Screening for silver resistant *silE* gene in *Klebsiella pneumoniae* isolated from wound and burn patients

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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November 2017

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DECLARATION

This is to declare that the research work embodying the results in this thesis entitled "Screening for silver-resistant *silE* gene in *Klebsiella pneumoniae* isolated from wound and burn patient" submitted by Jakia Rahman. It has been carried out by the undersigned under the joint supervision of Dr. Kaiissar Mannoor, Scientist and Head of ideSHi (Institute of Developing Science and Health Initiatives); and Professor M. Mahboob Hossain, Coordinator of Microbiology Program, Department of Mathematics and Natural Sciences of BRAC University. It is further declared that the research work presented here is original and submitted for the partial fulfillment for the degree of Bachelors of Science in Microbiology, BRAC University, Dhaka, Bangladesh.

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DEDICATED TO

My Greatest Strength

'My Beloved Family'

Acknowledgement

First of all, I am grateful to the Almighty for blessing me with the opportunity of performing this research work successfully and submitting this paper. I would like to express my sincere and immense gratitude to Prof. A. A. Ziauddin Ahmad, former Chairperson and prof. A F M Yusuf Haider , Department of Mathematics and Natural Sciences, BRAC University, for allowing me and emboldening me to complete this thesis work seamlessly at Institute for developing science and health initiatives (ideSHi) Laboratory; Prof. Naiyyum Choudhury, Chairman at Bangladesh Atomic Energy Regulatory Authority (Ex. Coordinator of the Microbiology and Biotechnology Programmed, Department of Mathematics and Natural Sciences) and Prof. M. Mahboob Hossain, Department of Mathematics and Natural Sciences.

I would like to give my heartiest thanks to Dr. Firdausi Qadri, Acting Senior Director of Infectious Disease Division, icddr,b and Executive Director, Institute for Developing Science & Health Initiatives (ideSHi), for giving me the excellent opportunity to complete my thesis in laboratory. I am highly indebted to my Supervisor Dr. Md. Kaiissar Mannoor, Scientist and Head, of ideSHi for his inspiration, precious suggestions, supervision, cooperation for completing my thesis work, and for all the opportunities, I was given to conduct my research. Guiding me and solved my problems, which I have faced while doing the thesis.

My earnest admiration goes to the respective researcher of ideSHi laboratory including Dr. Sazzadul Islam, Suprovath Kumar Sarker, Tariqul Islam, Golam Sarower Bhuyain, Mohammad Amir Hossain, Mohammad Al Mahmud-Un-Nabi and Asifuzzaman Rahat for their continuous support and advice for my thesis work. Without their help it would be really difficult for me to complete my thesis.
I am lucky to have met Noorjahan Maliha, Shagoofa Rakshanda and Dr. Nusrat Sultana here and I am thankful to them for their immense love, care and unyielding support.

I also wish to express my humble regards to Tanzila Khan, Sameeya Tasnim Aurna, Kazi Sarjana Safain, Farhana Manzoor for their constant support and positivity towards my work. Last and foremost, I take this opportunity to acknowledge my cordial gratitude to my loving family for their constant support and encouragement throughout my life to achieve my goals.

Jakia Rahman
November 2017

ABSTRACT

Background: Bacteria are one-celled organisms cause various infectious disease. Wound site is more susceptible to the pathogenic bacteria and those bacteria becoming resistant to antibiotic day by day, a serious threat to public health. Silver may be a useful as a prophylactic or therapeutic agent for the prevention of wound colonization by organisms that impede healing, including antibiotic-resistant bacteria. It has been a choice antibacterial for use in wound dressings and therapeutics on account of its acknowledged low toxicity. However, concerns are being raised associated with the overuse of silver and the consequent emergence of silver resistant bacterial strains. In previous study, it was found that isolated *Klebsiella pneumoniae* (KPN) from nasal swab found to be resistant to silver. The aim of this study is to observe the frequency of KPN in burn and wound infected hospitalized patient of Bangladesh, identify the silver resistant KPN, identify the *silE* gene in silver resistant KPN strains and determine their MIC for silver.

Methodology: Specimens were collected from hospitalized patient admitted in burn and wound unit of Shaheed Suhrawardy Medical College. The samples were used for microbiological culture targeting KPN strains. To confirm KPN colony morphology, Gram staining, biochemical tests and Analytical profile index (API) were performed. Resistance for $AgNO_3$ of the isolated KPN was tested spectrophotometrically by dilution method. Polymerase chain reaction (PCR) using *silE* gene primers was carried out in Silver resistant KPN strains and further *silE* gene was confirmed by nucleotide sequencing in PCR positive samples. Finally, MIC of the resistant strains was determined spectrophotometrically using dilution method.

Results: A total number of 5 KPN strains were found in 15 collected specimens (burn/wound). Out of 5 KPN 2 strains found resistant in dilution method and PCR results targeting *silE* gene was also positive for these 2 strains. Product size for *silE* gene is about 424bp which matched with the primer amplified product (resistant 2 KPN). Nucleotide sequencing and subsequent BLASTn analysis that maximum identity and lower e-value shown in the BLASTn where query cover for KPN was 99% which indicates 99 out of 100 sequence lengths is covered by this search and results indicated that the amplified sequence belongs to the *silE* subclass. The MIC against silver nitrate was 4 mg/L and 3 mg/L for resistant KPN strains.

Discussion and conclusion: From this study, it has been found for the very first time in Bangladeshi perspective establish data based on the frequency of KPN in burn and wound infected hospitalized patient in Bangladesh, identify the silver resistant KPN and determine their MIC for silver and to identify the *silE* gene in silver resistant KPN strains with a possible evidence.

In summary, silver act as antimicrobial agent for wound infection and it is proposed that hygiene should be emphasized and targeted towards those applications that have demonstrable benefits in wound care. It would be appropriate for future studies to determine the actual prevalence of these genes and meet the final purpose of this study 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.

Table of Contents

Results

Discussion

Conclusion

List of figures

List of tables

List of flowcharts

LIST OF ABBREVIATIONS

API	Analytical Profile Index
AST	Antibiotic Sensitivity Testing
Ag	Silver
AgNO3	Silver nitrate
AgSD	Silver sulfadiazine
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
E-value	Expected value
ESBL	Extended Spectrum beta-lactamase
IEDCR	Institute of Epidemiology, Disease Control and Research
KPN	*Klebsiella pneumoniae*
kb	Kilobytes
MIC	Minimum Inhibitory Concentration
nm	Nanometer
OD	Optical Density
ORF	Open Reading frame
PCR	Polymerase chain reaction
rpm	Revolutions per minute
Sil gene	Silver resistance gene
UV light	Ultraviolet light
µl	Microliter

Introduction

1.1 Background:

Bacteria is the simplest living form on earth today are abundant for over 2 billion years before eukaryotes appeared in the world. Largest group of bacteria belongs to the group of prokaryotes. It has complex structure. Based on their, bacterial divided into two groups one is gram positive bacteria and another one is gram negative bacteria. Bacteria play a major role in modern medicine and agriculture, and have profound ecological impact. However, some pathogenic bacteria are involved in causing severe diseases such as diarrhea, Measles etc. which are related with high mobility rate. Wound site more susceptible to bacterial infection. These infections trigger the body's immune system and cause inflammation and tissue damage within the skin or wound and slow the healing process. In this study, try to discussed the severity the burn wound infection. Now a days, burns are one of the most common and devastating forms of trauma. Burn wound infection is problematic because it delays healing, encourages scarring and may result in bacteremia, sepsis or multiple-organ dysfunction syndrome (organ failure) whereby organs from several systems are unable to maintain homeostasis on their own. Resultant increased hospital stay due to wound infection has been estimated at 7-10 days, increasing hospitalization costs by 20%. Beside of this, fungi and virus are the most common pathogens of burn wounds. These microbes form multi-species biofilms on burn wounds within 48 – 72 hours of injury (Church et al., 2006). Organisms originate from the patient's own skin, gut and respiratory flora, as well as from contact with contaminated health care environments and workers (Alkaabi, 2013) such as burn wound infections due to zygomycetes such as *Rhizopus* spp. *Mucor* spp., and herpesvirus group although very rare. For treatment and diagnosis is most reliably made by histological examination or culture of burn wound biopsy specimens and requiring immediate medical attention. For inhibiting these bacterial wound infections, there is some primary care prescribed that is called antibiotic. "Antibiotics" are used as powerful medicine that can fight against bacterial infections. If it is used properly then antibiotic can save life lives by killing bacteria and destroy the bacterial regulatory system such as Penicillin was successfully used as a medicine for controlling pathogenic bacteria. From the penicillin, other antibiotics are produced like ampicillin, cephalosporin etc. But in recent time, there is a growing problem of antibiotic resistance. It means high rate antibiotic unable to resist pathogenic bacteria. According to the (2011) national survey of infectious-disease specialists, conducted by the IDSA Emerging Infections Network, found that more than 60% of participants had seen untreatable bacterial infection within the prior year (Robert, 2011). Such as

silver used as antimicrobial agent which is used wound infection(Sondi & Salopek-Sondi, 2004) and work against a broad range of micro-organisms and day by day silver is included commercially available and health care product. Most importantly, silver is an ideal wound dressing can both promote wound healing and prevent bacterial infection. The increased use of silver dressings has occurred because alternatives are required to replace antibiotics in the management of infected wounds. Here, potential dressing prepared by incorporating an optimized concentration(MIC) of silver which will exhibit good biocompatibility and appropriate porosity as well as effective antibacterial activity to accelerate wound healing. However, it is a matter of concern that, the over use of silver and the consequent emergence of bacterial resistant has being raised. Since, 2006 it was documented that silver is not act as strong antibiotic by observing biochemicals test and molecular basis. Moreover, there is no information and data regarding the silver resistant and Minimum inhibitory concentration disease severity.

So, in this report, paper represent review to assess the likelihood of widespread resistance to silver and the potential for silver to induce cross-resistance to antibiotics, find out the friendly environment efficient method to determine the efficacy of ionic silver against pathogenic wound infection. In addition, it also correlate and hypothesized that the optimized silver (Silver Nitrate) can promote wound healing and efficiently kill bacteria.

1.2 Spectrum of pathogens associated with wound/burn infection:

Burn/Wound infection is the mostly related with nosocomial infection. it accounts for significant patient morbidity, prolonged hospital stays, and increased costs (Classen et al., 1992).Enterobacteriaceae family more predominant in hospitalized nosocomial infection. Many of the traditional or familiar bacteria are found in this family such as *Salmonella*, *Escherichia coli*, *Yersinia pestis*, *Klebsiella*, and *Shigella*. Other disease-causing bacteria in this family include *Proteus*, *Enterobacter*, *Serratia*, and *Citrobacter*. This family is the only representative in the order Enterobacteriales of the class Gammaproteobacteria in the phylum Proteobacteria (Williams et al., 2010). Members of the Enterobacteriaceae are rod-shaped. They typically appear as medium to large-sized grey colonies on blood agar, although some can express pigments. These organisms have simple nutritional requirements and MacConkey agar is used to isolate and differentiate organisms of Enterobacteriaceae family (Pink colored colonies of lactose fermenter-coliforms and pale colored colonies of Non-lactose fermenter).

1.2.1 Antimicrobial Resistance:

In recent time, antimicrobial resistance increasing day by day such as Carbapenem-resistant or carbapenamase-producing Enterobacteriaceae have been reported worldwide and are major threats for global well-being. According to healthcare-associated infections (HAIs) reported the frequency of selected antimicrobial resistance patterns among pathogens such as: Carbapenem-resistant Enterobacteriaceae (CRE) are usually resistant to all β-lactam agents as well as most other classes of antimicrobial agents, *Escherichia coli* (10%), *Pseudomonas aeruginosa* (8%), *Klebsiella pneumoniae* (6%) (Hidron et al., 2008). *Klebsiella pneumoniae* carbapenemase (KPC) is one of the most common mechanism of carbapenem resistance (Donowitz & Mandell, 1988) .

1.2.2 Klebsiella spp. as Nosocomial Pathogens:

Bacteria belonging to the genus *Klebsiella* frequently cause human nosocomial infections. In particular, the medically most important Klebsiella species, *Klebsiella pneumoniae*, accounts for a significant proportion of hospital-acquired urinary tract infections, pneumonia, septicemias, and soft tissue infections. The principal pathogenic reservoirs for transmission of *Klebsiella* are the gastrointestinal tract and the hands of hospital personnel. Because of their ability to spread rapidly in the hospital environment, these bacteria tend to cause nosocomial outbreaks. Hospital outbreaks of multidrug-resistant *Klebsiella spp*., especially those in neonatal wards, are often caused by new types of strains, the so-called extended-spectrum-β-lactamase (ESBL) producers. The incidence of ESBL-producing strains among clinical *Klebsiella* isolates has been steadily increasing over the past years. The resulting limitations on the therapeutic options demand new measures for the management of *Klebsiella* hospital infections. While the different typing methods are useful epidemiological tools for infection control, recent findings about *Klebsiella* virulence factors have provided new insights into the pathogenic strategies of these bacteria. *Klebsiella* pathogenicity factors such as capsules or lipopolysaccharides are presently considered to be promising candidates for vaccination efforts that may serve as immunological infection control measures (Weinstein et al., 2005).

1.3 Silver:

Information regarding antibiotic/resistance pattern of pathogenic organisms are lacking in Bangladesh. As a result, empirical antibiotic intervention is given to the patient which results in increased antibiotic resistance.

Silver is most important antimicrobial agent (Kim et al., 2007) . It uses throughout the world day by day.

1.3.1 Use of silver:

The use of silver in wound management can be traced back to the 18th century, during which silver nitrate (AgNO3) was used in the treatment of ulcers (Klasen, 2000). The antimicrobial activity of the silver ions was first identified in the 19th century. Silver began to be used for the management of burn patients in the 1960s, this time in the form of 0.5% AgNO3 solution (Price & Wood, 1966). AgNO3 was combined with a sulphonamide antibiotic in 1968 to produce silver sulfadiazine (SSD) cream, which created a broader spectrum silver-based antibacterial that continued to be prescribed mostly for the management of burns (George et al., 1997). More recently, clinicians have turned to wound dressings that incorporate varying levels of silver, because the emergence and increase of antibiotic-resistant bacteria have resulted in clinical limitations in the prescription of antibiotics (Gemmell et al., 2006) .

1.3.2 Silver products and delivery modalities:

Elemental silver requires ionization for antimicrobial efficacy. Silver ion is a highly reactive species, readily binding to negatively charged proteins, RNA, DNA, chloride ions, and so on. This property lies at the heart of its antibacterial mechanism but also complicates delivery to the wound bed, because it is readily bound to proteins within the complex wound fluid. Different silver delivery systems exist, including those that deliver silver from ionic compounds, such as silver calcium phosphate and silver chloride, and those that deliver silver from metallic compounds, such as nanocrystalline silver (Kirsner et al., 2001). However, the difficulties with many current topical silver antimicrobials lie in their low silver release levels, the limited number of silver species released, the lack of penetration, the rapid consumption of silver ions, and the presence of nitrate or cream bases that are pro-inflammatory negatively affecting wound healing. Other issues include staining, electrolyte imbalance, and patient discomfort. Over the past few years, there has been a

rapid increase in the number of silver dressings made available to physicians to address these issues (Atiyeh et al., 2007). Various available silver products may be summarized as follows:

1.3.2.1 Absorption of silver:

Silver is absorbed into the human body and enters the systemic circulation as a protein complex to be eliminated by the liver and kidneys. Silver metabolism is modulated by induction and binding to metallothioneins. This complex mitigates the cellular toxicity of silver and contributes to tissue repair. Silver allergy is a known contra-indication for using silver in medical devices or antibiotic textiles.

1.3.2.2 Silver proteins:

Consist of silver complexed to small proteins in order to improve stability in solution. These however proved to possess much less antibacterial action than pure ionic silver and were rapidly replaced by silver salts in the 1960s (Atiyeh et al., 2007).

1.3.2.3 Inorganic silver compound:

Inorganic silver compound is germicidal and hence have been used extensively in the field of medicine. These compounds denature proteins by binding to the reactive groups of proteins resulting in their precipitation. They inactivate enzymes by reacting with the sulfhydryl groups to form hemi silver sulfides. They also react with the amino-, carboxyl-, phosphate-, and imidazole-groups and diminish the activities of lactate dehydrogenase and glutathione peroxidase.

1.3.2.4 Silver salts:

Delivery system becomes more stable when positively charged silver ion is complexed to negatively charged ions (AgCl, AgNO3, AgSO4). These are following below: (Fox & Stanford, 1971).

1.3.2.5 Silver nitrate:

0.5% Silver nitrate is the standard and most popular silver salt solution used for topical burn wound therapy. Concentrations exceeding 1% silver nitrate are toxic to the tissues. Ionic silver solutions are highly bactericidal, with no reported resistance and have a beneficial effect in decreasing wound surface inflammation. The solutions, however, are unstable and when exposed to light

produce typical black stains therefore extremely unpractical. On the other hand, nitrate is toxic to wounds and to cells and appears to decrease healing off setting to some degree the beneficial antibacterial effect of silver. Moreover, the reduction of nitrate to nitrite causes oxidant induced cell damage. This effect is most likely the reason for the impaired re-epithelialization reported with its use in partial thickness burns or donor sites (Moyer et al., 1965).

1.3.2.6 Silver sulfadiazine:

In 1970, silver sulfadiazine was first introduced as a antimicrobial agent for tropical treatment to help prevent and treat wound infections in patients with serious burns (Alexander, 2009). Silver is complexed to propylene glycol, stearyl alcohol, and isopropyl myrislate and mixed with the antibiotic Sulfadiazine producing a combined formulation made from silver nitrate and sodium sulphadiazine by substituting a silver atom for a hydrogen atom in the sulphadiazine molecule. works by stopping the growth of bacteria that may infect an open wound. This helps to decrease the risk of the bacteria spreading to surrounding skin, or to the blood where it responsible for serious blood infection (sepsis). Silver sulfadiazine belongs to a class of drugs known as sulfa antibiotics (Stanford et al., 1969).

