"*Pseudomonas aeruginosa* characterization and analysis based on its antimicrobial resistant pattern collected from hospital wastewater and adjacent community water."

Submitted By

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A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE BACHELOR IN SCIENCE FOR MICROBIOLOGY

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Declaration:

This is the declaration of submission of the thesis research work to complete our bachelor's degree which is completed by our own original research work.

● This thesis does not contain any content which is previously published nor is it produced by a third party. It is cited correctly with accurate referencing.
● The thesis does not contain any material that has been accepted or submitted by any other degree or diploma at a university or institute.
● We have identified all sources of help and acknowledged the assistance.

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Index

Table of contents

<u>Contents</u>	<u>Page no.</u>

List of acronyms

- ★ **ATCC= American type culture collection**
- ★ **Bp= Base pairs**
- ★ **bla= Beta-lactamase gene**
- ★ **CLSI= Clinical and Laboratory Standard Institute**
- ★ **CTX-M= Active on cefotaxime**
- ★ **DNA= Deoxyribonucleic acid**
- ★ **EDTA= Ethylene diamine tetra acetic acid**
- ★ **ESBL= Extended spectrum β-lactamase**
- ★ **Et al. =et alia (and others)**
- ★ **MBL= Metallo-β-lactamase**
- ★ **MDR= Multidrug resistant**
- ★ **OXA= Oxacillinase**
- ★ **PCR= Polymerase chain Reaction**
- ★ **SHV=Sulfhydryl variable**
- ★ **TBE= Tris-Borate-EDTA**
- ★ **TE= Tris-EDTA**
- ★ **TEM= Temoniera**
- ★ **NDM= New-Delhi Metallo Beta-Lactamase**
- ★ **VIM= Verona integron-encoded metallo-β-lactamase**
- ★ **HWW= Hospital Wastewater**
- ★ **ICU= Intensive Care Unit**

Abstract

Introduction: The main purpose of this study is to identify the multidrug-resistant genes from some specific microorganisms. Among them, our selected organism was *Pseudomonas aeruginosa*. The highly known Carbapenem and beta-lactamase-producing *Pseudomonas aeruginosa* was given priority in this study to test how much resistance and sensitivity is related to this organism in the context of Bangladesh, particularly in the highly polluted and unhealthy conditions of Dhaka city. To conduct this study different water samples from hospital waste and its surrounding selected community tap has been collected. Hospital wastewater is one of the major sources of antibiotic-resistant pathogens that is becoming an alarming concern daily.

Method: This study has been from January 2023 to June 2023. A total of 54 confirmed *Pseudomonas aeruginosa* isolates were suspected from 78 isolates collected from hospital wastewater and adjacent community tap water**.** To identify exact isolates of *Pseudomonas aeruginosa, the* phenotypic technique was used. Also, the Antibiotic Susceptibility test helped to detect which antibiotics from different generations are susceptible or resistant against the specific isolate. Since biochemical procedures are less accurate than PCR and need additional resources and time besides these methods, we did further PCR tests to identify the resistant gene of ESBL (Extended spectrum β-lactamase) and MBLS (Metallo-beta-lactamase) after getting the multidrug-resistant isolates. ATCC strains are used as positive controls.

Result: There was a total of 78 samples taken from the nearby community water and the three hospitals, and 54 of those 78 samples contained *Pseudomonas aeruginosa* isolates that could be confirmed. There are 69.23% confirmed isolates. These 54 confirmed isolates showed resistance to different 12 antibiotics. The hospital water showed the most resistance towards amoxyclav (94%) and then towards cefixime and ceftriaxon (82%). Whereas, the community water isolates resistance was quite similar. The highest resistance they showed towards amoxiclav (90%) and the second highest is cefixime (82%). Among all these isolates 7 isolates were chosen that showed the most resistance and further identification of Multidrug Resistance were done. Among them, for Bla-CtxM and Bla-SHV, two isolates from the June sample had positive results, or

28.5% of the total and one isolate from the June sample tested positive for Bla-oxa 48, or 14.2% of the total.

Conclusion: The quality of drinking water, distribution lines, and waste management of hospital water have now become key concerns for the spread of antibiotic resistance among various species, particularly for *Pseudomonas aeruginosa*. So, the goal of this study is to find out if this multi-drug-resistant bacteria is present in the wastewater of the hospital and its adjacent community water.

Introduction:

Pseudomonas aeruginosa is well known for its adaptability and capacity to flourish in a variety of conditions (*Are Antibiotic-Resistant Pseudomonas aeruginosa Isolated from Hospitalised Patients Recovered in the Hospital Effluents? - ScienceDirect*, n.d.) It is a gram-negative bacterium. Another important characteristic of *P. aeruginosa* is its motility. Due to the existence of polar flagella, the bacterium is extremely motile and can move quickly through liquids, facilitating its colonization of diverse surfaces (Kazmierczak et al., 2015). *Pseudomonas aeruginosa* is an aerobically metabolizing organism, which means it needs oxygen to thrive and multiply (de Sousa et al., 2021). This metabolic feature helps it survive in a variety of conditions. The bacteria frequently produce pyocyanin. This pigment is not only responsible for the distinctive greenish color, but it may also be poisonous to host cells. Identification of this bacterim can be done through this pigment as pyocyanin producing strains are more virulent towards host cells than non-producing pyocyanin (Abdelaziz et al., 2023). *Pseudomonas aeruginosa* is able to create biofilms, which are bacterial populations encased in a barrier of extracellular matrix (Thi et al., 2020). The bacterium's capacity to resist antibiotics and endure in varied settings is facilitated by biofilms (Thi et al., 2020).

Infections caused by *Pseudomonas aeruginosa* can affect many different body systems, including the skin, soft tissues, urinary tract, and respiratory system. As it is an opportunistic pathogen that can infect people, especially those who have compromised immune systems or underlying medical disorders (Loveday et al., 2014). That means that they can affect patients who are admitted to the ICU because they are heavily immune compromised (*Pseudomonas and Related Infections - Infectious Diseases*, n.d.). *Pseudomonas aeruginosa* infections are a serious problem, particularly in hospital settings, where they can result in serious and occasionally fatal illnesses. *Pseudomonas aeruginosa* is frequently linked to healthcare-associated illnesses, particularly in hospitals and other medical facilities. Infection is more likely in patients who have invasive medical devices such as catheters and ventilators. People with cystic fibrosis are especially vulnerable to developing persistent *Pseudomonas aeruginosa* lung infections, which can compromise lung function (*Pseudomonas Aeruginosa Biofilms in Disease - PMC*, n.d.).

P. aeruginosa is a widespread hospital-acquired pathogen of the respiratory and urinary tracts in every department in the hospital, but especially in the intensive care units, where it is responsible for 15% of healthcare-associated infections. (Slekovec et al., 2012). Multiple drug-resistant *P. aeruginosa* strains have been related to hospital outbreaks, according to numerous reports. *P. aeruginosa* infections are extremely difficult to treat because of the organism's intrinsic resistance to numerous antibiotic classes as well as its capacity to acquire resistance to almost all effective drugs throughout therapy. Considering how frequently antibiotics are used in hospitals, this multidrug-resistant bacteria is most likely to have its roots there. Although horizontal gene transfer can also result in antibiotic resistance, chromosomal changes are the main source of it in *P. aeruginosa*. Also, this bacterium can cause keratitis in people who wear contact lenses and community-acquired illnesses like folliculitis and ear infections from recreational contact with bacterially contaminated water. Even though the exact cause of the contamination is unidentified it is probable that the natural surroundings, such as water and soil, function as a storage for the *P. aeruginosa* strains that affect persons with symptoms of cystic fibrosis.

Antimicrobial resistance has become one of the serious public health issues of this century. Excessive consumption and incorrect application of antibiotics is the main reason for the development of antibiotic resistance. The antimicrobial selection challenge is very severe in acute care hospitals. For example, 20-30% of European patients are being given antibiotics throughout their hospitalization. (Hocquet et al., 2016). As a consequence, hospitals are major places for the growth and expansion of antimicrobial-resistant bacteria (ARB) and function as their natural surroundings. ARB leaves hospitals through wastewater systems in addition to colonized patients. If healthcare facility effluents are dumped directly into the wastewater network without first being treated, this situation could get worse. As a result, enormous volumes of antimicrobials are dumped into wastewater, exerting constant selection pressure on the ARB. Disinfectants with antibacterial properties and heavy metals may both help ARB remain persistent in the wastewater microbiota. Antimicrobial variation promotes the horizontal transfer of resistance genes from one to another species.

