a by-product of tryptophan metabolism, relies upon the chemical reaction

between indole and p-dimethylaminobenzaldehyde (DMAB) under acidic conditions to produce the red dye rosindole.

Tryptophan broth of 5 ml in each test tube was prepared by autoclaving at 15 psi, 121◦C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes and the tubes were incubated for 48 hours at 37◦C. Following incubation, five drops of Kovac's reagent were added. Then the colour of the cultures was examined and the results were recorded. Formation of a rose red ring at the top of the liquid surface indicates a positive result. A negative result can have a yellow or brown layer [15].

3.6.3.3 Methyl red test

This test determines whether the microbe performs mixed acids fermentation when supplied glucose. Mixed acids fermentation results in accumulation of a variety of acids and a significant drop in the pH of the medium. In the mixed acid fermentation pathway, glucose is fermented and produces several organic acids (lactic, acetic, succinic, and formic acids). After adding reagent, if the pH indicator (methyl red) is added to an aliquot of the culture broth and the pH is below 4.4, a red colour will appear. If the MR turns yellow (or no colour change), the pH is above 6.0 and the mixed acid fermentation pathway has not been utilized.

Using sterile technique, bacterial isolates were inoculated into 5 ml of dextrose phosphate broth (MR-VP broth) and incubated at 37^0C for 48 hours. After 48 hour incubation, 5 drops of methyl red reagent was added directly into the medium and the pH was tested[14].

3.6.3.4VogesProskauer test

This test is used to identify bacteria capable of 2, 3butanediol fermentation following mixed-acid fermentation. Many enteric organisms can overcome the buffering capacity of the media by producing large quantities of a stable acid end product, thus lowering the pH. Some organisms do not produce stable acid end products and instead further metabolize acids to more neutral end products like 2,3 butanediol. The production of 2, 3-butanediol is thus indirectly detected when the pathway intermediate acetoin reacts with reagents to turn cherry red. The catalyst here is alpha-napthyol and guanidine group. The addition of 40% KOH and a 5% solution of alpha-naphthol in

absolute ethanol (Barritt's reagent) will detect the presence of acetoin—and it represents positive result where no colour change indicates negative.

Experimental samples were inoculated into 5 ml of dextrose phosphate broth and incubated at 37^0C for 48 hours. After incubation, 10 drops of Barritt's reagent A was added, immediately followed by 10 more drops of Barritt's B reagent and the cultures were shaken. The cultures were then kept aside for 15 minutes for the reaction to occur. After 15 minutes, the colour of the cultures was examined and the results were recorded [14].

3.6.3.5 Citrate utilization test

Citrate utilization test is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source. In organisms capable of utilizing citrate as a carbon source, the enzyme citrasehydrolyzes citrate into oxaoloacetic acid and acetic acid. The oxaloacetic acid is then hydrolyzed into pyruvic acid and carbon dioxide. If carbon dioxide is produced, it reacts with components of the medium to produce an alkaline compound (e.g. sodium carbonate). The alkaline pH turns the pH indicator (bromthymol blue) from forest green to Prussian blue.

Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the test tubes by means of a streak inoculation method with an inoculating needle and the tubes were incubated for 48 hours at 37◦C [14].

3.5.3.6 MIU (Motility- Indole- Urease) test

MIU Medium Base is used to detect motility, urease and indole production in single tube. Casein enzymatic hydrolysate provides amino acids and other nitrogenous substances. Sodium chloride maintains osmotic equilibrium. Dextrose is fermentable carbohydrate. Phenol red is the pH indicator which turns pink- red in alkaline conditions. Motility and urease reactions are read before testing Indole production. Motile organisms show either diffused growth or turbidity extending away from stab inoculation line while non-motile organisms grow along the stab line. Organisms that utilize urea produce ammonia which makes the medium alkaline, showing pink-red colour by change in the phenol red indicator. Indole is produced from tryptophan present in Casein enzymatic hydrolysates. The indole produced combines with the aldehyde present in the Kovac's reagent to form a red complex.

Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes by means of a stab inoculation method with an inoculating needle and the tubes were then incubated for 24 hours at 37◦C [16].

3.6.3.7 Nitrate reduction test

This test determines whether the microbe produces the enzymes nitrate reductase and nitrite reductase. The two enzymes catalyze two reactions involved in converting starting compound nitrate into end product nitrogen gas. If a bacterium producing nitrate reductase is grown in a medium containing nitrate, the enzyme converts the nitrate to nitrite. Nitrite reacts with certain chemicals to yield a red-coloured product. If the bacterium also produces nitrite reductase, nitrogen gas will be liberated. Bubbles collecting in an inverted Durham tube indicate that nitrogen has been produced [17].

Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 24 to 48 hours at 37◦C[14].

3.6.3.8 Catalase test

The catalase test facilitates the detection of the enzyme catalase in bacteria. The function of this enzyme is to detoxify hydrogen peroxide (H_2O_2), which is formed from the superoxide radical by superoxide dismutase. The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen.

A drop of hydrogen peroxide was taken on an autoclaved glass slide.A single colony of experimental bacteria was placed on the reagent drop and the result was observed immediately. Rapid production of O_2 bubbles indicates positive result; the absence of bubble formation is a negative catalase test.

3.6.3.9 Oxidase test

The oxidase test determines whether a microbe can oxidize certain aromatic amines, for example, p –aminodimethylaniline oxalate, to form coloured end products. When present, the cytochrome c oxidase oxidizes the reagent to purple colour end product. When the enzyme is not present, the reagent remains reduced and is colourless.

One or two drops of oxidase reagent (p- aminodimethylaniline oxalate) were added on a filter paper. Using a sterile wooden tooth pick, small amount of experimental bacteria was placed on the reagent. A development of pink, maroon, and violet to purple and finally black coloration within a few seconds indicate positive result. No colour change, or a light pink colouration on the colonies, is indicative of the absence of oxidase activity [14].

3.6.3.10 Analytical Profile Index (API 20Efrom bioMerieux, Inc.)

The Analytical Profile Index (API) system is one of the simplified test kits used for the identification of bacteria. Three API kits are available for identifying Enterobacteria-a screening kit of 10tests (10S),a basic set of 20 tests (20E) and for further characterization of an organism a kit of 50 tests (50E). The 20E kit contains all the tests of the 10S but only a few tests are in both the 20E and 50E. The individual tests consist of dehydrated chemicals in a set of plastic cupules (moulded to a strip of plastic) which are inoculated with a bacterial suspension. The development of this system of cupules has been described by Janin (1977).The inoculated whole API® 20E test kit strips were incubated overnight at 37°C in an aerobic incubator. After the incubation period, test results are used to construct a 7-digits profile. Using this profile, the identity of the bacterium was derived from the database with the relevant cumulative profile code book or software [18].

TESTS	SUBSTRATE	REACTION TESTED	NEGATIVE RESULTS	POSITIVE RESULTS
ONPG	ONPG	beta-galactosidase	colorless	yellow
ADH	arginine	arginine dihydrolase	yellow	red/orange
LDC	lysine	lysine decarboxylase	yellow	red/orange
ODC	ornithine	ornithine decarboxylase	yellow	red/orange
CIT	citrate	citrate utilization	pale green/yellow	blue-green/blue
H2S	Na thiosulfate	H2S production	colorless/gray	black deposit
URE	urea	urea hydrolysis	yellow	red/orange
TDA	tryptophan	deaminase	yellow	brown-red
IND	tryptophan	indole production	yellow	red (2 min.)
VP	Na pyruvate	acetoin production	colorless	pink/red(10 min.)
GEL	charcoal gelatin	gelatinase	No diffusion of black	black diffuse
GLU	glucose	fermentation/oxidation	blue/blue-green	yellow
MAN	mannitol	fermentation/oxidation	blue/blue-green	yellow
INO	inositol	fermentation/oxidation	blue/blue-green	yellow
SOR	sorbitol	fermentation/oxidation	blue/blue-green	yellow
RHA	rhamnose	fermentation/oxidation	blue/blue-green	yellow
SAC	sucrose	fermentation/oxidation	blue/blue-green	yellow
MEL	melibiose	fermentation/oxidation	blue/blue-green	yellow
AMY	amygdalin	fermentation/oxidation	blue/blue-green	yellow
ARA	arabinose	fermentation/oxidation	blue/blue-green	yellow
OX	oxidase	oxidase	colorless/yellow	violet

Table 3.1: API 20E (bioMerieux,Inc.) Reading Table

3.7 DNA Extraction and template preparation for PCR assays

DNA for PCR was extracted in three different ways using boiling method. DNA from the eleven known *K. pneumoniae* strains obtained from ideSHi repository was extracted from colonies cultured on agar media. The DNA of the rest ten clinical samples were extracted directly from the samples and from enrichment broth for rapid identification of *Klebsiella pneumonie.* The supernatant of the bacterial solution, after lysing by boiling were used for PCR.

3.7.1 DNA Extraction from colonies grown on agar media:

Two colonies of overnight growth bacteria were used. The colonies were put in a 1.5 ml microcentrifuge tube containing 100µl autoclaved PBS (Phosphate buffer saline) using a sterile platinum loop. The suspension was then vortexed for 15 seconds until homogenization. After that, the suspension was allowed to heat at 95-99°C in a Water Bath (WiseBath, USA) for 10 minutes to release the DNA. Tubes were then immediately transferred into ice, kept for one minute and centrifuged at 13,000 rpm (rotation per minute) for 10 minutes. The pellet was discarded and supernatant was transferred into another RNase and DNase free microcentrifuge tube. The supernatant contains the template DNA which was ready to use as PCR template.

3.7.2 DNA Extraction from Enrichment broth:

Eleven separate enrichment broths were prepared by adding800µl Tood Hewitt media and 200µl FBS (Fetal bovine serum) in Cryo vials (Sigma-Aldrich). Then, 40µl STGG media containing the original samples collected from diverse sources (wound swab, pus, sputum, nasal swab) were added to each separate enrichment broth. The samples were incubated for 4 hours at 37^0C in aerobic condition. Following incubation, the suspensions were centrifuged at 12,000 rpm for 10 minutes; the cell pellet was collected and dissolved in 500 µl NaCl (0.9%). Next, 50 µl of suspensions were transferred into a microcentrifuge tube for microscopic observation to detect the viability of the organism and rest of the dissolved pellet was centrifuged at 20,000 g for 1 minutes. Afterward, the suspension were boiled for ten minutes, kept in ice for 1 minute and then were centrifuged at 13,000 rpm for 10 minutes. The supernatant contains the DNA and transferred into 1.5 ml DNA/RNS free microcentrifuge tube and stored at -20°C for future use.

3.7.2.1 Preparation of Enrichment Broth:

Enrichment Broth was prepared by adding 800µlTood Hewitt media and 200µl Fetal Bovine Serum (FBS) in a Cryo Vial.

TODD HEWITT BROTH (Enrichment broth): Todd Hewitt Broth is a liquid medium recommended for use in qualitative procedures for cultivation and isolation of most pathogenic organisms (most specifically used for beta-hemolytic

streptococci).Peptones and heart infusion supply nitrogenous compounds and amino acids essential for the growth of organism and prevent the formation of proteinase; type-specific M protein production is inhibited by proteinase. Dextrose is an energy source. Disodium phosphate and sodium carbonate are buffers which counteract the acidity produced during the fermentation of dextrose.

FETAL BOVINE SERUM (FBS): Fetal bovine serum (FBS) is a ubiquitously used essential supplement in cell culture media. FBS is a cocktail of proteins, vitamins, carbohydrates, lipids, hormones, growth factors, minerals and trace elements and is used as an universal growth supplement effective for most types of human and animal (including insect) cells.

3.7.3. Direct DNA Extraction:

200 µl of STGG media containing the original samples collected from diverse sources (wound swab, pus, sputum, nasal swab) were centrifuged at a high speed (14,000 rpm) to separate the cells. The pellet was collected and dissolved in 200 µl PBS and boiled for 10 minutes and then kept in ice for 1 minute. The cell debris was removed by centrifugation at 13,000 rpm for 10 minutes, and the supernatant was saved for PCR.

3.8 Polymerase chain reaction

3.8.1 Principle

Polymerase Chain Reaction (PCR) is a key technique in molecular genetics that permits the analysis of any short sequence of DNA. It is used to amplify specific regions of a DNA strand that lies between the known sequences. The PCR is commonly carried out in a reaction volume of 15-100 µl in small reaction tubes (0.2-0.5 ml volumes) in a thermal cycler. The thermal cycler allows heating and cooling of the reaction tubes to control the temperature required at each reaction step. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase, which the method is named after, are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA

template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase (an enzyme originally isolated from the bacterium *Thermus aquaticus*). This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample through a defined series of temperature steps.

The templates DNA are first denatured by heating in the presence of the two oligonucleotides and the four dNTPs. The reaction mixtures are then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerase. The cycle of denaturation, annealing and DNA synthesis is then repeated for 30-40 cycles, each successive cycle essentially doubles the amount of the DNA product[19].