1.3.2.7 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).

1.3.3 Silver products efficacy:

Very few randomized prospective studies on the use of silver have been published (Cutting et al., 2007)however, the role and the mechanism of action of silver ions in vivo continue to provide a steady contribution to the surgical literature. For silver to be biologically active, it must be in a soluble form such as Ag+ or Ag0 clusters (Dunn & Edwards-Jones, 2004) and any silver dressing efficacy is determined by total available soluble silver, not total silver in the dressing. Ag0 is the metallic or uncharged form of silver found in crystalline, including nanocrystalline, silver

structures. In solution, it exists in a sub-crystalline form, less than eight atoms in size. Ag+ is the familiar ionic form present in silver nitrate, silver sulfadiazine and other ionic silver compounds. In wound management, silver quantities should be sufficient to provide sustained bactericidal action (Dunn & Edwards-Jones, 2004). Since there is no point in having a long duration of activity if the low concentration may result in the development of resistance, maintaining an adequate concentration of silver in a dressing over time has been a challenge. Metallic-coated dressings release silver over a long period. Silver nitrate has a high concentration of silver but no residual activity necessitating very frequent applications. Silver sulfadiazine, on the other hand, provides an adequate concentration of silver but has limited residual activity. Thus, silver dressings appears to alter the biological properties of the solution, including both antimicrobial and anti-inflammatory activity (Taylor et al., 2005).

1.3.4 Silver in health care antimicrobial effects and safety in use:

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. The antimicrobial action of silver or silver compounds is proportional to the bioactive silver ion (Ag^{+}) released and its availability to interact with bacterial or fungal cell membranes. Silver metal and inorganic silver compounds ionize in the presence of water, body fluids or tissue exudates. The silver ion is biologically active and readily interacts with proteins, amino acid residues, free anions and receptors on mammalian and eukaryotic cell membranes. Bacterial (and probably fungal) sensitivity to silver is genetically determined and relates to the levels of intracellular silver uptake and its ability to interact and irreversibly denature key enzyme systems. Silver exhibits low toxicity in the human body, and minimal risk is expected due to clinical exposure by inhalation, ingestion, dermal application or through the urological or hematogenous route. Chronic ingestion or inhalation of silver preparations (especially colloidal silver) can lead to deposition of silver metal/silver sulphide particles in the skin (argyria), eye (argyrosis) and other organs. These are not life-threatening conditions but cosmetically undesirable (Lansdown, 2006).

1.3.5 Mode of action of silver:

Plasmid pMG101 is a 180-kb IncH1 silver resistance plasmid that also confers resistance to mercury and tellurite, and to several antibiotics. Although silver sulfadiazine-resistant bacteria have occasionally been observed elsewhere in burn ward infections. The region of pMG101 that determines increased resistance to Ag(I) was cloned and sequenced (GenBank accession AF067954) (Gupta et al., 1999). 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* ORF105*silAB* ORF96 *silC, silSR* ,*silE*. In this study, trying to show the gene pattern of *silE* gene (Lo, 2001).

the silver resistance determinant (Fig. 1.1), the first gene *silE* encodes a periplasmic Ag(I)-binding protein (*silE*) (Fig. 1.1). Two parallel membrane Ag (I) efflux pumps (*SilCBA* and *SilP*) are encoded. The central six genes (*silA* through *silS*) produce products. *silE* is a small periplasmic protein that is 47% identical to PcoE (pcoE is a homologous of *silE*), of the gram negative bacterial plasmid copper resistance system. The *silE* polypeptide has its first 20 amino acids removed on movement across the membrane to the periplasm and is synthesized only during growth in the presence of Ag(I) (Gupta, 1999). *silE* and pcoE DNAs and transcriptional promoters and regulatory sites (Fig. 1.1) are homologous but the sequences upstream of *silE* and pcoE are transcribed separately. The remaining six ORFs in the Ag+ resistance system are transcribed divergently from *silRSE* (Gupta et al., 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, etal. , 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).

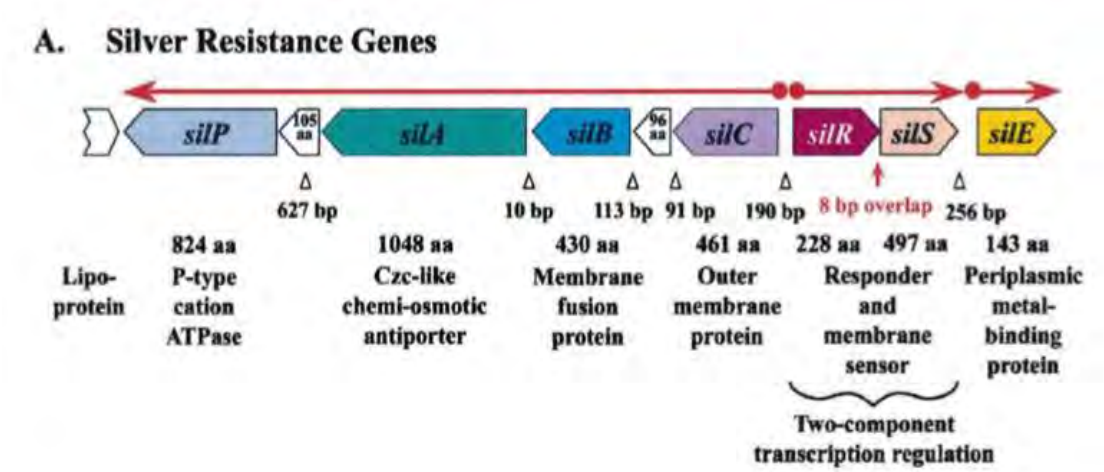


Figure 1.1 Silver Resistant Genes

Silver nanoparticles are able to physically interact with the cell surface of various bacteria. This is particularly important in the case of Gram-negative bacteria where numerous studies have observed the adhesion and accumulation of silver nanoparticles to the bacterial surface. The silver ions bind to the protein and nucleic acid negatively charged, causing structural changes and deformations in the wall, in the membranes and in the nucleic acids of the bacterial cell. AgNPs (silver nanoparticles) can damage cell membranes leading to structural changes, which render bacteria more permeable.

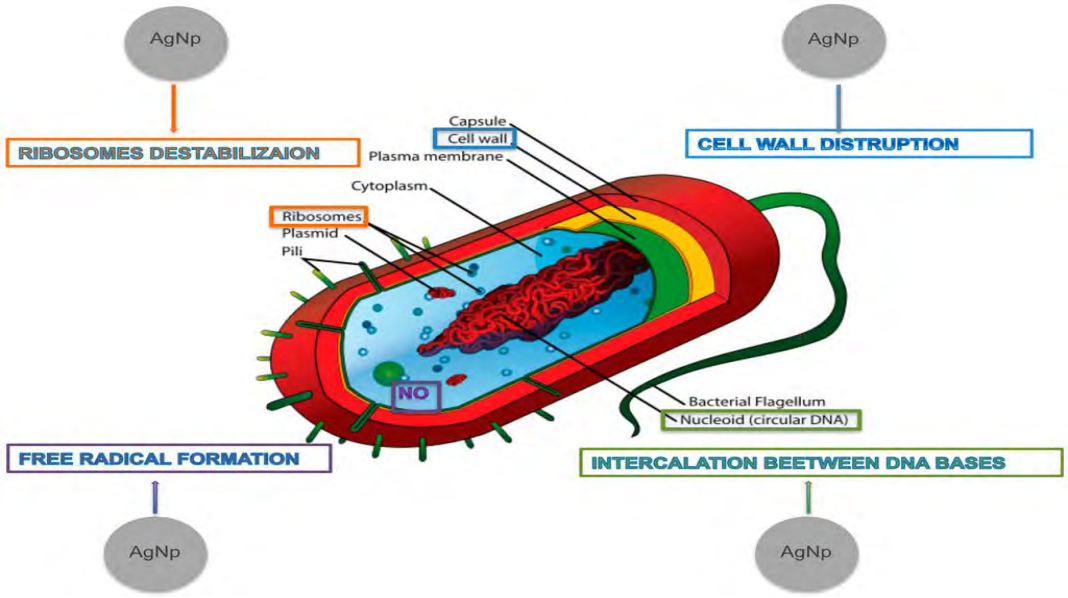


Figure 1.2 Mechanisms of AgNPs'

This effect is highly influenced by the nanoparticles size, shape and concentration. AgNPs accumulation on the membrane cell creates gaps in the integrity of the bilayer which predisposes it to a permeability increase and finally bacterial cell death. Several studies have shown that AgNP activity is strongly dependent on the size. In fact, the bactericidal activity of AgNPs of smaller dimensions (<30 nm) was found to be optimal against *Staphylococcus aureus* and *Klebsiella pneumoniae*. Smaller nanoparticles seem to have a superior ability to penetrate into bacteria. In fact, the interactions with the membranes and any resulting damage, which may lead to cell death. As a consequence, ribosomes may be denatured with inhibition of protein synthesis, as well as translation and transcription can be blocked by the binding with the genetic material of the bacterial cell. Protein synthesis has been shown to be altered by treatment with AgNPs and proteomic data have shown an accumulation of immature precursors of membrane proteins resulting in destabilization of the composition of the outer membrane (Franci et al., 2015).

1.3.6 Antibiotic Resistance against Silver:

Silver products have been known to have inhibitory and bactericidal effects. However, silver-resistant bacteria have been found repeatedly in environments where silver toxicity might be expected to select for resistance, in particular from burn wards of hospitals where silver salts (silver nitrate but especially silver sulfadiazine) are used as antiseptics to treat burns such as. *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus.* So, Resistance to antimicrobial agents by pathogenic bacteria has emerged in recent years and is a major health problem. It is known that silver resistance bacteria contains a total of nine genes (Lo, 2001). But in many studies, it was found that *silE* gene is more frequent that other genes and highly significant that *silE* is more susceptible to silver (Parikh, 2008) and MIC(minimum inhibitory concentration) also varied in case of silver resistant bacteria. But in previous study, it was found that *silE* involvement was more significant at ideSHi (unpublished data).

In ideSHi's study (unpublished data),found that *SilE* gene is more predominant in *Klebsiella pneumoniae* and it is identified that *Klebsiella pneumoniae* acquired in nosocomial infection. But in Bangladeshi perspective, there is no information or data regarding the silver resistant bacteria in case of wound/burn of hospitalized patient. So, in this study trying to figure out some major issue/objectives which are given below:

1.4 Aim of this Study:

1. To observe the frequency of KPN in burn and wound infected patient of Bangladesh
2. To identify the silver resistant KPN and determine their MIC for silver
3. To identify the *SilE* gene in silver resistant KPN strains

Methodology

2.1 Working laboratory:

Laboratory works were performed in the ideSHi Laboratory, IPH Building, Mohakhali, Dhaka. This Laboratory has got the Biosafety Level 2 (BSL-2) facility. All the microbiological works were done inside Biological Safety Cabinet.

2.2 Study duration:

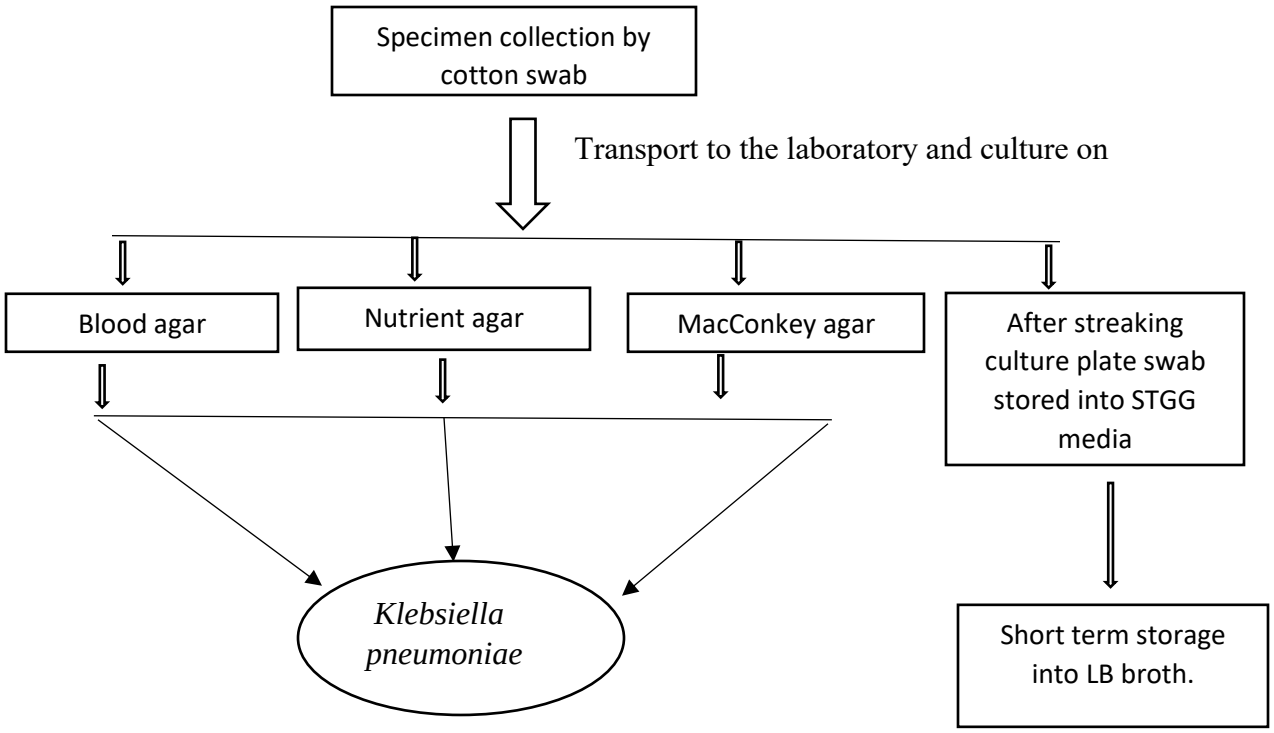
This study was carried out from January 2017 to November 2017

2.3 Sample collection:

In this study, specimens were collected from Burn and Plastic unit of Shaheed Suhrawardy Medical College (ShSMC), Bangladesh. A total number of samples were collected from pus/wound site of the burn/wound patients admitted to the respective unit by performing hand hygiene and wearing clean glove.

2.4 Microbiological culture of the isolated organisms and storage:

Rubbing pus/wound by using sterile cotton swabs. Then swabs were carefully placed in sterile test tubes for transport. After that in lab it was streak on blood agar, nutrient agar and MacConkey agar plate for microbiological culture of the isolated colony and stored in STGG media at -70ºC storage for long term use. Next day, observed the culture plate and isolated (KPN) *Klebsiella pneumoniae* organisms and stock for further used. For short term use, Luria broth could be used. Bacterial culture gave into LB and shaking at 30 min for 33000 rpm. Then 800µl LB containing sample mixed up with 200µl glycerol and vortex it properly. Thus, stored at -70ºC for further used. Samples were identified routinely to distinguish *Klebsiella pneumoniae*. The cultural properties of the organisms were observed and recorded.



Flow chart 2.1: Flow chart of microbiological culture of the isolated organisms and storage.

2.5 Phenotypic characterization:

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

2.5.1 Analysis of colony morphology:

Isolated five KPN samples were culture on MacConkey agar plate. MacConkey agar plate is one kind solid media. It is selective for gram negative bacteria and give pink colonies since *Klebsiella*

pneumoniae is gram negative bacteria and it can easily grow on MacConkey agar plate. Based on key features of these bacterial colonies serve as an important criteria for their identification.

2.5.2 Gram Staining:

Gram staining is a common technique used to differentiate two large groups of bacteria- gram positive and gram negative based on their different cell wall constituents.

2.5.2.1 Materials Required: These are the materials which are used for gram staining:

- Clean glass slides, Inoculating loop, Saline, 18 to 24-hour cultures of organisms, Bunsen burner, Crystal Violet (the primary stain), Iodine, the mordant, A decolorizer made of acetone and alcohol (95%), Safranin (the counterstain), Microscope, Immersion oil.

2.5.2.2 Procedure:

1. Preparation of the glass microscopic slide

Grease or oil free slides were essential for the preparation of microbial smears. Grease or oil from the fingers on the slides were removed by washing the slides. Wipe the slides with spirit or alcohol. After cleaning, dry the slides and place them until ready for use.

2. Labeling of the slides

label the slide with the initials of the name of the organism on the edge of the slide.

3. Preparation of the smear

Two individual sterile glass slides were taken onto which an appropriate amount of normal saline was taken. A single colony from the culture plate of *KPN (Klebsiella pneumoniae)* were taken with the sterile loops and mixed with the saline on the slides. The slides were air dried and then films were fixed on the slide by passing them through the Bunsen burner two or three times without exposing the dried film directly to the flame. Smear appears as a thin whitish layer or film after heat-fixing. Heat fixing is an important part of gram staining because it kills the bacteria in the smear, firmly adheres the smear to the slide, and allows the sample to more readily take up stains.

5. Gram Stain Procedure

Place slide with heat fixed smear on staining tray. The slides were flooded with crystal violet solution for up to one minute. After one minute, the slides were washed off with distilled water and drained. Then slides were then flooded with Gram's Iodine solution and left for one minute, then washed off and drained. After that the slides were again flooded with decolorize agent 95% ethyl alcohol or acetone acetone and immediately rinse with water. Lastly, gently flood with safranin to counter-stain and let stand for 45 seconds.

2.5.3 Biochemical test:

Staining gives valuable records as to bacterial morphology, gram response, and presence of such structures as capsule and endospores. Past that, however, microscopic observation gives little additional information as to the genus and species of a specific bacterium. To determine this biochemical test works efficiently. Biochemical tests are the tests used for the identification of bacteria species based on the differences in the biochemical activities of different bacteria. All the biochemical tests were carried out at ideSHi laboratory. And the recommended biochemical tests like- Citrate utilization test, Triple Sugar Iron (TSI) test, Oxidase test, Catalase test, Motility Indole Urease (MIU) test etc. Before starting the process of any biochemical identification test, bacterial cultures were grown on MacConkey agar plates in the incubator at 37°C.

