Analysis of multidrug resistant *Klebsiella pneumoniae* and *Enterobacter aggalomerans* isolates from under five children suffering from acute respiratory infections in Dhaka, Bangladesh.

SAMINA KABIR CHOWDHURY

STUDENT ID: 11126001

DEPARTMENT OF MATHMATICS AND NATURAL SCIENCES

BRAC UNIVERSITY

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DECLARATION

I hereby certify that I am the only author of this thesis and that no part of this thesis has been published or submitted for publication. I hereby declare that the work entitled **"Analysis of multi-drug resistant *Klebsiella pneumoniae* and *Enterobacter aggalomerans* isolates from under 5 children suffering from acute respiratory infections in Dhaka, Bangladesh"**-is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made clearly in the text. I declare that this is a true copy of my thesis, including any final assessments, as permitted by my thesis supervisors and that this thesis has not been submitted for a higher degree to any other University or Institution.

Samina Kabir Chowdhury

Certified

Dr. Md. Kaiissar Mannoor
Supervisor
Scientist and Head
Institute Developing Science
& Health Initiatives (ideSHi)

Dr. Mahboob Hossain
Supervisor
Associate Professor
Department of Mathematics
& Natural Sciences
BRAC University

A special feeling of gratitude to my loving mom

I dedicate my dissertation work to my family. I also dedicate this dissertation to my university professors who have supported me throughout my undergraduate life. I will always thank them for all the approaches they have made to develop my professional and personal life.

I dedicate this work and give special thanks to my best friends and the people of ideSHi laboratory.

ACKNOWLEGDEMENT

THIS THESIS IS THE TERMINATION OF MY JOURNEY IN OBTAINING MY BACHELOR DEGREE.

Finally I could complete my undergraduate thesis project. However only my name appears on the cover of this dissertation, many people have contributed to its making. I obliged to all those people who have made this dissertation possible and because of whom my undergraduate experience has been one that I will reminisce forever.

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LIST OF ABBREVIATION

AST	Antibiotic Sensitivity / resistant Test
API	Analytical profile Index
ARI	Acute respiratory infections
ATP	Adenosine Triphosphate
CDC	Centers for Disease Control and Prevention
CT	Catalogue
CA	Citrate Agar
CLSI	Clinical and Laboratory Standards Institute
DMCH	Dhaka Medical College Hospital
DNA	Deoxyribonucleic acid
EtOH	Ethyl alcohol
Et-Br	Ethidium Bromide
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric Acid
IdeSHi	Institute for developing Science and Health initiatives
LRTI	Lower Respiratory Tract Infections
MDR	Multi-Drug Resistant
MHB	Mueller Hinton broth
MHA	Mueller Hinton Agar
Mac	MacConkey Agar
MIU	Motility Indole Urea
SDS	Sodium dodecyl sulfate
SSMCH	Shaheed Suhrawardy Medical College Hospital
TE	Tris EDTA buffer

TSI	Triple Sugar Iron agar
URTI	Upper Respiratory Tract Infections
UNICEF	United Nations Children's Fund
WHO	World Health Organization

ABSTRACT

Emergence of resistance against multiple types or classes of antibiotics among bacterial pathogens associated with Acute Respiratory Infection (ARI) is a global concern. Since, ARI still remains the major cause of child death worldwide, this loss of effectiveness of antibiotics can cause severe health problems of children suffering from acute respiratory infections. Acute respiratory infection (ARI) is the leading cause of mortality and morbidity among <5 children in developing countries like Bangladesh. In this study we focused on elucidating the correlation between antibiotic resistance and plasmids in two multi-drug resistant bacterial respiratory isolates obtained from an ARI surveillance study conducted in Dhaka. From 196 nasal swab specimens collected for a systematic ARI surveillance study from under-five patients presented to Dhaka Medical College Hospital (DMCH) and Shaheed Suhrawardy Medical College Hospital (SSMCH), Two isolates of multidrug resistant (MDR) bacteria-one *Klebsiella pneumoniae* and *Enterobacter aggalomerans* were found. The isolates were identified and confirmed by microbiological culture and appropriate biochemical tests, and their multi-drug resistance characteristics were confirmed by disc diffusion method. Plasmid curing was performed on the two MDR isolates using Ethidium bromide, SDS, and elevated temperature curing methods, and antibiotic sensitivity testing was performed to check its effect on their multi-drug resistance. Both isolates were found resistant towards ampicillin, azithromycin, levofloxacin, amoxicillin, tobramycin, cefixime, cefotaxime, penicillin, gentamycin, imipenem, trimethoprim and gentamycin. The minimum inhibitory concentration (MICs) of Ethidium bromide was determined. Loss of plasmid by Ethidium bromide treatment correlated with loss of resistance to antibiotics in cured strains. Plasmid curing with SDS produced similar results. Curing plasmid DNA by elevated temperature (45°C) produced significant changes in *Enterobacter aggalomerans* cells but had little effect on *K. pneumoniae*. The results of this study can help direct the mechanistic investigation of multi-drug resistance in respiratory pathogens explore newer avenues.

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Acute respiratory tract infections are among the most common illnesses all around the world. It is a severe infection that precludes normal breathing function. It usually arises as a viral infection in the nose, trachea (windpipe), or lungs. If the infection is not cured, it can spread to the entire respiratory system. Severity of Acute respiratory infection may result in death. Correspondingly, acute respiratory infections are transmissible, which means they can spread from one person to another.

Children are particularly susceptible to acute respiratory infections (ARI). According to the World Health Organization, ARI kill an estimated 2.6 million children annually worldwide (WHO, 1986).

ARI contributes to 2-4% of all deaths in children <5 years of age in developed countries, and 19% to 21% in the Eastern Mediterranean, Africa, and South East Asia regions (Emmelin, 2007).

ARI causes 10%-15% of all infant deaths in developed countries. This figure rises to 27% in the developing world. The total number of acute respiratory infection episodes in <5 children has been estimated to range between 500 and 900 million a year in developing countries. In the absence of appropriate treatment, 15-25% of all children with pneumonia would die (UNICEF, 1992).

Acute respiratory infection is considered as one of the commonest cause of death and sickness among <5 children in developing countries including Bangladesh. A joint WHO and UNICEF statement says that acute respiratory infections, diarrheal diseases and malnutrition are the major reasons of illness and death in children in the developing countries (WHO, 1986).

Health information unit of national health services of Bangladesh show that the frequency of ARI among all outpatients in a rural health complex was 18.2%, of which 16% were children between 0-5 years of age (Akbar, 1986).

According to a statistical report the total morbidity percentage due to ARI was 8.8% (Bangladesh Health Services Report , 1990).

According to the 2011 report of Bangladesh Demographic and Health Survey (BDHS) the morbidity percentage of children under-five with symptoms of ARI is about 6% (Associates, 2011). In recent, emerging threats of ARI causing multidrug resistant gram negative bacteria are mounting the mortality and morbidity rate of child < 5. ARIs causing bacteria that are inherently resistant or that can acquire resistance are surviving and replacing the drug-susceptible bacteria. This is really a matter of concern that the number of < 5 aged children is infected by the multidrug resistant ARI causing microorganisms.

The study under question here is an extension of the work on acute respiratory and enteric infection of under-five children in Dhaka already going on at Institute for Developing Science and Health Initiatives (ideSHi). Two multidrug resistant ARI causing gram negative isolates, namely *Klebsiella pneumoniae* and *Enterobacter aggalomerans* were found which are resistant against 11 and more antibiotics.

1.2 Acute respiratory infections (ARIs)

Acute respiratory infections (ARIs) are classified as upper respiratory tract infections (URIs) or lower respiratory tract infections (LRIs).

1.2.1 Upper Respiratory Tract Infections

Upper Respiratory Tract Infections include rhinitis (common cold), sinusitis, ear infections, acute pharyngitis or tonsillopharyngitis, epiglottitis, and laryngitis—of which ear infections and pharyngitis cause more severe complications including deafness and acute rheumatic fever; respectively. The vast majority of URI's have viral etiology. Rhinoviruses account for 25 to 30 percent of URIs; respiratory syncytial viruses (RSVs), Para influenza and influenza viruses, human metapneumovirus, and adenoviruses for 25 to 35 percent; corona viruses for 10 percent; and unidentified viruses for the remainder (Denny, 1995). Because most URIs is self-limiting, their complications are more important than the infections. Acute viral infections predispose children to bacterial infections of the sinuses and middle ear (Berman, 1995), and aspiration of infected secretions and cells can result in LRIs.

1.2.1.1 Pathogenesis: Pathogenesis occurs by inhalation of droplets after which organisms gain entry to the respiratory tract and invade the mucosa. Epithelial destruction may result, along with redness, edema, hemorrhage and sometimes an exudate.

1.2.1.2 Clinical Manifestations:

Acute upper respiratory tract infections include rhinitis, pharyngitis/tonsillitis and laryngitis, often referred to as a common cold, and their complications: sinusitis, ear infection and sometimes bronchitis (though bronchi are classified as part of the lower respiratory tract).Symptoms of URTIs commonly include cough, sore throat, runny nose, nasal congestion, headache, low grade fever, facial pressure and sneezing. Onset of symptoms usually begins 1–3 days after exposure. The illness usually lasts 7–10 days.

Group A beta hemolytic streptococcal pharyngitis/tonsillitis (strep throat) typically presents with a sudden onset of sore throat, pain with swallowing and fever. Strep throat does not usually cause runny nose, voice changes, or cough.

1.2.2 Lower respiratory tract infections

Pneumonia and bronchiolitis are the common lower respiratory tract infections (LRIs) in children. The respiratory rate is considered as main clinical sign for diagnosing acute LRIs in children who are coughing and breathing promptly. The presence of lower chest wall indrawing identifies more severe disease (Mulholland et al.1992; Shann et al., 1984).

1.2.2.1 Pathogenesis: Organisms enter the distal airway by inhalation or aspiration. The pathogen multiplies in or on the epithelium, causing inflammation, increased mucus secretion, and impaired mucociliary function; other lung functions may also be affected. In severe bronchiolitis, inflammation and necrosis of the epithelium may block small airways leading to airway obstruction.

1.2.2.2 Clinical Manifestations:

Lower respiratory tract infection (LRTI), while often used as a synonym for pneumonia, can also be applied to other types of infection including lung abscess and acute bronchitis. Symptoms include shortness of breath, weakness, fever, coughing and fatigue.

There are a number of infections that can affect the lower respiratory tract. As it mentioned earlier, the two most common are bronchitis and pneumonia (Therapeutic Guidelines; 2006), but Influenza can affects both the upper and lower respiratory tracts.

As of 2010, lower respiratory infections cause about 2.8 million deaths down from 3.4 million in 1990 (Therapeutic guidelines, 2000).

In 2013, LRTIs caused about 2.7 million deaths, whereas in the year of 1990 about 3.4 million deaths occurred .This was 4.8% of all deaths in 2013 (Disease Study 2013).

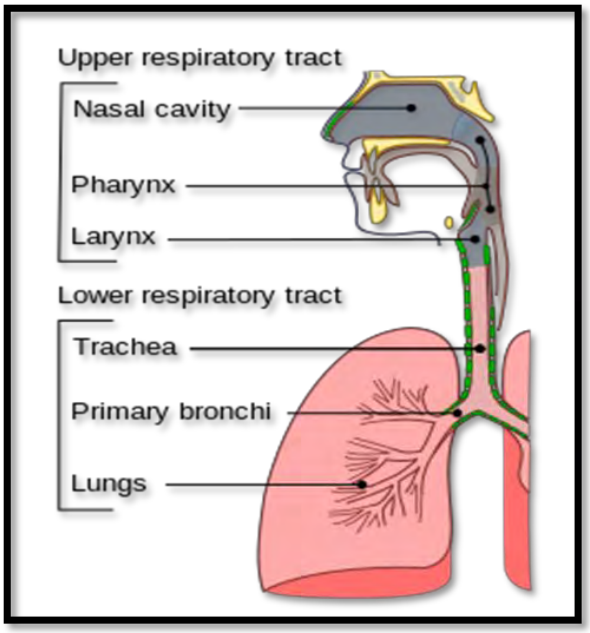


Figure 1.1: Upper and lower respiratory tract of human body.

1.3 Understanding gram negative and gram positive bacteria

According to the Gram staining theory, bacteria can be differentiated by the chemical and physical properties of bacterial cell walls by detecting the presence of peptidoglycan layer.

Gram-positive bacteria take up the crystal violet stain used in the Gram staining, and then appear purple-colored when seen through a microscope. Gram positive bacteria consisting of thick peptidoglycan layer in the bacterial cell wall retain the stain after it is washed away from the rest of the sample, in the decolorization stage of the test.

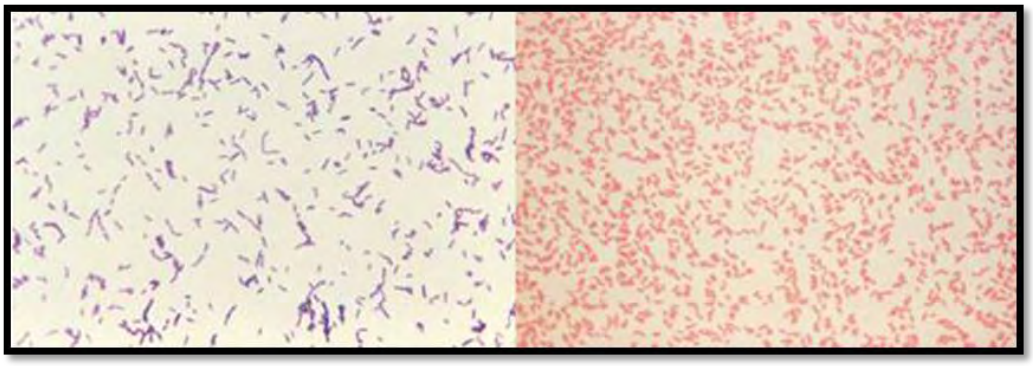


Figure 1.2: The image on the left shows Gram-positive bacteria, which retain a purple stain. The image on the right is Gram-negative bacteria, which appear pink or red. (Image courtesy: CDC).

Gram-negative bacteria cannot retain the violet stain after the decolorization step; alcohol used in this stage degrades the outer membrane of gram-negative cells making the cell wall more porous and incapable of retaining the crystal violet stain. Their peptidoglycan layer is much thinner and sandwiched between an inner cell membrane and a bacterial outer membrane, causing them to take up the counter stain (safranin or fuchsine) and appear red or pink.

Both structurally and chemically, Gram negative cell walls are more complex than gram positive cell walls.

1.4 Etiology of ARI's

The causative agents of ARIs include a wide variety of microorganisms such as *Streptococcus pneumoniae, Haemophilus influenzae*, and *Moraxella catarrhalis* (Bartlett, 1995). *Streptococcus pneumoniae* (gram positive), usually called pneumococcus and some of gram negative bacteria especially *Klebsiella pneumoniae* and *Haemophilus influenza* are the most common cause of bacterial pneumonia worldwide. *Klebsiella pneumoniae* responsible for about 30 percent of the total pneumonia case load with at least half of the cases occurring in the developing world. The Infectious Diseases Society of America (IDSA) has identified six pathogens as particular threats of multidrug resistance. *Klebsiella pneumoniae* is one of them, (Boucher et al., 2004). *Haemophilus influenza* type b (Hib) is involved in as many as 20 percent of the world's severe pneumonia cases. These bacteria, which also cause meningitis and other severe infections, were

responsible for 7.9 million cases of pneumonia in 2000, resulting in the deaths of an estimated 300,000 children age under five.

Infants can obtain lung infections before birth by breathing infected amniotic fluid or through a blood-borne infection through the placenta. Infants can also inhale contaminated fluid from the vagina at birth. The most prevalent pathogen causing ARIs in newborns is *Streptococcus agalactiae,* also known as group-*B streptococcus (*GBS). GBS causes more than half of ARIs in the first week after birth (Webber et al., 1990). Other bacterial causes of neonatal infection include *Listeria monocytogenes* and a variety of mycobacteria. Another group is *Chlamydia trachomatis*, acquired at birth but not causing pneumonia until two to four weeks later.

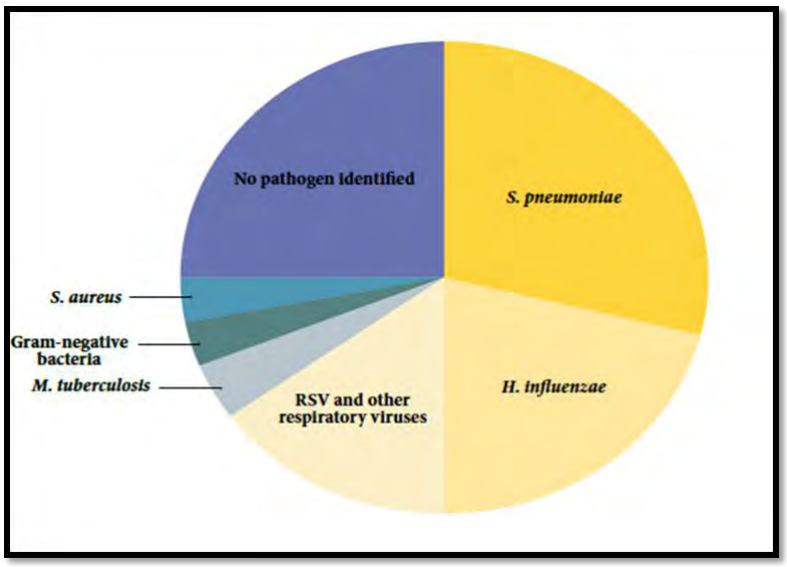


Figure 1.3: Bacterial pathogens responsible for pneumonia (ARI). (Ref: World lung foundation)

In older infants, infection can occur due to exposure to microorganisms, with common bacterial causes including *Streptococcus pneumoniae, Escherichia coli, Klebsiella pneumoniae, Moraxella catarrhalis* and *Staphylococcus aureus*. While children older than one month tend to be at risk for the same microorganisms as adults, children under five are much less likely to have pneumonia caused by *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* or *Legionella pneumophila*. In contrast, older children and teenagers are more likely to acquire *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* than adults (Wubbel et al., 1999).