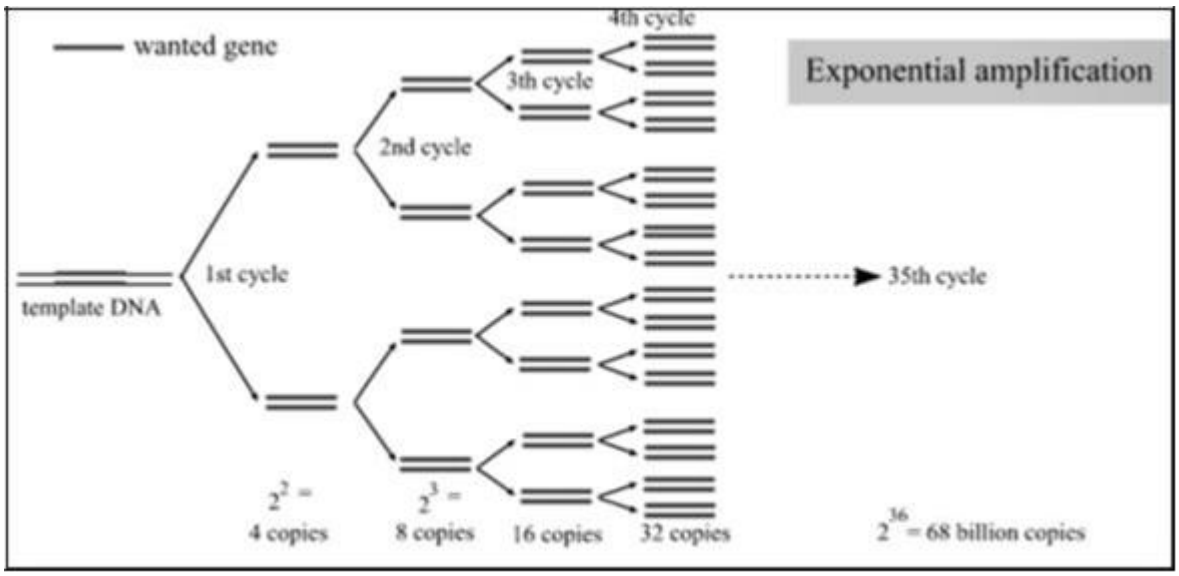


Figure 3.2: Polymerase chain reaction (Andy Vierstraete, 1999)

3.8.2 Equipment and Supplies for polymerase chain reaction

3.8.2.1 Equipment

- PCR machine
- Electrophoresis machine
- Pipettes
- Micro-centrifuge tubes
- PCR cabinet
- Refrigerators (-20°C, +4°C)

3.8.2.2 Reagents, Master Mix and Thermal Cycle Profile

The reagents that were used for polymerase chain reaction are given along with their storage temperature in Table 3.2. PCR Master Mix and the Thermal cycle profile are given in Table 3.3 and in Table 3.4 respectively

Table 3.2: Reagents used for PCR and their storage temperature

Reagents	**Temperature**
10X Buffer with MgCl2	-20^{0}C
2.5 mMdNTPs	-20^{0}C
Primer (Forward and reverse)	-20^{0}C
Taq polymerase (rTaq)	-20^{0}C
Q-solution (Qiagen)	-20^{0}C
Template DNA	-20^{0}C
Nuclease-free water	Room temperature

Table 3.3: PCR Master Mix

PCR Reagents	**Amount**
10X buffer with MgCl2	1.0 µl
2.5 mMdNTPs	1.0 µl
Q solution	1.0µl
Forward primer (NM3_F)	0.5µl
Reverse primer (NM3-R)	0.5µl
Taq polymerase	0.1µl

Nuclease free water	4.9µl
Template DNA	1.0 µl
Total volume	**10 µl**

Table 3.4: The Thermal Cycle

PCR condition	Temperature	Time	
Initial denaturation	94°C	3minutes	
Denaturation	94°C	30 seconds	**35 Cycles**
Annealing	58°C	30 seconds	
Elongation	72°C	30 seconds	
Final elongation	72°C	10 minutes	
	10°C	∞	

3.8.2.3 Starting materials

Isolated DNA from experimental bacterial sample was used as starting material.

3.8.3 Procedure

3.8.3.1 Primer designing

All database searching was done through the website of the National Center for Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov/. All published 16S rRNA gene sequences for all *Klebsielleae* (and closely related organisms) were retrieved from NCBI. The most representative 16S rRNA sequences were determined for each species. Such a database of representative sequences was used to align closely related *Klebsiella* species to detect specific positions in the 16S rRNA gene for the identification of *Klebsiella pneumoaiae*[20].Multiple sequence alignment was done by using CLUSTALW software. (http://www.genome.jp/tools-bin/clustalw).

Figure 3.3: Multiple sequence alignment result.

ClustalW generated the multiple sequence alignment needed to find the conserved sequences in *Klebsiella* and closely-related species. A particular portion of *Klebsiella* sequence (highlighted in figure 3.3) was selected in order to design forward primer that is different from all other bacterial species.

Subsequently, the specificity of the designed primers was confirmed by further analysis using the software Primer-BLAST, which compares nucleotide sequences against user-selected database to avoid primer pairs causing non-specific binding and amplifications[21].

Primer-BLAST URL: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

This software combines BLAST with a global alignment algorithm to ensure a full primer-target alignment and is sensitive enough to detect targets that have a significant number of mismatches to primers. Primer-BLAST allows users to design new target-specific primers in one step as well as to check the specificity of pre-existing primers. Primer-BLAST also supports placing primers based on exon/intron locations and excluding single nucleotide polymorphism (SNP) sites in primers.

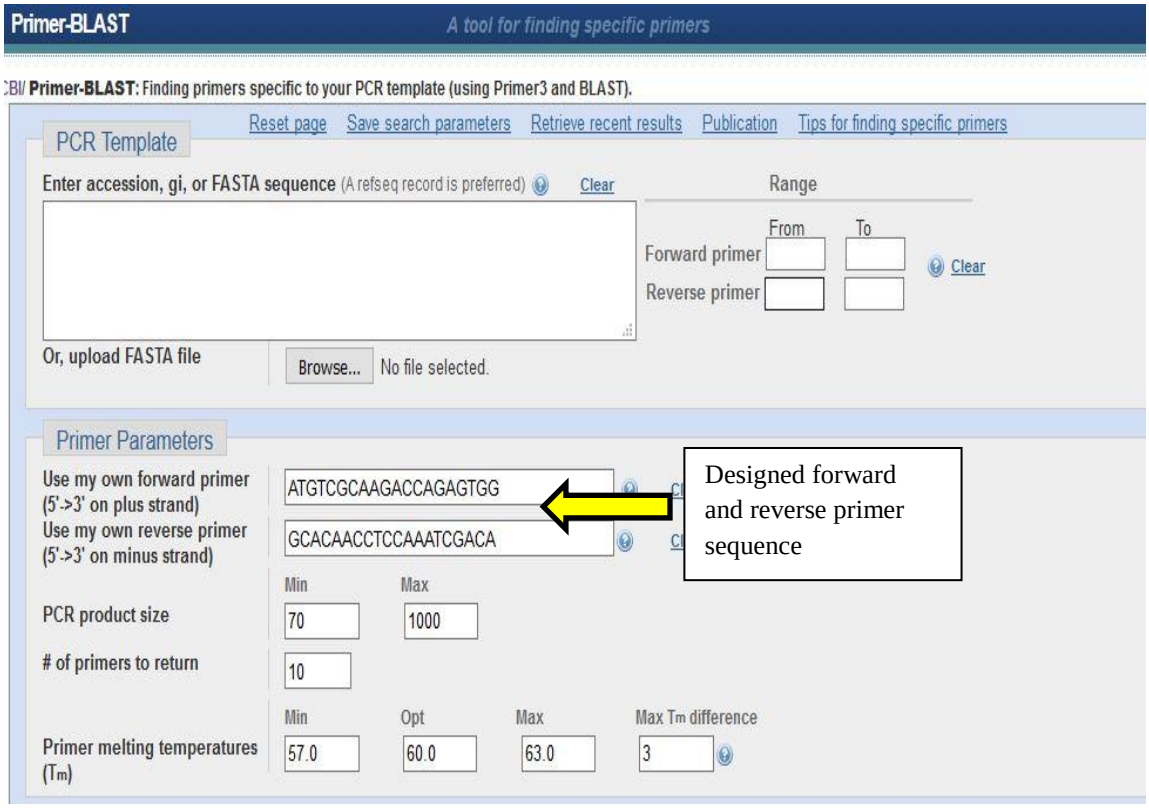


Figure 3.4: Designed primer sequence was entered into primer-blast software.

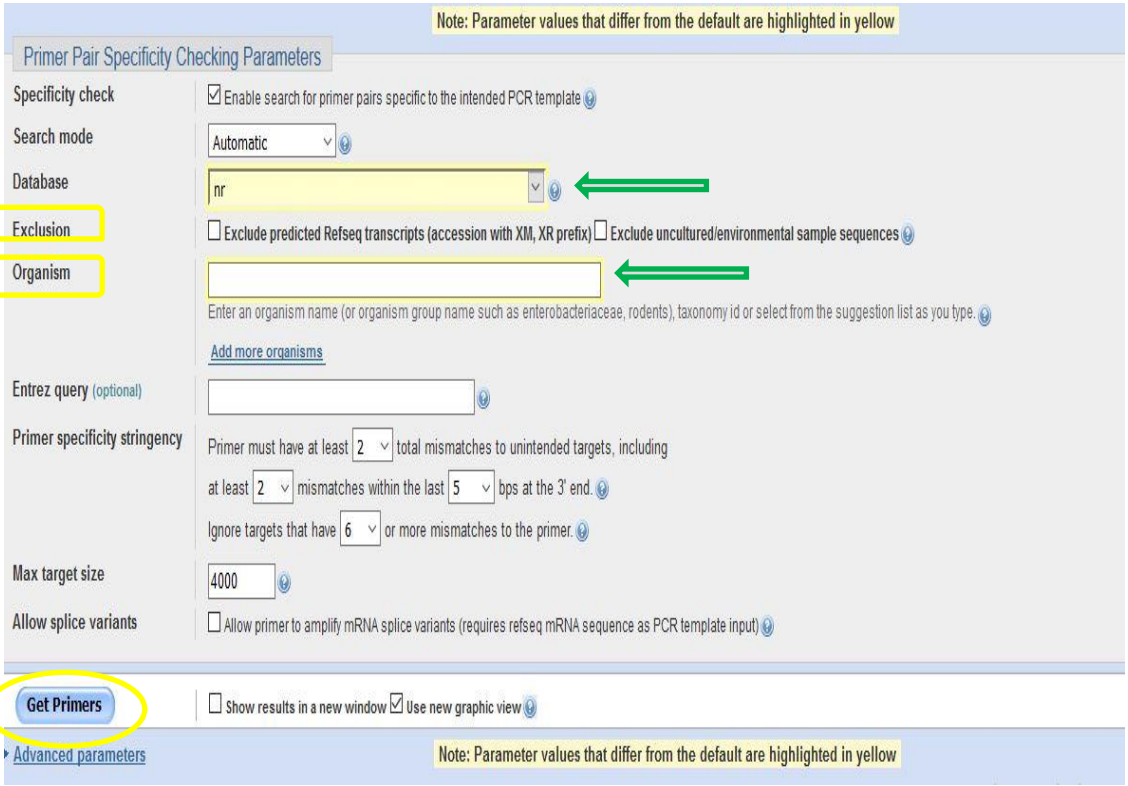


Figure 3.5: Parameters were carefully selected to get desired results

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ATGTCGCAAGACCAGAGTGG	20	60.04	55.00	3.00	3.00
Reverse primer	GCACAACCTCCAAATCGACA	20	58.77	50.00	4.00	0.00

Products on target templates

>MF678855.1 Klebsiella pneumoniae strain Y90 16S ribosomal RNA gene, partial sequence

```
product length = 658
Forward primer  1    ATGTCGCAAGACCAGAGTGG  20
Template        151  ..............A.....  170

Reverse primer  1    GCACAACCTCCAAATCGACA  20
Template        808  ....................  789
```

>MF973087.1 Klebsiella pneumoniae strain KC-PI-HB1 16S ribosomal RNA gene, partial sequence

```
product length = 658
Forward primer  1    ATGTCGCAAGACCAGAGTGG  20
Template        170  ..............A.....  189

Reverse primer  1    GCACAACCTCCAAATCGACA  20
Template        827  ....................  808
```

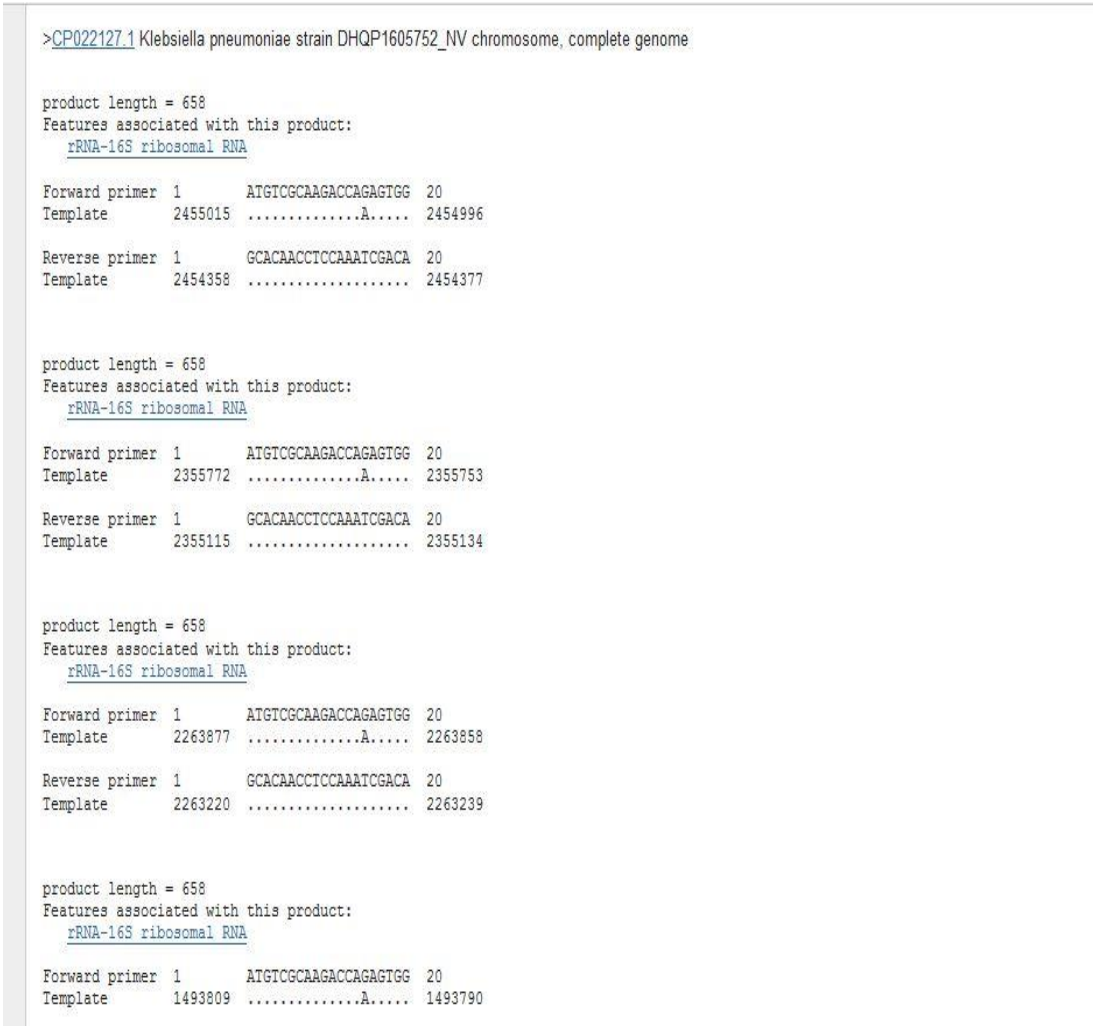
>CP022573.1 Klebsiella pneumoniae strain BIC-1, complete genome

```
product length = 658
Features associated with this product:
   succinate-semialdehyde dehydrogenase

   rRNA-16S ribosomal RNA

Forward primer  1        ATGTCGCAAGACCAGAGTGG  20
Template        3135659  ..............A.....  3135678

Reverse primer  1        GCACAACCTCCAAATCGACA  20
Template        3136316  ....................  3136297
```



>CP022127.1 Klebsiella pneumoniae strain DHQP1605752_NV chromosome, complete genome

product length = 658
Features associated with this product:
rRNA-16S ribosomal RNA

Forward primer	1	ATGTCGCAAGACCAGAGTGG	20
Template	2455015	.....A.....	2454996
Reverse primer	1	GCACAACCTCCAAATCGACA	20
Template	2454358	.....	2454377

product length = 658
Features associated with this product:
rRNA-16S ribosomal RNA

Forward primer	1	ATGTCGCAAGACCAGAGTGG	20
Template	2355772	.....A.....	2355753
Reverse primer	1	GCACAACCTCCAAATCGACA	20
Template	2355115	.....	2355134

product length = 658
Features associated with this product:
rRNA-16S ribosomal RNA

Forward primer	1	ATGTCGCAAGACCAGAGTGG	20
Template	2263877	.....A.....	2263858
Reverse primer	1	GCACAACCTCCAAATCGACA	20
Template	2263220	.....	2263239

product length = 658
Features associated with this product:
rRNA-16S ribosomal RNA

Forward primer	1	ATGTCGCAAGACCAGAGTGG	20
Template	1493809	.....A.....	1493790

Figure 3.6-3.7: The specificity of the designed primers was confirmed

Figure 3.4-3.7: Shows the results obtained from Primer-BLAST tool of NCBI. The designed primer (named as KP_16S_NM3) showed good specificity among all primers analysed, generating alignments exclusively against distinct sequences of *Klebsiella pneumoniae* species and no matches were found with other bacterial species.