2.5. 3.1 TSI (Triple Sugar- Iron agar) test:

Triple Sugar Iron Agar (TSI Agar) is utilized for the separation of gram-negative enteric bacilli in view of carbohydrate fermentation furthermore, the generation of hydrogen sulfide. TSI Agar contains three sugars (dextrose, lactose and sucrose), phenol red for detecting carbohydrate fermentation and ferrous ammonium sulfate for detection of hydrogen sulfide production (indicated by blackening in the butt of the tube). Carbohydrate fermentation is demonstrated by the creation of gas and an adjustment in the shade of the pH pointer from red to yellow. 1.0 % lactose/1.0% sucrose causes a large amount of acid turns both butt and slant yellow, thus indicating the ability of the culture to ferment either lactose or sucrose. Ferrous sulfate is present by indicating H_2S formation. Phenol red act as an indicator of acidification (It is yellow in acidic condition and red under alkaline conditions). It also contains Peptone which acts as source of nitrogen.

- For the test of TSI, considered a single bacterial colony of the bacterium to be tested was picked up from each MacConkey plates by a sterile straight needle and stabbed through the center of the medium to the bottom of the tube.
- Then streaking on the surface of the agar slant into the TSI containing dextrose, lactose and sucrose.
- Cap of the tube was loosened and incubated at 37°C for overnight and were examined after 24 hours for carbohydrate fermentation, CO_2 and H_2S production.

Figure 2.1: TSI media

2.5.3.2 Citrate utilization test:

The Citrate Utilization test is used to identify bacteria which utilize as one of their starting products of metabolism a compound called citrate (ionized form of citric acid).

- For citrate test, firstly made citrate medium prepared. The medium used is Simmons citrate agar slant. It contains mineral salts, sodium citrate for carbon, and ammonium phosphate for its nitrogen source. The p*H* indicator is brom thymol blue, which is green at neutral p*H*, yellow at acidic p*H* <6.0 and turns blue at alkaline (basic) p*H* >7.6.

- Considered a single pure bacterial colony of the bacterium to be tested was picked up from each MacConkey plates by a sterile straight needle. Then transferred aseptically to a sterile tube of Simmons citrate agar.
- The inoculated tube was incubated at 35-37 C for 24 hours and the results were determined. Abundant growth on the slant and a change from green to blue in the medium indicates a positive test for growth using citrate. If there is no color change then it was indicated negative result.

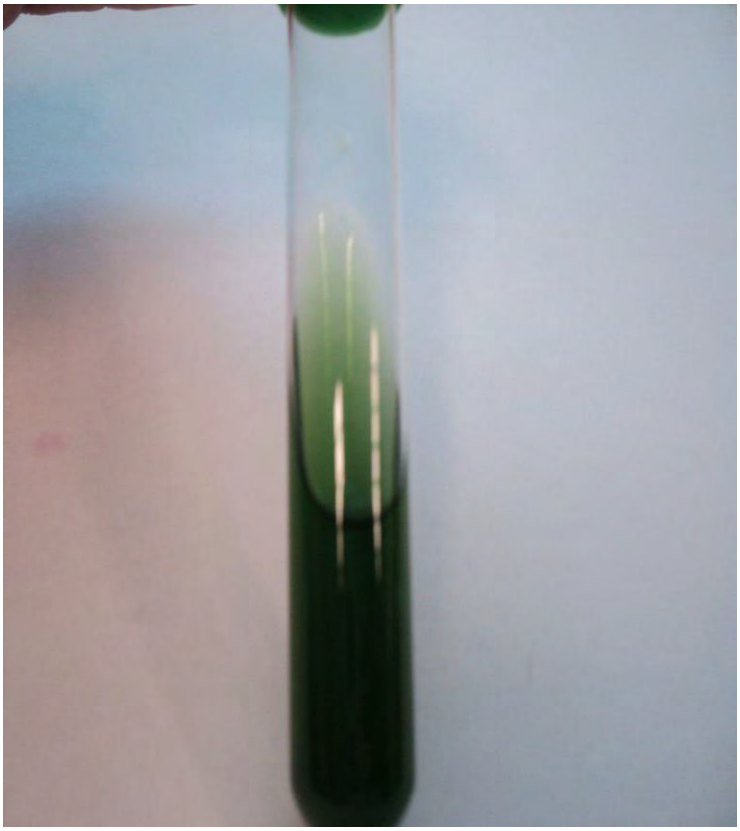


Figure 2.2: citrate media

2.5.3.3 Motility Indole Urease (MIU) test:

MIU medium is used for checking motility, urease production and indole production. MIU contains casein enzymic hydrolysate provide amino acids and other nitrogenous substances. Sodium chloride maintains osmotic equilibrium. Dextrose is fermentable carbohydrate. Phenol red is the pH indicator which turns pink- red in alkaline conditions.

- For MIU test, single pure bacterial cultures are stab-inoculated by the help of straight sterile needle throughout the center of the media. An indole reagent paper was attached at the top of the test tube and close the test tube by the help of cotton.
- Then it was incubated overnight at 37°C. Motility test was positive if organisms show either diffused growth or turbidity extending away from stab inoculation line while nonmotile organisms grow along the stabline. 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. A negative result was indicated by a yellow layer.

Figure 2.3: Motility indole urease (MIU)

2.5.3.4 Catalase test:

- At first, label the slide with the initials of the name of the organisms on the edge of the slide.
- Then one drop of catalase test reagent (hydrogen peroxide H_2O_2) was placed on a sterile glass slide.
- An isolate from a MacConkey agar plate was picked up with a sterile toothpick and placed on to the reagent drop.
- This was done with each of the bacterium to be tested. An immediate bubble formation indicates a positive result and compare with control.

2.5.3.5 Oxidase test:

- Before performing Analytical Profile Index of test microorganism, oxidase test must be carried out. Soak a small piece of filter paper in 1% Kovac's oxidase (tetra methyl-p-phenylenediamine dihydrochloride) reagent and let dry.

Figure 2.4: Oxidase reagent that was used in oxidase test.

- Use a loop and pick a well-isolated colony from a fresh (18- to 24-hour culture) bacterial plate and rub onto treated filter paper and observed for color changes. Microorganisms are oxidase positive when the color changes to dark purple within 5 to 10 seconds. Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 seconds. Microorganisms are oxidase negative if the color does not change or it takes longer than 2 minutes.

2.5.6 Phenotypic detection of the isolate (KPN):

Phenotypic detection of Silver resistant *Klebsiella pneumoniae* culture in broth. Firstly, preparing fresh silver nitrate stock solution for phenotypic identification of silver resistant bacteria. Stock solution must be maintained at specific concentration. Then inoculation of *Klebsiella pneumoniae* sample at 1mg/L of Agno3 in LB broth. Next day after 24- hour incubation, it was found that two samples were able to grow out of rest samples in presence of silver. Further, the resistant *Klebsiella pneumoniae* bacteria were used for silver resistant *silE* gene investigation before that performing antibiotic sensitivity test.

2.5.7 Antibiotic Sensitivity test(AST):

Antibiotic sensitivity test or Disk diffusion is a method which is used to detect which organisms are sensitive to which antibiotics. Antibiotics represent a major class of antimicrobial agents. By considering these antibiotics are biochemicals produced by microorganisms that inhibit the growth of, or kill, other microorganisms. Moreover, antibiotics must exhibit selective toxicity because they are produced by one microorganism and exert varying degrees of toxicity against others.

Figure 2.5: Mueller Hinton agar plate.

For AST, Mueller Hinton agar plate is used. Mueller Hinton Media contains Beef Extract, Acid Hydrolysate of Casein, Starch and Agar. Beef Extract and Acid Hydrolysate of Casein provide nitrogen, vitamins, carbon, amino acids, sulphur and other essential nutrients. Starch is added to absorb any toxic metabolites produced. Starch hydrolysis yields dextrose, which serves as a source of energy. Agar is the solidifying agent.

At first, Pure Colonies of sample are taken from MacConkey agar plate using sterile loop. Then colonies of the bacteria are inoculated into 1ml of saline and vortex to mix properly. After that incubated at 37˚C for 1-2 hours so that bacteria can grow inside of the saline.

Then Mueller- Hinton (MH) plates were labelled. Beside of this, sterile cotton swabs were dipped into saline. Then the MH plates were lawn with the cotton swab so that bacteria were spread evenly to the plates. It was repeated 3 times at 90 degree rotation. 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. Then plates are incubated at 37˚C for 24 hours. After incubation zone of inhibition is measured in mm.

In this investigation, 13 distinctive economically accessible anti-microbials were utilized. The anti-toxins were chosen for the Antimicrobial Sensitivity Test as those are indicated for the identification of *Klebsiella pneumoniae*as indicated by CLSI (Clinical and Laboratory Standards Institute) Guideline as changed in 2015. Thirteen anti-microbial plates which were utilized for AST alongside their zone measurement for *Klebsiella pneumoniae* are given below:

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

Serial No	Antibiotic (Antimicrobial agent)	Disc Code	Concentration	Result interpretation		
				Resistant <or=mm	Intermediate Mm	Sensitive >or=mm
1.	Amikacin	AK	30 µg	≤14	15-16	≥17
2.	Azithromycin	AZM	15 µg	≤13	14-17	≥18
3.	Carbapenem	CAR	100 µg	≤19	20-22	≥23
4.	Cefixime	CFM	5 µg	≤15	16-18	≥19
5.	Ceftriaxone	CRO	30 µg	≤19	20-22	≥23
6.	Ciprofloxacin	CIP	5 µg	≤15	16-20	≥21
7.	Imipenem	IMP	10µg	≤13	14-15	≥16
8.	Meropenem	MEM	10µg	≤13	14-15	≥16
9.	Netilmicin	NET	30 µg	≤12	13-14	≥15
10.	Pipercilin+ Tazobactam	TZP	100 µg	≤17	18-20	≥21
11.	Polymyxin B	PB	15µg	≤11	12-13	≥14
12.	Tobramycin	TOB	10 µg	≤12	13-14	≥15
13.	Gentamycin	CN	10 µg	≤12	13-14	≥15

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

Table 2.2: Pathogen specific *Klebsiella spp.* drug lists

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

2.5.8 Analytical Profile Index:

The Analytical Profile Index (API) is a mini -biochemical test which was used to distinguish between groups of closely related bacteria for their identification. There are 20 biochemical tests which are compiled together in a single test strip. Different test panels are prepared in dehydrated forms which are reconstituted upon addition of bacterial suspensions. The inoculated whole API® 20E test kit strips were incubated overnight at 37°C in an aerobic incubator. After the incubation period, test results are used to construct a 7 -digits profile. Using this profile, the identity of the bacterium was derived from the database with the relevant cumulative profile code book or software.

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. H2S : 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)

2.6 Genotypic detection of silver resistance genes:

To establish this report, it is necessary to detect silver resistance gene *silE* by following molecular technique. It was carried out by conventional PCR and analyzing the sequence.

2.6.1 Designing a PCR primer:

From the nucleotide database of National Centre for Biotechnology Information (NCBI), the DNA sequences of silver resistance genes were recovered. FASTA arrangement was then used for analyzing DNA sequence. To run this program by utilizing Primer-BLAST. BLAST means Basic Local Alignment Search Tool,this program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance.

2.6.2 DNA sequencing software- primer blast:

Primer-3 plus, Primer-Blast

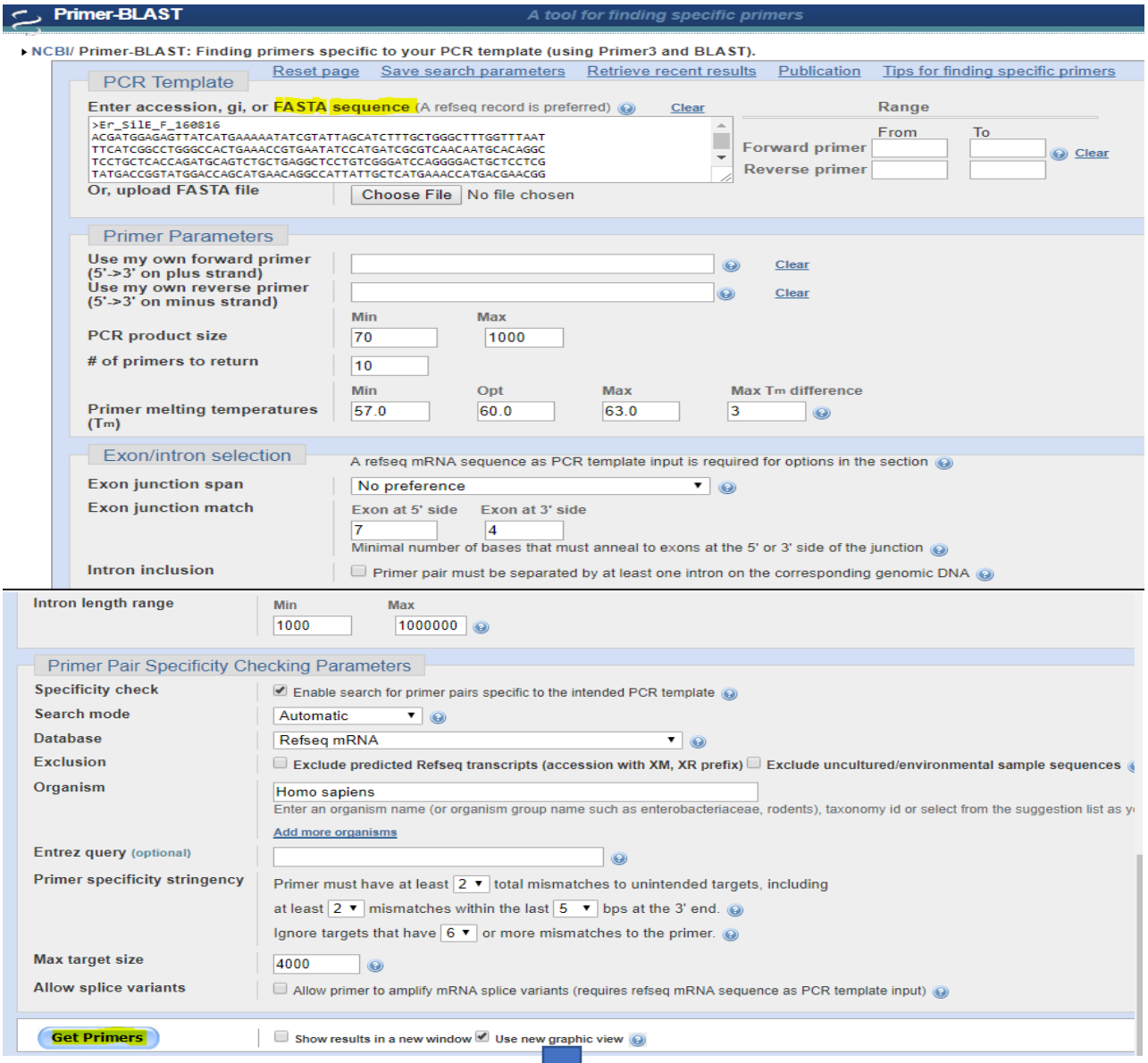
Primer-BLAST, a general-purpose public tool that helps users in designing target-specific primers. Primer-BLAST offers flexibility to accommodate different primer design needs. Users can either design new primers or check the specificity of pre-existing primers. Notably, Primer-

BLAST incorporates a global alignment mechanism and is designed to be very sensitive in detecting potential amplification targets. In this study, primer blast is used to find out the specific primer for PCR (polymerase chain reaction) that primer can detect specific gene of silver resistant bacteria.

2.6.2.1 Tool- primer blast URL: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

2.6.2.2 Method:

- Enter into this link https://www.ncbi.nlm.nih.gov/tools/primer-blast/
- There is a box where desire whole Fasta sequence input.
- Other parameters can be changed if it is necessary.
- Lastly, click on the “Get primer” box. And waited for a while, thus it will give desire result.


