PCR Identification of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* Isolated from Drinking Water Provided in Tea Stalls and Road-side Restaurants of Dhaka North City Corporation (DNCC) and their Antibiotic Resistance Pattern

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A thesis submitted to the Department of Mathematics and Natural Sciences (MNS) in partial fulfillment of the requirements for the degree of
Bachelor of Science in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing 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 material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Approval

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Abstract/ Executive Summary

This study aimed to identify two specific bacterial species, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, in the drinking water available at food stalls and roadside restaurants in Dhaka North City Corporation (DNCC) and analyze their antibiotic resistance patterns. A total of 16 water samples were collected from six different locations, with at least two samples from each location.

The water samples were enriched in LB and spread plated on KPC agar media to screen for suspected *K. pneumoniae* isolates and on Cetrimide agar for *P. aeruginosa*. DNA was extracted from each isolate, and PCR amplification was performed using specific primers for molecular identification. Out of 321 isolates, 180 were confirmed by PCR amplification. Subsequently, the antimicrobial resistance of these isolates was assessed using 12 antibiotics.

Alarmingly, all *90 Pseudomonas aeruginosa* isolates exhibited resistance to Amoxicillin, Cefixime, and Amoxiclav. Significant resistance was also observed for Kanamycin (71.1%) and Tetracyclines (25.5%). Among the *Klebsiella pneumoniae* isolates, all 90 strains were completely resistant to Amoxicillin. Furthermore, these bacteria displayed varying degrees of resistance to Cefixime (82.2%), Colistin (48.8%), Azithromycin (30%), Ciprofloxacin (15.5%), and Ceftriaxone (6.6%).

Keywords: *Pseudomonas aeruginosa*; *Klebsiella pneumoniae*; Antibiotic Resistance;

Drinking water.

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List of Acronyms

NA	Nutrient Agar
MDR	Multidrug Resistant
KPC	*Klebsiella Pneumoniae* Carbapenemase
MAC	MacConkey Agar
MHA	Mueller Hinton Agar
LB	Luria Broth
TE	Tris-EDTA
EtBr	Ethidium Bromide
CLSI	Clinical and Laboratory Standards Institute
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
DNCC	Dhaka North City Corporation

Chapter 1

Introduction and Literature Review

1.1 Introduction

With a staggering population of 23 million (*Dhaka, Bangladesh Metro Area Population 1950-2023*, n.d.), Dhaka is not just the capital of Bangladesh but also the fourth most densely populated place on Earth. It's a city where people strive just to survive on a daily basis and provide for their families. Safe drinking water has been scarce for the mass people who consume food from the tea stalls and road side restaurants. For the people with low income, tea stalls and road side restaurants were always the primary choice for daily meal intake. In the last 10 years, the total number of these food stalls in Bangladesh have doubled with a number of 344,687 (*Tea Stalls, Restaurants Doubled in 10yrs*, n.d.). A recent study conducted in Dhaka city regarding the purified water jars found 5 alarming bacterial species (Chowdhury & Hossain, 2018). Microbial contamination in the commercially supplied water jars were also found, where 11 different bacterial species were isolated in a study based on Chittagong city (Mina et al., 2018). Another study found 7 different bacterial species from the municipal tap water in Dhaka (Islam et al., 2011). As the source of the drinking water at these tea stalls and roadside restaurants ranges from tap water to commercially available water jars, it poses a great health risk for the people who consume this contaminated water.

Resistance against available antimicrobial drugs of these microorganisms is a growing concern for the current world. Resistant bacteria can spread their ability of resistance by transferring their DNA material by a biological method called Horizontal Gene Transfer (HGT). The existence of bacteria that are resistant in drinking water is very alarming. As the bacteria can easily transfer their resistant genes, they eventually end up creating an army of resistant bacteria everywhere.

1.2 *Pseudomonas aeruginosa*

Pseudomonas is a genus of Gram-negative, aerobic–facultatively anaerobic, encapsulated, rod-shaped bacteria which attained its name from Greek origins, where pseudo means “false” and monas means “unit”. German botanist Walter Migula first named this genus of bacteria “*Pseudomonas*” in 1894, though he never clarified the etymology of the term (“Etymologia,” 2012). This genus of bacteria is infectious and is known to cause diseases in plants, animals and humans.

Pseudomonas is known for its clinical significance as an opportunistic pathogen which has the ability to cause a broad spectrum of infections, especially in those with weakened immune systems or underlying medical problems. It is the most typical pathogen to be found in patients who have spent more than a week in the hospital, and it has a notorious reputation for frequently causing a variety of nosocomial infections (Reynolds & Kollef, 2021). Additionally, *Pseudomonas aeruginosa* is a subspecies of this bacteria that can infect the eyes and ears of people whose immune systems are already compromised. This subspecies is a common cause of infection and is growing increasingly resistant to antibiotics. It can cause skin rashes and other irritations if it's in the water source (*Information About Pseudomonas In Drinking Water*, n.d.).

1.2.1 *Pseudomonas aeruginosa* in Drinking Water

In aquatic environments, *P. aeruginosa* is widely distributed and has the ability to multiply even on the surface of organic materials and filters that come into contact with water. It is known to survive even in deionized or distilled water (van der Kooij et al., 1982; Warburton et al., 1994). As a result, it can be found in both oligotrophic or low-nutrient settings as well as high-nutrient ones like sewage and the human body. A very big concern is that it is particularly good at forming biofilms and if it grows in large quantities, it can affect the color, flavor, odor,

and turbidity of drinking water. Once created, biofilms in artificial water systems can be challenging to get rid of since they require the application of a bio-dispersant and/or physical removal before disinfection (*Explore Drinking Water Quality*, n.d.).

Because the presence of this bacteria is so common everywhere, removing *P. aeruginosa* from our food and water is not only impractical, but doing so would also result in "disinfection byproducts" that are more dangerous than the species itself (Hardalo & Edberg, 1997). When the germs are introduced through the main supply or by using incorrect cleaning or installation techniques, they might contaminate water coolers and vending machines. The sporadic or low flow rates of these kinds of devices often allow the bacteria to stick to the inside pipework surfaces, form a biofilm as a defense mechanism, and start to multiply (*Explore Drinking Water Quality*, n.d.).

Because of its exceptional ability to adapt to a wide variety of temperatures, low nutrient requirements, and unique environmental adaptation, *P. aeruginosa* is frequently observed to live for months in damp settings. Since it has a temperature tolerance of 4 to 42°C, it can withstand a wide range of water media, including drinking water, distilled water, running and standing water, pool water, hot baths, etc (Vukić Lušić et al., 2021). *Pseudomonas*, if discovered in a water system, is typically an indicator that the water quality is not what it should be. It tends to attract other bacteria, some of which remain hidden within it (Greaves, n.d.). From a study published in 2014, it was seen that *P. aeruginosa* promotes *E. coli* biofilm formation in a limited nutrient environment (Culotti & Packman, 2014).

1.2.2 *Pseudomonas aeruginosa* and Antibiotic Resistance

Pseudomonas infections are difficult to treat and even fatal. In order to avoid being treated by the antibiotics used to cure the diseases they cause these bacteria are constantly developing

new evasion techniques. Antibiotic resistance develops when bacteria develop resistance to the drugs meant to eradicate them. If these microbes develop resistance to three or more distinct antibiotic classes, they may turn into multidrug-resistant (MDR) organisms.

It has been observed that *Pseudomonas aeruginosa* is resistant to several antibiotics, such as β-lactams, quinolones, and aminoglycosides and has become a "superbug" (Breidenstein et al., 2011; Hancock & Speert, 2000). There are three main types of antibiotic resistance mechanisms in *P. aeruginosa*: intrinsic, acquired and adaptive. Because of its low outer membrane permeability, the development of efflux pumps that remove drugs from the cell, and the synthesis of enzymes that inactivate antibiotics, *P. aeruginosa* exhibits high intrinsic resistance. *P. aeruginosa* acquires its resistance through mutational changes or the horizontal transfer of resistance genes (Breidenstein et al., 2011). Lastly, the development of biofilm in the lungs of infected individuals is a component of adaptive resistance of *P. aeruginosa*; this biofilm functions as a diffusion barrier, preventing antibiotics from reaching the bacterial cells (Drenkard, 2003).

An estimated 32,600 hospitalized patients and 2,700 estimated deaths in the US in 2017 were linked to MDR *P. aeruginosa*, according to a 2019 study by the Centers for Disease Control and Prevention (CDC) (*Multidrug-Resistant Pseudomonas Aeruginosa*, n.d.). In this study, almost all antibiotics, including carbapenems, were found to be ineffective against *P. aeruginosa*, making it a severe threat. More alarmingly, there was evidence of a mobile genetic element producing the carbapenemase enzyme in 2-3% of these carbapenem resistant *P. aeruginosa* strains. The carbapenem antibiotics lose their effectiveness as a result of this enzyme. Furthermore, mobile genetic elements can be transferred between bacteria with ease, which causes resistance to emerge quickly and destroy these essential drugs (*Multidrug-*

Resistant Pseudomonas Aeruginosa, n.d.). A study conducted in Bangladesh on patients from Dhaka Medical College Hospital revealed that *P. aeruginosa* isolated from surgical wound infections was resistant to azithromycin (100%), ceftazidime (86.8%), and ciprofloxacin (75.5%) and when it came to respiratory infection isolates that were 100% resistant, ciprofloxacin, ceftazidime, and amikacin showed zero efficacy (Rashid et al., 2016).

1.2.3 Pathogenicity of *P. aeruginosa*

Pseudomonas aeruginosa is a pathogenic bacterium species that infects and causes disease in both plants and animals. It also causes a number of human illnesses, particularly in people with impaired immune systems, as well as several infections acquired in hospitals (Azam & Khan, 2019). *P. aeruginosa* can adapt to the unfavorable conditions in hosts and spread infection and disease by secreting a variety of virulence factors. Multidrug-resistant (MDR) opportunistic pathogens like *Pseudomonas aeruginosa* can cause short-term or acute infections as well as long-term chronic infections in immunocompromised patients who suffer from co-morbidity and a number of different health conditions such as cystic fibrosis, sepsis, trauma, cancer, burns and ventilator-associated pneumonia (VAP), which also includes COVID-1-related infections (S. Qin et al., 2022). One of the most significant virulence determinants when taking into account the pathogenicity of *P. aeruginosa* infections is the formation of biofilms. These biofilms can lead to persistent infections due to the bacteria's heightened resistance to environmental conditions, disinfectants, radiation treatments, antibiotics, and the immune system (Tuon et al., 2022).

1.3 *Klebsiella pneumoniae*

Klebsiella pneumoniae, is a gram-negative and rod-shaped bacterium. It’s also a non-motile, encapsulated, facultative anaerobe. It’s known as an opportunistic bacterium which doesn’t cause infection under normal conditions (Podschun & Ullmann, 1998). The genus *Klebsiella*

got its name after the German microbiologist Edwin Klebs (1834-1913). *K. pneumoniae* falls under the family of Enterobacteriaceae. They are commonly found in human gut microbiota (Ryan & Ray, 2003). They don't cause any infection while living inside the human intestines (Lin et al., 2012). But if *K. pneumoniae* is found inside the other sensitive parts of the body, it can be destructive and fatal. It can lead to a variety of diseases such as pneumonia, urinary tract infection, blood stream infection, meningitis and more (Paczosa & Mecsas, 2016).

Infections that occur in a hospital setting are called health care associated or hospital acquired infections, commonly known as nosocomial infections. *K. pneumoniae* is a common nosocomial pathogen (Yinnon et al., 1996). Generally, it colonizes human mucosal surfaces. It can also infect the oropharynx and gastrointestinal tract. Once within the human body, it can display elevated levels of pathogenicity and resistance to antibiotics. One of the most frequent causes of hospital-acquired pneumonia in the United States is thought to be *K. pneumoniae* pneumonia. Three percent to eight percent of nosocomial bacterial infections are caused by it (Ashurst & Dawson, 2023).

