Screening of silver resistance gene in clinical isolates and determination of Minimum Inhibitory Concentration (MIC) for silver nitrate

A Dissertation Submitted to the Department of Mathematics and Natural Sciences, BRAC University in Partial Fulfillment of the Requirement for the Degree of Bachelor of Science in Microbiology

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DECLARATION OF AUTHENTICITY

I, the undersigned, Kazi Sarjana Safain, declare that this dissertation is my original work, gathered and utilized especially to fulfill the purposes and objectives of this study, and has not been previously submitted to any other university for a higher degree. I also declare that the publications cited in this work have been personally consulted. It is further declared that this work has been carried out under joint supervision of Dr. Md. Kaiissar Mannoor, Senior Scientist and Head, institute for developing Science and Health initiatives (ideSHi), Mohakhali, Dhaka as External Supervisor and Dr. Mahboob Hossain, Professor, Department of Mathematics and Natural Sciences, BRAC University, Mohakhali, Dhaka as Internal Supervisor.

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DEDICATION

My humble effort I dedicate to my sweet and loving

Family

And

Well-wishers

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ABSTRACT

Bacterial resistance to antibiotics has increased rapidly within recent years and has become a serious threat to public health. Ionic silver exhibits antimicrobial activity against a broad range of micro-organisms. As a consequence, the use of silver is increasing rapidly in the field of wound care and a wide variety of silver-containing dressings are now common place. However, concerns are being raised associated with the overuse of silver and the consequent emergence of silver resistant bacterial strains. Hence, the aim of this study was to investigate silver resistance in *Klebsiella pneumoniae* isolates both phenotypically and genotypically, isolated from nasal swab specimens which were collected from hospitalized acute respiratory infection patients. Confirmation of bacterial isolates was done by a range of methods including Gram staining, biochemical tests and Analytical profile index (API). Minimum Inhibitory Concentration (MIC**)** was determined to detect silver resistance and finally, molecular techniques such as Polymerase chain reaction (PCR) and sequencing were used for genotypic confirmation. MIC data showed that out of eleven isolates, growth of two isolates were inhibited at silver nitrate concentrations of 10 mg/L. *SilE,* among nine silver resistance gene types (*silP, silE, silA, silB, silC, silR, silS, ORF105* and *silABC*), was identified by conventional PCR using gene specific primers. PCR result showed that one of the two isolates was positive for *silE*. The primer amplified product size was about 400 bp. Nucleotide sequencing and subsequent BLASTn analysis revealed 99% similarity with homologous bacterial strains harboring the resistance gene which includes *E. coli*, *Salmonella enterica* and *Klebsiella oxytoca* strains. From this study, it has been found for the very first time in Bangladesh that the clinically prevalent species *Klebsiella pneumoniae* harbored silver resistance gene, which provides a possible evidence for their unequal involvement in nosocomial and especially burn wound infections. Furthermore, it would be appropriate for future studies to assess the likelihood of widespread resistance to silver and the potential for silver to induce cross resistance to antibiotics, in light of its increasing usage within the healthcare setting in perspective of Bangladesh.

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LIST OF ABBREVIATIONS

API	Analytical Profile Index
AST	Antibiotic Sensitivity Testing
Ag	Silver
$AgNO_3$	Silver nitrate
AgSD	Silver sulfadiazine
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
CLSI	Clinical and Laboratory Standards Institute
CTX-M	Cefotaximase-Munich
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
E-value	Expected value
ESBL	Extended Spectrum beta-lactamase
HSP	High scoring pairs
IEDCR	Institute of Epidemiology, Disease Control and Research
kb	Kilobytes
MDR	Multi Drug Resistance
MIC	Minimum Inhibitory Concentration
$MgCl_2$	Magnesium Chloride
nm	Nanometer
OD	Optical Density
ORF	Open Reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
Sil gene	Silver resistance gene
Tm	Melting temperature
UTI	Urinary tract infection
UV light	Ultraviolet light
µl	Microliter

Unit 1

Introduction

1.1 Background

Bacteria are considered to be the oldest form of life on earth and they appeared more than 3.5 billion years ago. The environment was extremely hostile at the time of their appearance. For their survival, they had to evolve certain strategies to manage the toxic natural elements and chemical compounds nearby of their region. In addition, it was vital to find appropriate ecological niches to avoid desiccation, high osmotic pressure, radiation, and extreme pH changes. Although microscopic, bacteria harbor a machinery that is impressively versatile in its simplicity. Meanwhile, they have adapted to most global conditions and can live in areas where only a very small number of other organisms could survive (Sütterlin, 2015). To combat against these organisms, various types of medicines and antibiotics were developed. After the discovery of penicillin by Alexander Fleming in 1928, these antibiotics are being widely used for treatment against infection. However, bacteria are developing resistance against these antibiotics while they are being applied on them. The regularly prescribed antibiotics are not strong enough to kill or inhibit those organisms. As a result these multi drug resistant (MDR) organisms are now becoming one of the major public health concerns throughout the world. Thus antibiotic resistance has become a global health problem. Moreover, since the turn of the century, the consumption of biocides has increased. One of the most popular biocides is silver, which is used as a disinfectant and preservative in a wide and mounting range of health care and consumer products, e.g., silver-containing wound dressings, silver-treated catheters and textiles, and washing machines releasing silver ions. Although humans and animals are naturally exposed to silver at low concentrations through food and water, the exposure level in both hospitals and in the society is probably higher today than when the silver consumption was mainly confined to the photographic, battery, and car industries (Sütterlin et al., 2014). Wounds often provide a favorable environment for the colonization of micro-organisms. In order to improve the opportunity for wound healing, it is important to create conditions that are unfavorable to micro-organisms and favorable for the host repair mechanisms and topical antimicrobial agents are believed to facilitate this process. Antiseptic agents are now considered for the treatment of localized skin and wound infections because they have a lower propensity to induce bacterial resistance than antibiotics. However, concerns are being expressed regarding the overuse of silver and the possible

emergence of bacterial resistance to silver, particularly within the clinical environment. Silver-resistant bacteria have been reported since 1975 and research within this area is clearly increasing. A preliminary understanding of the genetics underlying silver resistance has been known since 1998. Clinical evidence of silver-resistant bacteria has been principally in hospitals, specifically in burn wards, where silver salts (in the form of silver nitrate) are used as antiseptic agents. Many clinicians and researchers have questioned whether the widespread usage of silver could lead to cross-resistance to antibiotics, as has been suggested with a number of biocides, specifically triclosan, chlorhexidine and quaternary ammonium compounds (QACs). However, in reference to the available evidence to date, this appears to represent an unjustifiable concern (Percival, Bowler, & Russell, 2005).

1.2 Bacteria of interest and the infections they can cause

1.2.1 The Enterobacteriaceae family

Several genera can be found in the Enterobacteriaceae family. These gram negative facultative anaerobic rods are widely distributed in soil, water, plants and intestines of animals and humans. Most important members of the family are the genera *Klebsiella* and *Enterobacter*. *Klebsiella* and *Enterobacter* can be found as commensals in humans but have their natural habitat in soil and on plants. Both genera can also cause infectious diseases, often in nosocomial contexts. *Klebsiella pneumoniae* and *Enterobacter cloacae* rank between fifth and ninth among the causative agents of septicaemia, but more frequent are the urinary tract infections. *Klebsiella* and *Enterobacter* are more resistant to beta lactams than *E. coli*, and, due to its ability to form biofilms, *K. pneumoniae* can be extremely difficult to eradicate from the lungs of patients with ventilator-associated pneumonia (Torres & Carlet, 2001).

1.3 Nosocomial infections and the liable pathogens

Nosocomial infections (NI) are common in burn patients due to the typical features of the disease: loss of the first line of defense against microbial invasion; presence of devitalized, avascularized tissue that provides a favorable environment for microbial growth; alterations in the specific and nonspecific components of the immune system; gastrointestinal translocation; and extended hospitalization and multiple invasive diagnostic and therapeutic procedures (Oncul et al., 2009). Complex reasons, but

mostly the widespread use of third generation cephalosporins in the last decade, have led to the emergence of new nosocomial pathogens in burns - the extended spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae, which were uncommon until recently (Kumar, Kashyap, Mishra, Agarwal, & Kaur, 2011). All these multi-drug-resistant organisms (MDRO) may cause infections which are particularly challenging to treat as there are few antimicrobial agents still effective to combat them. In terms of new antibiotics, the pipeline is virtually dry, especially for gram-negative bacteria. Efforts should therefore be directed toward preserving the activity of the available antimicrobials, combating antimicrobial resistance and providing effective infection control measures for prevention of colonization and infection of patients with MDRO strains (Chan, 2012). Of the 0.2 million nosocomial infections occurring each year in the United States, 50 to 60% are caused by antimicrobial-resistant strains of bacteria (Weinstein, 1998). Multiple surveillance studies have demonstrated that resistance among prevalent pathogens is increasing at an alarming rate, leading to greater patient morbidity and mortality from nosocomial infections. Among gram-positive organisms, the most important resistant pathogens are methicillin- (oxacillin-) resistant *Staphylococcus aureus*, β-lactam-resistant and multi drug resistant pneumococci, and vancomycin-resistant enterococci. Important causes of gram-negative resistance include extended-spectrum β-lactamases (ESBLs) in *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis*, high-level third-generation cephalosporin (Amp C) β-lactamase resistance among Enterobacter species and *Citrobacter freundii*, and multi drug resistance genes observed in *Pseudomonas aeruginosa*, Acinetobacter, and *Stenotrophomonas maltophilia* (Jones, 2001).

1.3.1 *Klebsiella* spp. as nosocomial pathogens

Klebsiella is well known to most clinicians as a cause of community-acquired bacterial pneumonia, occurring particularly in chronic alcoholics (Carpenter, 1990) and showing characteristic radiographic abnormalities (Felson, Rosenberg, & Hamburger Jr, 1949) due to a severe pyogenic infection which has a high fatality rate if untreated. The vast majority of *Klebsiella* infections, however, are associated with hospitalization. As opportunistic pathogens, *Klebsiella* spp. primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus or chronic pulmonary obstruction. Nosocomial *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae*, the medically most important species of

the genus. It is estimated that *Klebsiella* spp. cause 8% of all nosocomial bacterial infections in the United States and in Europe. No great geographical variations in frequency have been noted. In the United States, *Klebsiella* accounts for 3 to 7% of all nosocomial bacterial infections, placing them among the eight most important infectious pathogens in hospitals (Horan et al., 1988). The urinary tract is the most common site of infection. *Klebsiella* accounts for 6 to 17% of all nosocomial urinary tract infections (UTI) and shows an even higher incidence in specific groups of patients at risk, e.g., patients with neuropathic bladders or with diabetes mellitus (Bennett, Young, & Darrington, 1995). As a cause of nosocomial gram-negative bacteremia, *Klebsiella* is second only to *Escherichia coli* (Bryan, Reynolds, & Brenner, 1983). In pediatric wards, nosocomial *Klebsiella* infections are especially troublesome particularly in premature infants and intensive care units. *Klebsiella* species are often the pathogens involved in neonatal sepsis, in both early-manifestation and late-manifestation infections.

Due to the extensive spread of antibiotic-resistant strains, especially of extended-spectrum β-lactamase (ESBL)-producing strains, there has been renewed interest in *Klebsiella* infections (Podschun & Ullmann, 1998).

1.4 Antimicrobial resistance

The underlying mechanisms for the development and spread of antibiotic resistance are complex. Most resistance mechanisms are pre-existent. In order to become clinically significant they need to become incorporated into a pathogen. This can be achieved by means of genetic exchange, by translation of pre-existing genes that may be activated by selection or induction, or when mutational events extend the substrate range of resistance-mediating enzymes (Tenover, 2001).

1.4.1 Antimicrobial resistance due to global response systems

Bacterial cells have wide spectrum of mechanisms to use when reacting acutely to sudden environmental changes. The resistance mechanisms at play on the cellular level are, however, divided into only four groups: (1) reduced permeability of the cell wall (porin loss, active efflux), (2) modification of the antimicrobial substance (beta-lactamases), (3) modification of the target protein (PBP-changes), and (4) altered

metabolic route. For silver, the first two mechanisms, a loss of porins or an activation of efflux pumps, are of most interest.

Porins, or outer membrane proteins (Omps), are channels in the bacterial cell membrane of gram-negatives that allow substances needed for the bacterial cell metabolism to penetrate into the cell. Some antimicrobial substances enter the cell through the same porins, and a transcriptional down-regulation of these Omps reduces the intracellular concentration of antimicrobial agents and thus, results in decreased susceptibility (Livermore, 1992).

Efflux pumps are transport proteins that often use active transport to clear the cell from antibiotics or other harmful substances. Multidrug efflux pumps have been described, e.g. AcrB in *E. coli*, that rather unspecifically clears a wide range of substances (Nikaido & Takatsuka, 2009).

1.4.2 Antimicrobial resistance due to genetic exchange

The bacterial genome is characterized by a remarkable plasticity that is caused by horizontal gene transfer, genome rearrangements and the activity of mobile DNA elements. A common differentiation is made between the ability of mobilizing of genetic elements within a cell and between two different cells. Horizontal gene transfer means the transfer of genetic elements between bacteria by cell-to-cell contact through conjugation and transduction, or without cell-to-cell contact through transformation or phages. Pre-existing antibiotic resistance genes may become mobilized from the chromosomes of bacterial species with limited clinical significance and get introduced into important human pathogens. For instance, two plasmid mediated AmpC beta-lactamases have been mobilized from *Aeromonas* spp. and *Citrobacter freundii* and are now frequently isolated from clinical *E. coli* isolates (Jacoby & Han, 1996).

In order to accomplish horizontal gene transfer, genetic elements have to be mobilized. Most mobile elements, like transposons, integrons or genomic islands, can be integrated into other genetic elements, but not all are mobile by themselves. For instance, despite the fact that integrons can integrate themselves by an integrase, their mobilisation is achieved indirectly, often as parts of integron cassettes that are incorporated into transposons (Gillings et al., 2008). Transposons are able to move vertically, between the chromosome and extrachromosomal DNA, i.e. plasmids. Horizontal transfer of

transposons is accomplished by conjugative plasmids or phages (Brown-Jaque, Calero-Cáceres, & Muniesa, 2015).

1.5 Silver

1.5.1 Chemistry of Silver

Symbol: Ag. Atomic weight: 107.868, 51.35%. Ag107 and 48.65% Ag109; atomic number: 47; electron shells: 2, 8, 18, 18, 1, orbital: 4d10; covalent radius: 1.34 AY; atomic radius: 1.75 AY. Oxidation state: 1. Density (at 293 K): 10.5 g cm33. With Ag(I) cations, the question of solubility in clinical and environmental settings is crucial, especially with halides and other anions present.

Silver occurs naturally as two isotopes – Ag107 and Ag109 – in approximately similar proportions. It exhibits three oxidation states – Ag(I), Ag(II) and Ag(III) – but only compounds of the Ag(I) state are sufficiently stable to be of relevance as antibiotics in medical devices and textiles. Like all other metals, silver is an electron-positive element with the Ag cation showing a profound ability to interact with and bind proteins and anions in a medium. Additionally, Ag binds receptor groups on the surfaces of adjacent cells, bacteria and fungi/yeasts. Silver metal and the majority of silver compounds ionize in the presence of water, body fluids and tissue exudates to some extent to release Ag or other 'biologically active silver ions' for antibiotic action or absorption into adjacent human tissues. The chemistry of silver is not well documented, and accurate data on relative ionization rates for the compounds commonly used in medical devices are not available. Occasionally, documents fail to identify the nature of the chemical source of silver in products referring only to 'silver content' or 'ionic silver'. The ionizing capacity of the silver metal or silver compound is critical in comparing their antimicrobial activities and in predicting the possible toxicity or health risk. To be effective in killing pathogenic organisms, each silver source should release silver ions. The expressions 'activated' or 'hydro-activated' are used colloquially to denote the bioactive state of the silver ion. The silver cation binds strongly to electron donor groups of biological molecules containing sulphur (SH), oxygen and nitrogen (Lansdown, 2006).

1.5.2 Biological Properties of Silver

Silver is not a recognized trace metal but occurs in the human body at low concentrations (2.3µg/L) due to ingestion with food or drinking water, inhalation and occupational exposures. Blood silver (argyraemia) is a measure of silver exposure from all sources. Clearly, occupational exposure or medicinal use of silver as an antibiotic in wound dressings, indwelling catheters, cardiac devices and in orthopedic surgery will be associated with higher than normal blood levels and may be a safety concern. Uptake of silver through mucous membranes (urethra) or by the haematogenous route from indwelling catheters is not well documented. The percutaneous uptake of silver from medicated textiles is not known. Medicated fabrics like cellulose fibers, rayon etc. will be in contact with the human skin for prolonged periods. Silver ions released in the presence of sweat, sebum and any moisture accumulate on the skin surface and some will penetrate the superficial layers of the skin to precipitate as silver sulphide in the stratum corneum. Some will be bound by chloride ions in sweat but a minute proportion can be expected to penetrate into the circulation bound to albumins and other proteins. Hair and nail growth provides a route for the excretion of silver from the human body, but most will be eliminated via the liver and kidneys. Hot weather and high humidity leading to hyperhydration will promote silver uptake through the skin and mucous membranes, but toxic risks are predictably low, except in individuals sensitized to silver (Lansdown, 2006).

1.5.3 Wound microbiology and antimicrobial agents

Wounds often provide a favorable environment for the colonization of micro-organisms which may both delay healing and cause infection. Bacteria found in wounds originate primarily from the mouth and colon, and constitute a unique collection of organisms that are potentially pathogenic. Consequently, broad-spectrum antimicrobial agents are required to control these mixed species populations to minimize the opportunity for infection. This has been reflected in the increased usage of silver in wounds, principally due to the fact that silver is relatively safe and exhibits broad-spectrum antimicrobial activity (Percival et al., 2005).

