CHARACTERIZATION OF *Pseudomonas aeruginosa* FROM HOSPITAL SEWAGE WATER AND NEARBY COMMUNITY WATER BASED ON THEIR MULTI-DRUG-RESISTANT GENE

Submitted By

Rezwana Afrin Adiba
Student ID: 18126077

Riyan Mehzabin Ferdous
Student ID: 18126041

A dissertation submitted to the Department of Mathematics and Natural Sciences, in partial fulfilment of the requirements for the degree of Bachelor of Science in Microbiology

Department of Mathematics and Natural Sciences

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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.

Students' Full Name & Signature:

1. Rezwana Afrin Adiba
   Student ID: 18126077

2. Riyan Mehzabin Ferdous
   Student ID: 18126041

Approval:

The thesis/ project titled "*Characterization of Pseudomonas aeruginosa from Hospital Sewage Water and Nearby Community Water based on their Multi-Drug-Resistant Gene*" submitted by_

1. **Riyan Mehzabin Ferdous (18126041)**
2. **Rezwana Afrin Adiba (18126077)**

Of Batch Spring 2018 has been accepted as satisfactory in partial fulfilment of requirement for the degree of Bachelor of Science in Microbiology.

Examining Committee	**Full Name, Designation & Signature**
Supervisor (Member)	Akash Ahmed Senior Lecturer, Department of Mathematics& Natural Sciences, BRAC University
Program Director (Member)	Dr. Nadia Sultana Deen Associate Professor, Department of Mathematics and Natural Sciences BRAC University
Departmental Head (Chair)	A F M Yusuf Haider, PhD Professor & Chairperson, Department of Mathematics & Natural Sciences, BRAC University

ABSTRACT:

Introduction: Hospital wastewater is one of the most prominent sources of antibiotic-resistant pathogenic bacteria. This study aims to find multi-drug-resistant *Pseudomonas aeruginosa* from the hospital and nearby community water to find the organisms with Multidrug-resistant genes from hospital wastewater that get to mix with community water.

Method: The study was conducted with samples from November 2022 to March 2023 and a total of 43 different isolates of *Pseudomonas aeruginosa* were collected from hospital wastewater and surrounding community tap water. Firstly, the phenotype technique was used to identify the multi-drug resistant isolates. Further, following the Kirby-Bauer disk diffusion method, an antibiotic susceptibility test was performed. To identify the resistant gene of ESBL (Extended spectrum β-lactamase) and MBLS (Metallo-beta-lactamase) PCR method is further performed.

Result: A Total 65 Number of samples were collected from Three Hospital and their adjacent community water and Confirmed isolates of *Pseudomonas aeruginosa were* found in 43 of those 65 samples. The percentage of confirmed isolates is 27.95%. These 43 isolates were found resistant to Amikacin (15%), Tetracycline (46%), Amoxiclav (93%), Azithromycin (48%), Aztreonam (65%), Cefepime (32%), Cefixime (32%), Ceftazidime (100%), Chloramphenicol (11%), Ciprofloxacin (13%), Gentamicin (3%), Imipenem (11%). After that, 7 isolates were selected for further identification of Multidrug-resistant-Gene. The presence of *NDM*-1 was found in 3 isolates, *bla* $_{TEM}$ *was* found in 4 isolates, and *bla* $_{VIM}$ *was* found in one isolate after performing the PCR method using these 8 Genes.

Conclusion: In our study 43 target *Pseudomonas aeruginosa* confirmed isolates were found and among them 24 were hospital isolates and 19 were community isolates. 7 MDR isolates were selected, among them 4 isolates, 3 isolates, and 1 isolate positive for respectively bla_{TEM}, *NDM- 1, and* bla_{VIM} genes. This scenario shows how hospital waste contains organisms with resistant patterns and transmitting MDR microorganisms to nearby community water and spreading MDR genes. It is becoming a serious threat to the environment and public health, so it requires proper maintenance of hospital disposal to the environment and need proper methods to prevent these strains.