Table 3.5: Primers specific to 16srRNA gene of *Klebsiella pneumoniae* along with sequence, product length, melting temperature (Tm) and guanine-cytosine content (GC %)

Primer name	Primer sequence (5'-> 3')	GC content (%)	Product size	Tm [°C]
KP_16S F1 (NM3_F)	ATGTCGCAAGACCAGAGTGG	55.0	657	60.04
KP_16S R1 (NM3_R)	GCACAACCTCCAAATCGACA	50.0		58.77

3.8.3.2 PCR optimization

PCR optimization is a vital step in order to detect high efficiency PCR product and to avoid primer dimer formation as well as non-specific binding. To measure the success of PCR amplification, in this study, at first the DNA templates from positive controls along with other closely related bacterial species were amplified. Following amplification, the PCR products were run on a1% or 2% agarose gel and visualized by staining with GelRed showing desired results.

3.9 Agarose gel electrophoresis

3.9.1 Principle

The results of PCR experiments are checked by running a portion of the amplified reaction mixture in an agarose gel.An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool. At neutral or alkaline pH, the phosphate groups of DNA or PCR products give rise to a uniform negative charge. Agarose gel electrophoresis involves the use of a buffer to maintain the constant states of ionization since any change in pH would alter the charge on the DNA molecules to be separated or visualized. TBE buffer can be used as it gives good resolution and sharpband plus no microbes can grow if kept for a long time but at times the formation of precipitation can be its drawback. During electrophoresis, the gel is submersed in a

chamber containing a buffer solution and a positive and negative electrode. The DNA to be analysed is forced through the pores of the gel by the electrical current. Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black) through the pores in the gel. Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. The velocity of movement is inversely proportional to the molecular weight of the DNA molecule. Therefore, the largest molecules will have most difficulty passing through the pores, whereas the smallest molecules are relatively unhindered, moving the fastest. DNA itself is not visible within an agarose gel. The DNA will be visualized by the use of a dye that binds to DNA. Usually, 1% (w/v) gel is prepared for visualization of DNA fragments.

3.9.2 Equipment and reagents

Equipment

- Conical Flask
- Electric Balance
- Falcon tube
- Micropipettes
- Gel Electrophoresis Apparatus
- Gel Doc^{TM} XR^{+}(BioRad, USA)

Reagents

- Agarose powder
- 1X TBE buffer
- Gel Red (Intercalating nucleic acid fluorescent dye)
- Bromophenol Blue (Loading dye)
- DNA ladder (Marker DNA solution)

3.9.3 Procedure

Preparation of 1X TBE buffer

10X TBE buffer (pH-8.0) was prepared dissolving 108 g Tris-HCl (100 mM), 40 mL EDTA (0.5 M, pH-8.0) and 55 g Boric acid and then adjusting the final volume to 1 Liter by adding deionized water. It was stored at room temperature. To prepare 1X TBE buffer, 100 µL of 10X TBE buffer was mixed with 900 µL deionized water. It was stored at room temperature.

Preparation of gel mix

For making 1% (w/v) agarose gel, 0.5 g agarose powder was measured in the digital balance and 50 mL 1X TBE buffer was measured in a Falcon tube. Agarose powder was dissolved into 1X TBE buffer in a 250 mL conical flask and heated for about 2 minutes in the microwave. Then the molten agarose gel was allowed to cool at room temperature. 1.0 µL of GelRed was added, and the flask was swirled to mix. The melted agarose gel was poured carefully into the gel tray that had a comb fixed in place near one end so that loading wells could be formed in the gel. Then the gel was allowed to solidify by placing it at the room temperature for at least about 15-20 minutes. After the gel had solidified, the comb was removed gently, and the gel tray was placed within the electrophoresis tank. The tank was filled with 1X TBE buffer to submerge the gel. 2µL of 6X Loading Dye, bromophenol blue (dark blue color), was added to 5 volumes of amplified PCR product on a small piece of Parafilm and mixed by pipetting up and down before loading into the gel wells by micro-pipetting. To estimate the size of the PCR products, 1 µL of 1 kb Plus DNA ladder (Cat. No. 10787018, InvitrogenTM) that had a broad size range (75bp - 20kb) and a storage temperature of 4^0C was mixed with 2µL of bromophenol blue followed by loading it in a well at one end of the gel along with the experimental sample DNA in the other wells. Then the gel was subjected to electrophoresis. After running the gel at 150 V for 1 hour, the bands of PCR products were subsequently visualized using the Gel DocTM XR+ (BioRad, USA).

Precaution

While pouring the melted gel mix solution into the gel tray, care was taken so that no bubbles were formed.

3.10 PCR Purification

3.10.1 Principle

MinElute® PCR purification kit can be used to purify 5 µg PCR products (70 bp – 4 kb) using the column-based method. When sample is loaded on a spin column, dsDNA binds to the column. The adsorption of DNA to the membrane is efficient only in acidic condition at a pH < 7.5 that is maintained by the addition of binding buffer. An optional pH indicator added to the binding buffer allows easy determination of the optimal pH for DNA binding to the spin column. The binding buffer contains chaotropic salts, which disrupts protein structure by destabilizing hydrophobic interactions and removes all the DNA-binding proteins (such as - polymerase) and other contaminants. The high salt also decreases the negative charge on the DNA, allowing stronger interactions with the column membrane. Ethanol is added to the washing buffer addition of which efficiently removes dNTPs, primer dimers (which are usually around 70bp), salt, buffer or anything except dsDNA from the column. Centrifugation is done for complete removal of the residual ethanol from buffer PE. Finally, the neutral pH and higher affinity of nuclease-free water for dsDNA is utilized to elute dsDNA or PCR products from the column. All centrifugation steps are carried out at 17,900 x g (13000 rpm) in a conventional micro-centrifuge at room temperature (15-25oC, usually 21oC) (Source: Instruction manual for MinElute® PCR Purification Kit). Figure 3.8 shows the steps for purification of PCR products (Figure reprinted from www.bioneer.com.au).

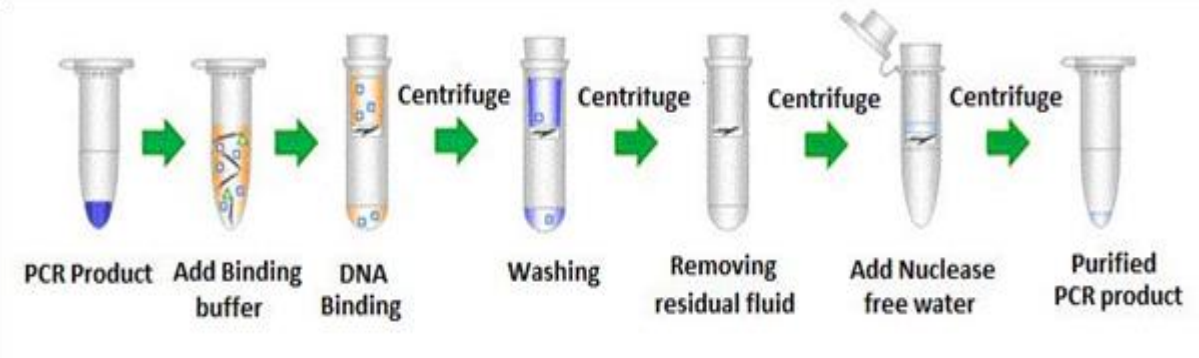


Fig 3.8: Steps involving PCR products purification

3.10.2 Equipment and reagents for purification of PCR products

Equipment

- Biosafety cabinet (Level II)
- Micro-centrifuge
- Water bath
- Spectrophotometer or EON ELISA reader (BioTek, USA)
- Refrigerator (-70^{o}C)

Materials

- MinElute Column
- 1 mL Pipettes
- 20-200 µL Pipettes
- 1-20 µL Pipettes
- Take3 plate (BioTek, USA)

Reagents

- MinElute® PCR Purification Kit (Cat. No. 28004 and 28006, QIAGEN, Germany)
  - Buffer PB
  - Buffer PE
- 3 M Sodium acetate (pH=5.0)
- Nuclease-free water

All reagents were stored according to the instructions on the label, given in Table 3.6.

Table 3.6: Storage temperature of Reagents and Materials used for PCR Purification

Name of Reagent and Material	Storage temperature
MinElute PCR Purification Kit	Room Temperature (15-25^{o}C)
Spin Columns	2-8^{o}C

3.10.3 Procedure

Before starting purification procedure, all the PCR products were spun and then transferred to separate micro centrifuge tubes. The next step involves adding 1:250 volume pH indicator-I to the Buffer PB. It should be noted that, pH indicator I was added to the entire buffer contents, not to buffer aliquots. Afterward, ethanol (96-100%) was added to Buffer PE concentrate before usein appropriate volume labelled on the bottle.

Now the main steps began by adding five volumes of Buffer PB to 1 volume of the PCR products and mixed properly. It was ensured so that the color of the mixture was yellow (similar to Buffer PB). If the color of the mixture was orange/violet, 10 µL 3 M sodium acetate (pH 5.0) was added and mixed well by pipetting up and then inverting. The mixture was transferred to a MinElute Column that was placed in a 2 mL collection tube. The preparation was allowed to stand at room temperature for 5 minutes so that it could pass through the column properly. Then it was centrifuged at 17,900 × g for 1 min. The flow-through was discarded, and the MinElute Column was placed back into the same collection tube. 750 µL Buffer PE was used to wash the column. The tube was allowed to stand at room temperature for 5 minutes and then centrifuged for 1 min. The flow-through was discarded and the column was placed back into the same collection tube followed by another centrifugation for another 1 min. Each column was then placed in a new 1.5 mL micro-centrifuge tube. Depending on the amount of PCR products, nuclease-free water was applied to the center of the column membrane for complete elution of the bound DNA and was allowed to stand at room temperature for 5 mins and then centrifuged at 17,900 x g for 1 min. Then the column was discarded as the purified PCR products were in the flow-through. The amount and purity of the dsDNA were measured using Take3 plate and spectrophotometer. The purified template were stored in the refrigerator (-70°C) before downstream analysis.

3.11. Measurement of DNA concentration and purity

DNA concentration was measured using NanoDrop™ 2000 spectrophotometer (Thermo Scientific™). Approximately 2µl of autoclaved filtered PBS was used as blank, 2µl of DNA sample was loaded onto the optical measurement surface and optical density (OD) of the sample was estimated spectrophotometrically. DNA concentration was measured at 260nmin ng/µl unit, where they have the highest absorbance. DNA purity was also evaluated by the ratio of absorbance at 260nm to 280nm.

3.12Sequencing

3.12.1 Principle

DNA sequencing enables us to perform a thorough analysis of DNA because it provides us with the most basic information of all the sequence of nucleotides. Sanger's method, which is also referred to as dideoxy sequencing or chain termination, first devised by Fred Sanger and colleagues in the mid-1970s.The key principle of the Sanger method was the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators. This process takes advantage of the ability of DNA polymerase to incorporate 2´, 3´-dideoxynucleotides—nucleotide base analogs that lack the 3´-hydroxyl group essential in phosphodiester bond formation

Applied Biosystems (ThermpFisher Scientific) fluorescence-based cycle sequencing system is an extension and refinement of Sanger dideoxy sequencing. Applied Biosystems automated DNA sequencing generally follows this flow:

1. Template preparation
2. Cycle sequencing
3. Purification after cycle sequencing
4. Capillary electrophoresis
5. Data analysis

Fluorescence-based automated cycle sequencing entails a DNA template, a sequencing primer (forward or reverse), a thermal stable DNA polymerase, deoxynucleotide triphosphate (dNTPs), sequencing buffer and fluorescent dye-labeled 2′,3′-

dideoxynucleotide triphosphates (ddNTPs). All the components are mixed and subjected to cycles of denaturation, annealing, and extension in a thermal cycler. DNA polymerase incorporates either a dNTP (A, C, G, or T) or the corresponding ddNTPs (nucleotide base analogs that lack the 3′-hydroxyl group essential for phosphodiester bond formation) at each step of chain extension depending on the relative concentration of both molecules. When a dNTP is added to the 3′ end, chain extension is continued. However, when a ddNTP terminator (either ddA, ddC, ddG, or ddT each tagged with a different fluorescent dye) is added to the 3´ end, chain elongation is terminated, forming labeled extension products of various lengths. Figure 3.9 shows the general overview of dye terminator cycle sequencing that was retrieved from www.appliedbiosystems.com.

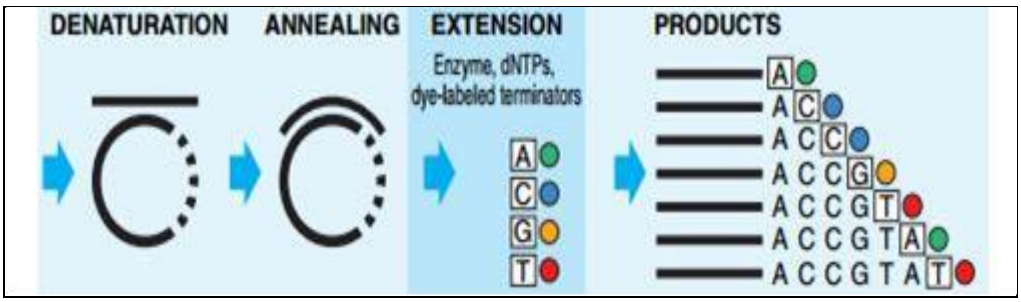


Figure 3.9: Diagram of dye terminator cycle sequencing.