1.5.4 Silver as antimicrobial agent

For centuries silver has been in use for the treatment of burns and chronic wounds. As early as 1000 B.C. silver was used to make water potable (Heggers et al., 2002). Silver

nitrate was used in its solid form and was known by different terms like, “Lunar caustic” in English, “Lapis infernale” in Latin and “Pierre infernale” in French (Klasen, 2000). In 1700, silver nitrate was used for the treatment of venereal diseases, fistulae from salivary glands, and bone and perianal abscesses (Klasen, 2000; Landsdown, 2002). In the 19th century, granulation tissues were removed using silver nitrate to allow epithelization and promote crust formation on the surface of wounds. Varying concentrations of silver nitrate was used to treat fresh burns (Castellano et al., 2007). In 1881, Carl S.F. Crede cured opthalmia neonatorum using silver nitrate eye drops. Crede's son, B. Crede designed silver impregnated dressings for skin grafting (Klasen, 2000; Landsdown, 2002). In the 1940s, after penicillin was introduced the use of silver for the treatment of bacterial infections minimized (Fraise, Lambert, & Maillard, 2008). Silver again came in picture in the 1960s when 0.5% silver nitrate was used for the treatment of burns. In 1968, silver nitrate was combined with sulfonamide to form silver sulfadiazine cream, which served as a broad-spectrum antibacterial agent and was used for the treatment of burns. Silver sulfadiazine is effective against bacteria like *E. coli, S. aureus, Klebsiella* sp., *Pseudomonas* sp. It also possesses some antifungal and antiviral activities (Fox & Modak, 1974). Recently, due to the emergence of antibiotic-resistant bacteria and limitations of the use of antibiotics, the clinicians have returned to silver wound dressings containing varying level of silver (Chopra, 2007).

1.5.5 Silver Compounds

In one form or another, silver and its compounds have long been used as antimicrobial agents. The most important silver compound currently in use is silver sulfadiazine (AgSD), although silver metal, silver acetate, silver nitrate, and silver protein are also being used. In recent years, silver compounds have been used to prevent the infection of burns and some eye infections and to destroy warts (McDonnell & Russell, 2001).

1.5.5.1 Metallic silver

The antimicrobial property of silver is related to the amount of silver and the rate of silver released. Silver in its metallic state is inert but it reacts with the moisture in the skin and the fluid of the wound and gets ionized. The ionized silver is highly reactive, as it binds to tissue proteins and brings structural changes in the bacterial cell wall and nuclear membrane leading to cell distortion and death. Silver also binds to bacterial

DNA and RNA by denaturing and inhibits bacterial replication (Lansdown, 2002; Castellano et al., 2007).

1.5.5.2 Silver sulfadiazine

Silver sulfadiazine (AgSD) is a combination of silver and sulfadiazine. AgSD is used as a 1% water-soluble cream. AgSD works as a broad-spectrum antibiotic. It is used especially for the treatment of burn wounds. AgSD serves as reservoir of silver in the wound and slowly liberates silver ions. All kinds of sulfa drugs have been tested in combination with silver but sulfadiazine was found to be most effective. AgSD binds to cell components including DNA and cause membrane damage (Atiyeh, Costagliola, Hayek, & Dibo, 2007). It achieves bacterial inhibition by binding to the base pairs in DNA helix and thus inhibits transcription. In similar way it also binds to phage DNA (Maple, Hamilton-Miller, & Brumfitt, 1992).

1.5.5.3 Silver zeolite

Silver zeolite is made by complexing alkaline earth metal with crystal aluminosilicate, which is partially replaced by silver ions using ion exchange method. In Japan, ceramics are manufactured coated with silver zeolite to apply antimicrobial property to their products. These ceramics are used for food preservation, disinfection of medical products, decontamination of materials (Kourai, Manabe, & Yamada, 1994).

1.5.5.4 Silver Nitrate

Silver nitrate is caustic and irritant at concentrations exceeding 1%; in contact with living tissue it can cause leakage of cellular electrolytes including sodium and potassium. However, it is an excellent antibacterial agent and at 0.5% is particularly effective in inhibiting *Pseudomonas aeruginosa* which can prove fatal in burn wounds. Silver nitrate is claimed to be superior to many other antibiotics including chlorhexidine and silver sulfadiazine, especially in eliminating more resistant strains of *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes* and *Klebsiella pneumoniae*. Silver nitrate compresses are claimed to reduce levels of infection in severe burns by up to 70% and significantly reduce mortality. It exhibits haemostatic properties and may be useful during minor surgery (Lansdown, 2006).

1.5.6 Uses of silver compounds in medicine and health

The increased use of silver dressings has occurred because alternatives are required to replace antibiotics in the management of infected wounds (Thomas & McCubbin, 2003). Silver-based dressings release different amounts of silver ions in different ways via different materials.

Figure 1.1: Example of frequently used silver based dressings (Sütterlin, 2015).

1.5.6.1 Topical antimicrobial agent for burns

The widest and best known use of silver preparations in medicine is as preferred antimicrobial agents for treatment of serious burns. A topical cream that contains 1% silver sulfadiazine plus 0.2% chlorhexidine digluconate in a water immiscible cream base is the most widely used product for human use and veterinary medicine, marketed as Silvazine in the USA (by Marion Hoechst-Russell Laboratories, Kansas City, MO, USA) and as Flamazine in other countries, largely in the UK (Smith and Nelson

Company; Roche), Canada and continental Europe. From the initial use of silver sulfadiazine creams, there has been more recent incorporation of the silver sufadiazine directly into bandages used on burned skin surfaces and similar large open wounds. Use of direct current electricity to accelerate the release of Ag(I) from the covering into the damaged tissue and then penetration into the tissue has been shown beneficial, although this appears without wide use. It is the silver, Ag(I), that is biocidal with sulfadiazine functioning to keep the Ag(I) in a stable form less subjected to blackening by reduction, than with applications of AgNO3, which also has been used effectively as a biocide on burns- with, however, the unwelcome side effect of turning the burned tissue black from reduced Ag(0) (Silver, 2003).

1.5.6.2 Bandages for trauma and diabetic wounds

Ag-coated bandages are increasingly being used to cover burn wounds and traumatic injuries of humans and large animals. Silver sulfadiazine-coated methacrylate sheet material that provides a stable base for sustained release of Ag(I) over days is also being investigated. Two new commercial products being used in North America are Acticoat and Silverlon. Acticoat is a silver-coated polyethylene polymer sheet that releases Ag(I) and 'Ag nanoparticles'. Silverlon is a product consisting of Ag-coated polyamide fibers. These silver-containing fabrics are easier to apply and remove from large burns and wounds than is the residue of a cream. It is clear that these products are effective clinically and that the released Ag is broadly bactericidal. It is less clear that the released Ag(I) provides a direct therapeutic benefit. Some studies seem to indicate more rapid wound healing with Ag present than with control coverings, while others conclude that it is only the maintenance of a moist sterile covering that is needed, and that the Ag(I) itself does not add to the benefit. Additional clinical uses of Ag(I) include aseptic coverings for plastic surgery, traumatic wounds, leg ulcers, skin grafts, incisions, abrasions, and minor cuts (Silver, 2003).

1.5.7 Mechanism of action

The exact mechanism of action of silver on the microbes is still not known but the possible mechanism of action of metallic silver, silver ions and silver nanoparticles have been suggested according to the morphological and structural changes found in the bacterial cells (Rai, Yadav, & Gade, 2009).

1.5.7.1 Mechanism of action of silver

The mechanism of action of silver is linked with its interaction with thiol group compounds found in the respiratory enzymes of bacterial cells. Silver binds to the bacterial cell wall and cell membrane and inhibits the respiration process (Klasen, 2000).

1.5.7.2 Mechanism of action of silver ions/$AgNO_3$

The silver ions show efficient antimicrobial property compared to other salts due to their extremely large surface area, which provides better contact with microorganisms. The ions get attached to the cell membrane and also penetrate inside the bacteria. The bacterial membrane contains sulfur-containing proteins and the silver ions interact with these proteins in the cell as well as with the phosphorus containing compounds like DNA. When silver ions enter the bacterial cell it forms a low molecular weight region in the center of the bacteria to which the bacteria conglomerates thus, protecting the DNA from the silver ions. The silver ions preferably attack the respiratory chain and cell division finally leading to cell death. The silver nanoparticles release silver ions in the bacterial cells, which enhance their bactericidal activity (Feng et al., 2000). Moreover, the possible mechanisms for silver nanoparticle synthesis and the bactericidal action of silver ions are shown in Fig.1.3. At lower concentrations of silver nitrate, respiratory nitrate reductase may be involved in the production of nanoparticles (Kalimuthu, Babu, Venkataraman, Bilal, & Gurunathan, 2008). But at higher concentrations, silver nitrate inhibits the action of proteins by reacting with their thiol groups and also binds with the DNA, thus arresting its replication (Matsumura, Yoshikata, Kunisaki, & Tsuchido, 2003). Catalase may also be inhibited by high concentrations of its substrate, hydrogen peroxide. Therefore, silver nitrate may induce apoptosis in bacteria through many ways. So it can be said that apoptosis is the major effect of the bactericidal action of silver nitrate (Pandian, Deepak, Kalishwaralal, Viswanathan, & Gurunathan, 2010). Also, it has been reported that heavy metals react with proteins by getting attached with the thiol group and the proteins get inactivated (Feng et al., 2000).

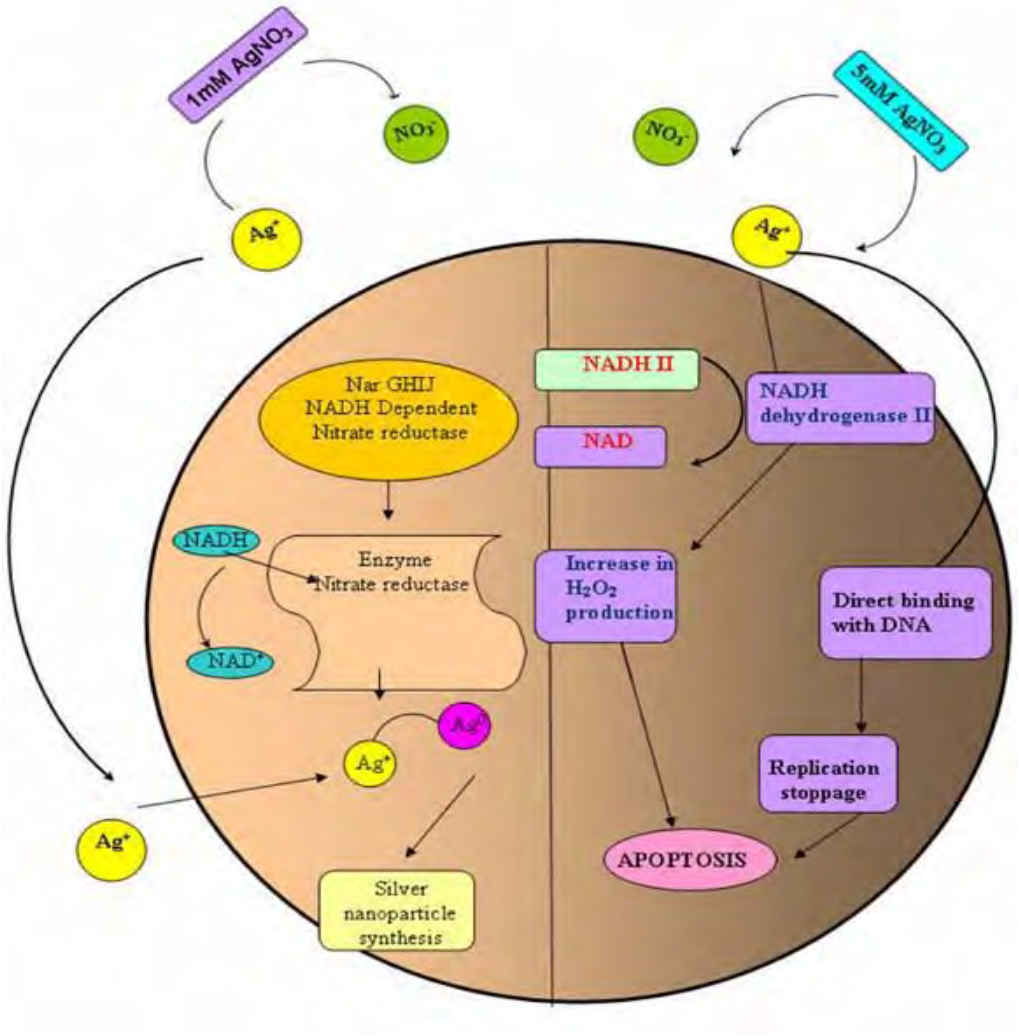

Figure 1.2: Possible mechanisms of the duality in functions of silver nitrate in bacteria. A) The left side of the figure shows the possible mechanism of silver nanoparticle synthesis at lower concentration which may involve nitrate reductase enzyme. B) The right side of the figure shows the possible mechanism for the induction of apoptosis by silver nitrate which may involve inactivation of thiol group containing proteins (e. g. NADH dehydrogenase II) and direct binding of silver to DNA thus stopping replication that certainly leading to apoptosis (Pandian et al., 2010).

1.5.8 Link between Ag^+ usage, resistance and antibiotics

Ag^+ resistance is most likely to be found in environments where greatest Ag^+ usage of silver containing products might be expected, such as in the dental setting where amalgams are known to contain 35% silver, burn units in hospitals or the use of silver-coated catheters (Sampath, Chowdhury, Caraos, & Modak, 1995). Some biocides disrupt cellular targets and subsequent mutations in these targets may confer low-level cross-resistance to certain antibiotics used in humans. Whilst a number of laboratory based studies have indicated a possible association between bacterial resistance to biocides and cross resistance to antibiotics, solid evidence is lacking. As such, this area

remains contentious. There does, however, appear to be some similarity between bacterial resistance to antibiotics and antiseptics. It is evident that bacteria tolerate biocides and antibiotics by employing the same types of cellular mechanism. Activation of the same efflux pumps can result in decreased bacterial susceptibility to both antibiotics and biocides. Enzymatic modification and destruction are commonly used by bacterial resistance mechanisms (Percival et al., 2005).

It is probable that the increasing use of biocides will eventually result in the selection of bacteria that are less susceptible. In fact, bacterial adaptation and resistance to biocides is certainly not a new phenomenon (Russell, 2004). Furthermore, the contribution of biocides to the development of bacterial antibiotic resistance has yet to be fully elucidated. Additional research is required to examine the modes of action of biocides and bacterial biocide resistance mechanisms, as well as to better characterize potential cross-resistance with antibiotics (Russell, 2003). It has been shown that most interactions between chemotherapeutic agents and microbial populations occur at very low concentrations (Baquero, 2001) and that low concentrations produce a substantial stress in bacterial populations that eventually influences the rate of variation and the diversity of adaptive responses leading to high levels of resistance. It is unclear whether the use of heavy metals, such as Ag^{+}, is contributing to the emergence and spread of antibiotic-resistant bacteria; however, this is unlikely (Barbosa & Levy, 2000). It is further examined that antibacterial-containing antiseptics or disinfectants can contribute to the selection and propagation of drug-resistant bacteria in the home environment. This finding, along with an indicated increased prevalence of potential pathogens in nonuser homes, provides a rational basis for the targeted use of efficacious formulations of antibacterial products to combat a spectrum of emerging and often antibiotic resistant bacterial pathogens that can present significant health risks in the home environment (Cole et al., 2003). The risks associated with the overuse of biocides has been overstated, and that it is now imperative that confidence is restored in products that form an essential part of domestic and hospital hygiene (Gilbert & McBain, 2004).

1.5.9 Pathogens which exhibit resistance to silver

Out of the 20,000 catalogued pathogens in existence (and millions more that remain uncatalogued), only a very few have been found to be silver-resistant. In fact, only about 19 pathogens have demonstrated an ability to develop silver resistance, and then, only

to varying degrees. According to the Immunogenic Research Foundation, these include sub-species of 19 pathogens, which have been shown to exhibit various degrees of resistance to the bactericidal effects of silver-based drugs and those organisms include *Acinetobacter baumani*, *Citrobacter freundii*, *Entamoeba histolytica* cysts, *Enterobacter cloacae*, Enterobacteriaceae, *Enterococcus hiraea*, *Escherichia coli*, *Helicobacter pylori*, *Klebsiella pneumoniae*, Mycobacteria, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* , *Pseudomonas Putida*, *Proteus mirabilis*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Thiobacillus ferro-oxidans*, *Thiobacillus thio-oxidans*, Vegetative *B. Cereus* Spores. In each case above, only some sub-species of the pathogens listed appear to be able to develop resistance to silver's antimicrobial qualities (Barwick, 2017).

1.5.10 Bacterial resistance to silver

Since the reintroduction of silver products as treatment alternatives for burn wounds, there has been an increasing number of reports on bacterial resistance to silver (Chopra, 2007). Silver-resistant bacteria have mainly been isolated from patients in burn care centers (Rosenkranz, Coward, Wlodkowski, & Carr, 1974), but some have also been isolated from the environment (Haefeli, Franklin, & Hardy, 1984). The physiological, biochemical, genetic and structural studies of the silver resistance determinant plasmid pMG101 established the molecular basis of silver resistance (Silver, Gupta, Matsui, & Lo, 1999) . Plasmid pMG101 is a 182 kb, transferable plasmid encoding resistance to silver (nine Open Reading frames [ORFs] in three transcriptional units), mercury, tellurite, ampicillin, chloramphenicol, tetracycline, streptomycin, and sulphonamide (Mchugh, Moellering, Hopkins, & Swartz, 1975). Silver resistance gene functions have been assigned based on homologous genes that encode resistance to other genes. The silver resistance cassette of genes encodes two silver efflux pumps (one an ATPase and the other chemisomotic) and two periplasmic Ag+ binding proteins (Silver, 2003).

1.5.11 Molecular genetics of silver resistance

Plasmid pMG101 is a 180-kb IncH1 silver resistance plasmid that also confers resistance to mercury and tellurite and to several antibiotics. The gene cluster for silver resistance contains a total of nine genes, seven of which were named and the two less-recognized open reading frames are still called ORFs : in order *silP, silA, silB, silC, silR, silS, silE,* ORF105 and *silABC* (ORF96) (Silver, 2003).

The silE gene determines an extracellular (periplasmic space) metal-binding protein of ten histidine residues implicated in Ag(I) binding. SilE is homologous to PcoE, of copper resistance. (Gupta, 1999). Upstream from silE and in the same orientation is a presumed two-component gene pair, silRS, encoding a transcriptional regulatory responder protein and a membrane sensor kinase, homologous to other members of the two-component family.

The remaining six ORFs in the Ag+ resistance system are transcribed divergently from silRSE (Gupta, 1999). The silCBA genes immediately upstream of silRS encode a presumed three-polypeptide chemiosmotic cation/proton antiporter that is a member of the resistance, nodulation and cell division (RND) family of transporters (Saier, Tam, Reizer, & Reizer, 1994). This protein complex consists of an inner-membrane proton/cation antiporter (SilA), a membrane fusion protein that spans the inner and outer membranes of gram-negative bacteria (SilB) and an outer-membrane protein (SilC). Between silC and silB, a 96 codon ORF of unassigned function was identified (Gupta, 1999).