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Content

List of ABBREVIATIONS

- **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 Waste Water
- **ICU=** Intensive Care Unit

Introduction:

Pseudomonas aeruginosa is a popular infectious microorganism that causes many diseases in the human body. It is a Gram-negative rod-shaped, motile bacterium. It's an obligatory anaerobic bacterium that doesn't require oxygen to grow. appears as a blue-green color in the cetrimide agar plate and gives a glow under UV light (in the presence of fluorescence. (Faure et al., 2018). This organism can cause nosocomial infections, pneumonia, otitis, etc. *Pseudomonas aeruginosa* is an opportunistic pathogen that can easily infect immune-compromised patients who are admitted to ICU or patients who are suffering from fatal diseases. This becomes a privileged option for the *Pseudomonas aeruginosa* to infect. (Mulcahy et al., 2014). Patients under ventilation treatment, Patients with urinary tract infections, and under treatment with catheters also get infected with this opportunistic pathogen. Patients who have cystic fibrosis and burn wounds, a chronic pulmonary disorder, and diabetics patients also easily get infected with *Pseudomonas aeruginosa*. (Mulcahy et al., 2014). Mortality rates are 20-25% for bone marrow transplant patients with *Pseudomonas aeruginosa* bacteremia. (Stryjewski & Sexton, 2003). Also, it is a phytopathogenic organism and prefers a moist environment to grow, thus it is cultured in a very high number from drains and similar semi-aquatic environments. (Talon et al., 1996) (Brisse et al., 2000).

It is considered one of the well-known organisms which have acquired multidrug resistance genes. Over the last few years, *Pseudomonas aeruginosa* has become resistant to many antibiotics. Multidrug drug-resistant bacteria are explained as one agent of bacteria that is resistant to more than one antibiotic. (Aijaz Shah et al., 2015). When bacteria become multidrug-resistant antibiotics; treatment does not work in the infected Patient. According to

the report of the European Antimicrobial Resistance Surveillance Network of the European Centre for Disease Prevention and Control (ECDC) in the European country among the findings they found at least one group which are antimicrobial resistant. However, they also found about 19.2% of isolates, which are resistant to more than one antimicrobial group.(Spagnolo et al., 2021). There are several reasons to adopt the antimicrobial resistance mechanism of *Pseudomonas aeruginosa* which are having lower membrane permeability, high expressed efflux pumps, etc. (Aijaz Shah et al., 2015).

In an epidemiological study, it has been reported that around 700,000 people have died due to the infection of antibiotic resistance each year, and among that in the European population, the resistance level was *Pseudomonas aeruginosa* 12.9%.(Qin et al., 2022). According to a study from the year 2005 it has been claimed that antimicrobial cleaning agents like soap, and detergents also cause microbes to get Antimicrobial drug-resistant as those agents have triclosan which primarily kills the microbes and after some time makes the organism antibiotic-resistant. The triclosan exposure can cause bacterial target mutation which leads to multidrug resistance expressed by efflux pumps.(Aiello et al., 2005).

Hospital wastewater contains many pathogenic micro-organisms including *Pseudomonas aeruginosa* because of the compromising quality of the water. Hospital wastewater comes from water used by patients, wards, surgery rooms, hospital laboratories, etc. (Majumder et al., 2021). Wastewater refers to any water whose quality has been compromised by human activities. It includes liquid waste discharged from domestic homes, agricultural commercial sectors, pharmaceutical sectors, and hospitals. Hospital wastewater (HWW) can contain dangerous and infectious elements which include pharmaceutical products, chemical hazardous substances, pathogens, etc. (Majumder et al., 2021). Moreover, all these elements in wastewater are harmful to the environment and the public. That is why hospital effluent treatment before the water discharge is very important. Mostly, hospital wastewater carries multidrug infectious bacteria and viruses. As the water used by ICU patients, surgery rooms, or hospital laboratories where several tests are done on patients. (Majumder et al., 2021). However, hospital wastewater is also released from the operation room using water, water from wards, or laboratories. (Faure et al., 2018). Even sewage water from the toilets is dangerous to the environment if it is released without treatment as infected patients use that and infected patients release the pathogenic organism with urine or stool. Also, wastewater has the potential ability for horizontal gene transfer and generate antibiotic-resistant bacteria because it contains mass amounts of nutrients and high microbial biomass. (Asfaw, 2018). Without the maintenance, of

