Isolation and Identification of *Pseudomonas aeruginosa* from River Water Adjacent to Mangrove Forest.

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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 of Microbiology

Department of Mathematics and Natural Sciences
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Abstract/ Executive Summary

Pseudomonas aeruginosa (P. aeruginosa) is a critical opportunistic pathogen known for causing a variety of infections, particularly in hospital settings, and is associated with high antibiotic resistance, including carbapenem resistance. Its persistence in recreational waters, such as lakes and rivers, poses health risks due to its adaptability and capacity to form biofilms, which protect it from disinfection. This pathogen can lead to skin infections, gastrointestinal issues, and other health concerns through contact with contaminated water during activities like swimming. In our study of the Pasur River, we examined water quality and the prevalence of *Pseudomonas aeruginosa*. The pH ranged from 5.8 to 6, indicating an acidic to neutral environment. Salinity varied from 0.1% to 0.8%, with higher salinity observed upstream, while the temperature remained consistent at 30-31°C. Out of eight samples, microbial counts measured in CFU/ml varied significantly, with Karamjal having the highest count at 171,250,000 CFU/ml and Dublar Chor the lowest at 7,625,000 CFU/ml. PCR analysis and gel electrophoresis confirmed 54 isolates as *Pseudomonas aeruginosa*, with most isolates found near tourist areas. Antimicrobial susceptibility testing revealed that among 39 isolates of *pseudomonas aeruginosa*, 51.79% were resistant to tetracycline and 58.97% were resistant to cefepime. The resistance percentage to imipenem was 53.85%, with an intermediate resistance of 23.08%, followed by 20.51% intermediate resistance to Meropenem. These findings highlight potential health risks due to bacterial contamination in river waters.

Keywords: *P. aeruginosa*, Beta-lactam, antibiotics, salinity, carbapenem. antibiotic resistant, AMR, ESAKAPE pathogens

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HAI	Hospital acquired Infection
AMR	Antimicrobial Resistance

Chapter 1

Introduction

1.1 Background

River waters can harbor a variety of opportunistic pathogens and antibiotic-resistant bacteria, posing significant health risks, especially in developing countries. The quality of river water has a direct impact on public health, with poor conditions leading to illnesses such as gastroenteritis, skin irritations, and respiratory issues (Stec et al., 2022). Water quality in recreational settings, such as public baths, beaches, and rivers, is crucial for preventing disease transmission. Contaminated water bodies are associated with various health risks, emphasizing the importance of maintaining safety standards and implementing effective sanitation practices (Rachma et al., 2021). Generic *E. coli* has been used as a reliable indicator of fecal contamination and associated health risks, helping identify pollution sources and assess public health threats (Butler et al., 2021). The risk of gastrointestinal illness is particularly high with recreational exposure to freshwater bodies, where primary contact activities, such as swimming, show higher adverse health outcomes compared to secondary or no-contact recreation (Gitter et al., 2020). Given the potential for recreational waters to harbor antibiotic-resistant bacteria, there is an urgent need to identify the infectious agent, its adaptability to the physicochemical condition of water bodies and their antimicrobial resistance (Nappier et al., 2020) and fill research gaps related to source tracking, human exposure, and standardized methodologies for risk assessment.

Pseudomonas aeruginosa, a member of the ESKAPE group alongside *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Enterobacter* species, is a gram-negative opportunistic bacterium that is involved in various infections including nosocomial infections and UTIs (De Oliveira et al., 2020). *Pseudomonas aeruginosa*

is renowned as a potent opportunistic pathogen known for its capacity to induce severe infections, especially in individuals with compromised immune systems. Its capability to thrive in different environments, combined with its resistance to antimicrobial agents, poses notable challenges in clinical settings. Within the realm of nosocomial infections, *P. aeruginosa* emerges as a significant pathogen distinguished by its ability to adapt to unfavorable conditions such as pH and osmolarity. Identified as one of the three primary pathogens of global apprehension by the World Health Organization (WHO) in 2017, this bacterium is also included in the Global Priority List of Antibiotic-Resistant Bacteria for the purpose of directing research, exploration, and innovation in new antibiotic development.

Ensuring the provision of universal healthcare is imperative in order to achieve a range of sustainable development goals (SDGs). Nonetheless, a significant challenge that is currently being encountered is antimicrobial resistance (AMR), which hinders our advancement towards these objectives. AMR arises when bacteria develop resistance to the therapeutics utilized for their treatment, primarily as a result of the inappropriate application of antimicrobials such as antibiotics in human beings, animals, plants, and veterinary medications. This resistance presents a substantial obstacle to the successful management of bacterial infections. The World Health Organization (WHO) delineated three levels of priority in addressing AMR in the year 2017: medium, high, and critical. Within the critical tier of priority, specific emphasis was placed on combating the Multidrug Resistance exhibited by gram-negative bacteria, particularly the ESKAPE group 2022- *Pseudomonas* was identified as having third generation carbapenem resistance.(Jasovský et al., 2016).

A lot of investigation was done on the urban water bodies in developing countries like Bangladesh. However, there is lack of evidence of microbiological and ex-situ physicochemical data of water bodies such as Pasur river of Khulna adjacent to Mangrove Forest which has also been used as a popular travel destination. Thus, in our study, we predicted that the Pasur River is possibly contaminated of *Pseudomonas aeruginosa* via multiple anthropogenic activities.

1.2 Literature Review

1.2.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, motile, aerobic bacterium from the Pseudomonadaceae family, characterized by oxidase and catalase positive reactions. This bacterium is classified as a critical pathogen by the World Health Organization due to its significant role in causing life-threatening infections, especially in immunocompromised individuals and hospital settings. It can lead to a variety of infections, including respiratory infections, bloodstream infections, wound infections, burn infections, urinary tract infections, otitis media, and meningitis. The wide range of infection types and its impact on vulnerable populations underscore the importance of continued research and targeted therapeutic approaches to address the challenges posed by Pse*udomonas aeruginosa* (Das, 2021).

1.2.2. Disease Caused by *Pseudomonas aeruginosa*

Community-acquired infections caused by *Pseudomonas aeruginosa* are rare but serious occurrences. While typically associated with nosocomial settings, instances of community-acquired infections have been reported. These infections can manifest in various forms, including pneumonia (Matsuki et al., 2022), urinary tract infections (Sando et al., 2021), and even meningitis (Cotran-Lenrow et al., n.d.). *P. aeruginosa* poses a significant threat due to its ability to form biofilms, leading to persistent infections with high antibiotic resistance (Vo et al., n.d.).

Pseudomonas aeruginosa is a common pathogen responsible for hospital-acquired infections (HAIs), particularly in Intensive Care Unit (ICU) settings, posing significant risks to patients with weakened immune systems or those dependent on medical devices like ventilators, catheters, and intravenous lines (Hernández et al., 2018). Hospital-acquired infections caused by this pathogen include ventilator-associated pneumonia, urinary tract infections, burn wound infections, and bloodstream infections, with a high prevalence of drug-resistant strains, including multidrug-resistant (MDR) and extensively drug-resistant (XDR), such as carbapenem-resistant variants. (Fazeli et al., 2012; Litwin et al., 2021; Maclean et al., 2022)

Among the common HAIs caused by *Pseudomonas aeruginosa*, ventilator-associated pneumonia (VAP) accounts for a significant portion, up to 40% of ICU cases. Bloodstream infections (BSI) are also prevalent, with *P. aeruginosa* being the most commonly identified species among gram-negative pathogens (Kalanuria et al., 2014). This bacterium also contributes to sepsis, with some studies indicating that it accounts for up to 46% of HAIs in specific hospital settings. Other notable infections include urinary tract infections, burn wound infections, ear infections, folliculitis, and cystic fibrosis-related infections (Qin et al., 2022).