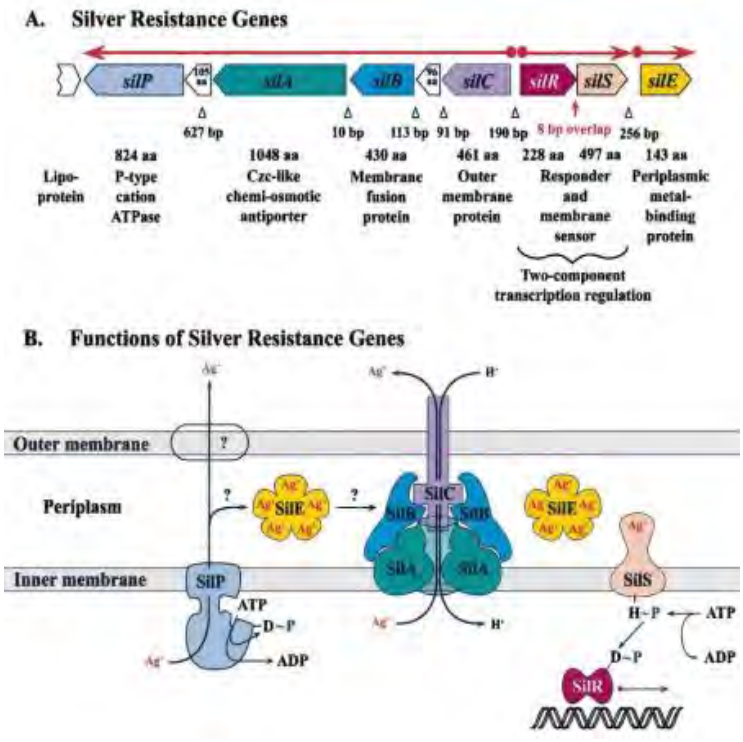

Figure 1.3: Silver resistance genes, transcripts and protein products. A: Top line shows the mRNAs. The open boxes indicate different genes or ORFs and their orientations. Nucleotides (nt) between genes and the sizes of gene products in amino acids (aa) are marked. B: The proposed function of each gene product, deduced from homologies to known proteins (Silver, 2003).

The product of the last gene of the Ag+ resistance determinant, SilP, is predicted to be a P-type ATPase, a member of the family of heavy-metal resistance ATPases (Rensing, Ghosh, & Rosen, 1999). The Ag+ resistance determinant is unique to date in encoding both a metal-binding protein and two biochemically different efflux mechanisms (Gupta, Phung, Taylor, & Silver, 2001).

A wide distribution of silver resistance homologous determinants, localized on plasmids or on the bacterial chromosomes might pose a threat toward effective use of silver compounds as antiseptics, analogous to the development of antibiotic-resistant bacteria when antibiotic usage increases (Salyers & Amabile-Cuevas, 1997).

1.5.11.1 The periplasmic Ag(I)-binding protein (*SilE*)

SilE is a small periplasmic Ag(I)-binding protein that binds Ag(I) ions specifically at the cell surface, presenting the first line of resistance against Ag(I) toxicity. The SilE protein is purified to homogeneity from bacterial periplasmic proteins and its sequence is confirmed by N-terminal amino acid sequencing (Gupta, Matsui, Lo, & Silver, 1999). Studies with purified SilE protein using atomic absorption spectroscopy (AAS) and inductive-coupled plasma analysis showed very high specificity for Ag(I) binding. The SilE protein contains 10 histidine residues that bind with the five Ag(I) cations. In contrast to other metal-binding proteins such as metallothionein, SilE has no cysteine residues. Binding of Ag(I) to the SilE protein brings about a usually large change in protein folding. Although the silE gene confers low level Ag(I) resistance by itself, it has never been found in nature without the other sil genes (Lo, 2000).

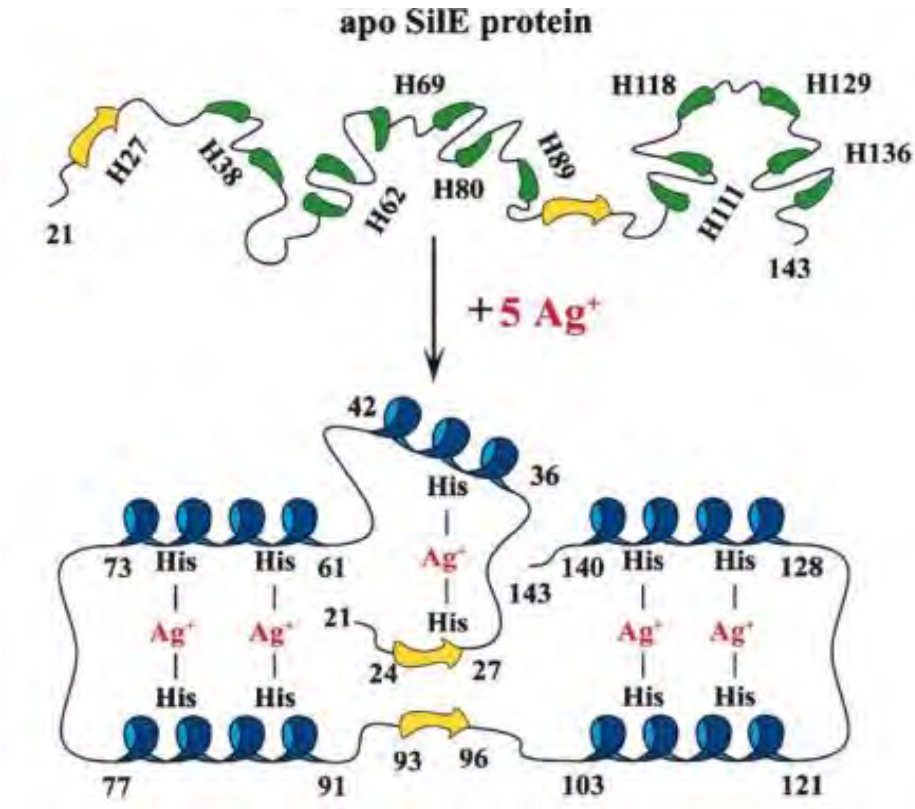

Figure 1.4: Model for Ag(I) binding and folding of the periplasmic Ag(I)- binding protein SilE. Top: 122-amino acid processed SilE protein after removal of 20-amino acid leader sequence with positions of the 10 histidine residues noted. Bottom: secondary structure predictions of K-helical (coils) and L-sheet (arrows) regions from standard software and predicted cross-linking of five Ag(I) cations by 10 histidines (Silver, 2003).

1.6 Objectives of this study

With the major problem of multi drug resistant bacteria and the increasing use of silver based dressings in health care and consumer products containing silver as a background, the overall purpose of this research was to fill in knowledge gaps concerning the existence, the mechanisms and the possible collateral damage of silver resistance. With a view to achieving this aim, the objectives of this study were set to:

- ✓ Perform the initial screening of clinical isolates for detection of bacterial resistance to silver;
- ✓ Determine the minimum inhibitory concentration (MIC) for silver nitrate in the resistant strains;
- ✓ Detect silver resistance gene (silE) genotypically.

Unit 2

Methodology

2.1 Working place:

The laboratory works of this study were conducted at the institute for developing Science and Health initiatives (ideSHi), Institute of Public Health building, Mohakhali, Dhaka. The laboratory has been set up to meet international BSL-2 requirements in order to carry out molecular, immunological and genetic studies.

2.2 Duration of the study

This study was carried out from October 2016 to May 2017.

2.3 Bacterial isolates

Eleven clinical isolates of *Klebsiella pneumoniae* that were preserved in -70°C freezer as glycerol stock was used for this study.

2.3.1 *Klebsiella pneumoniae* isolates

Isolates of *Klebsiella pneumoniae* were approved for the isolation of silver resistance. The isolates were obtained from the preserved bacterial stock of collected nasal swab samples which had been isolated from an acute respiratory infection surveillance project at ideSHi Laboratory.

2.3.2 Sample Preparation and Culturing

The isolates were taken from the STGG (a medium containing skim milk, tryptone, glucose and glycerin) stock culture from the -70°C. The isolates were first subcultered on MacConkey Agar plate (BD Difco™) using streak plate method. A drop of the stock culture was inoculated with a sterile platinum loop and streaked out using three quadrant streaking method and incubated for 18-24 hours at 35-37˚C in aerobic condition.

2.3.3 Confirmation of bacterial isolates

For confirming the bacterial isolates, several methods were followed. These methods include-

- ✓ Analysis of colony morphology
- ✓ Gram staining
- ✓ Biochemical tests
- ✓ Catalase test
- ✓ Oxidase test

- ✓ Analytical Profile Index (API® 20E)
- ✓ Antibiotic sensitivity test

2.3.3.1 Microbial isolation and identification

Well isolated colonies were used for the phenotypic identification of silver resistance.

2.3.3.2 Analysis of colony morphology

When grown on a variety of media, microorganisms exhibit differences in the macroscopic appearance of their growth. These differences are known as cultural characteristics, are used as a basis for separating microorganisms into taxonomic groups. Analysis of colony morphology helps to determine the cultural characteristics of microorganisms as an aid in identifying and classifying organisms into taxonomic groups. These demonstrate well isolated colonies and are evaluated by observing the size, pigmentation, form, margin and elevation of the colonies (Cappuccino & Sherman, 2008).

2.3.3.3 Gram staining

Grease or oil free slides are essential for the preparation of microbial smears. Grease or oil from the slides was removed by cleaning with a tissue paper. With a sterile cooled loop, isolated colonies along with a drop of normal saline were heat fixed on the centre of a glass slide. A satisfactory smear allows examination of the typical cellular arrangement and isolated cells. Then the slide with heat fixed smear was placed on staining tray. The smear was flooded with crystal violet for 1 minute. Later, the slide was gently rinsed with distilled water using a wash bottle. Then the slide was again flooded with Gram's iodine for 1 minute. It forms complex structure with crystal violet which tightly binds with the cell wall of the bacteria. The iodine solution was rinsed off with distilled water. After that the smear appears as a purple circle on the slide. The smear was then decolorized using 95% ethyl alcohol or acetone. It acts as the decolorizing agent so that it dehydrates the peptidoglycan layer through shrinking the slide was then tilted slightly and the alcohol drops were applied by drop for 5 to 10 seconds until the alcohol ran almost clear. The slide was immediately rinsed with deionized water.

Then the slide was gently flooded with safranin to counter-stain and allowed to stand for 45 seconds. It gives light red or pink colour in the cell wall of gram-negative bacteria

due to retention. Slide was blotted dry with a tissue paper and placed in an upright position. The smear was then viewed under a light-microscope using oil-immersion.

2.3.3.4 Biochemical tests

Biochemical tests were done to differentiate between closely related bacteria. The biochemical tests were performed according to the methods described and applied in Microbiology Laboratory of ideSHi. The biochemical tests that were done for the confirmation of bacteria were-

- Triple Sugar Iron (TSI) test
- Citrate utilization test
- Motility Indole Urease (MIU) test

2.3.3.4.1 Triple Sugar Iron Agar (TSI) Test

With a sterilized straight inoculation needle, the top of a well-isolated colony from MacConkey agar plate was picked up. The needle containing the colony was inoculated into TSI Agar by first stabbing through the centre of the medium to the bottom of the tube and then streaking on the surface of the agar slant. The cap was left on loosely and incubated at 35°C in ambient air for 18 to 24 hours to observe carbohydrate fermentation, CO_2 and H_2S production.

TSI Slant contains agar, pH sensitive dye (phenol red), 1% sucrose, 1% lactose and 0.1 %glucose, sodium thiosulphate together with ferrous sulphate or ferrous ammonium sulphate. All of these ingredients were mixed together and allowed to solidify in a test tube in a slanted angle. This slanted shape of this medium provides an array of surfaces that are either exposed to oxygen-containing air in varying degrees or not exposed to air. Bacteria fermenting any of these three sugars will produce acid as by-products. Therefore, the colour of the phenol red will turn red to yellow. Position of the colour change in the tube will determine the fermented sugar. For example, if the butt turns yellow, it represents that fermentation of glucose has occurred. If the whole agar turns yellow, it represents that all three sugars have been fermented and if it turns black, it confirms the production of hydrogen sulphide (H_2S) gas and production of bubbles confirms Carbon dioxide (CO_2) gas formation.

2.3.3.4.2 Citrate utilization test

A well isolated colony was taken using a sterilized straight needle. The needle containing the colony was inoculated into Simmons's citrate agar by first stabbing through the centre of the medium to the bottom of the tube and then streaking on the surface of the agar slant. Then it was incubated overnight at 37°C to detect if the organism had the ability to utilize citrate. Abundant growth on the slant and a change from green to blue in the medium indicates a positive test for growth using citrate and there would be no colour change for the negative result.

2.3.3.4.3 Motility Indole urease (MIU) test

Motility Indole urease (MIU) media is commonly used for the identification of gram negative bacteria of Enterobacteriaceae family. It is a single medium which is incorporated with three individual tests such as motility of the organism, indole production and presence of urease enzyme.

A sterile straight needle containing a well isolated colony from a freshly cultured media was inoculated in the Motility Indole urease (MIU) media making a single stab down the centre of the tube to about half the depth of the medium and an indole reagent paper was attached at the top of the test tube. Then it was incubated overnight at 37°C. If the organism is motile, it would typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque. Indole production will be determined by the indole reagent paper attached at the top of the test tube. Change of the paper colour into pink indicates a positive result. To detect the presence of urease enzyme, the top of the media is observed. If the organism is urease positive, it will degrade the urea present in the medium into ammonia. This ammonia will react with water to produce ammonium hydroxide and thus the colour of the medium will turn yellow to red or pink.

2.3.3.5 Catalase test

A small amount of bacterial colony was transferred to a surface of clean, dry glass slide using a loop. A drop (1 drop ~10 µl) of 30% H_2O_2 was placed on to the slide and mixed. Within 5-10 seconds the rapid evolution of oxygen as evidenced by bubbling specifies positive result. A negative result is no bubble or only a few scattered bubbles.

2.3.3.6 Oxidase test

The Oxidase test must be performed before performing Analytical Profile Index of test microorganism. A small amount of well isolated colony from a fresh bacterial culture was picked up and rubbed onto a filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride. Inoculated area of paper was observed for a colour change to deep blue or purple within 10-30 seconds. If the colour changes, it indicates positive result and no colour change indicates negative result.

2.3.3.7 Analytical Profile Index (API® 20E)

API 20 E is a standardized identification system for Enterobacteriaceae and other non-fastidious, Gram negative rods. The plastic strip holds twenty mini-test chambers containing dehydrated media having chemically-defined compositions for each test.

These tests include:

1. ONPG: test for β-galactosidase enzyme by hydrolysis of the substrate o-nitrophenyl-β -D-galactopyranoside
2. ADH: decarboxylation of the amino acid arginine by arginine dihydrolase
3. LDC: decarboxylations of the amino acid lysine by lysine decarboxylase
4. ODC: decarboxylations of the amino acid ornithine by ornithine decarboxylase
5. CIT: utilization of citrate as only carbon source
6. H_2S : production of hydrogen sulphide
7. URE: test for the enzyme urease
8. TDA (Tryptophan deaminase): detection of the enzyme tryptophan deaminase: Reagent to put- Ferric Chloride
9. IND: Indole Test-production of indole from tryptophan by the enzyme tryptophanase. Reagent- Indole is detected by addition of Kovac's reagent
10. VP: the Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway
11. GEL: test for the production of the enzyme gelatinase which liquefies gelatin
12. GLU: fermentation of glucose (hexose sugar)
13. MAN: fermentation of mannose (hexose sugar)
14. INO: fermentation of inositol (cyclic polyalcohol)
15. SOR: fermentation of sorbitol (alcohol sugar)

16. RHA: fermentation of rhamnose (methyl pentose sugar)
17. SAC: fermentation of sucrose (disaccharide)
18. MEL: fermentation of melibiose (disaccharide)
19. AMY: fermentation of amygdalin (glycoside)
20. ARA: fermentation of arabinose (pentose sugar)

2.3.3.7.1 Procedure

The microorganisms to be identified was first isolated on a culture medium adapted to the culture of Enterobacteriaceae and/or non-fastidious Gram-negative rods, according to standard microbiological techniques.

2.3.3.7.2 Preparation of the strip

An incubation box (tray and lid) was prepared and about 5 ml of distilled water or demineralized water was distributed into the honey- combed wells of the tray to create a humid atmosphere. The strain reference on the elongated flap of the tray was recorded. The strip was removed from its packaging. The strip was placed in the incubation box.

2.3.3.7.3 Preparation of the inoculums

An ampoule of API NaCl 0.85 % Medium (5 ml) was opened. By using a pipette, a well isolated colony was removed from an isolation plate of young cultures (18-24 hours old). Emulsification was then done cautiously to achieve a homogeneous bacterial suspension. Suspension was used immediately after preparation.

2.3.3.7.4 Inoculation of the strip

Using the same pipette, both tube and cupule of the tests CIT, VP and GEL were filled with the bacterial suspension. Only the tube (and not the cupule) of the other tests was filled. An anaerobic condition was created in the tests ADH, LDC, ODC, H_2S and URE by overlaying with mineral oil. Then the incubation box was closed and incubated at 36°C ± 2°C for 18-24 hours.

2.3.3.7.5 Reading and Interpretation

The strip was read by referring to the Reading Table after the incubation period was completed. When 3 or more tests (GLU test + or –) are positive, then all the spontaneous

reactions on the result sheet were recorded and then tests were revealed which required additional reagents.

The identity of the organism was derived from the database with the relevant API catalogue or apiweb^{TM}.

2.3.3.8 Antibiotic sensitivity test (AST)

Disk-diffusion method was used to determine the antibiotic susceptibility of these isolates. The media used in this test was Mueller-Hinton agar (MHA). Well isolated colonies (2-3 colonies) from MacConkey agar plate were picked up by a sterilized loop and suspended in 0.8% normal saline and then vortex was done for uniform mixing. Using an aseptic technique, a sterile swab was placed into the broth culture of a specific organism and then the excess liquid was gently removed by gently pressing or rotating the swab against the inside of the tube. Using the swab, the Mueller-Hinton agar plate was streaked to form a bacterial lawn. To obtain uniform growth, the plate was swabbed in one direction, then the plate was rotated at 90° and the plate was swabbed again in that direction. This rotation was repeated for 3 times.