Primer-BLAST
A tool for finding specific primers
NCBI/ Primer-BLAST: Finding primers specific to your PCR template (using Primer3 and BLAST).
Reset page
Save search parameters
Retrieve recent results
Publication
Tips for finding specific primers
PCR Template
Enter accession, gi, or FASTA sequence (A refseq record is preferred)
Clear
>Er_SilE_F_160816
ACGATGGAGAGTTATCATGAAAAATATCGTATTAGCATCTTTGCTGGGCTTTGGTTTAAT
TTCATCGGCCTGGGCCACTGAAACCGTGAATATCCATGATCGCGTCAACAATGCACAGGC
TCCTGCTCACCAGATGCAGTCTGCTGAGGCTCCTGTCGGGATCCAGGGGACTGCTCCTCG
TATGACCGGTATGGACCAGCATGAACAGGCCATTATTGCTCATGAAACCATGACGAACGG
Or, upload FASTA file
Choose File
No file chosen
Range
From
To
Forward primer
Reverse primer
Clear
Primer Parameters
Use my own forward primer (5'->3' on plus strand)
Use my own reverse primer (5'->3' on minus strand)
Clear
Clear
Min
Max
PCR product size
70
1000
# of primers to return
10
Min
Opt
Max
Max Tm difference
Primer melting temperatures (Tm)
57.0
60.0
63.0
3
Exon/intron selection
A refseq mRNA sequence as PCR template input is required for options in the section
Exon junction span
No preference
Exon junction match
Exon at 5' side
Exon at 3' side
7
4
Minimal number of bases that must anneal to exons at the 5' or 3' side of the junction
Intron inclusion
Primer pair must be separated by at least one intron on the corresponding genomic DNA
Intron length range
Min
Max
1000
1000000
Primer Pair Specificity Checking Parameters
Specificity check
Enable search for primer pairs specific to the intended PCR template
Search mode
Automatic
Database
Refseq mRNA
Exclusion
Exclude predicted Refseq transcripts (accession with XM, XR prefix)
Exclude uncultured/environmental sample sequences
Organism
Homo sapiens
Enter an organism name (or organism group name such as enterobacteriaceae, rodents), taxonomy id or select from the suggestion list as y
Add more organisms
Entrez query (optional)
Primer specificity stringency
Primer must have at least 2 total mismatches to unintended targets, including
at least 2 mismatches within the last 5 bps at the 3' end.
Ignore targets that have 6 or more mismatches to the primer.
Max target size
4000
Allow splice variants
Allow primer to amplify mRNA splice variants (requires refseq mRNA sequence as PCR template input)
Get Primers
Show results in a new window
Use new graphic view

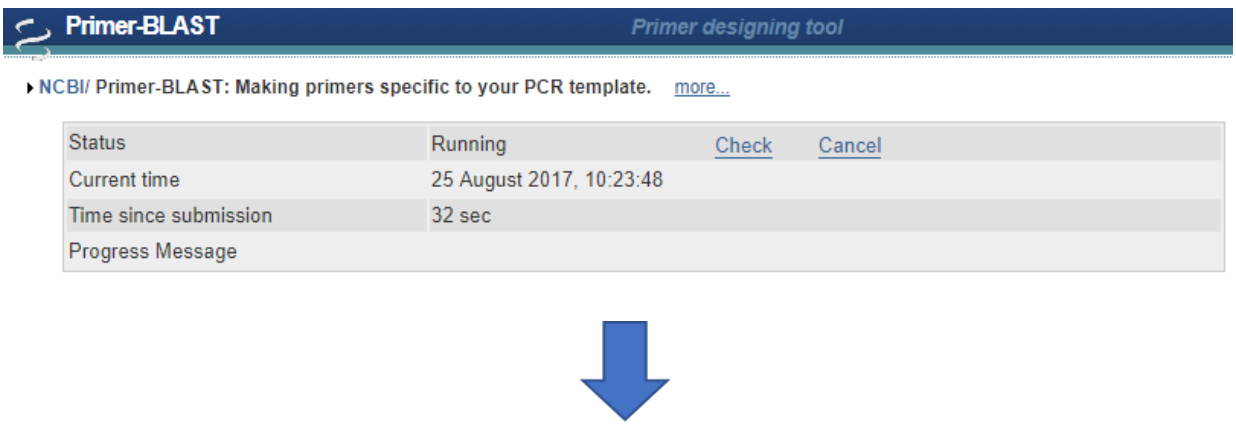

Primer-BLAST
Primer designing tool
NCBI/ Primer-BLAST: Making primers specific to your PCR template.
more...
Status
Running
Check
Cancel
Current time
25 August 2017, 10:23:48
Time since submission
32 sec
Progress Message

Table 2.3: Silver resistant gene *silE's* primers and their contents

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

2.6.2.3 Product size for silE gene:

Table 2.4:*silE* gene's product size.

Silver resistant gene	Product size
• ***silE***	**424bp (base pair)**

For detection gene *silE*, bacterial DNA must be isolated from the sample which is showing silver resistant characteristics in previous test.

2.7 Preparation of DNA by boiling lysis of bacteria isolated from the sample:

- DNA from isolated silver resistant bacteria KPN (*Klebsiella spp.)* sample was prepared by boiling method. With the pure sample of DNA, one can easily analysis the specific gene *silE* pattern by following PCR.
- For DNA extraction, sample was cultured overnight in MacConkey agar plate.

- Next day, single colony was taken with a sterile loop and dissolve into 100 microliter nuclease free water.
- Then vortex it and mixed it properly.
- After 1 to 2 minutes later then organisms containing Eppendorf dissolved into 100° C boiling water and waited for 10 minutes.
- After 10 minutes separated those Eppendorf and put into the ice immediately and kept for 1 minutes. This process is known as heat shock.
- Then centrifuge at 13000rpm for 10 min to settle down the pellet and collect the supernatant in another RNase and DNase free microcentrifuge tube and labeled it properly.
- In addition, stored at 20°C for a month or a year.

2.7.1 Measurement of DNA concentration and purity:

After DNA isolation, it is necessary to measure the concentration and check the purity as well. For this study, Nanodrop spectrophotometer was used in laboratory. Depending on the wavelength DNA absorb UV light more or less strongly. DNA concentration was measured by comparing with the blank. Here, nuclease free water was used as a blank at 2 microliters. After that, placed 2 microliters sample in nanodrop spectrophotometer and measure the concentration and purity. The concentration was measured in ng/µl. When assessing DNA purity, it is important to understand the A260/A280 ratio. DNA absorbs so strongly at 260 nm that it takes significant protein contamination to have a noticeable effect on the A260/A280 ratio. If the 260/280 ratio is about 2 or above then it is considered that there is high probability for getting pure DNA.

2.8 Polymerase chain reaction(PCR) for the amplification of extracted DNA:

After DNA purification, PCR technique was used for this study. Through this PCR it is easy to determine *Klebsiella pneumoniae* which is demonstrating resistance to silver. For this study, *SilE* gene pattern was considered. *silE* is silver resistant gene. And the main purpose of this technique is that amplify of a particular sequence of the DNA extracted from the sample which may contain *silE*. PCR method is preferring in this study because is a rapid, inexpensive process and simple way of copying specific DNA fragments from minute quantities of source DNA material, even when that source DNA is of relatively poor quality. This process was go through four stages. The basic principle of PCR, at first target double stranded DNA is denatured then primers are added then tag polymerase is used to bind the primer. Lastly renaturation occur and getting amplified DNA.

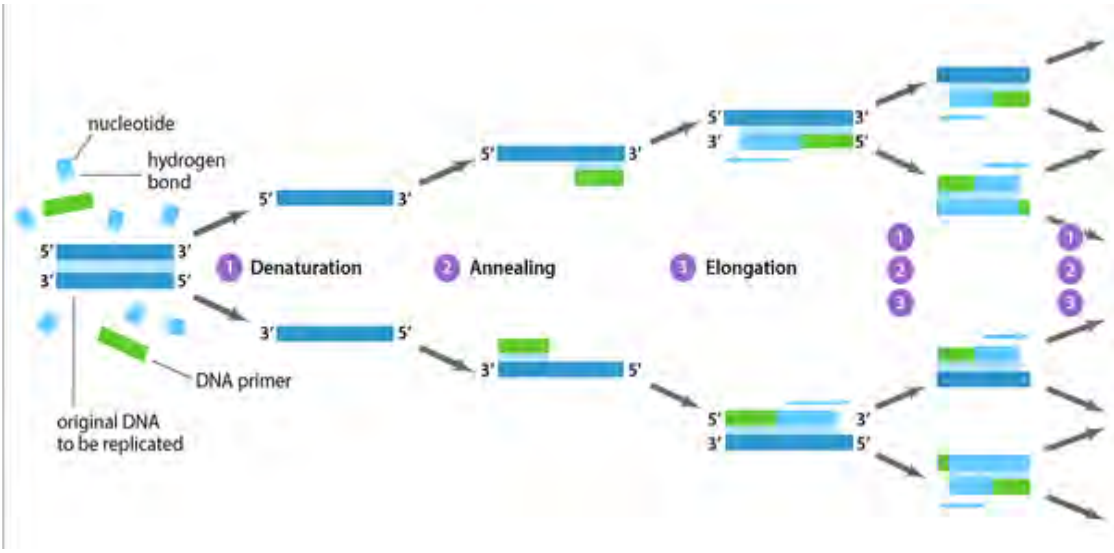


Figure2.6: Principle of polymerase chain reaction.

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

2.8.1 Conventional PCR:

a) Master-mix preparation: The master-mix for each sample to run PCR was prepared according to the following measurement:

Table 2.5: Components of PCR master mix

Components	Amount
10X buffer(Mg2+)	1 µl
2.5 mM dNTPs	1 µl
10 µM Forward primer	0.5 µl
10 µM Reverse primer	0.5 µl
Tag polymerase	0.1 µl
Nuclease free water	3.9 µl
DNA Template	1 µl
Total amount	**10µl**

Table 2.6: primers use in this study.

Primer name	Primer sequence (5'-> 3')	GC content	Product length	Temp. [°C]
silE (F)	GTACTCCCCCGGACATCACT AATT	50%		
silE(R)	GGCCAGACTGACCGTTATT	52.6%		

Product size

424 base pair

b) PCR thermal cycle setup: While conducting the PCR the thermal cycle was set up according to

the following:

Table 2.7: PCR thermal cycle with temperature and time.

PCR condition	Temperature	Time	
Initial denaturation	95°C	5 minutes	
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	∞	

The target DNA containing sequence to be amplified is heat denatured (around 94°C for 15 sec) in this temperature main DNA is separated its complementary strands, this process is called melting of target DNA. Annealing of Primers are added in excess and the temperature is lowered to about 55°C for 30 sec., as a result the primers form the hydrogen bonds and anneal to the DNA on both sides of the DNA sequence. Finally, different nucleoside triphosphate (dATP, dGTP, dCTP, dTTP) and a thermo-stable DNA polymerase are added. It helps in polymerization process of primers and, therefore, extends the primers (at 72°C) resulting in synthesis of multiple copies of target DNA sequence. After completion of all these steps in one cycle, again the second cycle is repeated following the same process. If 35 such cycles occur, then about one million copies of target DNA sequence are produced. At the end, in 10 degree Celsius the PCR product remain constant.

2.9 Agarose gel preparation and gel electrophoresis of PCR products:

Agarose gel electrophoresis is the easiest and most popular way of separating and analyzing DNA. Here, DNA molecules are separated on the basis of charge by applying an electric field to the electrophoretic apparatus. Shorter molecules migrate more easily and move faster than longer molecules through the pores of the gel and this process is called sieving. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band. The DNA can be visualized

in the gel by the addition of gel red. It is an intercalating agent which intercalates between nucleic acid bases and allows the convenient detection of DNA fragments in gel. Agarose is a polysaccharide obtained from the red algae Porphyra umbilicalis.

A 0.7% gel will show good separation for large DNA fragments (5-10kb) and a 2% gel will show good resolution for small fragments with size range of 0.2-1kb. Low percentage gels are very weak (Note: it may break when you lift them) but high percentage gels are usually brittle and do not set evenly. The volume of agarose required for a mini-gel preparation is around 30-50ml and for a larger gel, it is around 250ml. There is an important factor that works behind the gel electrophoresis that is voltage applied. The migration rate of the linear DNA fragments through agarose gel is proportional to the voltage applied to the system. As voltage increases, the speed of DNA also increases. But voltage should be limited because it heats and finally causes the gel to melt.

2.9.1 Agarose gel preparation:

1. 50ml TBE buffer and 0.5 gm agarose were added in a beaker and heat was applied.
2. Then agarose was dissolved in buffer.
3. After that, adding 1µl gel red into the buffer. Then let it cool down.
4. After that, chose an appropriate comb for sample slots in gel was chosen and gel was poured in the mold.
5. When the gel was settle down, the comb was removed carefully.
6. And place into the electric field. Now the gel was ready for input the PCR products for electrophoresis.

2.9.2 Detection of silver resistant band using PCR product by following gel electrophoresis:

1. At first took 2µl loading dye according the sample.
2. Then took 3µl sample (PCR product) was added into the specific dye and properly mixed it up and input the well.

3. In one well 6 µl ladder was added. Ladder is about 1Kb plus (Invitrogen, USA). Ladder is an important component because it will help to measure the extract desire band (silver resistant band- product size is about 400 bp)
4. Extra buffer added in the electric field if it is necessary.
5. Electrophoresis was done for 30-40 minutes at 150 volts.
6. Then the gel was loaded onto the gel dock and the results were analyzed.

2.10 DNA sequencing (Sanger method):

Fluorescence-based automated cycle sequencing entails a DNA template, a sequencing primer (forward or reverse), a thermostable DNA polymerase, deoxy nucleoside triphosphate (dNTPs), sequencing buffer and fluorescent dye-labeled 2′,3′-dideoxynucleotide triphosphates (ddNTPs). All the components are mixed and subjected to cycles of denaturation, annealing, and extension in a thermal cycler. DNA polymerase incorporates either a dNTP (A, C, G, or T) or the corresponding ddNTPs (nucleotide base analogs that lack the 3′-hydroxyl group essential for phosphodiester bond formation by DNA polymerase) at each step of chain extension depending on the relative concentrations of both molecules. When a dNTP is added to the 3′ end, chain extension is continued. However, when a ddNTP terminator (ddA, ddC, ddG, or ddT each tagged with different fluorescent dye) is added to the 3′end, chain elongation is terminated, forming labeled extension products of various lengths. The DNA samples were sequenced at IEDCR (Institute of Epidemiology, Disease Control and Research). The calculation of cycle sequencing was obtained according to the measured template concentration.

Table2.8: Master cycle components with amount.

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

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

Table2.9: Master cycle components with amount

PCR condition	Temperature	Time	
Initial denaturation	95°C	5 minutes	
Denaturation	95°C	30 seconds	25 Cycles
Annealing	55°C	30 seconds	
Extension	72°C	45 seconds	
Final extension	72°C	6 minutes	
	10°C	∞	

After completion of cycle sequencing, the reaction plate was centrifuged at 4100 rpm for 2 minutes. Then, 45µl of SAM solution and 10 µl of X-terminator (Applied Biosystems, USA) were added per 10µl volume. Both of the solution aid in removal of impurities by desalting as salts interfere 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 rpm for 2 minutes and the supernatant was collected for capillary electrophoresis. Ten 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.

2.10.1 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.

2.10.1.1 BLAST (Basic Local Alignment Search Tool):

An important goal of genomics is to determine if a particular sequence is like another sequence. This is accomplished by comparing the new sequence with sequences that have already been reported and stored in a database. This process is principally one that uses alignment procedures to uncover the "like" sequence in the database. The alignment process will uncover those regions that are identical or closely similar and those regions with little (or any) similarity. Conserved regions might represent motifs that are essential for function. Regions with little similarity could be less essential to function. In a sense, these alignments are used to determine if a database contains a potential homologous sequence to the newly derived sequence. Further, phylogenetic studies are necessary to determine the orthologous/paralogous nature of the two aligned sequences. This tool is available online at the National Centre for Biotechnology Information (NCBI) website (Lobo, 2008).

2.10.1.2 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.

2.11 Antimicrobial activity of Silver resistance:

Silver resistant sample further used to determine MIC means minimum inhibitor

2.11.1 Preparation of silver nitrate stock solution:

- Take silver nitrate reagent and 0.01698 g silver nitrate (AgNO3) was measured by using balance machine and kept into dry 15mL conical centrifuge tube.
- Autoclaved distilled water added up to 10 ml.

- Then it was mixed thoroughly to make a homogenous solution using a vortex machine.
- 15ml conical centrifuge tube must be wrapped by foil paper as silver nitrate is sensitive to light.
- And labeled it properly and kept it in normal condition.

2.11.2 Preparation of Luria-Bertani (LB) broth plates with different concentrations of silver nitrate:

Luria-bertani (LB), a nutritionally rich medium, is primarily used for the growth of bacteria. LB broth composed of three components. These are tryptone which it provides essential amino acids to the growing bacteria, meat extract, it provides a plethora of organic compounds and NaCl and it is essential for transport and osmotic balance.

- In this report, 200 ml LB was prepared.
- For this, tryptone and meat extract and NaCl were added one by one at specific amount.
- Then added distilled water up to 200 ml

Mixed it properly and autoclaved at 121º C for 45 minutes to 1 hour

2.11.3 MIC (minimal inhibitory concentration) of silver nitrate against silver resistant bacteria:

The **minimum inhibitory concentration** (**MIC**) is the lowest concentration of a chemical which prevents visible growth of a bacterium. MIC depends on the microorganism, the affected human being (in vivo only) and the antibiotic.

Here, the antibacterial activity of the silver nanoparticles was assessed by determining the minimal inhibitory concentration (MIC) and repeated this process three times. For these first of all it is necessary to make chemical solution in vitro at increasing concentrations, incubating the solutions with the separate batches of cultured bacteria, and measuring the results using agar dilution or broth microdilution. In addition, results have been graded into susceptible often called sensitive, intermediate, or resistant to a particular antibiotic by using a cut- off point by observing agar

diluted plates. But in case of broth solution result must be interpreted by observing by turbidity of that particular micro-organisms.

- In this report, MIC was done in LB broth by adding different concentration of silver nitrate.
- Take 3 set of gram vial. Each set contain 0 to 12 tubes. First set mark as control to compare and other 2 set marked with the KPN those were isolated and survived in presence of silver nitrate solution.
- Then each gram vial contains 2 ml LB and also added 20µl particular culture sample was added in particular gram vial.
- Then silver nitrate was added by increasing concentrations in all the set of gram vial.
- Then kept all the gram vial at shaker incubator at 250 rpm for 24 hours.
- After 24-hour incubation, took 100 µl sample into Elisa plate and measured OD (optical density) by the help of spectrophotometer.

2.12 Quality control:

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

2.12.1 Quality control for confirmation tests of the organisms:

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

Results

After performing the microbiological culture targeting KPN, a total number of 5 KPN strains out of 15 specimens were found, a frequency of 33%.

3.1 Colony morphology:

Isolated 5 KPN (*Klebsiella pneumoniae)* cultured on MacConkey agar plate. It was a selective media for the identification and conformation for gram negative bacteria. *Klebsiella pneumoniae* grow on MacConkey media after 24 -hour incubation. Based on its colony morphology some feature criteria analysis which were given below in a table 3.1

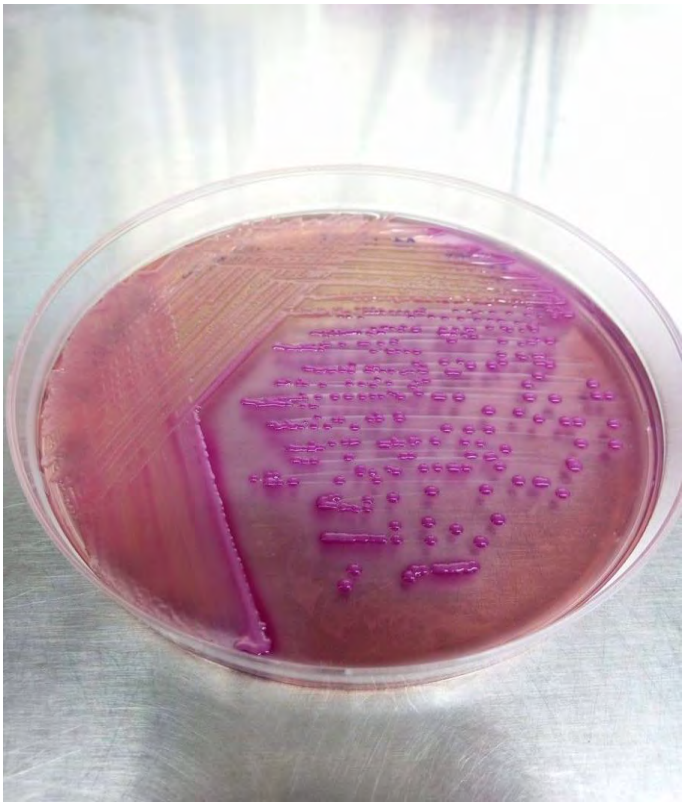


Figure 3.1: Observing colony morphology of isolated KPN (*Klebsiella pneumoniae)*

Table 3.1: Bacteria isolation from wound sample and observation of colony characteristics.