For automated separation and analysis of fluorescently labeled DNA fragments, the products are subjected to capillary electrophoresis that involves a denaturing polymer. It has largely replaced the polyacrylamide gel electrophoresis technique due to significant gains in workflow, throughput, and ease of use as there is no need to pour gels between two glass plates with capillary electrophoresis and more samples can be processed at once. A high voltage charge is applied to the buffered sequencing reaction that forces the negatively charged fragments into the capillaries (Applied Biosystems, 2009). Shortly before reaching the positive electrode, the fluorescently labelled DNA fragments, separated by molecular weight or size, move across the path of a laser beam. When excited by the laser beam, each fluorescent dye on the fragments emits light at a unique wavelength, and all four colours and therefore, all four bases, can be detected in one capillary injection using an optical detection device. Finally, the fluorescence signal is converted to digital data using Data Collection Software.

BigDye Terminators version 3.1 is formulated with dITP in place of dGTP to reduce peak compressions. They utilize a single dye molecule for efficient transfer of energy. A tremendously efficient energy transfer linker combines an energy donor dye (fluorescein) and an energy acceptor dye (dichlororhodamine).

3.12.2 Reagents and apparatus for sequencing

Apparatus

- Applied Biosystems 310 Genetic Analyzer
  - No. of capillaries: 1
  - Capillary Array Length: 47 or 61 cm
  - Sample capacity: 48 or 96 sample tubes
- Cycle sequencing machine Mastercycler® gradient)
- Centrifuge machine
- 8-tube PCR strip

Reagents

The reagents needed for sequencing are given along with their storage temperature in Table 3.7.

Table 3.7: Reagents needed for sequencing and their storage temperature

Reagents	Storage temperature
Sequencing buffer	4^oC
BigDye® Chain Terminator v3.1 Ready Reaction (RR) mix	-20^oC
Forward primer or Reverse primer	-20^oC
SAM solution (Applied Biosystems, USA)	4^oC
X-terminator solution (Applied Biosystems, USA)	4^oC

3.12.3 Cycle Sequencing Master-mix Preparation

Purified PCR products for all deficient samples were sent to Institute of Epidemiology, Disease Control, and Research (IEDCR), Dhaka, Bangladesh. Direct sequencing of specific regions of PCR products was done using BigDye Chain Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI PRISM 310 automated sequencer (Applied Biosystems, USA) according to

manufacturers' instruction. For each sample, a master-mix was prepared in an Eppendorf tube and mixed by vortexing. For each sample, 2.0 µL of 5X sequencing buffer and 0.5 µL of BigDye® chain terminator version 3.1 Ready Reaction mix and 0.3 µL of individual primer (forward or reverse) was added to an 8-tube PCR strip (Table 3.8).

Table 3.8: Reagents and their amounts used for Cycle Sequencing Master-mix Preparation.

Reagent	**Concentration**	**Amount of single reaction (µL)**
Sequencing buffer	5X	2
BigDye® Chain Terminator v3.1 RR mix	2.5X	0.5
Forward primer or Reverse primer	0.2 µM	0.3

The tubes containing the template were spun, and 10-20 ng/µL (depending on the band size and density) 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 vortexed and centrifuged at 4000 rpm for 3 minutes. Then the PCR strip was placed in the cycle sequencing machine Mastercycler® gradient (Cat. No. 4095-0015, USA Scientific) Thermal Cycler and subjected to following thermal cycling profile: pre-denaturation at 94oC for 1 minute; 25 cycles of denaturation at 94°C for 10 seconds, annealing at 58°C for 5 seconds and extension at 60°C for 4 minutes; and a final extension at 60°C for 10 minutes (Table 3.9)

Table 3.9: Cycle sequencing thermal condition cycle

Stage	Step	Temperature (^{O}C)	Duration	Cycle
I	1 (Initial denaturation)	94	1 min	
II	1 (Denaturation)	94	10 s	25
	2 (Annealing)	58	5 s	
	3 (Extension)	60	4 min	
	Final extension	60	10 min	
III	1 (Hold)	4	Hold	

After the completion of cycle sequencing, products were centrifuged for 2 mins at 4100g. Purification of cycle sequencing products were performed by mixing with 45 µL of SAM solution (Applied Biosystems, USA) and 10 µL of X-terminator solution (Applied Biosystems, USA) in an automated shaker for 30 minutes. 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, the narrow outer end of the micropipette tips was cut a little to make the opening a little wider. If any particulate was present in SAM solution, it was heated at 37^{o}C, mixed and cooled to room temperature before using it. The PCR strip was vortexed for 30 mins continuously (1500-2500 rpm). Following centrifugation at 4000 X g for 2minutes, 10 µL supernatants from each tube were transferred to a new 0.2 mL PCR tube strip. The tubes were subjected to capillary electrophoresis through (denaturing) POP-6^{TM} polymer in an automated sequencing machine ABI PRISM® 310 Genetic Analyzer (Sequencing by Sanger Method).

3.12.4 Sequence analysis

Sequencing data were analysed by ChromasLite 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

Chapter 4

Results

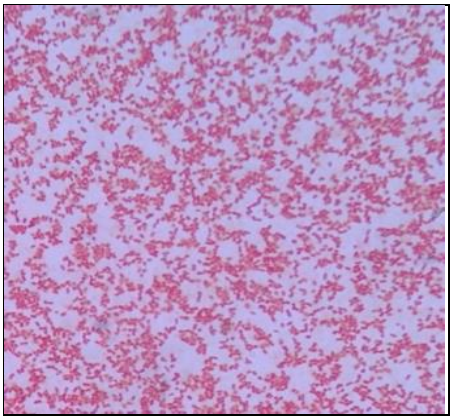
4. Results

4.1. Conformation of the strains by culture and biochemical tests

All the 21 clinical bacterial strains were reconfirmed using several laboratory tests.

4.1.1Gram staining results:

The experimental bacteria showed the pink colour of safranin stain suggesting that it was Gram negative rod as shown in Figure 4.3.



Cell wall type: Gram Negative

Shape: Rod Shaped (Bacillus)

Figure 4.1: Cell morphology was observed under microscopic oil emersion lens

4.1.2 Cultural characteristics on selective media

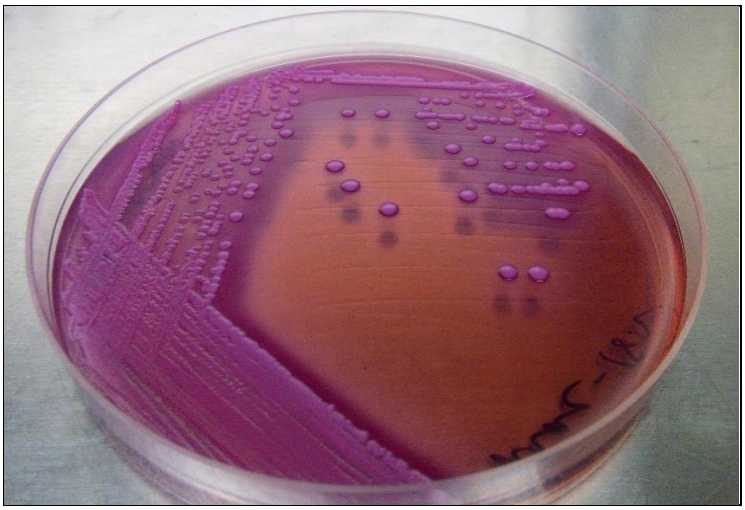


Figure 4.2: After 24 hours, MacConkey agar culture plate inoculated with experimental strains showed pink colonies with viscous/mucoid appearance.

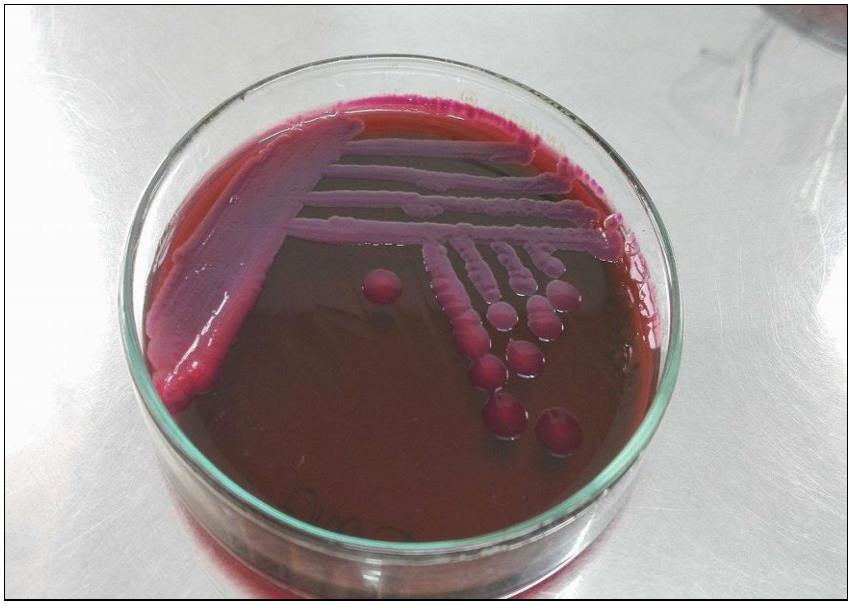


Figure 4.3: Purple and characteristic mucoid colonies were observed on EMB agar following 24 hours incubation.

Table 4.1: Colony Morphology on MacConkey and Eosin Methylene Blue (EMB) Agar

Agar Plate	Bacterial Colony Morphology						
	Size	**Form**	**Elevation**	**Margin**	**Colour**	**Texture**	**Appearance**
MacConkey	Medium	Circular	Convex	Entire	Pink	Mucoid	Shiny
EMB	Large	Circular	Umbonate	Entire	Purple	Mucoid	Glossy

4.1.3Biochemical Identification:

Experimental bacterial strains were subjected to several Biochemical Tests and all gave standard results of *Klebsiella pneumoniae*.The results are as follows:

Table 4.2 Biochemical Identification Results

IMVic				**Nitrate Reduction Test**	**Catalase Test**	**Oxidase Test**	**MIU Test**		**TSI (Triple Sugar Iron Test)**				
Indole Test	**Methyl Red Test**	**Voges-Proskaur Test**	**Citrate Utilization Test**				**Motility**	**Urease**	**Slant**	**Butt**	**Carbohydrate Fermentation**	**H_2S**	**CO_2 or Gas**
(-)	(-)	(+)	(+)	(+)	(+)	(-)	(-)	(+)	Yellow Acidic	Yellow Acidic	(+)	(-)	(+)

Here, (+) indicates positive result and (-) indicates negative test result

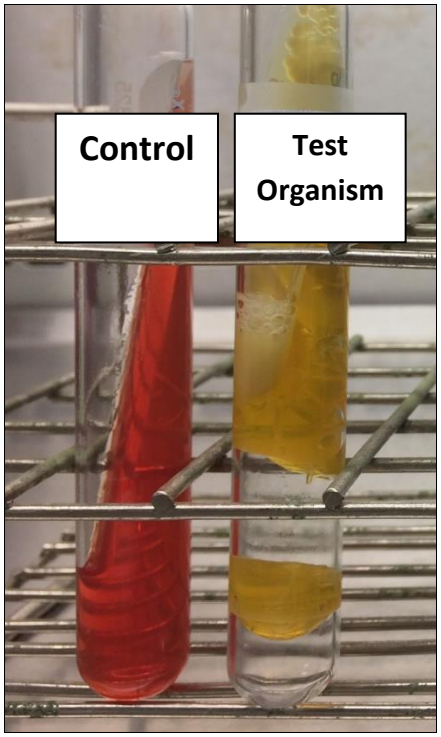


Figure 4.4: Result of Triple Sugar Iron Agar Test

Acidic slant and acidic butt (Yellow/yellow) indicating glucose, lactose and/or sucrose fermenter. Production of bubbles and cracks suggests that the organism generates gas.

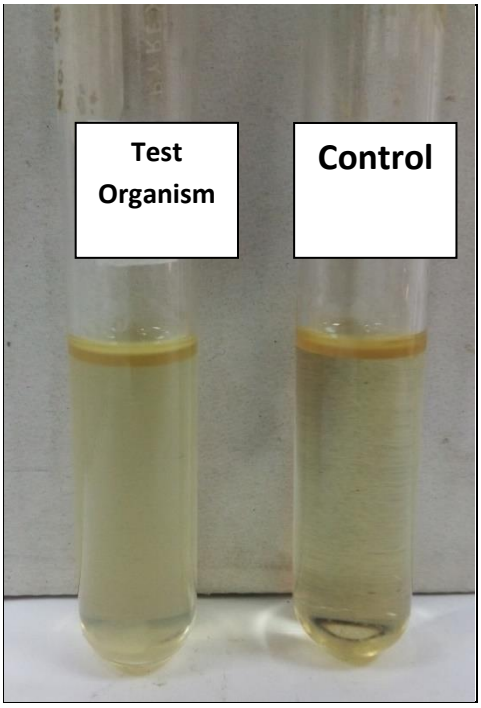


Figure 4.5: Organism showed indole negative result by retaining the original colour of media.