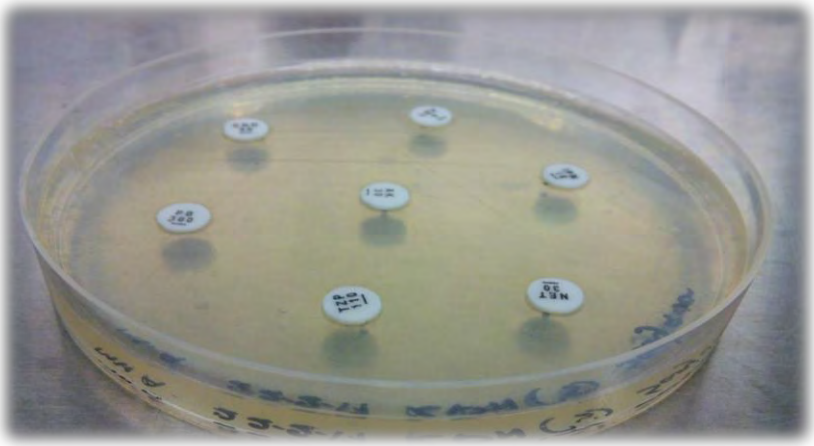

Figure 2.1: Placement of antibiotic discs on MHA containing bacterial lawn culture

The plate was then allowed to dry for approximately 5 minutes. The required antibiotic disks were then placed using one flame-sterilized forceps on the lawned surface of the Mueller- Hinton Agar. The disks were gently pressed using the forceps to the agar to ensure that the discs are attached to the agar. Plates were incubated overnight at an incubation temperature of 37 °C (98.6 °F) for 22-24 hours. Following overnight

incubation, zone sizes were measured from the edge of the disk to the end of the clear zone.

In this study, 13 different commercially available antibiotics were used. The antibiotics were selected for the Antimicrobial Sensitivity Test as those are specified for the detection of *Klebsiella pneumoniae* according to CLSI (Clinical and Laboratory Standards Institute) Guideline as modified in 2015. Thirteen antibiotic disks which were used for AST along with their zone diameter for *Klebsiella pneumoniae* are given below:

SL	Antibiotics Name	Short Name	Concentration	Result Interpretation		
				Sensitive	Intermediate	Resistant
1	Amikacin	AK	30 µg	**≥17**	**15-16**	**≤14**
3	Azithromycin	AZM	15 µg	**≥18**	**14-17**	**≤13**
4	Carbapenem	CAR	100 µg	**≥23**	**20-22**	**≤19**
5	Cefixime	CFM	5 µg	**≥19**	**16-18**	**≤15**
6	Ceftriaxone	CRO	30 µg	**≥23**	**20-22**	**≤19**
7	Ciprofloxacin	CIP	5 µg	**≥21**	**16-20**	**≤15**
8	Gentamycin	CN	10 µg	**≥15**	**13-14**	**≤12**
9	Imipenem	IMP	10 µg	**≥16**	**14-15**	**≤13**
10	Meropenem	MEM	10 µg	**≥16**	**14-15**	**≤13**
11	Netilmicin	NET	30 µg	**≥15**	**13-14**	**≤12**
12	Pipercilin+ Tazobactam	TZP	100 µg	**≥21**	**18-20**	**≤17**
13	Polymyxin B	PB	15 µg	**≥14**	**12-13**	**≤11**
14	Tobramycin	TOB	10 µg	**≥15**	**13-14**	**≤12**

Table 2.1: Zone diameter interpretation chart for *Enterobactericeae* **(Ref:** Clinical and Laboratory Standards Institute (CLSI), Zone Diameter for *Enterobactericeae*)

Pathogen specific drug lists for determining Antibiotic Sensitivity Test (AST) that is followed in ideSHi Laboratory:

Organism	Drug Lists		
	1st Generation	2nd Generation	3rd Generation
Klebsiella spp.	CN,TOB,CRO,CFM, CIP,IPM,MEM,AZM	AK,NET,TZP,CAR,PB	Lev

Table 2.2: Pathogen specific drug list for Antibiotic Sensitivity test

2.4 Phenotypic detection of Silver resistance

For phenotypic identification of resistance to silver, firstly a stock silver nitrate solution was made and then from that specific concentration, various concentrations of silver nitrate were prepared for determining the minimum inhibitory concentration (MIC) of silver nitrate in *Klebsiella pneumoniae.* Different concentrations ranging from 5 mg/L to 512 mg/L of silver nitrate were checked for detecting MIC.

2.4.1 Preparation of silver nitrate solution

- Using weighing machine 0.01698 g silver nitrate ($AgNO_3$) was measured and added into dry 15mL conical centrifuge tube
- The tube was filled with autoclaved deionized water up to 10 ml
- Then it was mixed thoroughly to make a homogenous solution using a vortex machine
- Since, silver nitrate is light sensitive material, the 15mL conical centrifuge tube containing silver nitrate solution was wrapped with an aluminium foil paper.

2.4.2 Preparation of MacConkey agar plates with different concentrations of silver nitrate

To asses MIC, a set of MacConkey agar plates were prepared with different concentrations of silver nitrate. Firstly, MacConkey agar media was prepared which is enough to fill the required petri dishes and then autoclaved in 121°C (Wise Clave, USA) for around 30 minutes. Autoclave was done for sterilizing the media that kills all microorganisms and their spores. After that, the media was allowed to cool. In the meantime, the sterile petri dishes were placed in the laminar airflow cabinet. Media was then evenly distributed into conical flasks in a way that each flask contains 20 ml media. The concentration of the stock silver nitrate solution was 10 mM. From the stock solution, silver nitrate solution at concentrations ranging from 5-512 mg/L were added in MacConkey media and were swirled vigorously for uniform mixing. The media was smoothly poured into plates until it covers the bottom. The plates were kept for several hours until media solidifies without moving them.

2.4.3 Minimum inhibitory concentration (MIC) of silver nitrate

The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that prevents visible growth of a bacterium. The MIC of a chemical is determined by preparing solutions of the chemical at different concentrations, incubating the solutions with the separate batches of cultured bacteria, and measuring the results using agar dilution method. To find out the MIC of silver nitrate, stock culture of *Klebsiella pneumoniae* isolates were kept out from the -80°C Ultra Low Temperature (ULT) freezer and allowed to thaw. Then 10 µl of the stock solution was taken and streaked into the MacConkey agar plates containing different concentrations of silver nitrate. While streaking was done, plates were incubated overnight at an incubation temperature of 37 °C (98.6 °F) for 22-24 hours in the incubator (Memmert, USA). After 24 hour of incubation, the MIC was recorded as the lowest concentration yielding no visible growth.

2.4.4 Quality control

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

2.4.4.1 Quality control for biochemical tests of the organisms

A negative control was set up for all the three types of biochemical tests that were performed. By doing this, we can check whether any contamination has occurred during the experiment or any external source is affecting the experiment or not. A negative control was set up which is the test tube containing the particular media with no inoculated organism and it was kept under the same condition as the other inoculated test tubes. Following overnight incubation, the results were compared with the negative control for verification.

2.4.4.2 Quality control for Minimum Inhibitory Concentration of silver nitrate

The quality control for the minimum inhibitory concentration of silver nitrate was established by keeping up a negative control and a positive control for the test organisms. From the MacConkey agar plates that were prepared, one concentration was set to 0 mg/L, which means no silver nitrate solution was added to that MacConkey agar. This concentration was set up as a positive control meaning that after streaking the particular organism in the plate, visible growth can be seen after 24 hour of incubation. The other plates containing silver nitrate was then compared with the positive control to verify the efficacy of silver nitrate on *Klebsiella pneumoniae*.

2.5 Genotypic detection of silver resistance genes

In order to confirm the presence of silver resistance gene in the bacterial isolates, molecular techniques such as PCR and sequencing were done.

2.5.1 Designing a PCR primer

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

2.5.1.1 Software

Primer-3 plus, Primer-Blast.

Primer-Blast is a widely used program for designing PCR primers (PCR = "Polymerase Chain Reaction"). PCR is an essential and ubiquitous tool in genetics and molecular biology. Primer-Blast can also design hybridization probes and sequencing primers. PCR is used for many different goals. Consequently, primer blast has many different input parameters that can be controlled and that tell exactly what characteristics make good primers for certain goals.
Primer-BLAST URL: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

2.5.1.2 Method

- **http://www.ncbi.nlm.nih.gov/tools/primer-blast/** was browsed
- Sequences were pasted
- Parameters were changed if necessary
- **Get primers** was clicked on and few minutes were required to get the result.

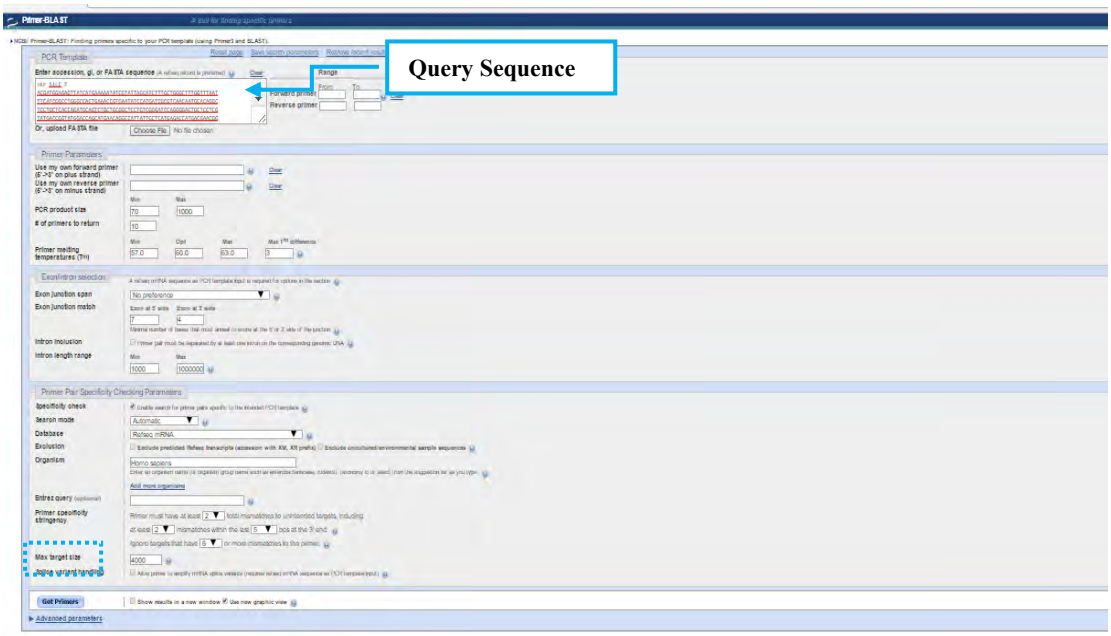

Figure 2.2: Uploading query sequence into Primer-BLAST

The name and sequence of the primers specific to the silE gene along with their melting temperature (Tm), and guanine-cytosine (GC %) content are given below:

Primer name	Primer sequence (5'-> 3')	GC content	Product length	Tm [°C]
silE (F)	GTACTCCCCCGGACATCACTAATT	50%	24	62.7
silE (R)	GGCCAGACTGACCGTTATT	52.6%	19	56.7

Table 2.3: Primers specific to silE gene and their melting temperature, size and GC content

Silver resistant gene	Product size
SilE	400 bp

Table 2.4: Desired PCR product size of silver resistant gene

2.5.2 Bacterial DNA extraction

DNA was extracted from the isolates of *Klebsiella pneumoniae.* The procedure performed in order to extract high quality DNA from the bacterial isolates is given below-

- ✓ One separated single colony was taken from MacConkey agar media by using a sterile platinum loop
- ✓ It was suspended in a 1.5 ml Microcentrifuge tube containing 100 µl autoclaved PBS then waited for 1-2 minutes

- ✓ The suspension was then vortexed for 15 seconds until homogenization
- ✓ The tube was heated at 95-99°C in a Water bath (WiseBath, USA) for 10 minutes
- ✓ It was immediately kept in ice for 1 minute
- ✓ The suspension was then centrifuged at 13000 rpm for 10 minutes
- ✓ The supernatant was transferred into another RNase and DNase free microcentrifuge tube
- ✓ The separated supernatant was used as the PCR template and the pellet was discarded. The extracted DNA was stored at -20°C for later use.

2.5.3 Measurement of DNA concentration and purity

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

2.5.4 Polymerase chain reaction for the amplification of extracted DNA

These *Klebsiella pneumoniae* isolates demonstrating resistance to silver were screened for the presence of silE gene using gene-specific Polymerase Chain Reaction. PCR is a powerful technique in molecular biology that uses Taq DNA Polymerase enzyme to amplify the quantity of a DNA sample. At first, high temperatures in the denaturation phase denature the double stranded template into single strands. Then in the annealing phase, short oligonucleotides called primers bind to the template strands. After that, the Taq Polymerase binds to the primers and starts forming new strands by adding complementary nucleotides and thus forming double stranded DNA again. This occurs in the renaturation phase.

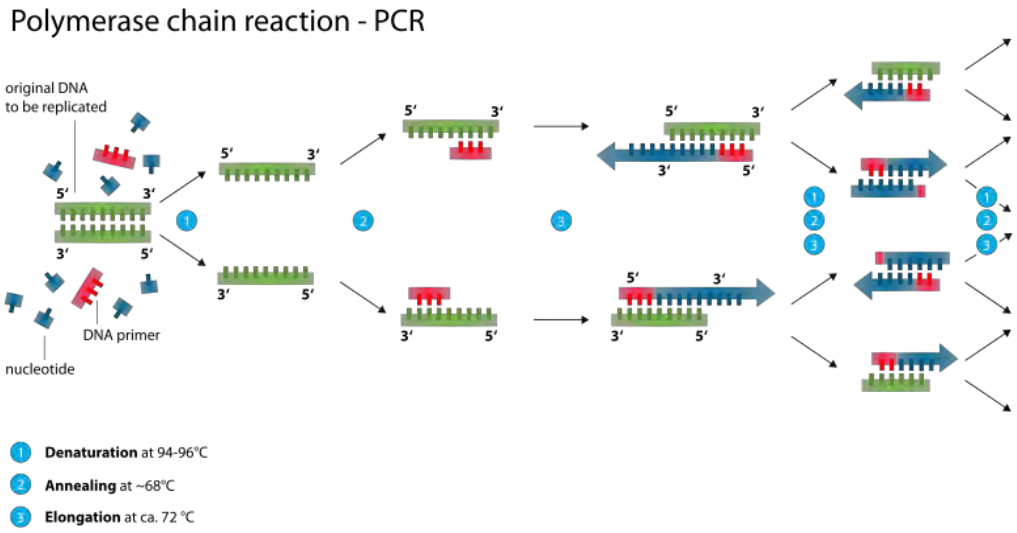

Figure 2.3: Principle of Polymerase chain reaction

(Source: https://www.thinglink.com/scene/764167700015480833)

2.5.5 Conventional PCR

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

- 1 µl 10X PCR buffer with $MgCl^{2+}$ (Clontech, code no:R001A)
- 1 µl of dNTPs mixture (2.5 mM)
- 0.5 µl forward and reverse primers
- 0.1 µl of Taq polymerase (Clontech, code no: R001A)
- 3.9 µl Nuclease Free Water
- 3 µl of template DNA

Component	Amount
2.5 mM dNTPs	1 µl
10X buffer(Mg^{2+})	1 µl
Forward primer	.5 µl
Reverse primer	.5 µl
Taq polymerase	.1 µl
Nuclease free water	3.9 µl
Template DNA	3 µl
Total volume	**10 µl**

Table 2.5: PCR master mix

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

PCR condition	Temperature	Time	
Initial denaturation	95°C	5minutes	
Denaturation	95°C	30 seconds	35 Cycles
Annealing	55°C	30 seconds	
Extension	72°C	45 seconds	
Final extension	72°C	6 minutes	
	10°C	∞	

Table 2.6: Thermal cycling profile

The annealing temperature (55°C) is related to the melting temperature (Tm) of the primers and must be determined for each primer pair used in PCR. During the extension step (72°C), the polymerase extends the primer to form a nascent DNA strand. This process is repeated multiple times (35 cycles) and because each new strand can also serve as a template for the primers, the region of interest is amplified exponentially. The final step of the PCR is generally a longer, single temperature step (6 min at 72°C) that allows for the completion of any partial copies and the clearance of all replication machinery from the nascent DNA. Once the PCR is complete, the thermal cycler is set to 4°-10°C to maintain product integrity until such time as the tubes can be removed from the machine.

2.5.6 Agarose gel preparation and electrophoresis

Agarose gel electrophoresis is a standard laboratory procedure used to separate amplified PCR product into bands based on size. Amplified DNA is applied in wells in the gel close to the negative electrode. In the presence of an electrical field, negatively charged DNA moves toward the positive pole through the small holes that make up the gel matrix. These holes allow the shorter fragments of DNA to migrate faster than their longer counterparts. Once the reaction is complete, the length of the amplified DNA can be accurately determined by comparing with a DNA ladder.

Amplified PCR products were analysed by electrophoresis on 1% agarose gel. 0.50 g agarose (Ultrapure, Invitrogen, USA) was dissolved in 50 ml 1X TBE buffer and heated to dissolve in a microwave oven for about 1-2 minutes. The mixture was then allowed to cool down at room temperature. To the cooled agarose gel, 2 µl of Gel red (Biotium,

Cat no: 41003, USA) was added. The gel was then poured on the gel casting tray previously set with the comb and allowed to solidify. While pouring the melted gel mix solution into the gel tray, care was taken so that no bubbles were formed.

2.5.6.1 Detection of PCR product using agarose gel electrophoresis

4 µl of the PCR product was mixed with 2 µl of loading dye and was loaded into the individual wells of the gel. A ladder of size 1Kb plus (Invitrogen, USA) was used to ensure amplification of the desired gene and measure the exact product size which was estimated to be within 1,500 bp. Amplified PCR products were electrophoresed at 150 volts for 1 hour. The separated DNA bands were observed on a Gel documentation system (Bio-Rad, USA) under Ultraviolet light.

2.5.7 Gel purification of PCR products

Gel purification allows isolation and purification of DNA fragments based on size. After observing the presence of the desired DNA in the PCR amplicon, DNA was purified from the agarose gel using QIAGEN gel extraction kit. Before purification, approximately 30 µl of PCR products along with the loading dye were loaded and electrophoresis was done. For getting smoother separation of DNA bands, the gel was run at 80 volts for 1.5 hours. The procedure performed in order to purify gel is given below:

- The DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The size of the gel slice was minimized by removing extra agarose.
- Then the excised gel was weighed in an Electronic balance machine (Mega, Japan standard).
- 3 volume of buffer QG was added to 1volume of gel.
- To dissolve the gel properly, it was incubated at 50°C for 10 minutes with periodic vortexing.
- After the gel has dissolved, the colour of the mixture was observed if it was yellow or not. If the colour turns orange or violet, 10 µl of 3 M sodium acetate is mixed.
- Then, 1 gel volume of isopropanol was added to the sample to increase the yield of DNA fragments.
- Then a QIA quick spin column was placed in a provided 2 ml collection tube.