As this is an opportunistic bacterium, patients with weak immune systems are prone to this infection (Magill et al., 2014). Also, sick or injured individuals who are already undergoing medical procedures or consuming immunosuppressive drugs can get affected. If there is an outbreak of this nosocomial infection in a hospital setting, it can spread rapidly including neonatal wards leaving the new born children at risk. Nosocomial infections like several UTIs, pneumonia, septicemias, and soft tissue infections are related to *K. pneumoniae* (Podschun & Ullmann, 1998). Nowadays, a number of *Klebsiella* species are considered to be highly significant pathogens when studying nosocomial infections.

1.3.1 *Klebsiella pneumoniae* in Drinking Water

Multiple health risks to humans are associated with the presence of *Klebsiella pneumoniae* in drinking water. Though *Klebsiella* species are ubiquitous in nature, safe drinking water should not contain traces of microorganisms like *K. pneumoniae*. Generally, they are not considered as harmful microbes for healthy people ("Microbial Fact Sheets," 2022) but they can cause fatal infections in sick or immunocompromised individuals. Patients undergoing immunosuppressive drugs can get infected by this bacterium that may lead to fatal consequences.

The common source of *K. pneumoniae* is the normal gut flora of warm-blooded animals, including humans (*Klebsiella Pneumoniae in Healthcare Settings | HAI | CDC*, n.d.). So, the presence of this bacteria is also common in human feces. If this bacterium is found in drinking water, there is a chance that the water had fecal contamination. To confirm fecal contamination, indicator bacteria like fecal coliform test is necessary. As *K. pneumoniae* belongs to the fecal coliform group of bacteria, presence of this bacteria indicates potential fecal contamination of the water (Water--Eph-Dw, n.d.). Therefore, it poses a great health risk for the consumers.

1.3.2 *Klebsiella pneumoniae* and Antibiotic Resistance

K. pneumoniae strains often cause multidrug-resistant gram-negative bacterial infections (*Mackie & McCartney Practical Medical Microbiology*, 1996). It's a commonly found pathogen, which causes infections of the digestive tract, surgical wounds acquired in hospitals, and community-onset infections. In a hospital setting, this might result in a nosocomial infection outbreak (Qin J. et al., 2017). Globally, antimicrobial drug-resistant *K. pneumoniae* now makes up 70% of the population, and the percentage of infection-related deaths has increased to 40%–70% (Iredell et al., 2016). In recent times, *K. pneumoniae* that is resistant to multiple drugs and *K. pneumoniae* that is resistant to carbapenem have emerged as major global

public health concerns. The term "Superbug" has been used in a recent study that identified 47 antibiotic resistance genes because of the bacterium's widespread and multidrug resistance (Sleiman et al., 2021). In recent years, it has grown its ability to exhibit drug resistance against antibiotics like carbapenems, cephalosporins and colistin. A recent study based on ICU patients suffering from lung disease found a distressingly higher prevalence of *K. pneumoniae* colistin resistance. The resistance to colistin was only 8% in 2018 which spiked to 72% in the year 2022 (Sharma et al., 2023). Another study in Bangladesh found resistant *K. pneumoniae* from clinical samples which showed resistance against most ciprofloxacin, piperacillin, β-lactam antibiotics, cotrimoxazole, carbapenem, aminoglycosides, and tazobactam (Aminul et al., 2021). Developing nations such as Bangladesh, where food and water is limited, the occurrence of *K. pneumoniae* strains that are resistant to multiple antibiotics is becoming an increasingly worrisome issue for public health (Effah et al., 2020).

1.3.3 Pathogenicity of *K. pneumoniae*

For the immunocompromised individuals, *K. pneumoniae* is a significantly important opportunistic bacteria (Rock et al., 2014). *Klebsiella* species can cause bacteremia, the presence of bacteria inside the bloodstream. These species have been isolated from several unusual infections. For example, septic arthritis, peritonitis, fascial space infections of the head and neck, and endocarditis (Canada, 2011). *Klebsiella* species stick to the upper respiratory tract epithelial cells, endothelial cells or uroepithelial cells and infect them. One of the main culprits in nosocomial and community-acquired pneumonia is *K. pneumoniae*. It can also cause lung abscesses. The upper lobe of the lungs is prone to infection by this bacterium. This pathogen can cause brain abscesses and community-acquired meningitis, which can present with headaches, fever, seizures, and even septic shocks. Urinary tract infection, which can be life-threatening, is frequently caused by *K. pneumoniae* (Everest, 2007). It’s also a significant causative agent for pyrogenic liver abscesses.

1.4 Resistant bacteria and Horizontal Gene Transfer

Genes are transferred from one organism to another through a biological process called Horizontal Gene Transfer (HGT). It's also known as lateral gene transfer where genetic material is exchanged between organisms that are not parents and offspring. The vertical gene transfer involves transfer of the genetic information through reproduction, whereas the HGT occurs between organisms of the same or different species that may not be closely related. Horizontal gene transfer can result in enormous genetic diversity in asexually reproducing organisms.

Horizontal gene transfer takes place by 3 well understood mechanisms, transformation, conjugation and transduction. Transformation involves bacteria taking up genetic material of lysed or degraded bacteria from their environment. These extracellular DNA often carry plasmid DNA or fragmented DNA that may carry the antibiotic resistant genes (Overballe-Petersen et al., 2013). Conjugation refers to the transfer of genetic material directly from cell to cell through physical contact. A structure called pilus connects two bacteria and makes the transfer possible. Finally, the transduction method involves bacteriophage that moves DNA from one bacterium to another.

HGT played a great role behind the evolution of many organisms (Gyles & Boerlin, 2014). It is shifting perspectives on bacterial evolution more significantly while also influencing scientific understanding of higher order evolution (Ochman et al., 2005).

The primary mechanism by which bacteria acquire resistance to antibiotics is horizontal gene transfer (Huddleston, 2014). It helps the bacteria gain resistance and plays an important role in its evolution to tackle the novel antibiotics that are being developed.

Infectious diseases that are caused by bacteria that are resistant to drugs are a crucial threat to public health. Between the same genus or different species, their resistant genes can be

transmitted. Commensal and opportunistic bacteria have been shown to exhibit these traits when they colonize human intestines (Porse et al., 2017). Through transformation, transmission of resistant genes can occur easily. A study found that, under normal conditions, transformation of E. coli cells by plasmid DNA is possible. It means the E. coli living inside the human gut can also take up extracellular DNA that may contain an antimicrobial resistant gene (Sun, 2018). Mild bacteriophage is used as the carrier that transfers the chromosomal and non-chromosomal DNA from one bacterium to another in the transduction method of HGT. Studies suggest that coexistence of phages with resistant genes and bacteria in the same ecological environment is possible. Therefore, on the spread of drug resistant genes, phages may play a crucial role (Voigt et al., 2021).

1.5 Antibiotics and its different classes

Antibiotics are a class of medications used to treat bacterial infections by either killing bacteria or inhibiting their growth. (*Antibiotic Resistance*, n.d.) They are a vital tool in modern medicine, playing a crucial role in treating various bacterial illnesses and preventing the spread of infectious diseases. Antibiotics work by targeting specific bacterial structures or functions, disrupting the bacteria's ability to survive or reproduce (Kohanski et al., 2010).

Antibiotics are categorized into several classes based on their chemical structure, mechanism of action, and the specific types of bacteria they target. In our study, the eight different classes of antibiotics used were Penicillins and Penicillin combination drugs (amoxicillin, amoxiclav), Carbapenems (meropenem), Cephalosporins (cefixime, ceftriaxone, cefepime), Aminoglycosides (kanamycin), Fluoroquinolones (ciprofloxacin), Macrolides (azithromycin), Polypeptides (colistin), Tetracyclines (tetracycline). Here are some major classes of antibiotics that were used in our study:

- Penicillins: The first discovered antibiotics, penicillins, are characterized by the presence of a beta-lactam ring in their structure. This class includes penicillin, amoxicillin, and ampicillin, and they work by interfering with bacterial cell wall synthesis. (Lobanovska & Pilla, 2017)

- Cephalosporins: Similar to penicillins, cephalosporins also contain a beta-lactam ring but have a different structure. They are effective against a broad spectrum of bacteria and are often used as an alternative to penicillins. Cephalosporins include cefixime, cefotaxime, ceftizoxime, ceftriaxone, ceftazidime of third generation and cefepime of fourth generation. (Das et al., 2019)

- Carbapenems: These antibiotics belong to the beta-lactam group and share structural similarities with penicillins and cephalosporins. However, carbapenems are distinct in their resistance to degradation by many bacterial enzymes, making them highly effective against bacteria that produce beta-lactamases, enzymes that often allow resistance to other beta-lactam antibiotics. Commonly used carbapenems include imipenem, meropenem, ertapenem, and doripenem. They are reserved for treating serious infections or situations where other classes of antibiotics may be less effective, such as infections caused by multidrug-resistant bacteria. (Papp-Wallace et al., 2011)

- Polypeptides (Polymyxins): These are a class of antibiotics known for their activity against Gram-negative bacteria, including many multidrug-resistant strains. Colistin and polymyxin B are the two primary members of the polymyxin class. These antibiotics work by disrupting the bacterial cell membrane, leading to increased permeability and eventual cell death. Polymyxins are particularly effective against

bacteria that have developed resistance to other classes of antibiotics and so are among a reserved class of antibiotics. (Trimble et al., 2016)

- Tetracyclines: Tetracyclines, such as doxycycline and minocycline, inhibit bacterial protein synthesis by binding to the bacterial ribosome. They are effective against a wide range of bacteria but should be used cautiously in children and pregnant women due to potential side effects on developing teeth and bones. (Chopra & Roberts, 2001)

1.5.1 Mechanism of Antibiotic Resistance in Bacteria

Antimicrobial drug resistance in bacteria is a complex and ever evolving process. It allows the bacteria to survive and multiply in the presence of antibiotics. These specialized drugs are made in a manner so that they have the power to eradicate or stop bacterial growth. There are several mechanisms through which the bacteria can acquire resistance against antibiotics. They can even develop multiple mechanisms simultaneously to become resistant.

Obtaining resistant genes is most commonly achieved through horizontal gene transfer where a donor bacterium receives an extracellular DNA material that may contain a resistant gene (Von Wintersdorff et al., 2016). In enzymatic inactivation, bacteria chemically modify antibiotics by the production of enzymes (Schaenzer & Wright, 2020). These enzymes have the ability to inactivate antibiotics. For instance, β-lactamase enzymes can modify β-lactam antibiotics like penicillin which makes them ineffective. Certain bacteria have the ability to change the antibiotics' target sites (*Resistance Mechanisms – Antibiotic Resistance – ReAct*, 2021). This technique lets them change the target sites where antibiotics typically bind to bacterial cells. The development of efflux pumps allows the bacteria to release antibiotics from the cell which ultimately reduces the concentration of the drug from the lethal levels (Soto,

2013). Bacteria can modify the permeability of their cell walls or membranes which can reduce the entry of antibiotics into the cells (University of Bristol, 2020). It limits the antibiotic's ability to reach the target. Bacteria have the ability to form biofilms, which are protective matrices of extracellular substances. Reaching to bacterial cells by penetrating the biofilms makes it harder for the antibiotics (*The Role of Bacterial Biofilms in Antimicrobial Resistance | ASM.org*, n.d.). Random mutations can also make the bacteria resistant to specific antibiotics. Eventually, these resistant genes get survival advantages and proliferate in nature. Even in some cases, bacteria can form a symbiotic relationship with other bacteria that will produce antibiotic degrading enzymes and save each other from destruction (*How Do Germs Become Resistant?*, 2022).

1.5.2 Multiple Drug Resistance (MDR)

Multiple drug resistance or Multidrug Resistance (MDR) refers to the ability of microorganisms, such as bacteria, viruses, or cancer cells, to withstand the effects of multiple drugs designed to inhibit or kill them. A type of microorganism is classified as MDR when it exhibits resistance to at least one antimicrobial drug across three or more antimicrobial categories (Magiorakos et al., 2012). This phenomenon poses a significant challenge in the fields of medicine and public health, as it complicates the treatment of various infectious diseases and cancers. MDR can arise through various mechanisms, including genetic mutations that allow resistance to multiple drugs, as well as the exchange of resistance genes through horizontal gene transfer (HGT) between different microorganisms (Munita & Arias, 2016). The overuse and misuse of antibiotics in healthcare and agriculture can contribute to the development of multidrug-resistant strains which highlights the importance of responsible drug use and the development of new therapeutic strategies.