The basis of reporting on resistance to antibiotics in hospital wastewater is the identification of resistance genes for antibiotics or bacterial cultures. Because of the substantial amount of

research studies on this topic and the variety of strategies employed to identify ARB, our project concentrated on the most prevalent multidrug-resistant bacteria (MDRB) recovered in hospital wastewater and the possible impacts of their release into the wastewater supply on ARB the study of epidemiology.

Due to the production and spread of bacteria resistant to drugs that have acquired novel mechanisms of resistance, antimicrobial resistance continues to represent an obstacle to our capacity to treat prevalent diseases. The WHO discovered 32 antibiotics under clinical trials that address the WHO essential pathogen list, just six of which were designated as novel in 2019. (*Antimicrobial Resistance*, n.d.)   In the United States in 2017, multidrug-resistant bacteria *Pseudomonas aeruginosa* caused approximately 32,600 infections across hospitalized patients and 2,700 deaths. (*Pseudomonas Aeruginosa Infection | HAI | CDC*, 2023). The Antibiotic Resistance Laboratory Network operated by the Centres for Disease Control and Prevention (CDC) found an epidemic of carbapenem-resistant *P. aeruginosa* with an uncommon kind of resistance in 2018. (CDC, 2022).

Hospitalized patients who have *Pseudomonas aeruginosa* bacteremia face significant difficulties. But as of now, it's unknown what causes mortality in Chinese inpatients with *P. aeruginosa* bacteremia. A retrospective multicenter study examined 215 individuals with culture-confirmed *P. aeruginosa* bacterial infections in five Chinese healthcare centers from 2012 to 2019. (Zhang et al., 2020). Over the research period, 61 (28.4%) of the 215 patients with *P. aeruginosa* bacterial infection died. The independent risk variables of death were coronary artery disease blood transfusions (OR=5.855, P0.001) and carbapenem-resistant *P. aeruginosa* (CRPA) phenotype (OR=4.485, P=0.038), according to a logistic multivariable modeling approach. In addition, both the CRPA and MDRPA (Multi-drug Resistant *Pseudomonas. aeruginosa)* phenotypes of *P. aeruginosa* were shown to be strongly related to 5-day survival. (Zhang et al., 2020).

Furthermore, Gram-negative infections tend to exhibit carbapenem resistance, which is a persistent problem in worldwide public health. This kind of antibiotic resistance is generating major outbreaks and drastically reducing treatment options, especially when it is caused by genes expressing transferable carbapenemases. Beta-lactam antibiotics, which include penicillins,

cephalosporins, monobactams, and carbapenems, are among the most commonly used antibiotics globally. (Meletis, n.d.). The penicillin-binding proteins (PBPs), which are responsible for creating the cell walls of bacteria, connect to and disable all of them in the same way.

Moreover, each one of them has a beta-lactam ring. Beta-lactams having broad antibacterial effects, such as carbapenems, are the strongest inhibitors against Gram-negative as well as Gram-positive bacteria. They have a distinct molecular composition because they include both a carbapenem along a beta-lactam ring. It's important to note that some animals naturally resist carbapenems. Clinically significant bacteria rarely have intrinsic carbapenem resistance; instead, the majority of them develop resistance to carbapenem either through mutational pathways or horizontal gene transfer.

As mentioned above, one of the most common causes of nosocomial infections is *Pseudomonas aeruginosa* which shows antibiotic resistance for many reasons. One of them is horizontal gene transfer. Based on genome sequencing, the vast majority of *P. aeruginosa* strains contain a substantial number of accessory genes clustered in genomic islands. These genes are required for *P. aeruginosa* to colonize new ecology settings that have significant antibiotic usage, including hospitals, or to survive host infection by providing pathogenicity factors. Pathogenicity island 1 (PAPI-1) of *P. aeruginosa* contains multiple potential virulence factors, including toxins, biofilm genes, and antibiotic resistance features.(Dangla-Pélissier et al., 2021). The process driving this change is currently unclear, although the integrative and conjugative element (ICE) PAPI-1 may be transferred horizontally using conjugation with a specific GI-T4SS.

In people with weakened immune systems, *Pseudomonas aeruginosa* is a human-borne pathogen that leads to severe chronic as well as acute diseases. Its capacity to form antibiotic-resistant biofilms is a factor that accounts for its widely recognized persistent nature in medical environments. A biofilm is a structure that is composed mostly of autogenic extracellular polymeric components that serve as a framework to keep bacteria intact on surfaces, shield them from external stressors, inhibit phagocytosis, and eventually allow them to colonize and survive over time. The World Health Organization identified *P. aeruginosa* as one of the most dangerous bacteria in 2017 and designated it as the top pathogen for novel antibiotic research and

development. (Thi et al., 2020). Since *P. aeruginosa* is extremely adaptable and innately resistant to drugs, conventional antimicrobial treatments such as antibiotics typically do not have enough efficiency, resulting in increased mortality. The ability of *P. aeruginosa* to create biofilms, which protects them from environmental stressors and inhibits phagocytosis, allows them to colonize and remain for lengthy periods of time, limiting therapy for these infections. This capacity is supported by quorum sensing, an effective cell-to-cell interaction method employed by *P. aeruginosa*'s microbial populations. As a result, highly organized biofilms can form, which are common in persons with chronic infections such as chronic bronchitis, chronic infections of wounds, and chronic rhinosinusitis. Biofilms are considered to cause more than 90% of chronic wound infections, which contribute considerably to poor wound healing. Chronic infections of wounds impacted roughly 6.5 million individuals in the United States resulting in a substantial health-care burden and damaging economic effects estimated at more than US$25 billion per year (Thi et al., 2020). As a result, identifying *P. aeruginosa* infections as soon as feasible is crucial, before biofilm development improves the bacteria's sensitivity to drugs.

Material and Method:

Sample Collection: Samples were collected from three hospitals- the National Institute of Cancer Research and Hospital (NICRH), Dedicated COVID-19 Hospital (DNCC), and Bangladesh Shishu Hospital. Also, the community water was collected from the adjacent three different areas of the hospital which were 200 meters distant places. The samples were collected three times in a month and all of them were collected from January 2023 to June 2023. Every week, specimens were delivered to the MNS Department's research lab at BRAC University. Within six hours samples were processed.
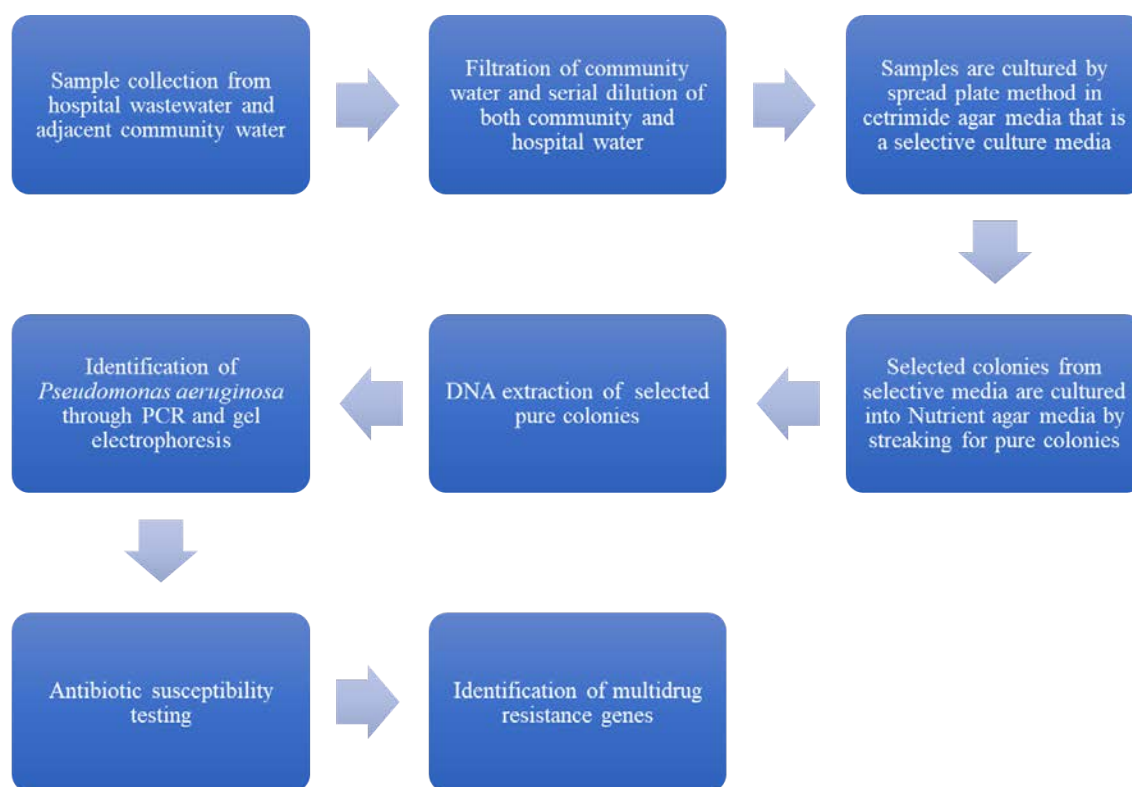
Workflow:

Figure-1: The Workflow of The Working Pattern in the Laboratory

Processing of Sample:

Hospital samples were directly processed by serial dilution. To reduce a dense population of cells to a suitable concentration, a series of successive dilutions is utilized. Serial dilution was conducted from 10^{-1} to 10^{-7}. From two dilution factors 10^{-2}, 10^{-4}, and from direct raw sample Spread Plate Technique was applied. Also, samples were cultured into Cetrimide agar media. This agar media is selective for *Pseudomonas aeruginosa* which helps to grow this specific bacterium. Moreover, this media enhances the production of *Pseudomonas* pigments. They are-

pyocyanin and pyoverdin. The colonies from this bacterium on this agar media will show a blue-green pigmented color under the UV light. The culture plates were kept in the incubator at 37°C for 24 to 48 hours after doing the Spread Plate technique. Then the plates were observed under the UV light which will show the color. If the colonies glow in blue-green color it means they are *Pseudomonas aeruginosa.*

On the other hand, the community water samples were processed with the Membrane Filtration Method. 100ml of samples were filtered with 0.45-µm of Whatman filter paper and transferred into a sterilized falcon which is filled with TSB (Tryptic Soy Broth). Tryptic Soy Broth (Soybean-Casein Digest Medium) is a liquid enrichment media that supports the development of a wide range of microorganisms especially facultative anaerobic bacteria like *Pseudomonas aeruginosa*. After that, A shaker incubator was used to keep the samples with TSB enrichment media overnight at 37^0 C. Next, it was processed for serial dilution from 10^{-1} to 10^{-7} From two dilution factors 10^{-2}, and $10^{-4,}$ and from a direct raw sample Spread Plate Technique was applied. Also, samples were cultured into Cetrimide agar media which is selective for *P. aeruginosa.* Next, the culture plates were kept in an incubator at 37°C for 24-48 hours.

Following observation, particular colonies are chosen that show a blue-green glow under UV light from each Cetrimide agar media plate. To acquire pure colonies of the specified isolates, the chosen colonies were cultivated on Nutrient Agar media using the Streak Plate Technique. Later, the method of boiling was used to extract DNA from single colonies of the selected isolates. The DNA extraction of isolates will also be subjected to PCR, which is essential for an accurate identification of *Pseudomonas aeruginosa*.

DNA Extraction: DNA extraction is a procedure that helps to purify DNA from cell membranes, debris, proteins, and other components by chemical or enzymatic methods. It is a crucial technique to find the DNA from a specific organism. This is also called the purification method because it removes cell debris and unwanted materials. For this, the boiling method was applied because it is very rapid, effective, cheap, and less time-consuming than other standard ones. It has several steps such as

- From the NA plate, 2-3 pure isolated colonies were selected with a sterile loop to

continue the further method.

- It requires inoculating the colonies to 150µl 1X TE buffer which means in a microcentrifuge tube, 10 mM Tris-HCL, 1 mM EDTA, and pH 8.0 these products are mixed up.
- Need to vortex the solution so that the colonies can mix with the buffer properly.
- The microcentrifuge tubes were boiled in the water bath for 15 minutes at 100°C.
- After 15 minutes the tubes were centrifuged for 5 minutes at 13,000 rpm.
- Then the supernatant was separated carefully from the pellet to another sterile microcentrifuge tube and stored the DNA at -20°C.

PCR (Polymeric Chain Reaction):

PCR (Polymeric Chain Reaction) is a technique for amplifying millions or billions of copies of DNA segments. The replication of DNA by enzymes is the basis of this process. In PCR, primer-mediated enzymes are employed to amplify a small part of DNA. DNA Polymerase plays the role of generating new complementary DNA strands to the template of DNA. The only pre-existing 3'-OH group will be added by the DNA polymerase. Therefore, a primer is crucial. More nucleotides are connected to the 3' prime end of DNA polymerase.

Primer Name and Sequence	Target organism	Annealing temperature	Base pair	Reference
PA-SS-R TCCTTAGAGTGCCCACCCG	*Pseudomonas aeruginosa*	58°C	956	(Spilker et al., 2004)
PA-SS-F GGGGGATCTTCGGACCTCA				

Preparation of PCR Product:

- First, we need to add Master- Mix which is 6.5µl.

- Then the Nuclease free water is required which is 2.5µl.

- The two primers were added which are PA-SS-R (Reverse Primer) = 1µl and PA-SS-F (Forward Primer) = 1µl

- After mixing them up DNA Template was added which was 2µl

  Total 13µl.

Each PCR tube contains 13µl products. 11µl PCR mix and 2µl DNA template.

All the products needed to mix properly.

After that, the products undergo the PCR process. (Spilker et al., 2004).

PCR Condition for *Pseudomonas aeruginosa*:

Initial Denaturation: 95°C for 2 minutes.

Denaturation: Denaturation occurs when a reaction mixture gets heated to 94°C for approximately 20 seconds. Single-stranded DNA arises when the hydrogen bonds connecting the two strands of DNA are broken. At the moment, individual strands of DNA are used as a template to produce new strands of DNA. To guarantee the separation of both strands, the temperature must be maintained over a longer period of time. They are the beginning point for the synthesis of DNA.

Annealing: The reaction temperature is decreased to 58° C after about 20 seconds. At this moment, the primers attach to their appropriate sequences on the template DNA. Primers are single-stranded DNA sequences that range in length from 20 to 30 nucleotides. They are the beginning point for the synthesis of DNA. As the two separated strands run in opposite directions, two primers are used: a forward primer and a reverse primer.

Elongation: At this stage, the temperature is raised to 72°C for 40 seconds. The DNA polymerase enzyme joins the nucleotides to the 3' end of the primer. As a result, the DNA expands in the 5' to 3' direction. Under optimum conditions, DNA polymerase generates around 1000 bp every minute. It attaches to the primer and continues to add nucleotides to the single strand of DNA. As a result, a two-stranded DNA molecule is created. These processes are repeated 20-40 times in order to produce a high number of DNA sequences of interest in a short period of time.

Final Extension: It is conducted for 1 minute immediately following the last PCR cycle at a temperature of 72° C (the temperature range necessary for optimum activity of most polymerases used in PCR) in order to ensure that the remaining single-stranded DNA is completely elongated.

After PCR amplification was done, the target organism's desired band size was determined by using the Gel electrophoresis technique.

Gel Composition:

- ❖ 1X TBE buffer =100ml (50X TBE =2 ml + D.H$_2$0 =98ml) was used.
- ❖ Agarose (1.5%) = 1.5 gram was used to prepare the gel.
- ❖ Ethidium Bromide (Etbr) = 3μl (Because DNA is colorless, so a non-reactive dye is used to see the DNA bands in gel electrophoresis).
- ❖ After solidifying the gel, 4μl-6μl of PCR product was loaded into the gel along with a 100bp ladder, positive control, and negative control.
- ❖ The whole procedure was run at 110 volume for 60 minutes.