- For binding of DNA, the sample solution was added to the QIA quick column and then centrifuged at 17,900 g for 1 minute.
- The flow through was then discarded.
- After discarding the flow- through, 0.5 ml of QG buffer was added and centrifuged for 1 minute to remove all the traces of agarose. This step is only required when the DNA will subsequently be used for direct sequencing.
- Later, for washing 750 µl of PE buffer was added to the QIA quick column and centrifuged for 1 minute at 17,900 x G.
- It was centrifuged twice and the flow through was discarded to remove the whole of ethanol as it will precipitate the DNA. Ethanol is PCR inhibitor and it will precipitate the product, that's why it is done twice. Residual ethanol from buffer PE will not be completely removed unless the flow through is discarded before this additional centrifugation.
- The QIA quick column was placed into a clean 1.5 ml microcentrifuge tube directly onto the white membrane without touching.
- To elute DNA, 50 µL of nuclease free water was added to the centre of the QIA quick membrane which was then centrifuged for 1 minute at maximum speed. Water is added according to the concentration.
- The column was then discarded and the supernatant containing the extracted PCR product was stored at +4°C for downstream use.

2.6 DNA sequencing (Sanger sequencing method)

DNA sequencing is the process of reading nucleotide bases in a DNA molecule. During Sanger sequencing, DNA polymerase copy single stranded DNA templates by adding nucleotides to a growing chain. During Sanger sequencing, DNA polymerases copy single-stranded DNA templates by adding nucleotides to a growing chain (extension product). Chain elongation occurs at the 3' end of a primer, an oligonucleotide that anneals to the template. The deoxynucleotides added to the extension product are selected by base-pair matching to the template. The extension product grows by the formation of a phosphodiester bridge between the 3'-hydroxyl group on the primer and the 5'-phosphate group of the incoming deoxynucleotide (Watson et al. 1987). Growth occurs in the 5' -> 3' direction. DNA polymerases can also incorporate analogues of nucleotide bases. The dideoxy method of DNA sequencing developed by Sanger takes

advantage of this characteristic by using 2', 3'-dideoxynucleotides as substrates. When dideoxynucleotides are incorporated at the 3' end of the growing chain, chain elongation is terminated selectively at A, C, G, or T. This is because once the dideoxynucleotide is incorporated, the chain lacks a 3'-hydroxyl group so further elongation of the chain is prevented.The DNA samples were sent at IEDCR (Institute of Epidemiology, Disease Control and Research) for sequencing. The calculation of cycle sequencing was obtained according to the measured template concentration.

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

Table 2.7: Master cycle components with amount

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

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

Table 2.8: Thermal cycling profile for cycle sequencing

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

2.6.1 Sequence analysis

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

2.6.2 BLAST (Basic Local Alignment Search Tool)

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

2.6.2.1 Method

- ⇨ https://blast.ncbi.nlm.nih.gov/Blast.cgi was browsed
- ⇨ Sequences were pasted
- ⇨ Parameters were changed if necessary
- ⇨ **BLAST** was clicked on and few minutes were required to get the result.

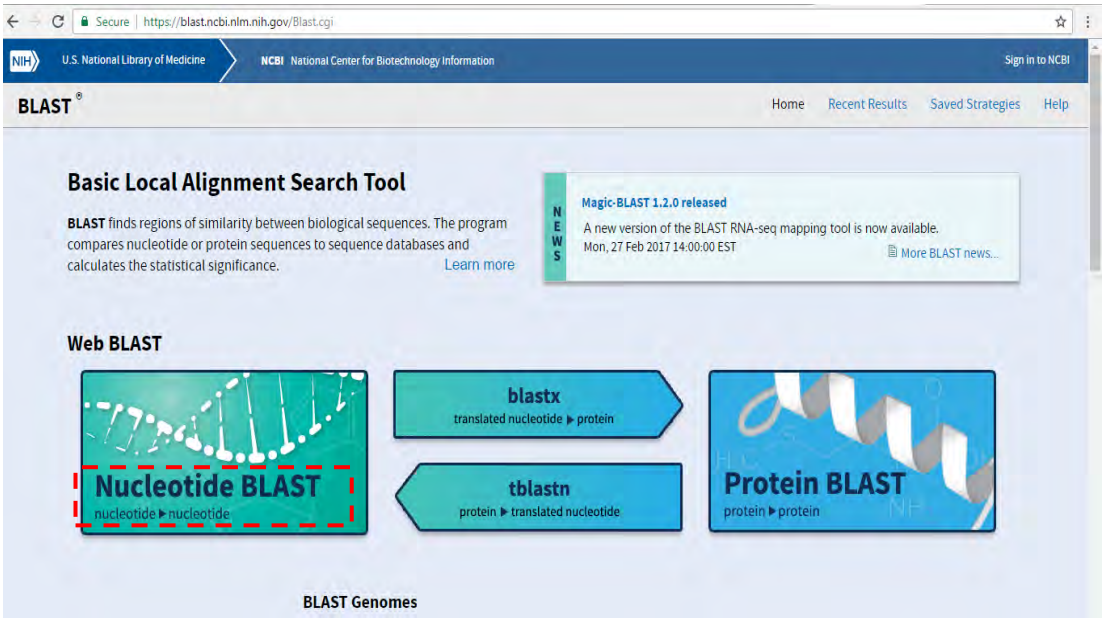

Figure 2.4: BLAST Homepage

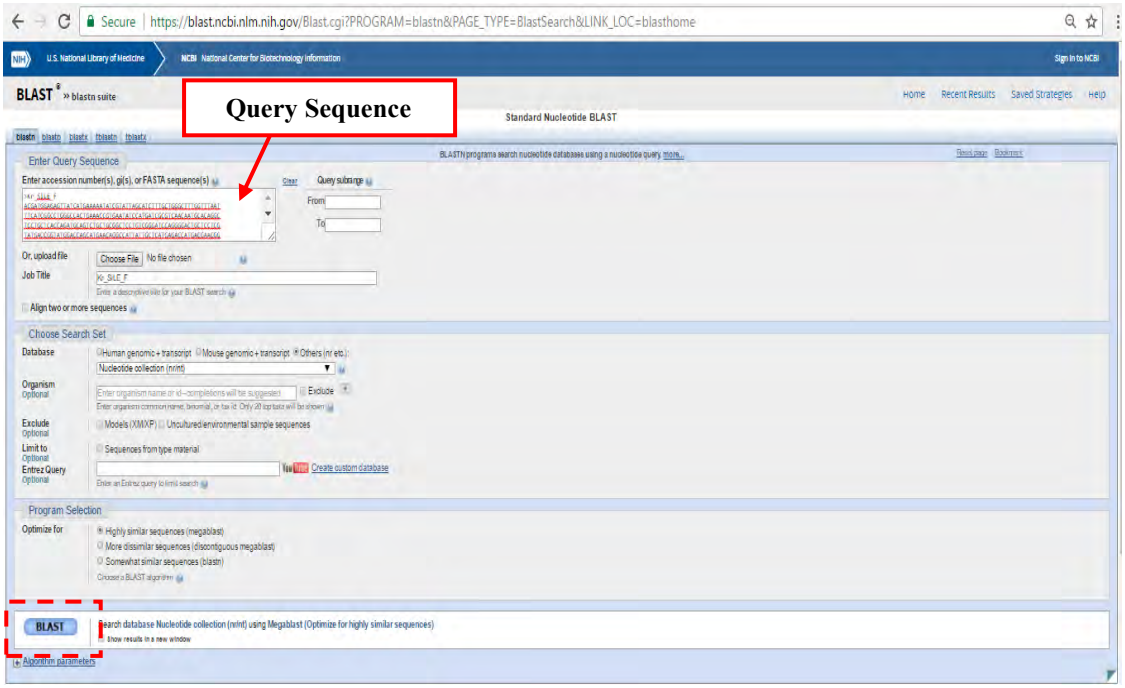

Figure 2.5: Entering query sequence into BLASTn

Unit 3

Results

3.1 Confirmation of the bacterial isolates

The isolates were confirmed using different laboratory tests. The test results were observed and recorded.

3.1.1 Colony morphology of *Klebsiella pneumoniae* isolates:

After 24 hours incubation of *Klebsiella pneumoniae* isolates on MacConkey agar plate, the colony appearance and morphology were observed for the confirmation of bacterial isolates.

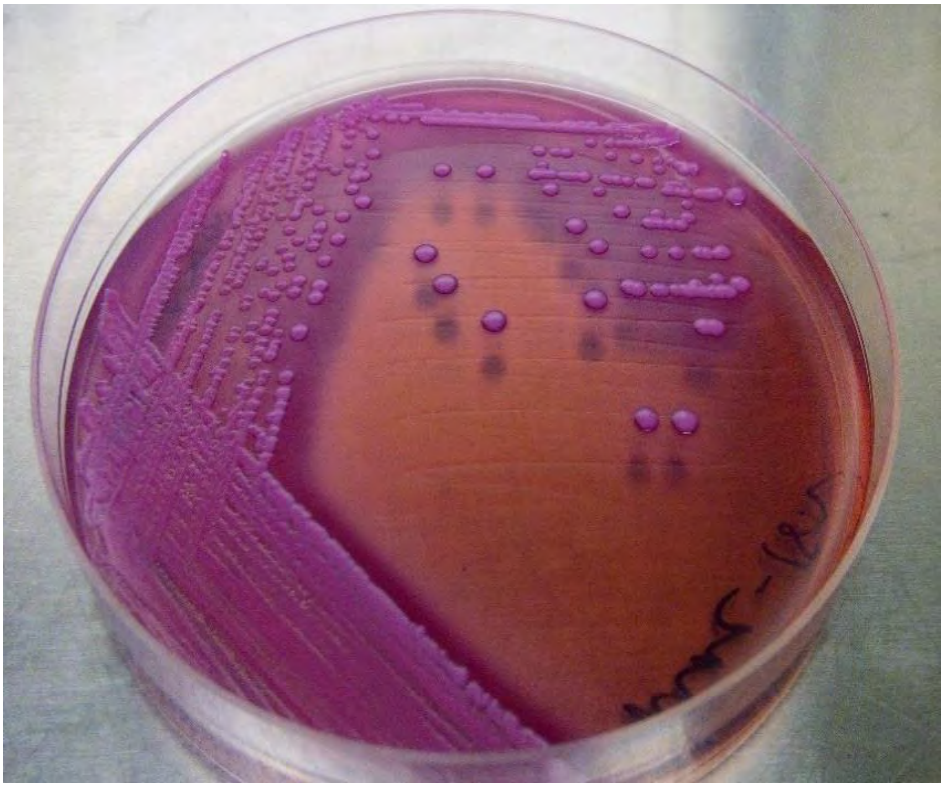

Figure 3.1: Colony appearance of *Klebsiella pneumoniae* on MacConkey agar plate

Bacterial isolate	Colony Characteristics					
	Size	Margin	Elevation	Pigment	Form	Consistency
Klebsiella pneumoniae	Medium	Entire	Pulvinate	Pink	Circular	Smooth

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

3.1.2 Gram staining results

Gram staining result of the isolates were observed under microscopic oil emersion lens for the confirmation of cellular morphology and arrangement. All the isolates retained the pink color denoting that they were gram negative rods as shown in the following figure.

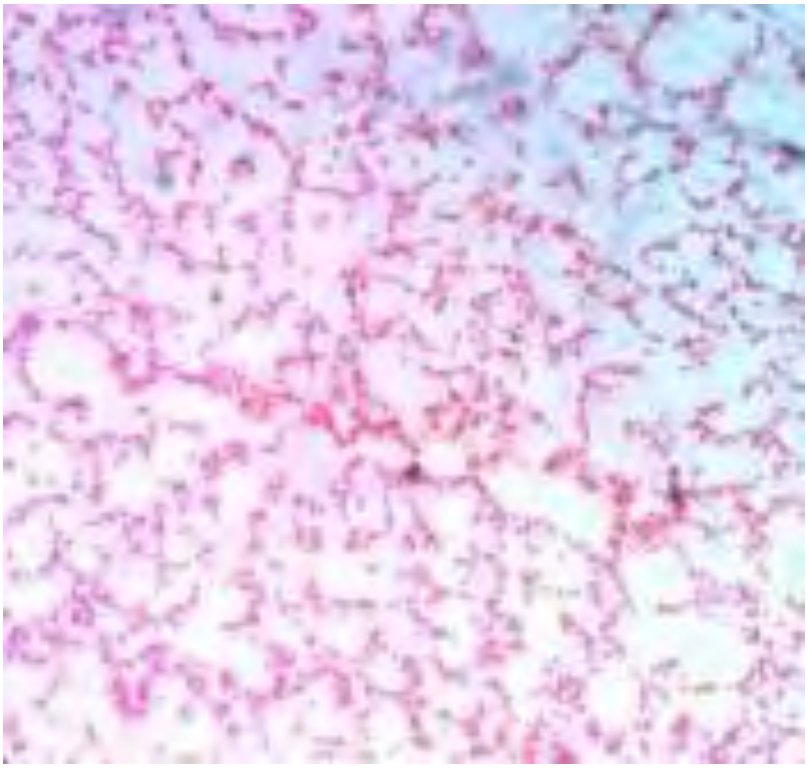

Figure 3.2: Microscopic characteristics of *Klebsiella pneumoniae* after Gram staining

3.1.3 Biochemical Identification

Isolates showed pattern of biochemical reactions typical for *K. pneumoniae*.

3.1.3.1 Triple Sugar Iron (TSI) Test

This is a differential medium containing lactose, sucrose, a small amount of glucose (dextrose), ferrous sulphate and the pH indicator phenol red. This is used to differentiate enteric bacteria based on the ability to reduce sulphur and ferment carbohydrates. The following result shows that the *Klebsiella pneumoniae* isolates were able to ferment lactose and/or sucrose and turned the slant into acidic and also all of them were able to ferment glucose which turned the butt of the TSI tube acidic. These organisms were capable of producing gas too after fermentation of the sugars. But no productions of hydrogen sulphate gas (H_2S) were observed.

Organisms	**Characteristics**			
	Slant	**Butt**	**Gas production**	**H_2S production**
Negative Control	Alkaline	Alkaline	Negative	Negative
Klebsiella pneumoniae	Acid	Acid	Positive	Negative

Table 3.2: TSI Test result of Negative Control and *Klebsiella pneumoniae*

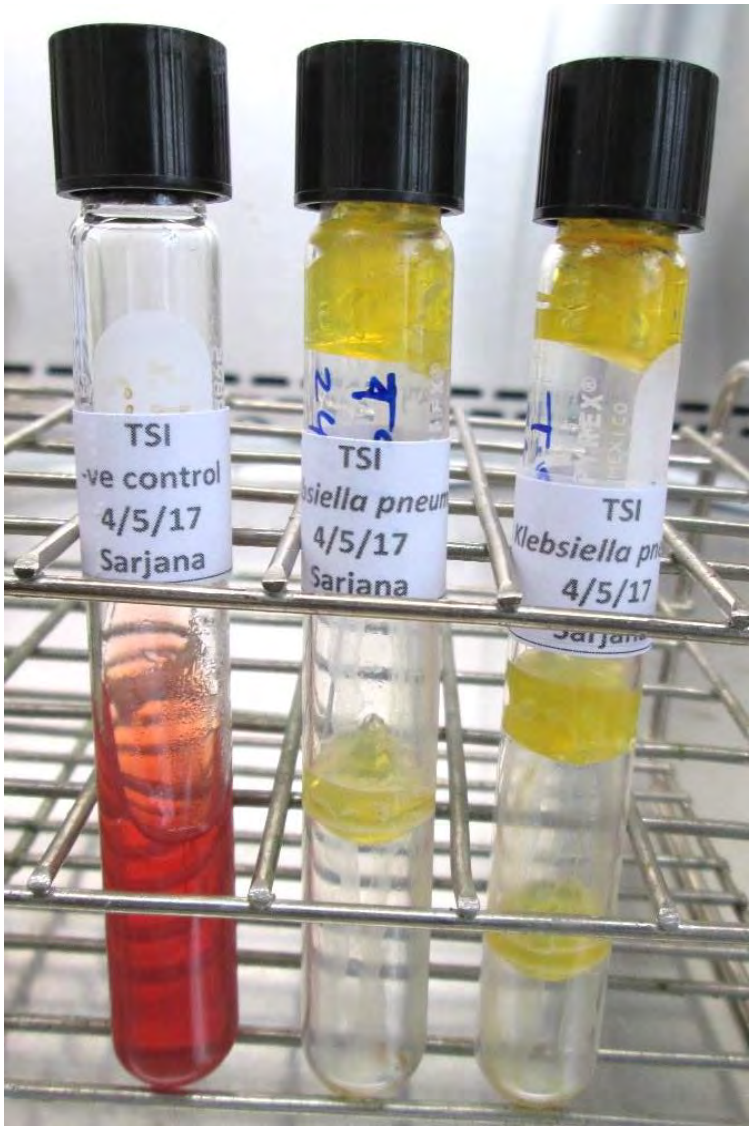

Figure 3.3: TSI Test result of Negative Control and *Klebsiella pneumoniae*

3.1.3.2 Citrate utilization test

Citrate agar is used to test an organism's ability to utilize citrate as a source of energy. The medium contains citrate as the sole carbon source and inorganic ammonium salts ($NH_4H_2PO_4$) as the sole source of nitrogen. The following result shows that the *Klebsiella pneumoniae* isolates were able to utilise citrate as a result the colour of the agar turned green to blue.