Rhino and corona viruses are the most predominant viral agent that caused ARIs, followed by parainfluenza viruses, respiratory syncytial virus (RSV), HMPV and HBoV.

1.5 Antibacterial resistance

Antibacterial resistance is an alarming issue now. Spreading of resistant organisms in developing countries is one of the major reasons that are increasing the problem of antibiotic resistance. Several factors such as unregulated dispensing and manufacture of antibacterial, shortened antibacterial therapy, inadequate access to effective drugs and sometimes drugs of questionable quality and overall poverty are likely to be contributing to antibacterial resistance (WHO, 2000).

Antibiotic resistance is a natural occurrence. When an antibiotic is used, bacteria that can resist that antibiotic have chance of survival than those that are "susceptible." Susceptible bacteria are killed or inhibited by an antibiotic, resulting in a pressure for the survival of resistant strains of bacteria. Some bacteria are naturally resistant to certain types of antibiotics. However, bacteria may also become resistant in two ways:

1) By a genetic mutation or

2) By acquiring resistance from another bacterium.

1.5.1 Genetic mutation

Mutations are the infrequent spontaneous changes of genetic sequence and are thought to occur in about one in one million to one in ten million cells. Diverse genetic mutations yield different types of resistance. Some mutations support the bacteria to produce potent chemicals (enzymes) that inactivate antibiotics, while other mutations eliminate or distort the cell target that the antibiotic attacks. Still others restricted their portals of entry mainly by cell's porin proteins dose not allowing antibiotics into the cell, and others manufacture pumping mechanisms that export the antibiotic back outside which have entered into the cell cytoplasm so it never reaches its target.

1.5.2 Transformation of genetic material

Bacteria can acquire antibiotic resistance genes from other bacteria in several ways. By a process called "conjugation," bacteria can transfer its genetic material, including genes encoding

resistance to antibiotics (found on plasmids and transposons) from one bacterium to another. Viruses are another supportive mechanism for passing resistance traits between bacteria. The resistance traits from one bacterium are packaged into the head portion of the virus. The virus then injects the resistance traits into any new bacteria it attacks. Bacteria also have the capability to acquire naked, "free" DNA from their environment.

Any bacteria that acquire resistance genes, whether by spontaneous mutation or genetic exchange with other bacteria, have the ability to resist one or more antibiotics. Because bacteria can collect multiple resistance traits over time, they can become resistant to many different groups of antibiotics.

Genetically, antibiotic resistance spreads through bacteria populations both "vertically," when new generations inherit antibiotic resistance genes, and "horizontally," when bacteria share or exchange segments of genetic material with other bacteria. Horizontal gene transfer can even occur between different bacterial species. Naturally, antibiotic resistance spreads as bacteria themselves move from place to place; humans can pass the resistant bacteria to others by coughing, sneezing or contact with unwashed hands.

The recent higher-levels of antibiotic-resistant bacteria are ascribed to the overuse and abuse of antibiotics. Patients sometimes take or suggested antibiotics unnecessarily, to treat viral illnesses like the common cold.

1.6 Objectives of the study

1) Identification of the respiratory isolates by microbiological culture method.
2) Reconfirmation of multi-drug resistance of *Klebsiella pneumonia* and *Enterobacter aggalomerans* isolates by antibiogram.
3) Plasmid curing of the MDR strains and checking its effect on the multi-drug resistance of the strains.

CHAPTER 2: LITERATURE REVIEW

2.1 Plasmid curing of bacteria

Zaman et al. (2010) investigated the plasmid eliminating abilities of Ethidium bromide and sodium dodecyl sulfate on multi drug resistant *Escherichia coli* from urinary tract infection specimens. They used three different concentrations of each curing agent (Et-Br and SDS) and found that frequencies of cured cells were 14.29 % (with 100 µg/ml), 21.05 % (with 100 µg/ml), 17.65 % (with 125 µg/ml) for Ethidium bromide and 7.4 % (with 10 % w/v) and 6.67 % (with 10 % w/v) for sodium dodecyl sulfate. There were no cured cells from for 75 µg/ml Ethidium bromide and 8 and 12 % SDS. When profiles of wild type and plasmid cured strains were analyzed by electrophoresis they found that varying sizes plasmid bands yielded for wild type cells, but none was obtained for Et-Br cured cells.

Another study which was conducted with *Pseudomonas aeruginosa* showed that no cured cells of *Pseudomonas aeruginosa* were obtained from 700 µg/ml Ethidium bromide treatment whereas in the treatment done for three incubation times using SDS at a concentration of 1% (W/V) with different incubation periods as curing agent, the bacterial colonies that lost their antibiotics resistance appeared with different curing rates. The results showed 75 - 100% of curing for streptomycin and gentamycin with little effect on erythromycin and trimethoprim (0 - 8%), while SDS had variable effect on other antibiotics resistance in some isolates. Curing of plasmid DNA by elevated temperature (45°C) revealed high percentage of curing for all antibiotics resistance. The percentages of bacterial colonies that lost their antibiotic resistance were 55- 100% for curing to all antibiotic resistance in the six tested isolates (Hayfaa et. al., 2013).

Another study showed that curing of *Acinetobacter baumanii* plasmids by 400 µg/ml Ethidium bromide was highly effective. Complete curing was achieved at 400 µg/ml of EtBr however lower concentration of 300 µg/ml also showed significant results (Pahwa et al., 2012).

Curing of *Klebsiella pneumoniae* by using sodium dodecyl sulphate proved that the curing frequency about $6.25 \times 10^{-4.}$ which was very effective in curing the plasmid with a relatively high (Mansi et al., 2000).

Plasmid curing experiment was done by Raja et al (2009), where they cured *E. coli* plasmid by using ethidium bromide, acridine orange, novobiocin, SDS and temperature at 40 °C .in their experiment they found that plasmid curing was achieved only by growing the strain treatment with 10 percent SDS while other curing agents and temperature at 40 °C were not effective.

In another study, it was found that 1% of sodium dodecyl sulfate with different incubation, bacterial colonies that lost their antibiotics resistance appeared with different curing rates. The results showed 100% of curing for the antibiotic such as streptomycin resistance whiles for chloramphenicol and tetracycline the plasmid curing varied 17% to 83% and little effect of SDS on ampicillin and trimethoprime resistance in some isolates. In addition, curing of plasmid DNA by elevated temperature (45°C) revealed high percentage of curing for all antibiotics resistance .The percentages of bacterial colonies that lost their antibiotic resistance were more than 60% and reached to 100% for curing the ampicillin resistance in the tested isolates (Khalid, 2005).

CHAPTER 3: METHODS AND MATERIALS

3.1 Study place

This study was conducted at the laboratory facility of Institute for developing Science and Health initiatives (IdeSHi).

3.2 Period of Study

This study was carried out between January 2015 and June 2015.

3.3 Flow Diagram of the Study Design

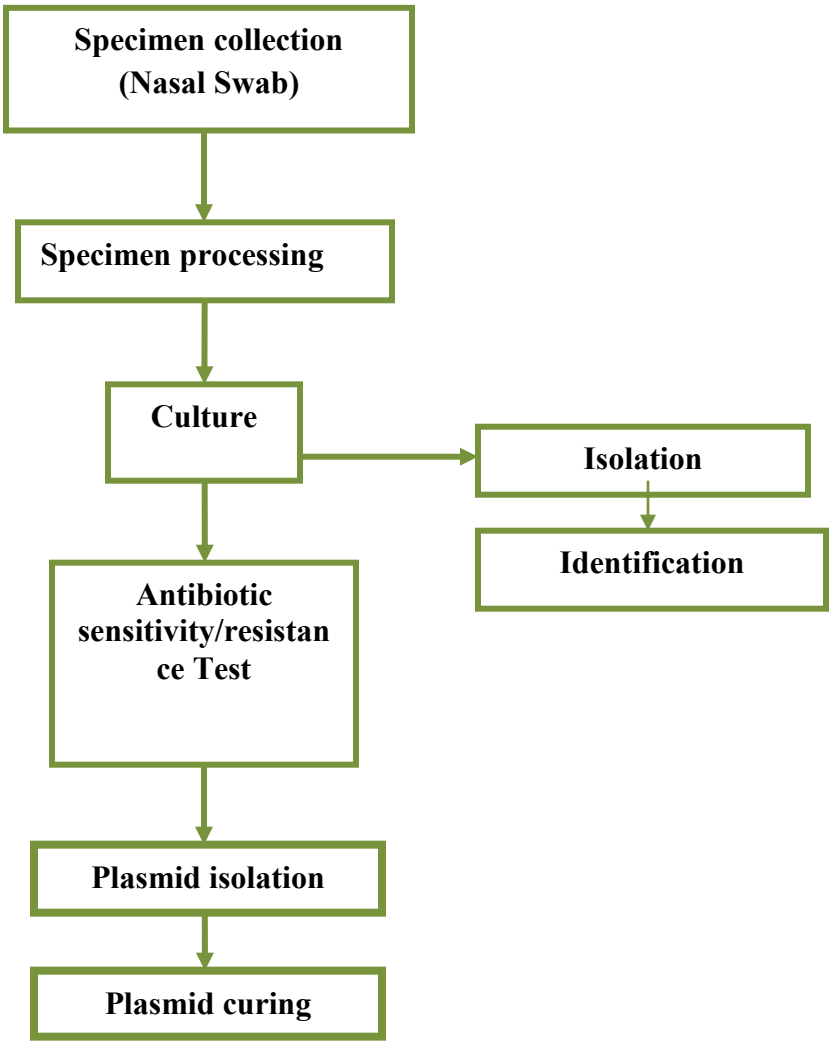


Figure 3.1: Flowchart of the study design.

3.4 Specimen processing

3.4.1 Preparation

Specimens stored at -70°C freezer were brought out and allowed to thaw.

3.4.2 Culture

3.4.2.1 Materials

1. MacConkey agar medium
2. P200 pipette
3. Inoculating loop
4. Bunsen burner

3.4.2.2 Procedure

By using a P200 pipette and sterile tip, MacConkey Agar media (For gram negative bacteria) was inoculated with a drop (10 µl- 1 loop) of the NS (Nasal Swab)-STGG and streaking out using four quadrant streaking method and incubated for 18-24 hours at 35-37˚C in aerobic condition.

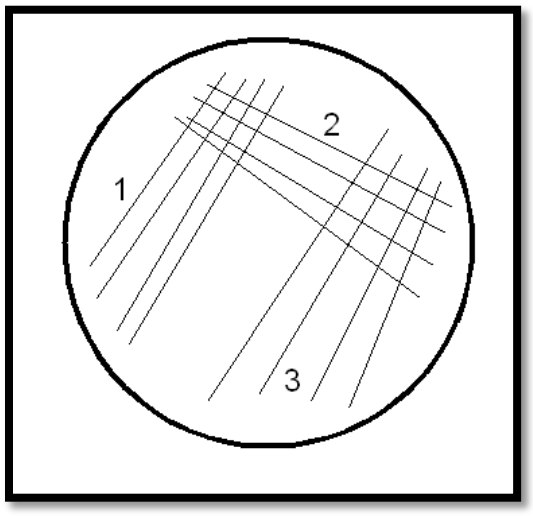


Figure 3.2: Conventional method of Streaking (ref: cappuccino, microbiology laboratory manual).

3.5 Isolation and Identification

3.5.1 Colony morphology

3.5 1.1 Equipment and materials

1. Microscope
2. Inoculating loop
3. Kim wipe

3.5.1.2 Procedure

A plate which has well-isolated colonies were used and the largest colonies were observed with the naked eye to determine general shape and pigmentation. a dissecting/stereoscopic microscope was used to get more details. The plate was then placed RIGHTSIDE UP on the stage, leaving the petri dish cover ON to protect from self-contamination. Two different magnifications were used to observe the plates—10X and 20X: To remove water condensation on the lid cover, a Kim Wipe was used. In order to determine consistency, an inoculating loop was used to pick up the colony.

3.5.2 Gram staining

3.5.2.1 Materials and reagent

Glass slides, Bunsen burner, Crystal violet, Grams iodine solution, Acetone and Safranin.

3.5.2.2 Procedure:

Slide containing specimen fixed by heat by passing it over a heat source, such as a flame, with the help of forceps. The slide was passed very quickly through the flame to avoid excessive heat. Then the slide was placed on the staining tray. The fixed smear was flooded with crystal violet solution and allowed to remain for 1 minute. The crystal violet was rinsed off with distilled water. The slide was flooded with grams iodine solution mainly mordant and allowed to remain for one minute. The iodine solution was rinsed off with distilled water. The smear was decolorized with acetone for 30 second and immediately washed with distilled water. The smear was stained with safranin and allowed to wait for 30 seconds. After that safranin was rinsed off

with distilled or tap water. Slide was then dried on bibulous paper or absorbent paper and placed in an upright position. Results of the staining procedure were observed under the microscope using oil immersion.

3.6 Biochemical Test

Biochemical tests were performed according to the methods described and applied in Microbiology Laboratory of IdeSHi. The biochemical tests carried out were Triple sugar Iron Test, Citrate Utilization and Motility, Indole and Urea test.

Table 3.1: Biochemical test result sheet is followed to identify respiratory disease causing microorganisms in ideSHi laboratory:

Name of organisms	TSI				MIU			Other Biochemical Test
	Slant	Butt	H_2S	Gas	Mot	Ind	Urea	Cit
Klebsiella pneumoniae	A	A	-	+	-	-	-	+

*K = alkaline, A= Acid, K/A=alkaline Acid.

*TSI= triple sugar iron agar, MIU=motility Indole Urea, CA=Citrate agar.

3.6.1 Equipment's and materials

1. Inoculating needle
2. Rack or test tube holder
3. Bunsen burner
4. Fresh culture
5. Incubator (37°C)

3.6.2 Procedure

3.6.2.1 TSI (Triple Sugar Iron Agar):

The inoculating needle was sterilized in the blue flame of the Bunsen burner till it turned into red hot and then allowed to cool. Using aseptic

technique, single colony of the organism was taken from fresh culture in MacConkey with the platinum needle. A sterile TSI slant tube was taken from the rack, the cap was removed and the neck of the tube was again burnt into flame to maintain its sterility. The needle containing colony was stabbed into the medium up to the butt of the TSI tube, and then streak the needle back and forth along the surface of the slant. Again the neck of the TSI tube was burnt into the flame and capped it and placed it in the test tube rack. Then the caps of the TSI were loosened, incubated at 37^{o}C for 18 to 24 hours to observe carbohydrate fermentation, CO_2 and H_2S production. Glucose fermentation was shown by a yellow butt and red slant. Gas Produce if any, was seen trapped inside medium. Detection of H_2S production was identified by black precipitation in the butt of the tube. Carbon di oxide gas production was indicated by splitting and cracking of the medium.

3.6.2.2 Citrate utilization Test:

Bacterial single colony was picked from the MacConkey agar plate culture by using a platinum needle and inoculated into the slope of Simmons's citrate agar (Oxoid Ltd, England) and incubated overnight at 37˚C.If the organism had the ability to utilize citrate, the medium changed its color from green to Prussian blue; a negative slant would have no growth of bacteria and would remain green.

3.6.2.3 MIU (Motility, Indole and Urea):

Motile organism grows out from the stabbed line throughout the medium. Indole and urea positive reaction is indicated by the formation of pink color.

3.6.2.4 Motility Test Agar

Motility test agar was used for the segregation of microorganisms on the basis of motility. Motility Agar was soft agar in a test tube without a slanted surface. Cells were stab-inoculated into the agar (the top surface was not inoculated). Non-motile bacteria would have grown only where they were inoculated. Motile bacteria would have grown along the stab

and would have swum out away from the stabbed area. Thus, a negative result was indicated by growth in a distinct zone directly along the stab. A positive result was indicated by diffuse (cloudy growth), especially at the top and bottom of the stab. By using an inoculating platinum needle a stab inoculation (about 2/3 of the way into the agar) was made from stock culture. Then the test tube was then incubated at appropriate temperature for 24 to 48 hours (up to 72 hours). After that culture was observed by holding it up to a light source.