Gel Electrophoresis method:

The goal of this experiment is to separate, identify, and purify DNA depending on its size using the Agarose Gel Electrophoresis method. The first step of the procedure involved setting up the electrophoresis apparatus and casting the gel. For casting the gel, 1.5% Agarose gel was used following mixing with 3μl of EtBr so that DNA bands could be visualized under UV light. The very warm agarose gel had been poured onto the casting tray and a comb was used to make wells in the gel. After the solidification of the gel, the comb was taken out carefully so that the gel would not break. Then, 1X TBE buffer =100ml (50X TBE =2ml + d.h20 =98ml) was poured slowly onto the chamber until it covered the gel and filled up the chamber. This helped to maintain the pH of the medium. After that, 4 μl of samples were loaded in the wells. The loading dye helped to track the rate of movement of the DNA samples. One well had been kept empty for the DNA ladder marker and each of the samples was loaded in the other wells. The DNA ladder was loaded in the first well. Then, the apparatus was ready to operate and an electric field had been applied. After applying 110 voltage for 40 minutes, the power was turned off and the result was observed under UV light. DNA fragments are negatively charged, so they have been seen to

migrate toward the positive electrode. As EtBr was applied, DNA bands were seen to glow under UV light which helped to visualize the DNA samples.

Antimicrobial Susceptibility Testing:

Antibiotic susceptibility testing is a method that is performed to evaluate the efficacy of several drugs against a specific bacterial strain. This test has another name which is the Kirby-Bauer Disc Diffusion method. This technique is widely used to establish whether an antibiotic is sensitive by eliminating the target organism or which antibiotic inhibits the development of susceptible bacteria, as antibiotic-resistant bacteria are capable of adapting to their surroundings and function as a source of resistance to antibiotics. The goal of this test is to identify the best drug for treating a disease caused by a specific bacterium. The disc diffusion method includes placing small discs impregnated with various antibiotics on a Mueller-Hinton agar plate.

Freshly grown bacteria were required for the test, and a bacterial inoculum was developed by suspending one loop of the pure colony in 7 ml sterile saline and adjusting turbidity using 0.5 McFarland standard. This approach made use of Muller Hinton agar medium. To distribute the bacterium, sterile swabs of cotton were used and suspended throughout the agar media, and the antibiotic discs were carefully put in the agar medium so that no antibiotics overlapped. Then the plates were incubated overnight at 37°C for 24 hours. All of the plates must be lawned confluently in order to observe the right zone of the antibiotics.

Result Interpretation: After 24 hours of incubation, the inhibition zone was characterized as a clean zone with no microbial growth. Millimeters were used to measure the zones. Sometimes an additional zone might be visible, indicating that the inner zone was measured. The test organisms were categorized as resistant, sensitive, or intermediate to the respective antibiotics using the CLSI guideline standard chart.

These are the antibiotics used in the Antimicrobial Susceptibility Test. Amikacin (AK30) mm, Amoxiclav (AMC30) mm, Azithromycin (AMZ15) mm, Aztreonam (AT30) mm, Cefepime (CPM30) mm, Ceftriaxone (CTR30) mm, Cefixime (CFM10) mm, Chloramphenicol (C30) mm, Ciprofloxacin (CIP5) mm, Gentamicin (GEN10) mm, Imipenem (IMP10) mm, Tetracycline (TE30) mm.

The following primers were used in this study against the Multiple drug-resistance genes of *Pseudomonas aeruginosa*:

After analysis of the antibiotic-susceptible findings of all positive isolates, 7 isolates were chosen to determine the existence of multiple drug-resistance genes. These are the multiple drug-resistant genes were selected:

Gene Name	Primer Sequence	Base Pair	Reference
NDM- 1	F - 5'- GGTTTGGCGATCTGGTTTTC - 3'	621bp	(Agarwal et al., 2018)
	R - 5'- CGGAATGGCTCATCACGATC - 3'		
Bla $_{OXA-48}$	F – 5' – TTGGTGGCATCGATTATCGG – 3'	733bp	(Shoja et al., 2017)
	R – 5' – GAGCACTTCTTTTGTGATGGC – 3'		
Bla $_{IMP}$	F – 5' - GAAGGCGTTTATGTTCATAC -3'	587 bp	(Shams et al.,2018)
	R – 5' - GTATGTTTCAAGAGTGATGC - 3'		
Bla $_{KPC}$	F – 5' – CATTCAAGGGCTTTCTTGCTGC - 3'	498bp	(Mushi et al.,2014)
	R – 5' - ACGACGGCATAGTCATTTGC - 3'		
Bla $_{TEM}$	F – 5' - AAAATTCTTGAAGACG - 3'	1100bp	(Sharma et al.,2010)
	R – 5' - TTACCAATGCTTAATCA- 3'		
Bla $_{SHV}$	F – 5' - TACCATGAGCGATAACAGCG- 3'	450bp	(Doosti et al.,2015)
	R – 5' - GATTTGCTGATTTCGCTCGG- 3'		
Bla $_{CTX-M}$	F – 5' - ACGCTGTTGTTAGGAAGTG - 3'	759bp	(Zhang et al.,2021)
	R – 5' - TTGAGGCTGGGTGAAGT - 3'		
Bla $_{VIM}$	F – 5' – GGTGTTTGGTCGCATATCGCAA – 3'	502bp	(Shoja et al.,2017)

20

| R – 5' – ATTCAGCCAGATCGGCATCGGC – 3' | |

Result:

The total 54 confirmed *Pseudomonas aeruginosa* isolates were suspected from 78 isolates. The total confirmed isolates showed 69.23%. From hospital wastewater, 34 isolates were identified and adjacent community tap water showed confirmed 20 isolates. The rate showed 62.96% from the hospital but 37.03% from the community water sample.

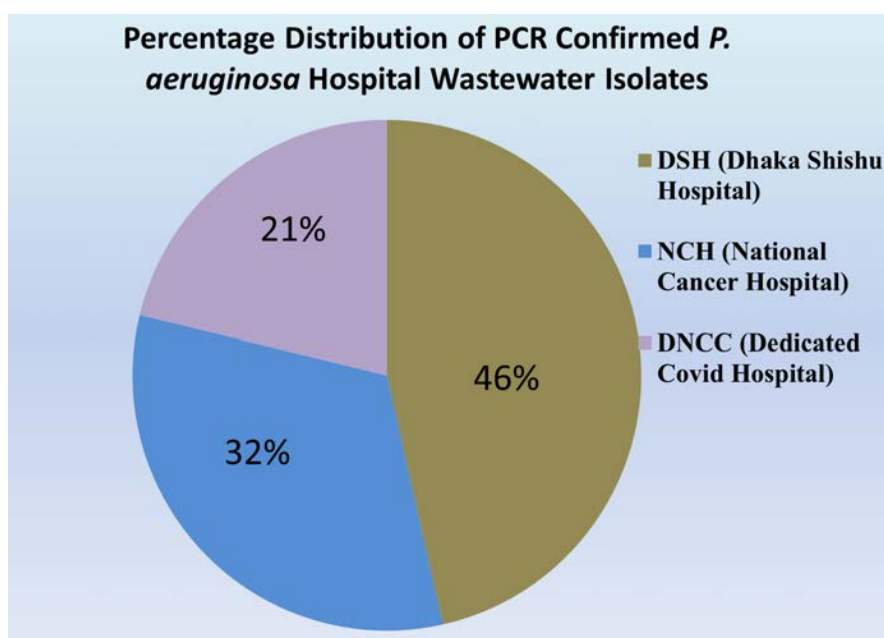**Identification of confirmed isolates from the hospital and community water sample:**
Pseudomonas aeruginosa

Figure-2: Percentage Distribution of PCR Confirmed *P. aeruginosa* Hospital Water Isolates