Organisms	Reaction
Negative Control	Negative
Klebsiella pneumoniae	Positive

Table 3.3: Citrate utilization test for Negative Control and *Klebsiella pneumoniae*

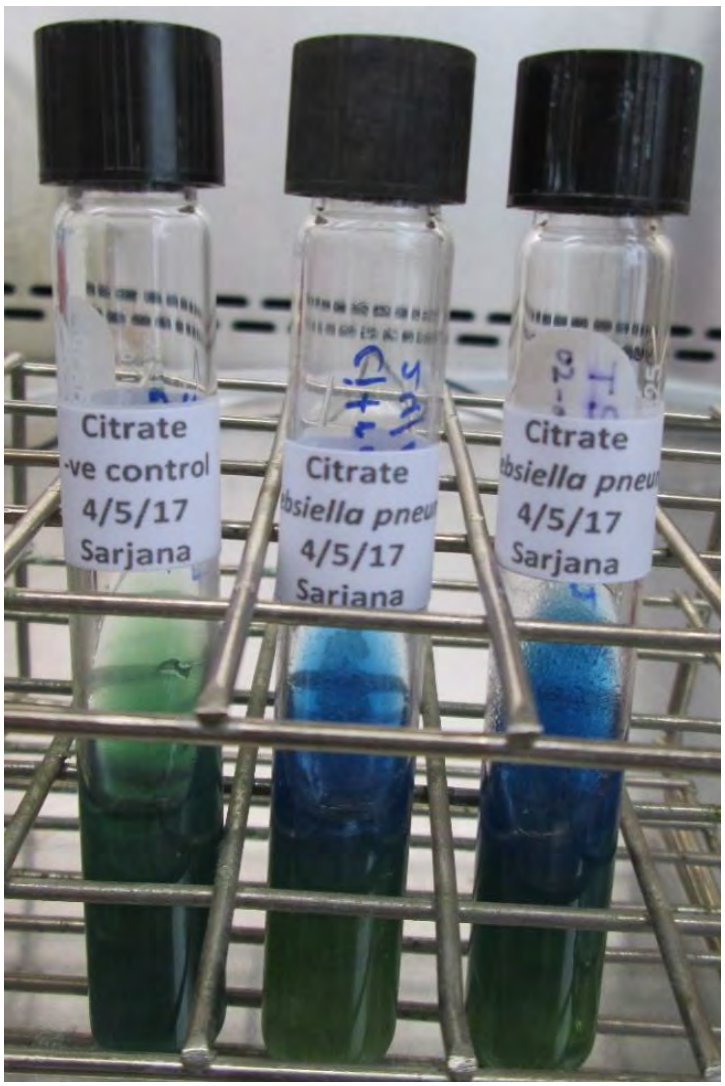

Figure 3.4: Citrate Utilization Test result of Negative Control and *Klebsiella pneumoniae*

3.1.3.3 Motility Indole urease (MIU) test

MIU Medium Base is formulated to detect motility, urease and indole production in single tube. 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 enzymic hydrolysate. The indole produced combines with the aldehyde present in the Kovac's reagent to form a red complex. The following table shows MIU results of the referred isolates together with a negative control and from the Figure 3.5 we can see that the organisms were motility negative, urease positive and indole negative.

Organisms	Characteristics		
	Motility	**Indole**	**Urease**
Negative Control	Negative	Negative	Negative
Klebsiella pneumoniae	Negative	Negative	Positive

Table 3.4: MIU Test result for Negative Control and *Klebsiella pneumoniae*

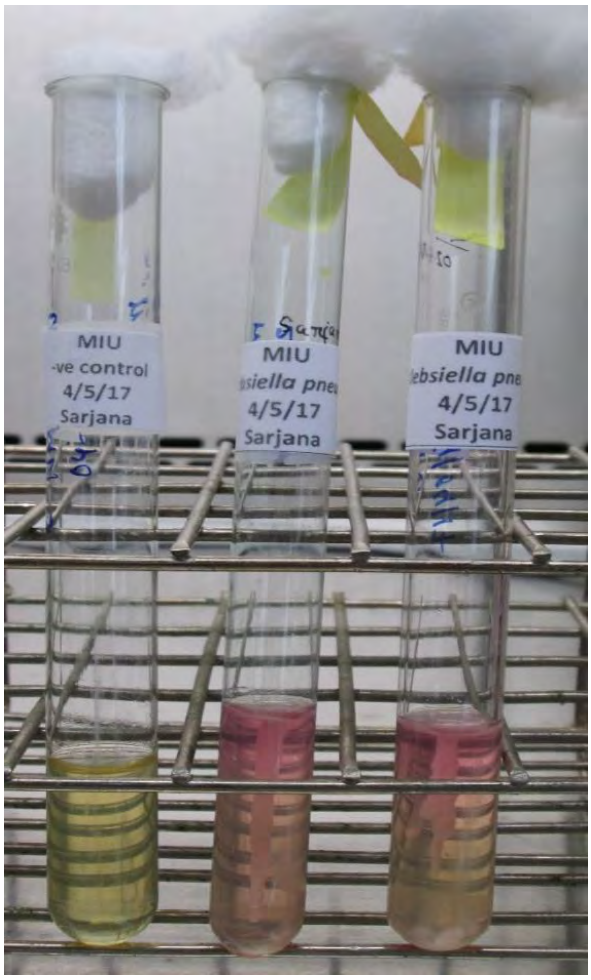

Figure 3.5: MIU Test result of Negative Control and *Klebsiella pneumoniae*

3.1.4 Catalase test results

Catalase test results of the isolates showed negative results which confirmed that these isolates were gram negative microorganisms and belonged to Enterobacteriaceae family.

Organisms	Catalase test results
Negative control	Negative
Klebsiella pneumoniae	Negative

Table 3.5: Catalase test result of Negative control and *Klebsiella pneumoniae*

Figure 3.6: Catalase test showing negative result having no bubbles

3.1.5 Oxidase test results

Negative oxidase results of the *Klebsiella pneumoniae* isolates firmly confirmed that these organisms were gram negative and a member of *Enterobacteriaceae* family. These negative result also suggested that these organisms did not have the cytochrome c oxidase that oxidizes the test reagent.

Organisms	Oxidase test results
Negative control	Negative
Klebsiella pneumoniae	Negative

Table 3.6: Oxidase test result of Negative control and *Klebsiella pneumoniae*

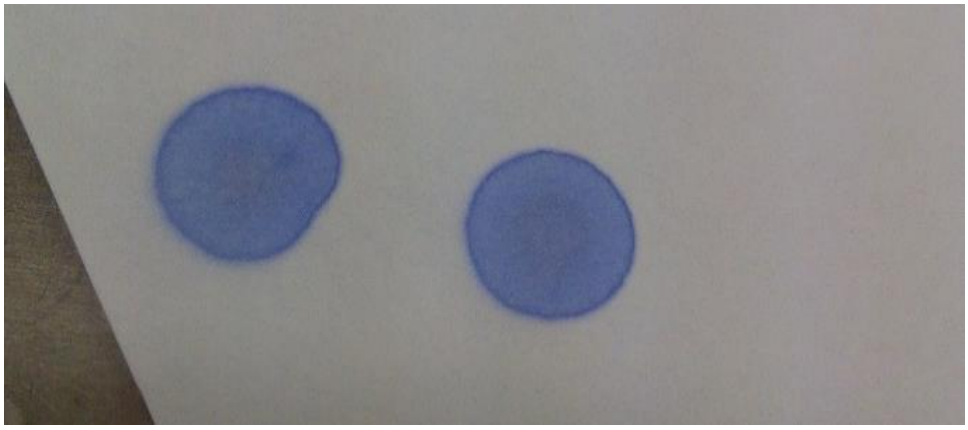

Figure 3.7: Oxidase test showing negative result

3.1.6 Analytical Profile Index

Analytical Profile Index (API) is series of cupules containing various freeze dried reagents and color indicators designed for biochemical tests. It allows fast identification of Enterobacteriaceae and other non-fastidious, gram-negative rods which uses 21 miniaturized biochemical tests and a database. According to the results of a set of biochemical tests in API® 20 E strip the reference isolates were identified to be *K. pneumoniae*. The results are shown in Figure 3.8 and Table 3.7.

Tests	Result
ONPG	Positive
ADH	Negative
LDC	Negative
ODC	Negative
CIT	Positive
H_2S	Negative
URE	Positive
TDA	Negative
IND	Negative
VP	Positive
GEL	Negative
GLU	Positive
MAN	Positive
SOR	Positive
RHA	Positive
SAC	Positive
MEL	Positive
AMY	Positive
ARA	Positive
INO	Positive
OX	Negative

Table 3.7: Analytical Profile Index test results of the isolates

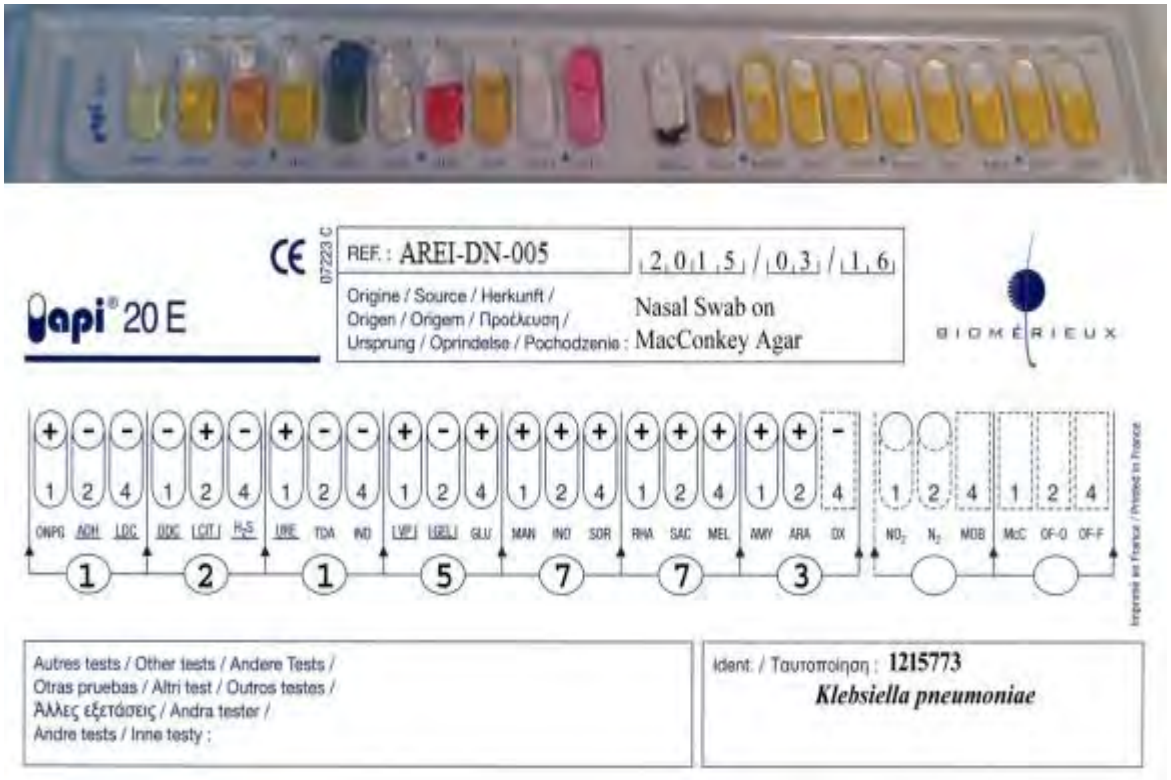

Figure 3.8: Identification of *Klebsiella pneumoniae* using API® 20 E

3.1.7 Antibiotic sensitivity test (AST)

Antibiotic Sensitivity Test gives us an idea about the resistivity or sensitivity pattern of an organism. This allows us to choose an effective antibiotic against that organism during an infection. To find out the resistivity pattern of the eleven reference *Klebsiella pneumoniae* isolates used in this study, AST was done. The result of AST is given in Table 3.8.

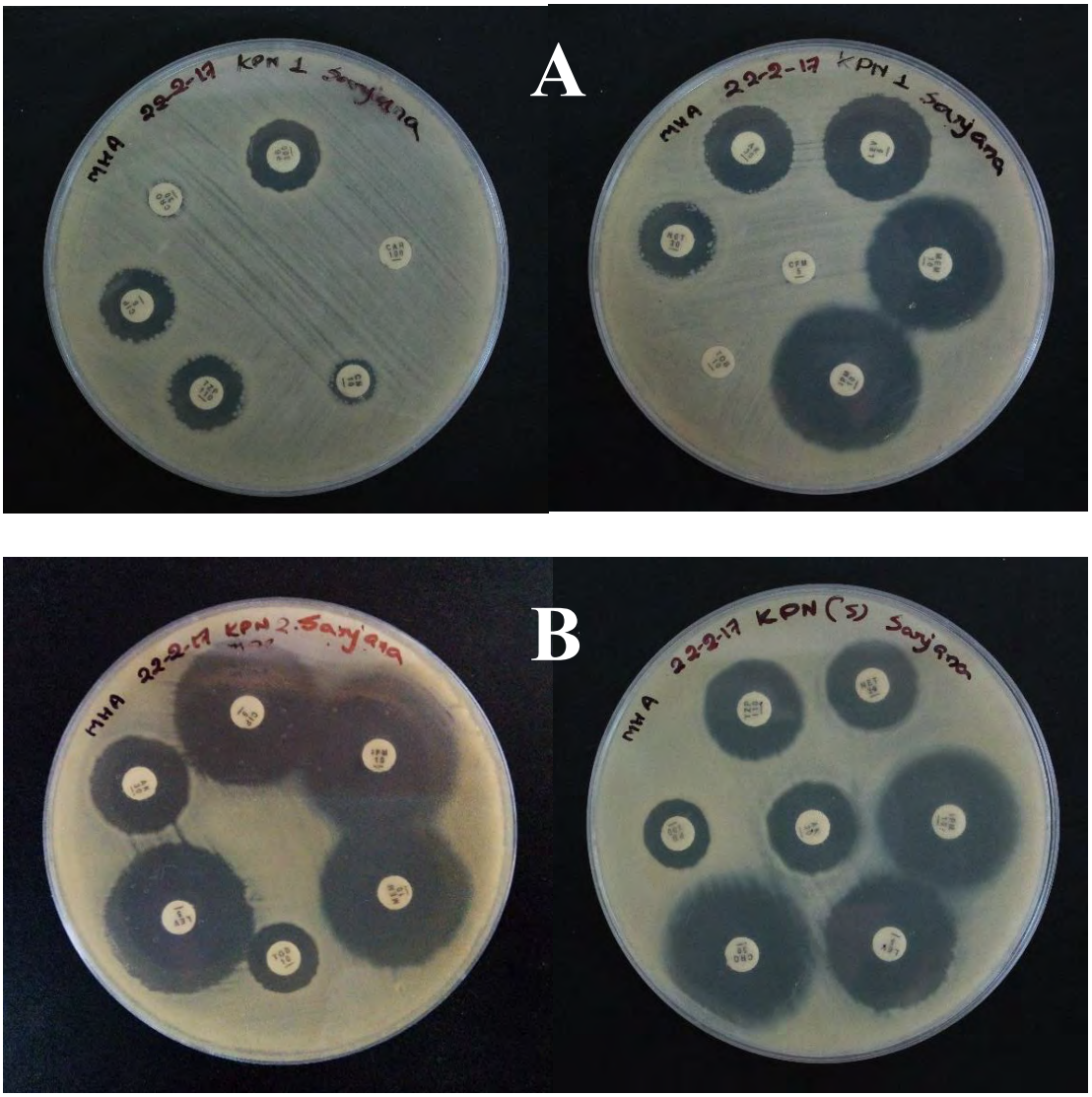

Figure 3.9: Antibiotic susceptibility test result of (A) MDR *Klebsiella pneumoniae*; (B) Sensitive *Klebsiella pneumoniae.*

SL No.	First generation antibiotics								Second generation antibiotics			
	CN	TOB	CRO	CFM	CIP	IMP	MEM	AZI	AK	NET	TZP	CAR
1	S	S	S	S	S	S	S	R	S	S	S	R
	22	19	26	23	24	17	18	9	17	17	19	6
2	S	R	R	R	S	S	S	R				
	18	17	28	22	24	19	22	8				
3	S	S	S	S	S	S	S	R				
	20	19	30	26	25	27	30	10				
4	R	R	R	R	R	S	S	R	R	R	R	R
	6	12	10	6	19	27	30	6	23	19	10	6
5	S	S	S	S	S	S	S	R				
	18	20	28	22	23	30	29	8				
6	S	R	R	R	R	S	S	R				
	20	11	16	13	14	26	25	8				
7	S	S	S	S	S	S	S	R				
	18	20	22	20	24	18	24	6				
8	S	S	S	S	S	S	S	R				
	20	22	26	22	24	26	24	9				
9	R	R	R	R	R	S	S	R				
	10	8	6	6	14	32	20	10				
10	S	S	R	R	R	S	S	R				
	20	16	6	6	18	26	22	10				
11	R	R	R	R	R	S	S	R				
	6	6	6	6	8	24	20	6				

Table 3.8: Antibiotic sensitivity test results of the eleven *Klebsiella pneumoniae* isolates (S= sensitive, R= resistant)

From the obtained result, it can be said that one of the isolates of *Klebsiella pneumoniae* was resistant to multiple antibiotics and one of them was extremely drug sensitive according to CLSI guideline.

