Bacteriological Study of Hospital Waste Water: Microbial Isolation, Identification, and Analysis of the Antimicrobial Resistance Pattern

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Microbiology

Department of Mathematics and Natural Sciences

BRAC University

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Declaration

It is hereby declared that,

1. The thesis submitted, titled **"Bacteriological Study of Hospital Waste Water: Microbial Isolation, Identification and Analysis of the Antimicrobial Resistance Pattern,"** is our own original work while completing a degree at BRAC University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain matter that has been accepted, submitted, or any other degree or diploma at a university or other institution.

4. We have acknowledged all main sources of help.

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of Summer 2020 and Fall 2020 semester respectively have been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology in Summer, 2025.

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Ethics Statement

This research is conducted under the ethical standards of the Microbiology Program of the Department and Natural Sciences, BRAC University. All the laboratory procedures are carried out under the supervision of qualified personnel and the biosafety compliant environment with proper protocol. Standard ethical practices for handling microbial samples are strictly followed throughout the work. No other living organisms were directly involved. All data that are collected and analyzed with honesty and dedicatedly and there is no fabrication and manipulation of results. The findings of the thesis are an accurate representation of the work which is conducted during the entire period.

Abstract

Antimicrobials are used to prevent and treat bacterial infections, which are often released into the watery environment via wastewater from the hospital without being metabolized properly. This untreated water often becomes a breeding ground for bacteria, some of which can resist several antibiotics because of the high use of such drugs in hospitals. Thus, the primary target of our study was to isolate the microbes, identify and determine the antimicrobial activity in Dhaka. In total, 12 hospital wastewater samples were collected from 4 different tertiary hospital drainage systems. The hospitals are National Institute of Diseases of the Chest and Hospital (NIDCH), National Institute of Cancer Research and Hospital (NICRH), Bangabandhu Sheikh Mujib Medical University (BSMMU) or Post Graduate (P.G Hospital) and National Institute of Ear, Nose and Throat (ENT Hospital). After sample collection, culture techniques were employed to isolate and identify the microorganisms present and do the antimicrobial susceptibility test to find out the drug resistant. In this study, mostly *Klebsiella pneumoniae (K. pneumoniae), Escherichia coli (E. coli), Staphylococcus aureus (S. aures), Acinetobacter baumannii (A. baumanni), Pseudomonas aeruginosa (P. aeruginosa), Vibrio spp., Shigella spp.* and *Salmonella spp.*, *Klebsiella pneumoniae is* 87.88% resistant to Imipenem and 18% to Gentamicin and Ciprofloxacin. In the case of *E. coli,* Cefixime is 100% resistant and Amikacin is 41.17%. *S. aureus* is 10% resistant to Azithromycin and 33.33% resistant for *Shigella spp.* For *P. aeruginosa,* Imipenem is 37.5% resistant and 40% for *A. baumannii. A. baumannii* is 10% resistant to Tetracycline. Hospital wastewater is fully covered with different types of antibiotic-resistant bacteria such as *S. aureus, K. pneumoniae,* non-typhoidal *Salmonella spp. and E. coli,* which is a great threat to public health globally. Untreated hospital drainage effluents and frequent consumption of antimicrobial supplements without proper prescription of individuals play a vital role in developing antimicrobial resistance. In every healthcare sector, wastewater needs to be treated properly, reduce malpractice of using antibiotics and raise public awareness to resolve AMR issue globally.

Keywords:

Waste-water; Hospital; CFU; PCR; Gel electrophoresis; AST; AMR;

Dedication

To my parents, siblings and supervisor.

Acknowledgement

We would like to begin by expressing our sincere gratitude to our Creator, whose mercy allowed us successfully to complete our bachelor's degrees. Secondly, we would like to express our utmost gratitude and appreciation to our parents for their continuous support and encouragement throughout the period of study so do our undergraduate thesis. Their emotional support and all possible materialistic support eventually help me to overcome our ups and downs. Thirdly, we are equally appreciative of our supervisor, Akash Ahmed, Senior Lecturer, Department of Mathematics and Natural Sciences, for his constant guidance and advice, encouragement and solutions to our difficulties that we have faced in our thesis. Additionally, we would like to acknowledge our supervisor's guidance that actually helped us to overcome most of the challenges regarding thesis, and for this, and obviously everything, we would be tremendously grateful to him forever. This person has given us not only support for the thesis but also given us mental support whenever we felt we couldn't do it. Then, we would like to give special thanks to BRAC University Microbiology & Biotechnology Laboratory's respected Laboratory Officers for their constant support and cooperation in completing the laboratory work for the thesis. All lab assistants there were immensely helpful, so they deserve special thanks too at the same time. Finally, we are pleased to express our sincere gratitude, especially to our Research Assistant back then Ifthikhar Zaman for his immersive support and we would like to mention, Teaching Assistants Zawad Al Saiyan and Rifat Shubarna Tamanna with due respect for their advice over the time. All the other research assistants, teaching assistants and all other thesis students of CDAB Laboratory for their help, they deserve special mention as well. We would love to mention the names of our fellow groups TZA, SNS and RKS without whom this journey wouldn't be enjoyable. Special shout out to Somrat, Sristy, Ayesha, Zuhaer and Nini who have given us mental support throughout the journey. We want to mention another friend of ours, Sadman Shakib who has helped us in various ways, and we are thankful for that.

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Acronyms

AMR	Antimicrobial Resistance
AST	Antibiotic Susceptibility Test
MDR	Multi-Drug Resistance
PCR	Polymerase Chain Reaction
HWW	Hospital Waste Water
CLSI	Clinical and Laboratory Standards Institute

Chapter 1

Introduction

1.1 Background

Antimicrobial resistance (AMR) has appeared as one of the most pass mark able global threats to public health (Prestinaci et al., 2015). Often inappropriate and excessive use of antibiotics has significantly contributed to the development and proliferation of antibiotic resistant bacterial strains in the health care settings (Ferri et al., 2015). One of the major drivers of antibiotic resistance is the overuse and misuse of antibiotics, particularly when sub - therapeutic does come out in touch with the environment and accelerates the evolution of resistance within hospital environments (Hocquet, D. et al.,2016).

In a hospital, each day typically consumes around 40-60 liters of water, which generates substantial volumes of wastewater (Zhang et al., 2020). These outcomes differ based on several factors like hospital beds, health care facilities, range of services offered and efficiency of onsite wastewater management. In hospital drainage, wastewater comes from different sources like operation rooms, patient wards, laboratories and pharmaceutical waste areas, administrative units and kitchens. These mixtures represent a complex mixture of biological and chemical contamination (Fekadu et al., 2015). So, this wastewater needs to be treated properly, otherwise, this will disseminate resistance traits into the environment and human population as it joins with different sewage, rivers and other water sources (Korzeniewska & Harnisz, 2019).

Hospital wastewater is highly indicated as a significant but as usual neglected source of antimicrobial resistance (Le et al., 2016). Due to the presence of high concentrations of antibiotics and pharmaceuticals that boost the selection and persistence of resistance pathogenic bacteria, it acts as a reservoir for antibiotic resistance of bacteria (Khawaja, T. et al.). AMR development can be accelerated if the sub- lethal metabolites are introduced into wastewater. So, hospital wastewater plays a role as a significant route for antibiotics to enter into the environment, which contains different biological substances like human waste with unmetabolized antimicrobials (Rita L. et al.). Hospital wastewater contains different pathogenic bacteria such as *E. coli, P. aeruginosa, Klebsiella pneumoniae.* Which comes from the patients used medicine as

debris or contamination of medical equipment. According to a WHO report, hospital wastewater is a main global source of antimicrobial resistance (Cantón R,2013). For example, resistance rates from the hospital wastewater are 30.1% in Pakistan (Mekengo BM, 2021).

To develop target strategies for reducing the AMR influence into the environment, it is very important to understand the resistance profile of the bacteria in the wastewater. There are critical gaps in supplying the proper information about the AMR (Nguyen et al., 2021). Though there are lots of articles and studies on hospital wastewater, people are still neglecting this significant issue. Dhaka is the capital of Bangladesh, despite that, this developing area's hospitals are not aware of the wastewater which is globally spreading the AMR. Thus, the principal aim of our research was to determine the antimicrobial resistance profile of bacteria from the hospital wastewater from National Institute of Ear, Nose & Throat Hospital (ENT), National Institute of Diseases of the Chest & Hospital (NIDCH), National Institute of Cancer Research and hospital (NICRH), Bangabandhu Sheikh Mujibur Medical University (BSMMU) in Dhaka city, Bangladesh.

1.2 Literature Review

A wide range of pathogenic organisms, chemical surplus, disinfectants and pharmaceutical products are commonly found in hospital wastewater, and it makes this a potent public health danger. The risk of antimicrobial resistance in the environment is increasing by the bacteriological assessment of the flow. In many studies, it has been shown that many significant bacteria in hospital wastewater have shown antibiotic resistance to an intolerable level. By observing different studies, it has confirmed the occupancy of clinically important bacteria in waste waters of hospitals. From the hospital wastewater, different pathogens were consistently found, such as *Salmonella spp., Shigella spp., Klebsiella pneumoniae. Acinetobacter baumannii, Pseudomonas aeruginosa, Vibrio spp., Staphylococcus aureus, Escherichia coli, Streptococcus spp.* And these are found to contain multiple resistance genes and virulence factors (Kümmerer, 2009). The study has also shown that resistance against first-line antibiotics and last-resort antibiotics like ciprofloxacin, amikacin, tetracycline, imipenem and cefixime are commonly carried by these bacteria. Antibiotics that are released from hospital flows help in the survival of resistant strains. It also enables horizontal transfer of resistant genes from those trains to other microorganisms.

Enteric pathogens like *Salmonella spp*. are one of the common pathogens that we found from hospital wastewater. This is a gram-negative facultative anaerobic bacterium. It causes food-borne illnesses such as typhoid fever (*S. Typhi*), and gastroenteritis (*S. Typhimurium, S. Enteritidis*). From the infected patients, it usually enters the hospital wastewater. It is usually resistant to fluoroquinolones, cephalosporins, and other commonly used antibiotics (Kumar, 2025). In the hospitals of Ethiopia, 16.14% *Salmonella spp.* is found. This pathogen is not so frequently found in hospital wastewater, which is also shown in the study.

Another enteric pathogen is *Shigella spp.* which is found in hospital wastewater. It causes a very fatal disease like bloody diarrhea disease, which may cause bloody stool. So, we find it from fecal contamination

of the infectant in the hospital wastewater. It is commonly resistant to trimethoprim-sulfamethoxazole, ampicillin, and fluoroquinolones (Lampel, 2015). In the hospitals of Ethiopia, *Shigella spp.* showed resistance against azithromycin, ciprofloxacin and tetracycline just like the study showed us.

Klebsiella pneumoniae is a gram-negative and non-motile rod. They usually cause pneumonia, UTIs and systemic infections, mainly in immunocompromised patients. As they can survive well in an aquatic environment, we can easily find them in hospital wastewater. It is known for producing carbapenem which makes infections tough to treat (Arnold, 2011). In the hospitals of West Africa, in the wastewater 32.1% *Klebsiella pneumoniae.* can be found, in Indonesia, 67% and in Ethiopia, the rate is 22%. In the hospitals of South Asia and also in the study that has been done, the resistance of *Klebsiella pneumoniae* against Gentamicin, Tetracycline and Cefixime which are very high.

E. coli is a facultative anaerobic bacterium. It is usually found in human and animal intestines. It can cause diarrhea, sepsis and UTIs. Many strains of this bacterium are multidrug resistant, which also includes Extended-Spectrum Beta-Lactamase. It is carbapenem resistant as well (Aljohni, 2025). In the hospital wastewater of West Africa, the percentage of founding *E. coli* is 42.7%, in Indonesia, 60% *E. coli* and in Ethiopia it is 39%. Also, *E. coli* showed high resistance to Cefixime, Gentamicin and Tetracycline which are seen in the wastewater of Asian hospitals as well. In the hospitals of European countries, specifically in Romania, *E. coli* has also shown resistance against carbapenem.

Vibrio spp. is a gram-negative bacterium that is a facultative anaerobic bacterium. It is usually resistant to tetracycline, sulfonamides and fluoroquinolones (Onohuean, 2022). *Vibrio spp.* can cause different diseases like cholera and gastroenteritis. It showed resistance against Tetracycline, Ciprofloxacin and Gentamicin in Asian countries and in this study as well. But it did not show any resistance against Imipenem which is similar to other studies. As *vibrio spp.* can change the resistant pattern to environmental and clinical organisms, it is a matter of concern.

Pseudomonas aeruginosa is a Gram-negative bacterium. Its adaptability is high in the environment. It affects burns, wounds and the respiratory tracts as it is an opportunistic bacterium. As it has the ability to survive in moist, it is usually found in hospital wastewater. It is resistant to multiple antibiotics. This bacteria's resistance rates against Piperacillin and Amikacin are higher as shown in this study and in Asian countries. In Indonesia, the wastewater of hospitals contains 36% carbapenem resistant *Pseudomonas aeruginosa*.

Acinetobacter baumannii is a non-motile, Gram-negative bacterium. It is also associated with pneumonia. It also causes bloodstream infections and affects wounds in the ICU. It is commonly resistant to aminoglycosides and colistin (Farajnia, 2014). Amikacin and Piperacillin are highly resistant to *Acinetobacter baumannii* which is also found in Asian countries' hospital wastewater. In Indonesia, 71% resistance level against carbapenem which is very crucial. Though a wide range of *Acinetobacter baumannii* is found in West Africa, the resistance level is moderate.

Staphylococcus aureus is a Gram-positive bacterium that causes illnesses like skin infections, sepsis and endocarditis. It is resistant to Methicillin which makes it tough to treat (Turner, 2019). In the wastewater of South Africa hospital wastewater, it showed a high level of resistance against Gentamicin, Ciprofloxacin and Amikacin.

In hospital wastewater, the rate of antimicrobial resistant bacteria growth is shown to be an alarming concern now all over the world. As the hospital wastewater is working as a reservoir of multi-drug-resistant bacteria, a consistent pattern is observed all over the different geographical states. The most concerning matter is it is shown resistance against the most prescribed antibiotic and the last-resort antibiotics.

1.3 Objectives

The key objective of the study is to observe the bacteriological profile and antimicrobial resistance (AMR) patterns of hospital housing area wastewater which is basically unrelated in four selected government hospitals in Dhaka, Bangladesh where all types of patients come and take treatment all over the county. As samples hospital wastewater was chosen as it contains patient body fluids, lab waste, and antimicrobial agents' rich residues from wards and toilets, which hold a highly concentrated reservoir of MDR strains of bacteria. Hospital wastewater contains high antimicrobial residue load and microbial contamination, unlike clinical isolates or surface and water, and such wastewater-oriented AMR surveillance studies are partially common in African and European countries while it is still rare in Bangladesh. The aim of this study is to fill the gap and support AMR pattern observation to think about the global AMR issue more precisely.

Chapter 2

Methodology

2.1 Study design and settings:

The research helps us to find the ubiquity of the presence of *Salmonella spp., Shigella spp., Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Vibrio spp., Staphylococcus aureus, and Escherichia coli* in hospital wastewater of different hospitals of Dhaka city. We collected the samples from four different government hospitals in four different months to observe the antibiotic resistance profile of those bacteria. We have taken our samples from National Institute of Ear, Nose, Throat Hospital (ENT), and National Institute of Diseases of the Chest & Hospital (NIDCH), National Institute of Cancer Research & Hospital (NICRH), Bangabandhu Sheikh Mujib Medical University (BSMMU) or P.G. Hospital.

2.2 Study Place:

The study took place in the labs of BRAC University, which include the Microbiology lab and the Molecular lab.

2.3 Sample Collection:

The sample was collected through a sample bottle, which was sterilized beforehand by the autoclave machine of our lab. Proper permission was taken from the hospital authority to get the wastewater from the hospitals inside drain. Then we did the serial dilution and inoculated our diluted sample in different media. Media were incubated at 37 degrees Celsius to initiate the bacterial growth.