3.6.2.5 Indole Test

Five gram of Para-dimethylaminobenzaldehyde was added to a sterile beaker. With a sterile glass pipette 25ml of methanol was taken to the beaker and mixed properly. After mixing 10ml of phosphoric acid was added drop by drop with a sterile glass pipette to the beaker and mixed carefully. A 0.45 µm filter was used to filter sterilization of the media and transfer it to sterile bottle. The prepared Indole reagent stored at 4°C.Sterile filter paper cut into 3 cm in length and 0.5 cm of wide by maintaining all the aseptic condition. The filter paper was taken into a sterile petri dish and 1ml of the indole reagent was added to the filter paper pieces. Then the filter papers were incubated overnight. One piece of overnight incubated filter paper was placed on the top of urease containing motility agar test tube. The indole reagent containing filter paper would change into pink color if the inoculated colony was a gram negative *Enterobacteriaceae*, in case of gram-positive it would have remained unchanged.

3.6.2.6 Oxidase Test

The Oxidase test must be performed before performing Analytical profile Index of test microorganism. From a fresh bacterial culture a well isolated colony was picked by using a platinum loop and rubbed onto a small piece of filter paper containing drops of N NN' N' tetra methyl p phenylenediamine dihydrochloride reagent. Inoculated area

of paper was kept for observing a color change within 10-30 seconds. Microorganisms are Oxidase positive when color changes to purple color (indophenols) within 10 seconds and microorganisms are Oxidase negative if color does not change.

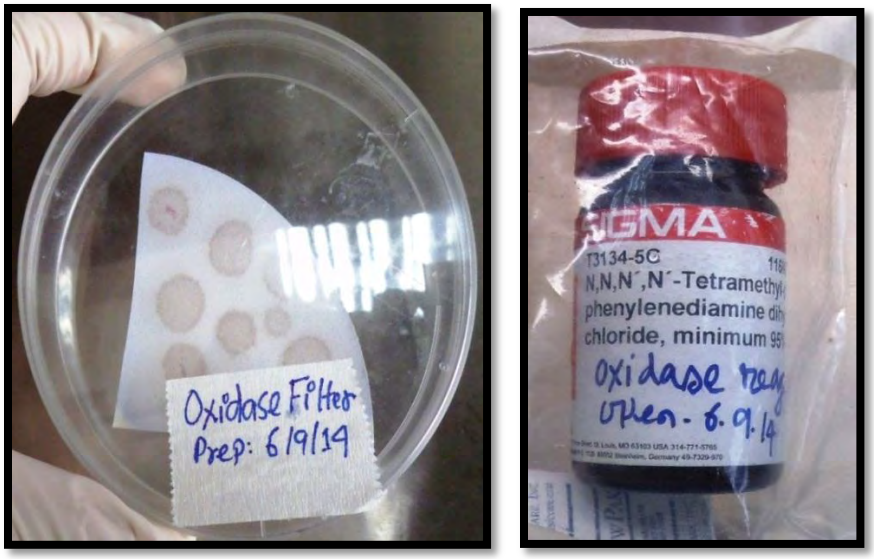


Figure 3.3: Oxidase reagent containing filter paper and N NN' N' tetramethyl p phenylenediamine dihydrochloride reagent (SIGMA).

3.7 Analytical Profile Index (Enterobacteriaceae) for genus label identification

The whole procedure was done in compliance with the protocol that has provided by the API 20 E test kit by bioMérieux®.

3.7.1 Aim

To identify bacterial isolates using commercial biochemical test kits (bioMérieux API).

3.7.2 Principle

The API 20 E strip consists of 20 micro tubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension that reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of

reagents. The reactions are read according to the Reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software.

3.7.3 Procedure

3.7.3.1 Preparation of the strip

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

3.7.3.2 Preparation of the inoculums

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

3.7.3.3 Inoculation of the strip

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

3.7.4 Reading and Interpretation

3.7.4.1 Reading the strip

1. The strip was read by referring to the Reading Table after the incubation period was completed.

2. When 3 or more tests (GLU test + or –) are positive, then all the spontaneous reactions on the result sheet were recorded and then tests were revealed which required additional reagents.

3.7.4.2 Interpretation

Identification was obtained with the numerical profile by the help of an expertise.

Table 3.2: API Reading Table which were used to interpret API test results

TEST	Active ingredients	Reactions	Negative	Positive
ONPG	2 -nitrophenyl ßD galactopyranoside	ß galactosidase	colorless	Yellow
ADH	L- arginine	Arginine dihydrolase	yellow	Red/orange
LDC	L- lysine	Lysine decarboxylase	yellow	Red/orange
ODC	L- orthonithine	Orthinine decarboxylase	yellow	Red/orange
CIT	Trisodium citrate	Citrate utilization	yellow	blue
H_2S	Sodium thiosulfate	H_2S production	Colorless	Black
URE	urea	Urease	yellow	red
TDA	L- tryptophane	Tryptophane deaminase	yellow	reddish brown
IND	L-tryptophane	Indole production	colorless	pink
VP	sodium pyruvate	acetoin	colorless	Pink
GEL	Gelatin	gelatinase	no diffusion	black
GLU	D -glucose	glucose	Blue	Yellow
MAN	D -mannitol	mannitol	Blue	yellow
INO	inositol	inositol	Blue	yellow
SOR	D -sorbitol	sorbitol	Blue	yellow
RHA	L-rhamnose	rhamnose	Blue	yellow
SAC	D-sucrose	sucrose	Blue	yellow
MEL	D-melibiose	melibiose	Blue	yellow
AMY	amygdalin	amygdalin	Blue	yellow

ARA	L-arabinose	arabinose	Blue	yellow
OX	Cytochrome oxidase	Cytochrome oxidase	colorless	purple

3.8 Antibiotic Sensitivity Test

Antibiotic susceptibility testing was performed in accordance with CLSI (Clinical Laboratories Standard Institutes) guidelines.

Table 3.3: List of Antibiotics that were used to perform antibiotic sensitivity test

Name of the Antibiotic	Disc potency	OXOID ,England
Sulphomethoxazole/ trimethoprim (SXT)	25 µg	CT0052B
Cefotaxime (CTX)	30 µg	CT0166B
Ampicillin (Amp)	10µg	CT0003B
Ceftazidime (CAZ)	30µg	CT0412B
Penicillin G(P)	10µg	SP028-1CT
Amoxycillin /Clavulanic acid (AMC)	30µg	CT0223B
Tetracycline (TE)	30µg	CT0054B
Netilmicin (NET)	30µg	CT0225B
Piperacillin/Tazobactam (TZP)	110µg	CT0725B
Carbenicillin(CAR)	100µg	CT0006B
Nalidixic acid (NA)	30µg	CT0031B
Azithromycin(AZM)	15µg	CT0906B
Amikacin (AK)	30µg	CT0107B
Imipenem(IPM)	10µg	CT0455B
Ciprofloxacin (CIP)	5 µg	CT0425
Cefixime (CFM)	5 µg	CT0653B
Ceftriaxone(CRO)	30µg	CT0417B
Tobramycin (TOB)	10µg	CT0056B
Gentamicin (GEN)	10µg	CT0024B
Meropenem (MEM)	10µg	CT0774B
Levofloxacin (LEV)	5 µg	CT1587B
Vancomycin(VA)	30 µg	CT00588

Figure 3.4: Antibiotic discs (oxoid, England)

Table 3.4: Pathogen specific drug lists for determining Antibiotic Sensitivity Test (AST) that is followed in ideSHi Laboratory**:**

Name of the species	**Antibiotic Sensitivity Test drug lists**		
***Enterobacter* and *Klebsiella spp*.**	**1st line**	**2nd line**	**3rd line**
	Cn,Tob,Cro,Cfm,Cip Ipm,Mem,Azi	Ak,Net,Tzp,Car,Pb	Lev

*****In accordance to CLSI standards

3.8.3 Enrichment of the culture

Fresh bacterial single colony of the *Enterobacter aggalomerans* and *Klebsiella pneumoniae* were inoculated in cryovial tubes containing l ml of Mueller Hinton Broth (MHB) and kept for approximately 4 hours to be enriched.

3.8.4 Procedure for Performing the Disk Diffusion Test

Within 15 minutes after adjusting the turbidity of the inoculums suspension, a sterile cotton swab was dipped into the adjusted suspension. Swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This removed excess fluid from the swab. Then

the dried surface of an MHA plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, the plate was rotating approximately 60° each time to ensure an even distribution of inoculums. As a final step, the rim of the agar was swabbed. Then the lid ajar was left for three to five minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks.

3.8.4.1 Placement of the antibiotic disks: Two sterile disks were placed on the surface of an agar plate, using a forceps. The forceps was sterilized by immersing the forceps in alcohol then igniting it. The disks were gently pressed with the forceps to ensure complete contact with the agar surface. The disks were placed away from the edge of the plates so that it is easily measured. Once all disks are in place, the plates were inverted, and placed them in a 37 °C incubator for 24 hour.

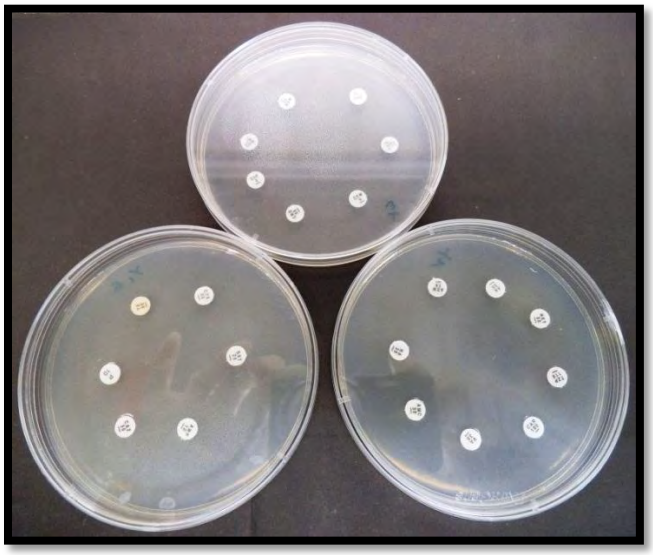


Figure 3.5: Placement of antibiotic discs on MHA containing bacterial lawn culture.

3.8.4.2 Measuring zone sizes

Following incubation, the zone sizes were measured precisely using a ruler. All measurements were made while viewing the back of the petri dish. The zone size was recorded on the recording sheet.

Table 3.5 Zone Diameter Interpretive Standards*

Name of the Antibiotic	Resistant mm* or less	Intermediate mm*	Sensitive mm* or more
Sulphomethoxazole/ trimethoprim (SXT)	≤10	11-15	≥16
Cefotaxime (CTX)	≤14	15-22	≥23
Ampicillin (Amp)	≤13	14--16	≥17
Ceftazidime (CAZ)	<14	15-17	>18
Penicillin G(P)	-	-	-
Amoxycillin (AMC)	≤13	14-17	≥18
Tetracycline (TE)	≤14	15-18	≥19
Netilmicin (NET)	12	13-14	15
Piperacillin (TZP)	≤17	18-20	≥21
Carbenicillin(CAR)	≤19	20-22	≥23
Nalidixic acid (NA)	≤13	14-18	≥19
Azithromycin(AZM)	-	-	-
Amikacin (AK)	≤14	15-16	≥17
Imipenem (IPM)	≤13	14-15	≥18
Ciprofloxacin (CIP)	≤15	16-20	≥21
Cefixime (CFM)	≤15	15-18	≥19
Ceftriaxone(CRO)	≤13	14-20	>21
Tobramycin (TOB)	≤12	13-14	≥15
Gentamicin (GEN)	≤12	13-14	≥16
Meropenem (MEM)	≤13	14-15	≥16
Levofloxacin (LEV)	≤13	14-16	≥17
Vancomycin (VA)	-	-	-

*In accordance to performance standards for Antimicrobial Disk Susceptibility Tests, CLSI (formerly NCCLS)

*mm= millimeter

3.9 Plasmid isolation (Birnboim, 1979)

3.9.1 A test tube containing 3-5mls of LB (which contains the antibiotic selective for the bacterial culture) was inoculated with a single isolated colony picked from an LB agar plate which contains the selective antibiotic and grown the culture O/N at 37^{o}C with shaking.

3.9.2 1.5ml of the O/N culture was centrifuged in a microfuge tube for 1 min at 14,000 rpm. Then the supernatant was discarded. The bacterial pellet was re-suspended in 200 µl of Solution I (see appendix).

3.9.3 200 µl of Solution II (see appendix IV) was added and mixed by inverting the tube gently.

3.9.4 Again 200 µl of Solution III (see appendix IV) was added and mixed by inverting the tube gently. A white precipitate was formed. Tubes were centrifuged for 10min at 14,000rpm. Then the supernatant was transferred to a fresh tube. Transfer any of the white pellets was avoided carefully.

3.9.5 900ul of 100% ETOH (Ethyl alcohol) was added to the supernatant. It was mixed well by inverting the tube several times. Then the eppendorf tubes were centrifuged at 14,000rpm for 20min. Removed and discarded supernatant. To the DNA pellet 100 µl of ice cold 75%ETOH was added. Again the tubes were centrifuged for 30sec. After that the supernatant were removed and discarded. All of the EtOH has been removed from the pellet. To remove supernatant completely the pellet was air dried 10-30 minutes.

3.9.6 The pellet was re-suspended in 50ul of sterile ddH2O or TE buffer. Then the plasmid sample was stored at -20°C until it was run in electrophoresis gel.

3.10 Gel electrophoresis

Gel electrophoresis was done to observe desired plasmid band after isolating plasmid from the test organisms.

Table 3.6 Equipment's and materials that were needed for Gel electrophoresis

Materials	Instrument
1. Agarose	1.Electrophoresis chamber
2. TE Buffer	2. Casting tray
3. Bromophenol blue	3. Combs
4. DNA stain	4. power supply
5. 1 kb DNA ladder standard	

3.10.2 Preparing the Agarose gel

3.10.2.1 About 0.4g Agarose powder was measured and added it to a 100 ml flask. 50 ml TE Buffer was added to the flask. Then the agarose was melted in a microwave until the solution becomes clear. Then the solution was left to cool to about 50-55°C.

3.10.2.2 The ends of the casting tray were sealed with two layers of tape. The combs were placed in the gel casting tray.

3.10.2.3 The melted agarose solution poured into the casting tray and letting for cool until it was solid. When it appeared milky white then carefully the combs were pulled out and removed the tape.

3.10.2.4 Then the gel was placed in the electrophoresis chamber. Enough TE Buffer was added so that there was about 2-3 mm of buffer over the gel.

3.10.3 Loading the gel

310.3.1 About 3 µl of bromophenol blue was loaded with 7 µl of DNA samples. Order of each sample were recorded that would be loaded on the gel.

310.3.2 Samples with the DNA dye were tipped carefully into separate wells in the gel. Then the DNA ladder standard was poured into at least one well of each row on the gel.

3.10.4 Running the gel

3.10.4.1 The lid was placed on the gel box, connecting the electrodes. The electrode wires were connected to the power supply, making sure the positive (red) and negative (black) are correctly connected. Then the power supply was turned to about 120 volts.

3.10.4.2 To make sure that the current was running through the buffer, the system was checked by looking bubbles forming on each electrode. Then the power was let to run until the blue dye approaches the end of the gel.

3.10.4.3 Then the power was turned off and the wires were disconnected from the power supply. The lid was removed of the electrophoresis chamber. Gel was separated from the tray by using gloves.

3.10.4.4 Then the plasmid DNA bands were observed on Gel DocTM XR^{+} (BioRad,USA) machine.

3.11 Plasmid curing

Curing experiments were performed using sodium dodecyl sulfate (SDS), Ethidium bromide and elevated temperature (45°C) as curing agents.

3.11.1 Plasmid curing by using Ethidium Bromide

In this study modified Et-Br plasmid curing method was used. Modified procedure was found to be more convenient than the established methods .Several concentrations of Et-Br (0.1 mg/ml) were added to 5 ml of LB broth. Antibiotic resistant *bacterial* culture was inoculated to LB broth having Et-Br. Broth containing MDR strains were kept in a shaker incubator (37°C) for incubation (24 hrs.).After incubation, this culture was swabbed into the MHA Plates. Again antibiogram was performed in accordance with CLSI. Plates were incubated for 24 hrs at 37^{o}C.

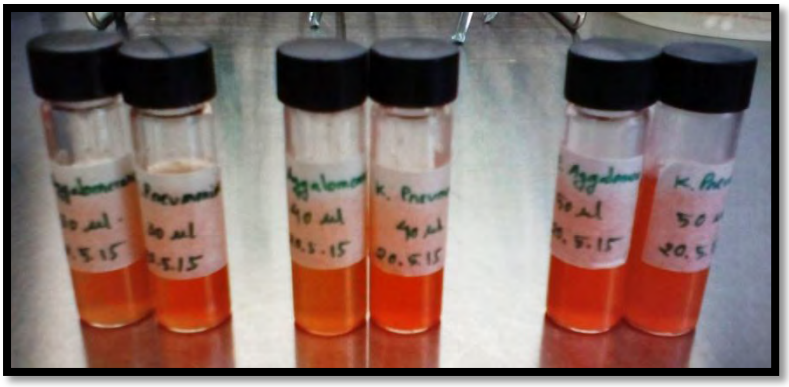


Figure 3.6: Plasmid curing of *E. aggalomerans* and *K. pneumoniae* by using different concentration of Ethidium bromide.