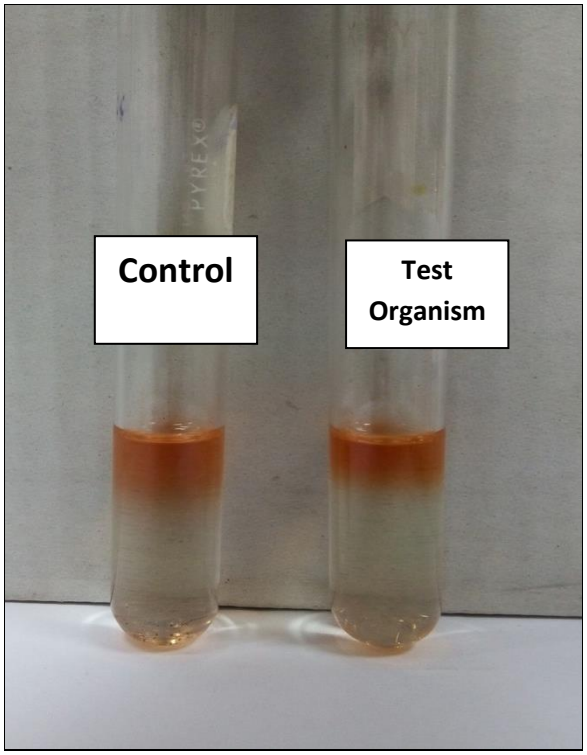


Figure 4.6: Methyl red negative result was observed indicating that the organism is unable to utilize the mixed acid fermentation pathway

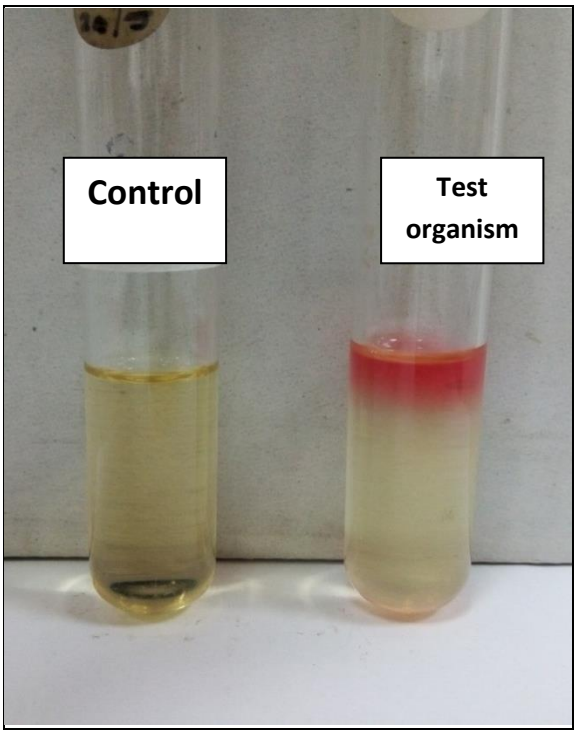


Figure 4.7: Formation of cherry-red colour indicates Voges-Proskauer positive result

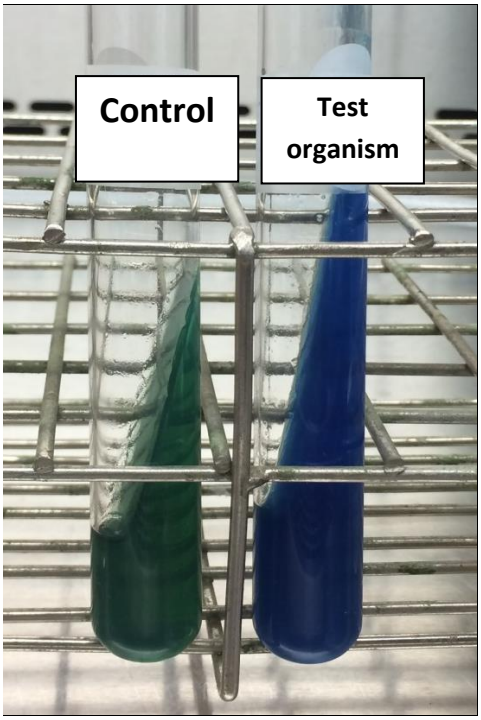


Figure 4.8: Result of Citrate utilization test.

Growth is visible on the slant surface and the medium turned an intense Prussian blue indicating the organism can utilize citrate as the sole carbon and energy source.

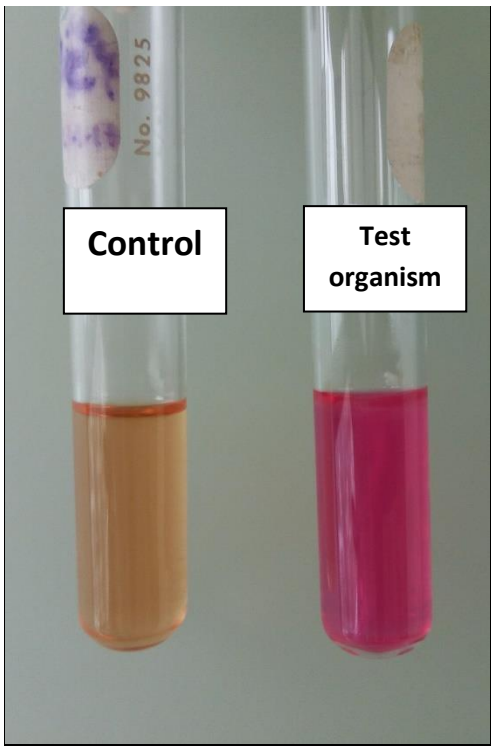


Figure 4.9: Non motile, urease positive result observed in MIU media

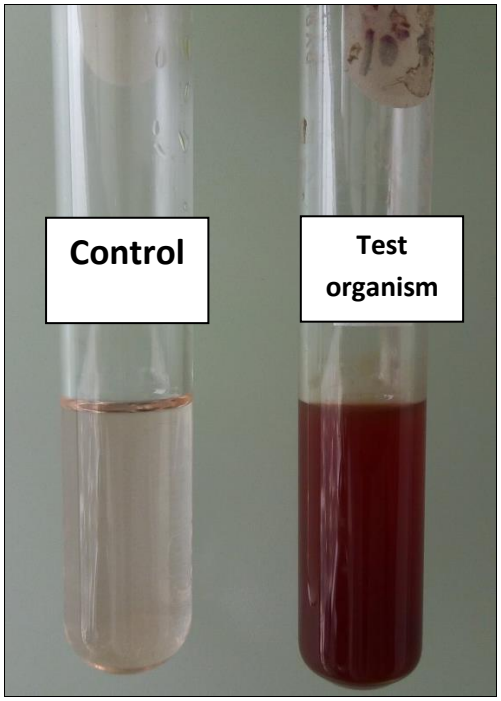


Figure 4.10: Experimental organism showed Nitrate Reduction Positive result

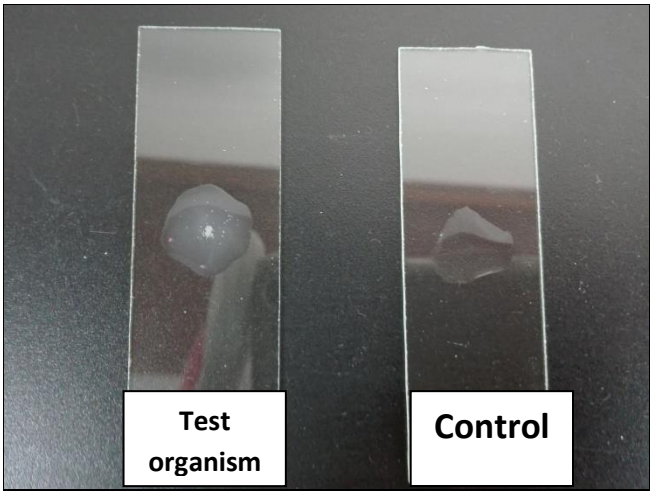


Figure 4.11: Organism shows positive catalase activity by the rapid formation of O2 bubbles

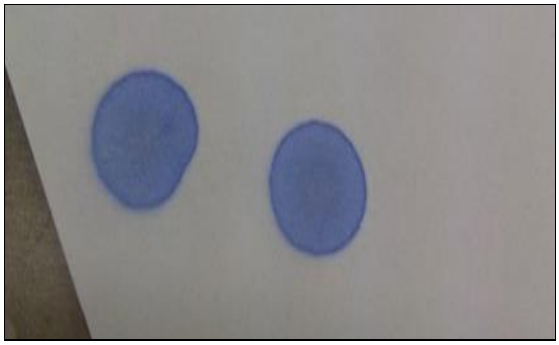


Figure 4.12: Test organism showing negative oxidase activity

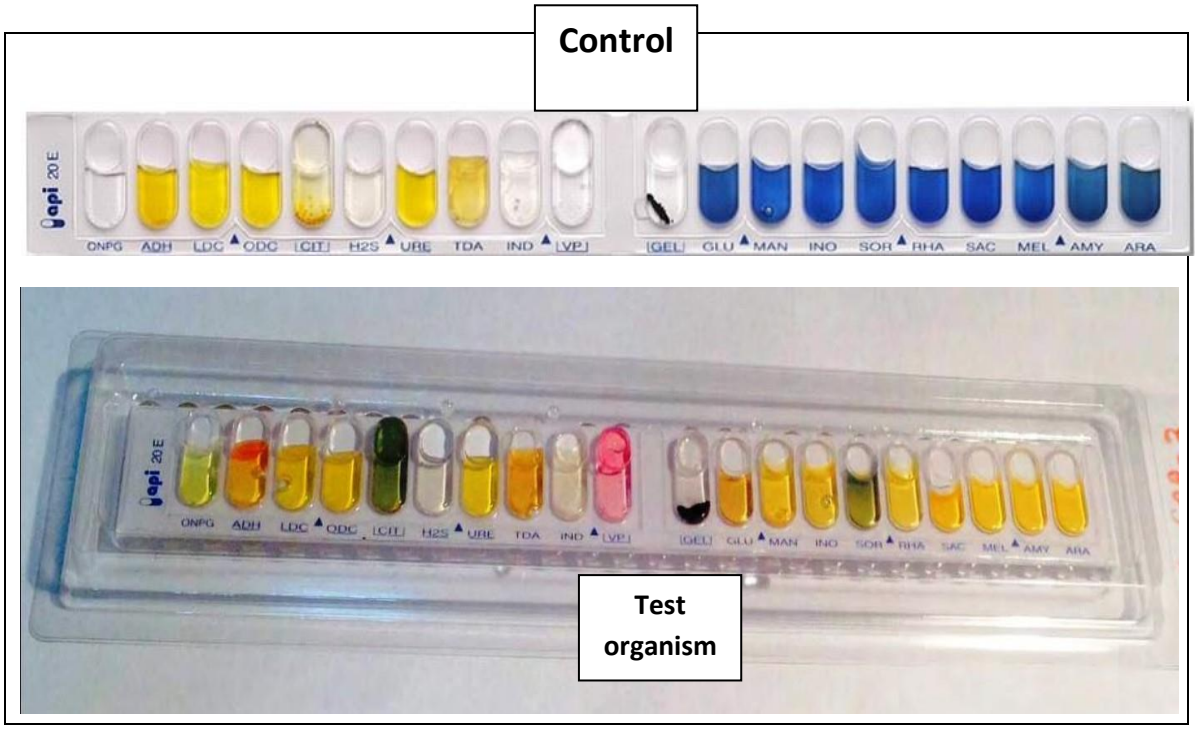


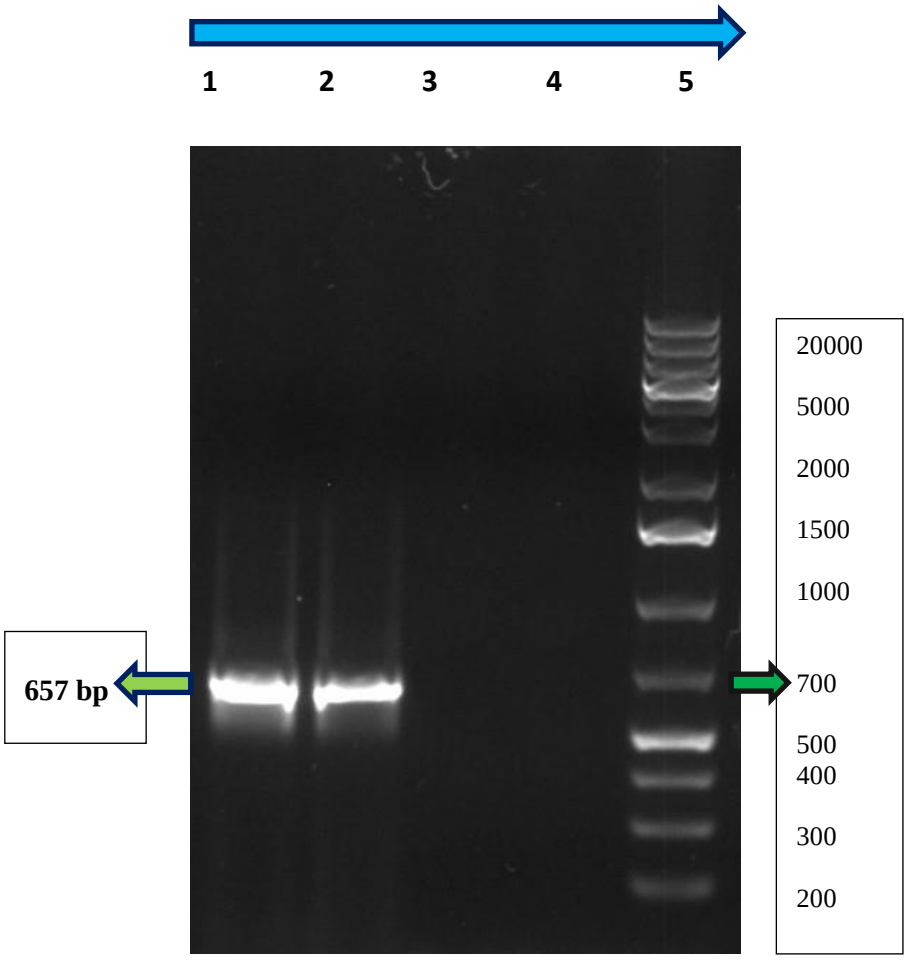
Figure 4.13: Identification of investigational organism using API® 20 E kit that exhibited typical reactions for*Klebsiella pneumoniae*

All the clinical isolates obtained from ideSHi repository and BUHS were reconfirmed by culturing on selective media and routine biochemical tests including API® 20 E kit based identification. All of them showed standard results for *Klebsiellapneumonie*and were subjected to PCR based identification which was the main focus of this study.

4.2 Genotypic characterizations of the isolates by PCR:

4.2.1 Verifying the efficency of the designed primer:

For primer verification, eleven positive controls for *Klebsiella pneumoniae* was used. PCR was carried out to amplify 16S rRNA gene with two primers: KP_16S F1 (NM3_F)-ATGTCGCAAGACCAGAGTGG and KP_16S R1 (NM3_R)-GCACAACCTCCAAATCGACA. PCR product size was found to be around 657bp by agarose gel electrophoresis.



1. *Klebsiellapneumoniae* 01
2. *Klebsiellapneumoniae* 02
3. *Streptococccuspneumoniae* (Neg control)
4. Nuclease free water
5. 1kb marker

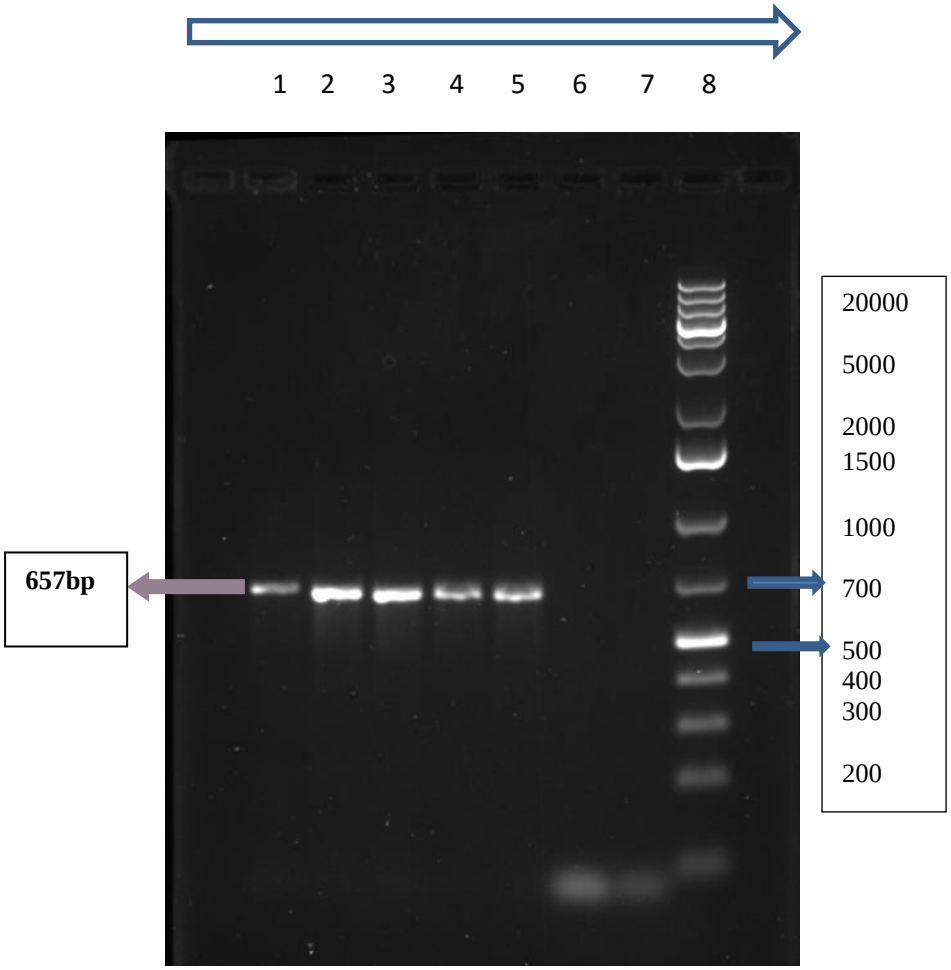
Fig: 4.14: A representative picture of PCR products after gel electrophoresis for the eleven *Klebsiella pneumoniae* positive strains for primer verification.