the proper protocol to release hospital wastewater is very important and when it doesn't follow properly the multidrug-resistant pathogenic organisms carried by the hospital wastewater further get mixed with the nearby community water of that hospital and resistant genes of microbes can cause illness to the people who are having that water also, those people can also become multi-drug-resistant as well. (Kaur et al., 2020). Mostly sewage or discarded water and community water has different pipelines, still the organisms are somehow getting exposed, and their resistant genes are spreading around the community through the water.

In the research findings we have found similar strains with similar resistance patterns of antibiotics in both hospital wastewater and community water. Community water stands for the water that supplies households for their regular use. Mostly hospital-surrounded community water can get contaminated by the wastewater of the hospital. Though the sewage line and water supply line are different but somehow the contamination is spreading to community water from hospital water and for that reason the same kind of strains are observed in the research work where we tried to find the *Pseudomonas aeruginosa* in our finding.

The *Pseudomonas aeruginosa* found in the hospital wastewater are mostly resistant also they help the rest of the non-resistant organisms to become resistant within the environment and to survive and for that with the help of their regulatory gene, they develop virulence factor metabolism and easily develop a resistant mechanism for antibiotics which cause the antibiotic fails its mechanism against resistant genes after entering the host. After entering the host body *Pseudomonas aeruginosa* also manages to survive by adapting to long-term sustainability. *Pseudomonas aeruginosa* has intrinsic and extrinsic resistant patterns, and the intrinsic mechanism comes from chromosomes that do not get affected by the presence of antibiotic exposure. The Extrinsic resistant mechanism is the adaptation of resistant genes. Resistant genes like ESBL and Carbapenems are increasing rapidly worldwide. (Pfeifer et al., 2010). These genes are plasmid-encoded and Most of the Plasmids become resistant by the conjugative method. The Conjugative method is a replicative process that produces copies of the plasmid in both recipient and donor cells. (Bennett, 2008). In the South and Southeast Asian subcontinent, plasmid-encoded Metallo-beta-lactamase is becoming endemic day by day. (Boyd et al., 2020). Horizontal gene transfer occurs due to the transfer of genetic information from one organism to another and through this antibiotic-resistant gene transfer bacteria. (Soucy et al., 2015)

Bacteria adapt resistant genes by up-taking DNA from other bacteria. This gene transfer is also known as lateral gene transfer as an organism transfers its genetic material to another organism that is not its offspring. This gene transformation can occur in three ways.

A Transformation is a form of genetic recombination in which a DNA fragment from a degraded bacterium enters a competent recipient bacterium and is exchanged for a piece of DNA of the recipient. Transformation usually involves only homologous recombination, the recombination of homologous DNA regions having nearly the same nucleotide sequences. Typically, this involves similar bacterial strains or strains of the same bacterial species. In this process, DNA fragments of both strands from dead cells get bound with the DNA binding protein on the surface of the competent living recipient bacterium. (Soucy et al., 2015)

For transduction gene transfer, DNA fragments transfer through bacteriophage from one bacterium to another. Lastly, for conjugation gene transfer is common in gram-negative bacteria, and DNA transfers from one living bacterium to another living recipient bacterium through cell to cell. Conjugation is involved by a plasmid or transposon and through the sex pilus one bacterium gets attached to another one and conducts the recombination of gene transfer to another bacteria. (Soucy et al., 2015).