3.11.2 Curing of plasmid by sodium dodecyl sulfate (Reference: Tomoeda et al. (1974)

The original Tomoeda plasmid curing method by SDS was slightly changed due to some unavailability of reagents. Test tube containing 5 ml of nutrient broth (MHB) was prepared by adding appropriate antibiotic (Ampicillin) at a final concentration (50 µg/ml), then inoculated with single colony of both isolates, and incubated at 37°C for 24. Then the culture was inoculated into the MH broth (MHB) containing 0.5, 1, 2 , 2.5,3,3.5 and 4% SDS (W/V) which were then incubated at 37°C for 24 h. Then Antibiogram was performed according to the CLSI guidelines and incubated at 37°C for 24 h, until the result appeared.

Figure 3.7: Plasmid curing of *E. aggalomerans* and *K. pneumoniae* by using different concentration of Sodium dodecyl sulfate (SDS).

3.11.3 Plasmid curing by physical agents (elevated temperature) (ref: Kheder, 2002)

Single colony of both isolates was inoculated into 10 ml of Mueller Hinton broth (MHB). After incubation at 37°C for24 h, 0.2 ml of bacterial culture was inoculated into 10 ml of fresh nutrient broth and incubated at an elevated temperature of 45°C for 24 h with shaking at 100 rpm. Dilutions were performed up to 10^{-3} dilutions, then 0.1 ml of the each three dilutions were spread on plates of MacConkey to observe whether the microorganisms were survived or not after treated with intercalating agent Ethidium bromide. Then the survival colonies were enriched in MHB about 4 hrs. Then the enriched cultures were plated onto MH agar which contain different antibiotics and at 37°C for 24 h.

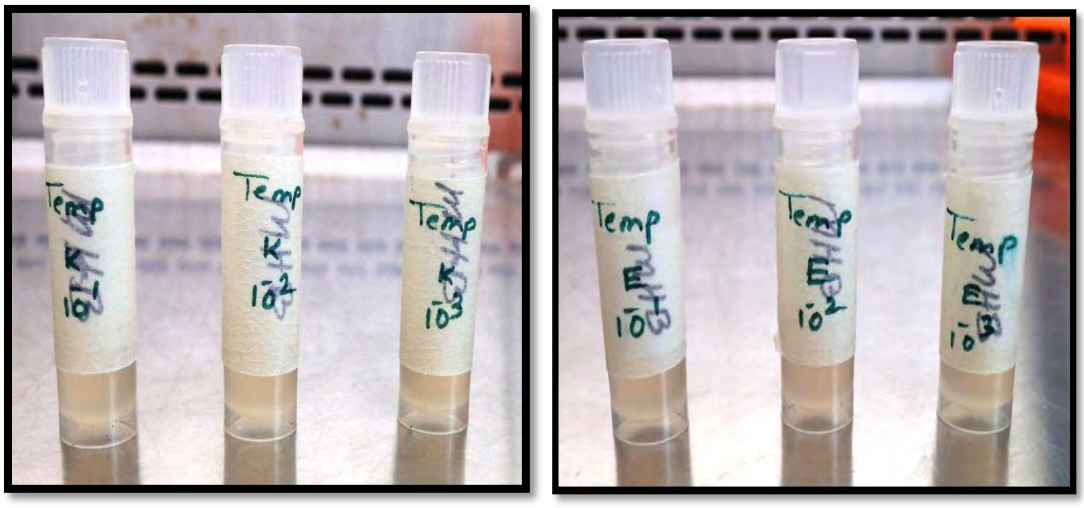


Figure 3.8: Enriched culture of MDR *K. pneumoniae* and *E. aggalomerans* treated in elevated temperature in MHB.

CHAPTER 4: RESULTS

4.1 Isolation of gram negative bacteria:

The nasal swab samples were collected randomly from the pediatric ward of Dhaka Medical College Hospital (DMCH) and Shaheed Suhrawardy Medical College Hospital (SSMCH) and the research work was performed in ideSHi laboratory. Two multi-drug resistant strains were isolated.

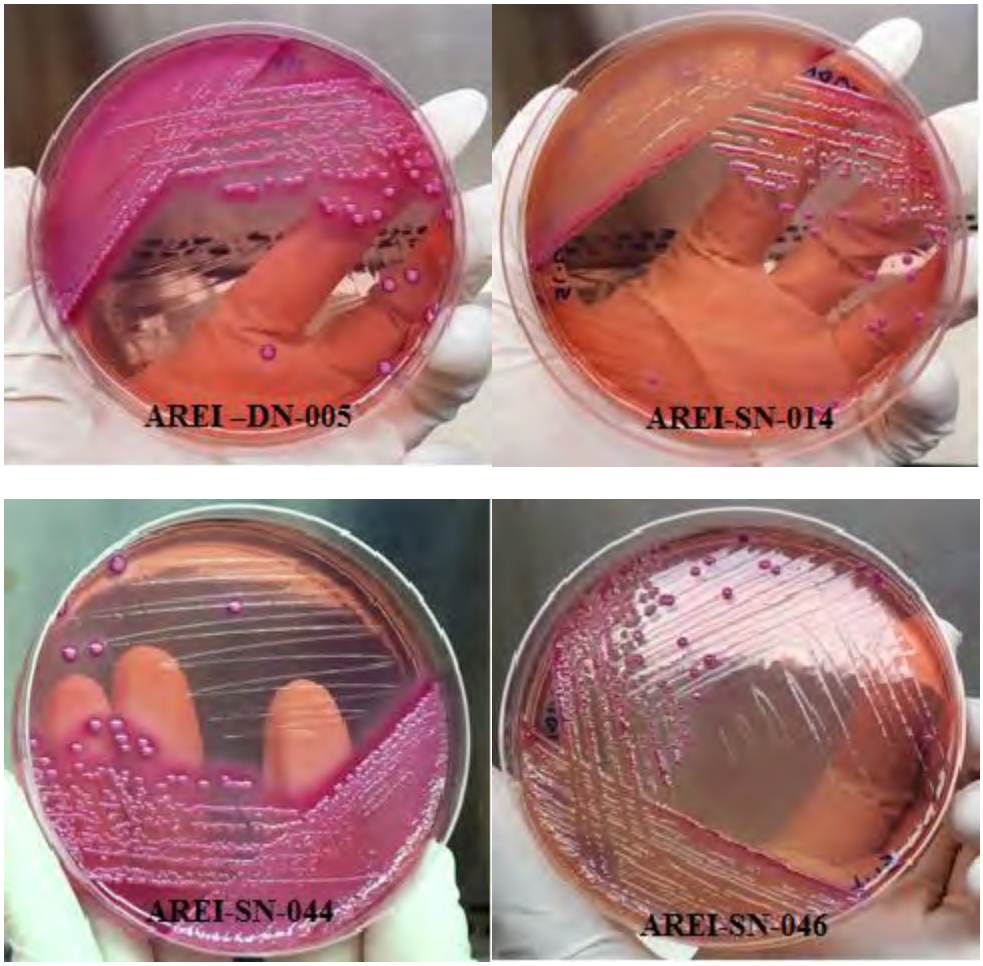


Figure 4.1: Bacterial isolates from nasal samples were collected from patients (age < 5) admitted in DMCH and SSMCH.

4.2 Characterization of bacterial isolates

Table 4.1 Colony characteristics of the isolates on MacConkey agar medium

Isolate ID	Colony characteristics				
	Size	**Form**	**Color**	**margin**	**Elevation**
AREI–DN-005	Small	Circular (mucoid encapsulated)	pink	Entire	Raised
AREI-SN-014	pinpoint	Circular (mucoid encapsulated)	pink	Entire	Convex
AREI-SN-044	Small	Circular (mucoid encapsulated)	pink	Entire	Raised
AREI-SN-046	Pinpoint	Circular (mucoid encapsulated)	pink	Entire	Convex

AREI-DN-005 and AREI-SN-044 both isolates had formed small sized mucoid encapsulated pink colored colonies on the medium. Whereas AREI-SN-014 and AREI-SN-046 isolates had formed pinpoint like pink colonies on the medium. AREI DN 005 and AREI -SN -044 had convex while AREI-SN-014 and AREI-SN-046 isolates had raised colonies.

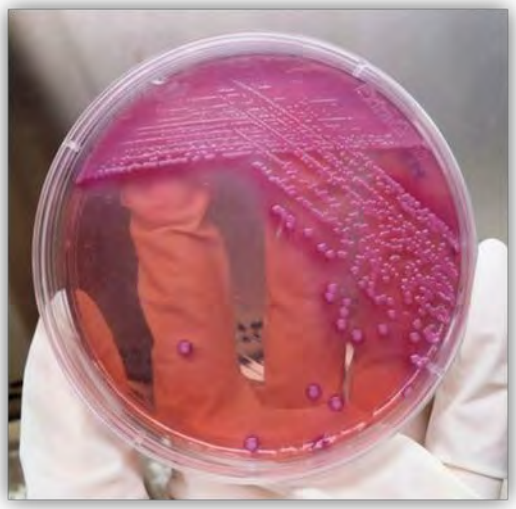
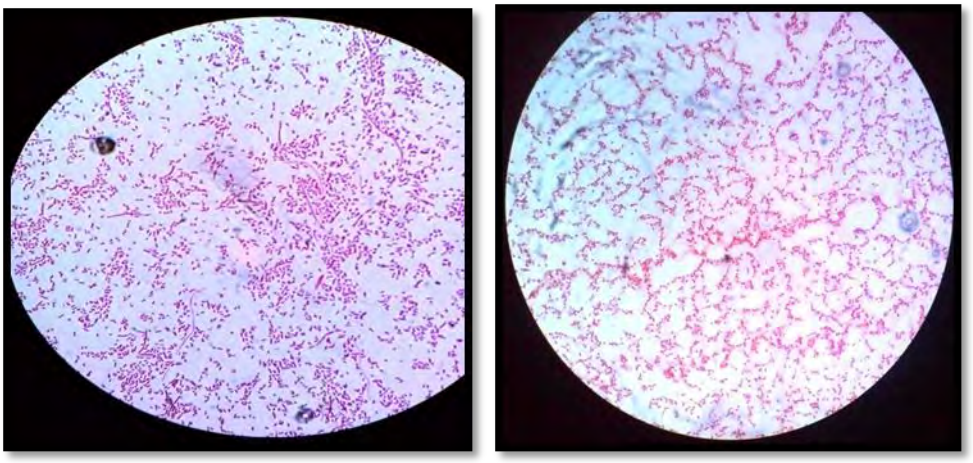


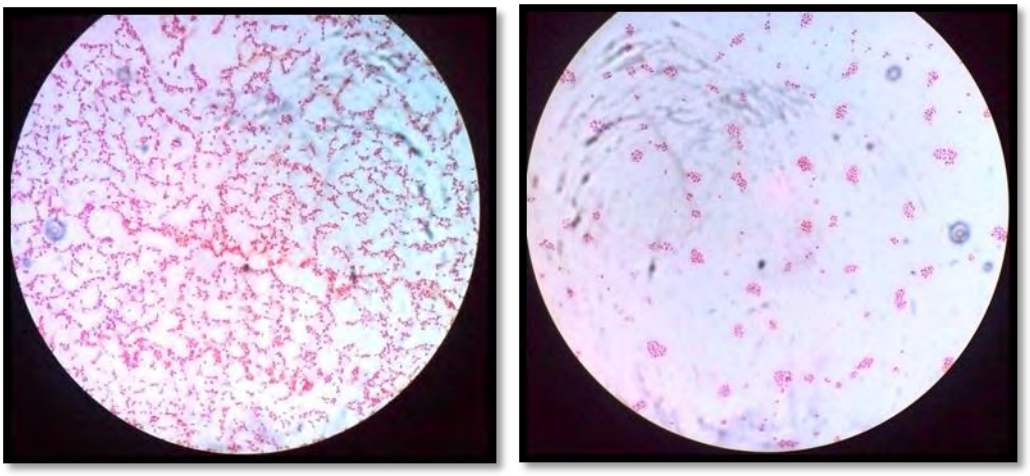
Figure 4.2: Bacterial colonies on MacConkey agar media. Pink color indicated that the colonies were lactose fermenter.

4.2.2 Morphological characterization

The isolates' morphological features were determined by Gram staining. All the isolates retained the pink color implying that they were Gram negative rods as shown in figure



AREI –DN-005 **AREI-SN-014**



AREI-SN-044 **AREI-SN-046**

Figure 4.3: Gram stain photographs of the four respiratory bacterial isolates.

4.3 Biochemical Test Results

Table 4.2 Results of Biochemical Test of the isolates collected from MacConkey agar medium

Isolate Id	Biochemical test								Plausible organism
	TSI				MIU				
	Slant	butt	H_2S	Gas	Motility	Ind	U	Cit	
AREI –DN-005	**A**	**A**	**-**	+	**-**	**-**	**-**	+	*K. pneumoniae*
AREI-SN-014	**A**	**A**	**-**	+	+	**-**	**-**	+	*Enterobacteriaceae spp.*
AREI-SN-044	**A**	**A**	**-**	+	**-**	**-**	**-**	+	*K. pneumoniae*
AREI-SN-046	**A**	**A**	**-**	+	+	**-**	**-**	+	*Enterobacteriaceae spp.*

***A=acidic, (+) =positive reaction, (-) =negative reaction**

In Table 4.2, AREI –DN-005 and AREI-SN-044 both isolates had produced acid and gas in TSI but had not produced H_2S gas on TSI. Both of these isolates had shown negative results in MIU but had shown positive results in Citrate utilization test.

Biochemical test results of AREI –DN-005 and AREI-SN-044 isolates were suggested that the isolates were K .pneumoniae.

AREI-SN-014 and AREI-SN-046 isolates had produced acid and gas in TSI and showed positive results in citrate utilization test. AREI-SN-014 and AREI-SN-046 had shown motility positive in MIU.

After observing their biochemical test results, it was hard to make any presumption of these organisms because both of these isolates showed similar biochemical test results as *K. pneumoniae* isolates but AREI-SN-014 and AREI-SN-046 isolates were motility positive whereas *K. pneumoniae* isolates were motility negative. It was suggested that AREI-SN-014 and AREI-SN-046 isolates were belonged to *Enterobacteriaceae spp.* Further tests (API) were done for confirmation.

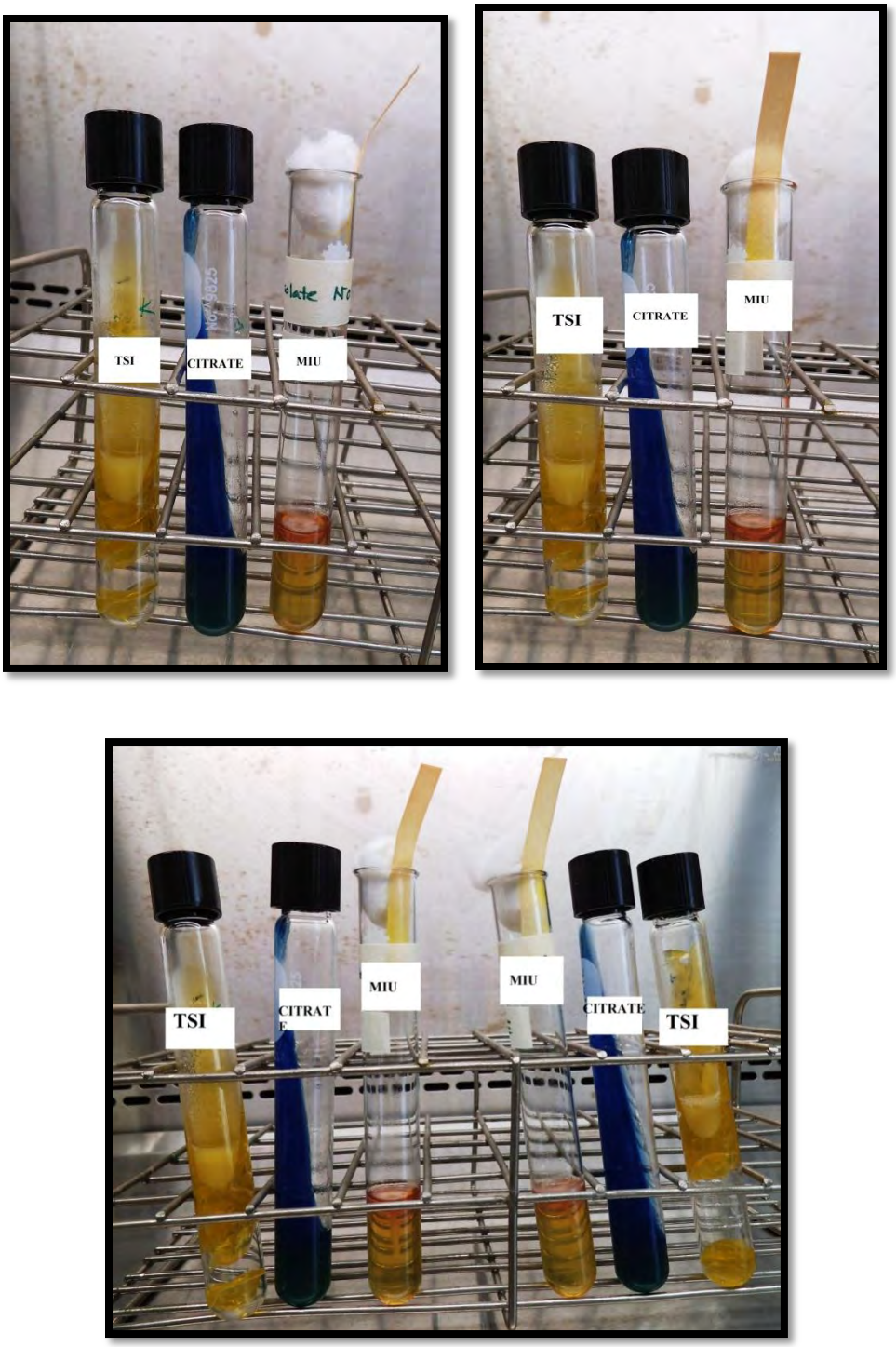


Figure 4.4: Biochemical test results of four isolates (AREI- DN- 005 on the left, AREI-SN-014 on right side (top) and AREI-SN-044, AREI-SN-046 respectively on the below).