The rise of drug-resistant phenotypes has exacerbated the clinical challenge, as these strains, particularly carbapenem-resistant ones, are more difficult to treat. In 2020, an increase in the rate of hospital-onset (HO) infections caused by *Pseudomonas aeruginosa,* particularly carbapenem-resistant strains, was observed compared to 2019 (de Souza et al., n.d.). This emphasizes the need for enhanced infection control measures and ongoing surveillance to curb the spread of resistant strains.

1.2.3. Epidemiology of *Pseudomonas aeruginosa* in river water

The epidemiology of *Pseudomonas aeruginosa* in water bodies, such as lakes, recreational water, and rivers, is an important area of research that highlights the potential risks to human

health. Studies have shown that *Pseudomonas aeruginosa* can persist and survive in these environments, primarily due to its ability to form biofilms and adapt to diverse environmental conditions. These biofilms serve as a protective matrix, allowing the bacteria to resist disinfection and survive for extended periods of time (Maclean et al., 2022). The adaptation of *Pseudomonas aeruginosa* to salinity, pH, and temperature in water environments is crucial for its survival and growth (Zhao et al., 2018). The quality of water in rivers is crucial for the maintenance of ecosystem health and the provision of safe drinking water for human populations. A study investigated the prevalence of bacteria in river water from various locations. They collected water samples from different points along the river and analyzed them for the presence of bacteria using standard microbiological methods. These studies have also highlighted the potential for *Pseudomonas aeruginosa* to cause infections in individuals who come into contact with contaminated water during recreational activities such as swimming or water sports (Nursyirwani et al., 2019).

1.2.4. Bacterial infection due to the recreational activities

Bacterial infections linked to recreational activities encompass a range of potential sources, including water, pets, and travel. Exposure to contaminated environments, such as lakes, pools, animals, and international travel destinations, increases the risk of infection (Ayi, 2015). Waterborne infections are particularly notable, as they can result from contact with water containing feces, organic and inorganic particles, or due to sedimentation-resuspension processes that allow bacteria to persist (Doménech-Sánchez et al., 2008).

Among the common bacterial infections related to recreational water activities, *Pseudomonas aeruginosa* stands out as a frequent causative agent. Infections with this pathogen can occur through skin contact, ingestion, or other exposures to contaminated water sources, such as inadequately disinfected indoor swimming pools. *Pseudomonas aeruginosa* folliculitis, for

instance, is a skin infection often associated with recreational water activities and can be linked to improper maintenance of pool hygiene ("Poor-Quality Water in Swimming Pools Associated with a Substantial Risk of Otitis Externa Due to Pseudomonas Aeruginosa.," 2004). These infections can also extend beyond the skin, affecting the eyes, urinary tract, lungs, and gastrointestinal system (Stec et al., 2022).

1.2.4. Antibiotics

Antibiotics are categorized according to the cellular components or systems they target, as well as by their mechanism of action—whether they kill cells (bactericidal) or merely inhibit cell growth (bacteriostatic). Bactericidal antibiotics primarily disrupt critical cellular processes such as DNA synthesis, RNA synthesis, cell wall synthesis, or protein synthesis. The mechanism by which these antibiotics cause cell death begins when the drug molecule physically interacts with its specific target in the bacterium, triggering a cascade of biochemical, molecular, and structural changes within the bacterial cell. The complexity of this process is highlighted by the involvement of various genetic and biochemical pathways.

Research also indicates that antibiotic-induced cell death can occur due to double-stranded DNA breaks, interruption of DNA-dependent RNA synthesis, damage to the cell envelope, and impacts on cellular energy production and protein synthesis (Kohanski et al., 2010).

1.2.5 Beta-Lactam Antibiotics

β-lactam antibiotics are considered the most extensively utilized class of antibacterial agents. Various derivatives of this class, including penicillin, cephalosporins, monobactams, and carbapenems, have been developed to broaden their range of activity and address resistance issues. The mechanism by which β-lactam antibiotics function involves covalent bonding with penicillin-binding proteins (PBPs), crucial enzymes for cross-linking peptidoglycan in

bacterial cell walls. This binding process interferes with cell wall synthesis, ultimately causing bacterial cell death.

β-lactams mimic the D-Ala-D-Ala dipeptide in peptidoglycan, enabling them to attach to the active site serine in PBPs and form an inactive acyl-enzyme complex. Certain β-lactams, such as ceftaroline, have the capability to bind to an allosteric site on PBP2a in Staphylococcus aureus, enhancing the antibiotic's efficacy. Resistance to β-lactams is mainly attributed to the activity of bacterial β-lactamase enzymes, which hydrolyze the β-lactam ring. (Bush & Bradford, 2016). Due to the variation in chemical structure of the beta-lactam ring, the following antibiotics have been prescribed- cephalosporins, beta-lactamase inhibitors, penicillins, monobactams, cephamycin, and carbapenems (ur Rahman et al., 2018).

Chapter 2

Materials and Methods

2.1. Study Design

The study was assessed during mid-August of 2023 in Pasur river of Khulna, Bangladesh. It is a southwestern river originating from the Sundarbans, a vast mangrove forest, and stretches approximately 50 kilometers before merging with the Bay of Bengal.

2.2. Sample collection

Total 12 samples (6 water and 6 sediment samples) were collected from the following areas- Dublar chor (21.7728° N, 89.5592°E), Karamjal (22.430105 N, 89.593732 E), Rainbow Eco Resort (22.4749° N, 89.5817° E), Harbaria (22.2979° N, 89.6146° E), Mongla port (22°28'59.4"N 89°35'18.7"E), and Sunmoon Shrimp Hatchery (22°29'40.6"N 89°34'56.2"E) on 17th August, 2023.