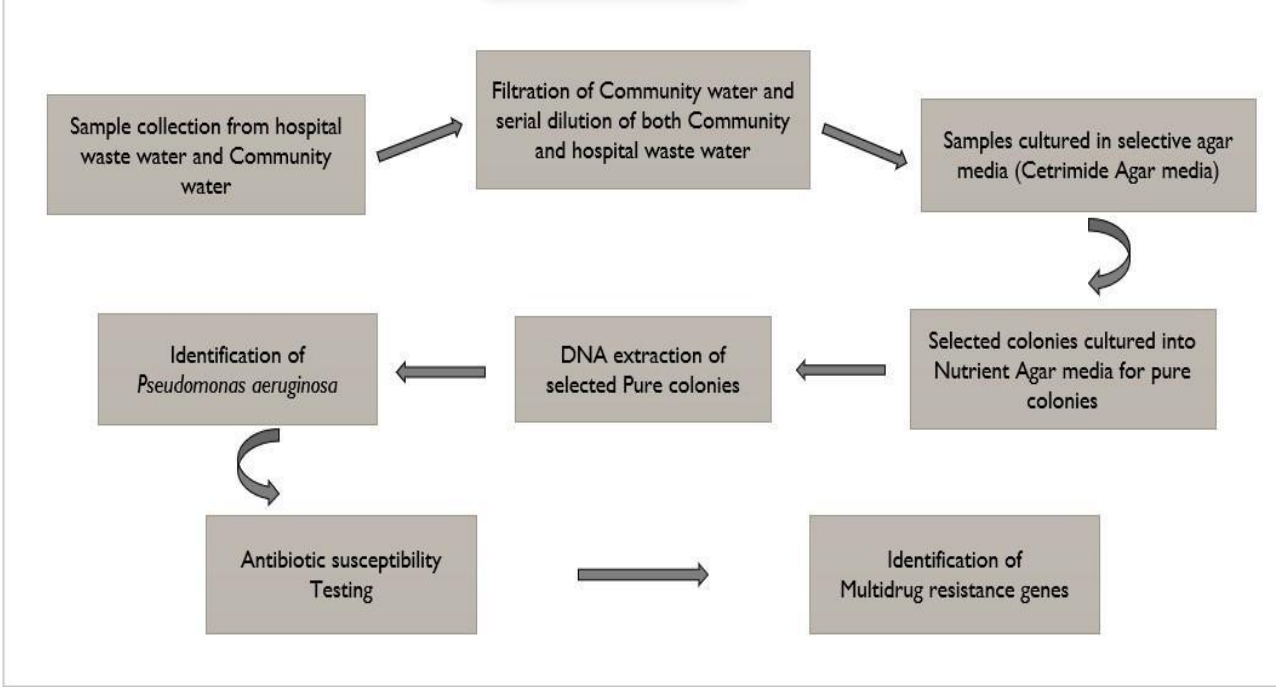
For the infection caused by *Pseudomonas aeruginosa* Beta lactam antibiotic carbapenem, is used for the treatment. In our finding of *Pseudomonas aeruginosa* from the hospital wastewater and community water because of its resistant genes mostly, we found the organism get resistant against amoxiclav, azithromycin, cefepime, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, and tetracycline. Among these beta-lactam antibiotics work by involving along with the cell wall of the organism and creating a covalent bond with the Penicillin-binding proteins (PBs). (Pachori et al., 2019). Carbapenems contain beta-lactam rings and they have the capacity to inhibit bacterial cell walls by binding with penicillin-binding protein. To treat *Pseudomonas aeruginosa* carbapenem is used but the resistance level of this antibiotic is globally increasing which is a matter of concern. Resistance patterns of *Pseudomonas aeruginosa* are appearing from multiple sectors like mutation, protein loss of outer membrane, enzyme production and multidrug efflux system. (Fang et al., 2022). The Outer membrane porin D (oprD) mutation in *Pseudomonas aeruginosa* is responsible for the reducing sensitivity of carbapenem but this mutation cannot be horizontally transferred to other genes. (Fang et al., 2022). Because of this high resistance of *Pseudomonas aeruginosa*, infection caused by this organism has a high mortality risk and for this reason, cystic fibrosis patients and

immunocompromised patients attacked by the *Pseudomonas aeruginosa* suffer extremely as antibiotics fail to work. (Moradali et al., 2017).