4.4 Oxidase Test

Table 4.3 Oxidase test results of the isolates are given below

Isolate ID	Name of the organism	Oxidase Test result
AREI –DN-005	*K. pneumoniae*	Negative
AREI-SN-014	*Enterobacteriaceae spp.*	Negative
AREI-SN-044	*K. pneumoniae*	Negative
AREI-SN-046	*Enterobacteriaceae spp.*	Negative

Oxidase test results (table 4.3) suggested that all of four nasal swab sample isolates showed negative results in oxidase test. A negative result in oxidase test meant that the test organisms were gram negative microorganisms and belonged to the *Enterobacteriaceae* family.

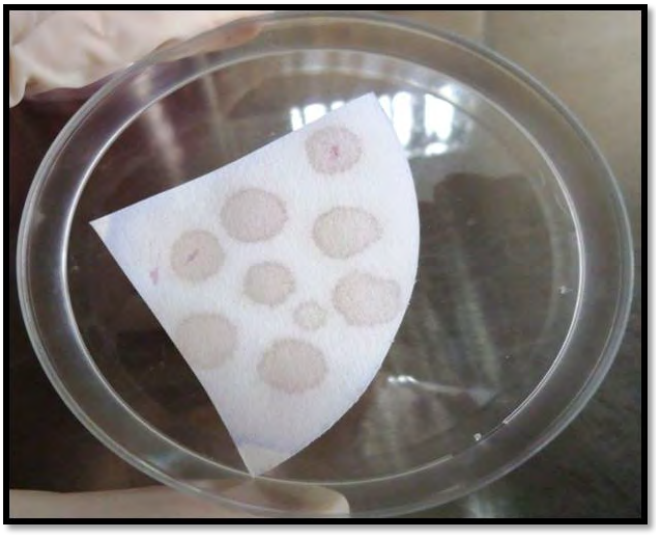


Figure 4.6: Inoculated area of filter paper containing oxidase reagent was kept for 10-30 seconds to observe a color change. After 20 seconds later no significant color changes had noticed and the test microorganisms were considered as oxidase negative.

4.5 Analytical Profile Index (*Enterobacteriaceae*) for genus label identification

Table 4.4 Analytical Profile Index test results of the isolates

Test	AREI – DN-005	AREI-SN-014	AREI-SN-044	AREI-SN-046
ONPG	(+)ve	(+)ve	(+)ve	(+)ve
ADH	(-)ve	(-)ve	(-)ve	(-)ve
LDC	(-)ve	(-)ve	(-)ve	(-)ve
ODC	(-)ve	(-)ve	(-)ve	(-)ve
CIT	(+)ve	(+)ve	(+)ve	(+)ve
H_2S	(-)ve	(-)ve	(-)ve	(-)ve
URE	(+)ve	(-)ve	(+)ve	(-)ve
TDA	(-)ve	(-)ve	(-)ve	(-)ve
IND	(-)ve	(-)ve	(-)ve	(-)ve
VP	(+)ve	(+)ve	(+)ve	(+)ve
GEL	(-)ve	(-)ve	(-)ve	(-)ve
GLU	(+)ve	(+)ve	(+)ve	(+)ve
MAN	(+)ve	(+)ve	(+)ve	(+)ve
SOR	(+)ve	(-)ve	(+)ve	(-)ve
RHA	(+)ve	(+)ve	(+)ve	(+)ve
SAC	(+)ve	(+)ve	(+)ve	(+)ve
MEL	(+)ve	(+)ve	(+)ve	(+)ve
AMY	(+)ve	(+)ve	(+)ve	(+)ve
ARA	(+)ve	(+)ve	(+)ve	(+)ve
INO	(+)ve	(+)ve	(+)ve	(+)ve
OX	(-)ve	(-)ve	(-)ve	(-)ve
Suggested species	*Klebsiella pneumoniae*	*Enterobacter aggalomerans*	*Klebsiella pneumoniae*	*Enterobacter aggalomerans*

API test results (Table 4.4) suggested that AREI –DN-005 and AREI-SN-044 isolates were *Klebsiella pneumoniae* while AREI-SN-014 and AREI-SN-046 isolates were *Enterobacter aggalomerans.*

Figure 4.7: Identification of *Klebsiella pneumoniae* (AREI-SN-044) isolate by using API-20E test kit.

Figure 4.8: Identification of *Enterobacter aggalomerans* (AREI-SN-046) isolate by using API-20E test kit.

4.6 Antibiotic sensitivity test (AST)

After performing antibiogram/AST, two multidrug resistant (MDR) isolates were found among these four gram negative isolates. **Table 4.5** Antibiotic sensitivity test results of four isolates.

Name of the organism	***Klebsiella pneumoniae***			***Klebsiella pneumoniae***			***Enterobacter aggalomerans***			***Enterobacter aggalomerans***		
Isolate ID	**AREI-SN-044**			**AREI-DN-005**			**AREI-SN-046**			**AREI-SN-014**		
Antibiogram results	**Disc**	**Zone (mm)**	**pattern**	**Disc**	**Zone (mm)**	**pattern**	**Disc**	**Zone (mm)**	**pattern**	**Disc**	**Zone (mm)**	**pattern**
	AMP	**23**	**R**	**CFM**	**S**	**23**	**AK**	**0**	**R**	**AK**	**21**	**S**
	AZM	**6**	**R**	**CIP**	**S**	**24**	**AZM**	**12**	**R**	**AZM**	**8**	**R**
	CFM	**6**	**R**	**COT**	**S**	**27**	**CFM**	**0**	**R**	**AMO**	**24**	**S**
	CIP	**19**	**R**	**CRO**	**S**	**26**	**CIP**	**0**	**R**	**CFM**	**21**	**S**
	COT	**10**	**R**	**APM**	**S**	**30**	**CRO**	**0**	**R**	**CTX**	**22**	**S**
	FOX	**6**	**R**	**AZM**	**R**	**9**	**GM_{10}**	**0**	**R**	**CHL**	**23**	**S**
	IPM	**17**	**S**	**ERY**	**S**	**32**	**IPM**	**0**	**R**	**COT**	**28**	**S**
	MEM	**30**	**S**	**GM_{10}**	**S**	**22**	**LEV**	**14**	**S**	**CIP**	**23**	**S**
	NET	**19**	**R**	**IPM**	**S**	**17**	**MEM**	**0**	**R**	**CRO**	**27**	**S**
	TOB	**12**	**R**	**LEV**	**S**	**16**	**NET**	**0**	**R**	**ERY**	**26**	**S**
	TZP	**10**	**R**	**MEM**	**S**	**18**	**PB**	**11**	**S**	**GM_{10}**	**17**	**S**
	CAP	**6**	**R**	**PB**	**R**	**12**	**RF**	**0**	**R**	**LEV**	**24**	**S**
	PB	**10**	**R**	**TOB**	**S**	**19**	**TOB**	**0**	**R**	**MEM**	**17**	**S**
							TZP	**0**	**R**	**TOB**	**18**	**S**
										AML	**10**	**R**

***S= sensitive, R= resistant.**

Antibiotic test results had shown that (table 4.6) AREI-SN-044 isolate was resistant against 11 antibiotics and showed sensitivity to two antibiotics including Imipenem and Meropenem. AREI-DN-005 isolate showed resistance against two antibiotics but showed sensitivity to 11 antibiotics. AREI-SN-046 isolate was resistant to 12 antibiotics and sensitive to only two antibiotics. AREI-SN-014 isolate showed sensitivity against 12 antibiotics and resistant to two antibiotics.

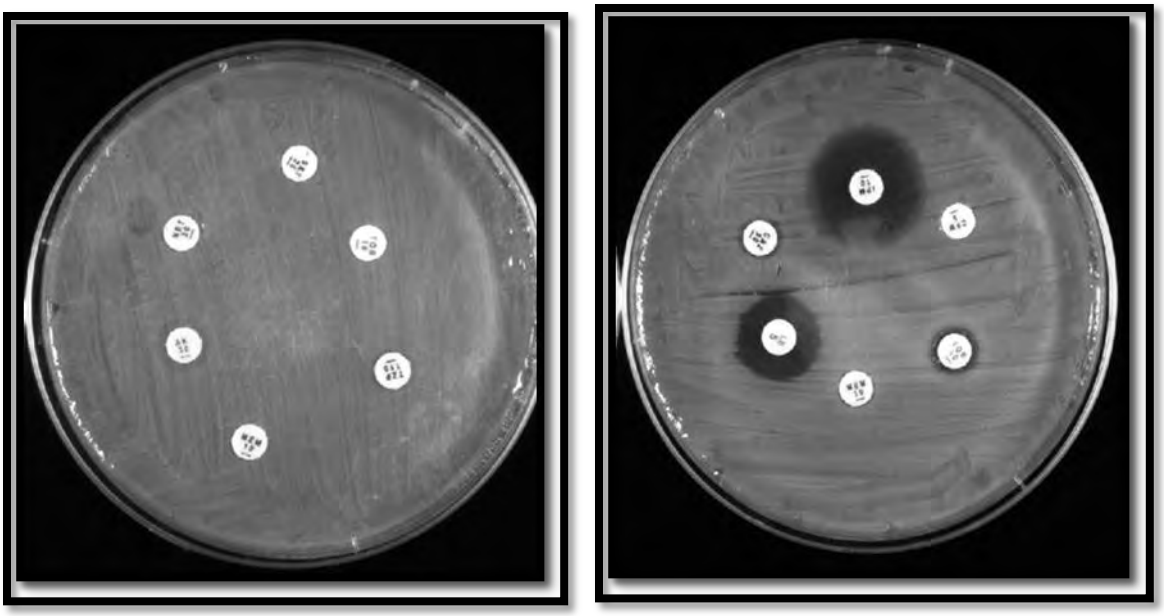


Figure 4.9: Antibiogram results of *Enterobacter aggalomerans*, AREI-SN-046 and AREI-SN-014 isolates.

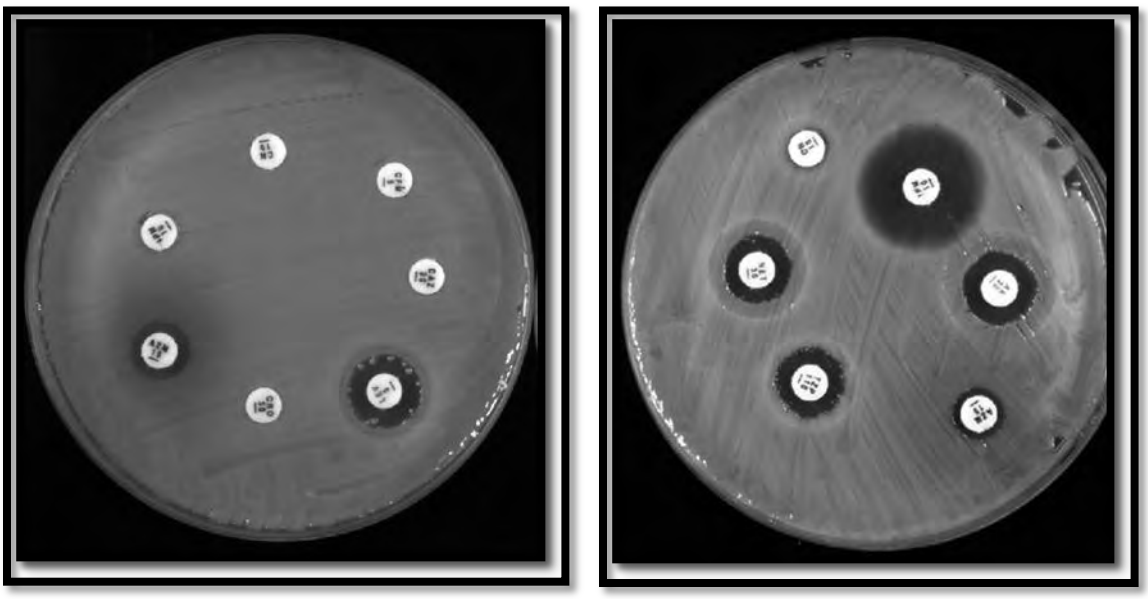


Figure 4.10: Antibiogram results of *Klebsiella pneumoniae*, AREI-SN-044 and AREI-DN-005 isolates.

Table 4.6 According to the antibiotic sensitivity test results two drug resistant isolates was found.

Isolate ID	Name of the organism	Sensitivity pattern
AREI-SN-044	*Klebsiella pneumoniae*	Partially multidrug resistant
AREI-DN-005	*Klebsiella pneumoniae*	Sensitive
AREI-SN-046	*Enterobacter aggalomerans*	Multidrug resistant
AREI-SN-014	*Enterobacter aggalomerans*	Sensitive

In the table above, an isolate resists more than 14 antibiotics called multidrug resistant whereas if it resists less than14 antibiotics is called partially multidrug resistant.

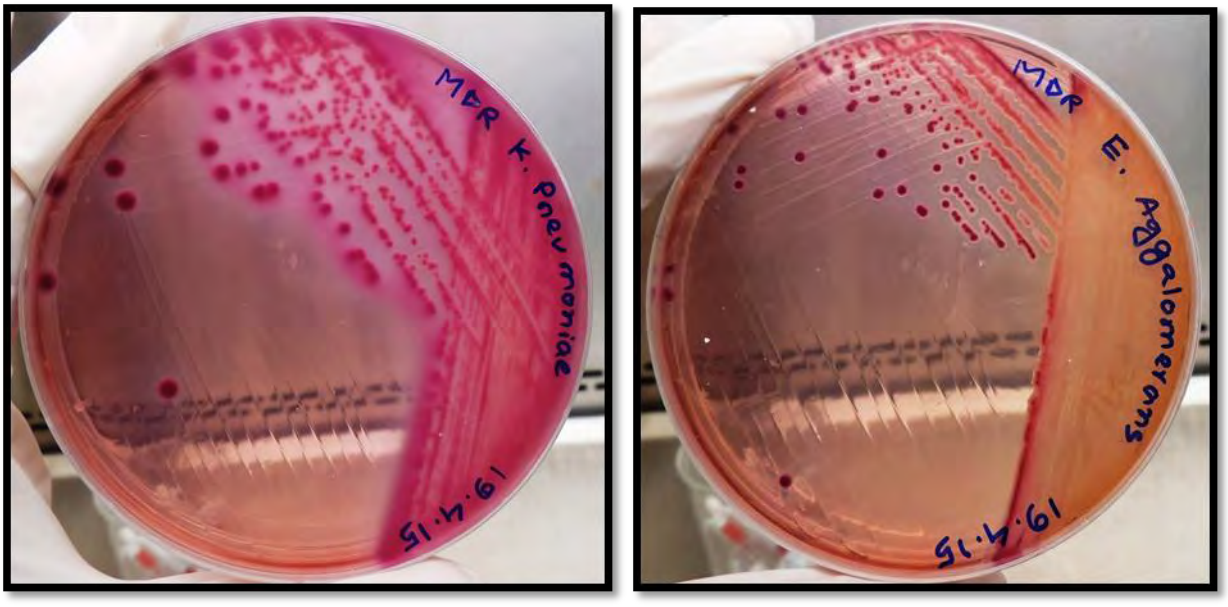


Figure 4.11: Partially multidrug resistant *Klebsiella pneumoniae* (AREI-SN-044) on left and multidrug *Enterobacter aggalomerans* (AREI-SN-046) on right side.

Table 4.7 To understand drug resistance pattern of these two isolates, a lists of antibiotic was prepared where antibiotics were classified into five classes according to their functional group.

Drug specificity	Class of antibiotics	Name of the drugs resisted by Test MDR organisms	
		***Klebsiella pneumoniae* (AREI-SN-044)**	***Enterobacter aggalomerans* (AREI-SN-046)**
1.Beta lactam ring containing group	Penicillin	Ampicillin, Mecellinum	Ampicillin, Piperacillin (Ureidopenicillin class)
	Cephalosporin	Cefixime,ceftotaxime(3rd generation)	Ceftazidime, Ceftriaxone,
	Carbapenem	Imipenem, Meropenem	Imipenem, Carbapenem (combination of Imipenem and Meropenem)
2. amino-modified glycoside	Amino glycoside	Amikacin, Netilimicin, Gentamicin, Tobramycin	Amikacin, Netilimicin, Gentamicin, Tobramycin
3.carboxylic acid moiety	Fluroquinolone		Ciprofloxacin, Norfloxacin, Levofloxacin
4. fluorine substituent	Quinolones	Ciprofloxacin , Norfloxacin	
5. macrolide ring containing group /macrocyclic lactone ring	Macrolide		Azithromycin
Combination of trimethoprim and sulfamehoxazole	Sulfonamide	Cotrimoxazole	

4.7 Molecular characterization

The quality of plasmid DNA extracted from the two multidrug resistant bacterial isolates was analyzed on a 1% agarose gel stained with gel red. Figure 4.12 showed that gel photographs of plasmid DNA from the two MDR bacteria isolates.