Among 34 isolates, 16 isolates were found from Bangladesh Shishu Hospital, 11 isolates were from National Cancer Hospital and 7 isolates were from Dedicated Covid Hospital.
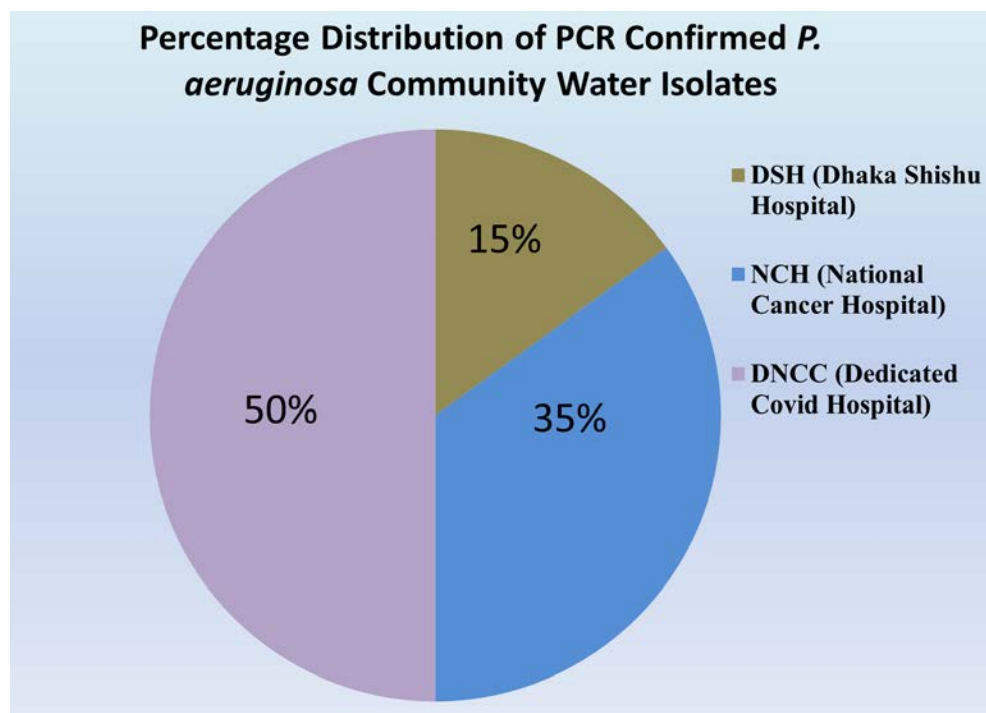
Figure 3: Percentage Distribution of PCR Confirmed *P. aeruginosa* Community Water Isolates

Among 20 isolates, 3 isolates were found from Bangladesh Shishu Hospital, 7 isolates were from National Cancer Hospital and 10 isolates were from Dedicated Covid Hospital.

Antimicrobial Susceptibility Testing: For 54 confirmed isolates, this method (Disc Diffusion Method) was used. 12 different types of antibiotic discs were used for each isolate.
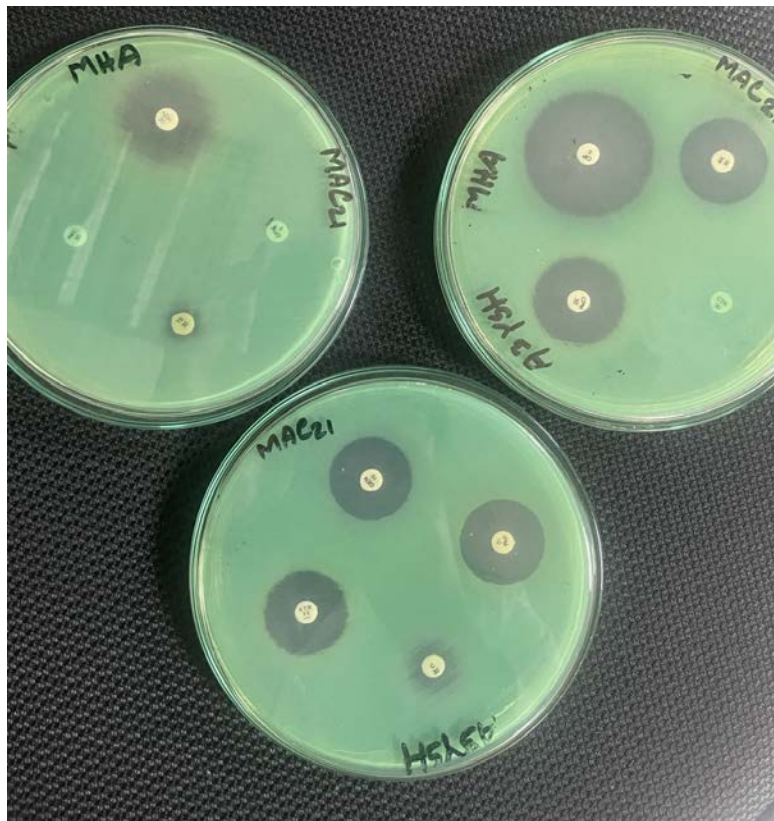
Figure 5: Zone of Inhibition in Mueller-Hinton Agar plate created by antibiotics

The test revealed that 94% of the isolates from hospital wastewater were resistant to the antibiotic amoxiclav (Amoxicillin + clavulanate) which was the highest. Cefixime and ceftriaxone were the second most resistant antibiotics, with a resistance rate of 82%. The majority of the hospital wastewater isolates showed resistance to all the antibiotics.
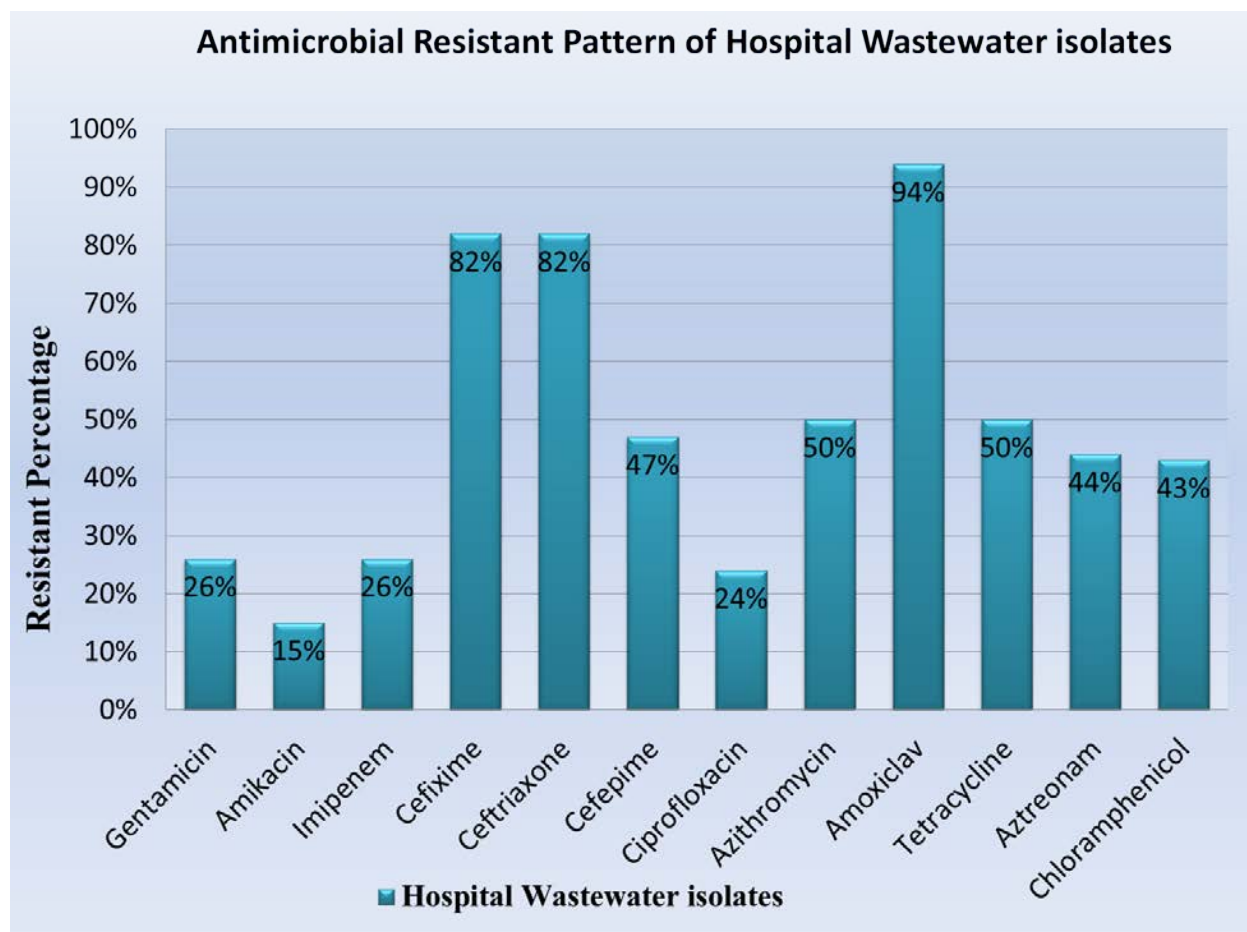
Figure 6: Antibiotic Resistant Pattern of Hospital Water Isolates