1.6 Contaminated Drinking Water

Drinking water, also known as potable water, is obtained from subsurface and surface sources and treated to meet federal and state regulations for human consumption. But unfortunately, different chemicals, bacteria, and radionuclides can contaminate surface waters as well as aquifers. These pathogens and contaminants can contaminate drinking water at the source or in the distribution system following treatment (*Water Contamination and Diseases | Drinking Water | Healthy Water | CDC*, 2022). In Bangladesh, arsenic is one of the main causes of drinking water contamination. If there is more arsenic present than the recommended 50 µg/L, it is deemed unsafe to consume (*Arsenic*, n.d.). Another major concern for public health is microbiological contamination of drinking water. Presence of coliforms or fecal coliforms indicates fecal contamination of the water that may lead to waterborne diseases like gastrointestinal illness, diarrhea, vomiting (Cabral, 2010; Gruber et al., 2014).

1.6.1 Consequences of Ingesting Contaminated Drinking Water

Drinking water contaminated to unsafe levels can have adverse effects on health, including reproductive and nervous system disorders as well as gastrointestinal disorders and even chronic diseases such as cancer (US EPA, 2017). Microbial contamination resulting from feces contamination poses the greatest risk to the safety of drinking water. As per the World Health Organization (WHO), in 2022, a minimum of 1.7 billion people worldwide drink from water sources contaminated by human waste or feces. Drinking water that has been exposed to microbial contamination can be responsible for critical diseases like diarrhea, cholera, typhoid, and dysentery. Each year approximately 505,000 deaths occur due to ingestion of microbially contaminated water (*Drinking-Water*, n.d.).

1.6.2 Condition of Drinking Water in Bangladesh

Bangladesh is among the world's most densely populated nations. Ensuring safe drinking water has been a significant challenge due to several contaminations like arsenic, microorganisms, etc. Drinking water contaminated with microorganisms pose a significant health risk to mass people. A study in 2008 found several harmful bacteria such as E. coli, Salmonella, coliforms, fecal coliforms from drinking water in Dhaka (Parveen et al., 2008). Another study found 5 critical bacteria from the commercially available water jars at the tea stalls and roadside restaurants in Dhaka city (Chowdhury & Hossain, 2018).

Presence of harmful bacteria in drinking water higher than the accepted level poses a great health risk for the consumers. The risk factor increases even more as most of the bacteria have grown resistance against several antibiotics that are available. Presence of bacteria resistant to antibiotics in drinking water indicates that these pathogenic microbes are spreading everywhere. An investigation in 2016 on the bacteriological quality of drinking water in Bangladesh found coliforms and fecal coliforms from water sources such as tube well, deep well, surface, etc. Among those, 80% isolates showed resistance against amoxicillin (10µg) (Parvez et al., 2016).

1.6.3 Methods of Contamination of Drinking Water

The main sources of fecal contamination in water are wildlife, non-collective sewage systems, combined sewage overflows (CSO), sewage treatment plants, and domesticated animals (manure spreading, pit stock overflow). The discharges from decontamination centers, hospitals, and other commercial establishments are among the point sources of microbial contamination of natural aquatic resources (Jung et al., 2014). Pathogen concentrations, persistence in bodies of water and biological reservoirs (i.e., sediments, aquatic plants) and pathogen transportability are only a few of the variables that affect the prevalence and

significance of pathogens in water (Pandey et al., 2014). The water can be contaminated by germs or chemicals at the source right after the treatment. Several sources are available from where the germs or the chemicals can enter into the water. For example, arsenic can contaminate groundwater. Toilet near deep well or tube well can transmit fecal contamination of the water. Storm water can also increase the load of the pollutants. If the water treatment facility does not maintain the guidelines of proper treatment, the commercially available packaged water can become unsafe for the consumers (Hasan et al., 2019; Müller et al., 2020).

1.7 Objectives of this Study

Some of the key goals and objectives of this study are summarized below.

1. Evaluate the microbiological drinking water quality in Dhaka's tea stalls and road-side restaurants
2. To get an overview of the antibiotic resistance pattern of the isolated strains in the water samples
3. Assess the potential public health risk posed by the presence of these bacteria and their antibiotic resistance in drinking water
4. Evaluate the risks posed by these multidrug-resistant bacterial strains in the environment by spreading their pathogenicity through Horizontal Gene Transfer (HGT)
5. To raise awareness among the public, tea stall owners, and roadside restaurant operators about the importance of water quality and hygiene
6. To provide information that can inform policy decisions related to food and water safety regulations in DNCC.

Chapter 2

Materials and Methods

2.1 Site of Sample Collection

From Dhaka North City Corporation, six distinct locations were selected for the collection of the drinking water samples. From each location, 2-4 tea stalls or roadside restaurants were picked randomly to collect the drinking water sample. The locations are- Mohakhali, Vatara, Uttara, Khilkhet, Mirpur 10, and Mohammadpur. These locations represent the overall bacteriological condition of drinking water in tea- stalls and road-side restaurants of DNCC.

Figure 1: Sample Collection Sites in Dhaka North City Corporation (DNCC)

2.2 Transportation of the samples

15ml falcon tubes were used to collect the drinking water samples. The falcon tubes were sterilized in the lab using the autoclave machine before collecting the water sample. After the collection, the falcon tubes were put inside zip-lock bags and brought to the lab in under 2 hours. Sterile gloves were used during the collection period to reduce the contamination of the samples.

2.3 Sample processing in the Lab

The sample water was enriched in LB broth for 18-24 hours. The enriched culture media was then diluted with saline water up to several dilution factors depending on their rate of growth in each selective and differential media. For *K. pneumoniae* the enriched culture solution was diluted up to a concentration of 10^{-4} and 10^{-5}. For *P. aeruginosa*, the concentration was 10^{-2} and 10^{-3}. The diluted culture solution was then spread-plated on KPC media for *K. pneumoniae* and Cetrimide for *P. aeruginosa* isolation. After the incubation period, the bacterial colonies were selected based on their morphology.

2.4 Flow chart of Sample Processing

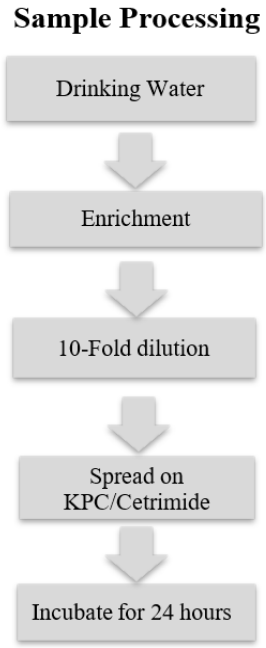

Figure 2: Flowchart of the sample processing

2.5 Screening of suspected *P. aeruginosa*

After the sample processing in the lab, the culture plates were left at 37°C for 24 hours for the incubation. The plates were examined for the suspected *P. aeruginosa* colonies. The presumptive positive colonies may appear as pigmented blue, blue-green, or non-pigmented. They will exhibit fluorescence at 250 nm (Ultraviolet ray) and a blue-green pigmentation. The following morphological characteristics were followed for the screening of *P. aeruginosa.*

2.6 Screening of suspected *K. pneumoniae*

After 24 hours of incubation at 37°C, the colonies gave metallic blue pigmentation in KPC media. The suspected *K. pneumoniae* colonies were picked from the culture plate maintaining those criteria. For further confirmation, these colonies were streaked on MacConkey Agar plates where *K. pneumoniae* colonies appear as large, mucoid with pink pigmentation.

2.7 Sample enrichment

To increase the bacterial load, the sample is added to an enrichment media which helps to grow the bacteria in number. LB broth was used in this case to increase the number of bacteria present in the drinking water sample. 2 ml of raw water sample was mixed with 10 ml of LB broth in a 15ml falcon tube and placed inside the shaker incubator at 37℃ for 24 hours.

2.8 Serial dilution

Serial dilution is a laboratory technique where the concentration of a rich culture is decreased in a progressive stepwise manner. It is used to lower the concentration of the culture solution. In our work, the 10-fold serial dilution method was used where each step in the dilution series results in a tenfold reduction in concentration. It means the original solution is getting diluted by a factor of 10 at each step. 9 ml of saline water was used to dilute 1 ml of our enriched water sample. The water samples were diluted from 10^{-1} to 10^{-5} for spread plating.

2.9 Spread plate method

The Spread plate method is a microbiological technique to quantify the number of viable microorganisms from a liquid sample. The sample is spread onto a solid agar medium for microbial growth. After serial dilution, 100µl of 10^{-2} and 10^{-3} dilution of enriched water samples were spread on large Cetrimide agar plates. 100µl of 10^{-4} and 10^{-5} dilution of enriched water samples were spread on large MAC and KPC agar plates.

2.10 Streak plate method

The Streak plate method is a microbiological technique to obtain pure colonies of bacteria from a mixed culture containing multiple types of bacteria. After the microbial growth in the spread plate method, suspected colonies were picked with a sterile loop and streaked onto the surface of the different agar medium following the rules of streak plate method. To avoid

contamination, the single colonies that gave fluorescence under UV light in Cetrimide agar plates were picked with a sterile loop and again streaked on Cetrimide plates to obtain pure colonies of suspected *P. aeruginosa*. Afterwards, the pure single colonies were transferred to Nutrient agar plates by streak plate method for further procedures. Like that, metallic blue colonies from KPC media were also transferred via streak plate method.

2.11 MacConkey Agar

MacConkey agar is a selective and differential culture medium that is used to isolate and distinguish between several types of gram-negative bacteria, especially Enterobacteriaceae. It's a selective media as it inhibits the growth of gram-positive bacteria. It's also a differential media as it can differentiate among several Enterobacteriaceae. *K. pneumoniae* colonies give large, mucoid, pink colonies in this media.

2.12 KPC Agar

In clinical microbiology laboratories, KPC media, also known as *Klebsiella pneumoniae* carbapenemase media, is a specialized culture medium used to identify carbapenem-resistant Enterobacteriaceae, particularly those containing the *Klebsiella pneumoniae* carbapenemase (KPC) gene. KPC is a selective and differential chromogenic culture medium. *K. pneumoniae* appear as metallic blue colonies.

2.13 Cetrimide Agar

Cetrimide agar is a selective and differential growth medium that is frequently used in microbiology laboratories for the isolation and identification of *Pseudomonas aeruginosa*. The majority of bacteria are inhibited by cetrimide, with the exception of *Pseudomonas* species. Additionally, the medium includes a number of nutrients and markers that help distinguish *P. aeruginosa* from other *Pseudomonas* species. Pyocyanin, a bluish-green pigment made by *P.*

aeruginosa, gives its colonies on cetrimide agar its distinctive color. The colonies that exhibit fluorescence at UV light and blue-green pigmentation are considered presumptive positive for *P. aeruginosa*.

2.14 Müller Hinton Agar

Müller-Hinton Agar (MHA) is a growth medium which is mainly used for antimicrobial susceptibility testing. This media was first developed by John Howard Mueller and Jane Hinton in 1941. Kirby-Bauer Disc diffusion method is a popular and simple method of performing antibiotic sensitivity testing where this media is primarily used. Antibiotic discs are placed on to the bacteria inoculated agar plates to analyze the zone diameter according to CLSI guidelines.

2.15 Luria-Bertani Medium

Luria-Bertani medium is a popular nutrient broth to grow and multiply a wide range of bacteria. A large variety of bacterial strains can grow in the adaptable and nutrient-rich LB medium. It is especially helpful for bacterial proliferation in scientific settings, such as molecular biology experiments.