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

3.2 Gram staining:

Gram staining told about the cell arrangement and morphology under oil immersion microscope. Here it is found that the cell color is purple and they are in chain formed which were the characteristic of gram positive bacteria.

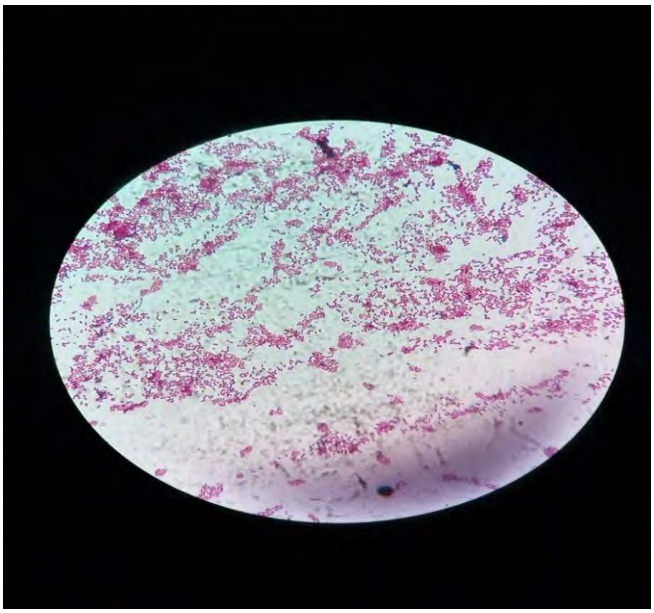


Figure3.2: Microscopic characteristics of Wound samples of isolated *Klebsiella pneumoniae* (KPN) after Gram staining

3.3 Biochemical test:

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

Sample type: Wound/Burn

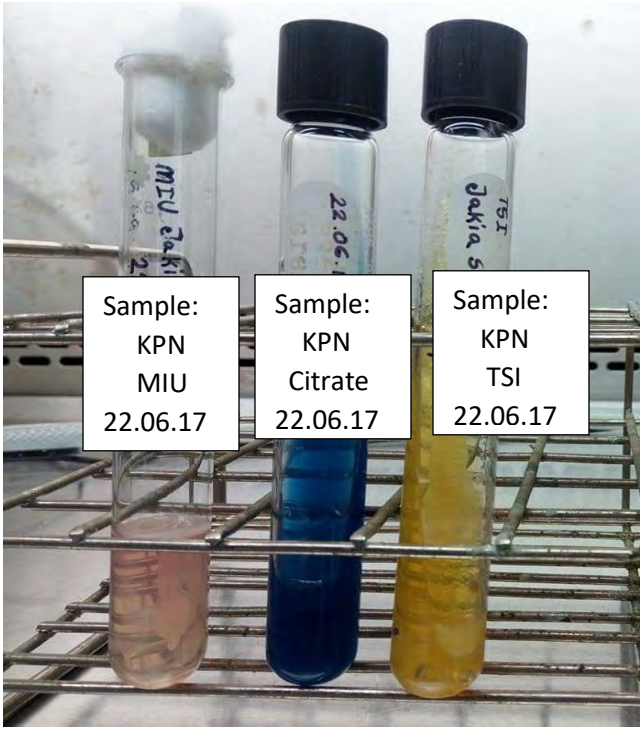


Figure 3.3: observation of biochemical test of wound sample of KPN (*Klebsiella pneumoniae)*

Sample ID	Reaction
• Wound swab Sample KPN, Sample was compared with control	• **TSI test:** positive (color change indicated acid produce. *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) • **Citrate test:** positive (Prussian blue, *Klebsiella pneumoniae* were able to utilized sole carbon source citrate) • **MIU test:** Indole (negative, indole does not combine with the aldehyde present in the Kovac's reagent to form a red complex)

	Urease (positive, Organisms that utilize urea), non-motile (non-motile organisms grow along the stab line)

Table 3.2: In case of sample KPN, biochemical test result analysis by comparing with control.

3.4 Oxidase test:

Through oxidase test, it would be very easy to detect whether organisms were oxidase positive or oxidase negative. Here, kovac's use as reagent.

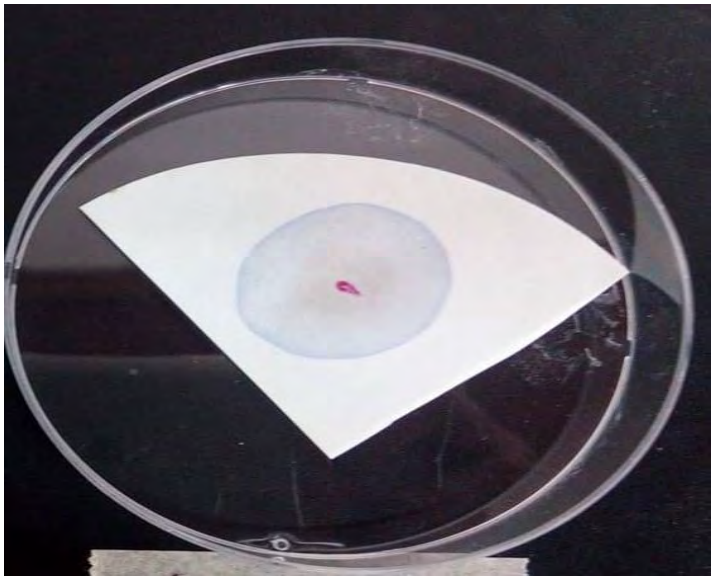


Figure 3.4: Oxidase test observation of five wound sample.

3.5 Catalase test:

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

Figure 3.5: Observation of catalyst test of KPN performing on contamination free glass slide.

Table3.3: Analysis catalyst test by comparing with control.

Sample ID	Reaction
Burn / wound swab: *Klebsiella pneumoniae*	Catalase test was positive by forming bubble. Here, *Klebsiella pneumoniae* react with hydrogen peroxide and bubble formed.

3.6 Phenotypic detection of the isolate (KPN):

Klebsiella pneumoniae were found at 1mg/L of $Agno_3$ in LB broth. It means two samples were able to grow out of the rest samples in presence of silver. Further, the resistant *Klebsiella pneumoniae* bacteria were used for silver resistant *silE* gene investigation before that we had to analysis antibiotic result of two isolated silver resistant bacteria.

3.7 Antibiotic test:

By antibiotic sensitivity test or Disk diffusion any one can detect which organisms are sensitive to which antibiotics. Here, getting antibiotic test result of two isolated silver resistant bacteria, which were given below. Based on this, diameters of the zone of inhibition are measured (including disk)

by using a metric scale or a sliding caliper. The measured zone diameter can be compared with a standard chart for obtaining the susceptible **(S)** and resistant **(R)** values [Table 2.2]. There are zone of intermediate resistance **(I)** which means that the antibiotic may not be sufficient enough to eradicate the organism.

Table 3.4 : Antibiotic resistant spectrum of two silver resistant KPN isolated in this study.

Sample ID:	**First line antibiotic**										**Second line antibiotic**		
W.S KPN	CN	TOB	CRO	CFM	CIP	IMP	MEM	AZM	AK	CT	NET	CAR	PB
	I 14	**I 14**	**R 6**	**R 6**	**R 17**	**S 25**	**I 21**	**R 11**	**I 16**	**S 11**	**S 18**	**R 6**	**S 12**
W.S KPN	**R 6**	**R 11**	**R 6**	**R 6**	**R 8**	**S 24**	**S 24**	**R 11**	**S 18**	**S 12**	**S 16**	**R 6**	**S 14**

3.8 API(Analytical Profile Index) test:

API stands for Analytical Profile Index and is a commercial system to identify different bacteria. One of the API systems is specific for differentiating between members of the Gram negative bacterial Family. API test strips consists of wells containing dehydrated substrates to detect enzymatic activity, usually related to fermentation of carbohydrate or catabolism of proteins or amino acids by the inoculated organisms. A bacterial suspension is used to rehydrate each of the wells and the strips are incubated. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. 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 3.6 and Table 3.5

Table 3.5: Analytical Profile Index test results of the isolates KPN

Klebsiella pneumoniae

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

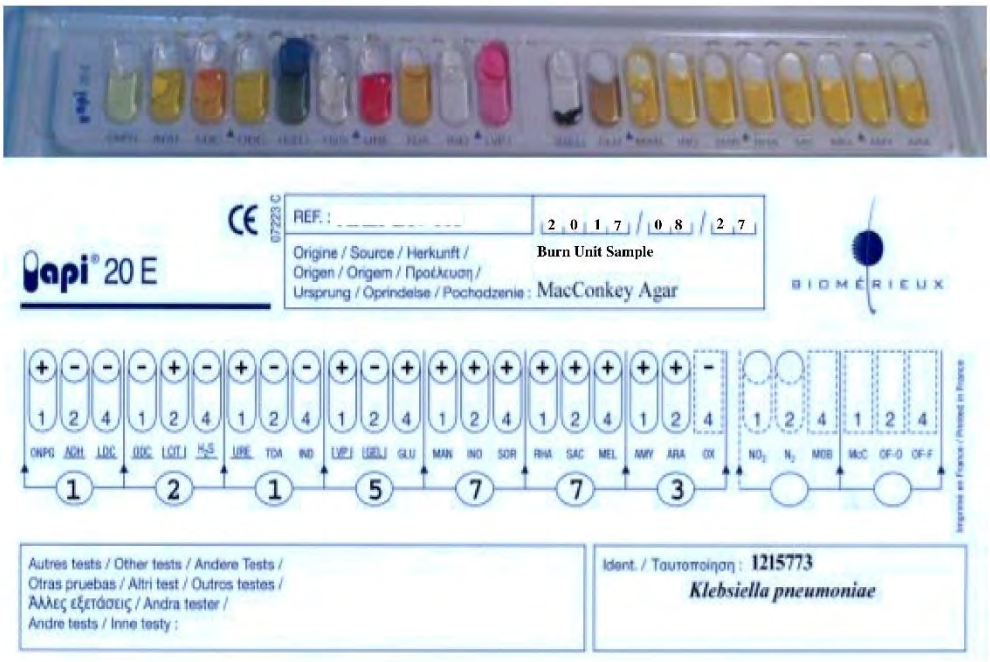


Figure 3.6: Identification of *Klebsiella pneumoniae* using API® 20 E

3.9 Genotypic detection:

In the phenotypic detection experiments, it was found that two isolates KPN among the five 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 Gene Ruler 1Kb Plus DNA Ladder by Invitrogen was used.

3.9.1 PCR (polymerase chain reaction) test:

PCR test dealing with amplified the specific DNA. Here, 5 sample of KPN were inputed *silE* gene was targeted and expected base pair was about 424 bp.Specific band must be compared with positive (P) control.After agarose gel electrophoresis, gel was observed under gel documentation system (BioRad).

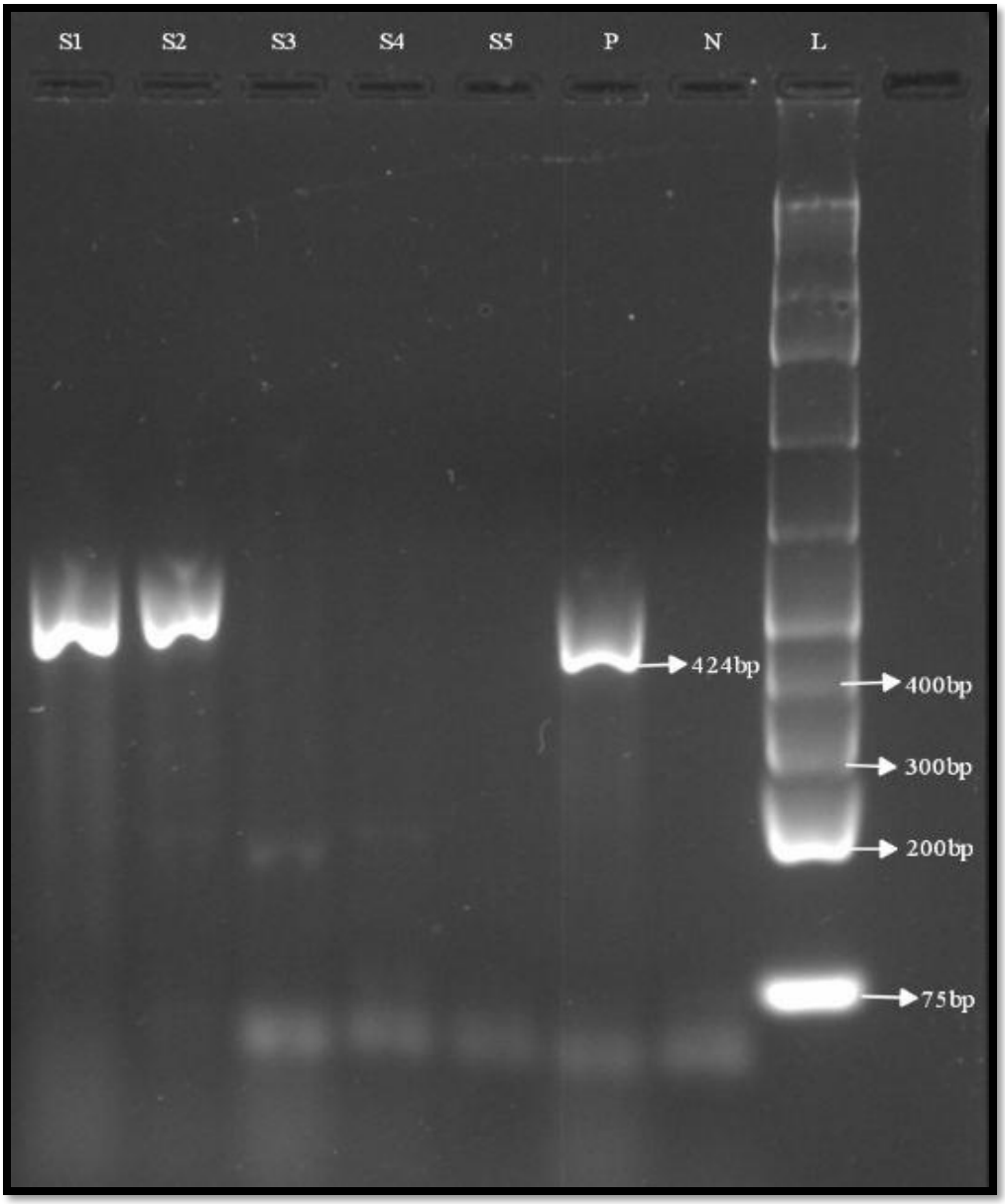


Figure 3.7: PCR amplification products of *silE* gene homologues resolved on Gel red stained 1% agarose gel. The desired band size (*silE* gene) for the gene specific primer is 424 bp (base pair). In the figure, lane 1: S1 indicate sample 1; lane 2: S2 indicate sample 2 ; lane 3: S3 indicate sample 3; lane 4: S4 indicate sample 4; lane 5 : S5 indicate sample 5; lane 6: P indicate positive control ; lane 7: N indicate negative control ; lane 8: 1Kb ladder (S1,S2,S3,S4,S5 = isolated five *Klebsiella pneumoniae* sample for genomic detection of *silE* gene. S1 and S2 contain *silE* gene that means isolated two silver resistant bacteria carry *silE* gene in their plasmid among the rest *Klebsiella pneumoniae*)

3.9.2 Purity and amount of purified PCR product:

Purity and the amount of purified DNA samples were measured by spectrophotometrically. The ratio of OD at 260 nm and 280 nm of the extracted DNA and the concentration of the PCR product was measured and recorded.