The test result represents that 90% of isolates from community water were resistant to the antibiotic amoxiclav (Amoxicillin + clavulanate) which was the highest. Cefixime was the second most resistant antibiotic, with a rate of 82%.
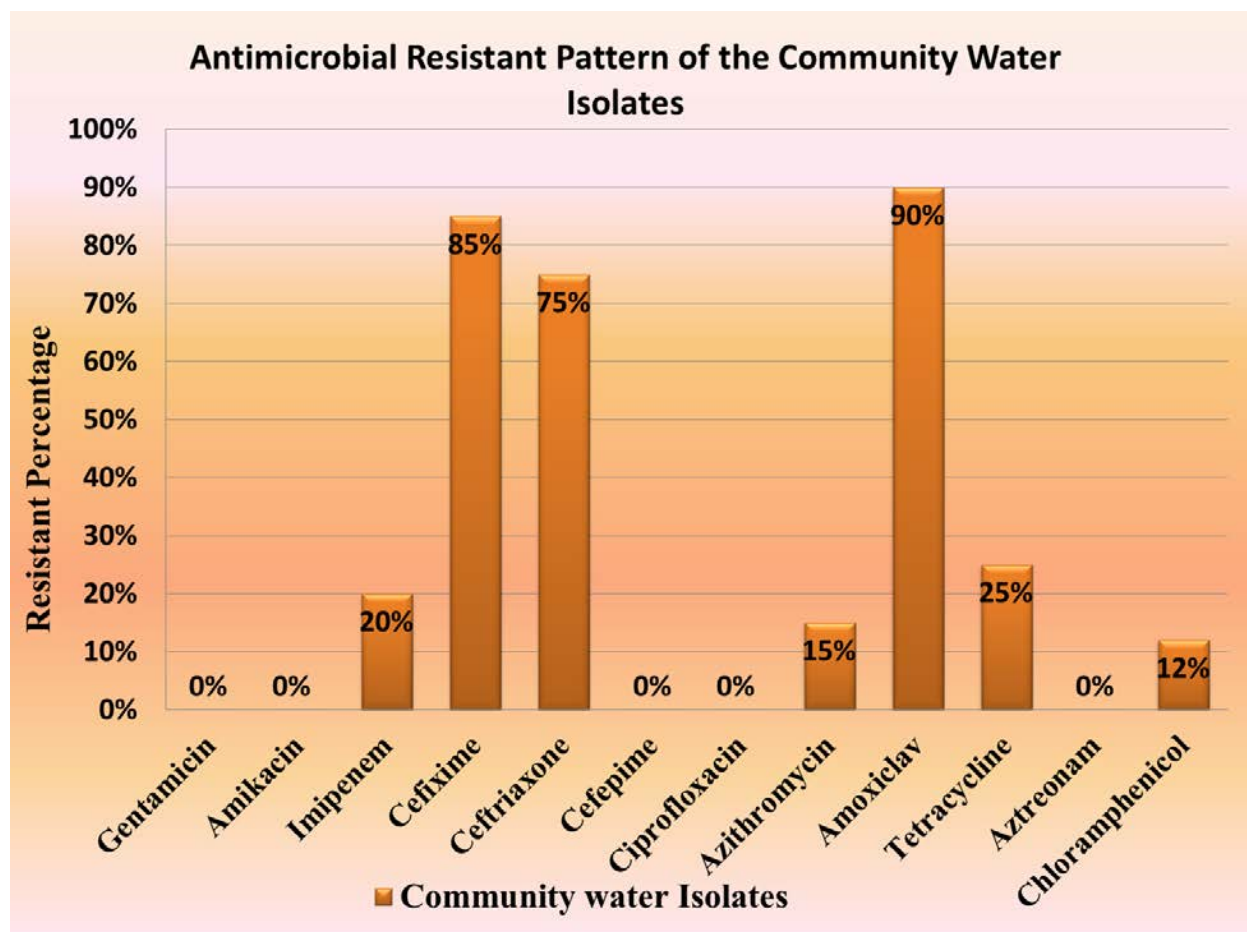
Figure-7: Antibiotic Resistant Pattern of Community Water Isolates

Comparison between the Antimicrobial Resistance Pattern of the Community Tap Water Isolates and the Hospital Wastewater Isolates:
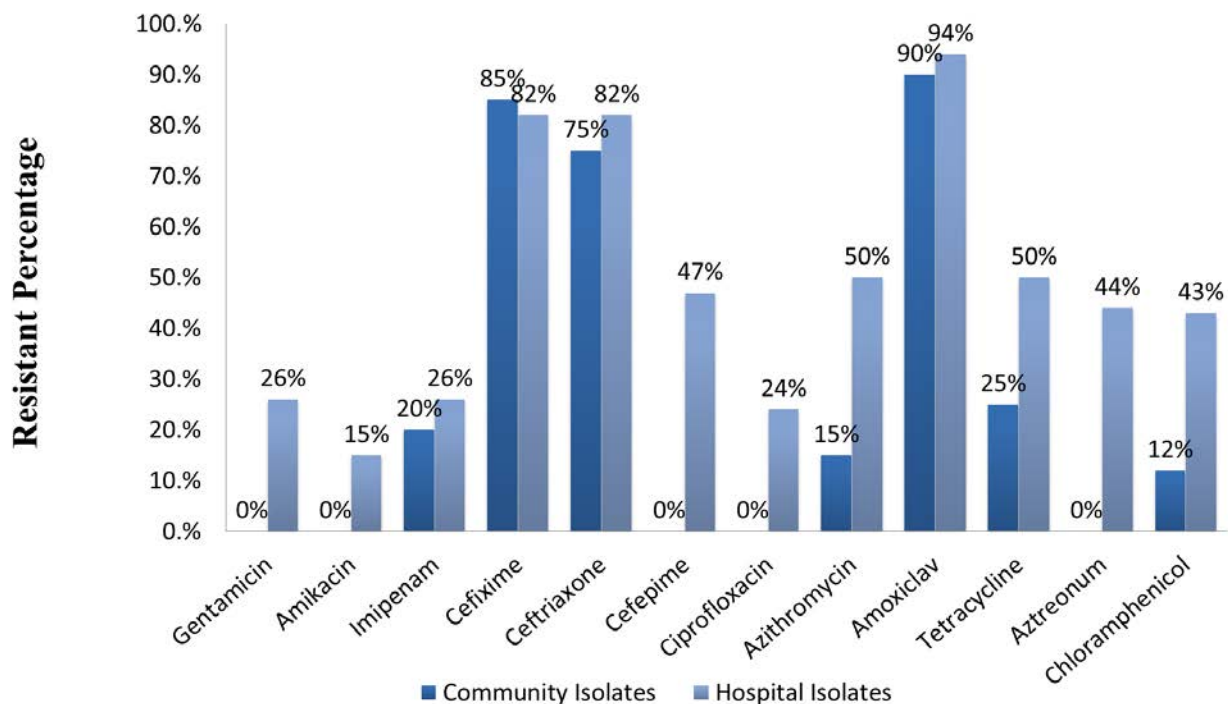

Figure-8: Comparison between the Antimicrobial Resistance Pattern of the Community Tap Water Isolates and the Hospital Wastewater Isolates

The highest resistant pattern was shown for Amoxiclav which was 94% for Hospital wastewater isolates and 90% for Community water isolates. The second highest-resistant antibiotic was Cefixime which showed 85% resistance to Community Isolates and 82% to Hospital Water Isolates. Moreover, Ceftriaxone antibiotic had the third highest resistant pattern which was 75% for Community isolates and 82% for Hospital isolates. A 50% Resistant pattern was shown for Azithromycin and Tetracycline from Hospital wastewater isolates.

The highest susceptibility was shown to Gentamicin, Amikacin, Cefepime, Ciprofloxacin, and Aztreonam which is 100% and isolates were from Community water. On the other hand, all the Hospital water isolates showed resistance to antibiotics but the highest susceptibility showed to Amikacin 85%, Ciprofloxacin 76%, Imipenem 74%, and Chloramphenicol 57%.