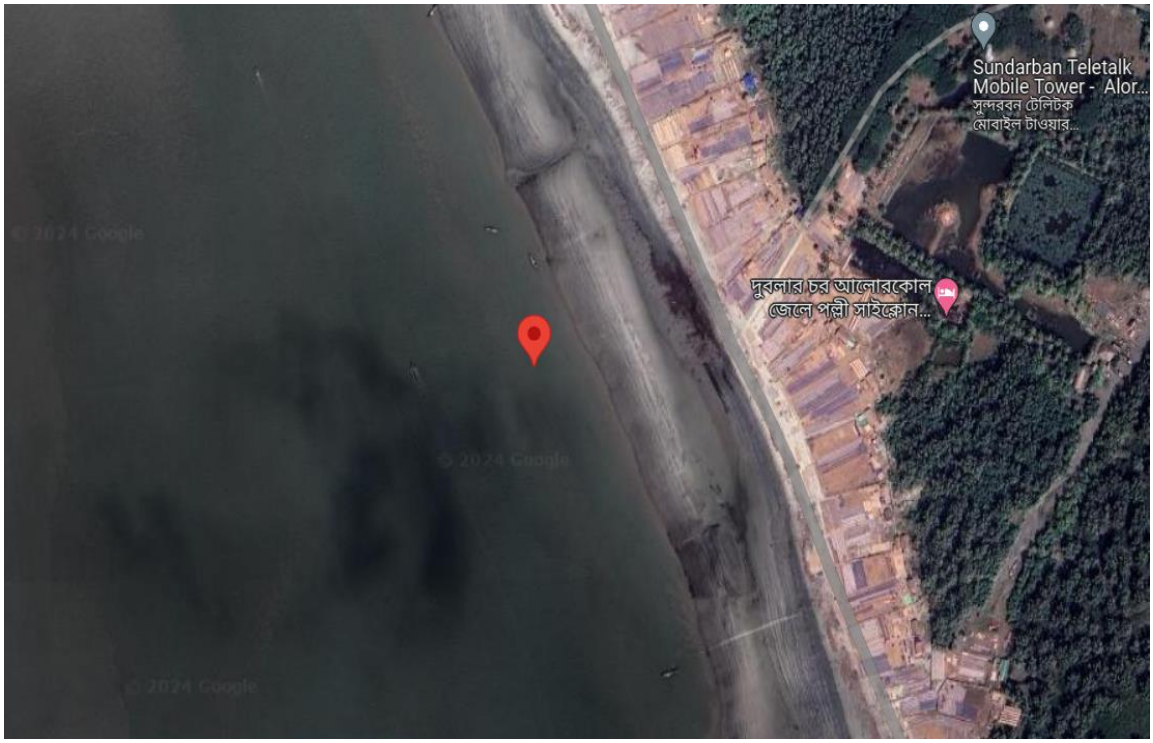

Figure 1: Dublar chor, Coordinates-21.7728° N, 89.5592°E

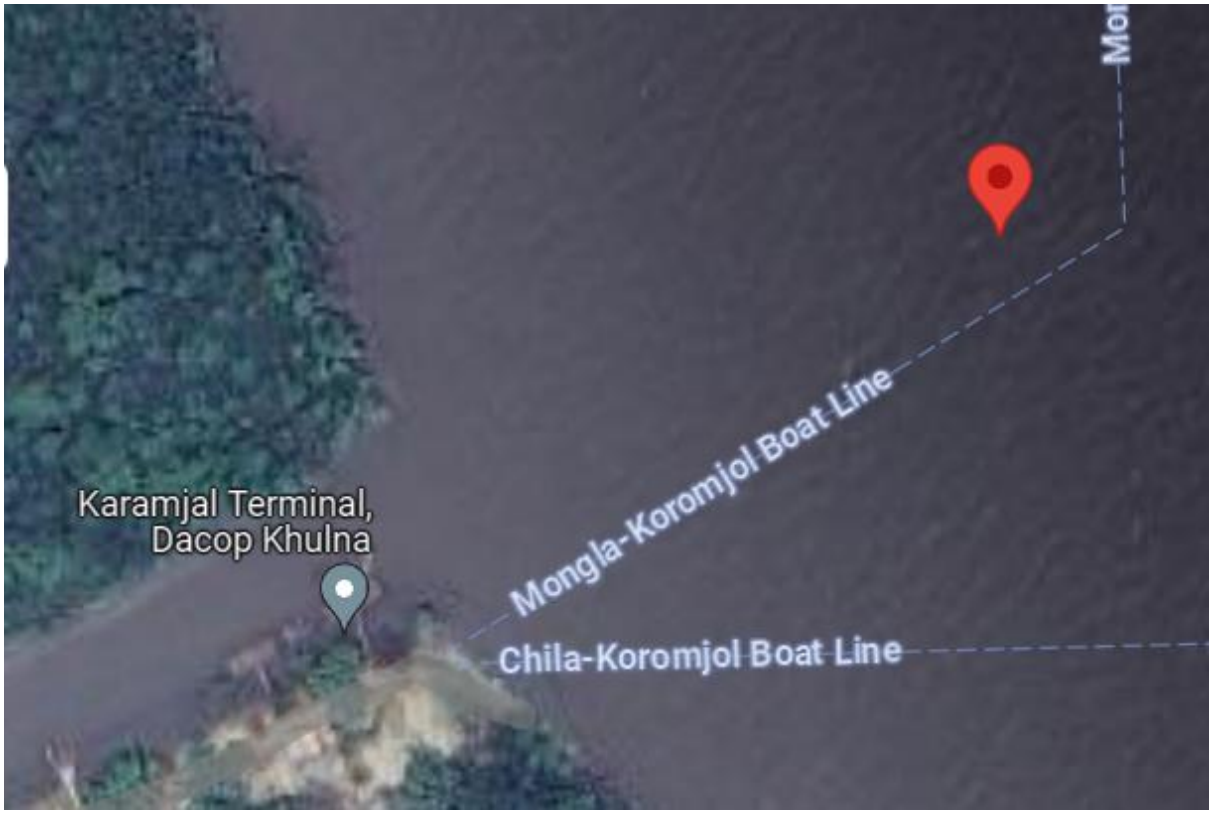

Figure 2: Karamjal, Coordinates- 22.430105 N, 89.593732 E

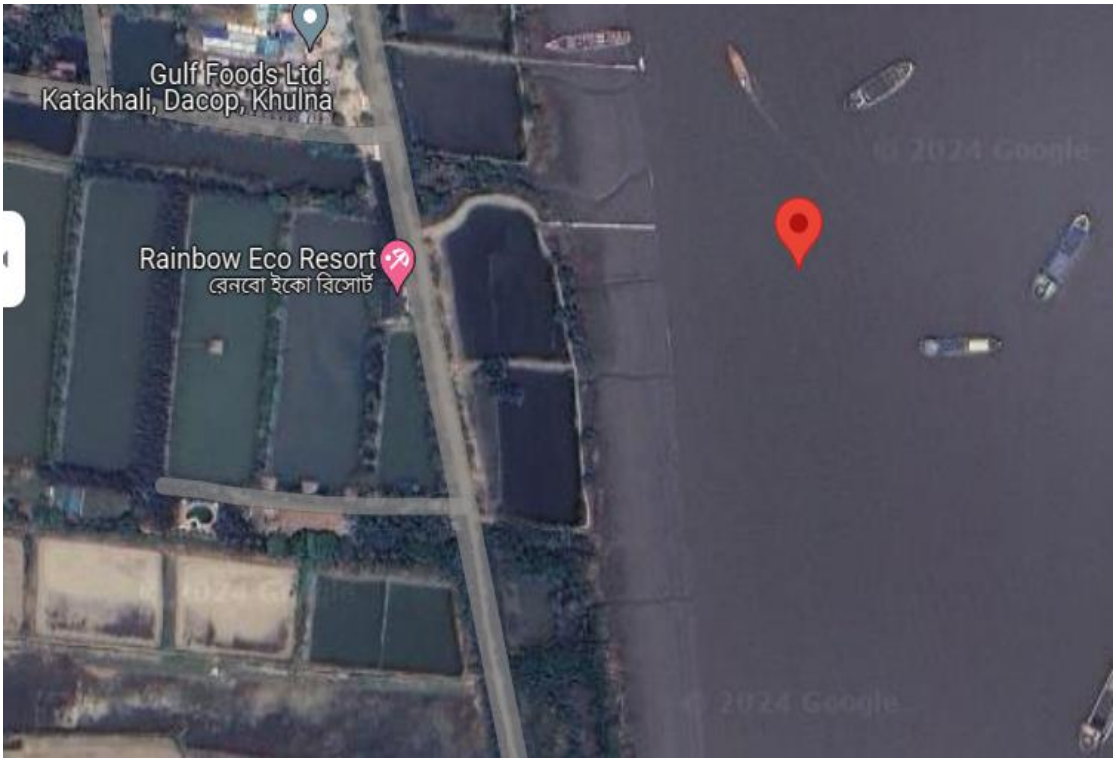

Figure 3: Rainbow Eco Resort, Coordinates-22.4749° N, 89.5817° E

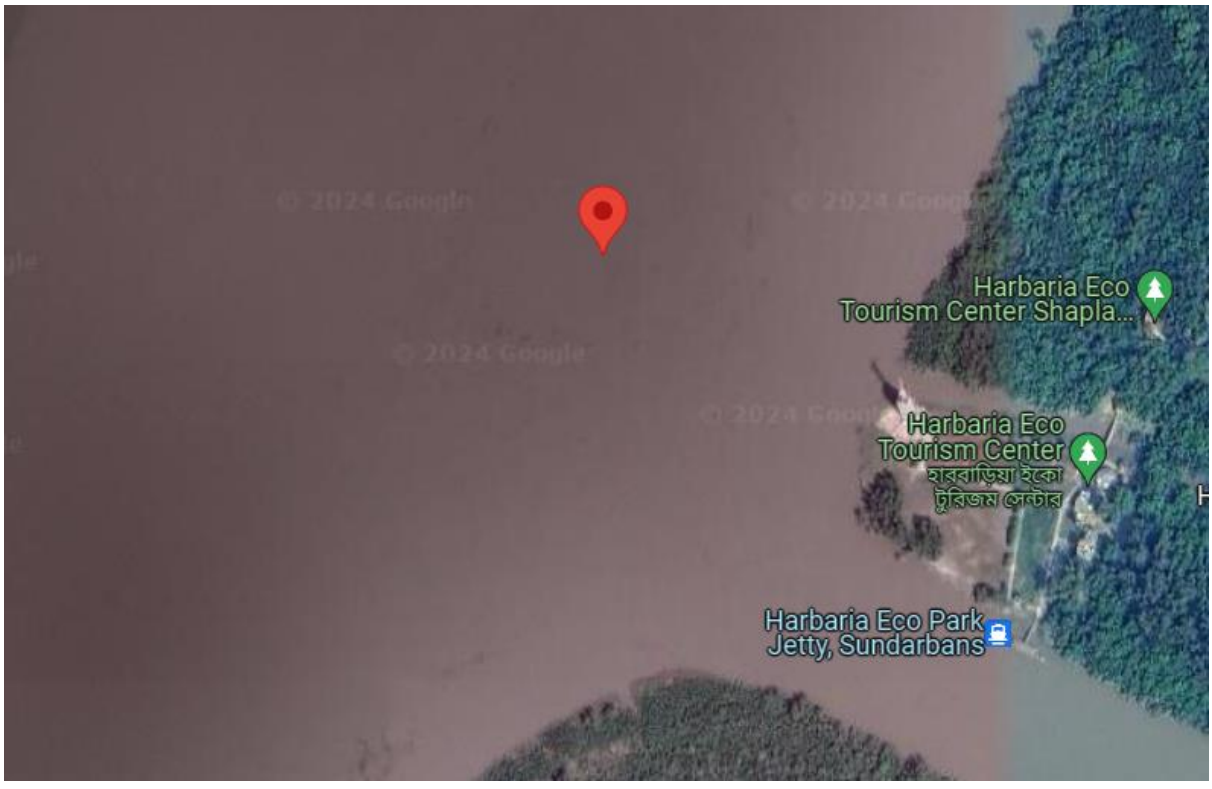

Figure 4: Harbaria, Cooridnates- 22.2979° N, 89.6146° E

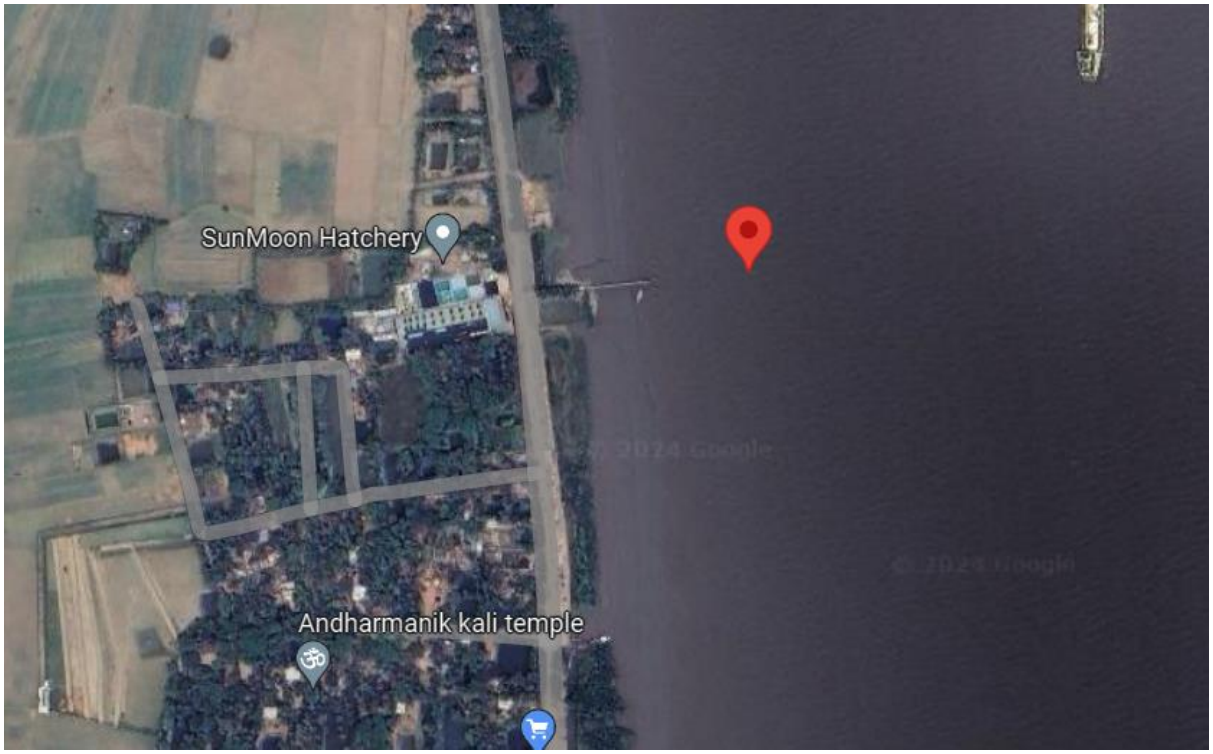

Figure 5: Mongla port, Coordinates- 22°28'59.4"N 89°35'18.7"E

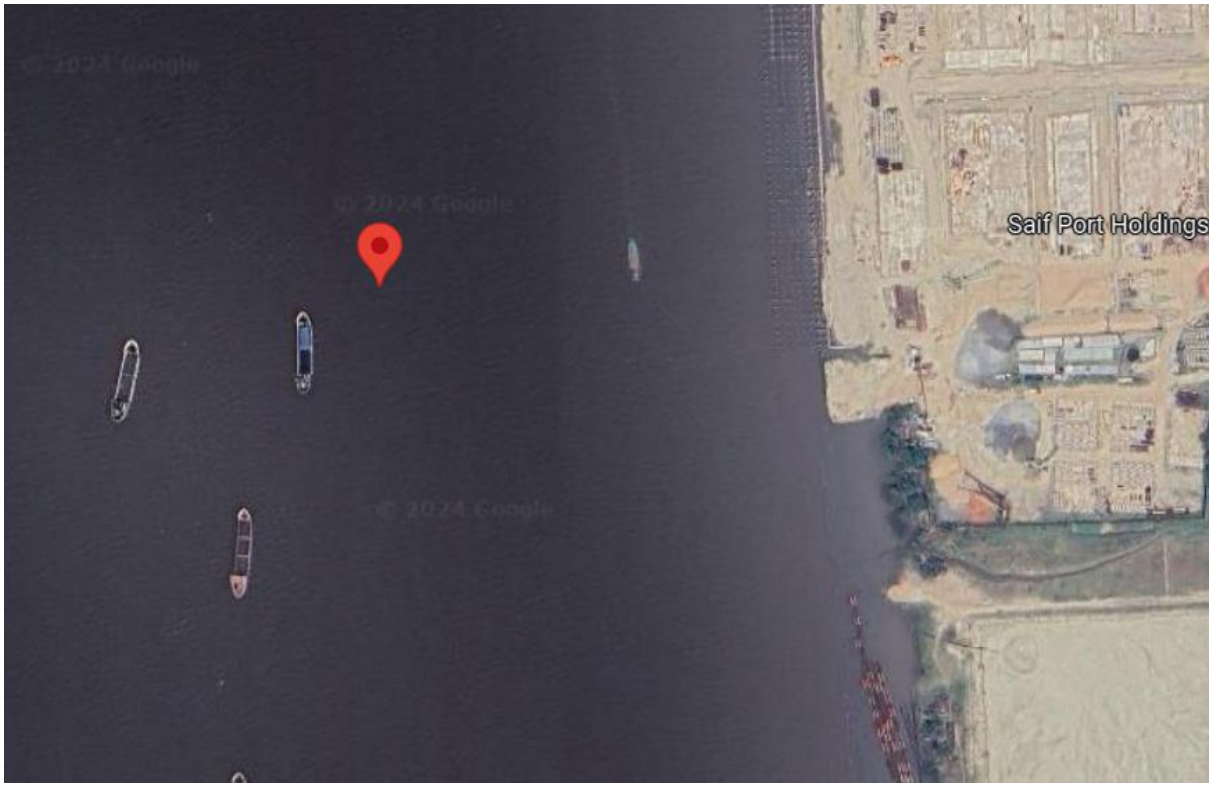

Figure 6: Sunmoon Shrimp Hatchery, Coordinates- 22°29'40.6"N 89°34'56.2"E

Subsequently, all the samples were immediately transferred to a cold chain maintaining the temperature of 4°C. Finally, by maintaining the temperature integrity, samples were transported to BRAC University Microbiology laboratory for further laboratory analysis within 24 hours.

2.3. Measuring Salinity, pH, Dissolved Oxygen (DO), and Temperature

Physicochemical parameters of water and sediments such as salinity, pH, dissolved Oxygen (DO), and temperature were assessed via digital sensors. To assess the salinity, TK285PLUS Pen-type Digital Sensor was used on the same day of sample collection. Similar ex-situ experiments were done for the dissolved oxygen, pH, and temperature. To measure DO, pH, and temperature, AS00370- Gravity: Analog Dissolved Oxygen Sensor and LJ-135 pH meter was used on site.

2.4. Water Sample

The river sample utilized in this study included both direct estuarine river water and sediment samples. The objective of the study was to isolate the target organism, *Pseudomonas aeruginosa*. Dilution was achieved by using 9 mL of autoclaved physiological saline solution (0.85%).