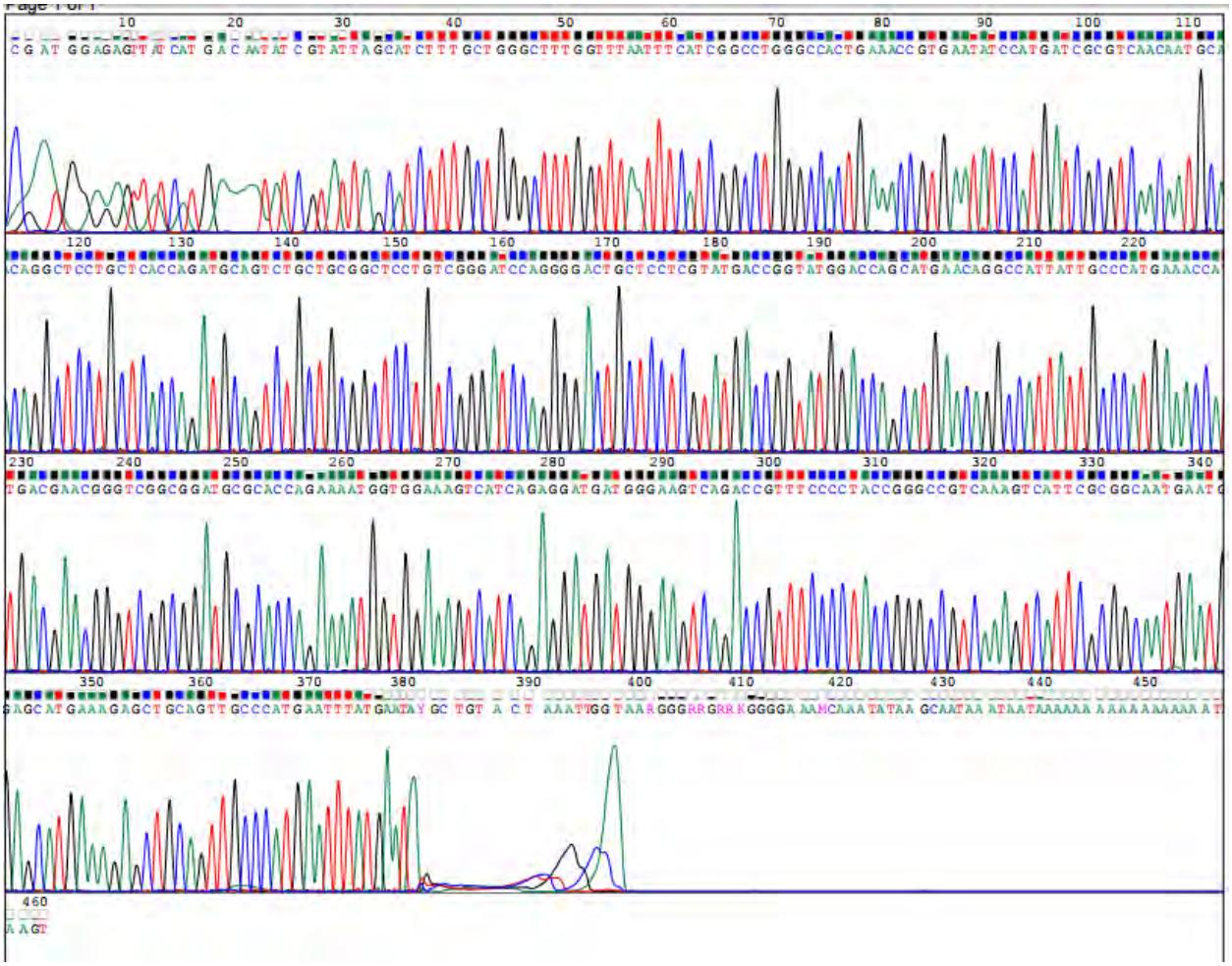
Table 3.6: Purity and the amount of purified DNA samples

Sample ID: *Klebsiella pneumoniae*	Nucleic Aicd Concentration	Purity(260/280 nm)
• KPN Sample	137.9 ng/µl	2.10 nm
• KPN Sample	157.6 ng/µl	2.00 nm

3.10 DNA sequencing result:

A positive PCR result for *silE* gene necessitated the final step which is known as gene sequencing for genotyping. Purified PCR product was important for sequencing and sequencing result in case of indentified *silE* gene containing KPN which were presented in figure- 3.8 to 3.13 figure

In figure-3.8, the chromatogram depicts the two-dimensional plot with the ordinate axis giving concentration in accordance to detector response (figure-3.6). Sequencing data were analyzed by Chromas Lite 2.1 tool which generated 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 3.8: Diagrammatic representation of a part of the chromatogram of sequence. Well-formed and distinctive single colored peaks and lack of background noises were observed, indicating refined sequencing with proper concentration of template and primer. In the chromatogram,considering the areas which were peaked for measuring the component concentration.

Representing nucleotide sequences or peptide sequences, in which base pair or amino acid are using single-letter codes is known as Fasta format. Using Bioedit software one can documented specific format. Sequencing data for the bacterial isolate one sample of *Klebsiella pneumoniae* (KPN) containing the *silE* gene is shown below in Figure: 3.8 and the gene sequence was analyzed by NCBI Nucleotide Basic local alignment search tool (BLAST).

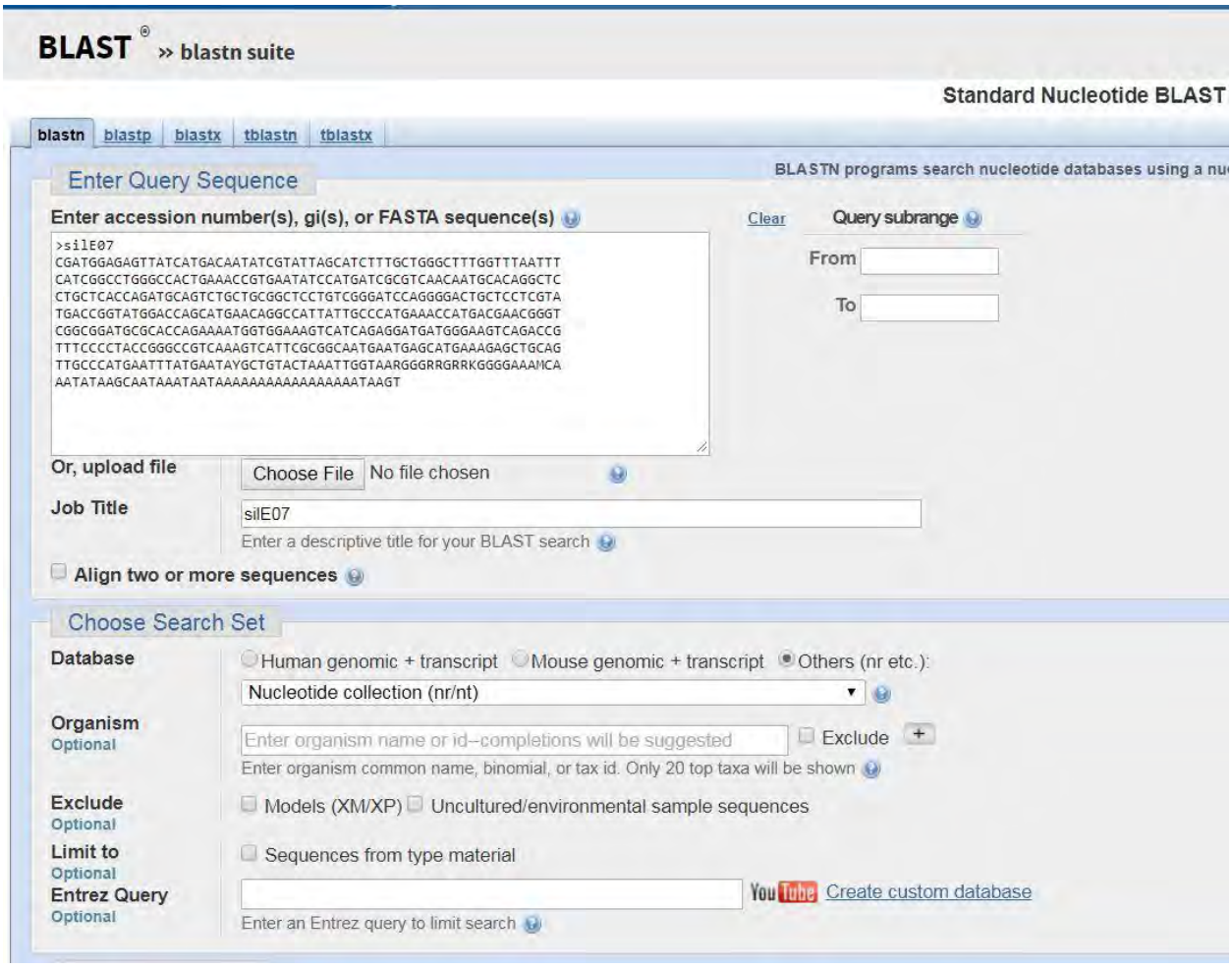


Figure3.9: Sequencing data obtained using *silE* specific forward primer

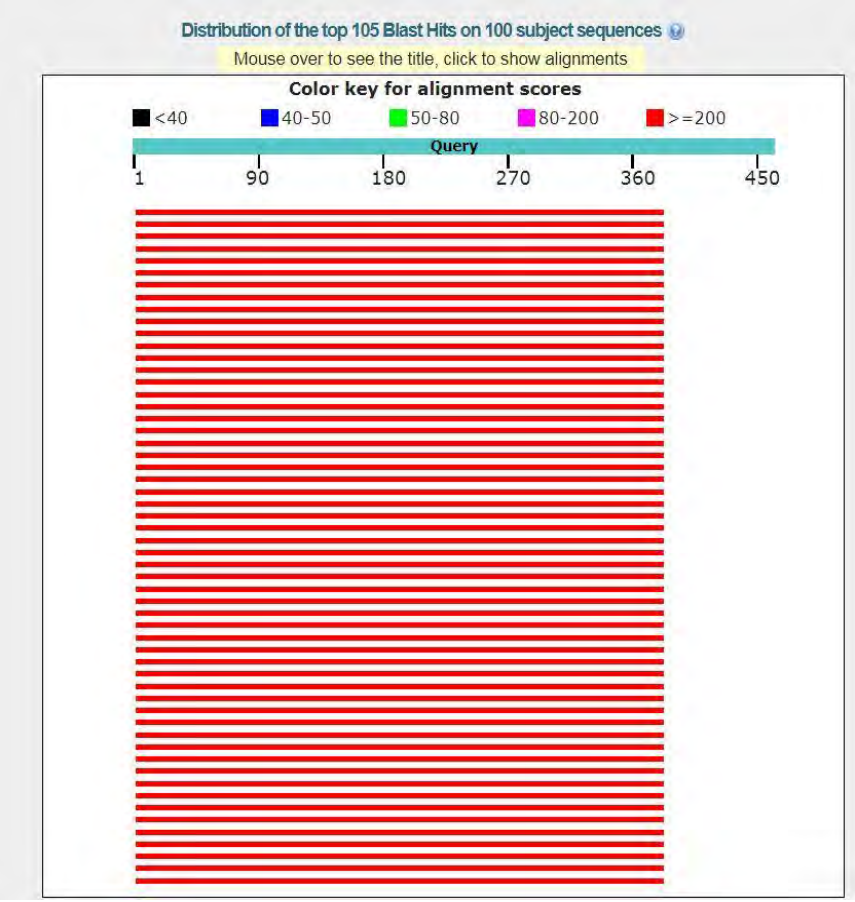
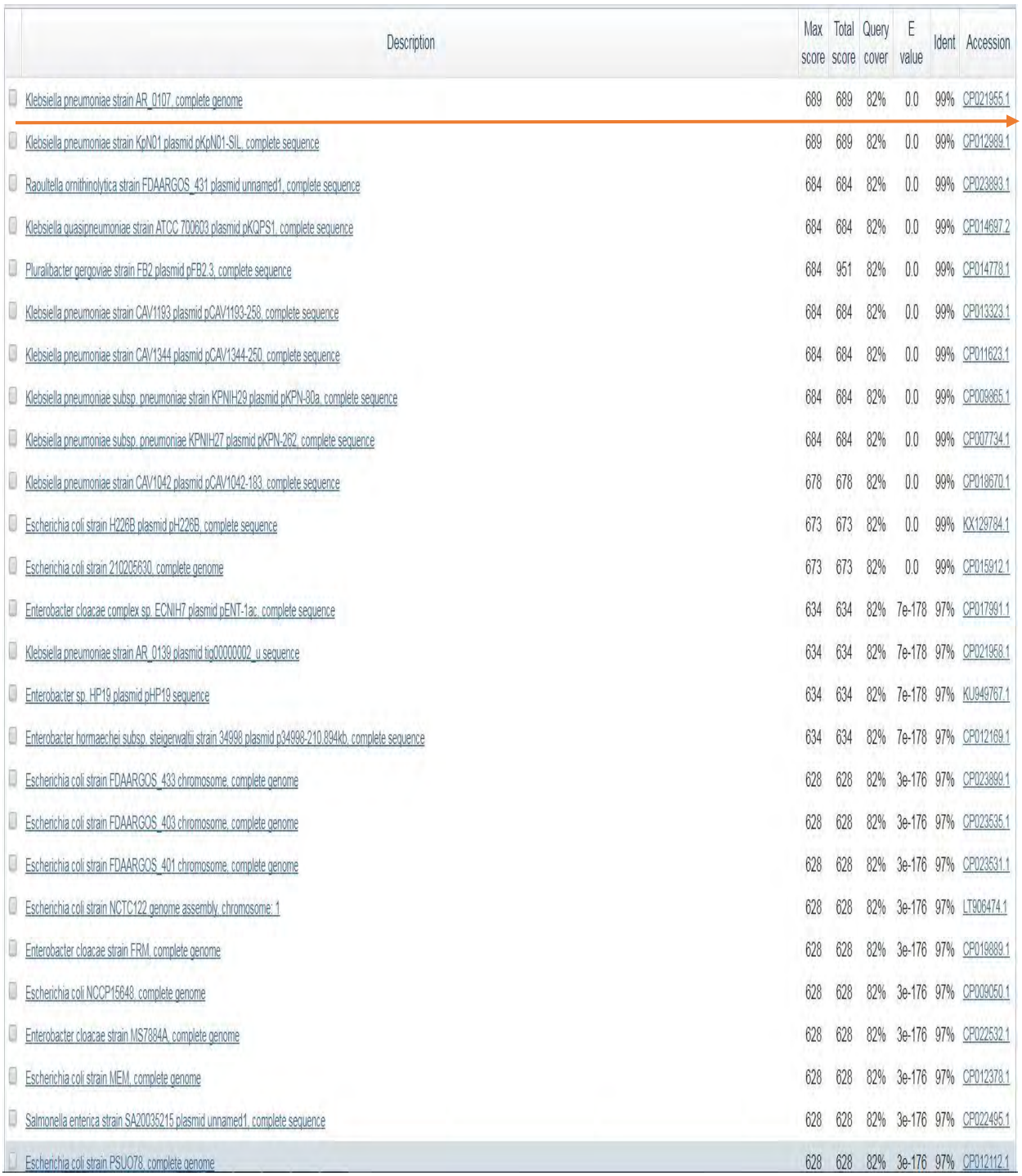


Figure 3.10: BLASTn result using the sequence of PCR product amplified by the *silE* specific forward primer of the silver resistant bacteria.



Description	Max score	Total score	Query cover	E value	Ident	Accession
Klebsiella pneumoniae strain AR_0107, complete genome	689	689	82%	0.0	99%	CP021955.1
Klebsiella pneumoniae strain KpN01 plasmid pKpN01-SIL, complete sequence	689	689	82%	0.0	99%	CP012989.1
Raoultella ornithinolytica strain FDAARGOS_431 plasmid unnamed1, complete sequence	684	684	82%	0.0	99%	CP023893.1
Klebsiella quasipneumoniae strain ATCC 700603 plasmid pKQPS1, complete sequence	684	684	82%	0.0	99%	CP014697.2
Pluralibacter gergoviae strain FB2 plasmid pFB2.3, complete sequence	684	951	82%	0.0	99%	CP014778.1
Klebsiella pneumoniae strain CAV1193 plasmid pCAV1193-258, complete sequence	684	684	82%	0.0	99%	CP013323.1
Klebsiella pneumoniae strain CAV1344 plasmid pCAV1344-250, complete sequence	684	684	82%	0.0	99%	CP011623.1
Klebsiella pneumoniae subsp. pneumoniae strain KPNIH29 plasmid pKPN-80a, complete sequence	684	684	82%	0.0	99%	CP009865.1
Klebsiella pneumoniae subsp. pneumoniae KPNIH27 plasmid pKPN-262, complete sequence	684	684	82%	0.0	99%	CP007734.1
Klebsiella pneumoniae strain CAV1042 plasmid pCAV1042-183, complete sequence	678	678	82%	0.0	99%	CP018670.1
Escherichia coli strain H226B plasmid pH226B, complete sequence	673	673	82%	0.0	99%	KX129784.1
Escherichia coli strain 210205630, complete genome	673	673	82%	0.0	99%	CP015912.1
Enterobacter cloacae complex sp. ECNIH7 plasmid pENT-1ac, complete sequence	634	634	82%	7e-178	97%	CP017991.1
Klebsiella pneumoniae strain AR_0139 plasmid tig00000002_u sequence	634	634	82%	7e-178	97%	CP021968.1
Enterobacter sp. HP19 plasmid pHP19 sequence	634	634	82%	7e-178	97%	KU949767.1
Enterobacter hormaechei subsp. steigerwaltii strain 34998 plasmid p34998-210.894kb, complete sequence	634	634	82%	7e-178	97%	CP012169.1
Escherichia coli strain FDAARGOS_433 chromosome, complete genome	628	628	82%	3e-176	97%	CP023899.1
Escherichia coli strain FDAARGOS_403 chromosome, complete genome	628	628	82%	3e-176	97%	CP023535.1
Escherichia coli strain FDAARGOS_401 chromosome, complete genome	628	628	82%	3e-176	97%	CP023531.1
Escherichia coli strain NCTC122 genome assembly, chromosome: 1	628	628	82%	3e-176	97%	LT906474.1
Enterobacter cloacae strain FRM, complete genome	628	628	82%	3e-176	97%	CP019889.1
Escherichia coli NCCP15648, complete genome	628	628	82%	3e-176	97%	CP009050.1
Enterobacter cloacae strain MS7884A, complete genome	628	628	82%	3e-176	97%	CP022532.1
Escherichia coli strain MEM, complete genome	628	628	82%	3e-176	97%	CP012378.1
Salmonella enterica strain SA20035215 plasmid unnamed1, complete sequence	628	628	82%	3e-176	97%	CP022495.1
Escherichia coli strain PSUO78, complete genome	628	628	82%	3e-176	97%	CP012112.1