Identification of MDR genes:

There were 8 carbapenems and ESBL genes were explored to know their presence. Such as- *NDM- 1*, Bla $_{TEM}$, Bla $_{OXA-48}$, Bla $_{IMP}$, Bla $_{CTX-M}$, Bla $_{KPC}$, Bla $_{SHV}$, Bla $_{VIM}$ these 8 carbapenems and ESBL genes. Also, 7 isolates were selected to detect the above multi-drug resistant genes. The 7 isolates showed the most resistance pattern towards antibiotics. To detect the gene, we followed PCR and gel electrophoresis methods. From the preserved DNA of isolates, it was run for Conventional PCR and specific primer sequences of genes. Later on, we found DNA bands from Gel Electrophoresis. 3 genes were detected such as Bla $_{OXA-48}$, Bla $_{CTX-M}$, and Bla $_{SHV.}$

- 2 isolates from the June sample showed positive results for Bla $_{CTX-M}$ and Bla $_{SHV}$ which is 28.5%.
- 1 isolate from the June sample showed a positive result towards Bla $_{OXA-48}$ which is 14.2%.

Positive results and percentages among them are given below:

Multi-Drug resistant Gene names	Number of positive isolates	Percentage of positive isolates
Bla $_{OXA-48}$	1	14.2%
Bla $_{CTX-M}$	2	28.5%
Bla $_{SHV}$	2	28.5%
NDM- 1	0	0
Bla $_{TEM}$	0	0
Bla $_{IMP}$	0	0
Bla $_{KPC}$	0	0
Bla $_{VIM}$	0	0

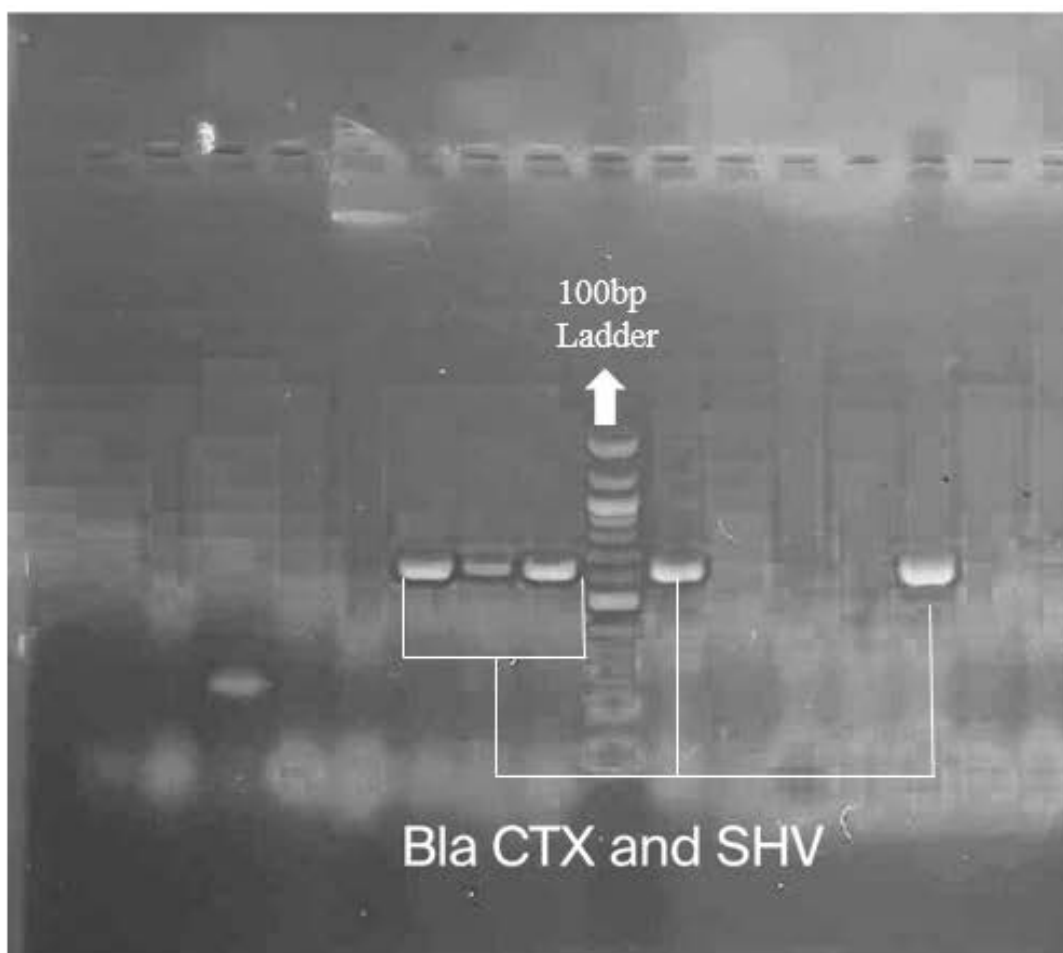**Gel Electrophoresis result of Multidrug-resistant gene:**

Figure-9: Positive bands are showing from two genes: Bla $_{CTX-M}$ and Bla $_{SHV}$

Discussion:

Antimicrobial resistance is one of the most serious public health issues today. The emergence of multi-drug resistance in Gram-negative bacteria, in particular, has become a severe concern for healthcare providers. In Gram-negative bacteria, resistance is primarily mediated by the synthesis of extended-spectrum-lactamases (ESBL), ampC-lactamases, and carbapenemases. (Schill et al., 2017). Antimicrobial resistance was often thought to only come from hospitals and other healthcare facilities. Hospital wastewater comprises a broad collection of disease-causing organisms and plays a significant role in the emergence of drug-resistant microorganisms in the surroundings, causing them to turn into emerging pollutants by amplifying, spreading, and remaining in the environment. For example, European research discovered that 1.5% of the microorganisms in hospital wastewater become MDR (multidrug-resistant).

In our research, we employed antibiotics from eight different categories for *Pseudomonas aeruginosa* which are- Aminoglycosides (Gentamicin/Streptomycin, Amikacin), Carbapenems (Imipenem/Meropenem), 3rd Generation Cephalosporins (Cefixime, Ceftazidime/Ceftriaxone), 4th Generation Cephalosporins (Cefepime), Fluoroquinolone (Ciprofloxacin/Levofloxacin), Macrolides (Azithromycin/ Erythromycin), Penicillin (Oxacillin, Amoxiclav/ Piperacillin+tazobactam, Tetracycline), Monobactam (Aztreonam, Chloramphenicol).

We collected a total of 78 water isolates from three different samples from hospitals and adjacent community areas in Dhaka, Bangladesh. The total confirmed isolates showed 69.23%, which is 54 isolates. Among them, 34 isolates (62.96%) were from hospitals, and 20 isolates (37.03%) were from community water samples. We got 16 confirmed isolates (47%) from Bangladesh Shishu Hospital, 11 confirmed isolates (32%) from the National Cancer Hospital, and 7 confirmed isolates (21%) respectively, from the Dedicated National COVID Hospital, we respectively got 3 isolates (15%), 7 isolates (35%) and 10 isolates (50%) from the community water sample of those hospital areas.

We found the percentage from AST results of Hospital isolates. Almost all the antibiotics showed a resistance pattern. Such as- 94% of isolates were resistant to Amoxiclav which involved in the category of Penicillin, 82% of isolates showed against Cefixime and Ceftriaxone (3rd Generation

Cephalosporins), 50% against Azithromycin and Tetracycline (Macrolides and Penicillin), 47% against Cefepime (4th Generation Cephalosporins), 44% against Aztreonam (Monobactam), 43% against Chloramphenicol (Monobactam), 26% against Gentamicin and Imipenem (Aminoglycosides and Carbapenems), 24% against Ciprofloxacin (Fluoroquinolone). Lastly, 15% showed against Amikacin (Aminoglycosides).

On the other hand, the percentage from AST results of Community isolates- 90% isolates were resistant against Amoxiclav which involves in the category of Penicillin, 85% isolates showed against Cefixime (3rd Generation Cephalosporins), 75% Ceftriaxone (3rd Generation Cephalosporins), 25% Tetracycline (Penicillin), 20% Imipenem (Carbapenems), 15% against Azithromycin (Macrolides), 12% against Chloramphenicol (Monobactam), 0% against Cefepime (4th Generation Cephalosporins), Aztreonam (Monobactam), Gentamicin and Amikacin (Aminoglycosides) and Ciprofloxacin (Fluoroquinolone).