3.2 Result of Minimum Inhibitory Concentration (MIC) of silver nitrate

The minimum inhibitory concentration of silver nitrate was determined by culturing the organisms in MacConkey agar plates containing different concentrations of silver nitrate. After overnight incubation of the plates at an incubation temperature of 37 °C, MIC was observed and recorded. The results are given below:

Organisms	Concentrations of silver nitrate (mg/L)							
	11	10	9	8	7	6	5	0
Control (no organism)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Klebsiella pneumoniae	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)

Table 3.9: Growth of the organisms in MacConkey agar with different concentrations of silver nitrate. *(-) = No growth, (+) = Growth

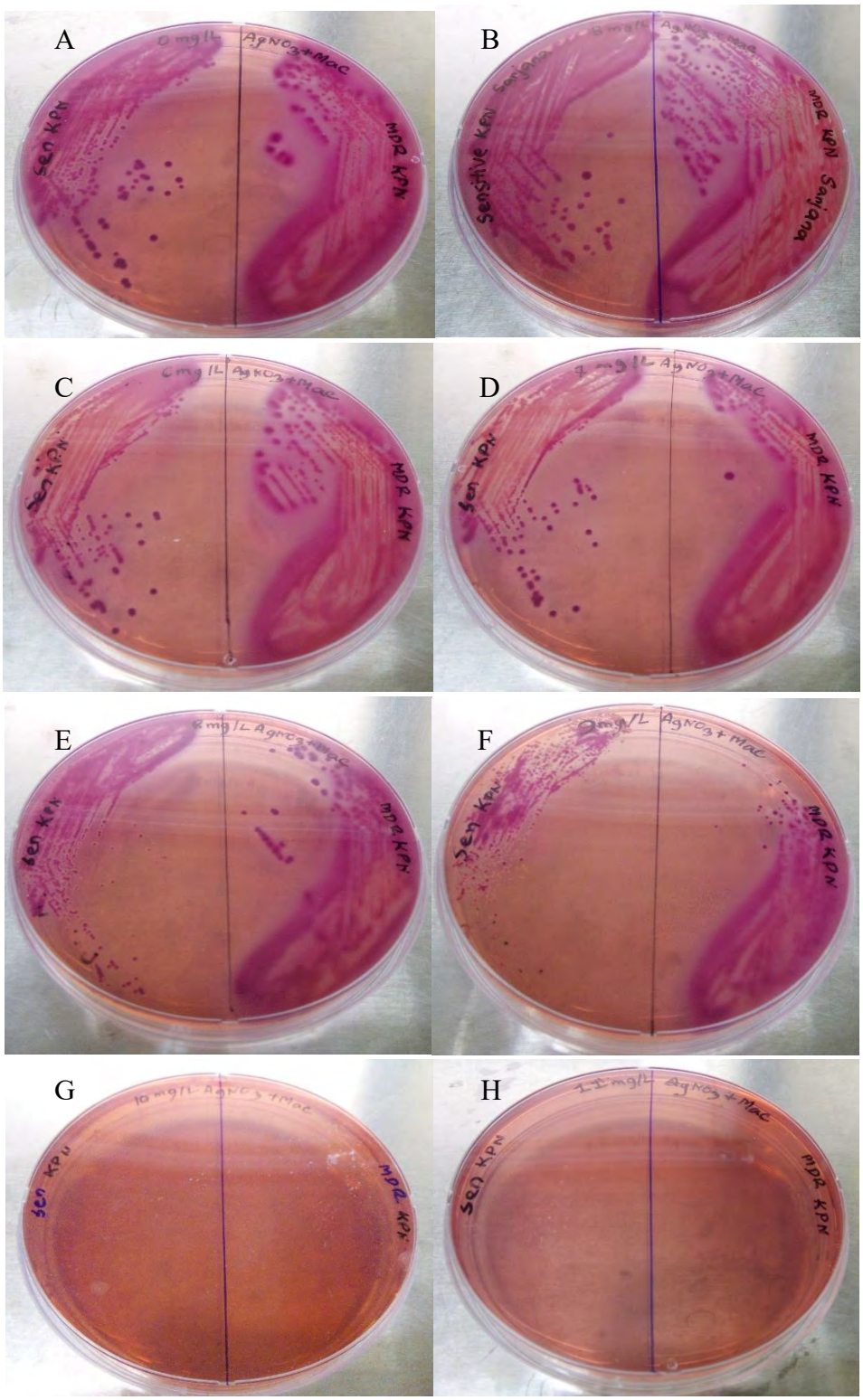

Figure 3.10: Checking the growth of *Klebsiella pneumoniae* isolates in MacConkey agar containing different concentrations of silver nitrate after overnight incubation. A. 0 mg/ml, B. 5 mg/ml, C. 6 mg/ml, D. 7 mg/ml, E. 8 mg/ml, F. 9 mg/ml, G. 10 mg/ml and H. 11 mg/ml

From the eleven bacterial isolates, only two isolates showed resistance to silver nitrate. Although sufficient growth was observed from the concentrations ranging from 0 mg/L to 9 mg/L, no growth was found in silver nitrate concentrations >9 mg/L. This result suggested that the MIC of silver nitrate for *Klebsiella pneumoniae* isolates was 10 mg/L.

3.3 Genotypic detection

In the phenotypic detection experiments, it was found that two isolates among the eleven isolates were resistant to silver nitrate. Therefore, we were interested to know whether those two isolates harbored silver resistance gene or not. To address this issue, we focused on silver resistance gene *SilE* as this was a preliminary study. In order to detect whether the PCR was successful, the amplified DNA was run in 1% agarose gel. To detect the size of the DNA band, the GeneRuler 1Kb Plus DNA Ladder by Invitrogen was used. DNA ladder is a solution of DNA molecules of different known lengths. When run alongside an unknown PCR product in an agarose gel, the ladder allows estimating the size of the unknown fragment by comparing it to the closest band in the ladder lane. Amplified products were analyzed with gel electrophoresis and interpreted visually. The band size (400 bp) of PCR product corresponded to the expected size of reported silE of silver resistance and this band was seen only in one strain of *Klebsiella pneumoniae* among the two. On the basis of partial sequence analysis, the silE homologue was identified as a periplasmic silver-binding protein.

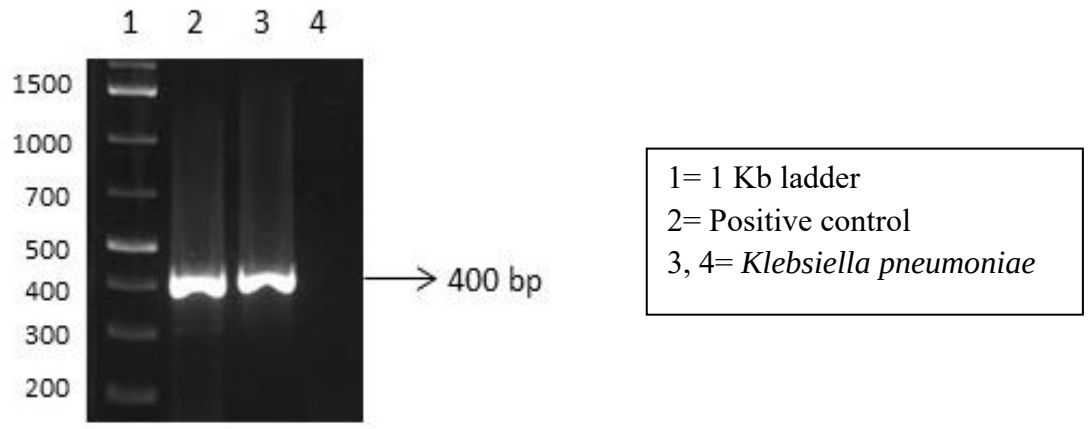

Figure 3.11: PCR amplification products of silE gene homologues resolved on Gel red stained 1% agarose gel. The desired band size (silE gene) for the gene specific primer is 400 bp. In the figure, lane 1: 1 Kb ladder; Lane 2: positive control and lane 3, 4: *Klebsiella pneumoniae*

3.3.1 Purity and amount of purified PCR product

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

Organisms	Nucleic Acid concentration	Purity (260/280 nm)
Klebsiella pneumoniae	46.3	1.80

Table 3.10- Purity and concentration of the PCR products

3.3.2 Sequencing results

The desired DNA bands for SilE gene was found and was subjected to sequencing. The sequencing data were analyzed by Chromas Lite 2.1 software**.** Chromas Lite is a handy and reliable application designed to read chromatogram files generated by sequencers. The software is able to process and export sequences without altering the original form of the data. This tool generates a four color chromatogram showing the result of sequencing run. Different bases are represented in different colors that are defined below:

1. **Adenosine= Green**
2. **Guanine= Black**
3. **Cytosine= Blue**
4. **Thymine= Red**

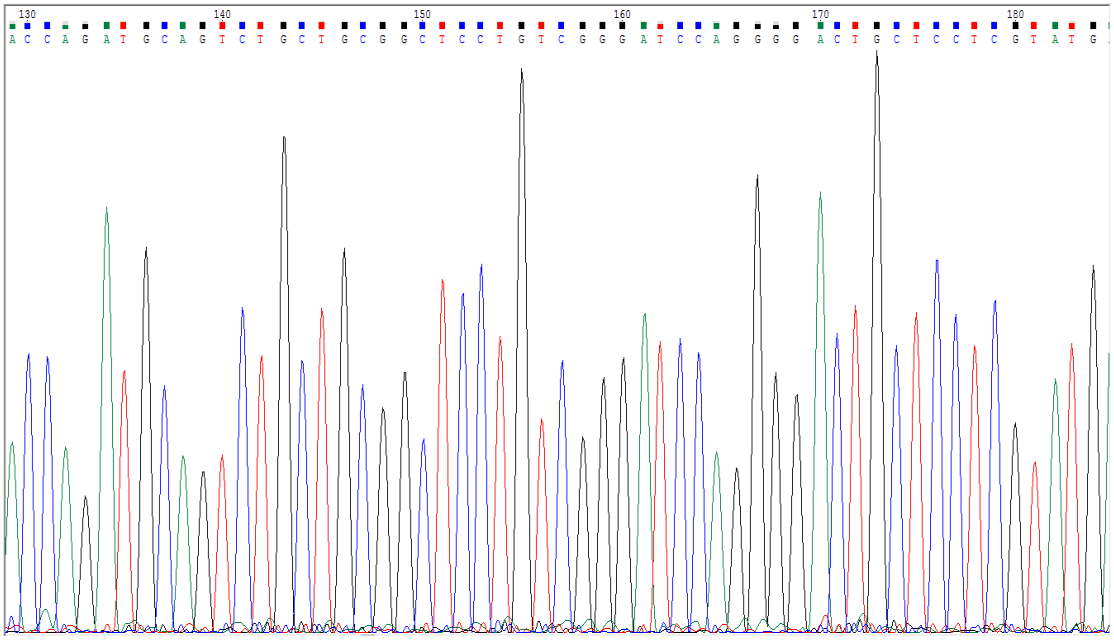

Figure 3.12: Diagrammatic representation of a part of the chromatogram of sequence.

3.3.3 Nucleotide sequence obtained

Nucleotide sequences are represented using single-letter codes in a text-based format which is known as FASTA format. A sequence in FASTA format begins with a single-line description, followed by lines of sequence data. The description line is distinguished from the sequence data by a greater-than (>) symbol in the first column. The FASTA formatted result for the bacterial strain containing silE gene is shown below-

```
>Kr_SiLE_F
ACGATGGAGAGTTATCATGAAAAATATCGTATTAGCATCTTTGCTGGGCTTTGGTTTAAT
TTCATCGGCCTGGGCCACTGAAACCGTGAATATCCATGATCGCGTCAACAATGCACAGGC
TCCTGCTCACCAGATGCAGTCTGCTGCGGCTCCTGTCGGGATCCAGGGGACTGCTCCTCG
TATGACCGGTATGGACCAGCATGAACAGGCCATTATTGCTCATGAGACCATGACGAACGG
CTCGGCGGATGCGCACCAGAAAATGGTGGAAAGTCATCAGAAGATGATGGGAAATAACAC
GGTATCCACGACCGTCCCGTCAACGTCTTACGCGGCGATGAATGAGCATGAAAGAGCAGC
GGTTGCCCATGAATTCATGAATAACGTTCACCGRCAAATCMTTYCACGCCGYYTKKKTTT
TTTTTTTTTTTTTTTTTTTTTTTTT
```

Figure 3.13: Sequencing data which consists of 447 nucleotide bases

3.3.4 Analysis of the obtained sequence

A graphic summary of the BLAST results for the target sequence was obtained where the top red bar indicated the query sequence. Database hits are shown aligned to the query, below the red bar. Of the aligned sequences, the most similar are shown closest to the query. In this case, all the hits are high scoring database matches that align to most of the query sequence. Alignment score is the summation of each specified aligned pair of bases or residues and their nulls in the alignment and the higher the alignment score, the better the alignment is. Here high alignment score indicates a significant alignments.

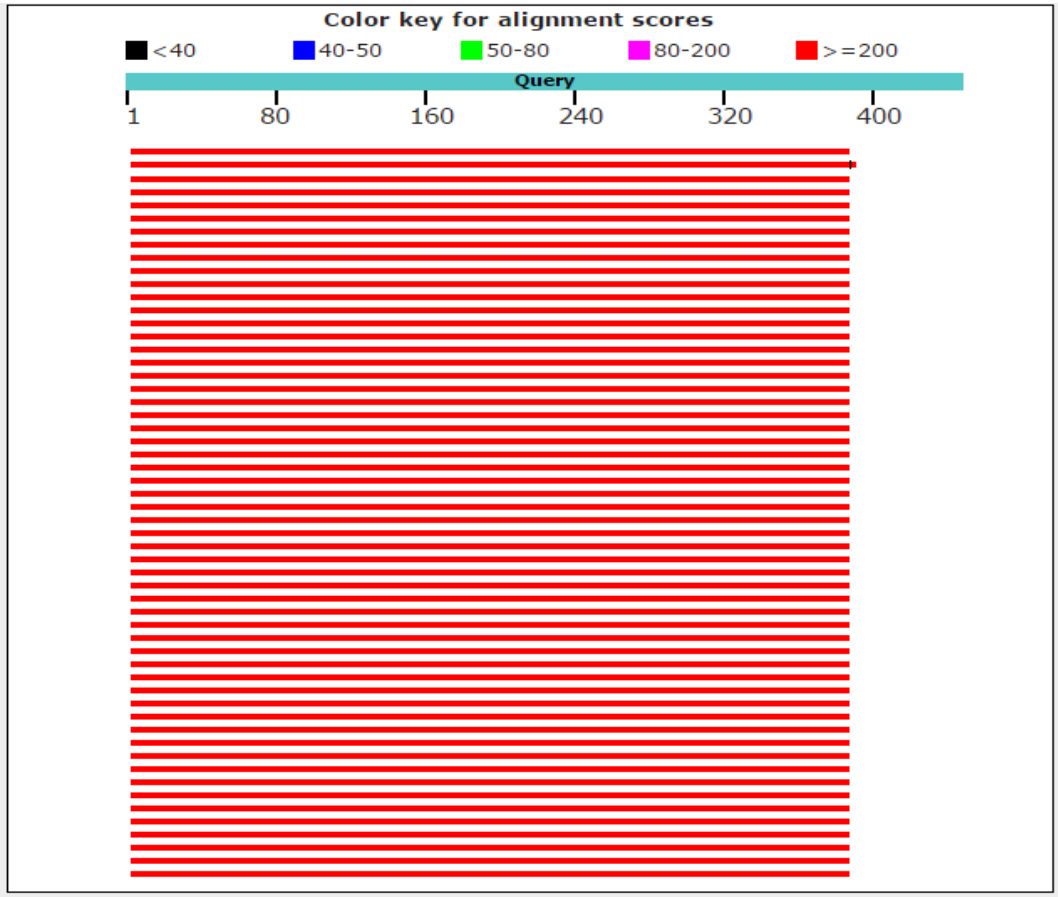

Figure 3.14: Graphic Summary of BLASTn result using the sequenced PCR product

The Descriptions table provides a summary of the database sequences identified by BLAST to be similar to the input query. Two selection controls at the top of the table, "All" and "None", allow for the quick selection and de-selection of matched database sequences. From left to right, the descriptions table columns provide information where Max Score is based on the overall score of HSPs (high scoring pairs) between sequences, similar to Expect Value. The higher the Max Score, the better the alignment between the hit and the query. Total Score is obtained by the sum of scores from all HSPs from the same database sequence. Query Coverage is the amount of the query sequence, expressed as a percent that overlaps the subject sequence. E (expected) Value describes the chance of randomly achieving the same alignment in a database of a particular size. An E Value is used to describe the significance (instead of a P value) of each sequence alignment hit to the query and Max Ident (Maximum Identity) is the highest percent identity for a set of aligned segments to the same subject sequence.

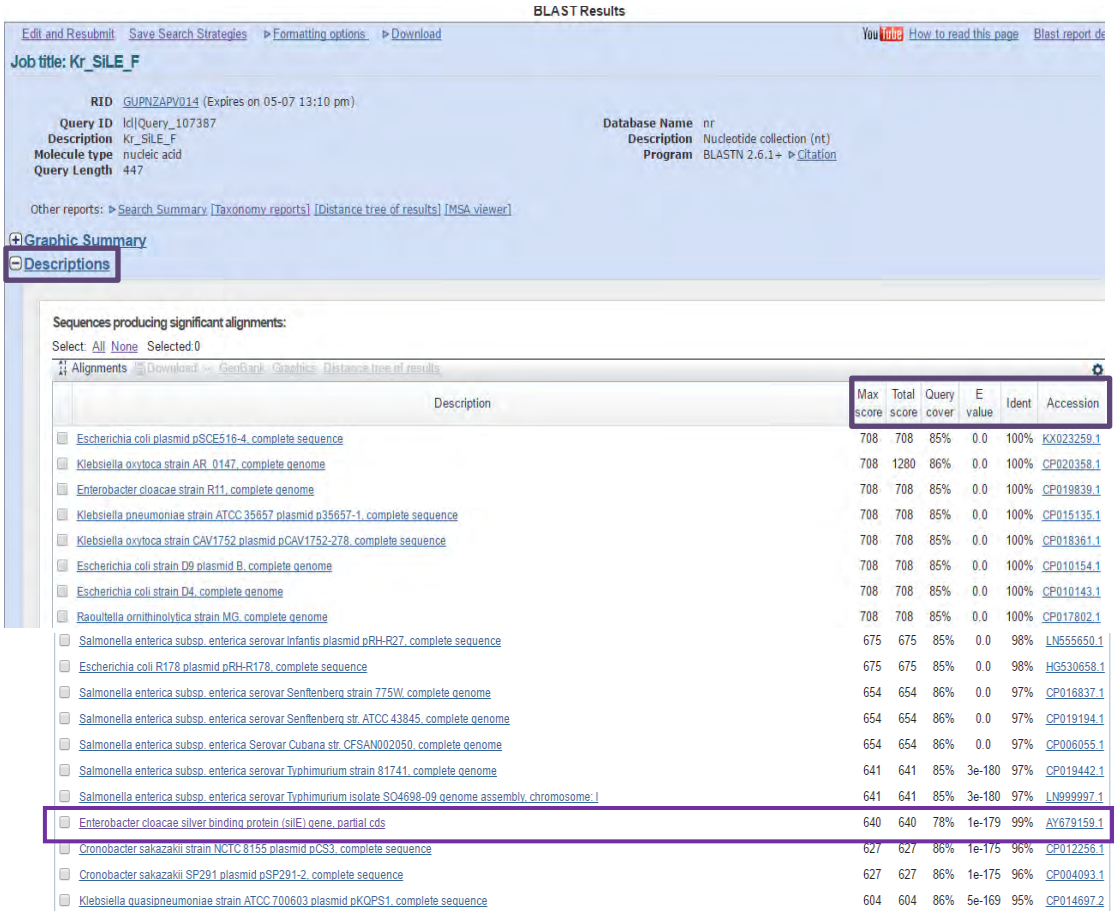

Figure 3.15: Top BLAST hits that produced significant alignments with query sequence

After BLASTn analysis, it was found that the resistant bacterial sequence matched with silE gene.