Figure 3.11: Click the *Klebsiella pneumoniae* strain complete plasmid

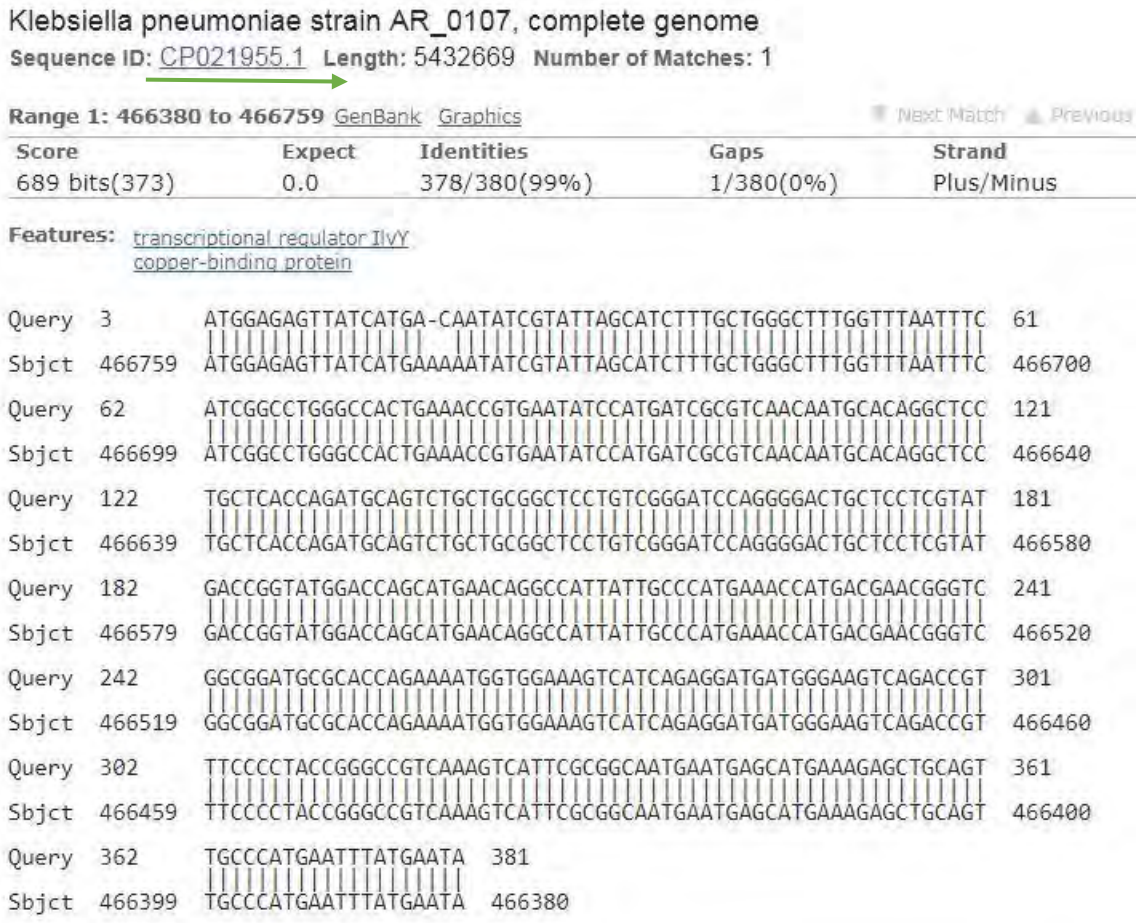


Figure 3.12

After BLASTn analysis it was found that the resistant bacterial sequence matched with SilE gene (figure-3.13). Here, it was easy to detect similarility between *silE* gene and complete genome of *Klebsiella pneumoniae* strain.

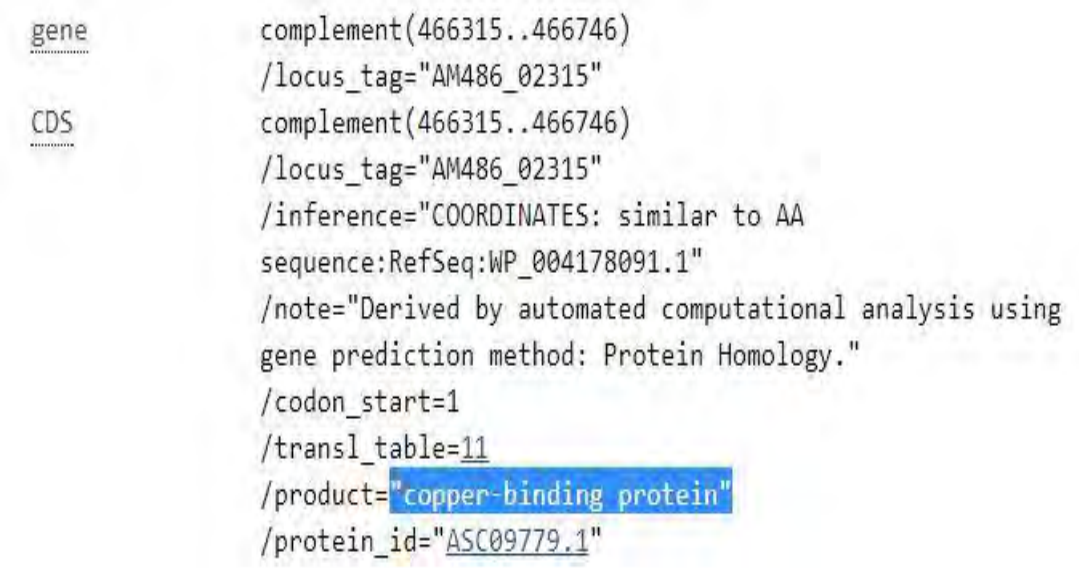


Figure 3.13

Figure 3.8 to 3.13:In summary, significant alignments with query sequence. The parameters are Maximum score, Total score, Query cover, E value and Accession number. Score is given according to the alignment number depending on the match, mismatch and gaps.Maximum identity and lower e-value shown in the BLASTn where query cover for *Klebsiella pneumoniae* is 99% which indicates 99 out of 100 sequence length is covered by this search and results indicated that the amplified sequence belongs to the SilE subclass.. Figure-3. illustrates the BLAST result of homologous sequences annotated as *Klebsiella pneumoniae* indicating that the primer pair had amplified the gene of interest.Gene range complement 466315 ..466746 where getting copper binding protein. It proved that *silE* belongs to this gene complement.

In case of another KPN, same process was found that getting desired result which were given below:

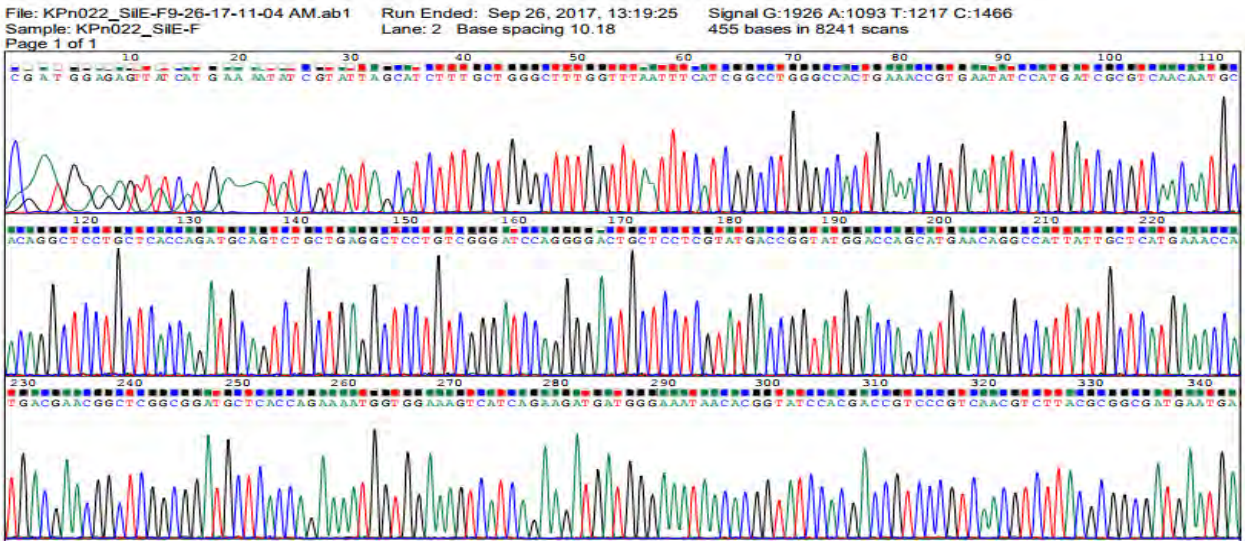
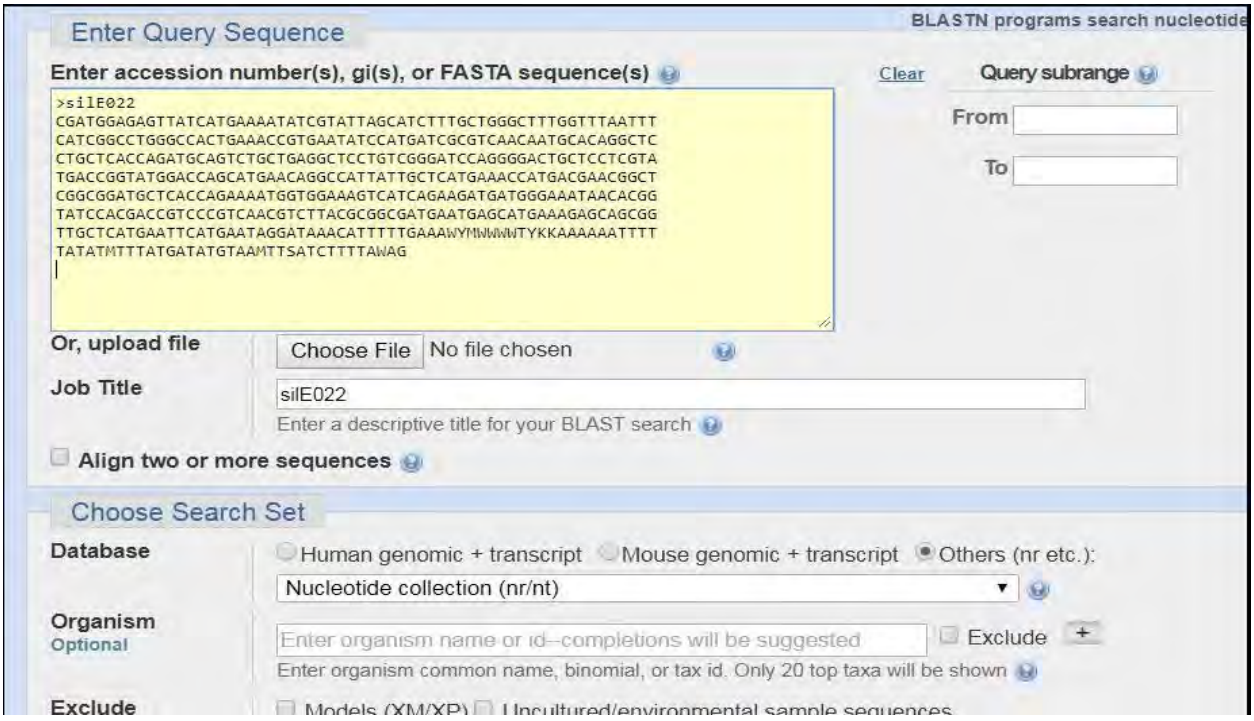
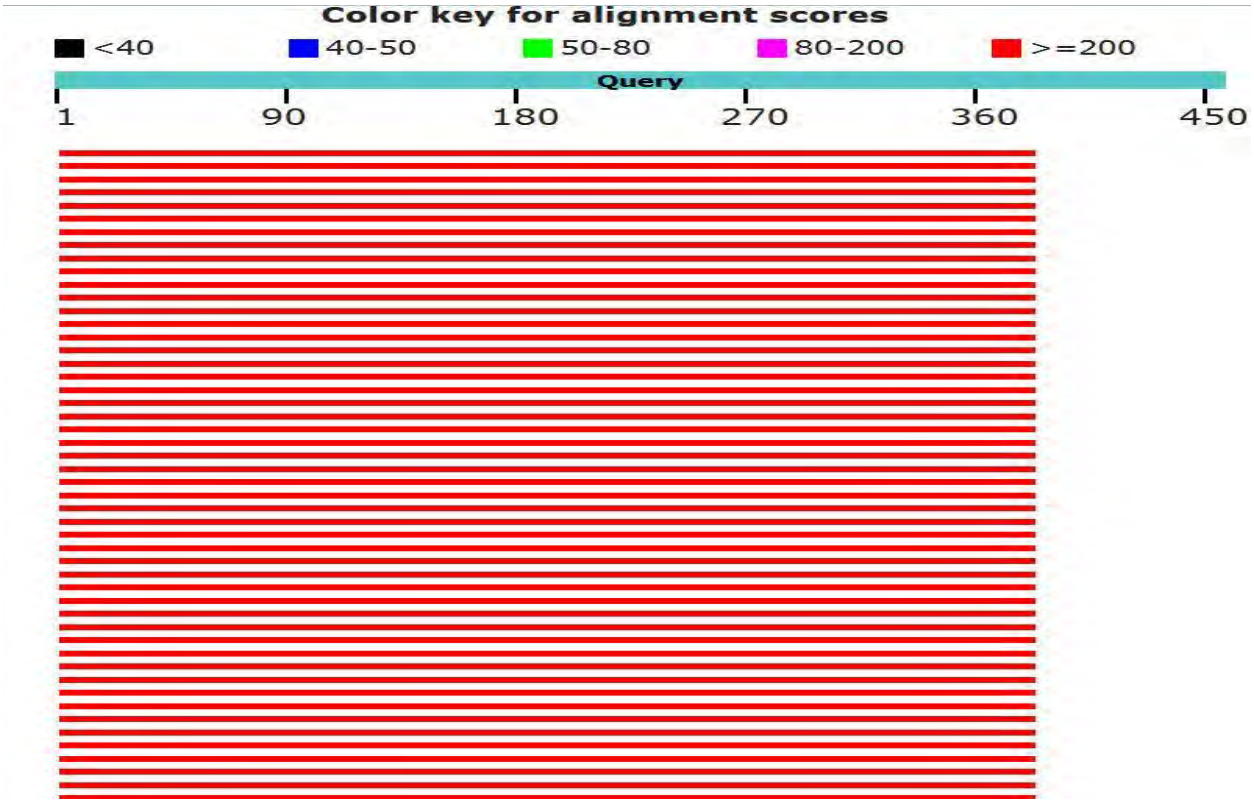


Figure 3.14: Diagrammatic representation of a part of the chromatogram of sequence

the gene sequence was analyzed by NCBI Nucleotide Basic local alignment search tool (BLAST) in case of another sample of KPN





Download GenBank Graphics

Klebsiella pneumoniae strain A64477 plasmid pKP64477a, complete sequence

Sequence ID: MF150084.1 **Length:** 228008 **Number of Matches:** 1

Range 1: 19049 to 19428 GenBank Graphics Next Match Previ

Score	Expect	Identities	Gaps	Strand
695 bits(376)	0.0	379/380(99%)	1/380(0%)	Plus/Minus

```
Query  3      ATGGAGAGTTATCATGAAAA-TATCGTATTAGCATCTTTGCTGGGCTTTGGTTTAATTTC  61
              |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  19428  ATGGAGAGTTATCATGAAAAATATCGTATTAGCATCTTTGCTGGGCTTTGGTTTAATTTC  19369

Query  62     ATCGGCCTGGGCCACTGAAACCGTGAATATCCATGATCGCGTCAACAATGCACAGGCTCC  121
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19368  ATCGGCCTGGGCCACTGAAACCGTGAATATCCATGATCGCGTCAACAATGCACAGGCTCC  19309

Query  122    TGCTCACCAGATGCAGTCTGCTGAGGCTCCTGTCGGGATCCAGGGGACTGCTCCTCGTAT  181
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19308  TGCTCACCAGATGCAGTCTGCTGAGGCTCCTGTCGGGATCCAGGGGACTGCTCCTCGTAT  19249

Query  182    GACCGGTATGGACCAGCATGAACAGGCCATTATTGCTCATGAAACCATGACGAACGGCTC  241
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19248  GACCGGTATGGACCAGCATGAACAGGCCATTATTGCTCATGAAACCATGACGAACGGCTC  19189

Query  242    GGCGGATGCTCACCAGAAAATGGTGGAAAGTCATCAGAAGATGATGGGAAATAACACGGT  301
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19188  GGCGGATGCTCACCAGAAAATGGTGGAAAGTCATCAGAAGATGATGGGAAATAACACGGT  19129

Query  302    ATCCACGACCGTCCCGTCAACGTCTTACGCGGCGATGAATGAGCATGAAAGAGCAGCGGT  361
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19128  ATCCACGACCGTCCCGTCAACGTCTTACGCGGCGATGAATGAGCATGAAAGAGCAGCGGT  19069
```

Figure 3.15

Figure 3.14 to 3.15: In summary, significant alignments with query sequence. The parameters are Maximum score, Total score, Query cover, E value and Accession number. Score is given according to the alignment number depending on the match, mismatch and gaps.Maximum identity and lower e-value shown in the BLASTn where query cover for *Klebsiella pneumoniae* is 99%

which indicates 99 out of 100 sequence length is covered by this search and results indicated that the amplified sequence belongs to the *silE* subclass.. Figure-3. illustrates the BLAST result of homologous sequences annotated as *Klebsiella pneumoniae* indicating that the primer pair had amplified the gene of interest.Gene range complement 18984..19532 where getting copper binding protein. It proved that *silE* belongs to this gene complement.