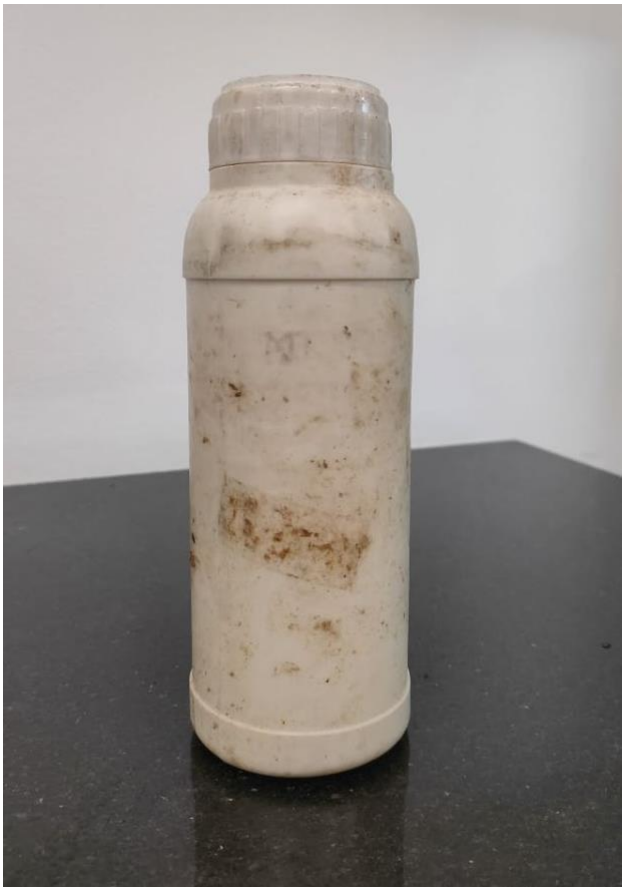

Figure 1: Sample Collection Autoclaved Bottle

2.4. Working Process:

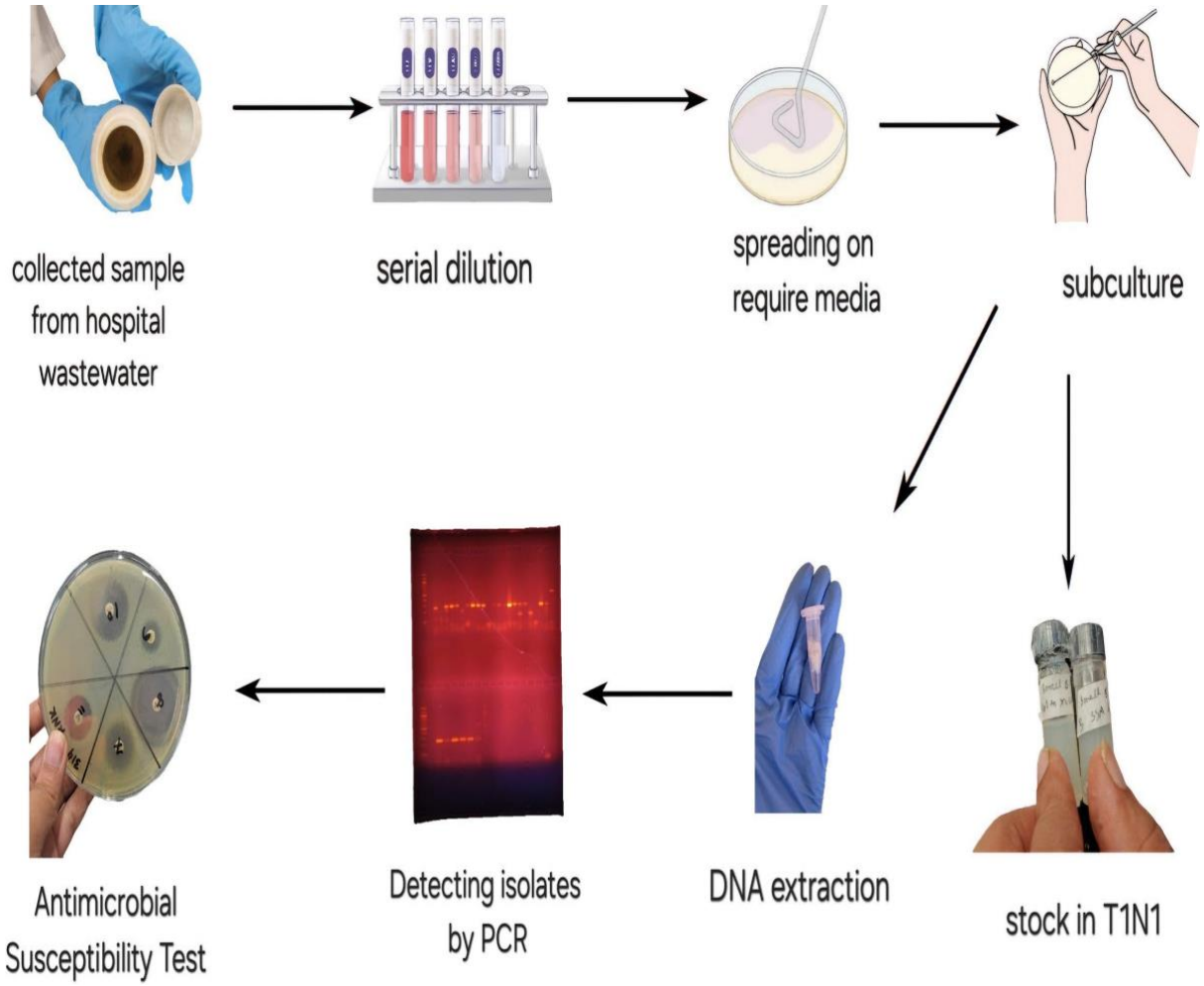

Figure 2: Workflow with scenario

2.5 Workflow chart:

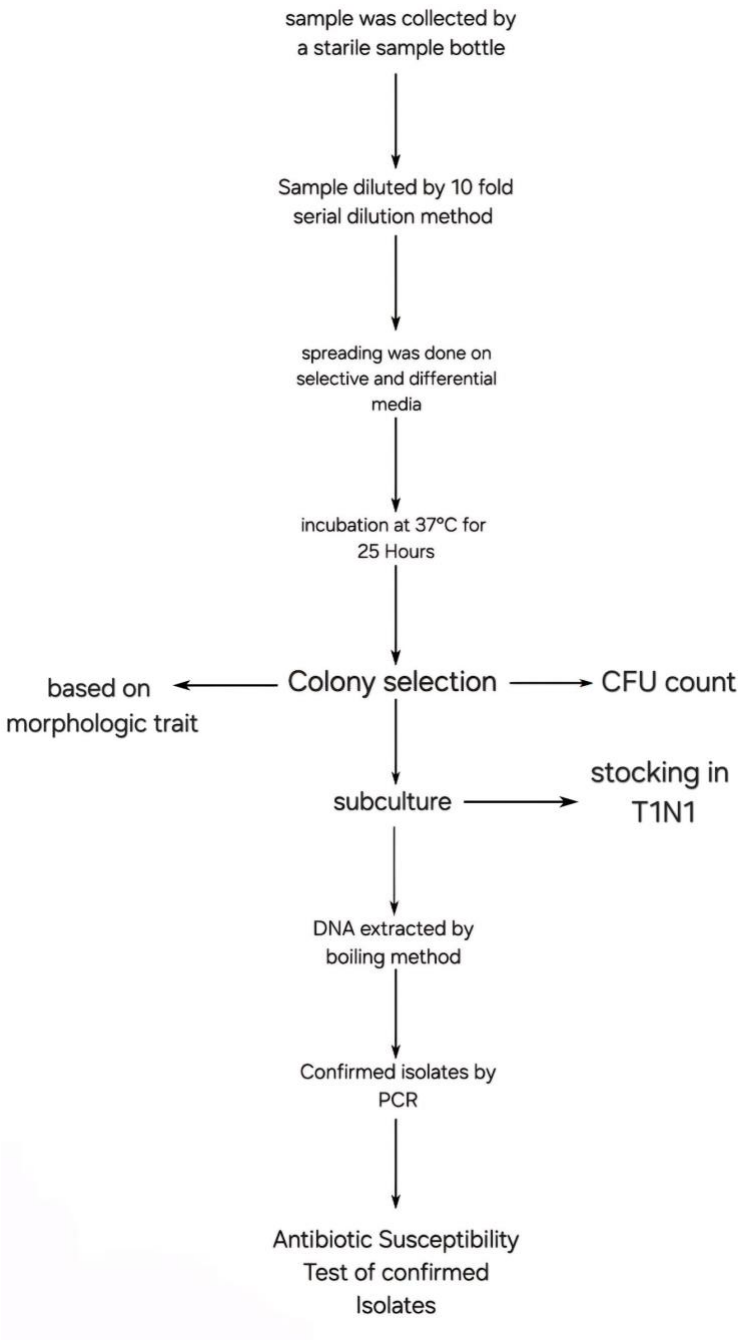

Figure 3: Work Flowchart of the study

2.6. Media Used for Isolation:

Culture media are such substances that we use to assist the growth of microorganisms. Different cultural media were used to get the desired microorganism. Different media were needed as every microorganism has its own requisition and needs to grow.

2.6.1 Xylose Lysine Deoxycholate (XLD):

Xylose Lysine Deoxycholate is a selective and differential media used to isolate and differentiate *Salmonella spp.* and *Shigella spp.* from samples. The growth of gram positive bacteria is inhibited by Sodium Deoxycholate of this media which makes it a selective media. As differential media, by utilizing Xylose, Lactose and Sucrose with Lysin where they work as fermenters. It also produces H2S which creates black centered colonies, indicating *Salmonella spp*.

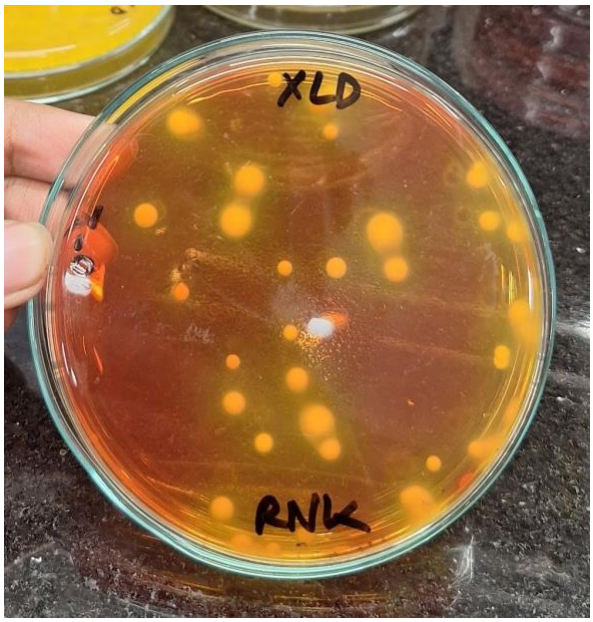

Figure 4: XLD Agar Median with Yellow, Red Colonies.

2.6.2 Salmonella-Shigella Agar (SSA) Media:

Salmonella-Shigella Agar is both selective and differential media which differentiates and isolates *Salmonella spp.* and *Shigella spp.* When we inoculate our sample in SS agar, it mainly inhibits the growth of gram-positive bacteria and sometimes the growth of gram - negative bacteria for convenient isolation of *Salmonella spp.* and *Shigella spp.* The major components of this agar are bile salt, sodium thiosulphate, sodium citrate and ferric citrate. As we have told before, it is a selective media, the growth of gram - positive bacteria is inhibited by bile salt and sodium citrate, which makes it selective for enteric pathogens. Hydrogen sulfate is also produced here with the help of sodium thiosulfate and ferric citrate.

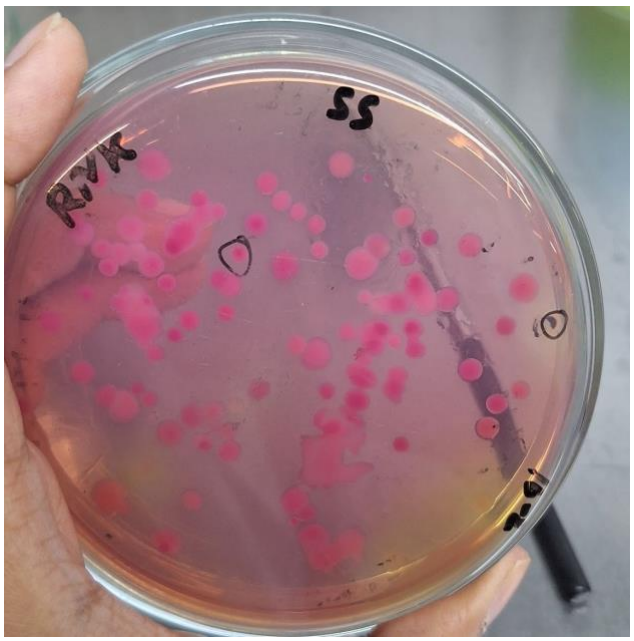

Figure 5: SSA agar media with pink colonies

2.6.3 Mannitol Salt Agar (MSA) Media:

Mannitol Salt Agar works as both selective and differential media. The high concentration of salt in MSA inhibits the production of most bacteria, which makes it a selective medium. In the case of differentiation, mannitol and phenol red play the key role. The fermentation of mannitol lowers the pH of the medium by acid production, which turns the phenol red to yellow. In other words, it differentiates between mannitol fermenting and non-fermenting. MSA media mainly isolates *Staphylococcus spp.* bacteria, potentially *Staphylococcus aureus*.

Figure 6: MSA agar media with yellow colonies

2.6.4 Eosin Methylene Blue Agar (EMB) Media:

Eosin Methylene Blue Agar is both selective medium and differential medium. The Eosin and Methylene blue in it dyes which prevents the growth of gram-positive bacteria and initiates easier growth of gram-negative bacteria. Lactose fermentation is the key of this media being a differential media. Lactose fermenting bacteria like *E. coli* ferment lactose and lowers the pH of the medium. This also makes the dye absorbed and can give green metallic sheen. On the other hand, lactose non-fermenters will give pink colonies or be colorless. The Enterobacteriaceae family particularly grows in this medium.

Figure 7: EMB agar media plate with black and metallic green colonies

2.6.5 Thiosulfate-Citrate-Bile Salts-Sucrose agar (TCBS) Media:

Thiosulfate-Citrate-Bile Salts-Sucrose agar is a selective medium. It potentially identifies *Vibrio spp.*, particularly *Vibrio Cholerae* and *Vibrio parahaemolyticus* from the sample. The pH plays an important role as its alkaline here, which inhibits the growth of Enterobacteriaceae and gram-positive bacteria. Sodium Chloride in these media initiates the growth of halophilic vibrio species as they're salt loving. Sucrose fermenter vibrio species give yellow colonies caused by acid production. The black and green colonies are caused by ferric sulfate, which detects Hydrogen Sulfate production.

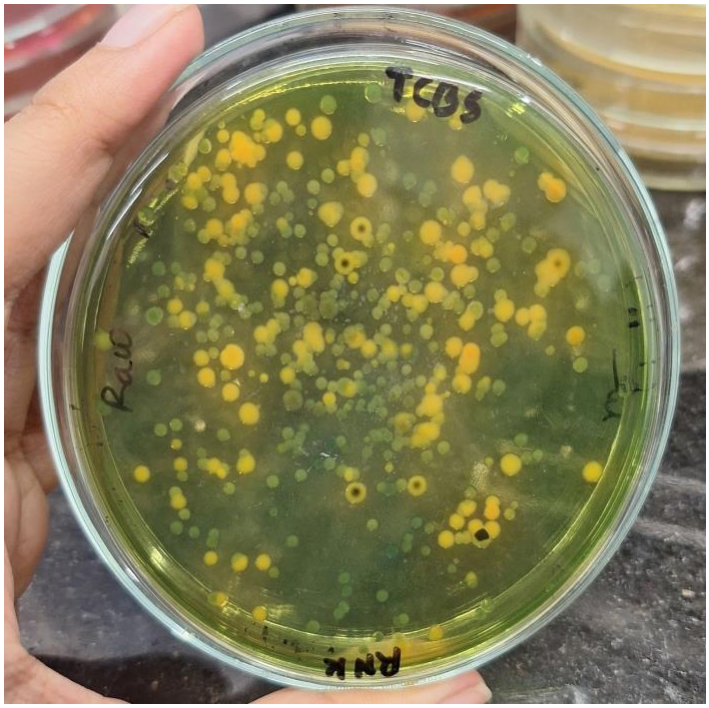

Figure 8: Vibrio spp. on TCBS media plate

2.6.6. Cetrimide Agar media:

Cetrimide agar media is a selective media which is a selective media. It mainly produces *Pseudomonas aeruginosa* which is a gram-negative bacterium. The key ingredient here is cetyltrimethylammonium bromide, an inhibitor that inhibits many bacteria.

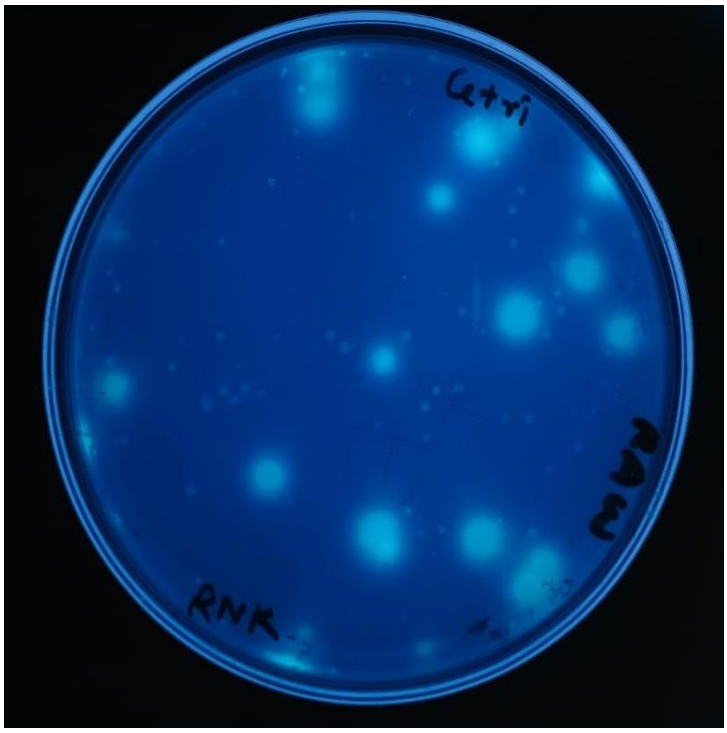

Figure 9: P. aeruginosa on Cetrimide agar media plate

2.6.7 UTI Agar media:

UTI Agar media is a chromogenic media, meaning it gives different colors according to the growth of specific media as indications. It is a differential medium. The chromogenic substrates here are cleaved by enzymes produced by certain bacteria, which give different colored colonies.