In the case of resistance of *Pseudomonas aeruginosa* in aminoglycoside antibiotics like gentamicin, and amikacin, bacteria bind with the 30s ribosome because of the alteration in the targeted site and inhibit protein synthesis. The methylase enzyme is responsible for the alteration. *Pseudomonas aeruginosa* infection has a rapid resistance pattern that also has been observed against fluoroquinolone antibiotics. According to research in south China among 256 isolated *Pseudomonas aeruginosa* strains 65 isolates developed the plasmid-mediated fluoroquinolone resistance pattern in their strain which has been detected performing the antibiotic susceptibility test and PCR confirmation. (Yang et al., n.d.). Strains of the organism were mostly resistant to piperacillin, piperacillin/tazobactam, ceftazidime, and amikacin. Resistance of the organism was mainly developed due to the mutation in DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE) genes. Quinolone resistance can also be developed due to the transformation of plasmid and mutation in the gene-regulating efflux pump when expression of the outer membrane of porin decreases. (Yang et al., n.d.).

Material and Method:

Work Flow:



Sample Collection: Samples were collected from 3 different hospitals which were **National Institute of Cancer Research and Hospital (NICRH), Dedicated COVID-19 Hospital**

(DNCC) and **Bangladesh Shishu Hospital**. The community water samples were collected from the tap water from the adjacent areas of these 3 hospitals. All the samples were collected from November 2022 to March 2023 each week of the month and transported to the laboratory in the cooler box and the samples were processed within 6 hours of collection at the research laboratory of the MNS department (Mathematics and Natural Science) of BRAC University.

Sample processing, isolation, and identification of *Pseudomonas aeruginosa:*

Hospital wastewater samples and community water samples were processed differently. For community water, 100ml of each sample was filtered through 0.45-µm filter paper and transferred the paper into Tryptic Soya Broth for overnight enrichment in a shaking incubator at 37°C. Then the TSB enrichment samples were processed for serial dilution (10-1- 10-7). 100µl of the diluted sample (10-2,10-4) and direct raw sample were cultured into Cetrimide Agar (selective for Pseudomonas species) by using the Spread Method. The culture plates were incubated at 37°C for 24-48hr.

The hospital wastewater was directly processed for serial dilution (10-1- 10-7) and 100µl of the diluted sample (10-2,10-4) and direct raw sample were cultured into Cetrimide Agar (selective for Pseudomonas species) by using the Spread Method. The culture plates were incubated at 37°C for 24-48hr.

After incubation, all the sample plates were observed under UV light. The specific strain of *Pseudomonas aeruginosa* shows blue-green pigment with irregular margin flat colonies under UV light.

After the observation, the specific colonies were selected from each positive Cetrimide plate. The selected colonies were cultured into Nutrient Agar media by the Streak method to obtain pure colonies of the specific isolates. Single colonies of the specific isolates were used for DNA extraction by boiling method. These DNA extractions further need to perform PCR which was required for the final identification of *Pseudomonas aeruginosa*.

DNA Extraction (Boiling Method) of the isolates:

DNA extraction and purification are very important steps that are used to find out the specific organisms. There are several methods established to extract and purify the DNA, but the

Boiling method is a very effective, rapid, simple, less time-consuming, and cheap method than other standard methods. (Ahmed* & Dablool, 2017).

- 2-3 pure isolated colonies were picked with a sterile loop and inoculate the colonies to 150µl 1X TE buffer (10mM Tris-HCL, 1mM EDTA, pH 8.0) in microcentrifuge tube.(Ahmed* & Dablool, 2017).
- Need to vortex the solution so that the colonies could 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 then centrifuge for 5 minutes at 13,000 rpm, so that all the particles could be set as a pellet form and pure DNA separated as supernatant form.
- Then supernatant was separated carefully from the pellet to another sterile microcentrifuge tube and stored the DNA at -20°C. (Ahmed* & Dablool, 2017).