2.16 Nutrient Agar

Nutrient agar is a non-selective, general purpose solid agar medium which facilitates the growth of a wide range of bacteria. It's easy to use and supports the growth of a variety of microorganisms.

2.17 Tryptone Salt Agar (T_1N_1)

Tryptone Salt agar commonly known as T_1N_1 medium is a nutrient medium to grow bacteria. This medium was used to make our bacterial stocks in the vials. Each isolate was stabbed in the T_1N_1 medium followed by sealing with sterilized paraffin oil.

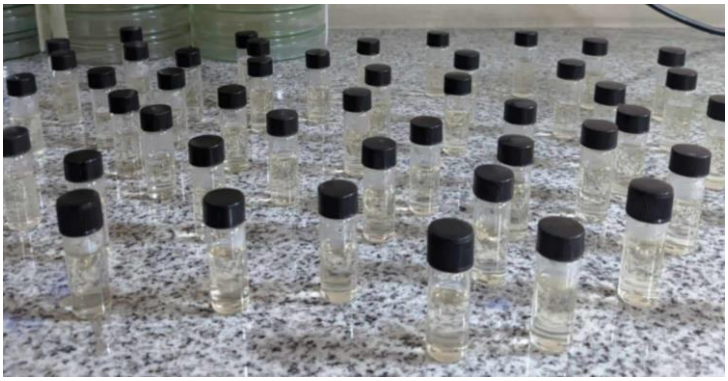

Figure 3: Stock preservation in T_1N_1

2.18 DNA Extraction

DNA extraction is a fundamental laboratory technique which is used to extract the DNA (deoxyribonucleic acid) from biological samples like cells, tissues, or microorganisms. The colony extraction method was used for DNA extraction. 150µl of TE buffer solution was added to a microcentrifuge tube (MCT). Then one loopful of fresh bacterial culture was added to that solution and mixed well. The MCT was placed in a heat block and boiled at 95°C for 15 minutes. Then it was centrifuged at 4°C at 8000 RPM for 10 minutes. 120µl of supernatant which contains DNA was transferred to another fresh MCT and was stored at -20°C after wrapping with parafilm. All the materials used were sterilized beforehand to avoid contamination.

2.19 PCR Amplification & Nucleotide sequence

Polymerase chain reaction (PCR) amplification is a popular molecular biology technique which is used to rapidly amplify DNA fragments and generate multiple copies of a specific DNA fragment. This powerful tool allows millions to billions of copies of a target region in the DNA which lets scientists run accurate tests and conduct genetic research. Template DNA has to be isolated from the target organism.

After the DNA extraction from the suspected *K. pneumoniae* and *P. aeruginosa*, PCR amplification was performed to further identify these bacteria. KPN primer was used to identify *K. pneumoniae*. PA-GS primer was used to confirm *Pseudomonas* genus and PA-SS primer was used to identify the species of *P. aeruginosa*.

Table 1: Selected primers for PCR amplification

Primer Name	**Nucleotide sequence**	**Target Organism**	**Temperature ℃**	**Product Size**	**Reference**
KPN	F-5'-TGCAGATAATTCACGCCCAG-3' R-5'-ACCCGCTGGACGCCAT-3'	*K. pneumoniae*	60	133bp	(Dong et al., 2015)
PA-GS	F-5-GACGGGTGAGTAATGCCTA-3' R-5'-CACTGGTGTTCCTTCCTATA-3'	*Pseudomonas* Species	54	618bp	(Spilker et al., 2004)
PA-SS	F-5'GGGGGATCTTCGGACCTCA-3' R-5-TCCTTAGAGTGCCCACCCG-3'	*Pseudomonas aeruginosa*	58	956bp	(Spilker et al., 2004)

For all the PCR reactions, the final volume was 13µl which contained 7.5µl of master mix, 2.5µl of nuclease free water, 0.5µl of forward primer, 0.5µl of reverse primer and finally 2µl of DNA template. Based on the primer, the following 3 different conditions were used to perform the PCR reaction.

Table 2: PCR Thermal Cycle Parameters: (KPN)

Step	**Temperature, ℃**	**Time**	
Initial Denaturation	94℃	10 minutes	1 X cycle
Denaturation	94℃	30 seconds	30 X cycle
Annealing	60℃	45 seconds	
Extension	72℃	45 seconds	
Final Extension	72℃	10 minutes	1 X cycle

Table 3: PCR Thermal Cycle Parameters: (PA-GS)

Step	**Temperature, ℃**	**Time**	
Initial Denaturation	95℃	2 minutes	1 X cycle
Denaturation	94℃	20 seconds	25 X cycle
Annealing	54℃	20 seconds	
Extension	72℃	40 seconds	

Final Extension	72℃	1 minute	1 X cycle

Table 4: PCR Thermal Cycle Parameters: (PA-SS)

Step	**Temperature, ℃**	**Time**	
Initial Denaturation	95℃	2 minutes	1 X cycle
Denaturation	94℃	20 seconds	30 X cycle
Annealing	58℃	20 seconds	
Extension	72℃	40 seconds	
Final Extension	72℃	5 minutes	1 X cycle

2.20 Gel Electrophoresis

Gel electrophoresis is a technique in molecular biology to separate and analyze molecules based on their size and charge. In our work, amplified PCR products were used to conduct this experiment. 1 to 1.5% agarose gel was prepared with 1% TAE buffer solution. Ethidium bromide was added and mixed well before pouring the gel into the casting tray. After forming

the wells, PCR products were loaded and the voltage was set to 90V for 45 minutes. Finally, the gel was viewed with UV light to detect the DNA bands.

2.21 Antimicrobial Susceptibility Test (AST)

An antibiotic susceptibility test, often referred to as AST, is a laboratory procedure used to assess the sensitivity of bacterial or fungal pathogens to various antibiotics or antimicrobial drugs. This test is essential in guiding healthcare professionals to choose the most effective treatment for infectious diseases. In our study, AST was performed in the Kirby-Bauer disk diffusion method, and for analysis of the results, the Clinical and Laboratory Standard Institute (CLSI) guidelines (2020) were followed.

The 11 antibiotics used in our study were Colistin (CL) 10mcg, Ciprofloxacin (CIP) 5mcg, Amoxicillin (AMX) 30mcg, Cefixime (CFM) 5mcg, Amoxiclav (AMC) (Amoxicillin 20 mcg/Clavulanic Acid 10mcg), Meropenem (MRP, MEM) 10mcg, Kanamycin (K) 30mcg, Tetracycline (TE) 30mcg, Cefepime (CPM) 30mcg, Azithromycin (AZM) 30mcg and Ceftriaxone (CTR, CRO) 30mcg. Their respective zone diameter interpretations are given below.

Zone diameter interpretative standards as per CLSI 2020 (3rd Edition)

Pseudomonas Aeruginosa

Table 5: Different Antibiotics Zone Diameter for *Pseudomonas aeruginosa*

Antibiotic Name	Disk Concentration	Symbol	Interpretative Categories and Zone Diameter, mm		
			Sensitive (S)	Intermediate (I)	Resistant (R)
Colistin	10mcg	CL	≥11	-	≤10
Ciprofloxacin	5mcg	CIP	≥25	19-24	≤18
Amoxicillin	30mcg	AMX	≥18	14-17	≤13
Cefixime	5mcg	CFM	≥19	16-18	≤15
Amoxiclav	30mcg (20/10mcg)	AMC	≥18	14-17	≤13

Meropenem	10mcg	MRP/MEM	≥19	16-18	≤15
Kanamycin	30mcg	K	≥18	14-17	≤13
Tetracycline	30mcg	TE	≥19	15-18	≤14
Cefepime	30mcg	CPM	≥18	15-17	≤14
Azithromycin	30mcg	AZM	≥18	14-17	≤13
Ceftriaxone	30mcg	CTR/CRO	≥21	14-20	≤13

Enterobacteriaceae (Klebsiella pneumoniae)

Table 6: Different Antibiotics Zone Diameter for *Klebsiella pneumoniae*

Antibiotic Name	Disk Concentration	Symbol	Interpretative Categories and Zone Diameter, mm		
			Sensitive (S)	Intermediate (I)	Resistant (R)
Colistin	10mcg	CL	≥14	12-13	≤11
Ciprofloxacin	5mcg	CIP	≥26	22-25	≤21
Amoxicillin	30mcg	AMX	≥17	14-16	≤13
Cefixime	5mcg	CFM	≥19	16-18	≤15
Amoxiclav	30mcg (20/10mcg)	AMC	≥18	14-17	≤13
Meropenem	10mcg	MRP/MEM	≥23	20-22	≤19
Kanamycin	30mcg	K	≥18	14-17	≤13
Tetracycline	30mcg	TE	≥15	12-14	≤11
Cefepime	30mcg	CPM	≥25	19-24	≤18
Azithromycin	30mcg	AZM	≥18	14-17	≤13
Ceftriaxone	30mcg	CTR/CRO	≥23	20-22	≤19

Chapter 3

Result Interpretation and Observation

3.1 Isolation of *Pseudomonas*

During the research timeframe from May to September 2023, a total of 16 water samples were collected from the available drinking water sources of tea-stalls and road-side restaurants in 6 major locations of Dhaka North City Corporation. The water samples were enriched by submerging in 10 ml liquid LB media and incubated for 24 hours at 37℃ to promote the optimum growth of our organisms of interest. After incubation, for the isolation of *Pseudomonas*, the enriched sample diluted up to a concentration of 10^{-2} and 10^{-3} was prepared for spread plating in Cetrimide media which is highly specific for *Pseudomonas* species. *Pseudomonas* colonies were initially marked based on colony morphology in these media.

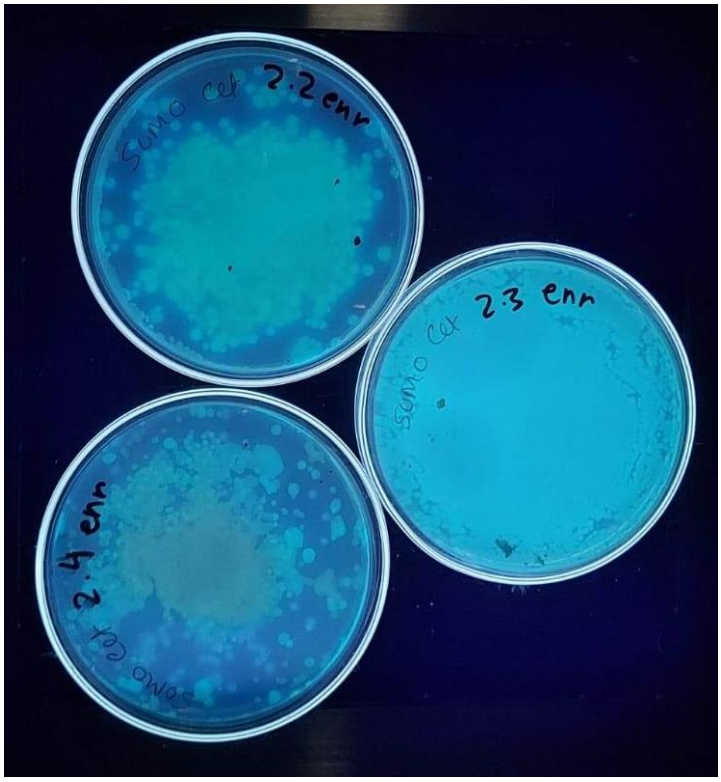

Figure 4: Luminescent growth on Cetrimide media after spread plating observed under UV light

After spread plating, the bacterial growth in the plates were observed under UV light and luminescence suggested the presence of *Pseudomonas* species. A few single colonies were then selected from the plate and then further streak plated for pure colonies. PCR was frequently performed to confirm whether or not these were *Pseudomonas*. From a total of 16 samples, 160 *Pseudomonas* species were isolated among which 90 were identified to be *Pseudomonas aeruginosa*.