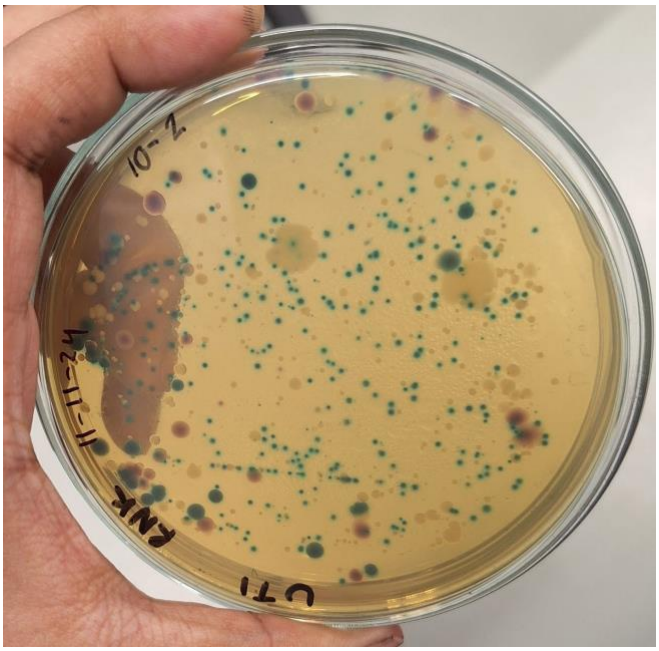

Figure 10: E. coli & K. pneumoniae on UTI agar media

2.6.8 LEEDS Agar media:

Leeds Acinetobacter media is both selective and differential medium. As it is both selective and differential media, it only facilitates the growth of *Acinetobacter spp*. At the same time, it inhibits the growth of other bacteria. The carbohydrate fermenting gives yellow colored colonies, and the bacteria that produce ammonia causes pink colored colonies.

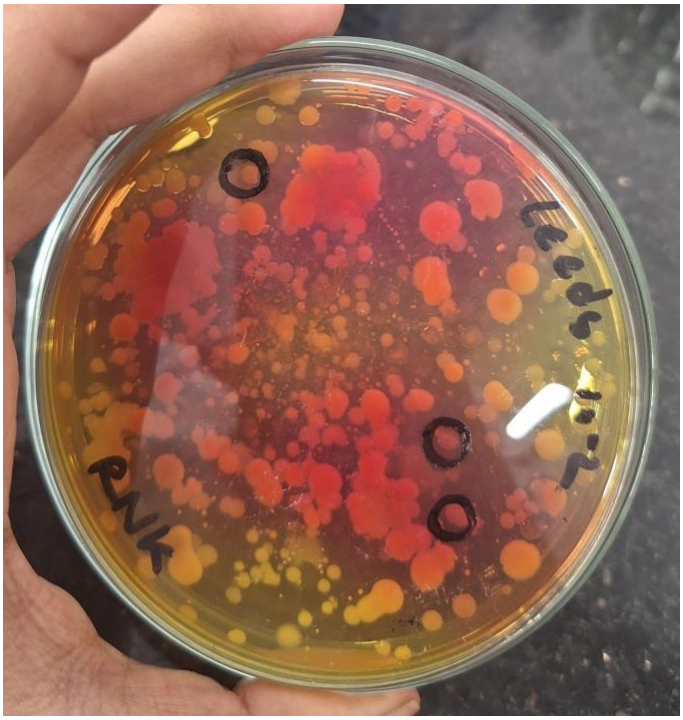

Figure 11: A. baumannii on Leeds agar media

2.6.9 MacConkey agar media:

MacConkey is both selective and differential medium. It is selective for Gram-negative and enteric bacteria. The crystal violet and bile salt in these media inhibit the growth of gram-positive bacteria, which allows the growth of gram-negative bacteria. The lactose fermentation factor makes it a differential medium. The bacteria which have the capability to ferment lactose will produce acid as a byproduct, which results in a lower pH the medium as a result it will give red or pink-colored colonies.

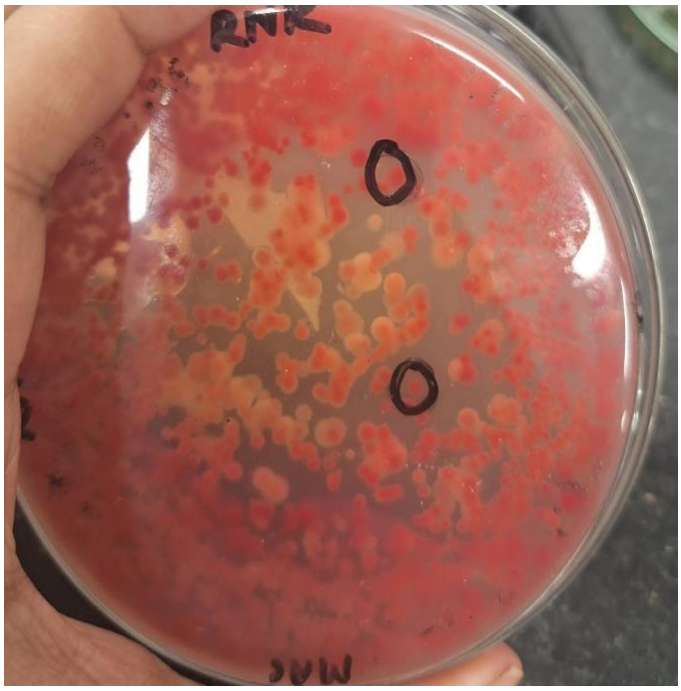

Figure 12: Gram - negative bacteria on MacConkey

2.6.10 *Klebsiella pneumoniae* Carbapenemase Agar (KPC) media:

KPC is both selective and differential medium. It inhibits the growth of other media except carbapenem-resistant *Enterobacteriaceae*. As it is a chromogenic agar, it produces different colors as the indication of different growths as it is a differential media.

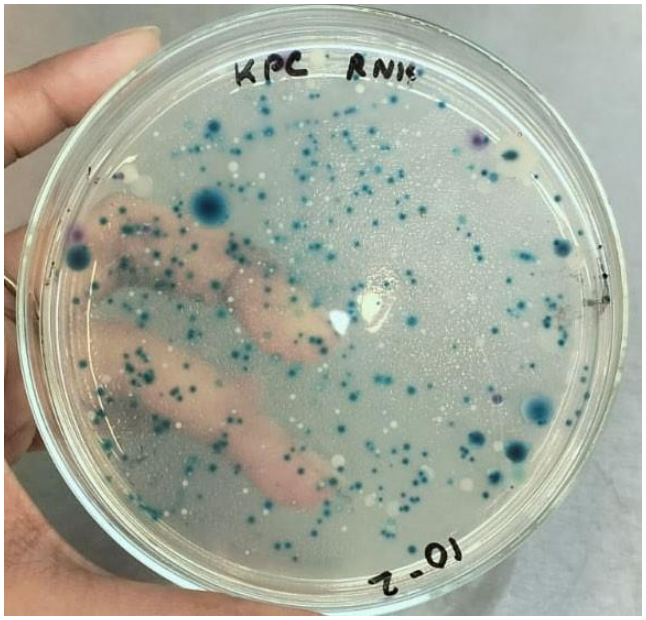

Figure 13: Carbapenem-resistant Enterobacteriaceae on KPC agar media

2.7 Additional Media Used:

2.7.1 Nutrient Agar:

A wide variety of non-fastidious bacteria grow on nutrient media as it is a solid media that has all the necessary nutrients to provide. The nutrients it contains are peptones, beef extract, sodium chloride and other nutrients. This media is used to subculture inoculated bacteria. To count total bacterial growth, this media is also used to viable microbial cell numbers in colony forming units.

2.7.2 Mueller Hinton Agar (MHA) Media:

For antibiotic susceptibility testing, Mueller Hinton agar is mainly used. Clinical and Laboratory Standards Institute chose Mueller Hinton Agar for various reasons, such as it supports the growth of a wide range of the non-fastidious microorganisms; this agar is low in sulfonamide, trimethoprim and tetracycline inhibitors. (Aryal, 2022)

2.8 Serial Dilution:

Serial dilution is a sequential process that includes diluting a solution multiple time. It includes an amount of the formerly used diluted solution into the next solution. Here, 9ml of saline solution was taken and add 1ml of our sample and vortex it. Then, in the second dilution, 1ml from the first dilution was taken and mixed with the second dilution's 9ml saline solution. By following these steps, tenfold serial dilutions were completed. This method is usually used to manage the concentration of the solution and to analyze and count the growth of microorganisms.

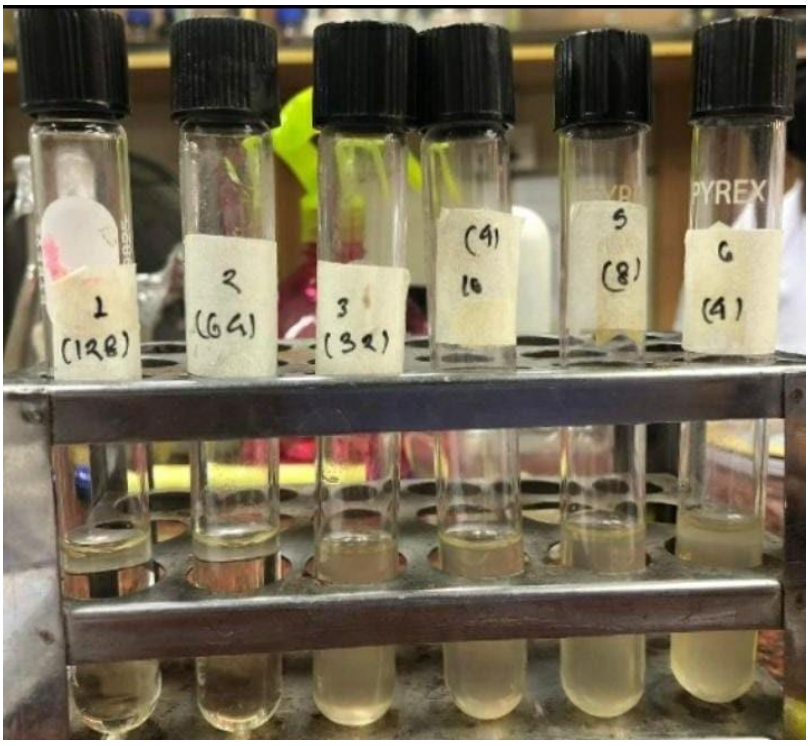

Figure 14: Serial Dilution picture up to 10^-6 fold

2.9 Molecular characterization:

Molecular characterization is such a technique to identify and classify isolates that implies other techniques like DNA sequencing, Polymerase Chain Reaction or PCR by scrutinizing their genetic material. It makes us understand the genetic composition and components of bacteria by providing powerful techniques with a wide range of applications in research settings.

2.9.1 DNA Extraction

DNA extraction is a purification method of DNA. By using this technique, DNA can be isolated from any physical or chemical sample. There are three steps in DNA extraction:

- The first step is lysis, where we break the cell to release the DNA,
- The next step is precipitation, in this step, proteins and other contaminants are removed,
- Once the precipitation is done, the DNA is purified by column based method.

This flowchart below shows the steps that were followed to get extracted DNA:

Took a loopful colony of 1ml into a 400ml TE buffer

⬇

Vortexed it until it mixed properly

⬇

Centrifuge the mixture at 13000 RPM for 10 minutes at 25 degrees.

⬇

Discard the supernatant and again mix the pellet with a 400 ml TE buffer.

⬇

Vortexed it until it mixed properly and put it in a 100 degrees water bath for 10 minutes.

⬇

ssCool down the mixture by putting it at room temperature and again centrifuge it at 10000 RPM for 10 minutes at 25 degrees Celsius.

⬇

Transfer 200ml supernatant and store it at -20 degrees Celsius.

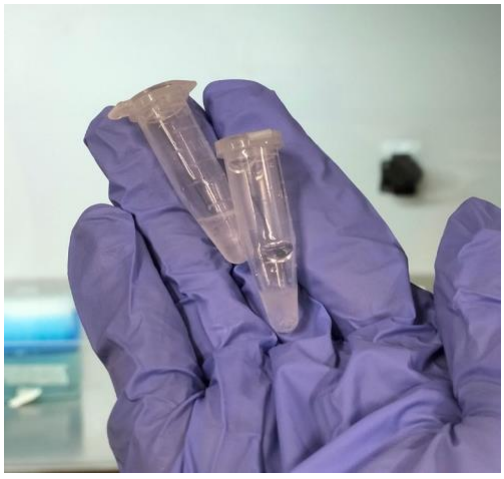

Figure 15: Extracted DNA stored at -20℃

2.9.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is such a method that is based on the enzymatic replication of DNA. In PCR, a small amount of DNA sequence's reprints is amplified in different cycles of temperatures in a row. It basically has three steps:

1. **Denaturation**: It is the initial stage of the thermocycler. In between the temperatures of 94-98 degrees Celsius, the mixture is heated for 30 seconds. It breaks the hydrogen bonds, which creates single stranded DNA. The single-stranded DNA we got will be working as templates now. The higher temperatures of denaturation are required to separate the double bond-stranded DNA. The steps and durations can change based on the type of templated. The change happens because different size's DNA may need longer or shorter incubation times.

2. **Annealing:** In this step, primers need to attach to the DNA template, so the temperature was too high. It needed to cool down, and now it will be 55-60 degrees Celsius. Here two single-stranded DNA containing the target region. Annealing temperature should be 3-5 degrees below melting temperature.

3. **Elongation:** The kind of DNA polymerase we will use in this experiment makes the decision for the temperature of this step. The number of targeted DNA sequences doubles at each extension. As all new strands along with the initial strand work as template, with each cycle, a particular targeted DNA region is amplified.

In this study, PCR was frequently done to detect microorganisms, respectively *Salmonella spp., Shigella spp., Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Vibrio spp., Staphylococcus aureus, Escherichia coli.* By using the process, we mentioned. We took 11ml of PCR mixture and 2ml of DNA template in PCR tubes and spun it to avoid any bubbles in the mixture. According to the targeted gene to the organisms and primer sequences, the cycles were put in the PCR machine. The table below shows all the information differs according to the microorganisms in PCR and gel electrophoresis.

Table 1: Primer used for PCR, PCR Sequence, Band Size, and PCR condition

Name of Bacteria	**Forward and Reverse Primer's Sequence**	**Product Size**	**PCR condition**	**Agarose Gel Electrophoresis Concentration**
Pseudomonas aeruginosa	PA-SS-F 5' - GGGGGATCTTCGGACCTCA-3' PA-SS-R 5'-TCCTTAGAGTGCCCACCCG-3'	956 bps	Initial denaturation: 95°C for 2 minutes, denaturation: 94°C for 20 seconds, annealing: 58°C for 20 seconds, and extension: 72°C for 40 seconds. Cycles: 25 cycles. Final cycle: 72°C for 1 minute.	1.5%
Staphylococcus aureus	nuc-F 5'-GCGATTGATGGTGATACGGTT-3' nuc-R 5'-AGCCAAGCCTTGACGAACTAAAGC-3'	279bps	Initial denaturation: 94°C for 4 minutes, Denaturation: 94°C for 1 minute, Annealing: 55°C	2%

			for 30 seconds, Extension: 72°C for 1.5 minutes. Cycles: 37 cycles. Final cycle: 72°C for 3.5 minutes.	
Acinetobacter baumannii	OXA-51-F 5′-TAATGCTTTGATCGGCCTTG-3′ OXA-51-R 5'-TGGATTGCACTTCATCTTGG-3'	353bps	Initial denaturation: 94°C for 5 minutes, Denaturation: 94°C for 1 minute, Annealing: 55°C for 1 minute, and extension: 72°C for 1 minute. Cycles: 30 cycles. Final cycle: 72°C for 10 minutes.	2%
Salmonella spp.	InV-A F 5'-CGG TGG TTT TAA GCG TAC TCT T-3' InV-A R 3'-CGA ATA TGC TCC ACA AGG TTA-5'	796bps	Initial Denaturation: 94°C, 1 minutes Denaturation: 94°C, 1 minute Annealing: 58.3°C, 30 seconds Elongation: 72°C, 30 seconds Final Elongation: 72°C, 7 minutes Cycle: 35	1.3%
Shigella spp.	GF 5'-TCCGTCATGCTGGAT	159bps	Initial Denaturation:	2%

	GAACGATGT-3’ GR 3’-ACAGTTCAGGATTGC CCGAGACACA-5’		95°C, 5 minutes Denaturation: 95°C, 30 seconds Annealing: 55°C, 1 minute Elongation: 72°C, 30 seconds Final Elongation: 72°C, 7 minutes Cycle: 30	
Vibrio spp.	VG F 5’-TGCAGATAATTCACG CCCAG-3’ VG R 3’-ACCCGCTGGACGCCA T-5’	689bps	Initial Denaturation: 94°C, 5 minutes Denaturation: 94°C, 30 seconds Annealing: 60°C, 30 seconds Elongation: 72°C, 30 seconds Final Elongation: 72°C, 10 minutes Cycle: 25	1.5%
Klebsiella pneumoniae	Pf-F 5’-ATT TGA AGA GGT TGC AAA CGA T-3′ Pr2-R 5’-CCG AAG ATG TTT CAC TTC TGA TT-3′	260bps	Initial Denaturation: 94°C, 10 minutes Denaturation: 94°C, 30 seconds Annealing: 57°C, 20 seconds Elongation: 72°C, 20 seconds Final Elongation: 72°C, 10 minutes Cycle: 35	2%

E. coli	ECO F 5'-GAC CTC GGT TTA GTT CAC AGA-3' ECO R 3'-CAC ACG CTG ACG CTG ACC A-5'	585bps	Initial Denaturation: 95℃, 5 minutes Denaturation: 94℃, 45 seconds Annealing: 45℃, 45 seconds Elongation: 45℃, 1 Min	1.5%

2.9.3 Antibiotic Susceptibility Test

By doing antibiotic susceptibility tests, it can be determined whether the bacteria are susceptible or resistant to the antibiotic. The resistant patterns of bacteria are very crucial. The most flexible method for this test is the Kirby-Bauer disk diffusion method. For this method, first we need to prepare the Muller-Hinton agar as it is low in sulfonamide, trimethoprim, and tetracycline inhibitors. 38ml of agar is added to 1 liter of distilled water. The antibiotic discs are infused on the plates where the microorganisms are lawned. After 24 hours of incubation at 37 degrees Celsius, the zones were measured in millimeters. Then we can decide whether it's susceptible or resistant according to the CSLI guidelines 2023.