All the eleven strains of *Klebsiella pneumoniae* showed positive results having the product size of 657bp. One strain of *Streptococccus pneumoniae* was used as negative control and was not detected by this primer (NM3). In addition to that, there was no non-specific band and the absence of DNA band in the well where nuclease free water was loaded, indicated that the PCR products were contamination free. Therefore, the results show that this primer (NM3) can specifically detect only the strains of *Klebsiella pneumoniae.*

4.2.2 Rapid identification of *K. pneumoniae* directly from STGG media and from Enrichment Broth:

In case of ten clinical isolates, DNA was extracted directly from STGG media (in which the samples were stored) and the organism were also subjected to four hours incubation in enrichment broth (ToodHwitt media+ Fetal Bovine Serum).These extraction methods escaped the streaking step which requires 24-48 hours of incubation. Following DNA extraction, PCR and gel electrophoresis, and all the bacterial isolates showed desired bands of 657bp indicating positive *Klebsiella pneuminiae* strains. The results are as follows:

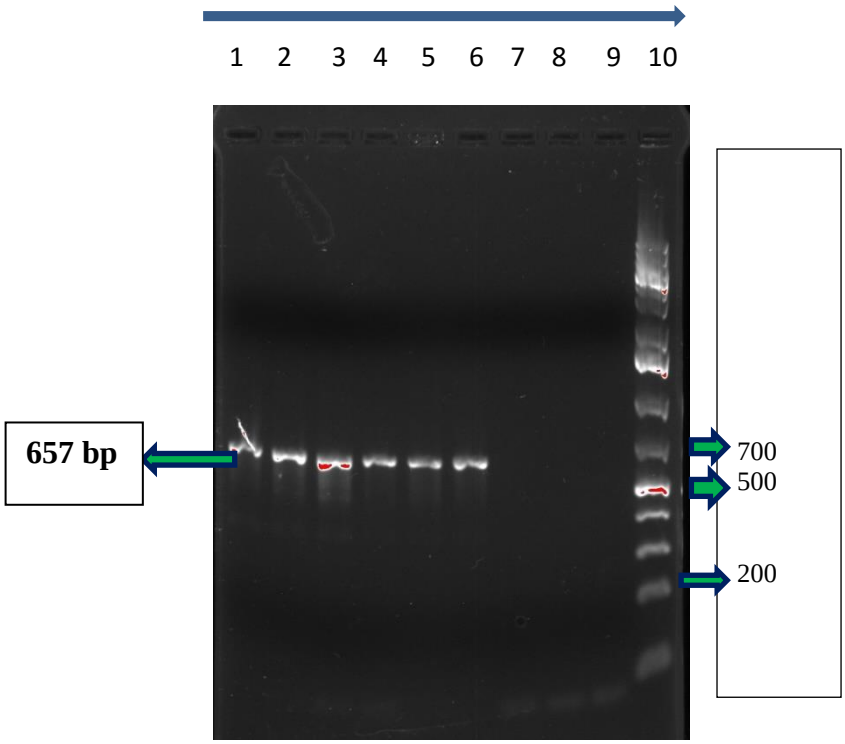
4.2.1 Direct DNA Extraction results:



1. Wound Swab 01
2. Wound swab 02
3. PUS sample 01
4. PUS sample 02
5. *Klebsiella pneumoniae* (positive control)
6. *Streptococcus pneumoniae* (Negative control)
7. Nuclease free water
8. 1kb marker

Figure 4.15: A representative picture showing the results obtained from Direct DNA Extraction followed by PCR and gel electrophoresis

4.2.2 DNA Extraction from Enrichment Broth



1. Wound Swab 01
2. Wound Swab 02
3. Wound Swab 03
4. PUS sample 01
5. PUS sample 02
6. *Klebsiella pneumoniae* (positive control)
7. *Streptococcus pneumoniae* (Negative control)
8. *Pseudomonas* (Negative control)
9. Nuclease free water
10. 1kb marker

Figure 4.16: A representative picture showing the results obtained from DNA Extraction from enrichment broth followed by PCR and gel electrophoresis

Estimation of DNA concentration and purity

For sequence analysis, the PCR products that have been confirmed as *Klebsiella pneumoniae*were purified by spin column based method, the purity and concentration of the DNA samples were measured spectrophotometrically by Nanodrop 2000 (Thermo scientific, USA).

Organism	**DNA Concentration (ng/µL)**	**Purity (260/280 nm)**
***Klebsiella pneumoniae*(positive control)**	27.5	1.87
PUS Sample (Direct Extraction)	21.0	1.84

4.3 Sequencing results:

4.3.1 Sequencing results for *Klebsiella pneumoniae* positive control:

Sequence analysis of *Klebsiella pneumoniae* positive strain used for PCR optimization:

Sequencing was done using column purified PCR products. For the sequencing, KP_16S F1 (NM3_F) primer was used. Sequencing data were analysed by ChromasLite 2.1 tool which generated a four colour chromatogram showing the result of sequencing run. Different bases are represented in different colours that are defined below:

1. **Adenosine=green**
2. **Guanine=black**
3. **Cytosine=blue**
4. **Thymine=red**

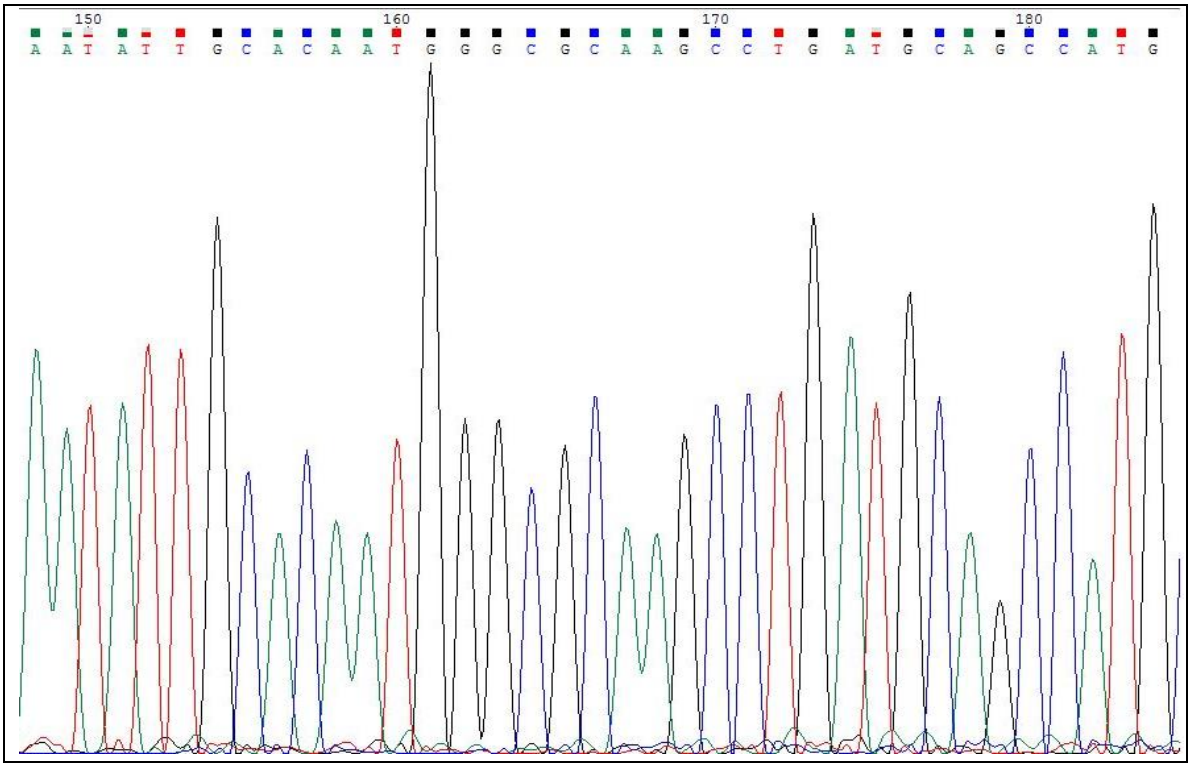


Figure 4.17: Diagrammatic representation of a part of the chromatogram of sequence. Evenly-spaced and distinctive single coloured peaks with a little baseline noise were observed. Peaks are readable.

Nucleotides sequences are represented using single-letter codes in a text-based format which is known as FASTA format. The specific format is documented through using Bioedit software. The FASTA formatted result for the bacterial strain containing 16S rRNA gene of *Klebsiella pneumoniae* is shown below in Figure 4.18.

Query Sequence	>Kpn32_NM3F ATYTGSCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTA AYGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCA GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGC GTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGG AAGGCGKTRAGGTTAATAACCTYRKCGATTGACGTTACCCGCAGAAG AAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG CAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCT GTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTC GAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTG TAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGC GGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAGCGTGGGGAGCA AACAGGATTAGATACCCTGGTAGTCCACGCCGTACKSMGTCMACAAA AAAAAAAAAAAATGAGGAAATTTTCCKMTTWSGTKGTRAKGRTATG YGARCMRMKRYRTGTAYTTRCYAS

Figure 4.18: Data obtained from software using 16S rRNA gene sequencing with *Klebsiella pneumoniae* specific forward primer.

Interpreting Nucleotide BLAST (blastn) output:

The Basic Local Alignment Search Tool (BLAST) is a program that can detect sequence similarity between a Query sequence and sequences within a database. Blastn compares a nucleotide query against a nucleotide sequence database.

After obtaining the FASTA formatted data, the query sequence was then submitted to NCBI, nucleotide databank and subjected to BLASTn.

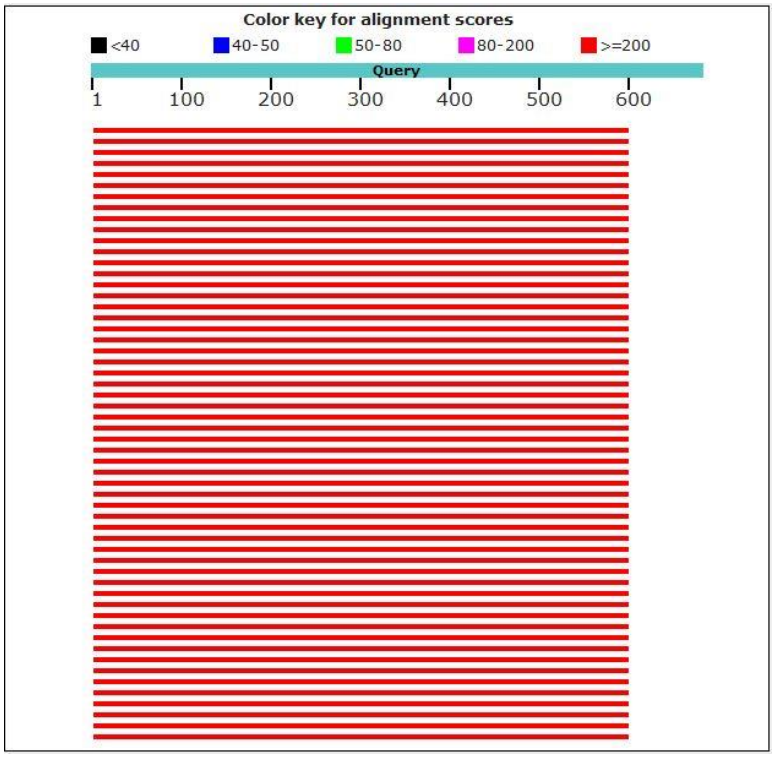


Figure 4.19: A graphical overview of all the Refseq 16S r RNA blastn hits for our query sequence.

The graphic summary shows alignments (as colored boxes) of database matches to Query sequence. The color of the boxes corresponds to the score (S) of the alignment, with red representing maximum matches with the query sequence and the highest alignment scores. Generally, the higher the alignment score, the more significant the hit.

Scrolling further down the output, we find a summary table that shows all the sequences in the Refseq database that show significant sequence homology to our sequence (Figure 4.20). By default, the results are sorted according to the Expect value (E-value) in ascending order.

Uncultured Klebsiella sp. clone F5feb.46 16S ribosomal RNA gene, partial sequence	1090	1090	93%	0.0	99%	GQ416015.1
Klebsiella pneumoniae strain Y90 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF678855.1
Klebsiella pneumoniae strain KL1 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	KY417867.1
Klebsiella sp. strain TR5 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF769336.1
Klebsiella pneumoniae strain ZG14 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767588.1
Klebsiella pneumoniae strain ZG16 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767583.1
Klebsiella pneumoniae strain ZG26 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767582.1
Klebsiella pneumoniae strain YYR54-1 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767579.1
Klebsiella pneumoniae strain YYR25 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767577.1
Klebsiella pneumoniae strain ZG2 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767574.1
Klebsiella pneumoniae strain QLR10-2 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767573.1
Klebsiella pneumoniae strain 721005, complete genome	1088	8669	92%	0.0	99%	CP022997.1
Klebsiella pneumoniae strain WMOK03 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	KX765858.1
Klebsiella pneumoniae strain WMOK 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	KX765857.1
Klebsiella pneumoniae strain 911021, complete genome	1088	8673	92%	0.0	99%	CP022882.1
Klebsiella pneumoniae strain K8 CIFRI 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF680516.1

Figure 4.20: Top 16 similar sequences resulted after alignments with query sequence.