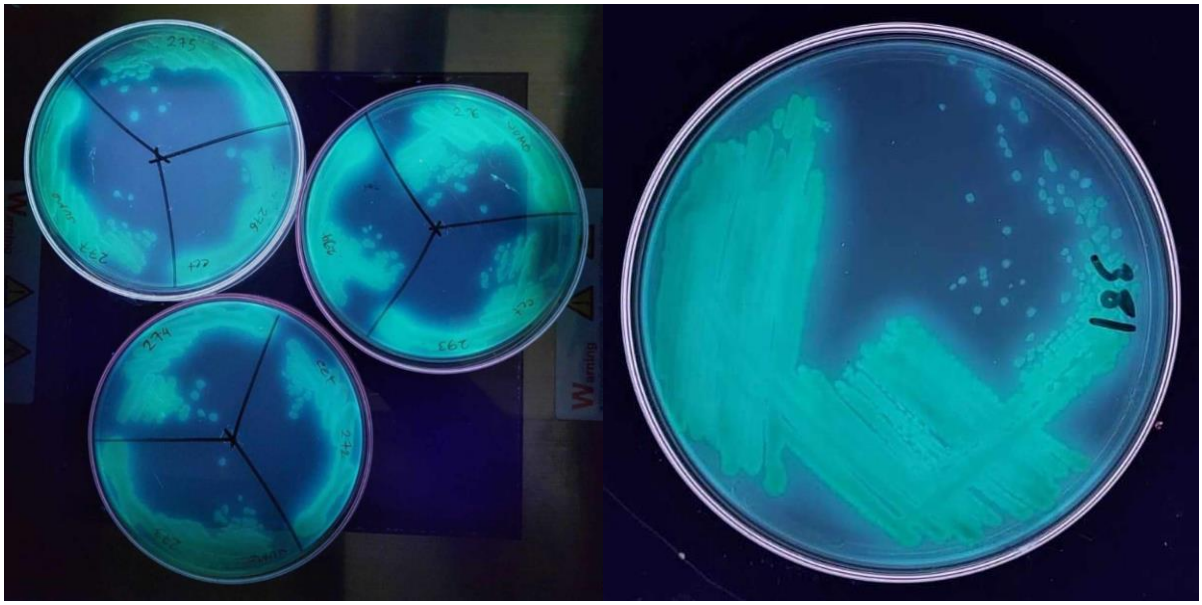

Figure 5: Single colonies from spread plates further streak plated in Cetrimide media showing pure luminescent colonies under UV light

3.2 Identification of *Pseudomonas aeruginosa* by PCR assay and Gel Electrophoresis

To confirm the *Pseudomonas* strains isolated by us, PCR was conducted using the 'PA-GS' primer first to identify *Pseudomonas* genus and then a species-specific primer 'PA-SS' was used on the confirmed *Pseudomonas* strains in order to identify *Pseudomonas aeruginosa* specifically. Among a total of 16 samples collected from May to September, 160 strains were isolated among which 105 were identified to belong to the *Pseudomonas* genus and among

those 90 were confirmed to be *Pseudomonas aeruginosa* specifically. After PCR, Agarose Gel Electrophoresis was conducted and the results were seen under a UV transilluminator. The results of the gel run are shown below:

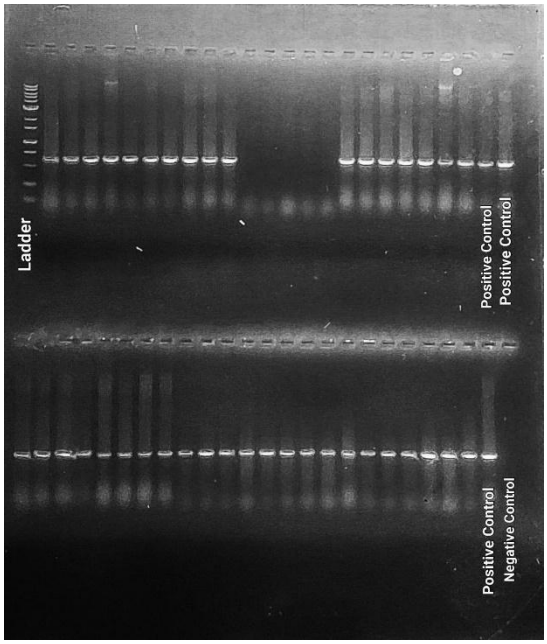

Figure 6: Gel electrophoresis results for P. aeruginosa confirmation

3.3 Isolation of *Klebsiella*

The 16 water samples collected were enriched by submerging in 10 ml liquid LB media and incubated for 24 hours in 37℃ to promote the optimum growth of the bacteria present in the water. After incubation, for the isolation of *Klebsiella*, the enriched sample diluted up to a concentration of 10^{-4} and 10^{-5} was prepared for spread plating in selective MAC and KPC media. *Klebsiella* colonies were initially marked based on colony morphology.

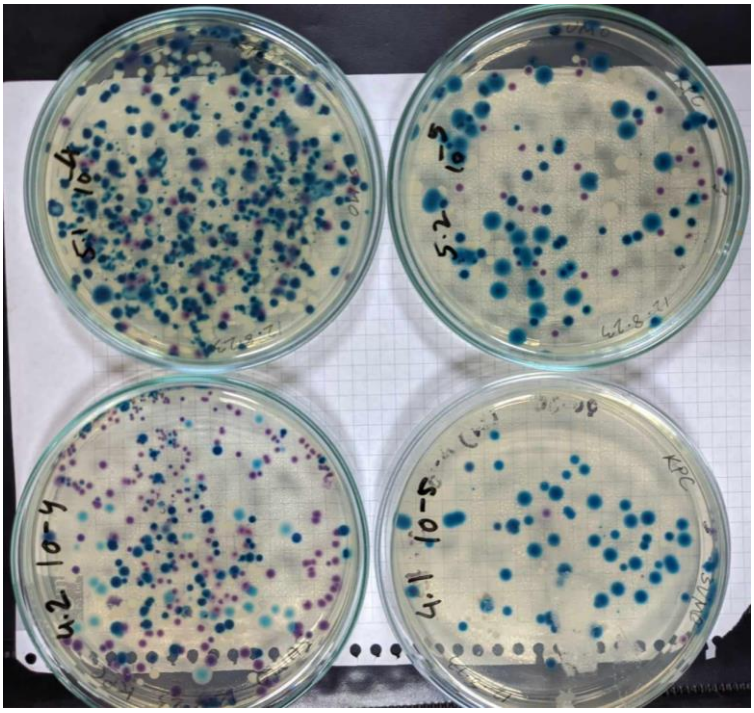

Figure 7: Metallic blue coloured colonies after spread plating on KPC agar

According to the characteristic morphology shown in both media, a few single colonies presumed to be *Klebsiella* species were then selected from the spread plate and then further streak plated for pure colonies. PCR was frequently performed to confirm whether or not these were *Klebsiella*. From a total of 16 samples, 161 *Klebsiella* species were isolated among which 90 were confirmed to be *Klebsiella pneumoniae* specifically.

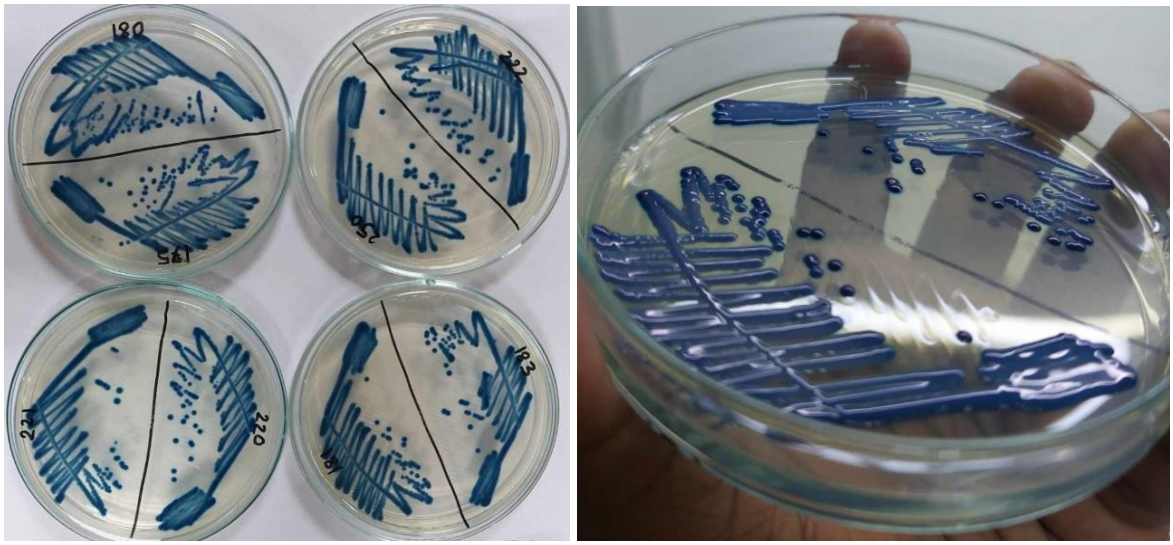

Figure 8: Single colonies from spread plates further streak plated in KPC media showing pure colonies of metallic blue colour

3.4 Identification of *Klebsiella* species by PCR assay and Gel Electrophoresis

To confirm the *Klebsiella* strains isolated by us, PCR was conducted using the species specific 'KPN' primer which identifies *Klebsiella pneumoniae* specifically. Among a total of 16 samples collected from May to September, 161 strains were isolated and among them, a total of 90 were confirmed to be *Klebsiella pneumoniae*. After PCR, Agarose Gel Electrophoresis was conducted and the results were seen under a UV transilluminator. The results of the gel run are shown below:

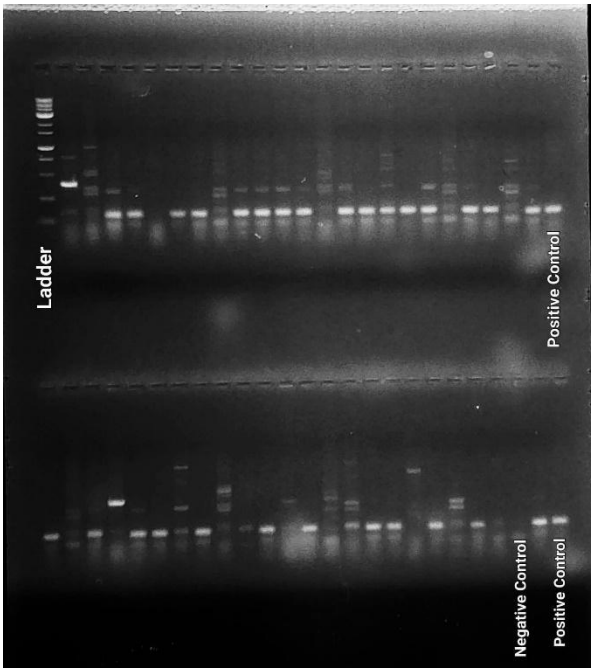

Figure 9: Gel electrophoresis results for K. pneumoniae confirmation

3.5 Interpretation of Antibiotic Susceptibility Test

After the species-specific PCR identification of the isolated organisms, antibiotic susceptibility test (AST) was conducted on the confirmed *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains. A total of 11 antibiotics were used for AST testing in the Kirby-Bauer disk diffusion method. After disk placement, the MHA plates were then incubated at a temperature of 37℃. 18-24 hours after incubation, reading of the plates was taken by measuring the zone diameters to determine whether the strains are Resistant (R), Intermediate (I) or Susceptible to the specific antibiotic dose according to standards of Clinical and Laboratory Standard Institute (CLSI) (2020). 72.2% *P. aeruginosa* and 51.1% *K. pneumoniae* isolates in the study were found to be multidrug resistant (MDR).