Chapter 3

Result

3.1 Colony Forming Unit

The study was done on different time periods respectively fall, spring and summer. From every sample, the CFU has been counted.

In Winter:

From the wastewater of National Institute of Ear, Nose, Throat Hospital, the count was 135000 CFU/ml

From the wastewater of, National Institute of Diseases of the Chest & Hospital, the count was 249000 CFU/ml

From the wastewater of National Institute of Cancer Research & Hospital, the count was 320000 CFU/ml

From, the wastewater of Sheikh Mujib Medical University, the count was 670000 CFU/ml

In Spring:

From the wastewater of National Institute of Ear, Nose, Throat Hospital, the count was 2030000 CFU/ml

From the wastewater of National Institute of Diseases of the Chest & Hospital, the count was 80000 CFU/ml

From, the wastewater of National Institute of Cancer Research & Hospital, the count was 235000 CFU/ml

From, the wastewater of Sheikh Mujib Medical University, the count was 340000 CFU/ml.

In Summer:

From the wastewater of National Institute of Ear, Nose, Throat Hospital, the count was 1670000 CFU/ml

From the wastewater of National Institute of Diseases of the Chest & Hospital, the count was 530000 CFU/ml

From the wastewater of National Institute of Cancer Research & Hospital, the count was 320000 CFU/ml

From the wastewater of Bangabandhu Sheikh Mujib Medical University, the count was 104000 CFU/ml.

Figure 16: CFU count on NA media plate

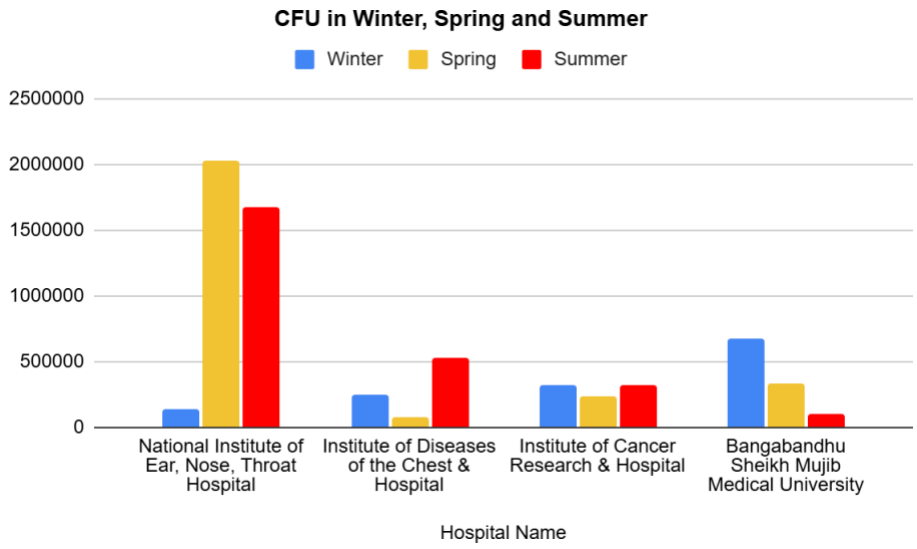

Figure 17: Graph of CFU count in graphs of 3 different seasons

The different CFU indicates different detection from different hospitals. The sharp increase rate of the National Institute of ENT is 15 times higher than in spring. A little less in the summer, but still the rate is high. In the case of the National Institute of Diseases of the Chest and Hospital, CFU count is not that high as it drops in spring and increases to a very low level in the summer. NICRH showed the same kind of result as well, but the rates were higher. Bangabandhu Sheikh Mujib Medical University shows a gradual fall from winter to summer. So, from the summary, it is visible that the winter has the lowest average CFU, and the spring has the highest average CFU, which is almost two times higher than winter. The CFU in summer is lower than spring but still higher.

3.2 PCR confirmation through agarose gel electrophoresis:

From the four hospital wastewater samples, 385 isolates were found. Among those 385 isolates, 134 positive isolates were detected by PCR. From the PCR confirmation through agarose gel electrophoresis, one *Salmonella spp.* isolate, three *Shigella spp.* isolate, five *Vibrio spp.* isolates, sixty-six *Klebsiella pneumoniae* isolates, eight *Pseudomonas aeruginosa* isolates, ten *Acinetobacter baumannii* isolates, seven *Staphylococcus aureus* isolates, thirty-four *Escherichia coli* isolates.

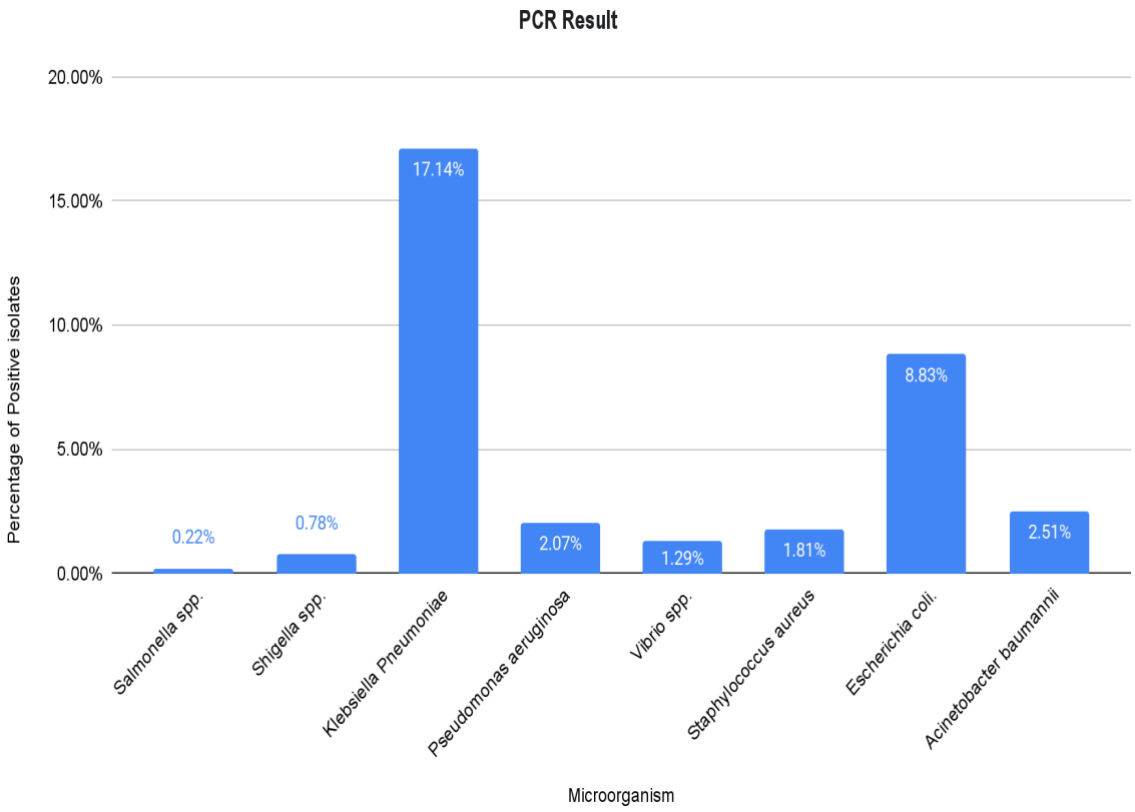

Figure 18: Graph of PCR confirmed isolates percentages

3.2.1 PCR and Agarose Gel Electrophoresis for identification of *Salmonella spp.*:

PCR was applied to suspected *Salmonella spp.* to get the desired bands. These bands were observed and confirmed under UV light by using agarose gel electrophoresis method. The desired band size for *Salmonella spp.* is 796. Among 31 *Salmonella spp.,* one positive result was found.

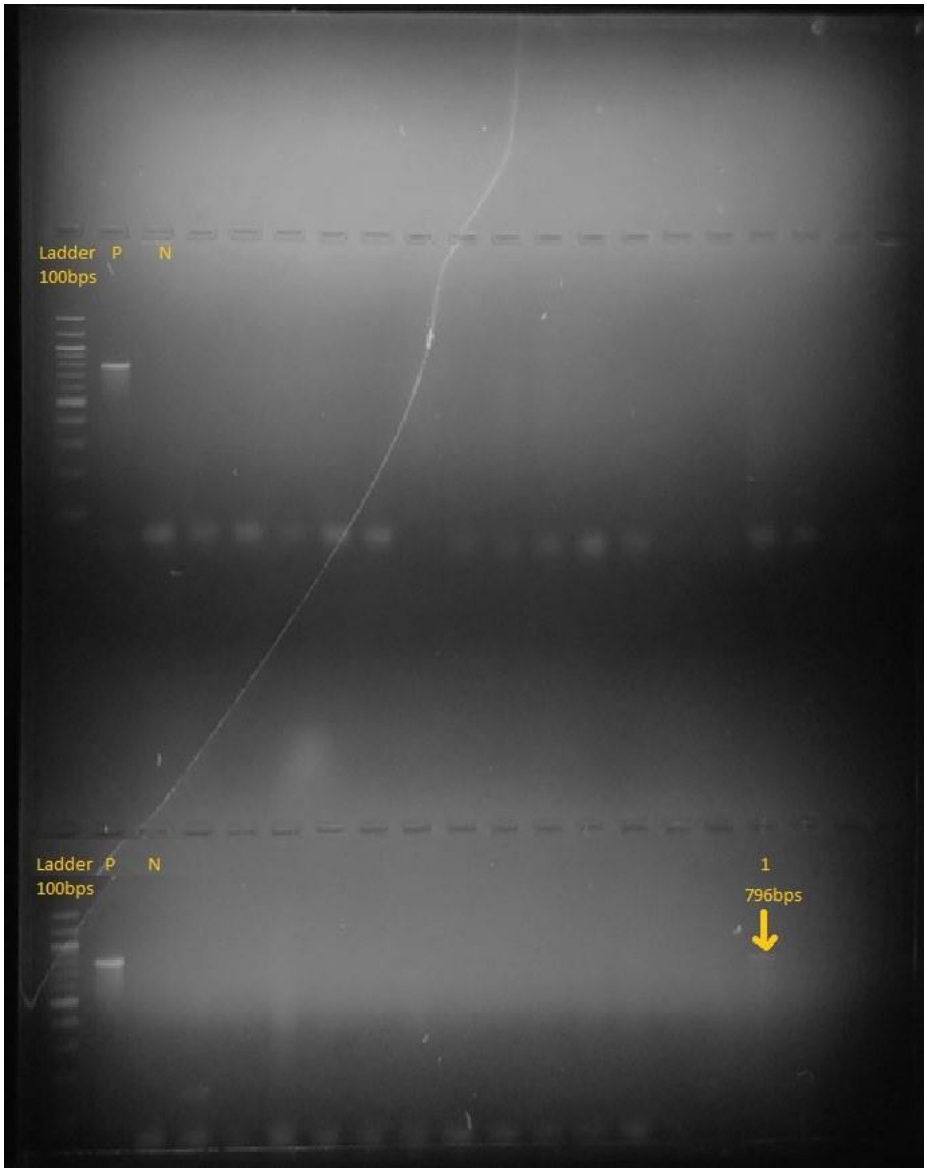

Figure 19: Salmonella spp. PCR result through gel run electrophoresis.

3.2.2 PCR and Agarose Gel Electrophoresis for identification of *Shigella spp.*:

PCR was applied on suspected *Shigella* to get the desired bands. These bands were observed and confirmed under UV light by using agarose gel electrophoresis method. The desired band size for *Shigella spp*. is 159. Among 31 *Shigella spp*. 3 positive results were found.

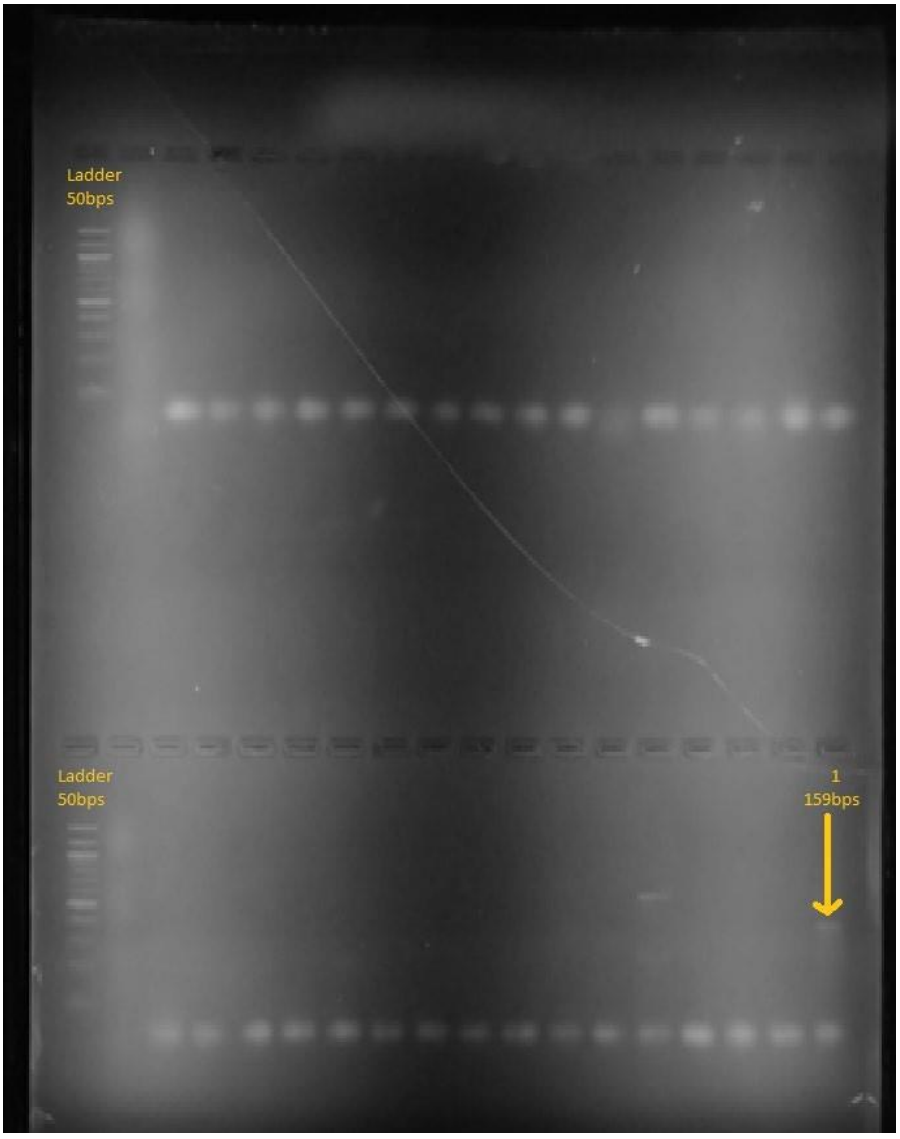

Figure 20: Shigella spp. PCR result through gel run electrophoresis.

3.2.3 PCR and Agarose Gel Electrophoresis for identification of *Klebsiella pneumoniae*:

PCR was applied to suspected *Klebsiella pneumoniae* to get the desired bands. These bands were observed and confirmed under UV light by using agarose gel electrophoresis method. The desired band size for *Klebsiella pneumoniae* is 260bps. Among 103 *Klebsiella pneumoniae*, 66 positive results were found.