Initially, saline solution at a concentration of 0.85% was prepared and dispensed into individual test tubes, each containing 9 mL, followed by autoclaving for sterilization purposes. For sediment samples, 1 gram of sediment was dispersed into the 9 mL of 0.85% saline solution and vortexed to ensure homogenization.

Subsequently, a 100-microliter aliquot of the river water sample was obtained from the falcon tube using a micropipette and introduced into the saline solution labeled as 10^{-1}.

A tenfold dilution series was then performed, with each dilution step involving mixing by vortexing to ensure thorough homogenization. Upon completion of the dilution process, the samples were spread onto selective media plates for further analysis and isolation.

2.5 Isolation of *Pseudomonas aeruginosa* on Selective Media from river water

The targeted organism was *Pseudomonas aeruginosa*, and for its isolation from both river water and sediment samples, different selective media were used. Since *P. aeruginosa* is known to cause urinary tract infections, HiChrome UTI Agar was selected because it distinguishes UTI-causing bacteria from other fecal and non-fecal bacteria, favoring the growth of the former. Being a gram-negative organism, HiChrome UTI Agar was suitable for isolating *Pseudomonas aeruginosa* from estuarine river water and sediment samples.

For the river water samples, each sample was serially diluted before being spread on HiChrome UTI Agar. Samples from the river water were processed within 18-24 hours of collection. After spreading, plates were incubated at 37°C for 24-48 hours.

2.6. Isolation of *Pseudomonas aeruginosa* on Selective Media from river sediment