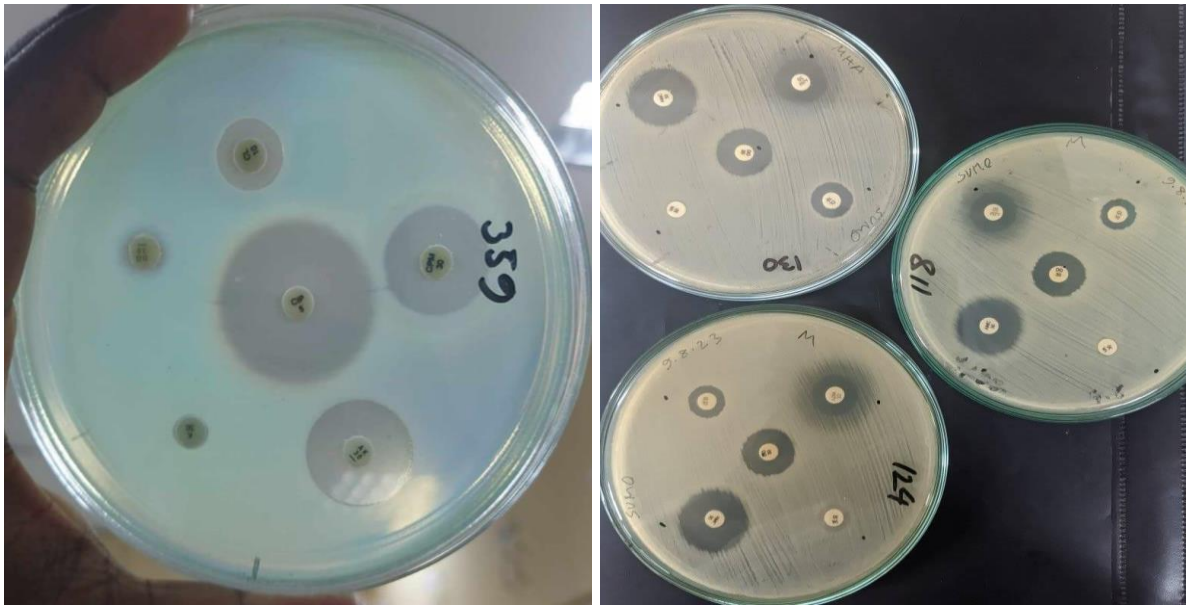

Figure 10: Inoculated MHA plates with the antibiotic-impregnated disks and their zone sizes after incubation

Antibiotic Resistance Pattern in the 90 *P. aeruginosa* isolates

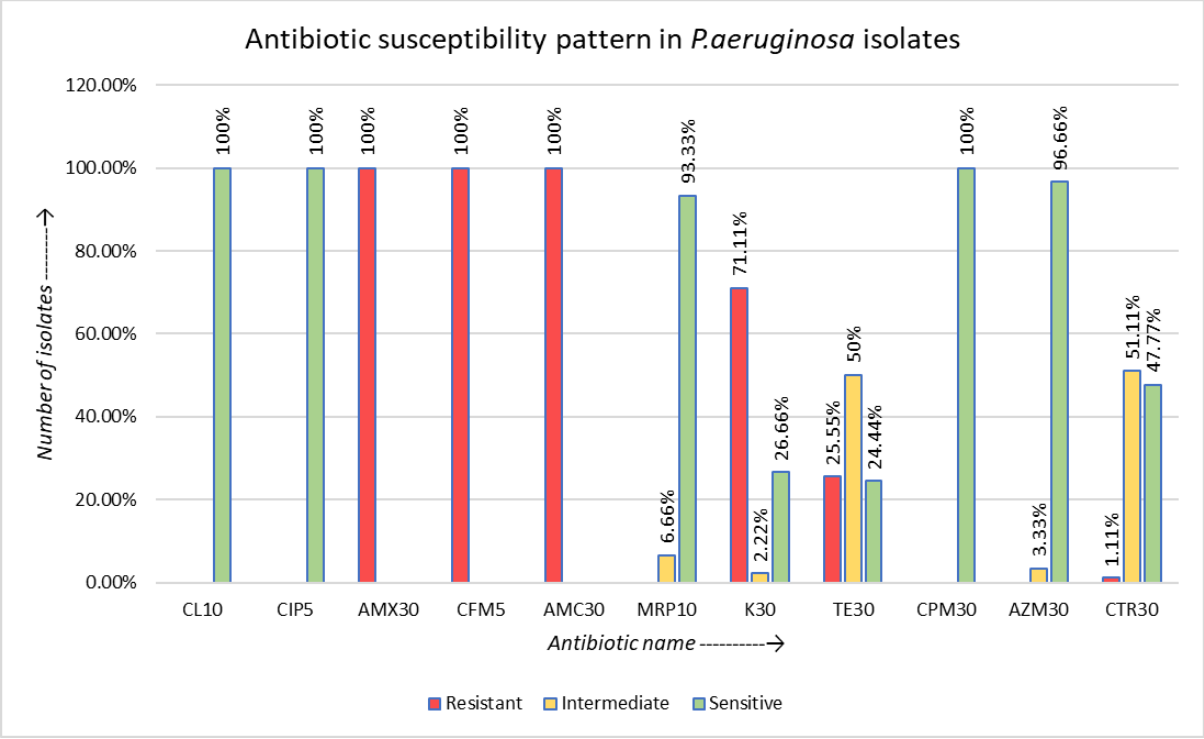

Figure 11: Graph showing the Antibiotic susceptibility pattern of 11 different antibiotics in P. aeruginosa isolates

From the bar chart above, it can be observed that in terms of susceptibility, all 90 isolates (100%) showed sensitivity to the antibiotic doses of Colistin (CL), Ciprofloxacin (CIP) and Cefepime (CPM). The next highest susceptibility is seen in case of the antibiotics Azithromycin (AZM) and Meropenem (MRP) which are 96.6% and 93.3% of the total population respectively. In case of Ceftriaxone (CTR), susceptibility was seen in 47.7% of the isolates, which is still quite high. For the rest of the antibiotics, the susceptibility rates were quite low, Kanamycin (K) (26.6%) and Tetracycline (TE) (24.4%).

Alarmingly, among the total population of *P. aeruginosa* isolates, all (100%) were resistant to the doses of Amoxicillin (AMX), Cefixime (CFM) and Amoxyclav (AMC). The next highest resistance is seen in case of the dose of Kanamycin (K), where 71.1% of the isolates were resistant. In the case of Tetracycline (TE), 25.5% of the total population of isolates showed resistance and for Ceftriaxone (CTR) only 1.1%.

Antibiotic Resistance Pattern in the 90 *K. pneumoniae* isolates

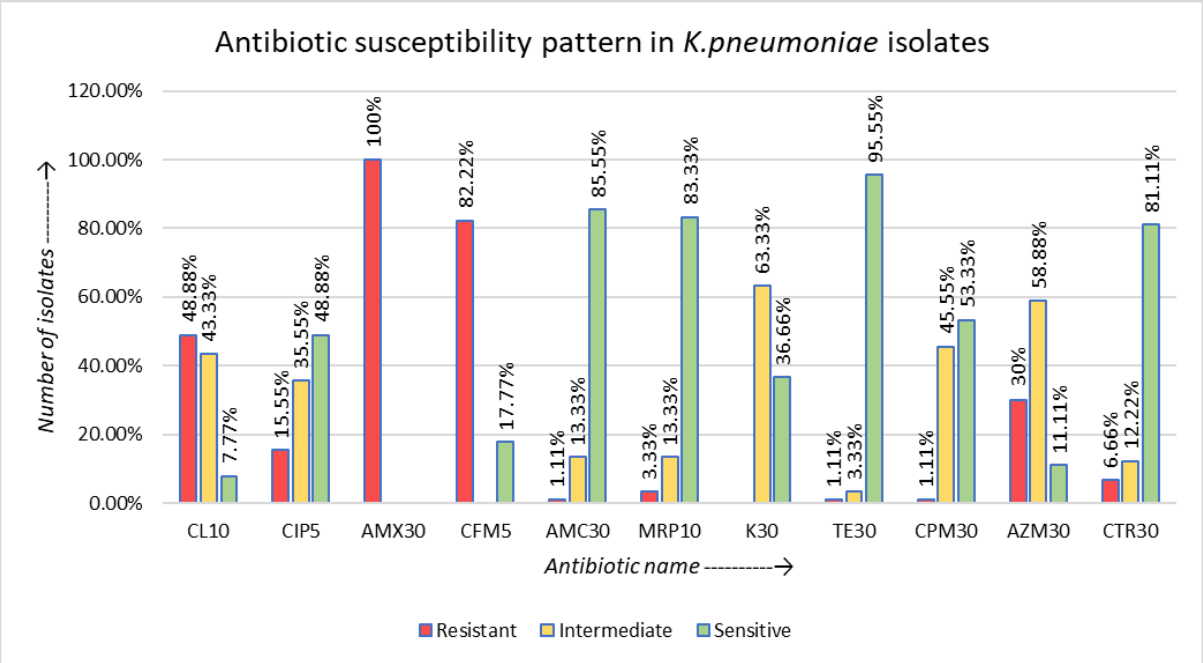

Figure 12: Graph showing the Antibiotic susceptibility pattern of 11 different antibiotics in K. pneumoniae isolates

In the above chart, it can be seen that the highest susceptibility can be observed in the case of Tetracycline (TE), to which 95.5% of the isolates were susceptible. The next highest susceptibility was shown to Amoxiclav (AMC), Meropenem (MRP) and Ceftriaxone (CTR), which had respectively 85.5%, 83.3% and 81.1% of the total isolates susceptible to it. Following these, Cefepime (CPM) and Ciprofloxacin (CIP) had 53.3% and 48.8% of the total isolates susceptible to it respectively. In the case of Kanamycin (K), the susceptibility rate was 36.6% and for Cefixime (CFM), 17.7%. The isolates showed very less sensitivity to Azithromycin (AZM) and Colistin (CL) which respectively had 11.1% and 7.7% of the total isolates susceptible to its doses.

All *K. pneumoniae* isolates (100%) in the study were seen to be resistant to the dose of Amoxicillin (AMX) which is a big concern. Second highest resistance was seen in Cefixime

(CFM), which was resistant in 82.2% of the total population of isolates. In case of Colistin (CL), 48.8% and in case of Azithromycin (AZM), 30% of the total number of isolates were seen to be resistant. For Ciprofloxacin (CIP), Ceftriaxone (CTR) and Meropenem (MRP) the rate of resistant isolates was quite low at 15.5%, 6.6% and 3.3% respectively. Lastly, it was seen that Amoxiclav (AMC), Tetracycline (TE) and Cefepime (CPM) each had about 1% of the isolates resistant to it.