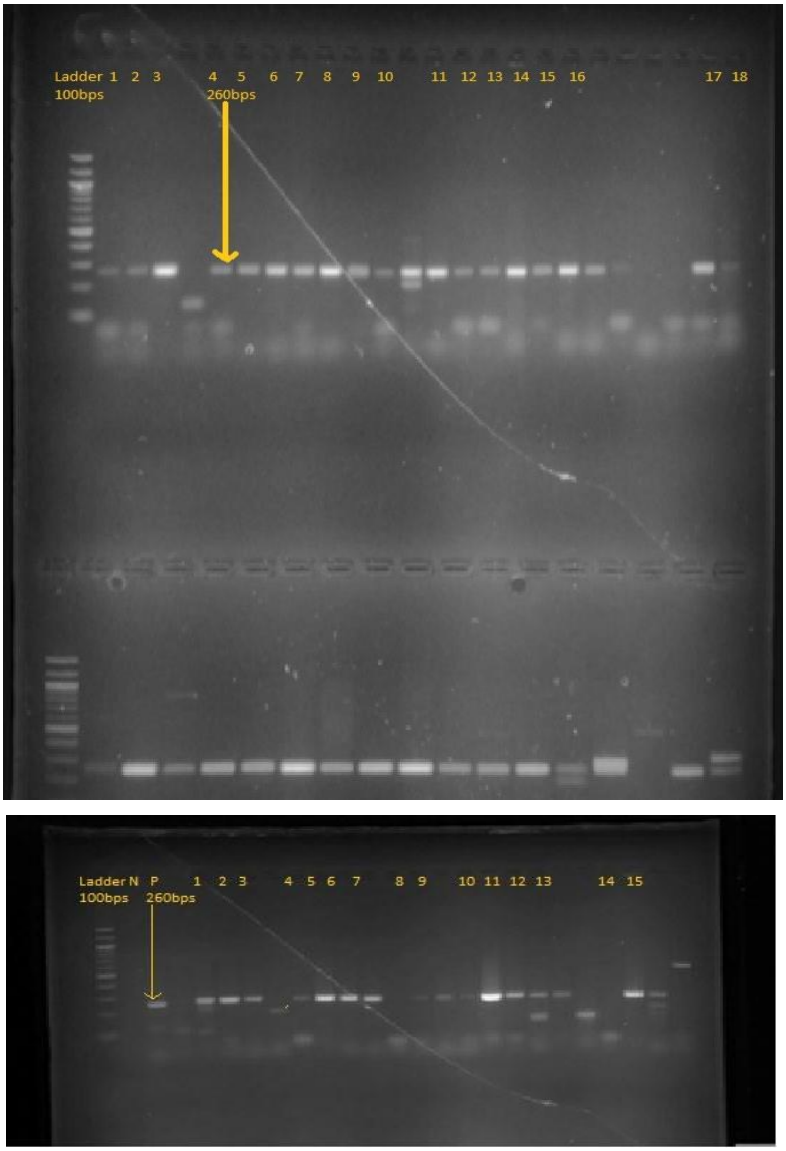

Figure 21: K. pneumoniae PCR result through gel run electrophoresis.

3.2.4 PCR and Agarose Gel Electrophoresis for identification of *Pseudomonas aeruginosa*:

PCR was applied on suspected *Pseudomonas aeruginosa* to get the desired bands. These bands were observed and confirmed under UV light by using agarose gel electrophoresis method. The desired band size for *Pseudomonas aeruginosa* is 956 bps. Among 36 *Pseudomonas aeruginosa***,** 8 positive results were found.

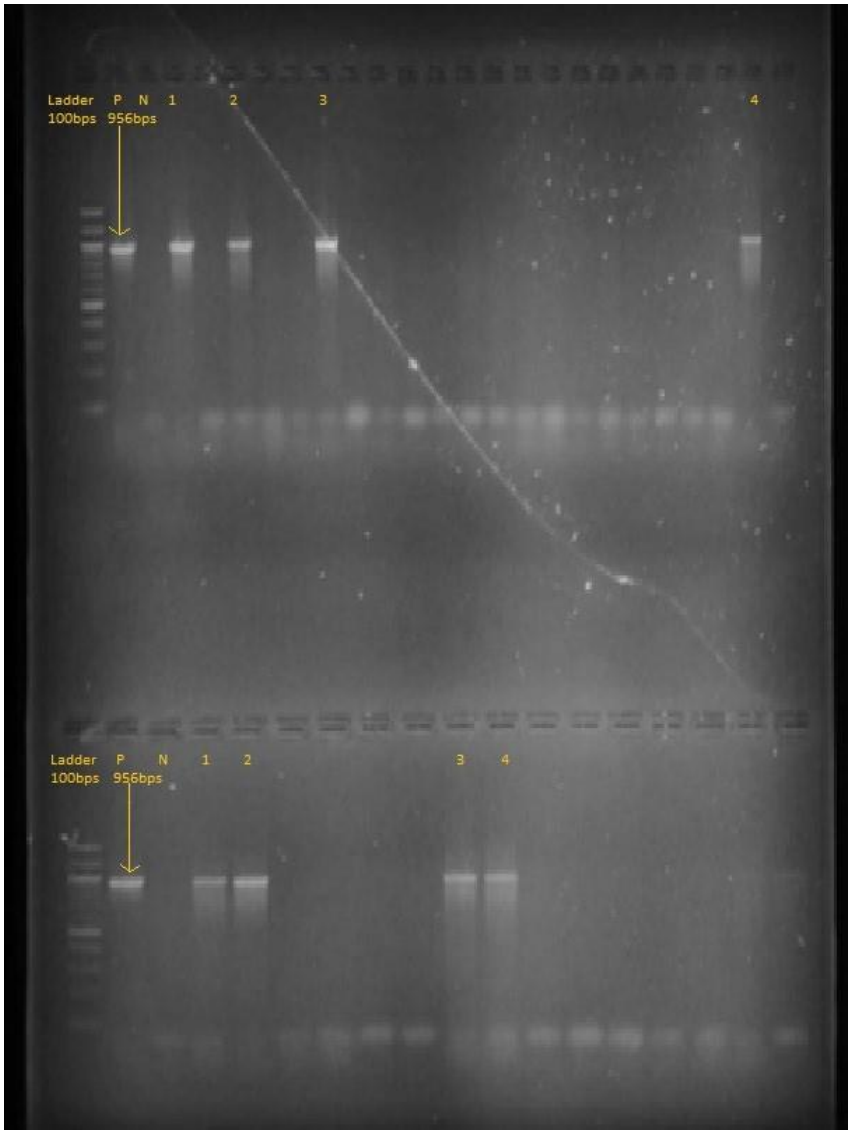

Figure 22: P. aeruginosa PCR result through gel run electrophoresis.

3.2.5 PCR and Agarose Gel Electrophoresis for identification of *Vibrio spp.*:

PCR was applied on suspected *Vibrio spp.* to get the desired bands. These bands were observed and confirmed under UV light by using agarose gel electrophoresis method. The desired band size for *Vibrio spp.* is 689. 5 *Vibrio spp.* positive results were found.

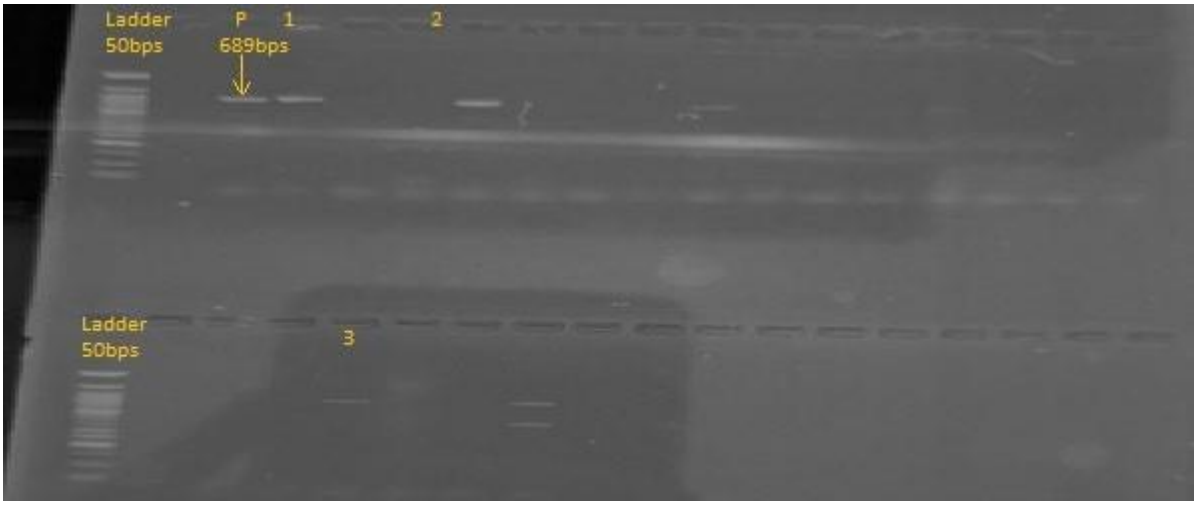

Figure 23: Vibrio spp. PCR result through gel run electrophoresis.

3.2.6 PCR and Agarose Gel Electrophoresis for identification *Acinetobacter baumannii*:

PCR was applied on suspected *Acinetobacter baumannii* to get the desired bands. These bands were observed and confirmed under UV light by using agarose gel electrophoresis method. The desired band size for *Acinetobacter baumannii* is 353bps. Among 35 *Acinetobacter baumannii,* 10 positive results were found.

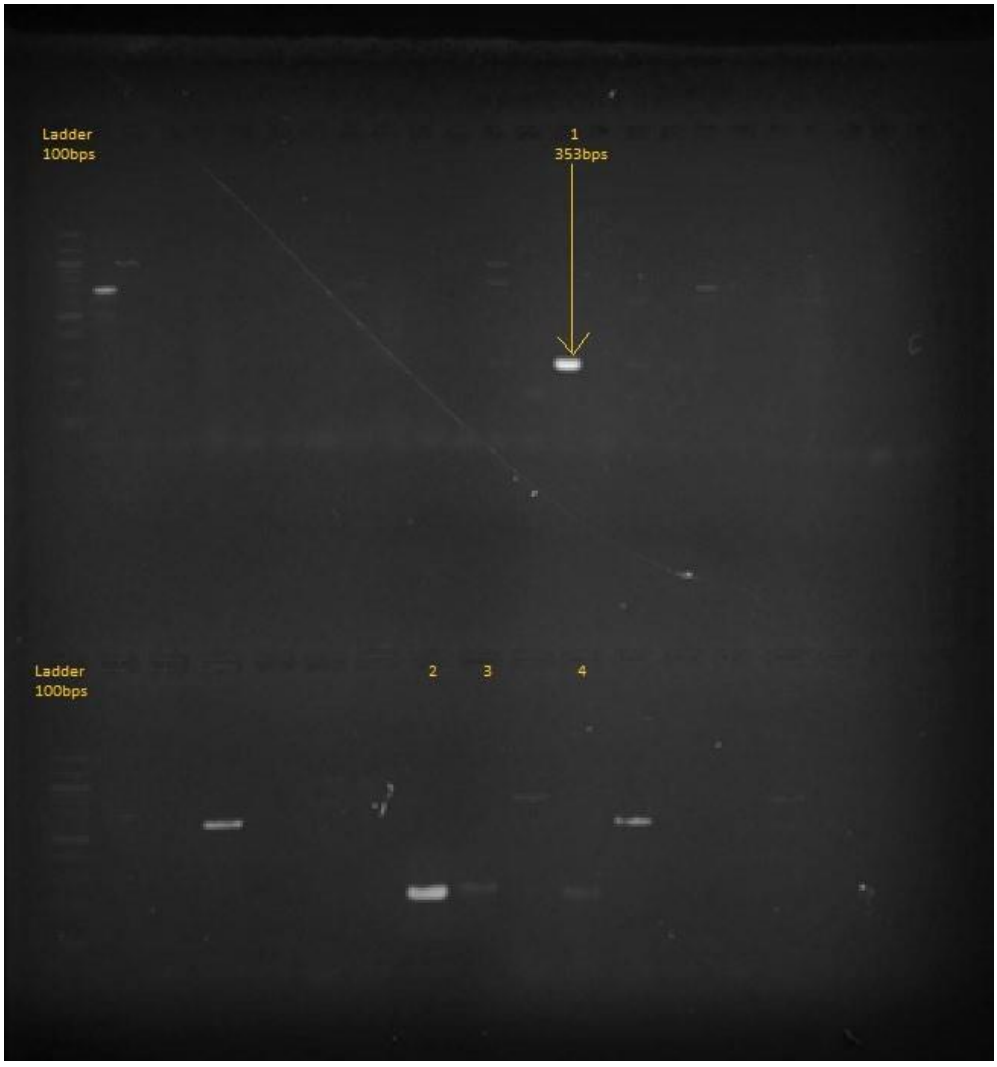

Figure 24: A. baumannii PCR result through gel run electrophoresis.

3.2.7 PCR and Agarose Gel Electrophoresis for identification *Staphylococcus aureus*:

PCR was applied on suspected *Staphylococcus aureus* to get the desired bands. These bands were observed and confirmed under UV light by using agarose gel electrophoresis method. The desired band size for *Staphylococcus aureus* is 279bps. Among 31 *Staphylococcus aureus*, 7 positive results were found.

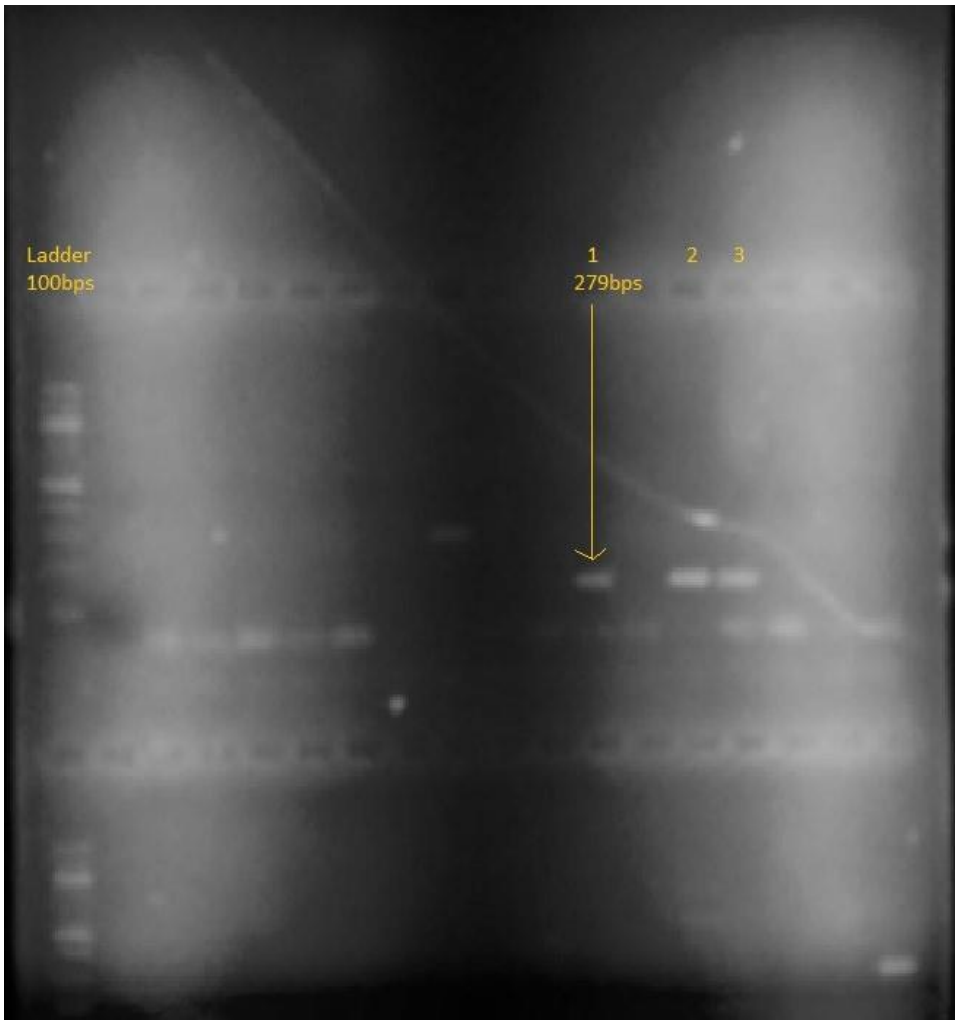

Figure 25: S. aureus PCR result through gel run electrophoresis.

3.2.8 PCR and Agarose Gel Electrophoresis for identification *Escherichia coli:*

PCR was applied on suspected *Escherichia coli to* get the desired bands. These bands were observed and confirmed under UV light by using agarose gel electrophoresis method. The desired band size for *Escherichia coli* is 585 bps. We have done PCR thrice to detect *Escherichia coli* from suspected isolates. Among 86 *Escherichia coli,* 34 positive results.

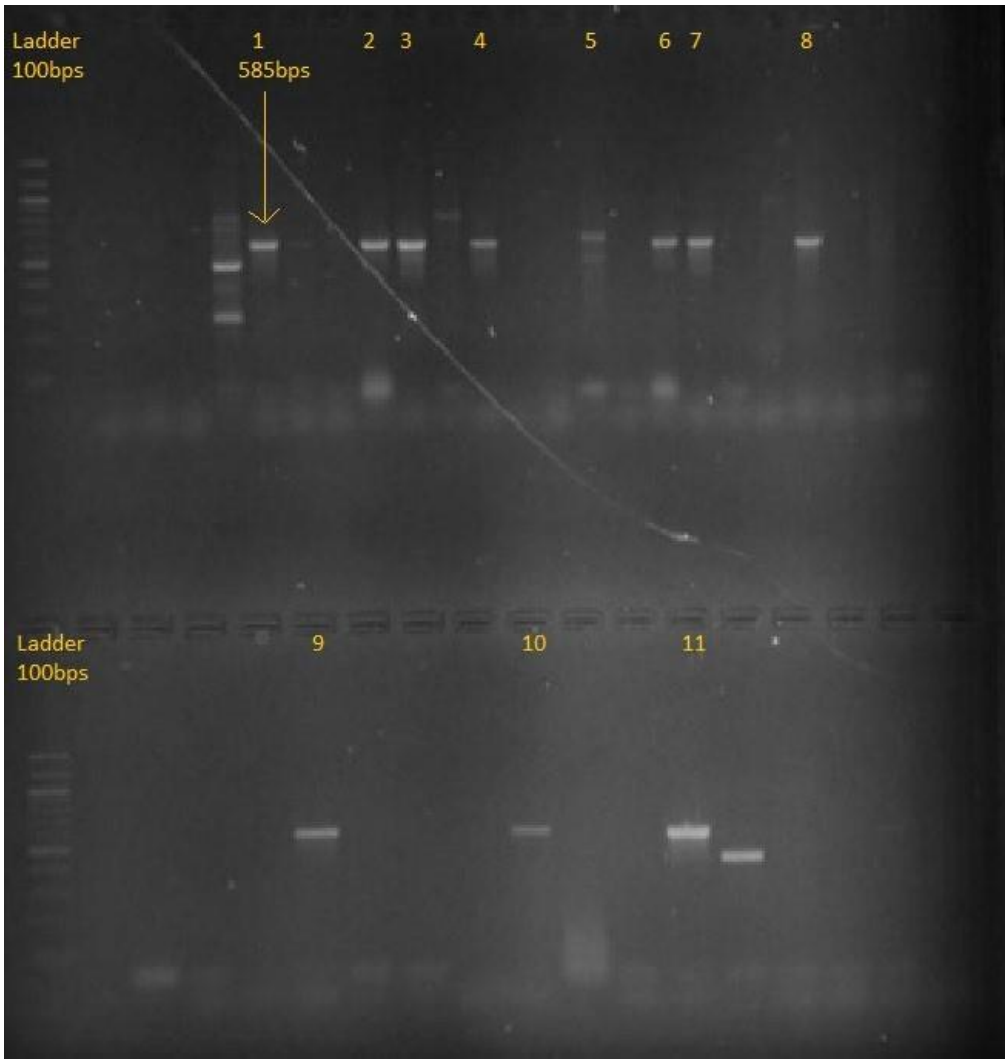

Figure 26.: E. coli PCR result through gel run electrophoresis.