For sediment samples, 1 gram of sediments were mixed in a 9 ml of test tube containing 0.85% of saline water, subsequently vortexed for homogenization. Similar to the water sample, each sample was serially diluted and was taken for spreading on the Hi-chrome UTI Agar. After spreading, plates were incubated at 37°C for 18-24 hours.

2.7. Preparation of Pure culture

As soon as the incubation period ended, the spread plates were observed to identify *Pseudomonas aeruginosa*. We looked for smooth, translucent, and large colonies measuring 2-4 mm in diameter on the HiChrome UTI Agar plates. Plates marked with presumptive *P. aeruginosa* colonies were selected for streaking on nutrient agar plates to isolate single and

pure colonies. After streaking on nutrient agar plates, they were incubated at 37°C for 18-24 hours. These processes were similar for both water and sediment samples.

2.8. Variables

The following variables were assessed for the water sample- Dissolved Oxygen, pH, Temperature, Salinity, Total aerobic count in CFU/ml. The Total aerobic bacteria were counted after the 24-48 hours of incubation period via direct plate count.

2.9. DNA extraction

After isolating the suspected target organism, DNA was extracted to confirm it as *P. aeruginosa*. DNA extraction is a process to purify DNA using physical and/or chemical methods from a sample, separating DNA from cell membranes, proteins, and other cellular components. Friedrich Miescher first isolated DNA in 1869 (Gupta, 2019). There are various techniques for DNA extraction using both physical and chemical methods. For our study, we used the boiling method to extract DNA because it was the easiest and less time-consuming method, requiring fewer reagents.