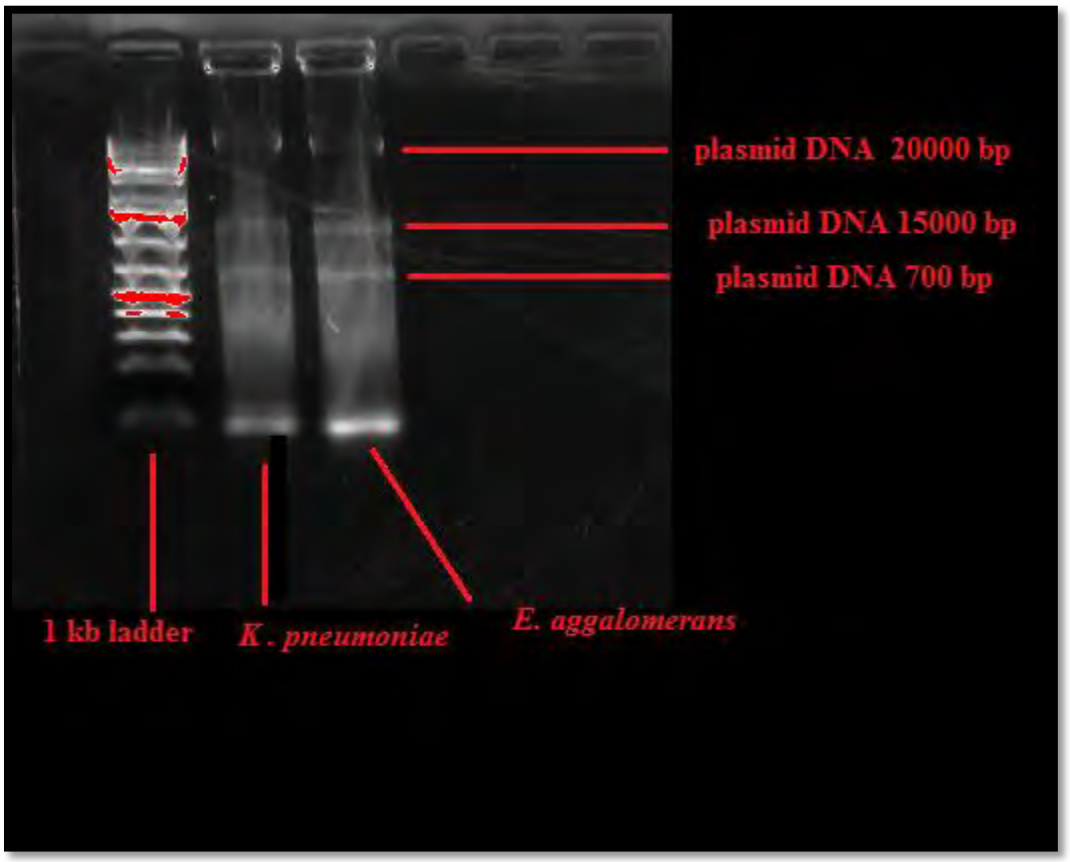


Figure 4.12: Agarose gel analysis of plasmid DNA from MDR *K. Pneumoniae* and MDR *E. aggalomerans*. First lane is the molecular ladder (1Kb ladder). Image had shown three plasmid DNA bands in both lanes which were approximately 20000 bp (0.02Mbp), 15000 bp (0.015 Mbp) and 700 bp (0.0007 Mbp) in size.

4.8 Plasmid Curing

4.8.1 Plasmid curing by Ethidium bromide

Table 4.8 Antibiotic sensitivity test results (before dilution)

Conc. Of EtBr (µg/ml)	*K. pneumoniae*		*E. aggalomerans*	
	IPM	**LEV**	**IPM**	**LEV**
	Clear Zone (mm)	Clear Zone (mm)	Clear Zone (mm)	Clear Zone (mm)
40	24	19	24	19
60	23	19	25	19
80	25	16	23	19
100	25	18	23	19
120	23	17	22	16
140	22	16	24	16
160	23	17	23	16

Both isolates had shown significant changes against Imipenem after cured by Et-Br. Before curing, both MDR isolates were resistant (of *K. pneumoniae* had 17 mm zone of inhibition where *E. aggalomerans* had 0 mm zone of inhibition) but after treated but using Et-Br both isolates became sensitive to imipenem. Both isolates had shown minor changes for levofloxacin (before curing, *E. aggalomerans* had 14 mm zone of inhibition but after treated with Et-Br it increased about 19 mm).

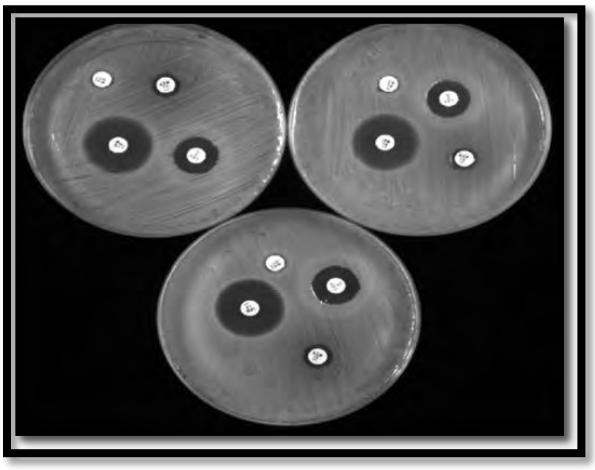


Figure 4.13: Antibiotic sensitivity test result of *E. aggalomerans* after treating with 120, 140 and 160 µg/ml Ethidium bromides.

Table 4.9 Antibiotic sensitivity test results (after performing serial dilution)

EtBr conc. (µg/ml)	Dilution	*E. aggalomerans*		*K. pneumoniae*	
		Antibiotic disc.	**Zone diameter (mm)**	**Antibiotic disc.**	**Zone diameter (mm)**
40	10^{-1}	IPM	26	IPM	25
		LEV	19	LEV	19
	10^{-3}	IPM	26	IPM	24
		LEV	19	TOB	R
60	10^{-1}	CRO	R	TOB	10
		TOB	8	CAZ	9
	10^{-3}	CN	R	P	R
		TZP	16	AK	17
80	10^{-1}	IPM	24	AZM	12
		P	R	CTX	R
	10^{-3}	MEM	R	TZP	15
		C	R	AZM	11
100	10^{-1}	AZM	11	CAR	R
		CFM	R	CTX	R
	10^{-3}	AZM	11	NET	R
		NET	14	AML	R
120	10^{-1}	TOB	R	AZM	12
		AML	R	NET	14
	10^{-3}	C	R	TZP	R
		CAR	R	CAR	Cont.
140	10^{-1}	TZP	11	MEM	24
		TOB	R	P	R
	10^{-3}	IPM	24	NA	13
		AML	R	TOB	7
160	10^{-1}	AZM	10	AK	14
		AK	15	AZM	40
	10^{-3}	MEM	27	CN	R
		CRO	R	CAZ	R

*Cont. = contaminated

Table 4.9 had shown AST results of two MDR isolates after performing serial dilution of Et-Br treated cultures. In this study, dilution of Et-Br treated cultures were performed up to 10^{-3} and first and third dilution were used to perform AST. It was done only for first and third dilution because of the minor differences between AST result of first and second dilution.

4.8.2 Plasmid curing by Sodium Dodecyl sulphate (SDS)

Table 4.10 Antibiotic sensitivity test results (no serial dilution was performed for SDS cured cultures).

SDS conc.	*K. pneumoniae*		*E. aggalomerans*			
0.5 %	**GEN**	**CIP**	**IPM**	**GEN**	**AZM**	
	12 mm	12 mm	25 mm	14mm	12mm	
1%	**CIP**	**GEN**	**IPM**	**GEN**	**AZM**	
	11mm	11mm	25 mm	11mm	10mm	
2%	**TOB**	**CIP**	**IPM**	**TOB**	**CIP**	**AZM**
	R	R	25 mm	R	R	11mm
2.5%	**CIP**	**TOB**	**IPM**	**TOB**	**CIP**	**AZM**
	R	R	27 mm	R	R	11mm
3%	**CN**		**IPM**			
	R		25 mm			
3.5%	**LEV**		**IPM**	**CN**		
	16 mm		25 mm	R		
4%	**LEV**		**IPM**	**AZM**		
	16mm		25 mm	11 mm		

In table 4.10, *K. pneumoniae* had not shown substantial changes after treated with different concentration of SDS. After 0.5%, 1%, 2% of SDS treatment isolates had shown 12 mm inhibition against gentamycin and ciprofloxacin, resistant against tobramycin. According to the CLSI zone interpretation guidelines it should be ≥16 mm and ≥21 mm zone of inhibition for tobramycin gentamycin, ciprofloxacin respectively. But isolate had shown minimal effect against levofloxacin after treated with 3.5% and 4% SDS.

E. aggalomerans isolate had shown about 25 mm inhibition against imipenem which is ≥18 mm that considered as sensitive according to the sensitivity patterns of CLSI guidelines. Isolate had shown intermediate effect against gentamycin after treated by 0.5% of SDS. From the treatment with 0.5, 1.2.2.5, 3, 3.5 and 4% SDS, *Klebsiella pneumoniae* had not shown significant effect against tobramycin, ciprofloxacin and azithromycin.

4.8.3 Plasmid curing by physical agent (temperature)

Table 4.11 Results of antibiotic sensitivity test before performing serial dilution.

Name of the organism	***K. pneumoniae***		***E. aggalomerans***	
	Disc	**Zone (mm)**	**Disc**	**Zone (mm)**
Antibiogram test results	AZM	9	IPM	24
	AMP	R	AK	15
	CFM	R	AZM	10
	NET	R	TOB	8
	TOB	R	TZP	14
	TZP	R	NET	12
			CRO	R

Table 4.11 had shown that *E. aggalomerans* had significant changes after treated with elevated temperature. *E. aggalomerans* were resistant to antibiotic IPM, AK, AZM, TOB, TZP and NET but after treated with elevated temperature it had not shown inhibition against those antibiotics. However *K. pneumoniae* had not shown remarkable changes after treated with elevated temperature.

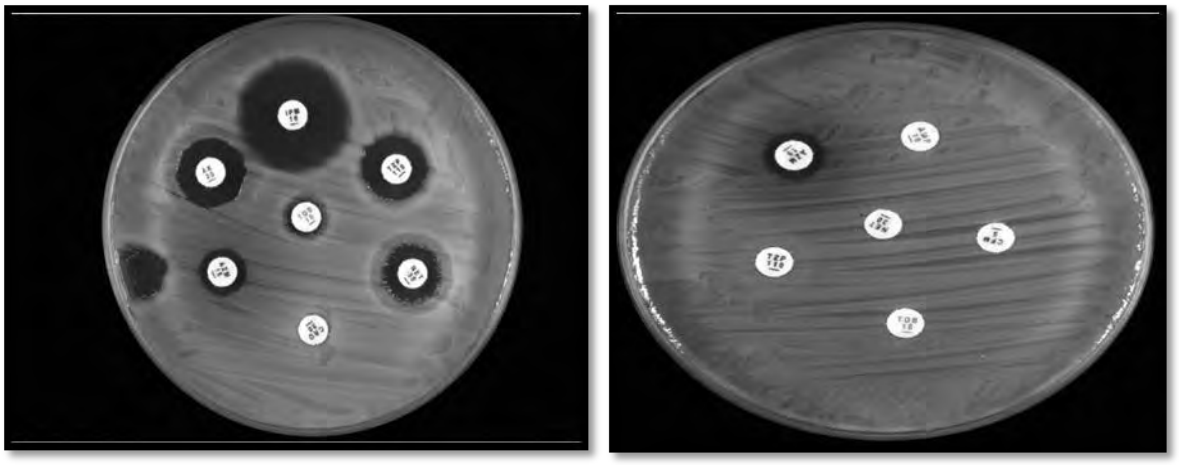


Figure 4.14: AST results of elevated temperature cured *Enterobacter aggalomerans* (AREI-SN-046) and *Klebsiella pneumoniae* (AREI-SN-044).

Table 4.12 Results of antibiogram after performing serial dilution.

Dilution	*K. pneumoniae*		*E. aggalomerans*	
10^{-1}	**Disc**	**Zone(mm)**	**Disc**	**Zone(mm)**
	AK	15	AK	R
	CRO	R	CRO	R
	TOB	9	TOB	R
10^{-2}	AZM	10	AZM	10
	CAR	R	CAR	R
	LEV	17	IPM	R
10^{-3}	NET	13	NET	R
	CFM	R	CFM	R
	TZP	14	TZP	R

Table 4.12 had shown different results of AST after performing serial dilutions of temperature cured *E. aggalomerans* cultures. Before performing serial dilution, temperature cured *E. aggalomerans* had shown inhibition against IPM =24 mm, AK=15mm, AZM=10mm, TOB=8mm, TZP=14mm, NET=12mm (table 4.11) but after serial dilution (Table 4.12) it had shown inhibition against those antibiotics.

K. pneumoniae had shown inhibition against AK=15 mm, Tob=9mm, Azm=10 mm, Lev=17 mm, Net= 13 mm and Tzp=14 mm. While in table 4.11, *K. pneumoniae* isolate was completely resistant to all of these selected antibiotics except Azithromycin.

According to the antibiogram result, MDR *Klebsiella pneumoniae* had shown comparatively positive results than MDR *Enterobacter aggalomerans* after performing serial dilution.

CHAPTER 5: DICUSSION

ARIs are the major cause of mortality among children aged less than 5 years, especially in developing countries. Worldwide, 20% mortality among children aged less than 5 years is attributed to respiratory tract infections (mostly pneumonia associated). Although the spread of ARI is worldwide, its impact is different in developed countries as opposed to developing ones. Whereas 1% to 3% of the deaths in children fewer than five years of age in developed countries are due to pneumonia, the disease causes 10% to 25% of these deaths in the developing countries. Infants have the highest risk of pneumonia in their first three months of life. Nearly 70-75% of all deaths in infants are due to pneumonia. In Bangladesh, it is estimated that one in five deaths of children under-five is due to pneumonia (UNICEF, 1986).

In the present study, total 196 nasal samples were collected from two renowned Medical college hospitals in Dhaka city and tested in Institute for developing science and health initiatives (IdeSHi) laboratory. Very few (about 4%) Gram-negative bacteria were isolated from 196 nasal swabs specimens of <5 children.

To isolate gram negative bacteria, nasal samples were inoculated onto MacConkey agar. The complete chart of colony characteristics of the isolates was given in table 4.1.The morphological characteristics of the isolates were determined by Gram staining. All the isolates retained the pink color of crystal violet stain indicating that they were Gram negative rods. Biochemical tests were performed separately for each of isolates. Biochemical tests confirmed two isolates as *K. pneumoniae* but two other isolates were needed to confirm in further confirmation test (table 4.2).

To identify the unknown isolates, analytical profile index test kits were used. Before performing API test, oxidase tests were performed. Oxidase test results confirmed that these four isolates were gram negative bacteria (table 4.3). The complete report of API test results was given in above tables. According to the API confirmation test results, two of four isolates were *E. aggalomerans* and remaining two were *K. pneumoniae.* Antibiotic sensitivity test results showed one isolate was resistant to more than 12 antibiotics and another one was resistant to 11 antibiotics. Two multi drug resistant isolates were obtained which were resistant to multiple

clinically used antibiotics, including extended-spectrum β-lactams, fluoroquinolone, aminoglycosides, trimethoprim, and sulfamethoxazoles. In the present study two sensitive isolates and two multidrug resistant (MDR) isolates were detected among these four gram negative isolates (table 4.7).

Molecular characterization of two MDR isolates were done after plasmid isolation which showed that both drug resistant isolates contained three plasmid DNA bands in both lanes , which were approximately 20000 bp (0.02Mbp), 15000 bp (0.015 Mbp) and 700 bp (0.0007 Mbp) base pair sized (figure: 4.7.1). Basically, *Klebsiella pneumoniae* have five plasmids, pKPN3, pKPN4, pKPN5, pKPN6, and pKPN7. Each of plasmid length is 0.18 Mbp, 0.11 Mbp, 0.089 Mbp, 0.0043 Mbp, and 0.0035 Mbp (Source:https://microbewiki.kenyon.edu/index.php/Klebsiella_pneumoniae). *Klebsiella pneumoniae* includes numerous mechanisms for antibiotic resistance, many of which are located on plasmid DNA (Hudson et al., 2014). Pinglei et al. (2011) reported *K. pneumoniae* genome sequence of HS11286 strain. It contains one chromosome (5.3 Mbp), three multidrug resistance plasmids (~110 kbp), including a carbapenemase producer, and three small plasmids (~3 kbp). *Klebsiella pneumoniae* carbapenemase (KPC)-producing bacteria are highly drug-resistant to Carbapenem grouped drug. That is the main reason that Carbapenem antibiotics are not effective against KPC-producing *K. pneumoniae* (Arnold et al, 2011). In the present study, we used 1kb molecular ladder which was for linear DNA. Usually plasmid DNA's are circular in sizes and ladder which was mainly used for linear DNA might have resultant dissimilarity between the original plasmid sizes and the plasmid sizes which we had found in our study after electrophoretic separation. Another possibility could be like that there might have more than five types of plasmid in *K. pneumoniae*. The more scientific investigation is required to prove this assumption. As very limited research were done on ARI causing *Enterobacter aggalomerans* that is why we were unable to define its plasmid sizes.