3.3 1. Antibiotic Susceptibility Patterns of isolates:

For the antibiotic susceptibility test, all the 134 positive isolates were taken. Among these 34 *Escherichia coli* positive isolates, 7 *Staphylococcus aureus* positive isolates, 10 *Acinetobacter Baumannii* positive isolates, 5 *vibrio spp.* Positive isolates, 8 *Pseudomonas aeruginosa* positive isolates, 66 *Klebsiella pneumoniae* positives isolates, 3 *Shigella spp.* Positive isolates, 1 *Salmonella spp.* positive isolates were detected. By the disc diffusion method, the susceptibility has been tested by measuring the zone of inhibition and detecting whether it is susceptible or resistant according to CSLI guidelines 2023.

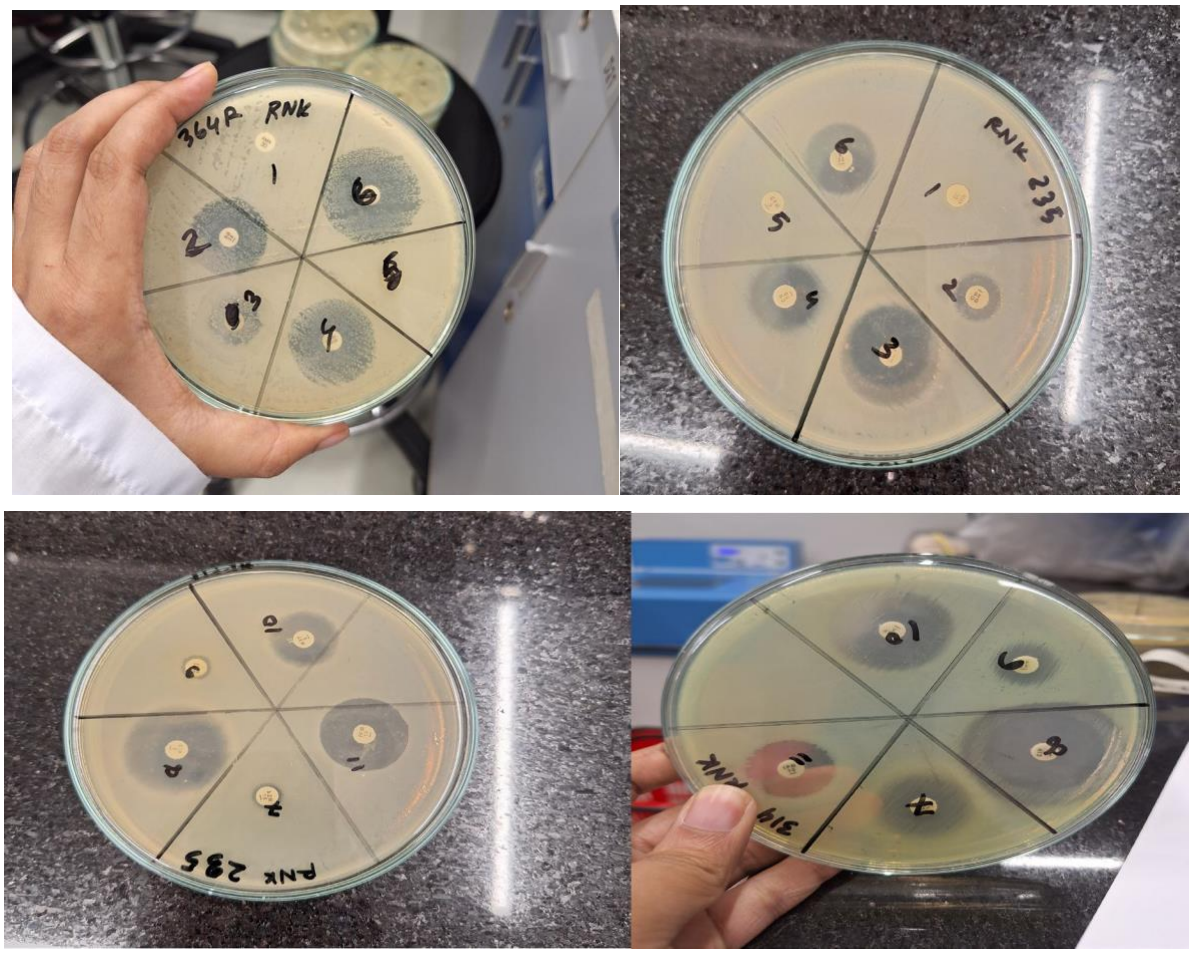

Figure 27: Zone of Inhibition (mm) of PCR confirmed isolates

3.3.1.1 Antibiotic susceptibility pattern of *Salmonella spp.*

One *Salmonella spp.* was confirmed through PCR and it underwent AST which showed resistance against Azithromycin, Tetracycline and Imipenem. It was susceptible against Ciprofloxacin. The resistance pattern of this bacterium:

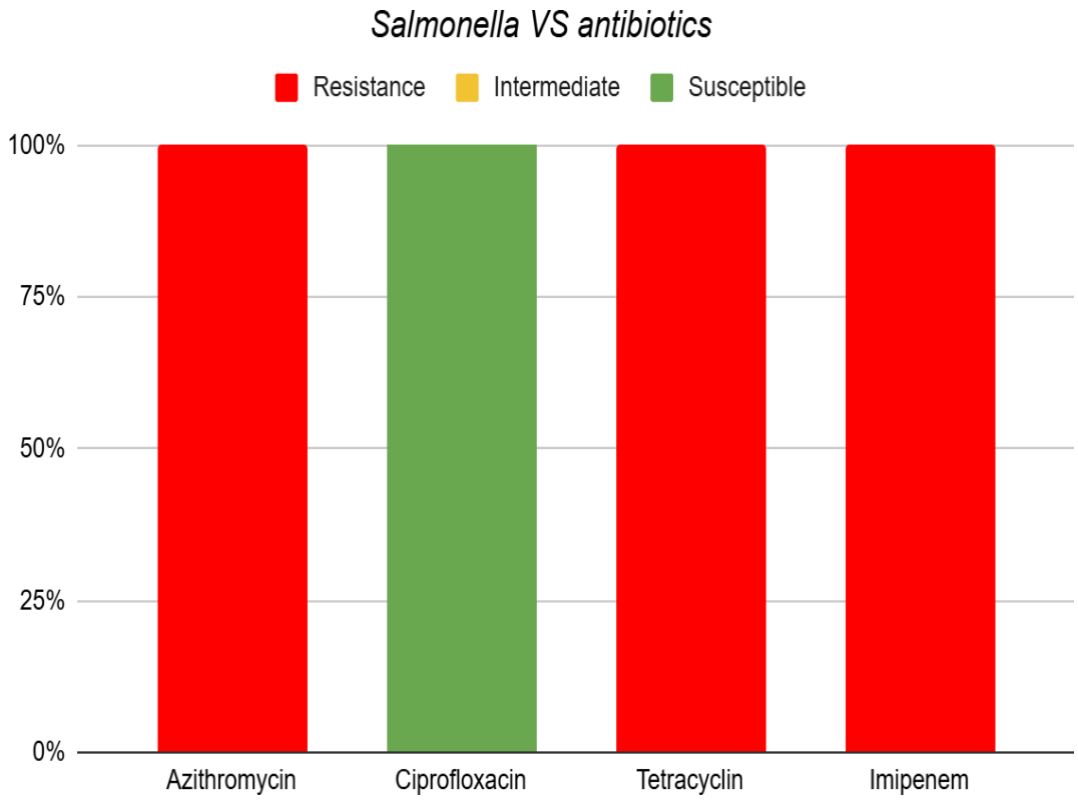

Figure 28: Bar chat of Salmonella spp. AST pattern

3.3.1.2 Antibiotic susceptibility pattern of *Shigella spp.:*

Almost the same results were found for all the antibiotics against *Shigella spp.* Only Imipenem is not resistant against it. The resistance pattern of this bacterium is:

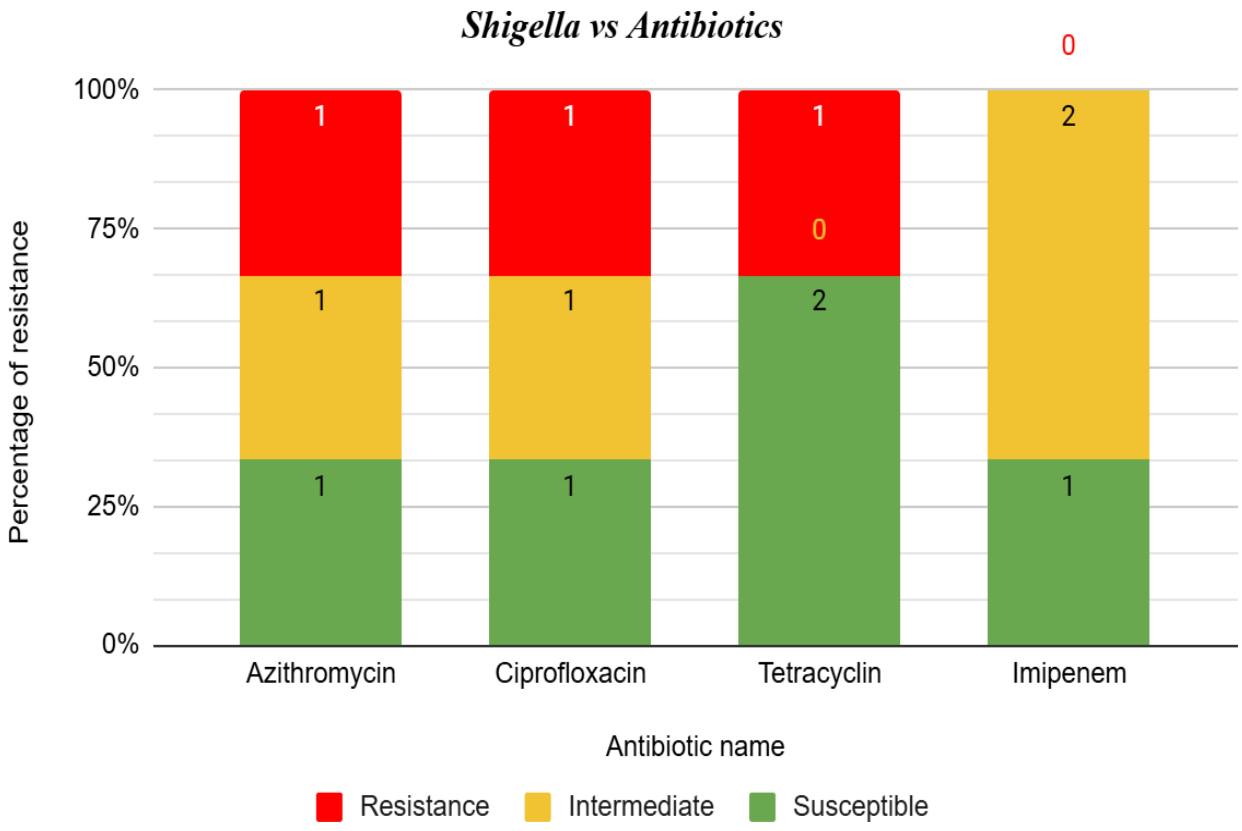

Figure 29: Bar chat of Shigella spp. AST pattern

3.3.1.3 Antibiotic susceptibility pattern of *Klebsiella pneumoniae:*

The minimum number of resistances against are *Klebsiella pneumoniae* Gentamicin, Ciprofloxacin and Imipenem which is 18%. On the other hand, the maximum number of resistances is *Klebsiella pneumoniae* against Cefixime which is 87.88%. The detailed resistance pattern of *Klebsiella pneumoniae* in the graph:

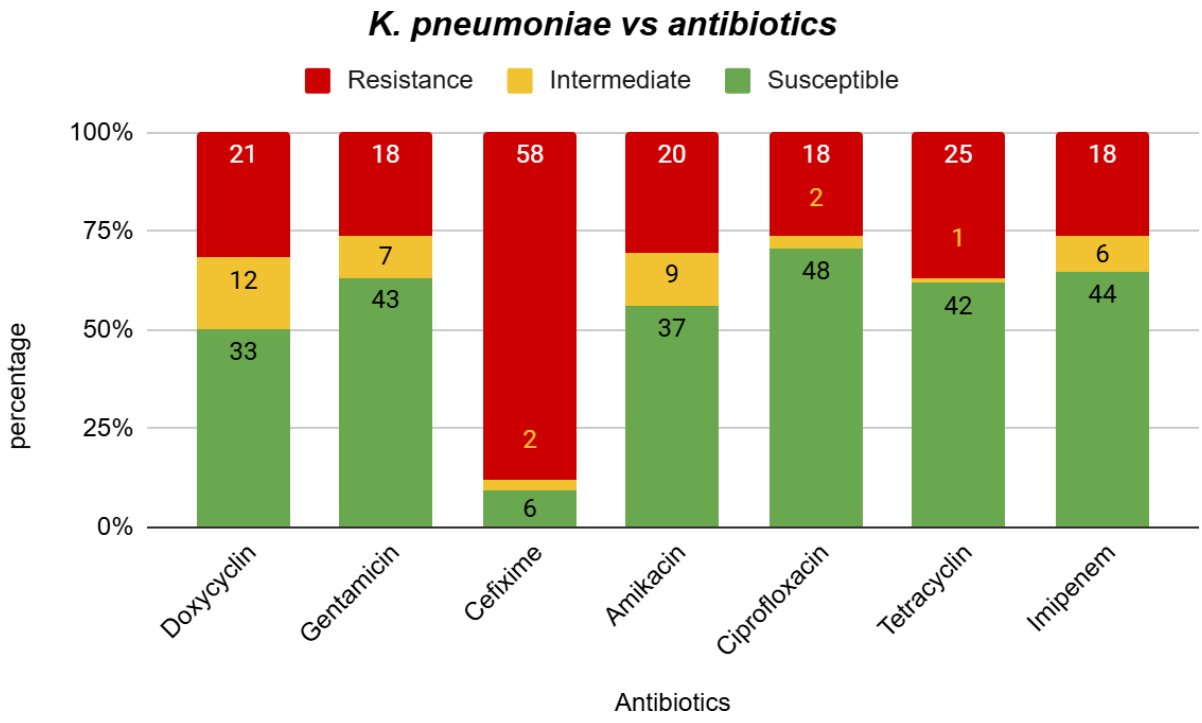

Figure 30: Bar chat of Klebsiella pneumoniae AST pattern

3.3.1.4 Antibiotic susceptibility pattern of *Acinetobacter baumannii:*

The minimum number of resistant against *Acinetobacter baumannii* are Doxycycline and Tetracycline which is 10%. On the other hand, the maximum number of resistant *Acinetobacter baumannii* is against Imipenem which is 40%. The detailed resistance pattern of *Acinetobacter baumannii* is in the graph:

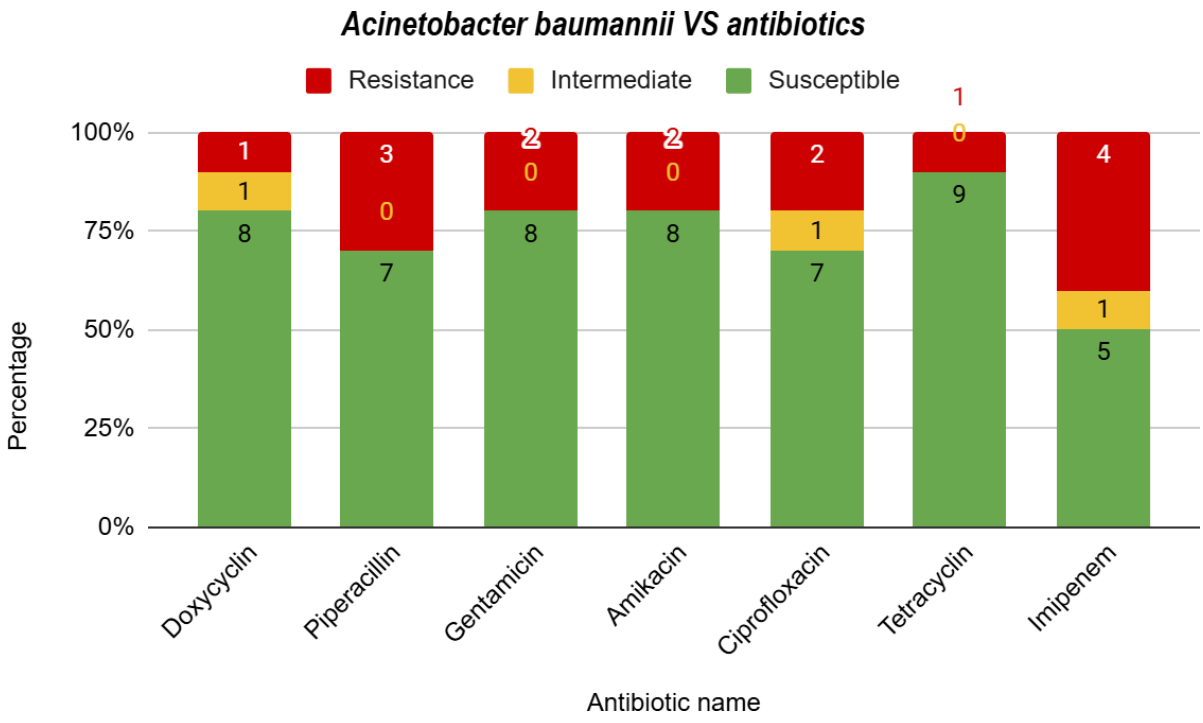

Figure 31: Bar chat of A. baumannii AST pattern

3.3.1.5. Antibiotic susceptibility pattern of *Pseudomonas aeruginosa*

The minimum number of resistant against *Pseudomonas aeruginosa* are Piperacillin and Imipenem which is 37.5%. On the other hand, the maximum number of resistant *Pseudomonas aeruginosa* is against Amikacin which is 87.5%. The detailed resistance pattern of *Pseudomonas aeruginosa* is:

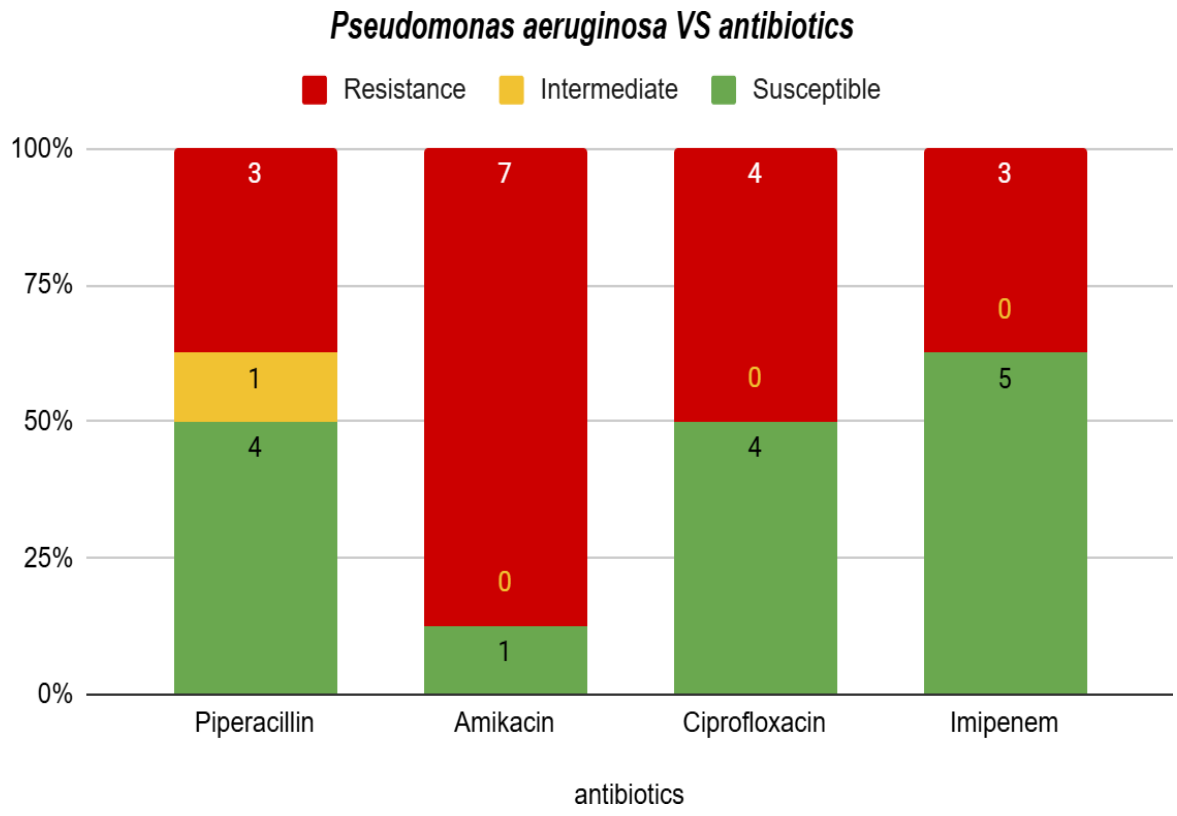

Figure 32: Bar chat of P. aeruginosa of AST pattern

3.3.1.6 Antibiotic susceptibility pattern of *Vibrio spp.*:

Vibrio spp. is mostly resistant to Piperacillin, Gentamicin, Amikacin, Ciprofloxacin and Tetracycline. The detailed resistance pattern of *vibrio spp.* is:

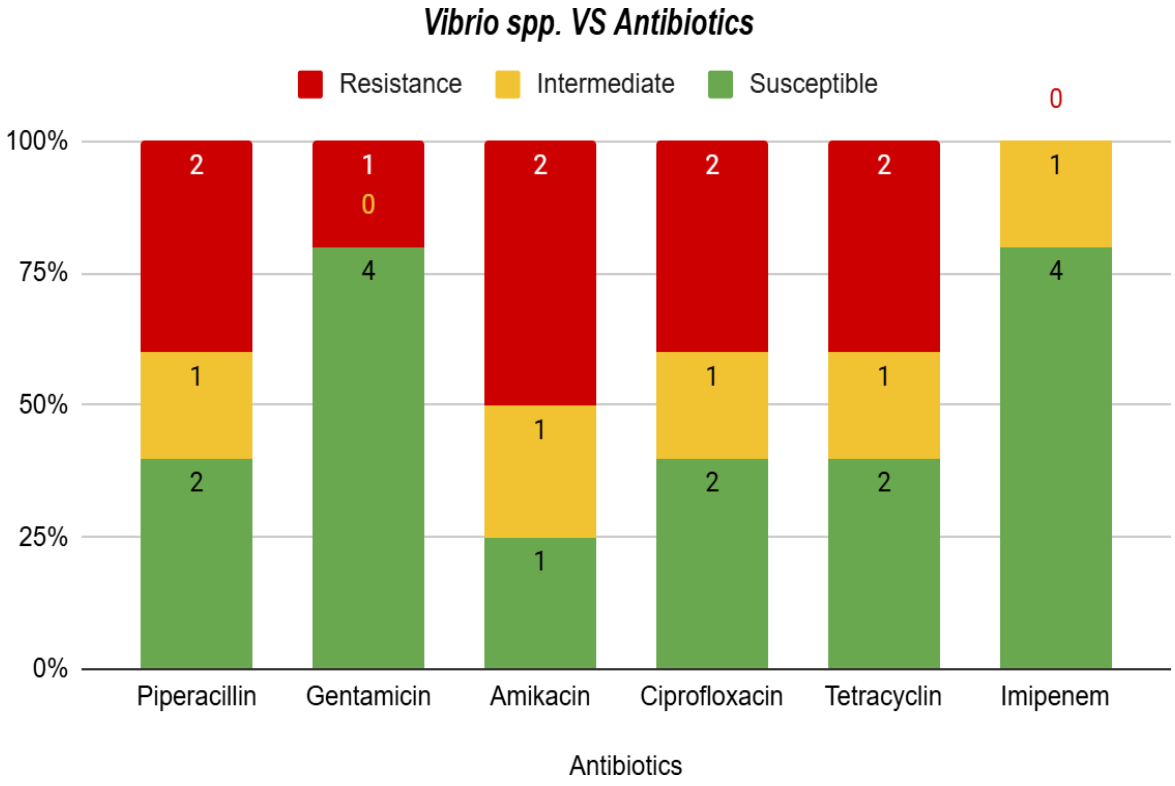

Figure 33: Bar chat of Vibrio spp. AST pattern

3.3.1.7 Antibiotic susceptibility pattern of *Staphylococcus aureus:*

Staphylococcus aureus is not that resistant to any antibiotics. The minimum number of resistant against *Staphylococcus aureus* is Azithromycin which is 14.28%. The detailed resistance pattern of *Staphylococcus aureus* is in the graph:

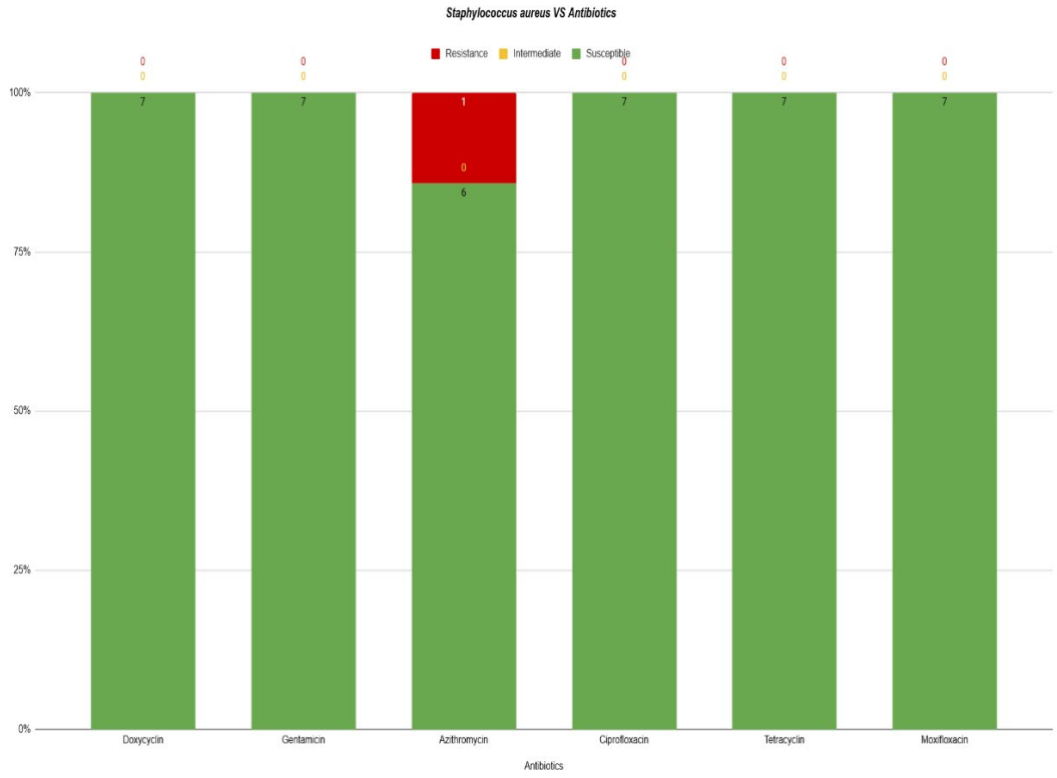

Figure 34: Bar chart of S. aureus AST pattern

3.3.1.8 Antibiotic susceptibility pattern of *Escherichia coli:*

The maximum number of resistant against *Escherichia coli* is Cefixime which is 100%. On the other hand, the minimum number of resistant *Escherichia coli* is against Amikacin which is 41.17%. The detail resistance pattern of *Escherichia coli* is:

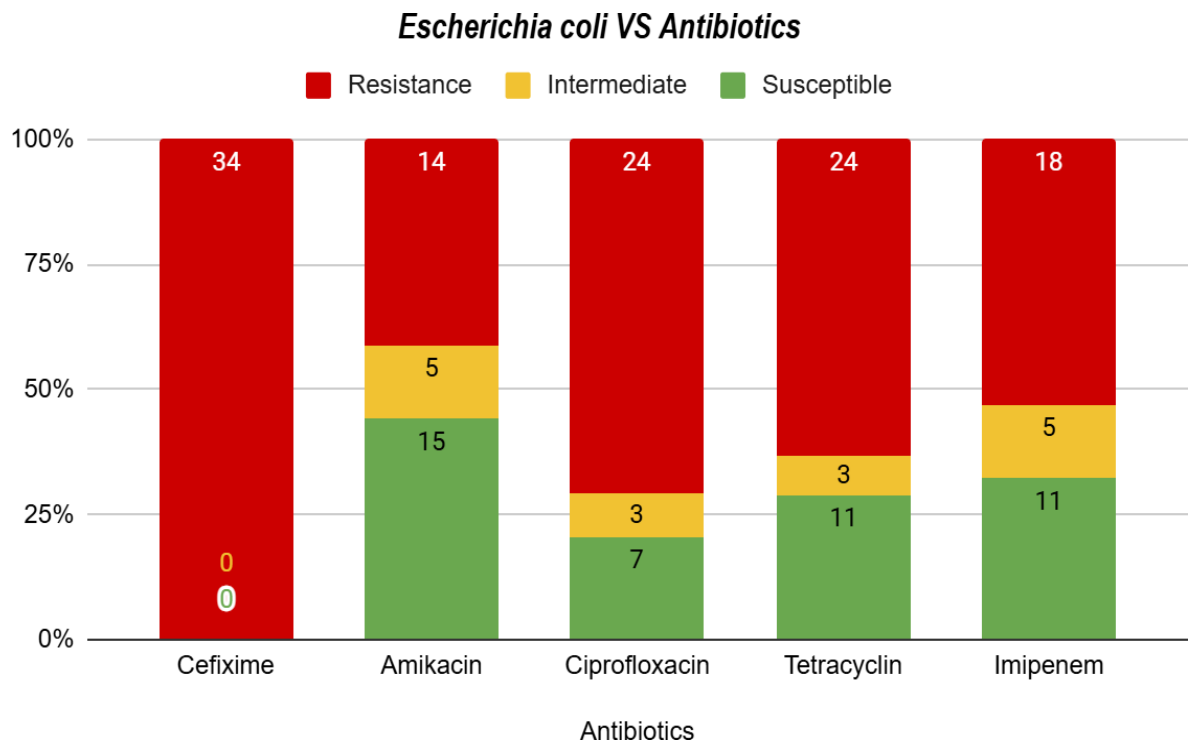

Figure 35: Bar chart of E. coli AST pattern

Chapter 4

Discussion

4.1 Discussion:

This study was an intensive bacteriological study of hospital wastewater from which isolated bacteria were identified and detected the antimicrobial resistance. We all know that in the wastewater of hospitals a significant diversity of microbial population is present. This microbial population includes pathogenic bacteria and opportunistic bacteria which are a real threat to us. The expansion of antimicrobial bacteria into the wastewater which spreads in the environment, is observed by the antimicrobial resistance bacteria's emergence. It shows not only the spread in the environment but also the prescient utilization of antibiotics. Furthermore, antibiotic resistance is one of the worst threats to public health (Endalamaw, 2024). Moreover, an increasing number of resistant bacteria is an outbound concern for the world.

All the microbes that are found, these are mainly constant discharges of different pharmaceutical substances, antibiotics, disinfectants and other things that are mainly used in the healthcare sector. The resistant strains get their potential survival immunity from the selective pressure of these substances. The bacteria we have isolated from hospital wastewater are *Klebsiella pneumonia (K. pneumonia), Escherichia coli (E. coli), Acinetobacter baumannii (A. baumannii), Pseudomonas aeruginosa (P. aeruginosa), Vibrio spp., Shigella spp. and Salmonella spp.* and these are most commonly associated with infections and other diseases.

Klebsiella peumoniae is one of the most common bacteria in hospital wastewater. From the study, we saw that there was a wide amount of *Klebsiella pneumoniae*. Among 66 Klebsiella *pneumoniae*, it showed, 27.27% resistant to Gentamicin, Imipenem and ciprofloxacin, 31.81% against Doxycycline, 37.88% to Tetracycline and 87.88% resistant to Cefixime. The study that

was conducted in India, *Klebsiella pneumoniae* showed 40-45% resistance against Gentamicin, 25-30% against Imipenem and Doxycycline, 50-60% against Tetracycline and 60-70% resistant to Cefixime. In the hospital wastewater of China, *Klebsiella pneumoniae* showed 35-40% resistance against Gentamicin, 25-30% against Imipenem and Doxycycline, 40-50% against Tetracycline and 68% resistant against Cefixime. In the hospitals of South Korea, in the wastewaters where we found the *Klebsiella pneumoniae*, 35-40% resistance against Gentamicin and Ciprofloxacin, 20-25% against Imipenem and Doxycycline, 40-45% against Tetracycline and 50-55% resistant against Cefixime. Again, if we look at the hospital wastewater of Indonesia, *Klebsiella pneumoniae* showed 40-45% resistance to Gentamicin, 20-30% to Imipenem and Doxycycline, 45-50% to Tetracycline and 50-55% resistant to Cefixime.