To perform the DNA extraction, 150 µL of 1X TE buffer was taken in a microcentrifuge tube (MCT). Then, a loopful of organisms was added to the TE buffer. The microcentrifuge tube was vortexed briefly for homogenization. Subsequently, it was heated in a dry heater at 100°C for 15 minutes. After heating, the microcentrifuge tubes were centrifuged for 5 minutes at 14,000 rpm. The supernatant was then collected into a separate centrifuge tube and stored at -20°C for further analysis.

2.10 Identification of *Pseudomonas aeruginosa* via Conventional Polymerase Chain Reaction (PCR) and Gel Electrophoresis

Polymerase Chain Reaction (PCR) is a technique used to make numerous copies of DNA or RNA by amplifying them. PCR is used for various purposes, such as paternity checks, identification of bacteria or viruses, and many more. Additionally, there are different kinds of PCR techniques available, such as conventional PCR, reverse transcriptase (RT-PCR), qualitative PCR, and quantitative PCR.

In our study, we used the conventional PCR technique to amplify our bacterial DNA because it was the easiest and most available method in our laboratory. To identify the targeted organism, *P. aeruginosa*, each PCR mixture tube contained 6.5 µl Master mix (Taq polymerase, buffer, dNTPs), 2.5 µl nuclease-free water, 1 µl of *P. aeruginosa*-specific primers that included both forward (PA-SS-F-GGGGGATCTTCGGACCTCA) and reverse primers (PA-SS-R TCCTTAGAGTGCCCACCCG), and 2 µl of previously extracted DNA of the sample isolates (Spilker et al., 2004).

After mixing all the elements, PCR tubes were kept on the PCR Machine. Then the PCR was done for 35 cycles on the following conditions: starting from Initialization at 94°C for 10 mins, Denaturation at 94°C for 40 secs, Annealing at 54°C for 45 secs, Extension at 72°C for 45 secs and ends on Final Extension at 72°C for 10 mins (Wang et al., 2022). After the completion of PCR, agarose gel electrophoresis was performed. Agarose gel electrophoresis is a method used to separate DNA fragments that vary in size. In our study, we used horizontal agarose gel electrophoresis to visualize the DNA amplified in the PCR technique. The amplified PCR products were run in a 1% agarose gel submerged in 1X TBE running buffer at 100 volts in an electrophoretic chamber for 45 minutes. The agarose gels were stained with ethidium bromide; typically, 4 µl of this was used in 100 ml of agarose gel for the clear visualization of the DNA bands of *P. aeruginosa*. In each well of the gel, 4 µl of the PCR products were inoculated. A previously characterized isolate of *P. aeruginosa* from the Pasur River was used as a positive control, and a 100 bp DNA ladder was used to confirm the band size. (Voytas, 1992) .

2.11. Phenotypic features of Antibiotic Susceptibility Test

To study the phenotypical scenario of the PCR Confirmed isolates one of the most used techniques Antibiotic Susceptibility Testing was done. Antimicrobial susceptibility testing (AST) and the detection of the target microorganism in the laboratory both yield crucial phenotypic information regarding an organism's morphological characteristics and antibiotic resistance (Coorevits et al., 2015). In our study, Disk Diffusion Antibiotic Susceptibility Testing was done on Mueller-Hinton Agar to identify if the isolates were carbapenem-producing *Pseudomonas aeruginosa* and cephalosporin-resistant isolates. To identify these isolates, the following five antibiotics were used: meropenem and imipenem from the carbapenem class, cefepime from the 4th generation cephalosporin class, and tetracycline and azithromycin from the macrolide class.

First, the PCR-confirmed P. aeruginosa isolates were cultured on nutrient agar. Then, from the pure culture, the isolates were taken and inoculated into 5 ml of 0.9% saline solution. The solution was vortexed and compared with the McFarland standard of 0.5. After that, freshly autoclaved cotton swab sticks were dipped into the saline solution and then spread evenly on the Mueller-Hinton Agar media. The lawn needs to be evenly spread; otherwise, the diffusion will not occur properly. After the lawn was prepared, the antibiotic discs were placed on the lawned plates and then incubated for 18-24 hours. After the incubation period, the zones of inhibition on the plates were measured to identify the sensitivity or resistance pattern. According to the CLSI and EUCAST standards, zones of inhibition were defined as susceptible (S), intermediate (I), or resistant (R). Various steps were followed to perform the disk diffusion method.(Coorevits et al., 2015)

Name of the Antibiotics	**Sensitive (mm)**	**Intermediate (mm)**	**Resistant (mm)**
Cefepime (CPM30)	>18	15-17	<14

Azithromycin (AZM30)	≥18	14-17	≤13
Tetracycline (TE30)	≥15	12-14	≤11
Meropenem (MRP10)	>19	16-18	<15
Imipenem (IMP10)	>19	16-18	<15

Table1: List of Antibiotics used in this study

Chapter 3

Results

3.1 Total Aerobic Microbial Count and Physicochemical Parameters

In-situ observation of physicochemical characteristics such as DO, salinity, temperature, and pH were assessed from six water samples. The results were summarized in the table. The pH values ranged from 5.8 to 6 indicating acidic to neutral nature of the river. Salinity of the assessed samples ranged from (0.05 - 0.5) %, with downstream regions having slighter salinity than the tourist spot. The temperature of the assessed samples was almost uniform, having a range of (30-31) °C.

Station Code	Station Name	Total aerobic count (CFU/ml)	pH	Salinity (%)	Temperature (°C)	Dissolved Oxygen (mg/L)
A	Dubalr Chor	76250	5.9	0.8	30.9	5.88
B	Shrimp Hatchery	1362500	6	0.5	31.3	4.5
C	Karamjal	1712500	5.8	0.3	30.8	3.9
D	Mongla Port	121,2500	5.9	0.1	30.8	5.1
E	Harbaria	1112500	6	0.4	31.2	4.8
F	Rainbow Eco-resort	98750	5.8	0.4	31.2	4.4

Table 2: List and value of variables (Total aerobic count and physiochemical parameters)

The mean bacteria count is **72479166.67** CFU/ml along with standard deviation of **71030425.55** CFU/ml

3.2 Isolation of *Pseudomonas aeruginosa*

A thorough collection of eight samples was conducted from six different locations along the Pasur River in mid-August 2023 as part of the study period. Four of these samples came from Mongla Port and rural areas, namely Dublar Chor along the river. The other four samples were from the riverfront area next to the popular tourist destination known as Karamjal, an eco-resort, and Harbaria. After the eight samples were analyzed, 64 isolates were determined to be possibly *Pseudomonas aeruginosa* based on the appearance of their colonies on differential media called HiChrome UTI agar. Of the isolates in this cohort, 33 were from the port, tourist hotspot, and local residences, while the remaining 31 isolates were found in other studied locations.