The above mentioned Descriptions table provides a summary of the database sequences identified by BLAST to be similar to the input query. The description table columns provide the following information:

- the description/title of matched database sequence
- the highest alignment score (Max score) from that database sequence
- the total alignment scores (Total score) from all alignment segment
- the percentage of query covered by alignment to the database sequence
- the best (lowest) Expect value (E value) of all alignments from that database sequence
- the highest percent identity (Max identity) of all query-subject alignments, and
- the Accession of the matched database sequence

In this output, Uncultured Klebsiella sp. clone F5feb.46 16S ribosomal RNA gene, partial sequence shows 99% identity with the query sequence.

Here,

Max score is 1090

Total score is 1090

E value is 0.0

Query cover is 93%

Clicking on the Score (bits) in the right column brings up the detailed alignment. Below is the output from clicking on the score of the most significant hit, which shows a perfect alignment at all the nucleotide positions. (Figure 4.21)

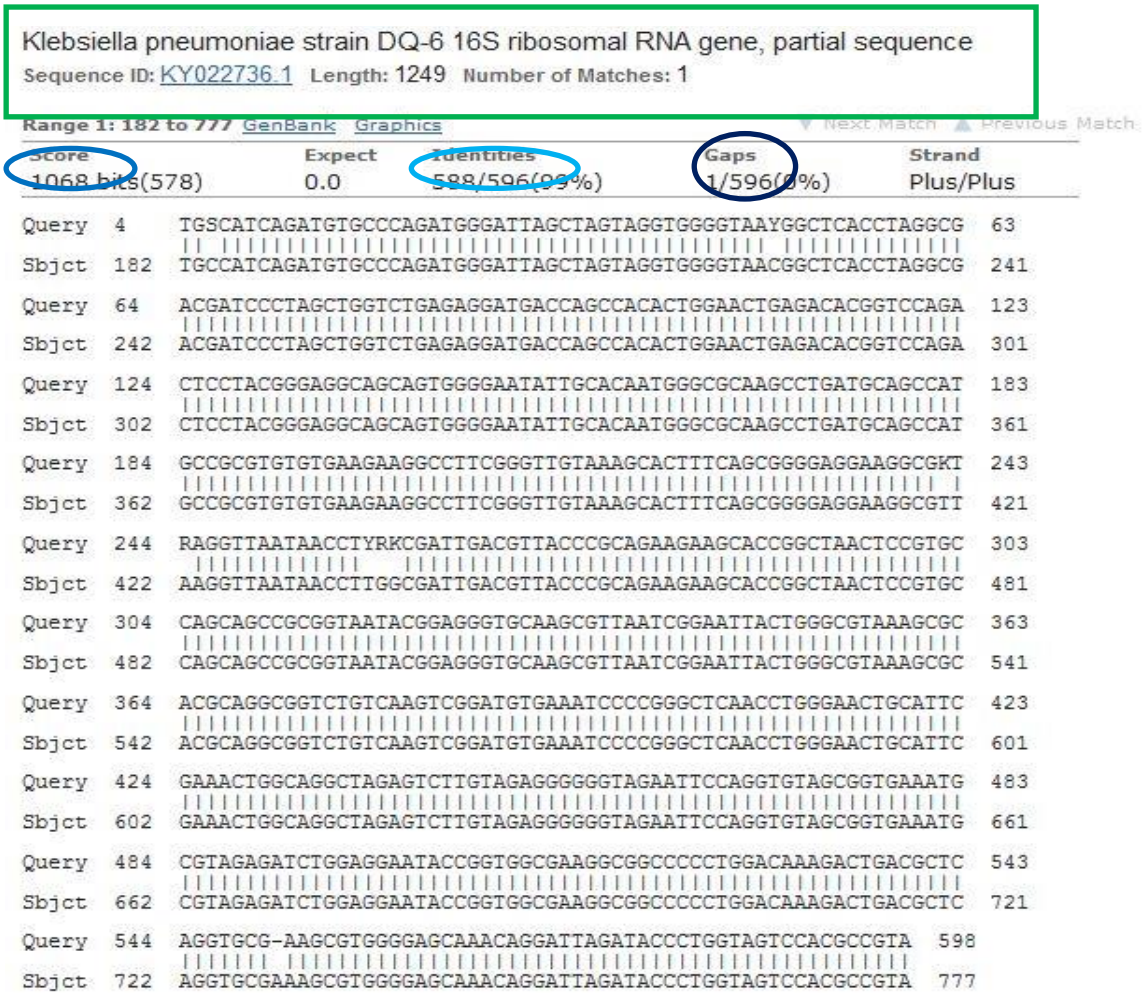


Figure 4.21: Part of the blastn alignment between the Query sequence and the subject Refseq16S rRNA sequence for *Klebsiella pneumoniae.*

Higher maximum identity and lower e-value shown in the BLASTn results indicated that the amplified sequence belong to the *Klebsiella pneumoniae* subsp.*pneumoniae*.

4.3.2 Sequencing results for direct extraction from Pus swab sample:

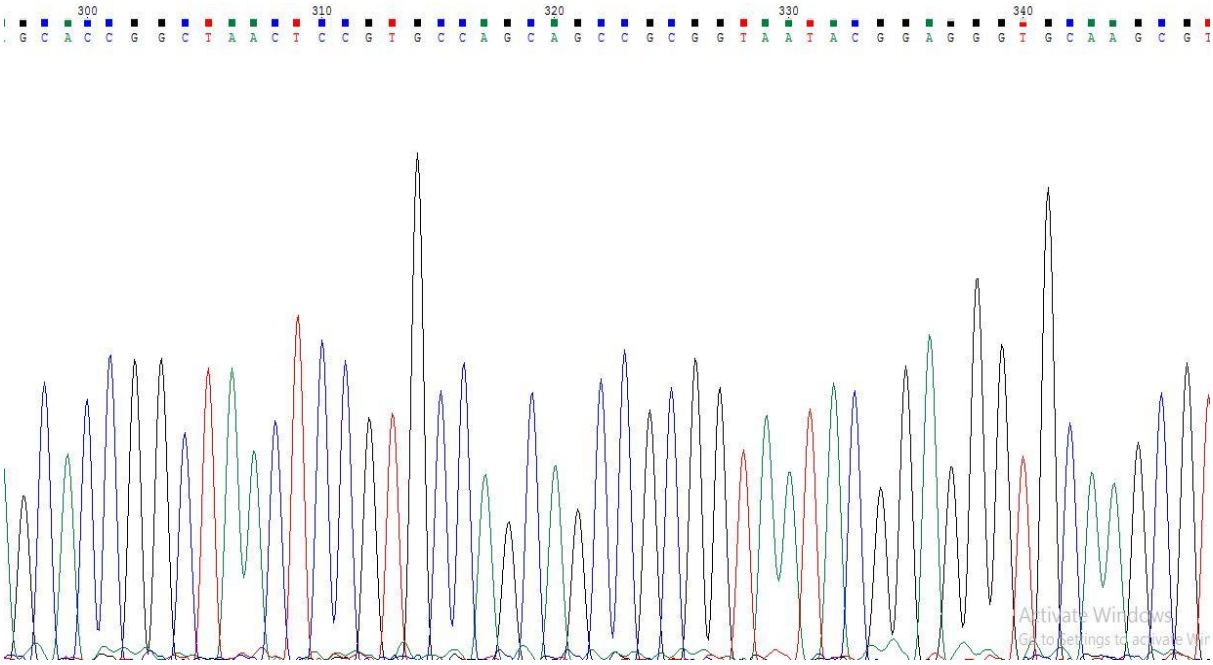


Figure 4.22: Diagrammatic representation of a part of the chromatogram of sequence. Evenly-spaced and distinctive single coloured peaks were observed. Background noise was present.

Nucleotides sequences are represented using single-letter codes in a text-based format which is known as FASTA format. The specific format is documented through using Bioedit software. The FASTA formatted result for the bacterial strain containing 16S rRNA gene of *Klebsiella pneumoniae* is shown below in Figure 4.23.

Query Sequence	>pus5334_NM3F TWCRTTCTGGMSKCWTKSCATCAGATGTGCCCAGATGGGATTAGCTA GTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTG AGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTA CGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAA GCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTA AAGCACTTTCAGCGGGGAGGAAGGCGRTRAGGTTAATAACCTYGKCG ATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG CCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGT AAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAACCCCGGGCT CAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAG GGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAG GAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAG GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA CGCCGTAAACGTGCTTTTTTWAAWWTTTTTTKMYAYCCA

Figure 4.23: Data obtained from software using 16S rRNA gene sequencing with *Klebsiella pneumoniae* specific forward primer.

Interpreting Nucleotide BLAST (blastn) output:

The Basic Local Alignment Search Tool (BLAST) is a program that can detect sequence similarity between a Query sequence and sequences within a database. Blastn compares a nucleotide query against a nucleotide sequence database.

After obtaining the FASTA formatted data, the query sequence was then submitted to NCBI, nucleotide databank and subjected to BLASTn.

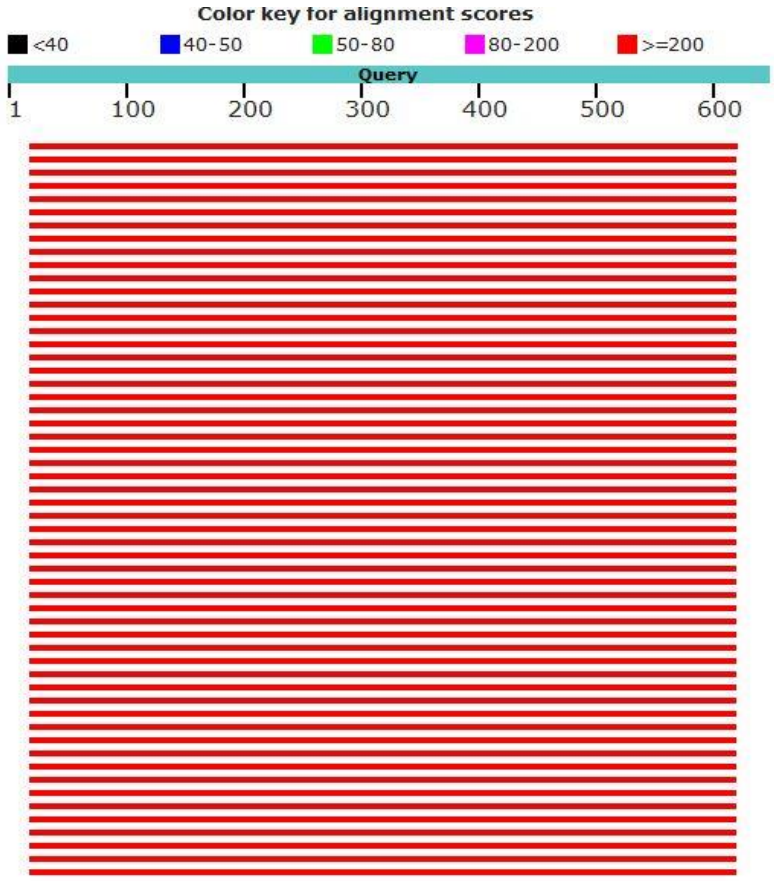


Figure 4.24: A graphical overview of all the Refseq 16S r RNA blastn hits for our query sequence.

The graphic summary shows alignments (as colored boxes) of database matches to Query sequence. The color of the boxes corresponds to the score (S) of the alignment, with red representing maximum matches with the query sequence and the highest alignment scores. Generally, the higher the alignment score, the more significant the hit.

Scrolling further down the output, we find a summary table that shows all the sequences in the Refseq database that show significant sequence homology to our sequence (Figure 4.25). By default, the results are sorted according to the Expect value (E-value) in ascending order.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Uncultured Klebsiella sp. clone F5feb.46 16S ribosomal RNA gene, partial sequence	1090	1090	93%	0.0	99%	GQ416015.1
Klebsiella sp. strain XN1 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	KY022426.1
Klebsiella pneumoniae strain Y90 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF678855.1
Klebsiella pneumoniae strain KL1 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	KY417867.1
Klebsiella sp. strain TR5 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF769336.1
Klebsiella pneumoniae strain ZG14 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767588.1
Klebsiella pneumoniae strain ZG16 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767583.1
Klebsiella pneumoniae strain ZG26 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767582.1
Klebsiella pneumoniae strain YYR54-1 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767579.1
Klebsiella pneumoniae strain YYR25 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767577.1
Klebsiella pneumoniae strain ZG2 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767574.1
Klebsiella pneumoniae strain QLR10-2 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF767573.1
Klebsiella pneumoniae strain 721005, complete genome	1088	8669	92%	0.0	99%	CP022997.1
Klebsiella pneumoniae strain WMOK03 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	KX765858.1
Klebsiella pneumoniae strain WMOK 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	KX765857.1
Klebsiella pneumoniae strain 911021, complete genome	1088	8673	92%	0.0	99%	CP022882.1
Klebsiella pneumoniae strain K8 CIFRI 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF680516.1
Klebsiella sp. strain MadaFrogSkinBac.DB-1560 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF525812.1
Klebsiella sp. strain MadaFrogSkinBac.DB-1528 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF525806.1
Klebsiella pneumoniae subsp. pneumoniae strain AUSMDU00008079, complete genome	1088	8662	92%	0.0	99%	CP022691.1
Klebsiella pneumoniae strain LZ 6 16S ribosomal RNA gene, partial sequence	1088	1088	92%	0.0	99%	MF593282.1

Figure 4.25: Top 20 similar sequences resulted after alignments with query sequence.

In this output, Uncultured Klebsiella sp. clone F5feb.46 16S ribosomal RNA gene, partial sequenceshows 99% identity with the query sequence.