In case of *Klebsiella pneumoniae*, Gentamicin was found in a lower percentage in this study compared to the resistance percentage shown in India, China, Indonesia and South Korea. Though it is in lower percentage than other countries, it is still a concerning matter as it is a first-line antibiotic that is given against *Klebsiella pneumoniae*. The resistant pattern of Imipenem is almost similar to the other countries like India, China, Indonesia and South Korea. The resistance rate of Doxycycline resistance is higher than the rates that were observed in India, China, Indonesia and South Korea. Though the rate is moderate, still it should be a matter of concern as it is a broad-spectrum given antibiotic. The resistant rate of Tetracycline is lower than India, China, Indonesia and South Korea. The resistance of Cefixime in this study is much higher than the studies that had taken place in Indonesia and South Korea. On the other hand, the studies of India and China are not much different but rates are lower. Though most of the antibiotics are moderately resistant to Klebsiella *pneumoniae*, it is a matter of concern as these are one of the broad-spectrum antibiotics

given against the infections caused by Klebsiella *pneumoniae*. The high rate of Cefixime is an alarming concern for this country as it is lower in rates in other countries.

These *Shigella spp.* isolates are almost similar to the results of study that took place in Ethiopia. From the study, it was found that *Shigella spp.* is 33% resistant to azithromycin, tetracycline and ciprofloxacin. Similarly, in Ethiopia, *Shigella* was found to be resistant to these antibiotics (Endalamaw, 2024). Also, in some hospitals of West Africa, *Shigella spp.* shows resistance to tetracycline (Kotey & Donkor, 2025). But this is not as frequently found in hospital wastewater. In the study it was seen that it was 33% resistant to Azithromycin, Ciprofloxacin and Tetracycline. On the other hand, no resistance shows against Imipenem. In the hospital's wastewater of India, 10-20% resistant to Azithromycin, 40-50% resistant to Ciprofloxacin and Tetracycline, 5-10% to Imipenem. The study was based on the hospital wastewater of China in which *Shigella spp.* showed 15-20% resistance to Azithromycin, 50-60% to Ciprofloxacin and Tetracycline, 5-10% to Imipenem. In the study that was held on Indonesian hospital wastewater, *Shigella spp.* was 5-15% resistant to Azithromycin and Imipenem, 50-60% to Tetracycline and Ciprofloxacin. About South Korea, the resistant patterns were like 5-15% resistant to Azithromycin and Imipenem. On the other hand, it is 30-50% resistant to Ciprofloxacin and Tetracycline.

The resistant pattern in *Shigella spp.* against Azithromycin is almost similar to the pattern of Ethiopia. On the other hand, the resistant rates of *Shigella spp.* are much higher than the resistant rates observed in Indonesia, China, South Korea and India. Again, the resistant pattern of *Shigella spp.* against Ciprofloxacin is similar to Ethiopia. But in the case of Indonesia, China, South Korea and India the resistance rate is a bit high. The same goes for the Tetracycline resistant pattern. This study did not show any resistance against Imipenem as it is given to Multi-Drug Resistant *Shigella*

spp. But in the case of other countries like Indonesia, China, South Korea and India, they showed less resistance rates against Imipenem. The resistant rate of Imipenem is very low and it should remain like this as overuse of this antibiotic can lead to higher resistance.

Again, in *Acinetobacter baumannii*, resistant to tetracycline and imipenem. The same findings were found in the hospitals of Ethiopia and in some European studies, it is found that it is Carbapenem-resistant. In some German hospitals, *Acinetobacter baumannii* is found resistant to imipenem as well. From the study, it has been found that *Acinetobacter baumannii* is 10% resistant to Doxycycline and Tetracycline, 30% resistant to Piperacillin, 20% resistant to Amikacin, Ciprofloxacin and Gentamicin, 40% resistant to Imipenem. The study that took place in India, *Acinetobacter baumannii* showed 40-50% resistance to piperacillin, Ciprofloxacin and Gentamicin, 50-60% to Tetracycline and Doxycycline, 30-40%Amikacin 50-60% to Tetracycline and 25-30% Imipenem. In the, hospital wastewater of China, *Acinetobacter baumannii* was 50-60% resistance to Piperacillin and Gentamicin, 45-55% to Ciprofloxacin and Doxycycline, 30-40% to Amikacin and Imipenem and 60-70% to Tetracycline. In the hospitals of South Korea, the wastewaters where we found the *Acinetobacter baumannii* was 40-55% resistance to Doxycycline, piperacillin and tetracycline, 30-40% Gentamicin and Ciprofloxacin, 25-30% to Amikacin and Imipenem, and 45-55% to Tetracycline. Again, if we look at the hospital wastewater of Indonesia, *Acinetobacter baumannii* was 40-45% resistance to Gentamicin, Doxycycline, Ciprofloxacin and Piperacillin, 50-66% to Tetracycline and 30-40% resistant to Imipenem and Amikacin.

The resistance pattern of Doxycycline against Acinetobacter *baumannii* is lower in this study than the other countries like China, Indonesia, South Korea and India. The resistance rates in China,

Indonesia, South Korea and India are comparatively very high which they should be concerned about. In the case of Tetracycline, the same resistant pattern is shown. The rate of resistance against *Acinetobacter baumannii* of Tetracycline is much lower than China, Indonesia, South Korea and India. Piperacillin also has a lower resistance rate in this study than the resistant rate of China, Indonesia, South Korea and India. Same for Amikacin and Ciprofloxacin, the resistant rates are higher in China, Indonesia, South Korea and India. On the other hand, in the case of Imipenem, the study showed higher resistance than the resistance shown in the hospital wastewater of China, Indonesia, South Korea and India. As we know that Imipenem is given against Multi-Drug Resistant bacteria, we should be concerned about the increasing resistant rate.

In the case of *Salmonella spp.*, our study showed 100% resistance to azithromycin, tetracycline and imipenem. On the other hand, from the studies that have been done in West Africa and Burkina Faso, it showed high resistance to tetracycline but very less to azithromycin and rare to imipenem (Kotey & Donkor, 2025). It was very less frequently found in Asian countries' hospital wastewaters.

The study showed that, *Vibrio spp.* was found to be 40% resistant to Piperacillin, Amikacin, Ciprofloxacin and Tetracycline and 20% to Gentamicin. On the other hand, it did not show any resistance against Imipenem. The study took place in the Hospitals of Delhi and Chennai of India, in the wastewater of those hospitals, *Vibrio spp.* showed 30-40% resistance to Piperacillin and Ciprofloxacin, 20-30% to Gentamicin and Tetracycline, 10-20% to Amikacin and 5-10% to Imipenem. In China, in the hospitals of Beijing wastewater, *Vibrio spp.* showed 25-35% to Ciprofloxacin and Tetracycline, 5-15% to Amikacin and Imipenem, 15-25% to Gentamicin and

30-40% to piperacillin. In case of Indonesian hospital wastewater, *Vibrio* spp. showed 20-35% resistance to Gentamicin and Tetracycline, 5-15% to Amikacin and Imipenem, 20-30% to Tetracycline and 40-50% resistant to Piperacillin. South Korea's wastewater of hospitals, *Vibrio spp.* showed 10-25% resistance to Gentamicin and Tetracycline, 5-10% to Amikacin and Imipenem, 25-35% to Piperacillin and Ciprofloxacin.

The resistance rates in this study of Piperacillin are similar to India and China against the *vibrio spp.* where these rates are slightly higher in Indonesia and South Korea. As Piperacillin is commonly used against *vibrio spp.* infections so it is a matter of concern. The resistance rate against Amikacin is higher in this study than India, China, Indonesia and South Korea. Amikacin is commonly given against Multi-Drug Resistant strains, so the higher rates are a matter of concern. The resistance rate of Ciprofloxacin is similar to India, China, Indonesia and South Korea. The resistant rate of tetracycline is higher than China, India and Indonesia as shown in the studies of those countries' wastewater. On the other hand, South Korea has the lowest resistance rate. The moderate resistance rate of Gentamicin is similar to all other countries. In the case of Imipenem, it didn't show any resistance rate in this study but lower rates in China, India, Indonesia and South Korea. The no resistance rate of Imipenem is a last resort treatment for *vibrio spp.* infection.

Pseudomonas aeruginosa is highly resistant to amikacin we have seen through our study. From the studies that have been done in Europe, the same result has been shown that it is frequently resistant to piperacillin, amikacin, gentamicin and imipenem. In the study, it is shown that *Pseudomonas aeruginosa* is 37.5% resistant to Piperacillin and Imipenem, 40% resistant to Amikacin. On the other hand, 87.5% are resistant to Ciprofloxacin. The study that was conducted in India, showed 20-30% resistance to Imipenem, 35-50% Amikacin and Ciprofloxacin and 50-

60% to Piperacillin. In the hospital wastewater of China, 24-35% resistance to Amikacin, 15-20% to Imipenem, 45-55% to Ciprofloxacin and 50-60% resistant to Piperacillin. In the hospitals of South Korea, in the wastewaters were *Pseudomonas aeruginosa*, 35-45% resistance to Ciprofloxacin, 10-20% to Imipenem, 25-32% to Amikacin, 40-50% to Piperacillin. Again, if we look at the hospital wastewater of Indonesia, 40-50% resistance to Ciprofloxacin and Amikacin, 20-30% to Imipenem and 50% to Piperacillin.

The study has shown moderate rate of resistance against Piperacillin of *Pseudomonas aeruginosa*. But resistance rates are higher in South Korea, China, India and Indonesia. It is found frequently resistant in Europe which is shown in the study as well but at a moderate level. The resistant rate of Amikacin is almost similar to India and Indonesia with the study shown. But the level of resistance in South Korea and China is lower than in our region. In the case of Imipenem, the study showed a lot higher resistance than it is in Asian countries like South Korea, China, India and Indonesia. On the other hand, the resistant rate of Imipenem is frequent in Europe which is similar to our region. According to the studies of Europe, *Pseudomonas aeruginosa* shows more resistance to carbapenem which includes Imipenem. The resistance level against Ciprofloxacin is higher in an intimidating. It has suppressed the global level of resistance against Ciprofloxacin as well.

In the case of *E. coli*, we have seen that it is 100% resistant to cefixime. In the studies that have taken place in Romania and European hospitals, it is highly resistant to Cefixime and moderately resistant to Amikacin as shown in our result as well (Gaşpar, 2021). In the study, it is shown that *E. coli* is 100% resistant against Cefixime, 70.59% against Ciprofloxacin and Tetracycline, 41.17% against Amikacin and 52.49% against Imipenem. This study has also been done in Asian countries. In India, it has taken place in Delhi and Chennai's few hospitals. According to that study, *E. coli* showed 50-60% resistance to tetracycline, 30-35% resistant to Cefixime, 30-40% to ciprofloxacin

and 15-20% to gentamicin. In China, *E. coli* was 40-45% resistant to tetracycline, 60-65% resistant to ampicillin, 30-40% resistant to cefixime. In South Korea, it is 25-30% resistant to Cefixime and 30-40% resistant to ampicillin. In the hospitals of Indonesia, it is 25-30% resistant to cefixime and 50-60% to tetracycline.

As this study has shown higher resistance of *E. coli* against Cefixime, it is also found in Europe and Romania and Amikacin has shown moderate resistance in the study, in Europe and in Romania. On the other hand, the other Asian country showed lower resistance rate of Cefixime. It also showed higher resistance against tetracycline and ciprofloxacin. The rate of resistance against Cefixime is at an alarming level. It is high time to take steps to fix it.

Staphylococcus aureus shows another resistance type. It has shown no resistance against Doxycycline, Gentamicin, Ciprofloxacin, Tetracycline and Moxifloxacin. It showed resistance against Azithromycin (14.28%) but at a lower rate of resistance. In the case of China, *Staphylococcus aureus* showed 5% resistance against Doxycycline, 10% resistance against Gentamicin, Ciprofloxacin and Tetracycline. In Indonesian hospital wastewater, *Staphylococcus aureus* showed 39% against Gentamicin, 14% against Ciprofloxacin and 43% resistance against Tetracycline. In South Korea, *Staphylococcus aureus* showed 35% resistance against Gentamicin, 29% resistance against Azithromycin, 30% resistance against Ciprofloxacin and Moxifloxacin and 42% resistance against Tetracycline.

The study showed the microbial resistance of microbes from the wastewater of four renowned government hospitals of Bangladesh. The antimicrobial resistance shown is at an alarming level. In some cases, the level has exceeded the other countries of Asia and Europe. From the study it is completely visible that it is high to develop the resistance level. By seeing the resistance level of Cefixime in *E. coli*, *Salmonella spp.* and *Klebsiella pneumoniae*, it is clearly in the advanced stage

of resistant development. The resistance level to last-resort antibiotics such as Imipenem is in a perceptible stage. The resistance level of Imipenem in *Acinetobacter baumannii* and *Pseudomonas aeruginosa* is at a warning level as these are multi-drug resistant organisms. The data of this study are also compared to some countries of Asia, Europe and West-Africa, it is clearly visible that the antimicrobial resistance is not just Bangladesh's issue. It is a global issue right now associated with public health. The reason behind this crucial issue is misuse and overuse of antibiotics. If anyone gets infected by these microorganisms and has diseases like Pneumonia, UTIs and bloodstream infections will be harder to treat because of the high resistant pattern of *Pseudomonas aeruginosa, E. coli* and *Klebsiella pneumoniae*. Due to treatment failures the fatality rates can be higher in these microorganisms. If we want to avoid these kinds of situations, we need to make strict rules in antibiotic use and the observation of hospital flows to control it from spreading in the environment.

Chapter 5

Strength and Limitations

5.1 Strength

This study represents a combination of culturing on selective media, polymerase chain reaction which is molecular level identification of organisms along with antimicrobial susceptibility testing. It provides a comprehensive approach to identify and characterize phenotypic observation of the organisms found in hospital wastewater whose drugs are resistant to selected eleven antimicrobial agents that are used frequently in the healthcare sector. The PCR method ensures accurate results of detecting targeted organisms of study which help not to create any misconception which may occur with only culture based technique back then. Antimicrobial resistance pattern shows intrinsic potential public health concerns caused by resistance organisms which are found in the hospital waste water. As samples were collected from the housing area of the four prime hospitals in Dhaka, where patient and lab waste are disposed of, gives relevance to healthcare associated environmental contamination data set. Even though this study has some limitations but growing dataset from this study on the open environmental source, as housing area includes MDR and/or XDR, superbugs comparison in future more precisely along with this study data which is going to be the support of making impactful awareness in Bangladesh regarding AMR issues.

5.2 Limitations

5.2.1. Sample Size

The sample size or numbers of isolates were limited for a few times of sampling time for those selected hospitals and obviously has some specific collection point and it holds that this study may not fully represent the diversity of microbial contamination potentially in other healthcare scopes and regions. Secondly, this study focused on only eight selected groups of bacteria but there are some other harmful pathogens too which are not included here.

5.2.2. Shortage of Resources

DNA extraction and PCR were performed on cultured isolates and from them many of them might be viable but non culturable (VBNC) might not have been detected using the current methodology which is followed for this study. On the other hand, due to shortage of resources, the number of antibiotics included in the AST pattern observation was limited. A broader pattern of using more antibiotics to check through the Kirby Bauer disc diffusion method on Muller Hinton Agar (MHA) media plates could provide a more detailed resistance profile, which may be explored in future studies.

Selected eleven antimicrobial agents were tested against the isolates because some other agents which were supposed to be used were unavailable during the time in the lab. Whereas PCR results show good results for targeted strains detection, results incorporating such techniques in future research would offer deeper insight into resistance levels and their transmission mechanisms.

5.2.3. MIC Test

One of the limitations of this study is that antimicrobial resistance bacteria were investigated by disc diffusion method and unable to perform Minimum Inhibitory Concentration (MIC) for better assessment of microbial susceptibility tests. Even more, biochemical tests could have been performed which is not included in this study as it was not the primary method.

Chapter 6

Conclusion

6.1 Conclusion

The study-research mainly focuses on the bacteriological quality and AMR patterns of the hospital wastewater from four major government hospitals in Dhaka, Bangladesh; where fundamental culturing methods, molecular techniques like PCR and antibiotic susceptibility tests have been followed so far. Consequently, the research successfully identified and characterized the prime fact, multi-drug resistance organisms (MDROs) that includes, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, Staphylococcus aureus, Shigella spp., salmonella spp.,* and *Vibrio spp.* Initially, among 385 isolates, 134 isolates came PCR confirmed isolates that matched the targeted strains of the study along with moderately high resistance ration. One of the most noticeable facts is, 100% resistance to cefixime in *E. coli* and 87.88% resistance to imipenem in *K. pneumonia*. These outcomes match with international literature that shows the increasing public health threat of AMR from hospital housing areas. On the other hand, the resistance level that is found here is higher than some reported similar studies from Asian countries like China, Indonesia, South Korea, Africa and the European continent as well. The discussion mainly represents that in hospitals, wastewater is a habitat containing resistance strains as it has antimicrobial agents, disinfectant, and biological waste. This claims that the housing areas are likely to be a source of environmental AMR species transmission. One of the most important facts of the study targeted wastewater from the housing drainage side of hospital premises. Moreover, this study filled a gap of local research knowledge by following culturing and molecular identification with phenotypic test approaches. The AMR pattern is observed in such a way that the data gathered by constructive experiments is highly relevant to public health and to raise environmental awareness in Bangladesh. It also ensured the necessary steps for balanced antimicrobial agents using policies, wastewater treatment, and introduced more

In future aspect based study to reduce the increasing resistance of bacteria. Additionally, high resistance antibiotics such as imipenem focused the potential failing of treatment in case of clinical infections in future if the rising point of environmental AMR remained unnoticed. So, this whole work underlines the need of continuous monitoring and offers a strong base for future nationwide effort to mitigate the situation.

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