PCR (Polymeric Chain Reaction) for final detection of *Pseudomonas aeruginosa*:

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				

Primers of specific *Pseudomonas* species (*Pseudomonas aeruginosa*):

Preparation of PCR Product:

- **Master-Mix = 6.5µl**
- **Nuclease free water = 2.5µl**
- **PA-SS-R (Reverse Primer) = 1µl**
- **PA-SS-F (Forward Primer) = 1µl**
- **DNA Template = 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: 94°C for 20 seconds.**
- **Annealing: 58°C for 20 seconds.**
- **Elongation: 72°C for 40 seconds.**
- **Final Extension: 72°C for 1 minute.**(Spilker et al., 2004)

After the PCR amplification, Gel electrophoresis method was done to observe the desired band size of the target organism.

Preparation of Gel Electrophoresis:

It is a very common and rapidly used technique to separate DNA fragments according to their size.

Gel Composition:

- 1X TBE buffer =100ml (50X TBE =2ml + d.h20 =98ml)
- Agarose (1.5%) = 1.5 gram
- Ethidium Bromide (Etbr) = 3µl (It is a non-reactive stain to visualise the DNA bands in gel electrophoresis as DNA is colourless).
- After solidifying the gel, 4µl-6µl each PCR product was loaded into the gel along with positive control, negative control and 100bp ladder.
- The whole procedure was run in 110 volume for 60 minutes.(Lee et al., 2012).

Antimicrobial Susceptibility Testing:

Once the target organism is identified and isolated from each sample by PCR process, the Antibiotic susceptibility profiles of the isolates was determined by the standard Kirby-Bauer disk diffusion method.(Nassar et al., 2019). This method is commonly used to determine which antibiotic is sensitive by killing the target organism or which antibiotic inhibits the growth of susceptible bacteria and antibiotic-resistant bacteria can adapt to their environment and serve as a source for its spread.(Nassar et al., 2019).

For the test freshly grown bacteria were needed and bacterial inoculum was prepared by suspending one loop of the pure colony into 7 ml sterile saline and turbidity was adjusted with 0.5 McFarland standard. Muller Hinton agar media was used for this method. Sterile cotton swabs were needed to loan all the bacterial suspension all over the agar media and all the antibiotic disks were placed into the agar media carefully so that any antibiotic overlaps with each other. Then the plates were kept for overnight incubation at 37°C for 24-48 hours. (Matuschek et al., 2014).

The following antibiotics were used for antibiotic susceptibility testing of *Pseudomonas aeruginosa* –

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.

Antibiotic zone interpretation-

For observing the correct zone of the antibiotics, all the plates must be lawn confluently. After 24-48 hours of incubation, the inhibition zone was interpreted where the clear zone was given and no growth of microorganisms was seen. The zones were measured in millimeters. Sometimes there might be seen double zone, for that inner zone, was measured.(Matuschek et al., 2014). By using the CLSI guideline standard chart it is classified that the test organisms were resistant, sensitive, or intermediate of the particular antibiotics.

Identification of Multiple drug resistant genes from selected isolates:

After observing the antibiotic susceptible results of all the positive isolates among them 7 isolates were selected to identify the presence of multiple drug-resistant genes.

Table of Selected multiple drug resistant genes:

Gene Name	Primer Sequence	Base Pair	Reference
NDM- 1	F - 5'- GGTTTGGCGATCTGGTTTTC - 3'	621bp	(Agarwal et al., 2018)
	R - 5'- CGGAATGGCTCATCACGATC - 3'		
bla VIM	F – 5' – GGTGTTTGGTCGCATATCGCAA – 3'	502bp	(Shoja et al., 2017)
	R – 5' – ATTCAGCCAGATCGGCATCGGC – 3'		
bla IMP	F – 5' - GAAGGCGTTTATGTTCATAC -3'	587bp	(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'		

Result:

Identification of *Pseudomonas aeruginosa:*

Total of 65 isolates were collected from 3 different hospitals and their surrounding community areas. *Pseudomonas aeruginosa* was found in 43 isolates, the percentage was 27.95%. Among them 24 isolates were confirmed from hospital wastewater samples and 19 isolates were confirmed from community water samples. The percentage between hospital and community water samples was 10.32% and 8.17% respectively.