In the present study, it was found that *K. pneumoniae* isolate was resistant to a combination drug of sulfonamide besides those five different groups of antibiotics. These two isolates *K. pneumoniae* and *E. aggalomerans* were resistant against the drugs containing Beta- lactam ring, amino glycoside, fluoroquinolone, quinolones and macrolide. These two extended-spectrum beta-lactamases (ESBL)-producing organisms were resistant against 10 beta lactam ring

containing drugs. There is a process called curing to observe whether the plasmids of bacterium contain the resistant gene of interest or not (Caro et al., 1984). Curing may occur naturally through cell division or by treating the cells with chemical and physical agents (Snyder et al, 1997).

In present study, several chemical treatment were used to cure plasmid of the isolates by using sodium dodecyl sulfate (SDS) and fluorescent dye Ethidium bromide for removing the plasmids from the bacteria. Physical treatment such as elevated temperature was also applied to eradicate plasmid from the organisms.

After treating in elevated temperature results (Table 4.11) showed that *E. aggalomerans* isolate was missing its plasmids containing resistance genes against imipenem but maintained resistance against all other antibiotics. Studies have been made on the effect of elevated temperature on DNA synthesis and plasmid curing. Among them, May et al. (1964) obtained a high frequency of loss after growing some bacterial strains at elevated temperature. Ahmad (1989) and Bennett (2008) reported that there is a clear effect of elevated temperature on curing the plasmid DNA content of isolates of *P. aeruginosa* which is in agreement with the results of this study.

In the meantime, *K .pneumoniae* isolate had maintained its resistance to Cefixime, azithromycin, piperacillin and tobramycin after being treated by elevated temperature. It may be occurred due to the location of its resistant genes on chromosomal DNA instead of plasmid DNA. A similar result was reported when Michael et al. worked with *P. aeruginosa* isolates. In their study, they had found that after treating with elevated temperature, isolates had not shown any changes. Later further investigation has showed that the resistant genes of *P. aeruginosa* were located on chromosomal DNA instead of plasmid DNA.

Plasmid curing by using Ethidium bromide in different concentration showed meaningful results for *E. aggalomerans*. It has become sensitive to beta lactam ring containing drugs however *K. pneumoniae* remained resistant. The result showed that the Ethidium bromide had no effect on curing of plasmid DNA carrying the levofloxacin, chloramphenicol, tobramycin, gentamycin. Piperacillin, penicillin, Meropenem, Carbenicillin, azithromycin, Cefixime and Netilmicin resistance genes, for both isolates. Rabab et al (2010) also found similar results while working with Et-Br for curing plasmid of *P. aeruginosa*.

Plasmid curing by different concentration of SDS also showed opposing results for *E. aggalomerans* and *K. pneumoniae*.

K. pneumoniae had not shown considerable changes after treated with different concentration of SDS. 0.5% SDS and its higher concentration had a significant effect on *E. aggalomerans.* SDS treated *E. aggalomerans* had showed sensitivity to imipenem but had less effect against gentamycin, tobramycin, ciprofloxacin and azithromycin. The cured plasmid of *E. aggalomerans* may be the R-plasmid which harbors most of antibiotics resistance genes. Adachi et al. (1972) had found that SDS was only effective in elimination of sex (F) and R plasmids in *E. coli* in a high frequency at concentration higher than 1%, and reported that the longer incubation times (24 to 72 hrs.), higher the frequency of sensitive.

Another possibility of less effect of SDS treatment is the copy number of plasmids or the amount of antibiotic- inactivating enzymes that may interfere. A study made on the effect of SDS treatment had (Sonstein and Baldwin, 1972) elucidated that the effectiveness of SDS may be related to plasmid copy number or the amount of the enzyme which inactivate antibiotics.

CONCLUSION AND FUTURE PERSPECTIVE

The presence of multidrug-resistant microorganisms associated with ARI's in children aged< 5 can cause a severe effect on infant health and increase the mortality and morbidity rate. High dosage or overuse of antibiotics has deteriorated the effectiveness of clinically used antibiotics. Nowadays for a simple complication during pregnancy women are suggested medication containing high dosage antibiotics which are affecting the health of developing fetus. In the present study, most of the nasal samples were collected from the patient aged 2 day to 6 months and most of them were suffering from typical pneumonia after their birth. Nevertheless, the presence of multidrug resistant bacteria implicated that medication which was provided to their mother to cure infection badly affects their immunity. The Immune system of these <5 children could not able to resist common bacterial infection which turned into multidrug resistance. The poor health condition of these <5 children also may contribute to causing ARIs among these ARIs patients admitted in hospitals. Unusual antibiotic usage should be firmly prohibited to avoid child health deterioration.

Strict and proper medication during pregnancy and after birth is needed to save the infant from the acute respiratory infection caused by multidrug- resistant bacteria.

This study has several imperfections. There was an unavailability of many materials that was needed while working with MDR strains. So results which were obtained may have dissimilarity with the results of other studies. Further research is needed to be carried out for better results. As we have found two multidrug resistant gram negative bacterial isolates, in future we will be proceed for next generation sequencing where appropriate primer sequence will be used to characterize the resistant genes which are contributing to becoming multidrug-resistant. A more comprehensive analysis will be done to investigate multidrug resistance pattern of ARI causing bacteria.

REFERENCES

1. **Emmelin A, Stig W (2007)** Indoor air pollution: a poverty-related cause of mortality among the children of the world. Chest 132(5):1615-1623.
2. **A joint WHO/UNICEF statement (1986)**. Basic principles for control of ARI in children in developing countries ARI in south-east Asia. SEARO Technical publication No.8. Delhi: WHO, 39-48.
3. **Akbar MS (1986).** Acute Respiratory infection. Leading Article. Bangladesh J. of child health; 10(2): 55-57.
4. **Directorate of Health Services. Bangladesh Health Services Report (1990)**. Dhaka: DGHS. 1990; P: 87.
5. **Associates, M.a. (2011).**Bangladesh demographic and health survey. Dhaka: Ministry of health, Bangladesh.
6. **Bartlett J G, Mundy L M (1995)** Community-acquired pneumonia. N Engl J Med.; 333:1618–1624.
7. **Trollfors B, Claesson B A (1997).** Childhood pneumonia: possibilities for aetiological diagnosis. Baillière's Clin Paediatr; 5:71–84.
8. **Gwatney, J.M., Sande, M.A., Austrian, R., Hendley, J.O. (1975).** Spread of Streptococcus Pneumoniae in families. Relation of transfer of *S. Pneumonia* to incidence of colds and serum antibody. J. Inf. Dis., 132, 62-68.
9. **Kamat, S.R., Godhindi, K.D., Shah. B.W., et. al. (1980).** Correlation of health morbidity to air pollution levels in Bombay City. Results of prospective 3 year survey at one year. J. Post. Grad. Med., 26, 46-61.

10. **Tager, I.B., Weirs, ST., Munoz, A., Rosner, B., Spizer, F.F. (1983):** Longitudinal study of the effects of maternal smoking on pulmonary function in children. N. Engl. J. Med. 309:699-703.
11. **Pandey, M.R., Sharma, P.R., New Pane, R.P. (1985):** Preliminary report of a community study of childhood ARI in Nepal. Eds-Douglas, R.M. and Kerby-Eaton, E. In Acute Respiratory Infections. Proceedings of an International Workshop. Sydney, August. 1984: University of Adelaide, Adelaide. 131-136.
12. **Brimhle Combe , F.S.W., Cruickshank, R., Mastus, Ph., Reed, D.D., Stewart, G.T., Sanderson, D. (1958);** Family studies, of respiratory infections. Br. Med. J., 1: 119-128.
13. **Denny F. W. Jr. (1995)** The Clinical Impact of Human Respiratory Virus Infections. American Journal of Respiratory and Critical Care Medicine.; 152(4, part 2):S4–12.
14. **Berman S (1995).** Otitis Media in Children. New England Journal of Medicine; 332(23):1560–65.
15. **Mulholland E. K., Simoes E. A., Castales M. O., McGrath E. J., Manalac E. M., Gove S. (1992)** Standardized Diagnosis of Pneumonia in Developing Countries. Pediatric Infectious Disease Journal; 11:77–81.
16. **Shann F., Gratten M., Germer S., Linnemann V., Hazlett D., Payne R (1984).** Aetiology of Pneumonia in Children in Goroka Hospital, Papua New Guinea. Lancet. 2(8402):537–41.
17. **Webber S, Wilkinson AR, Lindsell D, Hope PL, Dobson SR, Isaacs D (February 1990)**."Neonatal pneumonia". Archives of Disease in Childhood 65 (2): 207.

18. **Wubbel L, Muniz L, Ahmed A et al. (February 1999).** "Etiology and treatment of community-acquired pneumonia in ambulatory children". The Pediatric Infectious Disease Journal 18 (2): 98–104.
19. **Boucher, H. W., et al. (2004**). No Drugs: No ESKAPE! An update from the infectious diseases society of America. Clin. Infect. Dis. 48: 1-12.
20. **Rahman M M, Haq J A, Hossain M A, Sultana R, Islam OF, Islam A.H.M.S (2004).** Prevalence of extended spectrum- lactamase-producing Escherichia coli and Klebsiella pneumoniae in an urban hospital in Dhaka, Bangladesh. International Journal of Antimicrobial Agents; 24: 508 – 510.
21. **Kerr, McHale (2003).** "Flora of the Nose and Throat". Applications in General Microbiology. Edition 6. P.331-335.
22. **Donald, Paul J., Jack L. Gluckman, Dale H. Rice (1995).** The Sinuses. New York.
23. **M.U. Rasheed, Mohammed Awole (2007)**: *Staphylococcus* epidermidis: A Commensal Emerging as a Pathogen with Increasing Clinical Significance Especially in Nosocomial Infections. The Internet Journal of Microbiology.
24. **Elke K.H. Schweda, Brigitte Twelkmeyer, and Jianjun Li (August 2008)**. Invited review: –Profiling structural elements of short-chain lipopolysaccharide of non-typeable Haemophilus influenzae". Innate Immunity; 14: 199 - 211.
25. **Bouchet, V., Hood, D., Li, J., Brisson, J, et al. (2003). –**Host-Derived Sialic Acid Is Incorporated into Haemophilus influenzae Lipopolysaccharide and Is a Major Virulence Factor in Experimental Otitis Media".
26. **Ryan, KJ; Ray, CG, eds. (2004).** Sherris Medical Microbiology (4th ed.). McGraw Hill. ISBN 0-8385-8529-9.

27. **Davis T J, Matsen J M (1974)**. Prevalence and characteristics of Klebsiella species: relation to association with a hospital environment. J Infect Dis; 130:402–405.

28. **Kloos W E, Musselwhite M S (1975).** Distribution and persistence of Staphylococcus and Micrococcus species and other aerobic bacteria on human skin. Appl Microbiol; 30:381–395.

29. **Maton, Anthea; Jean, Hopkins Susan, Johnson Charles William, McLaughlin Maryanna Quon Warner David, LaHart Wright, Jill (2010).** Human Biology and Health. Englewood Cliffs: Prentice Hall. pp. 108–118.

30. **I. A. Kristensen and J. Olsen (2006),** South African Medical Journal 96 (7), 633.

31. **B. G. Williams, E. Eleanor, C. Boschi-Pinto, J. Bryce, and C. Dye (2002),** Lancet Infect Dis. 2, 25.

32. **B. Jennifer, B. P. Cynthia, S. Kenji, and R. E. Black (2005),** Lancet 365, 1147.

33. **A. Hadi**, Bulletin of the World Health Organization 81 (3), 183 (2003).

34. **Birnboim, H.C. and J. Doly (1979)**. A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. Nucleic Acids Research 7:1513-1523.

35. **Kheder AK (2002).** Studies on antibiotic resistance by plasmid of *Pseudomonas aeruginosa.* Ph.D. thesis. College of Education. University of Salahaddin, Iraq.

36. **Tomoeda M, Inuzuka M, Anto S, Konishi M (1974).**Curing action of sodium dodecyl sulfate on a Proteus mirabilis R+ strain. J. Bacteriol., 120(3): 1158-1163.

37. **Klein, Donald W.; Prescott, Lansing M.; Harley, John (2005).** Microbiology. Boston: McGraw-Hill Higher Education.

38. **Bergey, David H.; John G. Holt; Noel R. Krieg; Peter H.A. Sneath (1994).** Bergey's Manual of Determinative Bacteriology (9th Ed.). Lippincott Williams & Wilkins.

39. **Gene cloning and DNA analysis: An Introduction, T.A. Brown (2010**).6th edition. Published by Blackwell publishing.

40. **Adachi, H.; Nakano, M.; Inuzuka, M. and Tomoeda, M. (1972).** Specific role of sex pili in the effective eliminatory action of sodium dodecyle sulfate on sex and drug resistance factors in Escherichia coli. J. Bacteriol., 109(3): 1114-1124.

41. **Snyder, L. and Champness, W. (1997).** Molecular Genetics of Bacteria. American Society for Microbiology. ASM press Washington D.C.

42. **May JM, Houghton RH, Perret CJ (1964).** The effect of growth at elevated temperature of some heritable properties of S. aureus. J. Microbiol., 37: 157-169.

43. **Ahmad KD (1989).** The positive control of ilvC expression in E.coli K-12. Ph.D. Thesis, Univirsity of Durhum, England.

44. **Bennett PM (2008).** Plasmid encoded antibiotic resistance: acquisition and Transfer of antibiotic resistance genes in bacteria. Br. J. Pharmacol., 153(4):347-357.

45. **Rabab O. Radi and Fryad H. Rahman (2010)**. Study the effect of Ethidium bromide, SDS and elevated temperature on stability of multiple antibiotic resistance plasmids of Pseudomonas aeruginosa, Iraqi J. Biotech. 9(4): 797-811.

46. **Sonstein, S. A. and Baldwin, J. N. (1972).** Loss of the penicillinase plasmid after treatment of S. aureus with SDS. J. Bacteriol., 109: pp.262-266.

APPENDIX I

APP.I.I: Oxidase Test principle

During aerobic respiration in electron transport chain, cytochrome oxidase (The enzyme cytochrome c oxidase/ Complex IV (EC 1.9.3.1), is a bulky transmembrane protein complex found in bacteria and the mitochondrion of eukaryotes) catalyzes the oxidation of reduced cytochrome (Cytochromes are hemeproteins containing heme groups and are primarily responsible for the generation of ATP via electron transport.) by molecular oxygen that results the formation of H_2O and H_2O_2. The oxidase test is used to identify bacteria that produce cytochrome c oxidase. When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless.

Aerobic bacteria, some facultative anaerobes and microaerophiles exhibit Oxidase activity. Genera ***Neisseria*** and ***Pseduomonas*** are Oxidase positive and ***Enterobacteriaceae*** are Oxidase negative.

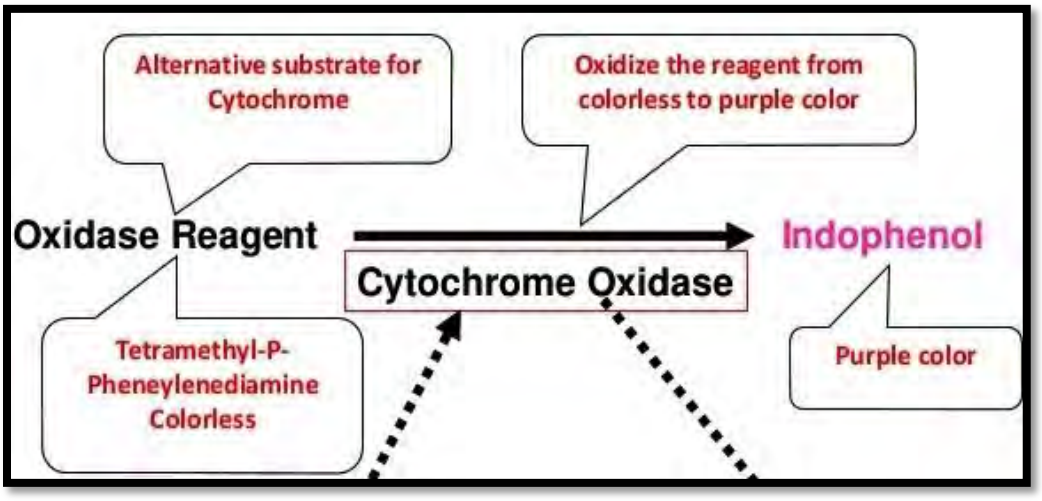


Figure (APP. I.I): Basic principle of oxidase test in diagram.

APP.I.II: Citrate Test

Some microorganisms are capable of using citrate as a carbon source in the absence of fermentable glucose or lactose for their energy. The ability depends on the presence of a citrate permease that facilitates the transport of the citrate in the cell. Citrate is the first major intermediate in the Krebs cycle and is produced by the condensation of active acetyl with oxalacetic acid. Citrate is acted on by the enzyme citrase which produces oxalacetic acid and acetate. These products are the enzymatically converted to pyruvic acid and carbon di oxide.