3.11 MIC (minimum inhibitory concentration) of silver nitrate against silver resistant bacteria result:

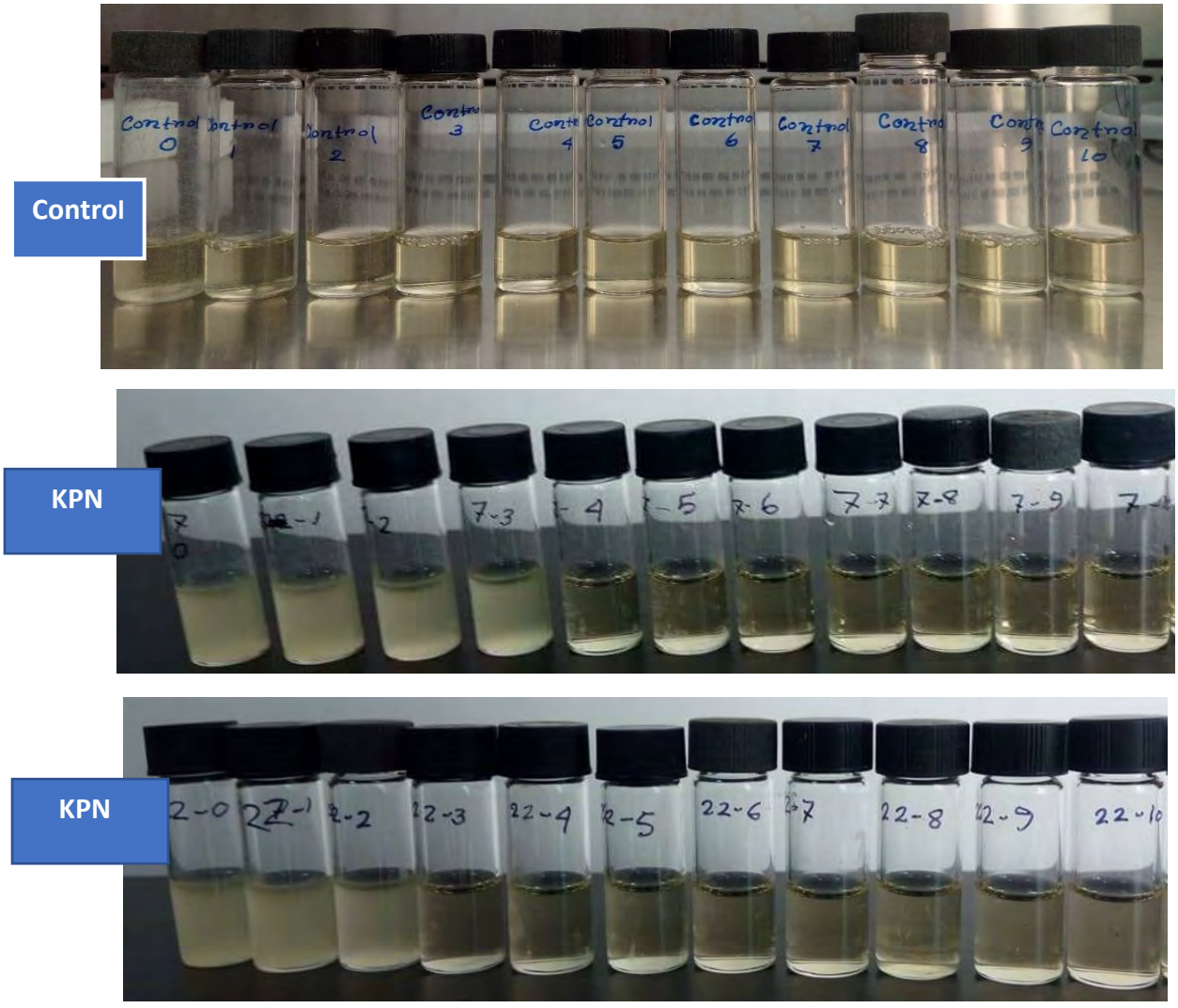


Figure 3.16 : Here, representing 3 set of gram vial where uper set indicate control and last two set indicate isolated wound sample KPN (*Klebsiella pneumoniae)* those were survived in silver nitrate solution.

By performing MIC test, it was easy to detect in which concentration of silver nitrate inhibited the growth of microorganisms. Here, showed that when the concentration of silver nirtate was low, microbes could be grow and turbidity proved that comapring with control. However, in case of higher concentration of silver nirtate bacteria did not grow at all.

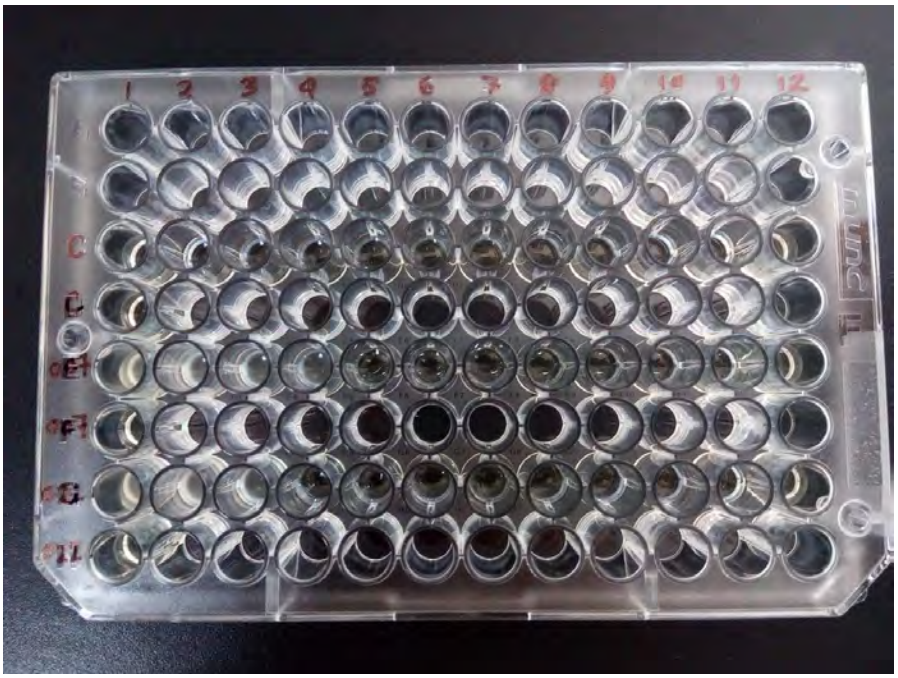


Figure 3.17: To figure out it was necessary to measure optical density (OD) by using spectrophotometer and the OD measurement were given below:

Table 3.7: Turbidimetric measurement (optical density) help to detect minimum inhibitory concentration (MIC) of silver nitrate that can inhibit the growth of bacteria

Sample ID	0	1	2	3	4	5	6	7	8	9	10
C	0.038	0.039	0.038	0.039	0.049	0.041	0.04	0.042	0.041	0.043	0.043
W.S KPN	**0.902**	**0.684**	**0.493**	**0.198**	0.043	0.049	0.042	0.042	0.045	0.045	0.044
W.S KPN	**0.973**	**0.629**	**0.601**	0.04	0.041	0.041	0.041	0.042	0.042	0.043	0.045

Discussion

4.1 Discussion:

Recently, antibiotic-resistant bacteria represent an increasing concern in wound infections. Wound colonization with these pathogenic organisms normally results in aggressive management of the wound complicated by a greatly limited choice of therapeutic antibiotics. Silver and other silver products are recognized as potential allies in combating these organisms in wounds. By using this topical chemotherapy has been fundamental in that regard and has helped to improve the survival of patients with major burns and to minimize the incidence of burn wound sepsis, a leading cause of mortality and morbidity in these patients. So, final aim is very cleared that, identified and screening of silver resistance gene in wound swab samples and management of burn and therapy is wound healing and epithelization as soon as possible in order to prevent infection and to reduce functional and aesthetic after effects.

In this study, specimens were collected and KPN were isolated from the collected specimens and confirmation of bacterial isolates was done on selective media. And the methods which were used in this study including Gram staining, biochemical tests and Analytical profile index (API). Beside of this, in this study, it was investigated silver resistant bacteria. Furthermore, molecular techniques such as PCR and nucleotide sequencing were used for genotypic confirmation whether silver resistant bacteria contain *silE* gene or not and lastly, minimum inhibitory concentration (MIC) was done to determine silver resistant bacteria in presence of silver nitrate and analysis the efficacy of silver nitrate treatment.

After done with methods, got desired result from the specific experiment. Getting 5 *Klebsiella pneumoniae* out of 15 wound/burn sample of hospitalized patient and observed colony morphology on MacConkey agar plate which was selective media for *Klebsiella spp*. Gram staining result deals with cellular morphology and arrangement by showing the pink color denoting that they were gram negative rods. Beside of this, biochemical test detects the biochemical characteristics such as oxidase negative, catalyst positive, produce acid in TSI, citrate test positive etc. by comparing with control, which proved that microorganism *Klebsiella pneumoniae* belong from the *Enterobactericeae* family.

Moreover, according to this study's aim got KPN and the frequency of the *Klebsiella pneumoniae* was about 33%. Nosocomial infection is an endemic problem encountered in hospitalized patients all over the world including Bangladesh. 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). Although the sample number about 15 which was less for determine the exact number of frequency if we got more sample than it was easily to investigate the actual frequency of *Klebsiella pneumoniae.* In the United States, *Klebsiella* accounts for 3 to 7% (Sirot, 1995) of all nosocomial bacterial infections, placing them among the eight most important infectious pathogens in hospitals (Podschun, 2001) whereas, in particular regions or hospitals in this study, the incidence of KPN can reach 33%.

After that, in this study found two silver resistant bacteria among these five isolated *Klebsiella pneumoniae* by using 1mg/L silver nitrate solution. As it was known to us that silver act as antimicrobial agent (Monteiro, 2009). Silver nitrate is widely used as antiseptics in medicine and health in Bangladesh. Ag coated bandages are used to cover burns and traumatic injuries. But many of the pathogenic bacteria growing antibiotic resistant day by day. Here, isolated two KPN also able to resist antibiotic for non-specific treatment. In result, performing antibiotic resistant spectrum of two silver resistant KPN isolated in this test and easily got information about the number of the resistant antibiotic such as: one KPN sample resistant to Azithromycin, Ceftriaxone, Ciprofloxacin, Cefixime, Carbapenem antibiotics. Another KPN resistant to Azithromycin, Ceftriaxone, Ciprofloxacin, Cefixime, Carbapenem, gentamycin, Tobramycin. In Bangladeshi perspective silver nitrate treatment is very non- specific that is why pathogenic KPN sample becoming resistant to antibiotic in case of burn/wound hospitalized infection treatment.

As silver resistance genes are plasmid-mediated and the isolates were collected from hospitalized burn/wound patients, so it was probable that silver resistance could easily be transferred among those isolates. Silver resistant bacteria contain 9 sil genes in their genome such as: *silP, silA, silB, silC, silR, silS, silE, ORF105 and silABC.* But in many studies, it was found that *silE* gene is more frequent that other genes and highly significant that *silE* is more susceptible to silver (parikh,2008). In this preliminary study, on wound/burn infection *silE* was selected for investigation. For genomic detection of silE was determined by PCR followed by Sanger sequencing. Where two isolated silver resistant bacteria contain *silE* gene in their genome 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 were 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.

In this study, another important aim is to investigate and analysis minimum inhibitory concentration of MIC. This protocol tries to build up first time in Bangladesh. 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). Through MIC one can determine in which amount silver nitrate solution were added to eliminate silver resistant bacteria and recover faster of wound infection. This minimum inhibitory concentration of MIC can vary in case of different resistant bacteria.

Most of the studies have produced different MIC data for AgNO3, 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). Here, identify the dose of MIC in non-specific treatment of wound/burn patient in Bangladesh perspective. If the antimicrobial dose level is increase, then the growth the pathogenic bacteria inhibited. By using this technique reduce the antimicrobial resistant bacteria and hospitalized infection and reduce cost, time as well. the Studies found that isolated KPN's minimum inhibitory concentration of silver nitrate accordingly, 4 mg/L and 3mg/L.

Another study on hospitalized site at ideSHi (unpublished data) investigate the MIC dose 10 mg/L in *Klebsiella pneumoniae* acquired from nosocomial infection. On the other hand, there are some drawback of this study, since the number of the sample size is small that is why did not get the actual frequency of KPN in burn/wound infection. Here, analysis and detection only *silE* gene among 9 gene silver resistant genes. In future, have to work with other sil gene 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.

So, all in all, it was stated the likelihood of widespread resistance to silver and the potential for silver to induce cross-resistance to antibiotics, find out the friendly environment efficient method to determine the efficacy of ionic silver against pathogenic wound infection. In addition, it also

correlate and hypothesized that the optimized silver (Silver Nitrate) can promote wound healing and efficiently kill bacteria.

Conclusion

5.1 Conclusion:

Antibacterial resistance developing from the use of silver biocides may well have been overstated. It is proposed that hygiene should be emphasized and targeted towards those applications that have demonstrable benefits in wound care (Gupta and silver,1998 and silver, Lo 1999). As it was a preliminary study, it would be appropriate for future studies to determine the actual prevalence of these genes and meet the final purpose of this study 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.

Bibliography

References

Alexander, J. W. (2009). History of the medical use of silver. *Surg Infect (Larchmt), 10*(3), 289-292.

Alkaabi, S. A. G. (2013). Bacterial Isolates and Their Antibiograms of Burn Wound Infections in Burns Specialist Hospital in Baghdad. *J. Baghdad for Sci, 10*(2), 331-340.

Atiyeh, B. S., Costagliola, M., Hayek, S. N., & Dibo, S. A. (2007). Effect of silver on burn wound infection control and healing: review of the literature. *burns, 33*(2), 139-148.

Church, D., Elsayed, S., Reid, O., Winston, B., & Lindsay, R. (2006). Burn wound infections. *Clinical microbiology reviews, 19*(2), 403-434.

Classen, D. C., Evans, R. S., Pestotnik, S. L., Horn, S. D., Menlove, R. L., & Burke, J. P. (1992). The timing of prophylactic administration of antibiotics and the risk of surgical-wound infection. *New England Journal of Medicine, 326*(5), 281-286.

Cutting, K., White, R., & Edmonds, M. (2007). The safety and efficacy of dressings with silver–addressing clinical concerns. *Int Wound J, 4*(2), 177-184.

Donowitz, G. R., & Mandell, G. L. (1988). Beta-lactam antibiotics. *New England Journal of Medicine, 318*(8), 490-500.

Dunn, K., & Edwards-Jones, V. (2004). The role of Acticoat™ with nanocrystalline silver in the management of burns. *burns, 30*, S1-S9.

Fox, C., & Stanford, J. (1971). Anti-bacterial action of silver sulphadiazine and DNA binding. *Research in burns. Bern: H. Huber publishers*, 133-138.

Franci, G., Falanga, A., Galdiero, S., Palomba, L., Rai, M., Morelli, G., & Galdiero, M. (2015). Silver nanoparticles as potential antibacterial agents. *Molecules, 20*(5), 8856-8874.

Gemmell, C. G., Edwards, D. I., Fraise, A. P., Gould, F. K., Ridgway, G. L., & Warren, R. E. (2006). Guidelines for the prophylaxis and treatment of methicillin-resistant Staphylococcus aureus (MRSA) infections in the UK. *Journal of Antimicrobial Chemotherapy, 57*(4), 589-608.

George, N., Faoagali, J., & Muller, M. (1997). Silvazine™(silver sulfadiazine and chlorhexidine) activity against 200 clinical isolates. *Burns, 23*(6), 493-495.

Gupta, A. (1999). RT-PCR: characterization of long multi-gene operons and multiple transcript gene clusters in bacteria. *BioTechniques, 27*(5), 966-970, 972.

Gupta, A., Matsui, K., Lo, J.-F., & Silver, S. (1999). Molecular basis for resistance to silver cations in Salmonella. *Nature medicine, 5*(2), 183-188.

Hidron, A. I., Edwards, J. R., Patel, J., Horan, T. C., Sievert, D. M., Pollock, D. A., & Fridkin, S. K. (2008). Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infection Control & Hospital Epidemiology, 29*(11), 996-1011.

Kim, J. S., Kuk, E., Yu, K. N., Kim, J.-H., Park, S. J., Lee, H. J., . . . Hwang, C.-Y. (2007). Antimicrobial effects of silver nanoparticles. *Nanomedicine: Nanotechnology, Biology and Medicine, 3*(1), 95-101.

Kirsner, R. S., Orsted, H., & Wright, J. B. (2001). The Role of Silver in Wound Healing Part 3 Matrix Metalloproteinases in Normal and Impaired Wound Healing: A Potential Role of Nanocrystalline Silver. *Wounds, 13*(3 SUPPL. C).

Klasen, H. (2000). Historical review of the use of silver in the treatment of burns. I. Early uses. *Burns, 26*(2), 117-130.

Lansdown, A. B. (2006). Silver in health care: antimicrobial effects and safety in use *Biofunctional textiles and the skin* (Vol. 33, pp. 17-34): Karger Publishers.

Lo, J. (2001). The silver binding protein SilE of plasmid-mediated silver resistance in Escherichia coli.

Moyer, C. A., BRENTANO, L., GRAVENS, D. L., MARGRAF, H. W., & MONAFO, W. W. (1965). Treatment of large human burns with 0.5% silver nitrate solution. *Archives of surgery, 90*(6), 812-867.

Price, W. R., & Wood, M. (1966). Silver nitrate burn dressing: treatment of seventy burned persons. *The American Journal of Surgery, 112*(5), 674-680.

Robert, J. G. (2011). IDSA Public Policy: Combating Antimicrobial Resistance: Policy Recommendations to Save Lives. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America, 52*(Suppl 5), S397.

Sondi, I., & Salopek-Sondi, B. (2004). Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. *Journal of colloid and interface science, 275*(1), 177-182.

Stanford, W., Bert, W. R., & Charles, L. F. (1969). Clinical experience with silver sulfadiazine, a new topical agent for control of pseudomonas infections in burns. *Journal of Trauma and Acute Care Surgery, 9*(5), 377-388.

Taylor, P., Ussher, A., & Burrell, R. (2005). Impact of heat on nanocrystalline silver dressings: Part I: Chemical and biological properties. *Biomaterials, 26*(35), 7221-7229.

Weinstein, R. A., Gaynes, R., Edwards, J. R., & System, N. N. I. S. (2005). Overview of nosocomial infections caused by gram-negative bacilli. *Clinical infectious diseases, 41*(6), 848-854.

Williams, K. P., Gillespie, J. J., Sobral, B. W., Nordberg, E. K., Snyder, E. E., Shallom, J. M., & Dickerman, A. W. (2010). Phylogeny of gammaproteobacteria. *Journal of bacteriology, 192*(9), 2305-2314.

Appendix

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)	Prepare up to 900ml for autoclave
NaCl (Sigma)	5	
Agar (Himedia, India)	4	
KH2PO4 (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 Paper	Fisherbrand®
Weighing Machine	OHAUS®
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