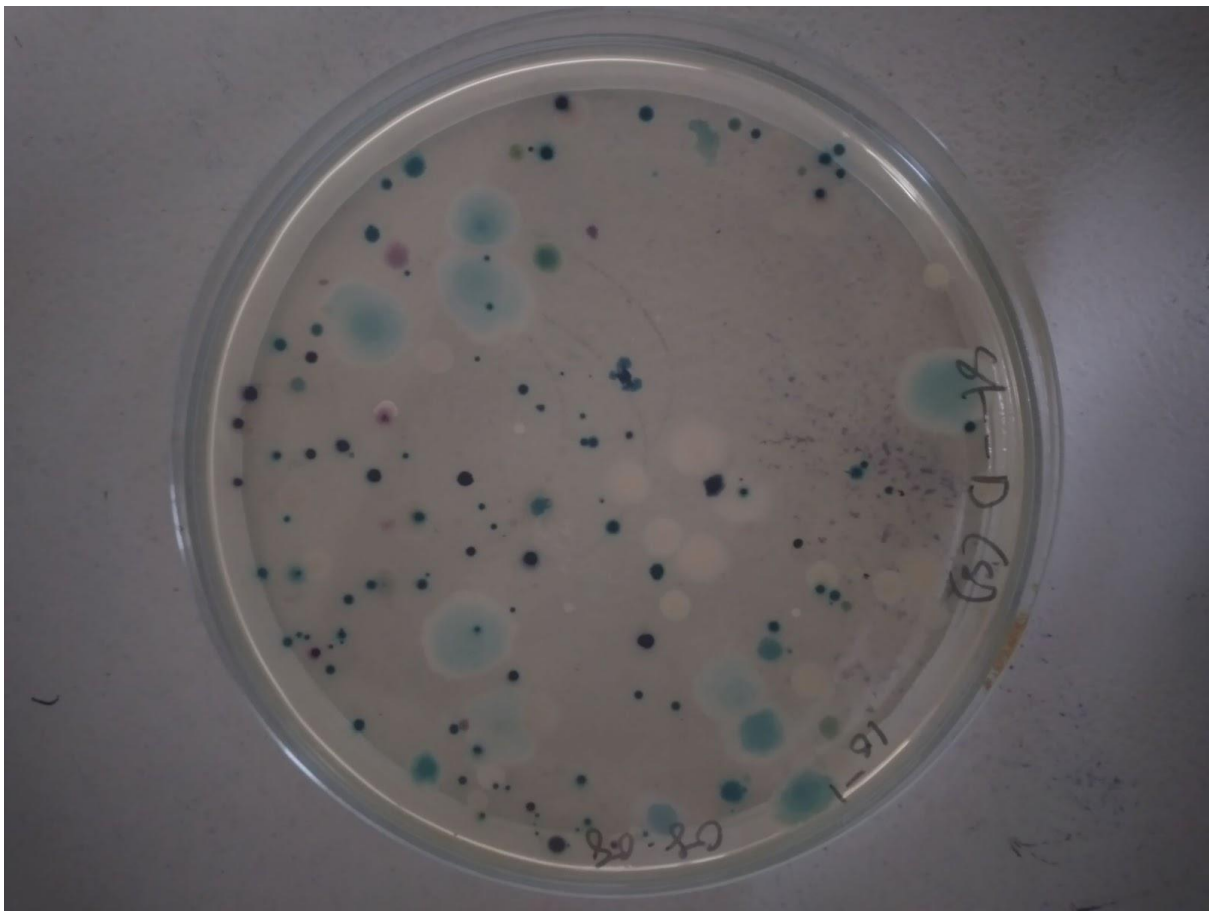

Figure 7: Greenish pigment (Suspected *Pseudomonas aeruginosa*) on Hi-chrome UTI agar media

Station Code	Station Name	Total Aerobic Count
A	Dublar Chor	76250 CFU/ml
B	Shrimp Hachery	136250 CFU/ml
C	Karamjal	1712500 CFU/ml
D	Mongla Port	121,2500 CFU/ml
E	Harbaria	1112500 CFU/ml
F	Rainbow Eco Resort	98750 CFU/ml

From the table, it is evidently clear that Karamjal has the highest total aerobic count (171,250,000 CFU/ml), indicating a significant microbiological load in the river water. With total aerobic values of 121,250,000 CFU/ml and 111,250,000 CFU/ml, respectively, Mongla Port and Harbaria follow closely behind, suggesting notable microbial populations in their water samples. With 13,625,000 CFU/ml, the Shrimp Hatchery has a moderately high microbial load. On the other hand, total aerobic values at Dublar Chor and Rainbow Eco Resort are lower at 7,625,000 CFU/ml and 9,875,000 CFU/ml, respectively, indicating the existence of microbial communities in the river water at these sites, albeit at a relatively lower concentration.

3.3 Confirmation of *Pseudomonas aeruginosa* via PCR analysis and Horizontal gel electrophoresis

Figure 9: Viewing the PCR product for the specific detection of *Pseudomonas aeruginosa.*

After the completion of Polymerase Chain reaction assay and subsequent Horizontal gel electrophoresis, the results were examined under a UV lamp. 54 of the 63 probable isolates that were examined during the experiment period, which began in mid-August 2023, had their identities as *Pseudomonas aeruginosa* verified by gel electrophoresis of PCR results. 21 of these confirmed isolates were found close to the mangrove forest's tourist area (Karamjhal), Harbaria and Dublar Chor, while the remaining 6 came from Mongla Port, 8 from the shrimp hatchery, and 12 from Rainbow Eco Resort. The attached above picture depicts the Horizontal agarose gel electrophoresis under a UV illuminator.

3.4. Resistance Pattern of *Pseudomonas aeruginosa*

This study examined the antibiotic susceptibility of 39 confirmed *Pseudomonas aeruginosa* isolates to cefepime, imipenem, meropenem, tetracycline, and azithromycin. The percentage of isolates classified as resistant, intermediate, or susceptible is displayed below:

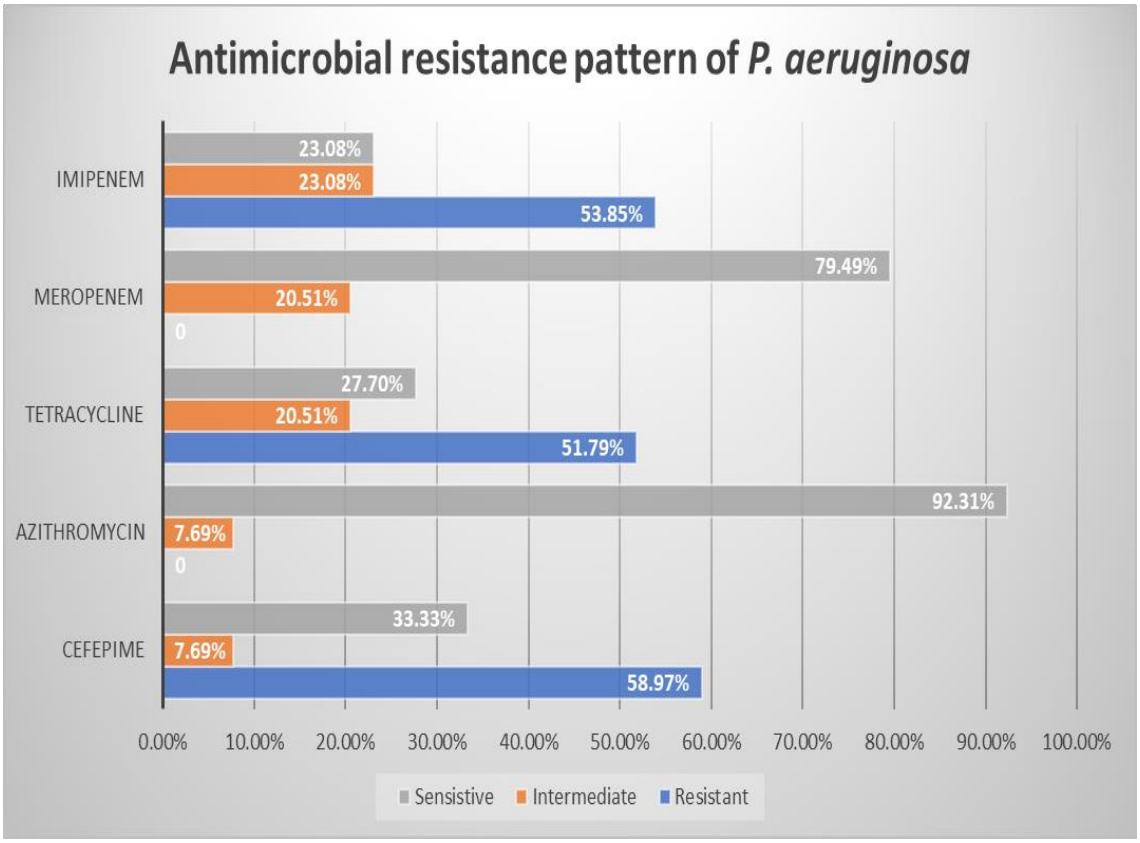

Figure10: Susceptibility Pattern of *P. aeruginosa* against beta-lactam and macrolide antibiotics.

The findings indicated a high degree of resistance to tetracycline (51.79%) and cefepime (58.97%). Positively, azithromycin showed excellent susceptibility (92.31%) and a very low resistance rate (0%). Promising outcomes were also displayed by meropenem, which had a high susceptibility rate (79.49%) and no resistance. However, the resistance percentage to imipenem was 53.85%, with an intermediate rate of 23.08%, followed by an intermediate rate of 20.51% for meropenem.

Chapter 4

Discussion

In this study, we examined the presence of *Pseudomonas aeruginosa* at multiple points of the Pushur River, a significant waterway adjacent to the Sundarbans mangrove forest, known for its diverse ecosystem and as a popular tourist destination. Along with microbiological analysis, physicochemical parameters such as pH, temperature, salinity, and dissolved oxygen were also measured to understand the adaptation of bacteria in the recreational water.

The high aerobic microbial count at Karamjal (171,250,000 CFU/ml) suggests that this popular tourist site has a significant microbiological load, posing potential health risks to both local residents and visitors. Exposure to contaminated environments, including lakes, pools, animals, and international travel destinations, raises the likelihood of infection (Ayi, 2015). Mongla Port and Harbaria also exhibited notable bacterial counts, indicating that these regions may require targeted monitoring to ensure public safety. Even though spot near Dublar Chor, a rural locality results in low aerobic microbial count, it showed greater salinity than the port, and popular tourist spot. This might not withstand the fact that salinity reduces the microbial load (Lew et al., 2022).

Microbiological analysis in the laboratory revealed a substantial presence of *Pseudomonas aeruginosa*, with 54 out of 63 possible isolates confirmed through PCR and horizontal gel electrophoresis. *Pseudomonas aeruginosa* is a prevalent cause of bacterial infections linked to recreational water activities – swimming or water sports (Nursyirwani et al., 2019). Infections from this pathogen can arise through skin contact, ingestion, or exposure to contaminated water, such as poorly disinfected indoor swimming pools. An example is *Pseudomonas aeruginosa* folliculitis, a skin infection commonly associated with recreational water activities ("Poor-Quality Water in Swimming Pools Associated with a Substantial Risk of Otitis Externa

Due to Pseudomonas Aeruginosa.," 2004). The antimicrobial susceptibility test results of *Pseudomonas aeruginosa* from the Pasur River pose a notable risk to the local population. The high resistance rates to tetracycline (51.79%) and cefepime (58.97%) suggest prevalent antibiotic resistance, likely exacerbated by environmental contamination. Notably, azithromycin showed exceptional susceptibility (92.31%) with no resistance, and meropenem demonstrated promising results (79.49% susceptibility), suggesting alternative therapeutic options. However, the substantial resistance to imipenem (53.85%) and its intermediate resistance (23.08%), alongside meropenem's intermediate rate (20.51%), underscore concerns regarding carbapenem efficacy. Given the river's usage by local residents for bathing and its popularity among tourists, the distribution of resistant *P. aeruginosa* strains could pose severe health risks. Further, the presence of numerous sources of contamination such as sewage systems, farms, and wastewater might contribute to the spread of antibiotic-resistant bacteria. These findings suggest the necessity for water quality monitoring and critical antimicrobial stewardship to mitigate the risks posed by resistant pathogens in such recreational environments. The epidemiology of *Pseudomonas aeruginosa* in recreational river water highlights the potential risks to human health, especially for those who take part in recreational activities and bathe daily in this river, particularly the rural population. Hence, it is noteworthy to mention the need for continued surveillance, improved sanitation practices, and implementation of preventive measures to reduce the risks associated with waterborne pathogens in the Pasur River and similar ecosystems.

Chapter 5

Limitation

This whole study was conducted on a particular river, the Pasur River, adjacent to the Mangrove Sundarbans of Khulna. If all the rivers adjacent to the Sundarbans were taken into

consideration, a stronger interpretation could be made. The accurate prevalence of *Pseudomonas aeruginosa* could be assessed undoubtedly. Furthermore, the in-situ experiment along with the ex-situ experiment for the salinity tolerance test could shed light on the survival pattern of our targeted bacteria, *Pseudomonas aeruginosa.*

In this study, only microbiological analysis was performed, but if the study focused on both microbiological and chemical analyses, a whole scenario could be analyzed. In that scenario, chemical elements, toxic materials, and microorganisms could all be identified from the study spots. Additionally, the unavailability of different antibiotics from different classes means that resistance patterns cannot be fully analyzed. There must be other classes of antibiotics for which the isolates may show a resistant or sensitive pattern. Moreover, due to the unavailability of the materials to identify whether the 54 isolates were pathogenic or not, we could not interpret our results on the basis of pathogenicity. If antibiotic-resistant isolates associated with pathogenicity were found, it could have given more immediate concern to install or optimize the water treatment system by the authorities.

Chapter 6

Conclusion

Our study verifies that *Pseudomonas aeruginosa* is present in the Pasur River. High bacterial counts indicate a possible health risk for rural people and even tourists participating in recreational activities, especially at well-known tourist destinations like Karamjal and local areas like Dublar Char. Areas with lower salinity levels may be more vulnerable to bacterial contamination, as indicated by the negative association found between salinity and bacterial counts. Our results highlight the necessity of better water quality management and continuous monitoring not only in recreational areas but also in overall river water quality where anthropogenic activities are frequent.

Local government agencies should prioritize enforcing good sanitation procedures and educating the public about the dangers of waterborne infections to safeguard public health. To decrease the number of opportunistic infections in recreational waters, further study is required to identify the sources of contamination. This study lays the groundwork for upcoming initiatives to provide secure and long-lasting recreational settings.

Chapter 7

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