Here,

Max score is 1090

Total score is 1090

E value is 0.0

Query cover is 93%

Clicking on the Score (bits) in the right column brings up the detailed alignment. Below is the output from clicking on the score of the most significant hit, which shows a perfect alignment at all the nucleotide positions. (Figure 4.26)

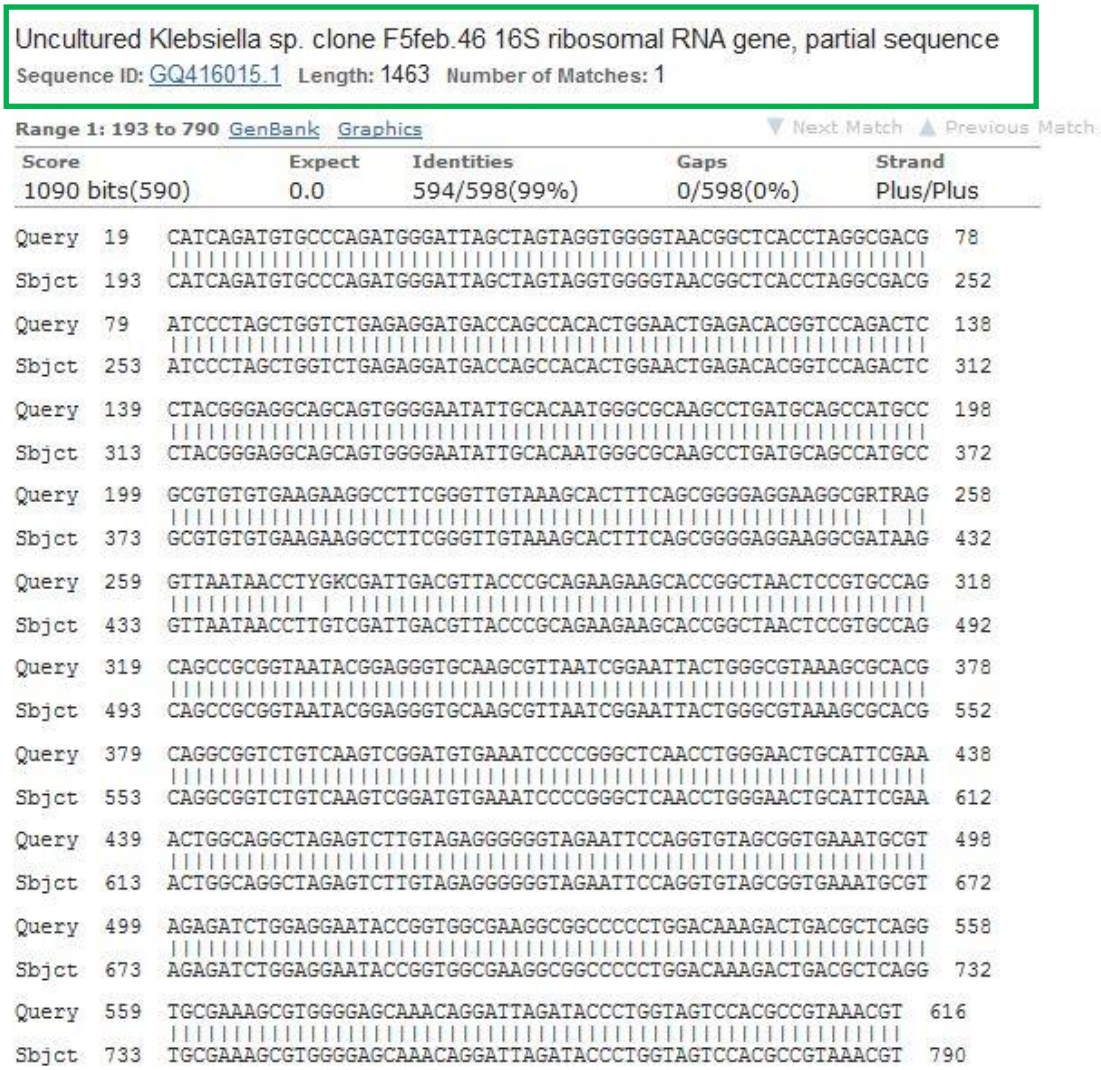


Figure 4.26: Part of the blastn alignment between the Query sequence and the subject Refseq 16S rRNA sequence for *Klebsiella pneumoniae.*

Higher maximum identity and lower e-value shown in the BLASTn results indicated that the amplified sequence belong to the *Klebsiella pneumoniae* subsp.*pneumoniae*.

The sequence results obtained from both the positive control and Pus swab sample indicate that, the PCR protocol could specifically amplify the 16S rRNA gene of *Klebsiella pneumoniae* from these samples representing 99% identity with the Reference database of *Klebsiella pneumoniae* of NCBI followed by BLASTn analysis.

It is also important to mention that, identification of target organism directly from the sample and from colonies on agar medium exhibited similar sequencing results.

Chapter 5

Discussion

DISCUSSION

The vast majority of *Klebsiella* infections, are associated with *Klebsiella pneumoniae* (KPN); clinically the most important *Klebsiella* species. The existing methods for detection of this organism include; conventional culture on selective media, biochemical tests, microscopic observation, serological testing etc. and these procedures are found to be time-consuming, less efficient and cumbersome. Therefore, it demands for a speedy diagnostic method for the identification of this opportunistic pathogen for rapid and specific intervention, ultimately to reduce the spread of infectious diseases.

The present study describes the use of a simple PCR based method targeting the 16S rRNA gene for the rapid identification of *Klebsiella pneumoniae*. The rRNA genes (16S, 23S, and 5S) are ideal candidates for bacterial identification, because they are highly conserved within the species [22,23]. PCR is widely used in many fields of microbiology, such as for direct bacterial detection in clinical specimens or for rapid identification of the cultured colonies. PCR can detect a small number of target organism by amplifying species-specific bacterial genes and bacteria can be identified by nucleotide sequence analysis of the PCR product followed by comparison of this sequence with known sequences stored in a database [24,25]. A number of PCR based assays have been developed targeting *Klebsiella pneumoniae, Corynebacterium kutscheri, Streptococcus pneumoniae, Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi* and numerous other pathogenic organisms as PCR offers higher sensitivity and specificity over traditional methods. But in most of these studies, a strain specific gene was targeted; however, mutation rate is quite high in these specific genes compared to evolutionarily conserved 16S rRNA genes. So that, primers targeting these specific genes might fail to identify the target organisms.

In the present study, a primer set targeting the 16S rRNA gene of *Klebsiella pneumoniae* was designed using CLUSTALW and Primer-BLAST software. The designed primer set using the PCR protocol described in this study has been able to identify the 16S rRNA region of *Klebsiella pneumoniae* directly and specifically in swab samples (both with and without enrichment broth) and in colonies grown on MacConkey agar plates.

Overall, the target nucleic acid fragments extracted from twenty-one clinical strains were specifically and efficiently amplified using the 16S rRNA gene of *K. pneumoniae* in this present investigation.

The primer was proved to be specific at 58°C, amplifying a 657bp product of the 16S rRNA gene of *Klebsiella pneumoniae* while avoiding the amplification of any other pathogenic bacterial species or related organisms (Figure 4.14). DNA extraction from colonies is a well-established method that is used in the microbiology laboratories for the preparation of DNA template. To overcome problems linked with application of PCR to clinical samples and to make the DNA extraction method quicker; two different techniques were employed for DNA extraction for the rest ten clinical isolates in this present analysis. At the start, DNA was extracted directly from the swab sample containing media and in another extraction method; initially the swab samples were briefly incubated (approximately 4 hours) in enrichment broth. After incubation, DNA extraction from enrichment broth was accomplished using appropriate protocol (described in section 3.7.2). In the conventional PCR based organism detection approach, DNA fragments are extracted from bacterial colonies grown on culture medium and then used in PCR. This process takes more time as organisms need to grow through time consuming culture methods where in this study, the DNA was extracted directly from the swab specimens and thus made it possible to deliver results within a day. Moreover, the other strategy where specimens were enriched in order to facilitate the organism's growth also shortens the time of *Klebsiella pneumoniae* detection. Together these methods do not require culturing the bacteria on agar medium thus saving a good deal of time. It has been estimated that by performing these DNA extraction methods, the results of the tests are readable within 4-8 hours. The process of identification of bacteria by direct DNA extraction from swab sample containing media that was used in the present study, including sample preparation, DNA extraction, PCR and gel electrophoresis, can be completed within ~4 hours however DNA extraction from enrichment broth required almost ~8 hours. PCR followed by Agarose gel electrophoresis showed clear bands having a product size of 657bp (Figure 4.15, 4.16). Similar results were obtained from these two extraction methods exhibiting that both the approaches could specifically detect the 16S rRNA region of *Klebsiella pneumoniae*. These results also demonstrate that, results were obtained more rapidly with direct extraction and enrichment broth than conventional culture-based DNA extraction method.

This PCR assay exhibited good specificity as no contamination was noted during the experiment as well as no false-positive signal was observed when the negative control (which comprised autoclaved nuclease free water and related organisms) was amplified in the reaction capillaries. These findings support the investigation of where their PCR assay for the rapid detection of *Klebsiella pneumoniae* demonstrated excellent sensitivity and specificity [26].

All the twenty-one clinical specimens were also subjected to biochemical characterization along with determination of colony morphology and cell morphology for the reconfirmation purpose. All the samples gave standard result for *Klebsiella pneumoniae* therefore indicating that, PCR results were concordant with traditional phenotypic identification systems. It is reported that when the results obtained from phenotypic methods were compared with PCR results, the real time PCR assay could show 100% concordance with phenotypic methods for the identification of *Klebsiella pneumoniae* [26, 27].

The PCR purified products were subjected to Sanger Sequencing. BLASTn analysis suggested 99% identity with the 16S r RNA gene of *Klebsiella pneumoniae*. Higher maximum identity and lower e-value shown in the BLASTn results indicated that the amplified sequence belong to the *Klebsiella pneumoniae* subsp. *pneumoniae*. 16S rRNA gene sequencing is a standard method in microbial taxonomy and can be applied directly on the amplified products of this PCR assay [20].

To the best of our knowledge, direct detection of *K. pneumoniae* from samples by PCR has been reported by a very few studies previously. Real-time PCR assay aiming the 16S rRNA gene for the direct detection of *K. pneumoniae* from positive blood culture bottles was described [26]. PCR techniques for detecting pathogenic organisms directly from clinical samples has been proved to be a very rapid method but it lacks the prior cultivation of the organism in order to alleviate interference from PCR inhibitors [29]. Moreover, more than one organism can be present in the sample in that case; it becomes difficult to specifically detect a particular organism through direct extraction.

Incubation of swab samples in enrichment broth was performed in order to increase the number of target bacteria, to increase the detection level of the target organism subsequently increasing the DNA yield. However in this investigation, non-selective enrichment broth (Tood Hewitt) was used. Besides, the bacterial count before and after incubating in enrichment broth was not calculated thus this study does not show any comparison between the enrichment broth extraction and direct extraction method in terms of bacterial multiplication.

Although *Klebsiella pneumoniae* infections can occur to anyone, it primarily attacks older adults and people with a chronic illness or compromised immune system. Among the infections caused by this pathogen; pneumonia, nosocomial infections and bloodstream infections are most devastating. Although, the epidemiological studies highlight the severity of *Klebsiella pneumoniae* infections but it is very unfortunate that in Bangladesh, there is no such accurate and reliable method exits for the identification of this serious pathogen. The phenotypic methods are time consuming and nonspecific whereas molecular detection methods are expensive. Being a developing country, here majority of the patients take health care facilities from government hospitals which lack the appropriate infrastructure to establish PCR technique and expert personnel to operate PCR. Some private hospitals and laboratories offer PCR technique based identification however, conventional PCR technique requires more than a working days to provide results and not affordable to most people of our country. In the meantime, the *Klebsiella pneumoniae* infection can progress to acute phase rendering treat to patients' lives. Therefore, in most cases, in our country, Physicians give empirical antibiotic treatment based on infection pattern, patient's history and radiographic observation avoiding the identification of the causative agent of the infection.

Klebsiella pneumoniae has three sub species (*Klebsiella pneumoniae* subsp. *pneumoniae*, *Klebsiella pneumoniae* subsp. *ozaenae*, *Klebsiella pneumoniae* subsp. *rhinoscleromatis*).This PCR protocol used in this study does not differentiate the subspecies of *Klebsiella pnumoniae* which possess similar 16S rRNA sequences. Therefore, along with additional in-depth analysis of the primer, a multiplex PCR protocol targeting both 16S rRNA gene and another specific gene of KPN are required. Additionally, the study would be more enriched if the research work would be conducted with larger sample size.

Chapter 6

Conclusion

CONCLUSION

In conclusion, the 16S rRNA gene based PCR assay presented in this study is simple, fast and can specifically detect *K. pneumoniae*. Identification of target organism in direct swab samples and brief incubation in enrichment broth has been proved to be rapid as results are readable in less than 4-8 hours of sampling. Using this procedure, samples can be processed in a relatively short amount of time eliminating the limitations of existing phenotypic identification systems.The 16S r RNA based PCR technique can proved to be useful in microbial detection and can be easily incorporated into any research laboratory capable of operating PCR. Future studies should enable us to develop a real-time, multiplex PCR assay for the identification of *Klebsiella pneumoniae* and allow differentiation among the *Klebsiella pneumoniae* subspecies and other *Klebsiella* species.

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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:

MacConkey Agar (Difco™):

Ingredients	Amount (g/L)
Peptone	17.0
Proteose Peptone	3.0
Lactose	10.0
Bile Salts No. 3	1.5
Sodium Chloride	5.0
Agar	13.5
Neutral Red	0.03
Crystal Violet	0.001

Eosine methylene blue agar (Oxoid, England)

Ingredients	Amount
Peptone	10.0
Sucrose	5.0
Lactose	5.0
Di-potassium phosphate	2.0
Eosin Y	0.14
Methylene blue	0.065
Agar	13.50

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

STGG Media

Ingredients	Amount
Skim milk powder	2.0 g
Oxoidtryptone soy broth	3.0 g
Glucose	0.5 g
Glycerol	10 ml
Distilled Water	100 ml

Luria-Bertani Broth:

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

Tryptone soy broth, (Oxoid, England)

Ingredients	Amount
Pancreatic digest of Casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Di-basic potassium phosphate	2.5
Glucose	2.5

MR-VP broth

Ingredients	Amount
Peptone	7 g
Dextrose	5 g
Potassium phosphate	5 g

Simmon's citrate agar (Oxoid, England)

Ingredients	Amount
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	0.2
Ammonium phosphate	0.8
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15.0
Bactobromthymol blue	0.08

Motility Indole Urease Agar (MIU)

Ingredients	Amount (g/L)	
NaCl(Sigma)	5	Prepare up to 900ml for **autoclave**
Agar (Himedia, India)	4	
KH_2PO_4 (Fisher Chemical, USA)	2	
Peptone (Himedia, India)	30	
Phenol Red (0.25%) (Sigma, India)	2 ml/L	
Urea (Amresco, USA)	20	Prepare up to 100ml for **filter sterilization**

Triple sugar iron agar (Himedia, India)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	10.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous sulfate	0.20
Sodium thiosulfate	0.30
Casein enzymatic hydrolysate	10.0
Yeast extract	3.0
Beef extract	3.0

APPENDIX-II

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

Kovac's reagent

5 g of para-dimethylaminobenzaldehyde was dissolved in 75 ml of amyl alcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored at 4^oC.

Methyl redreagent

0.1 g of methyl red was dissolved in 300 ml of 95% ethyl alcohol. Then distilled water was added to make the final volume 500 ml. This reagent was covered with aluminum foil and stored at 4^oC.

Barritt'sreagent

Solution A

5 g of alpha-naphthol was dissolved in 95% ethanol. This solution was covered with aluminum foil and stored at 4^{o}C.

Solution B

40 g of KOH was dissolved in distilled water. The solution became warm. After cooling to room temperature, creatine was dissolved by stirring. Distilled water was added. This solution was covered with aluminum foil and stored at4^{o}C.

Oxidasereagent

100 mg of N,N,N^{1},N^{1}-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4^{o}C.

Appendix-III
Instruments:

The equipment used throughout the study is 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