Distribution of *Pseudomonas aeruginosa* confirm isolates among the three different hospitals and the community areas-

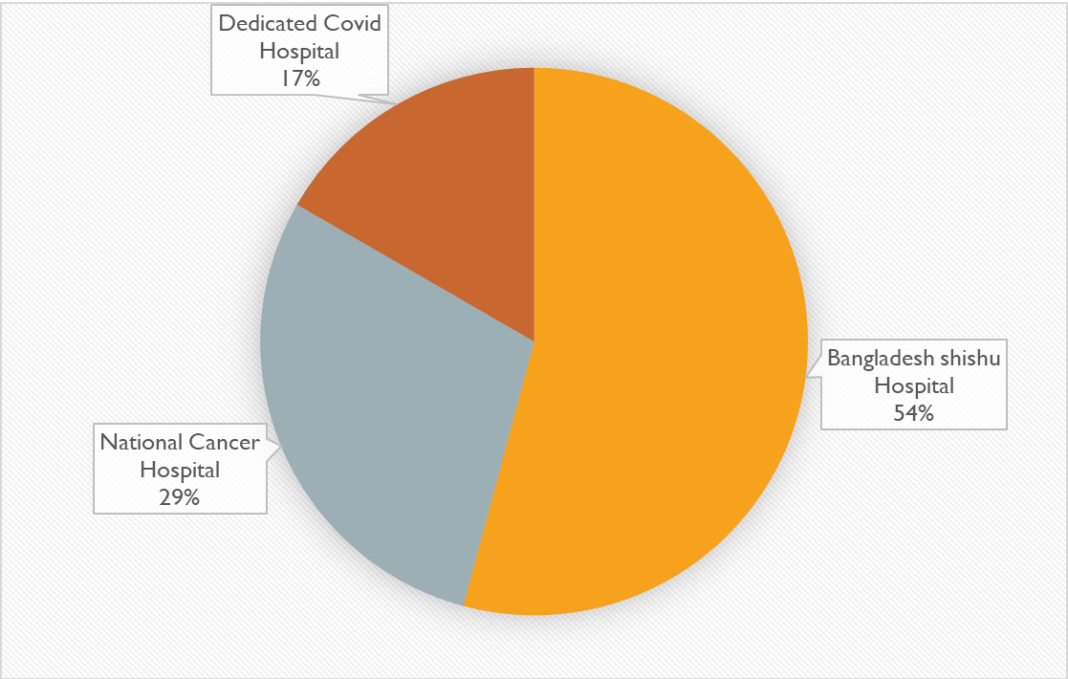


Figure 1: Percentage of positive isolates from each Hospital sample

Among 24 isolates, 13 isolates were found from Bangladesh Shishu Hospital, 7 isolates were from National Cancer Hospital and 4 isolates were from Dedicated Covid Hospital.