<u>*P. aeruginosa* Vs *K. pneumoniae* Antibiotic Resistance Comparison</u>

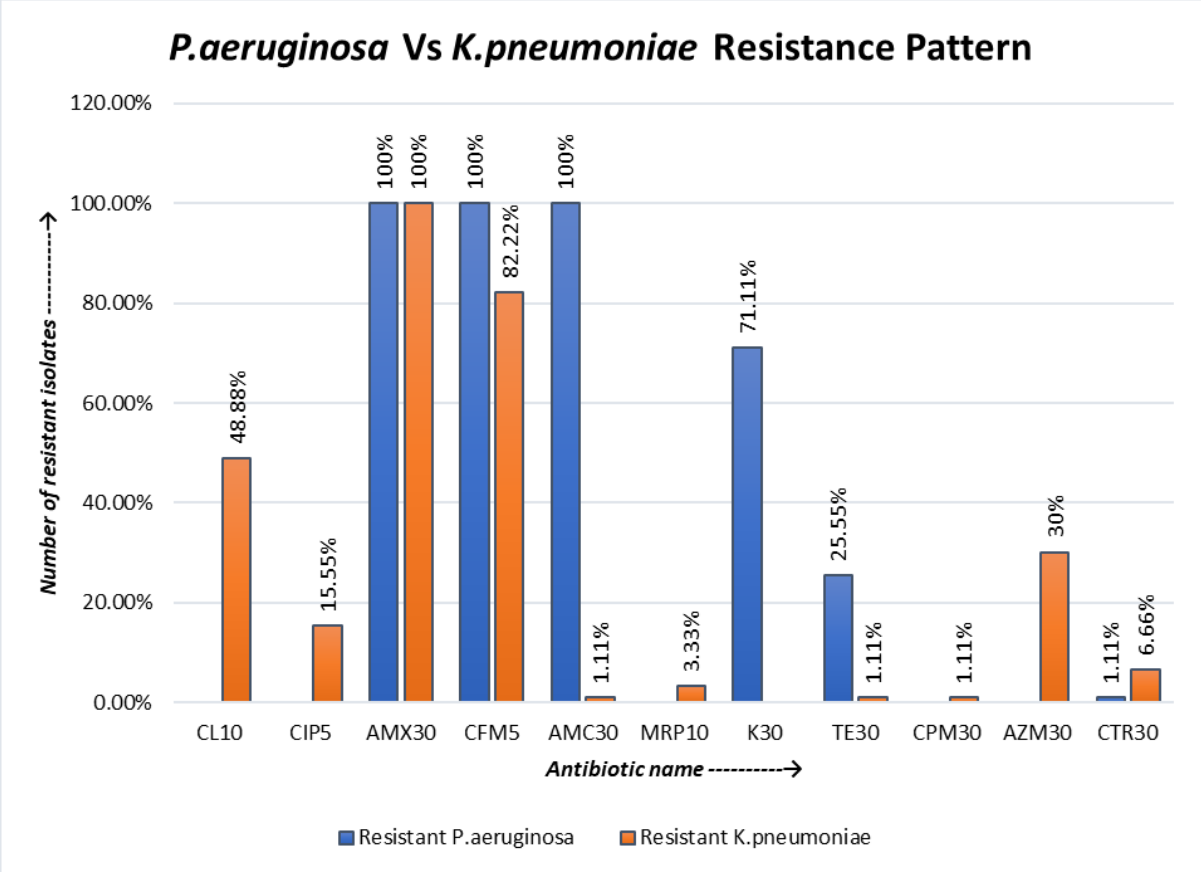

Figure 13: Graph showing a comparison in resistance pattern of the two organisms to different antibiotics

In the above graph, a comparison can be seen between the total number of resistant isolates found for the two organisms in response to each antibiotic used in the study.

In case of Colistin (CL), it can be seen that none of the *P. aeruginosa* isolates were resistant to it, whereas a total of 48.8%, so about half of the total *K. pneumoniae* isolates were resistant to its dose. For Ciprofloxacin (CIP), similarly there are no resistant *P. aeruginosa* isolates but 15.5% of the *K. pneumoniae* isolates were seen to be resistant. Amoxicillin (AMX) showed similar results in case of both organisms, as in both cases, all 90 isolates (100%) showed resistance. For Cefixime (CFM), both organisms showed considerably high resistance, as all 90 isolates (100%) of *P. aeruginosa* and 74 isolates (82.2%) of *K. pneumoniae* were resistant to it. In case of Amoxiclav (AMC), all (100%) *P. aeruginosa* isolates showed resistance but on the other hand, only 1 (1.1%) *K. pneumoniae* isolate was resistant. None of the *P. aeruginosa* isolates were seen to be resistant to Meropenem (MRP) but 3 (3.3%) of the *K. pneumoniae* isolates were resistant. Kanamycin (K) resistance was only observed in *P. aeruginosa* isolates and a considerable amount, a total of 64 isolates (71.1%) were resistant. For Tetracycline (TE) a small fraction of the population of both organisms were resistant, 25.55% *P. aeruginosa* and only 1.1% *K. pneumoniae* isolates were resistant. Cefepime (CPM) and Azithromycin (AZM) resistance were both only observed in *K. pneumoniae* isolates and 1 (1.1%) and 27 (30%) resistant organisms were found respectively. Lastly, in case of Ceftriaxone (CTR), a few resistant isolates were found in both cases, only 1.1% of *P. aeruginosa* and 6.6% *K. pneumoniae* isolates were found to show resistance.

Distribution of resistant *P. aeruginosa* isolates in different areas

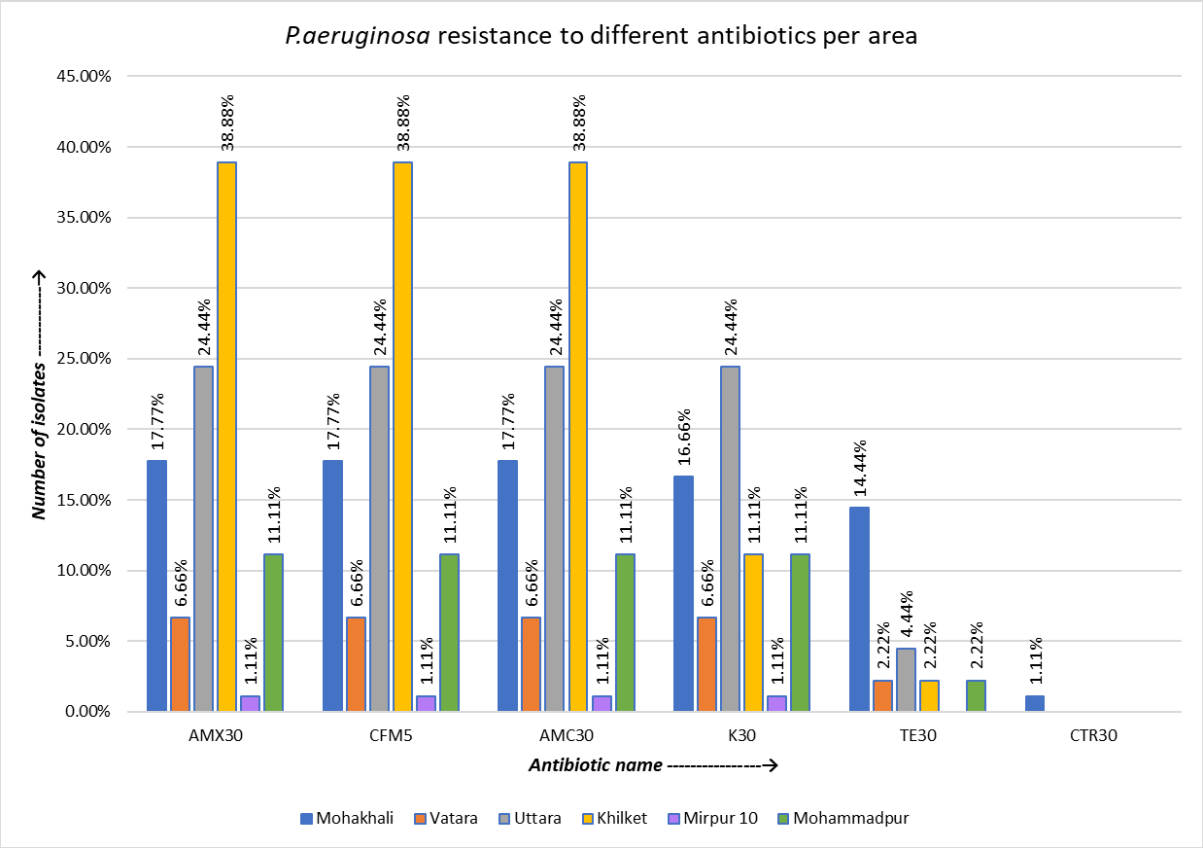

Figure 14: An area-wise comparison graph of the resistant P. aeruginosa isolates

In the above graph, a comparison of the *P. aeruginosa* isolates' resistance to different antibiotics based on the sample location is seen. In case of Amoxicillin resistance, the most resistant isolates were found in Khilkhet (38.8%), following which comes Uttara (24.4%), Mohakhali (17.7%) and Mohammadpur (11.1%). The least proportion of resistant isolates were seen in Vatara (6.6%) and Mirpur 10 (1.1%). For Cefixime and Amoxiclav doses, we can see identical results as above. In case of Kanamycin resistance however, the most resistant isolates were found in Uttara (24.4%), after which the most isolates can be seen in Mohakhali (16.6%). Khilkhet and Mohammadpur had around 11.1% of the total number of isolates each. Lastly, the least number of resistant isolates were seen in Vatara (6.6%) and Mirpur 10 (1.1%) as seen before. Tetracycline resistance was seen most in isolates from Mohakhali (14.4%). Very few isolates in the rest of the areas showed Tetracycline resistance- Uttara (4.4%), Vatara (2.2%),

Khilkhet (2.2%) and Mohammadpur (2.2%). Lastly, it can be seen that Ceftriaxone resistance was found in only 1 (1.1%) isolate from Mohakhali region.

Distribution of resistant *K. pneumoniae* strains in different areas

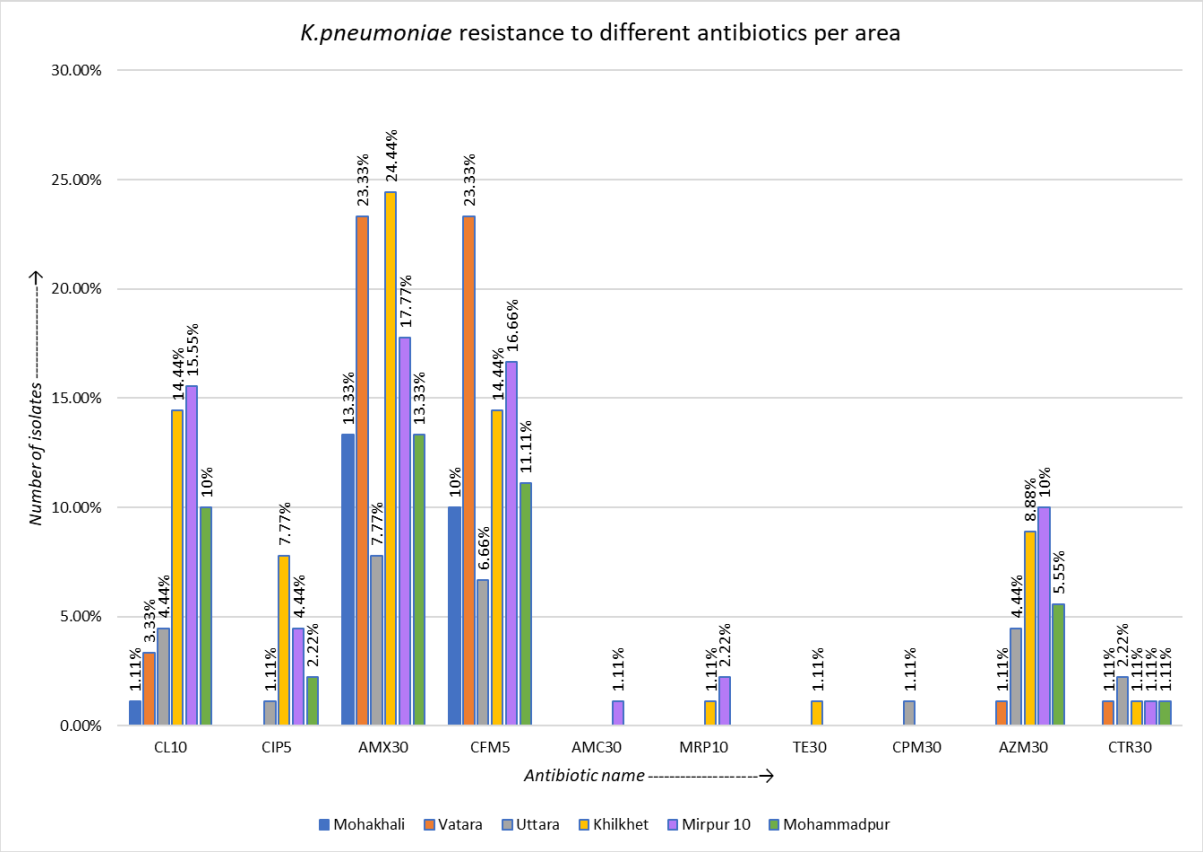

Figure 15: An area-wise comparison graph of the resistant K. pneumoniae

In the above graph, a comparison of the *K. pneumoniae* isolates' resistance to different antibiotics based on area is seen. In case of Colistin resistance, the highest peak can be observed in Mirpur 10 (15.5%), following which comes Khilhet (14.4%) and Mohammadpur (10%). Very few isolates were observed in the rest - Uttara (4.4%), Vatara (3.3%) and Mohakhali (1.1%). For Ciprofloxacin resistance, all areas yielded few isolates- Khilkhet (7.7%), Mirpur 10 (4.4%), Mohammadpur (2.2%) and Uttara (1.1%). Amoxicillin resistance showed high peaks in most areas, where the most resistant isolates were found in Khilhet (24.4%) and Vatara (23.3%). Other areas also had considerably high amounts of resistant isolates- Mirpur 10

(17.7%), Mohakhali (13.3%), Mohammadpur (13.3%) and Uttara (7.7%). In case of Cefixime, the highest peak was observed in Vatara (23.3%), following which comes Mirpur 10 (16.6%), Khilkhet (14.4%), Mohammadpur (11.1%), Mohakhali (10%) and Uttara (6.6%). Amoxicillin resistance was observed in only one isolate found in Mirpur 10, Meropenem resistance was found in one isolate from Khilkhet and 2 isolates from Mirpur10, Tetracycline resistance was found in only one isolate from Khilkhet and Cefepime resistance was seen in only one isolate from Uttara. For Azithromycin, the number of resistant isolates found in different areas were- Mirpur 10 (10%), Khilkhet (8.8%), Mohammadpur (5.5%), Uttara (4.4%) and Vatara (1.1%). Finally, in case of Ceftriaxone resistance, there were very few numbers of resistant isolates- two isolates found in Uttara, one in Vatara, one in Khilkhet, one in Mirpur 10 and one found in Mohammadpur area.