During this reaction the medium becomes alkaline – the carbon di oxide that is generated combines with sodium and water to form sodium carbonate, an alkaline product.

The presence of sodium carbonate changes the bromthymol blue indicator incorporated into the medium from green to Prussian blue. (**Cappuccino, microbiology lab manual)**

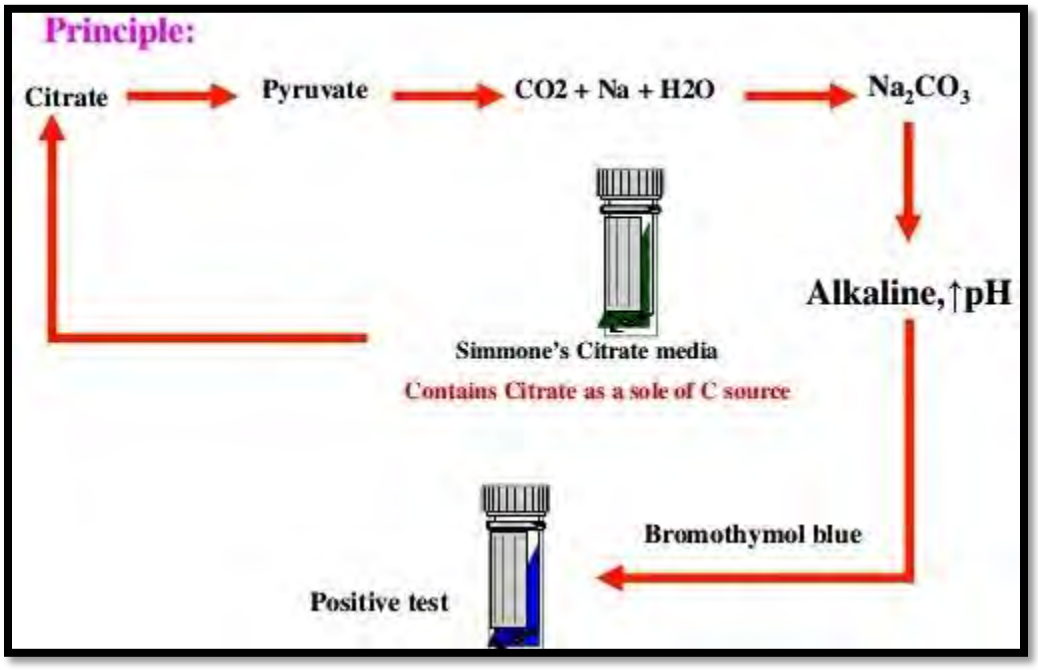


Figure (APP I.II): Schematic presentation of citrate test

APP.I.III: Indole Test

Indole test is used to determine the ability of an organism to spilt amino acid tryptophan to form the compound Indole. Tryptophan is hydrolyzed by tryptophanase to produce three possible end products – one of which is Indole. Indole production is detected from Para-dimethylaminobenzaldehyde reagent which mixture component is phosphoric acid and methanol; this reacts with indole to produce a colored compound. Indole test is a commonly used biochemical test. Indole test helps to differentiate Enterobacteriaceae and other genera.

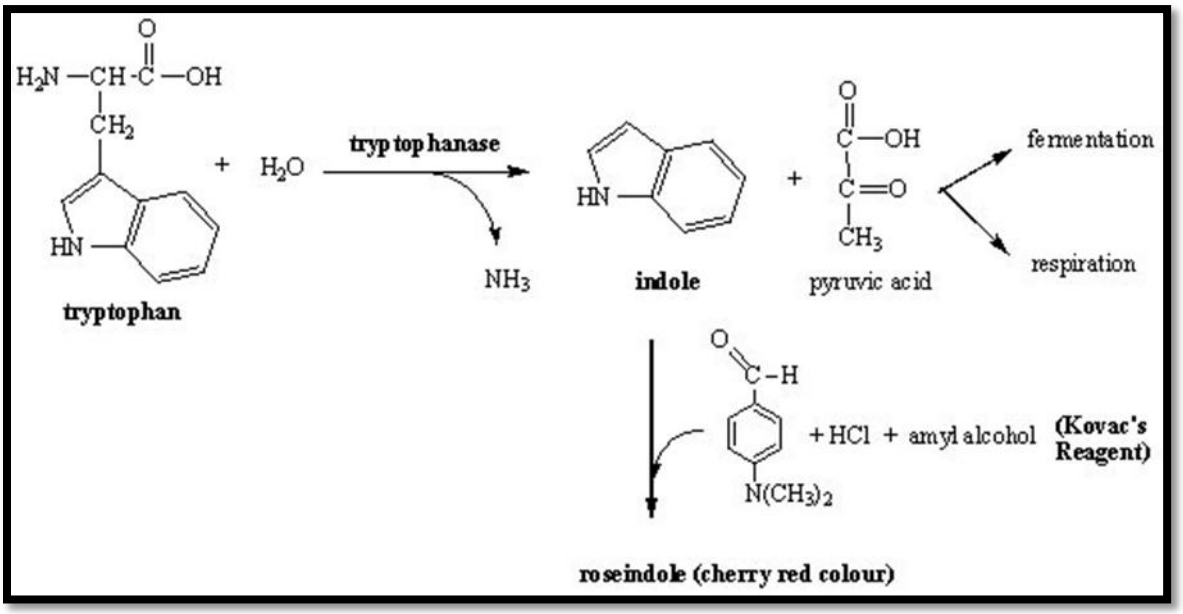


Figure (APP I.III): Reaction of Indole Test

APP.I.IV: Urease test

Urea is waste product excreted in urine by animals. Some enteric bacteria produce the enzyme urease, which splits the urea molecule into carbon dioxide and ammonia.

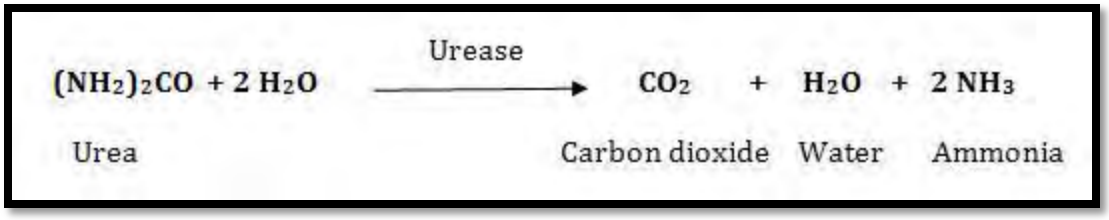


Figure (APP I.IV): Urease reaction

Urease, which is produced by some microorganisms, is an enzyme that is especially helpful in the identification of Proteus vulgaris, although other organisms may produce urease, their action on the substrate urea tends to be slower than that seen with Proteus species. Therefore this test serves to rapidly distinguish members of this genus from other lactose non fermenting enteric microorganisms.

Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end product ammonia. The presence of urease is detectable when the organisms are grown in a urea broth medium containing the pH indicator phenol red. As the substrate urea is split into its products, the presence of ammonia creates an alkaline environment that causes the phenol red to turn to deep pink. This is a positive reaction for the presence of urease. Failure of deep pink color to develop is evidence of a negative reaction (Reference: vlab.amrita.edu).

APPENDIX II

(APP.II.I): Media composition

Name of the medium	Composition	Direction
Triple Sugar Iron Difco™ Triple sugar Iron agar (Becton, Dickson and company) Base for the differentiation of gram negative enteric bacilli.	Approximate formula*per liter. 1. Beef extract =3.0 g 2. Yeast extracts =3.0 g 3. Pancreatic digest of casein =15.0 g 4. Proteose peptone no.3 = 5 g 5. Dextrose= 1 g 6. Lactose =10 g 7. Sucrose=10g 8. Ferrous sulfate =0 .2 g 9. Sodium chloride = 5g 10. Sodium thiosulfate =0.3 g 11. Agar= 12 g 12. Phenol red=0.024g *adjusted and/or supplemented as required to meet performance criteria. For laboratory use. Final pH 7.4	65 g of the powder was suspended in 1L of purified water. Then it mixed thoroughly. Heated with frequent agitation and boiled for 1 minute to completely dissolve the powder. Then it was dispensed into tubes and autoclaved at 121˚C for 15 minutes. Then the hot solution was cooled in a slanted position so that deep butts are formed. Test samples of the finished product for performance using stable, typical control outlets.
Simmons citrate agar (OXOID CM0155)	**Approx. composition per liter:** 1. Magnesium sulphate =0.2g 2. Ammonium dihydrogen phosphate= 0.2g 3. Sodium ammonium phosphate=0.8g 4. Sodium citrate, tribasic=2.0g 5. Sodium chloride=5.0g 6. Bromothymol blue=0.08g 7. Agar= 15.0g pH 7.0 ± 0.2 at 25°C	23 g of OXOID was suspended in 1 liter of distilled water and boiled until it dissolved completely. Then Sterilized by autoclaving at 121°C for 15 minutes. 5ml of the autoclaved media was added in test tubes and allowed to settle in a slanted position.

MIU (100ml)	**Preparation of gel** 1. NaCl= 0.5g 2. Agar= 0.4g 3. $KH_2PO_{2=}$0.2g 4. Peptone=3.0g **Preparation of 10ml phenol red (0.25%)** 0.025g of phenol red was added in 10ml of dH_2O **Preparation of urea solution (20%)** 2g of urea was added and made up to 10ml with dH_2O	**Method** 90ml of gel was prepared by adding dH_2O. 200µl of phenol red (0.25%) was added to the gel. Then the mixer was autoclaved. After autoclaving the solution was kept to cool down just before it solidify. Urea solution was sterilized by using filter sterilization. 10ml of prepared urea solution was added to the autoclaved 90ml mixture and was mixed them well.
Difco™ MacConkey Agar Base for the isolation and differentiation of enteric organisms**.**	Approximate formula* per liter 1. Pancreatic digest of gelatin =17 g 2. Peptones (meat and casein) =3 g 3. Lactose = 10 g 4. Bile salts no.3 =1.5g 5. Sodium chloride = 5 g 6. Agar =13.5 g 7. Neutral red =0.03 g 8. Crystal violet = 0.001 gm Final pH = 7.1 ±0.2	50 g of the powder was suspended in 1 l of purified water. Then it mixed thoroughly. Heated with frequent agitation and boiled for 1minute to completely dissolve the powder. Then the solution was autoclaved at 121°C for 15 minutes. Avoid overheating. Test samples of the finished product for performance using stable, typical control cultures.
Mueller Hinton Broth (OXOID CM0405) **and Agar**	500 g makes 23.8 liters 1. Typical formula *(g/l) 2. Beef, dehydrated infusion from =300 g 3. Casein hydrolysate= 17.5 g 4. Starch = 1.5 g 5. Divalent cation content of this lot 2.195 mg Ca^{++} per liter, 4.358 mg Mg^{++} per liter. For 100 ml broth agar is needed 1.5 gm.	For 100 ml MHB 2.1 gm of MH powder was added to dH_2O water. For Mueller Hinton Agar 1.5g/100ml was mixed with the 2.1 g of MH powder.

Bacto™ Todd Hewitt Broth Base for the cultivation of streptococci, pneumococci and other bacteria	Approximate formula *per liter 1. Heart infusion from 500g =3.1 g 2. Neopeptone =20 g 3. Dextrose =2 g 4. Sodium chloride =2 g 5. Disodium phosphate =0.4 g 6. Sodium carbonate-2.5 g Final pH 7.8 ±0.2	30 g of the powder was Dissolved in 1 liter if purified water. Autoclave at 121°C for 15 minutes. Test samples of finished product for performance using stable, typical control cultures.
Skim milk ,Trypton,glucose and glycerin (STGG) **(**for 100 ml broth)	1. Skim milk powder (OXOID LP0031) =2g 2. Trypton soya broth (OXOID CMO129) = 3g 3. Glucose =0.5 g/dextrose 4. Glycerol =10 ml 5. dH_2O =100 ml	First the solution was Autoclave, stirred and then pours into 1ml per cryovials.
Luria Bertani Broth	1. Trypton =10g/L 2. Yeast extracts =5 g/L 3. Sodium chloride =10g/L 4. dH_2O =950 ml 5. Agar= 75 g/L (for LB agar)	Preparation of LB broth for 1 Litter 1. We measured 10 g of Trypton, 5 g of Yeast Extract and 10 g of Sodium Chloride in a sterile conical flask. 2. Then we added around 950 ml of distilled water and mixed well to dissolve all the ingredients and added distilled water to make 1 liter of the solution followed by autoclave the solution at 121°C for 15 minutes to sterilize. 3. The prepared broth solution was stored in 4°C.

APPENDIX III

(APP. III.I): Gram staining Solution

Name of the reagent	*Component*	*Direction*
Crystal violet staining reagent	**Solution A for crystal violet staining reagent** Crystal violet (certified 90% dye content), 2g Ethanol, 95% (vol/vol), 20 ml **Solution B for crystal violet staining reagent** Ammonium oxalate, 0.8 g Distilled water, 80 ml	Solution A and B was mixed to obtain **crystal violet staining reagent.** Stored for 24 h and filtered through paper prior to use.
Mordant: Gram's Iodine	Iodine, 1.0 g Potassium iodide, 2.0 g distilled water, 300 ml	Iodine and potassium iodide were grinded in a mortar and water slowly with continuous grinding until the iodine was dissolved. Then the solution was stored in amber bottles.
Decolorizing Agent	Ethanol, 95% (vol/vol) ***Alternate Decolorizing Agent** Acetone, 50 ml Ethanol (95%), 50 ml	
Counter stain: Safranin	**Stock solution:** 2.5g Safranin O 100 ml 95% Ethanol Working Solution: 10 ml Stock Solution 90 ml Distilled water	

(APP.III.II): Reagents for Biochemical Test

Name of the reagent	Reagent and equipment that needed	Direction
Indole Reagent	1. Para dimethylamino benzaldegyde. 2. Phosphoric acid. 3. methanol 4. Filter paper. 5. 0.45 µm filter.	1. 5g of Para-dimethylaminobenzaldehyde was taken and added to a sterile beaker 2. With a sterile glass pipette take 25ml of methanol and to the beaker and mix properly. 3. After mixing 10ml of phosphoric acid was added drop by drop with a sterile glass pipette to the beaker and mixed carefully. 4. A 0.45 µm filter was taken for filter sterilization of the media and transferred it to sterile bottle. 5. The prepared Indole reagent was stored at 4°C. 6. Sterile filter paper was cut into 3 cm in length and 0.5 cm of wide by maintaining all the aseptic condition. 7. Then the filter was taken into a sterile petri dish and 1ml of the Indole reagent was added to the filter paper pieces. 8. Then the filter papers were incubated overnight and used it when it is dry.
Kovacs oxidase reagent	N N N' N' tetramethyl p phenylene diamine dihydrochloride reagent(SIGMA)	0.6-0.7 gm. of N N N' N' tetramethyl p phenylene diamine dihydrochloride reagent is mixed with 1 ml of normal saline.

APPENDIX IIV

(APP.IIV.I): Solution for plasmid isolation

BUFFERS	Component	Direction
Solution I	50mM Tris pH 8.0 with HCl, 10mM EDTA, 100ug/ml RNase A	For 1 liter: 6.06g Tris base was dissolved, 3.72g EDTA $2H_20$ in 800ml H_20. Adjust the pH to 8.0 with HCl. Adjusted the volume to 1 liter with ddH_20.
Solution II	200mM NaOH, 1%SDS	For 1 liter: 8.0g NaOH pellets were dissolved in 950ml of ddH_2O, 50ml of 20% SDS solution. The final volume was came out to 1 Liter
Solution III	3.0M Potassium Acetate, pH5.5	For 1 liter: 294.5g potassium acetate was dissolved in 500ml of H_2O. Then the pH adjusted to 5.5 with glacial acetic acid (~110ml). Then the volume was adjusted to 1 liter with ddH2O.
Tris EDTA buffer solution	TE 10mM Tris pH 8.0 with HCl, 1mM EDTA	For 1 liter: 1.21g Tris base was dissolved and 0.37g EDTA $2H_2O$ in 800ml of ddH_2O. Adjusted the pH to 8.0

APPENDIX V

APP.V.I: Instruments

The significant equipment's that used during the study are listed below

Name of the Instrument	Company
Molecular imager ,GelDoc™ XRT with image lab™ software	Bio-Rad ,USA
Micro oven	Miyako , japan
Powerpac™ basic	Bio-Rad ,USA
Jenway 3510 pH Meter	Keison,UK
Centrifuge machine	Thermo Fisher Scientific,USA
WisVen oven (60°C)	Witeg ,Germany
Olympus CKX41 inverted microscope	MSAC LTD ,UK
Kimwipes® EX-L Laboratory Wipes	Kimberly Clark Professional,USA
Incubator	Memmert, Germany
4°C Freezers ,Vest frost NFG 309	Vestfrost solution.com , Denmark
Wiseclave Autoclave	Witeg ,Germany
Lexicon® II Ultra-low Temperature Freezer (-80°C)	ESCOglobal,USA
Labculture® Class II, Type A2 Biological Safety Cabinets (E-Series)	ESCOglobal,USA
Weighing balance	Japan
WiseMix VM Vortex Mixer, WVM00010	Interlab ,NZ
Pipette 1000 µl	Gilson Inc,UK
Pipette 20-200 µl	Costar