An opportunistic pathogen is the *Pseudomonas aeruginosa* human pathogen. *Pseudomonas infections* are commonly treated with antibiotics such as beta-lactamases, aminoglycosides, and quinolones. Carbapenems are effective beta-lactam antibiotics for *P. aeruginosa,* which produces MBLs (metallo beta-lactamases) and is multidrug resistant. There have been numerous studies in recent months indicating that in many countries. Isolates from hospitals of *P. aeruginosa* and Gram-negative bacilli are developing carbapenem resistance. MBLs have been found in clinical isolates more frequently in recent years, extended treatment and severe infections are caused by strains that generate these enzymes. Japanese research found that patients who are infected with MBLs-producing *P. aeruginosa* required numerous medications, and infections caused by IMP-producing *P. aeruginosa* were more prevalent than those caused by Bla_{IMP}-negative *P. aeruginosa*. (Mrsalehian-et-al.-, 2010). In South America, Europe, and Asia *P. aeruginosa* strains have rapidly acquired MBL genes, increasing their resistance to carbapenems. As a result, the pattern of antibiotic use against multidrug-resistant *P. aeruginosa* has changed significantly.

Antimicrobial resistance to *P. aeruginosa* represents a severe problem, according to data from the National Healthcare Safety Network from 2015 to 2017. *P. aeruginosa* isolates from ICU patients were resistant to carbapenems at a rate of 26.3%, extended-spectrum cephalosporins at a

rate of 26.5%, and fluoroquinolones at a rate of 27.1%. (Reynolds & Kollef, 2021) In India, a prevalence of MBL in *P. aeruginosa* isolates ranging from 7.5% to 71% has been reported. (Kotwal et al., 2016) MBL prevalence in isolates of *P. aeruginosa* in India has been observed to range from 7.5% to 71%. According to one investigation, MDR strains were detected in 66.6% (100 of 150) of isolates. Among the 100 MDR strains of *P. aeruginosa* isolates, 54 (54%) were producers of ESBL and 21 (21%) were carbapenem-resistant. (Farhan et al., 2019). According to research, the Pan American Health Organization / World Health Organization (PAHO/WHO) obtained a report on infections at the surgical site due to antibiotic-resistant *Pseudomonas aeruginosa* after invasive operations conducted in Tijuana, Mexico, on February 12, 2019. In addition, on the 11th of February, 20 cases, 16 confirmed and 4 suspected, had been detected in nine states across the United States. (Carbapenem-Resistant Pseudomonas Aeruginosa Infection – Mexico, n.d.) *P. aeruginosa* nosocomial pneumonia shows a high frequency of MDR strains, with an International Multicenter Retrospective Research finding that 30.5% of nosocomial pneumonia related to *P. aeruginosa* were MDR-strains, which was linked with higher in-hospital mortality. (Reynolds & Kollef, 2021) Analysis from the International Nosocomial Infection Management Consortium study indicated antibiotic resistance rates of more than 40% in ICU patients for fluoroquinolones, piperacillin-tazobactam, and meropenem, while the research acknowledges that these resistance rates are greater than previously reported.(Reynolds & Kollef, 2021) MDR *P. aeruginosa* is an increasingly prevalent cause of death in burn patients, accounting for 86% of sepsis deaths in pediatric burn ICUs from 1999 to 2009, with *P. aeruginosa* being the causative bacteria 64% of that period.(Reynolds & Kollef, 2021)

Pseudomonas aeruginosa is a leading cause of dangerous nosocomial infections, and there has been a rise in cases of -lactam-resistant forms, making treatment tough and complicated. (Tam et al., 2007). According to a Mexican study, *Pseudomonas aeruginosa* is still being found in travelers with diseases who have surgery or invasive treatments at numerous Mexican healthcare facilities. These infections were caused by a strain of *P. aeruginosa* that generates the carbapenemase Verona integron-encoded metallo-lactamase (VIM). *P. aeruginosa* expressing VIM (VIM-CRPA) is commonly antibiotic-resistant and can cause difficult-to-treat infections. From our study, there was no Bla $_{VIM}$ gene found whereas we found three genes such as- Bla $_{SHV}$, and Bla $_{CTX}$ from two isolates and Bla $_{OXA-48}$ gene from one isolate. This bacterium also caused

disease in a burn hospital in Tehran, Iran. 48 (57.9%) of these strains were determined to be MBL producers, which was greater than the 2007-2008 research done by Mohammad Ali Bahar and the Shahid Motahari Hospital in Tehran, Iran. One of the most prevalent kinds of -lactam resistance is -lactamases. In *P. aeruginosa*, resistance to ceftazidime or cefepime may indicate ESBL resistance. Decreased outer-membrane permeability, efflux pumps, and the production of antibiotic-inactivating enzymes all contribute to *P. aeruginosa*'s intrinsic multidrug resistance. The great majority of ESBLs are SHV or TEM types produced from -lactamases with a restricted range of activity. The CTX-M gene was found in *Kluyvera* species and identified in *Enterobacteriaceae* and has subsequently been observed in many different parts of the world. Chronic *Pseudomonas aeruginosa* infection is linked to decreased lung function and a worse prognosis in cystic fibrosis patients. Novel cephalosporins, which are currently in clinical use, may be more effective against multi-drug-resistant bacteria.

Limitations of our Study:

All bacteria in nature are able to form biofilms when bacteria attach to diverse surfaces. Because *P. aeruginosa* is widely recognized for forming biofilms, it is an excellent model for studying biofilm development. *P. aeruginosa* has demonstrated growth slowly as detached cell aggregates under hypoxic and anoxic circumstances, which is equal to what has been seen in CF airways and severe wounds (Reynolds & Kollef, 2021). Additionally, *P. aeruginosa* successfully colonizes a wide range of surfaces, such as those found on medical devices (such as urinary catheters, implants, and contact lenses) and food-related machinery (such as mixing tanks, vats, and tubing). It is crucial to have a great awareness of the structure and composition of the biofilm, as well as the mechanism of molecular behind the antimicrobial resistance of bacteria acquired within a biofilm, for the purpose of developing efficient management, prevention, and, above all, eradication methods of biofilm-associated infections.

Numerous post-operative wound and lung infections have been linked to *Pseudomonas aeruginosa*. Numerous acquired resistance and virulence markers reveal *P. aeruginosa*'s survival strategy. Analysis of the entire genome has been found to be an effective method for determining how pathogenic this organism is. One of the most recent methods to research organisms' resistance mechanisms is whole-genome sequencing (WGS) (Madaha et al., 2020). This method delivers an enormous amount of information about the genes contained inside a pathogen since it can process many DNA sequences in parallel with high throughput, at a reduced cost, and in a quicker turnaround time (Madaha et al., 2020). It is an effective protocol that has completely changed how microbial genome research is done. There were limitations in our work where we could not identify the whole genome in our laboratory. But it would have been very effective if it was done as whole genome sequencing let us find out how the strains of the pathogen work and find out effective strategies to solve it.

Conclusion:

Pseudomonas aeruginosa isolates were collected from the selected hospitals and nearby community water, which was 69.23%. Among the isolates from hospital wastewater, we have found 62.96% isolates whereas from nearby community tap water isolates found 37.03%. There were 8 carbapenems and ESBL genes were explored to know their presence. Out of the 7 isolates for this MDR gene identification, Bla $_{OXA-48}$ was found in 14.2%, Bla $_{CTX-M}$ in 28.5%, and Bla $_{SHV}$ in 28.5% isolates. In the study, we have observed almost similar resistant patterns for both hospital wastewater and nearby community water in Antibiotic susceptibility tests. For instance, we have observed 92% amoxiclav resistance for hospital wastewater and 90% resistance for nearby community water which is almost of a similar pattern. It has been claimed that Hospital wastewater is the reservoir of Antimicrobial resistant organisms and following that we also found an Antimicrobial resistant gene in *Pseudomonas aeruginosa* from hospital wastewater.

From our study, we assumed that community water has become resistant to hospital wastewater. However, the findings are concerning for human health and life as the organisms are becoming multidrug resistant rapidly for inattentiveness and the MDR genes are spreading and becoming a high risk for the public health. For further studies of these MDR isolates, whole genome sequencing will be needed to know the gene expression and detect a range of variant types. Also, biofilm-producing bacteria are known to have stronger antibiotic resistance compared to non-biofilm-producing bacteria. So, it is also needed to study whether these isolates are biofilm producers or not. Treating the infections caused by biofilm-producer bacteria is very challenging, potentially resulting in treatment failure.

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