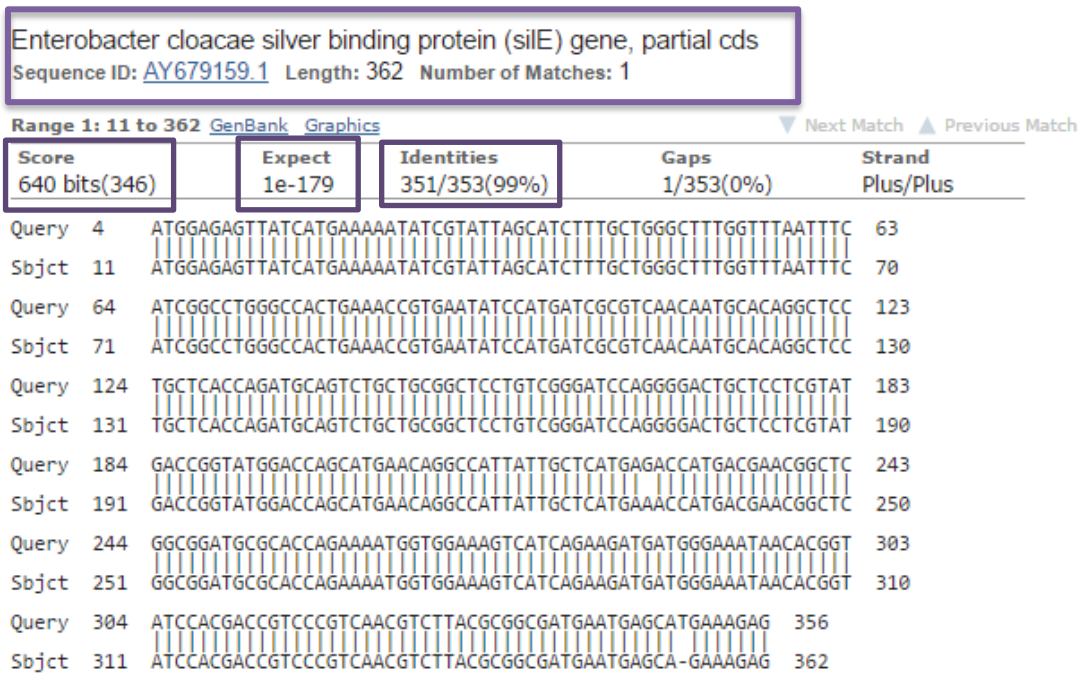

Figure 3.16: One of the matched sequence with silE gene

The quality of the alignment is represented by the Score (S).The score of an alignment is calculated as the sum of substitution and gap scores. Substitution scores are given by a look-up table (PAM, BLOSUM) whereas gap scores are assigned empirically. The significance of each alignment is computed as an E-value (Expectation value). The number of different alignments with scores equivalent to or better than S that are expected to occur in a database search by chance. The lower the E-value, the more significant the score. Hence, a sequence with low E-value is considered a good match as it is unlikely to occur by chance.

After doing the nucleotide analysis, maximum identity and low E-values were found. These maximum identity and lower E-value suggested that sequences were homologous and the amplified sequence belonged to the SilE subclass of silver resistance genes.

Unit 4

Discussion

Discussion

In recent decades, antibiotic resistance has become a global health problem. The decreasing effectiveness of antibiotics in treating common infections has quickened in recent years and with the emergence of untreatable strains of carbapenem resistant Enterobacteriaceae, we are almost at the dawn of a pre antibiotic era. In high-income countries, continued high rates of antibiotic use in hospitals, the community and agriculture have contributed to selection pressure that has sustained resistant strains, forcing a shift to more expensive and more broad-spectrum antibiotics. On the other hand, in low income and middle-income countries (LMICs), antibiotic use is being increased with rising incomes, high rates of hospitalization and high prevalence of hospital infections. Thus, resistance arises as a consequence of mutations in microbes and selection pressure from antibiotic usage that provides a competitive advantage for mutant strains, whereas suboptimum antibiotic doses help stepwise selection of resistance. Resistance genes are borne on chromosomal, and increasingly, on transmissible extrachromosomal elements (Laxminarayan et al., 2013). Furthermore, the comprehensive use of empirical therapy referred by specialists is very common in some developing countries like Bangladesh. Hence, antibiotic resistance is expanding and still no faster method is established to detect such resistance. As a result, in some serious incidents or accidents, silver-based dressings are used. Fighting an infection sooner rather than later is important to minimize morbidity, risk, and complications and so, silver compounds are frequently used in hospitals. Silver has a long and intriguing history as an antibiotic in human health care. It has been developed for use in water purification, wound care, bone prostheses, reconstructive orthopedic surgery, cardiac devices, catheters and surgical appliances (Lansdown, 2006). Burn wound mortality rates rapidly reduced to about one fourth by using silver compounds. A decade later, a series of studies reported silver resistance in Enterobacteriaceae. Since that time, an increasing number of outbreaks due to silver-resistant strains of Enterobacteriaceae have been reported (Kremer & Hoffmann, 2012). However, concerns are being expressed regarding the overuse of silver and the possible emergence of bacterial resistance to silver, particularly within the clinical environment (Percival, Bowler, & Russell, 2005). Though this is a major concern, no study related to silver resistance has yet been initiated in Bangladesh. Thus, this is the first study in Bangladesh to show phenotypic and genotypic evidence of bacterial resistance to silver.

In this study, eleven clinical isolates of *Klebsiella pneumoniae* were used for the detection of silver resistance. These isolates were isolated from nasal swab specimens which were collected from hospitalized acute respiratory infection patients. *Klebsiella pneumoniae* isolates were selected for this study as *Klebsiella* spp. are opportunistic pathogens and can give rise to severe diseases such as septicemia, pneumonia, urinary tract infections (UTI) and soft tissue infections. Typically, *Klebsiella* infections are nosocomial. The hospitalized and immunocompromised patients with underlying diseases is the main target of these bacteria. Thus, *Klebsiella* infections may serve as a paradigm of hospital-acquired infections. Their incidence of 5 to 7% of all hospital-acquired infections ranks them among the most important nosocomial pathogens and thus nosocomial *Klebsiella* infections continue to be a heavy burden on the life expectancy of patients as well as on the economy (Podschun & Ullmann, 1998). Nosocomial infection is an endemic problem encountered in hospitalized patients all over the world including Bangladesh. Estimates are that from 3% to 5% of patients leave the hospital having acquired infections, depending on case mix, hospital size, and other multiple factors (Schaberg, Culver, & Gaynes, 1991). Nosocomial bacterial infections of the respiratory tract occurs in 0.5% to 5.0% of hospitalized patients. The recognition that inhalation therapy equipment, especially reservoir nebulizers, is a potential source of infections has led to the development of effective decontamination techniques and there has been a resultant decrease in the prevalence of necrotizing bacillary pneumonia at autopsy. But the respiratory tracts of a high percentage of critically ill patients become colonized with gram negative bacilli, even with no definable environmental sources (Johanson, Pierce, Sanford, & Thomas, 1972). A cross-sectional study conducted in 1991 in the surgical wards of Dhaka Medical College Hospital showed that out of 240 patients, 72(38.0%) suffered from nosocomial infections of which 36.1% had wound infections and 23.6% had UTI. Prevalence of nosocomial infection in post-operative patients was found to be higher (49.0%) than preoperative patients (15.9%) (Azad, 2015). In one study, a two- to four fold increase in the colonization rates with *Klebsiella* was observed 2 weeks after admission to the hospital (Pollack et al., 1972) and such increase occurred primarily in patients receiving antibiotics, especially in persons receiving broad-spectrum or multiple antibiotics. Recent data from the U.S. National Healthcare Safety Network indicate that gram-negative bacteria are responsible for more than 30% of hospital-acquired infections and

these bacteria predominate in cases of ventilator-associated pneumonia (47%) and urinary tract infections (45%) (Hidron et al., 2008). Along with these statistical data and information about nosocomial infections, it is clear that *Klebsiella* spp. are one of the major pathogens in nosocomial infections. *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae* which is the most important species of the genus; hence *Klebsiella pneumoniae* isolates were selected for the detection of silver resistance in this study.

Silver nitrate is widely used as antiseptics in medicine and health in Bangladesh. Ag-coated bandages are used to cover burns and traumatic injuries. Silver-impregnated polymers are commonly used in medical devices, such as catheters to prevent the formation of bacterial biofilms. Besides these and other medical applications, silver compounds are employed in a variety of nonmedical products. For example, silver ions are used in disinfectants, water purifiers, and sterilizers in hospitals and hotels. Hence, a resistance mechanism against silver ions would serve as a potent hygienic fitness factor for bacteria, facilitating their survival in hospital environments and creating a potential infection risk for patients and thus promoting nosocomial infections (Kremer & Hoffmann, 2012). In perspective of Bangladesh, the widespread use of silver nitrate as an antimicrobial agent and the less availability of other silver compounds (such as such as silver sulfadiazine, silver zeolite and silver nanoparticles) have focused our attention to select silver nitrate for this investigation.

The present study was intended as there is no specific guideline of the standard minimum inhibitory concentrations (MIC) value of silver for *Klebsiella pneumoniae* isolates. MICs are considered the ‘Gold standard‘ for determining the susceptibility of organisms to antimicrobials (Andrews, 2001) and no study related to silver resistance had been performed in Bangladesh. To confirm the unusual silver resistance and to give a definite answer, MIC was detected. In order to confirm the MIC result, the method was repeated thrice. Out of 11 isolates, only 2 isolates displayed phenotypic silver resistance. In these 2 isolates, MIC was observed and recorded and thus, this study has presented a standard MIC value of silver nitrate at the concentration of 10 mg/L. Growth of organisms was observed at the concentrations below 10 mg/L. From Figure 3.12-3.17, it can be seen that though silver nitrate was present in MacConkey media in different concentrations, growth was observed and a decreasing growth pattern was

seen from the concentration ranging from 5 mg/L to 9 mg/L. Thus, the data gave us the initial idea about silver resistance isolates. Since other silver resistant strains have shown MIC values of up to 5440 mg/L like *Enterobacter cloacae* was found to be resistant to silver nitrate with a MIC value of >5440 mg/L (Ip, Lui, Chau, Lung, & Burd, 2006). Most of the studies have produced different MIC data for $AgNO_3$, and this demonstrates the extent of variation that currently exists with regard to the pharmacological parameters of silver. For instance, the results from other studies that explored MIC values for *Staphylococcus aureus* (around 100 strains) ranged from 8 to 80 mg/L (Ug & Ceylan, 2003). Similarly, the two largest studies examining silver ion MIC values for approximately 100 strains of *Pseudomonas aeruginosa* produced a range from 8 to 70 mg/L (Caroline, 2017). In case of *Morganella* spp., when grown in different concentrations of $AgNO_3$, the bacterium was able to grow in up to 84.94 mg/L $AgNO_3$. However, the growth rate decreased significantly at 101.922 mg/L, and no growth was observed above 101.922 mg/L. So, in that study the MIC was recorded to be at the concentration of 101.922 mg/L. The isolates from the current study appeared to have only low level phenotypic resistance to silver nitrate. In addition, as discussed by Chopra, there are no recognized breakpoints for determining true silver resistance, as with other antimicrobial therapies (Chopra, 2007). As the MIC values of this study highly varied from the other studies, so molecular approach was taken in order to confirm the presence of silver resistance gene. The gene detection methodology can contribute to the major breakthrough for screening, tracking and spread of the genes which are contributing to bacterial resistance to silver.

Investigation of the prevalence of silver resistance is important because of the possibility of plasmid transfer and hence cross-resistance in other bacteria. Chronic wounds are colonized and infected with an array of different bacteria. The wound, therefore, provides an ideal environment for the transfer of plasmids, which may contain silver resistance genes, between strains particularly as biofilms are becoming significant in this area (Davis, Richards, & Mullany, 2005). As silver resistance genes are plasmid-mediated and the isolates were collected from hospitalized patients, so it was probable that silver resistance could easily be transferred among those isolates. In this study, silver resistance was further analysed using molecular techniques such as polymerase chain reaction (PCR) followed by PCR product sequencing. Interestingly, the polymerase chain reaction and sequencing result for silver resistance gene revealed

the presence of silE gene in one isolate of *Klebsiella pneumoniae*. Among the nine silver resistance (sil) genes (*silP, silA, silB, silC, silR, silS, silE,* ORF105 and *silABC*), the presence of silE was determined by PCR followed by Sanger sequencing. Then the sequencing results were analyzed and the BLAST result for the target sequence was obtained. The nucleotide BLAST analysis represented the homologous bacteria harboring the resistant gene. BLAST result showed the genes that are believed to be part of the silver-resistant machinery in *Klebsiella pneumoniae* were highly similar to reported genes. The reported silE, which encodes a periplasmic silver-binding protein, revealed that they were 99% similar in nucleotide and amino acid composition; it also showed the presence of similar sequences in *E.coli*, *Salmonella enterica* and *Klebsiella oxytoca* isolates. As silver resistance genes are plasmid-mediated, the presence of same gene in different species may indicate a horizontal gene transfer or cross-resistance to antibiotics. Although bacterial growth was observed in two isolates phenotypically, when PCR was done, the desired DNA band was seen only in one of the two isolates of *Klebsiella pneumoniae.* Presence of other silver resistance (sil) genes might be a reason behind this finding. Other silver resistance genes such as *silP, silA, silB, silC, silR, silS,* ORF105 and *silABC*, might be involved in that strain and that's why no DNA band was observed. Whether there are indeed such associations is currently unknown and further investigation is needed to focus on this issue.

In 1983, Markowitz et al. reported a strong correlation between silver resistance and the presence of a typical "epidemic" plasmid pattern in *E. cloacae* outbreak strains causing infections in burn patients (Markowitz, Smith, & Williams, 1983). Irrespective of the medical impact of silver resistance in bacteria, the molecular genetics of silver resistance were not deciphered until the late nineties (Kremer & Hoffmann, 2012). Further evidence on silver resistance transpired from studies using *S. typhimurium*. A total number of 172 bacterial strains isolated from chronic wounds in humans and horses were screened for the presence of the silver resistance gene cassette. From the strains of *E. cloacae*, a total of 6 (2 from human, 4 from horse) bacteria were shown to contain silver resistance genes (Woods, Cochrane, & Percival, 2009). Gupta et al. described a silver resistance determinant composed of nine open reading frames (ORFs), of which seven have been named and two are still called ORFs. The unique feature of this determinant is the composition of two parallel membrane Ag(I) efflux pumps (silCBA and silP) and a periplasmic metal-binding protein (silE) (Gupta, Phung,

Taylor, & Silver, 2001). Among these genes, silE is a macromolecule which plays a major role in highly specific uptake of Ag+ ions from surrounding environment by providing histidine sites as primary candidate for Ag+ ions binding. Similarly, Parikh et al., showed the presence of SilE gene homologue in *Morganella* strains. This observation further strengthens the probable role of silver resistance genes and gene products in AgNPs synthesis in *Morganella* (Parikh et al., 2011). The isolates used in this study were examined in another study which revealed the existence of the $ESBL_A$ (Extended spectrum beta lactamase) subtype CTX-M-15. Similarly, another study showed that, among the CTX-M producers, 85% were detected as silE-positive isolates. The most common ESBL type among the carriers of silE gene was CTX-M-15 followed by CTX-M-14. Two of the three isolates with only one or two sil genes did not produce ESBL. Furthermore, silE-positive isolates are more frequently found to be resistant to antibiotics. By being associated with relatively recent types of CTX-M with more multi-resistant profiles, it is possible that the sil genes are co-selected by antibiotics to a larger extent (Sütterlin et al., 2014). Thus, silE gene is more frequently detected and more prevalent than the other sil genes. So, in this preliminary study, our choice of interest was to detect silE gene.

In this study it is shown that the silver resistance gene silE was present and functional in one of the clinical isolates. However, for better understanding of these genes, further investigation of the other eight silver resistance genes (*silP, silA, silB, silC, silR, silS,* ORF105 and *silABC*) is needed. Besides, this study was carried out using only *Klebsiella pneumoniae* isolates. It would be more appropriate if the other pathogens such as *Escherichia coli*, *Helicobacter pylori*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* which have been reported to harbor resistance to silver can be examined for detecting silver resistance. Moreover, further research and in-depth analysis are required to find out whether these genes are plasmid-mediated or not.

Genetic and phenotypic silver resistance was found in *Klebsiella pneumoniae* and it is clear that the presence of silE gene made it resistant. The existence of such genes might provide some explanation for the high infection rates in hospitalized patients. In this study, silver resistance was detected from the nasal swab specimens of hospitalized patients, so physicians should look forward to switching to a more powerful form of silver or simply increasing the dosage of silver in order to inhibit these silver-resistant

species. As silver compounds are not working and resistance genes are originating, it is in demand that new drugs are developed and invented immediately which is potent enough to work against such resistant bacteria.

With widespread use of silver in wound care, more potential pathogens are going to be exposed to this agent. In this context, therapeutic usage of silver compounds would not only be ineffective but could even be harmful, as they would select for more pathogenic strains. However, it remains to be seen whether such resistance will increase. Nevertheless, hygiene should be emphasized and targeted towards those applications which have demonstrable benefits. Moreover, it is high time to take measure to prevent the spread of these resistant genes by increasing awareness about health and hygiene and also by imposing restriction to random use of antibiotics and antiseptics. As it was a preliminary study, it would be appropriate for future studies to determine the actual prevalence of these genes within clinical and environmental settings to prevent their rapid spread.

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Appendices

APPENDIX-I

Media composition

The media composition used in this research is given below. All the media were autoclaved at 121°C for 30 minutes for sterilization.

1. 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

2. Mueller Hinton Agar (Oxoid, England)

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

3. Motility Indole Urease Agar

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**

4. Simmon's Citrate Agar (Oxoid, England)

Ingredients	Amount (g/L)
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium ammonium phosphate	0.8
Sodium citrate, tribasic	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0

5. Triple Sugar Iron Agar (Difco™)

Ingredients	Amount (g/L)
Beef Extract	3.0
Yeast Extract	3.0
Pancreatic Digest of Casein	15.0
Proteose Peptone No. 3	5.0
Dextrose	1.0
Lactose	10.0
Sucrose	10.0
Ferrous Sulfate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Agar	12.0
Phenol Red	0.024

APPENDIX-II

Instruments

List of the important equipment used throughout the study.

Name	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®
Take 3 plate	Bio-Tek
Thermal Cycler	INFINIGEN
Centrifuge Machine	Thermo SCIENTIFIC
Light Microscope	OLYMPUS CX41
Antibiotic disks	Oxoid
Gel documentation machine	Bio-Rad
Electronic balance machine	Mega