Chapter 4

Discussion & Conclusion

4.1 Discussion

Pseudomonas aeruginosa and *Klebsiella pneumoniae* are significant bacterial pathogens that are commonly found in various environmental samples. Even with the advancements in medical and surgical treatments, as well as the introduction of a wide range of antimicrobial agents in animal agriculture, the number of resistant isolates of these bacteria are seen to significantly increase in different water sources. This in turn, raises concerns that these water sources could serve as a vast reservoir for resistant microorganisms, potentially allowing the transfer of resistance genes back into disease-causing organisms in both humans and animals. As a result, this study was conducted to assess the prevalence of antibiotic-resistant isolates in surface water and to investigate their patterns of antibiotic resistance.

In this study, one such water source was analyzed, which is the available drinking water provided in tea stalls and road-side restaurants. In such settings, the common source of drinking water is usually the supplier-provided mineral water jars in case of tea-stalls and water-filters in case of road-side restaurants. It is presumed that in many cases, small food establishments such as these may not have stringent water quality monitoring programs in place. This can result in the use of water with unknown or suboptimal quality, potentially containing antibiotic-resistant bacteria. With such a concept in mind, our research aimed to speculate whether the tea stalls and street restaurants across various locations within the Dhaka North City Corporation (DNCC) were ensuring the safety and quality of the drinking water they serve.

From the water samples collected, a total of 321 isolates were screened, out of which 180 were verified by PCR amplification using species specific primers. There were a total of 90 confirmed *P. aeruginosa* and 90 confirmed *K. pneumoniae* across all six sample locations. The antimicrobial resistance of these isolates was then evaluated using 11 antibiotics.

In our study, all *Pseudomonas aeruginosa* isolates were found to be 100% resistant to **Amoxicillin and Amoxiclav (Amoxicillin/clavulanic acid)**. Similar results were seen in another study in Dhaka city regarding *P. aeruginosa* found in the surface water where the findings show a 100% resistance to Ampicillin (also a penicillin derived drug structurally similar to Amoxicillin) (Nasreen et al., 2015). In a study conducted on medical samples in Egypt, *P. aeruginosa* isolates found in respiratory tract infections were 100% resistant to Ampicillin, Amoxicillin and Amoxicillin/clavulanate (Amoxiclav) (Gad et al., 2008). In other places, high resistance of these antibiotics were also observed, such as in a medical sample study in Iran, Amoxicillin and Amoxiclav resistance were 87.8% and 96% respectively (RABIRAD et al., 2014) and in hospital samples from burn patients in North India, 63.3% ampicillin resistant P. aeruginosa isolates were found (Shahid et al., 2003).

All *P. aeruginosa* isolates in our study were also 100% resistant to **Cefixime** which bears similarity with a study conducted on clinical specimens from Dhaka Medical College Hospital (DMCH), where almost all *P. aeruginosa* isolates were resistant to Cefixime (93.3%) and many were resistant to Ceftazidime (86.8%) (also a third generation Cephalosporin drug similar to Cefixime) (Rashid et al., 2016). In a study in Iran, *P. aeruginosa* isolated from ICU-admitted COVID-19 patients were also found to be 100% resistant to Cefixime (Jamnani et al., 2022).

The *P. aeruginosa* isolates in the present study showed significant resistance to **Kanamycin** (71.1%) and this result aligns with the result of the study by Klastersky et al. that found that

Kanamycin had little effect on *P. aeruginosa* (Klastersky et al., 1974). In another study in Texas however, moderate resistance to Kanamycin (56%) were found in medical samples. (Holmes et al., 1974)

Our *P. aeruginosa* isolates showed 25.5% **Tetracycline** resistance but different results are seen in other studies, such as in the surface water study by Nasreen et al., the resistance to Tetracycline was was much higher (93.7%) (Nasreen et al., 2015) and even a study conducted on medical samples in Egypt showed high resistance to Tetracycline (89%) in *P. aeruginosa* isolates. (Gad et al., 2008)

All of the isolated *Klebsiella pneumoniae* strains in our study were totally resistant to **Amoxicillin** (100%) and this outcome is consistent with the study findings by Bouza and Cercenado that determined that *Klebsiella spp.* are intrinsically resistant to penicillin (Bouza & Cercenado, 2002). Similar results were obtained in other places, such as in a study on clinical samples in Pakistan, *K. pneumoniae* isolates were 100% resistant to Amoxicillin (Fatima et al., 2021) and in another study on ICU patients samples in North India, *K. pneumoniae* had complete resistance to Ampicillin and resistance to Amoxicillin increased considerably through the years from 2018 to 2022 (Sharma et al., 2023). However, contradicting results were found in a study in Turkey on *K Pneumoniae* strains from infected neonates where ampicillin was deemed to be the least effective (0%) (Aktas et al., 2002).

In the present study, the *K. pneumoniae* isolates also showed significant resistance to **Cefixime** (82.2%) and this result is congruent with Bouza and Cercenado's study findings where it was found that due to the synthesis of plasmid-mediated extended-spectrum beta-lactamases (ESBLs), *Klebsiella spp.* can develop resistance to third- and fourth-generation cephalosporins (Bouza & Cercenado, 2002). In a study on clinical samples in Pakistan, *K. pneumoniae* isolates

were 100% resistant to Cefixime similar to our result but it was also 100% resistant to another third-generation cephalosporin drug, Ceftriaxone (Fatima et al., 2021), which in our study was quite efficient against the *K. pneumoniae* isolates as they were highly susceptible to Ceftriaxone (81.1%). A reason for this could be the varied usage of cephalosporin drugs in different countries.

Our isolated *K. pneumoniae* isolates showed a moderate resistance to **Colistin** (48.8%), in other places however, an alarmingly high rate of colistin resistance in *K. pneumoniae* was discovered, such as in a recent study focused on ICU patients with lung illness in North India, Colistin resistance was just 8% in 2018, but shot up to 72% in 2022 (Sharma et al., 2023). This is probably because of the increased use of Colistin as a last-resort drug as the prevalence of multidrug-resistant *Klebsiella pneumoniae* has increased in recent years (Janssen et al., 2020). Another emerging problem is the wide use of Colistin as an antibiotic for poultry in Bangladesh, although it belongs to the reserve category of antibiotics (Sobur et al., 2019). According to a report by the Centers for Disease Control and Prevention (CDC) in 2016, this has become a huge problem worldwide because if this colistin resistance spreads to bacteria that have already developed resistance to every other antibiotic, these bacteria could lead to infections that are genuinely impossible to treat.

The antibiotics found to be most effective against our *P. aeruginosa* isolates were Colistin (100%), Ciprofloxacin (100%), Cefepime (100%), Azithromycin (96.6%) and Meropenem (93.3%) while the *K. pneumoniae* isolates showed the most sensitivity to Tetracycline (95.5%), Amoxiclav (85.5%), Meropenem (83.3%) and Ceftriaxone (81.1%). Meropenem is a carbapenem antibiotic which is a frequent drug of choice to cure critical Ventilator-associated

pneumonia (VAP) infections, a common nosocomial infection often caused by *P. aeruginosa* and *K. pneumoniae* in intensive care unit (ICU) patients. Therefore, the emergence of carbapenem-resistant organisms (CROs) which includes carbapenem-resistant Enterobacterales (CRE) and carbapenem-resistant *Pseudomonas aeruginosa* (CRPA), is a matter of serious concern in the modern world (Mohamed et al., 2021). Both the *P. aeruginosa* and *K. pneumoniae* isolates in our study were seen to be well susceptible to Meropenem which is a positive finding.

Even though a good number of antibiotics were effective against our isolated strains, it is still a very big concern that 72.2% *P. aeruginos*a and 51.1% *K. pneumoniae* strains of our study were found to be multidrug resistant (MDR). Being collected from a public drinking water source, these resistant bacteria pose a big threat as they can easily spread their resistance to other organisms in the environment through horizontal gene transfer (HGT). Furthermore, Bangladesh is a country experiencing a significant rise in antibiotic resistance. A primary reason for this being the prevalence of over-the-counter antibiotics for both humans and animals, coupled with insufficient training and adherence to healthcare standards among healthcare providers (HCPs) throughout the country (Rousham et al., 2019). In a technical report by Hossain (2015), it was determined that in retail drug stores of Bangladesh, most antibiotics are bought without a prescription (Hossain, 2015). In environments where antibiotics are frequently used, bacteria like *P. aeruginosa* and *K. pneumoniae* with resistance genes have a survival advantage. Studies from both community and hospital settings have found that the resistance against antimicrobial drugs is related to the amount of antibiotics that are being consumed (Costelloe et al., 2010; Tacconelli et al., 2009). As these bacteria multiply and shed resistant plasmids, they can expose other bacteria to selective pressure, promoting the development and spread of resistance.

To mitigate the spread of antibiotic resistance, it is crucial to implement strict infection control measures, rational antibiotic use, and surveillance of antibiotic-resistant strains. Additionally, research into novel antibiotics and alternative treatment strategies is essential to combat the growing threat of multidrug-resistant bacteria like *P. aeruginosa* and *K. pneumoniae*.

This study has some constraints that should be acknowledged. Firstly, our examination focused on samples collected from different areas of the Dhaka North City Corporation (DNCC) in Bangladesh, which may not be fully representative of the entire country. To gain a more comprehensive understanding of resistance pattern changes over time, a more extensive investigation should be conducted, involving a larger sample size and encompassing a broader range of regions and divisions. Additionally, if sample collection could be scheduled at a specific time each month, it would be possible to get a more detailed analysis of how various characteristics such as availability or resistance of the organisms vary on a month-by-month basis.

Furthermore, the unavailability of various antibiotics from distinct classes has made it impossible to determine the Antimicrobial Susceptibility Test (AST) results for other antibiotics. It is likely that there are several antibiotic classes for which the isolates may exhibit resistance patterns. This paints an incomplete picture of the resistance pattern of the organisms.

We have positive expectations for future progress in this study, with plans to expand the sample collection sites to outside Dhaka North City Corporation (DNCC) and thus cover a larger area of research with a larger sample size. In addition, we hope to extend the time-frame of the research and conduct a long-term monitoring of the presence and prevalence of these bacteria in the sample sites in Dhaka city which will help in analyzing seasonal variations, trends, and

how they might be influenced by environmental factors. In the future, our study aims to identify antimicrobial resistance genes (AMR) through PCR techniques and pinpoint the specific genes responsible for causing antibiotic resistance in the resistant isolates found. Additionally, if available, we look forward to comparing the antibiotic resistance profiles and genetic characteristics of the isolated strains with clinical isolates of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

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