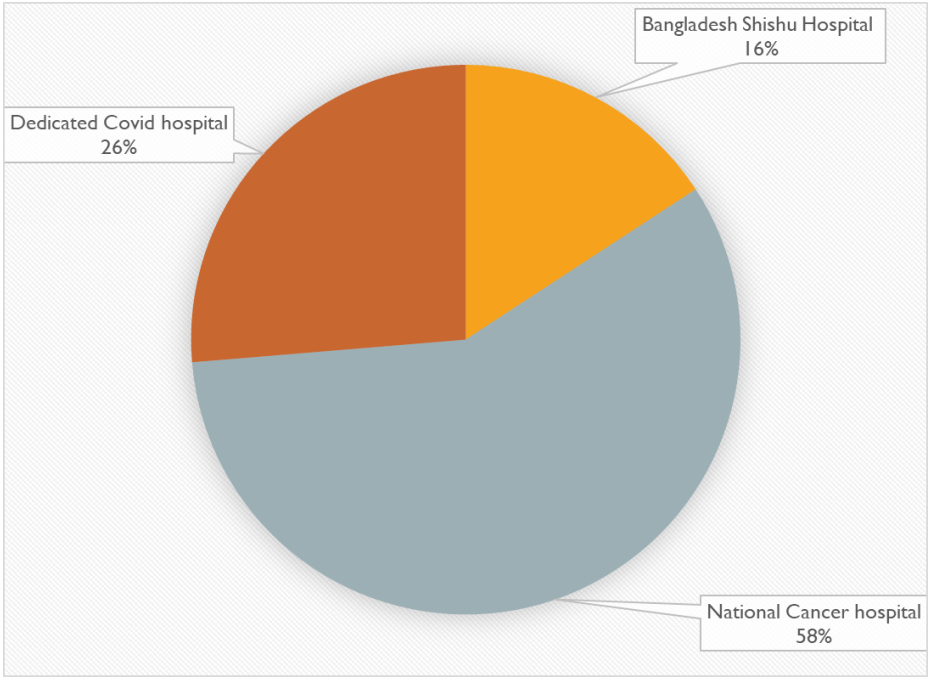


Figure 2: Percentage of positive isolates from each Community sample of Hospital areas

Among 19 isolates, 3 isolates were confirmed from Bangladesh Shishu Hospital, 11 isolates were from National Cancer Hospital and 5 isolates were from Dedicated Covid Hospital.

Antibiotic Susceptibility Test profile-

All 43 isolates were selected for further antibiotic susceptible tests. The Kirby-Bauer method was used for the isolates.

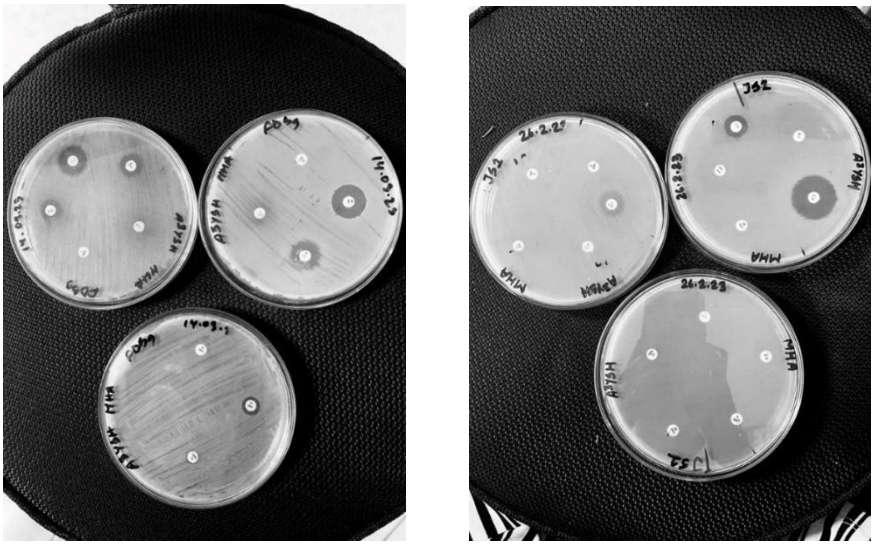


Figure 3: Antibiotic Zones in Mueller-Hinton Agar plate

From the test all the isolates from both hospital wastewater and community water were 100% resistant to Ceftazidime antibiotic. 2nd most resistant antibiotic was amoxiclav (Amoxicillin + clavulanate) which was 91.2%. The majority of the hospital waste water isolates showed resistance towards all the antibiotics.

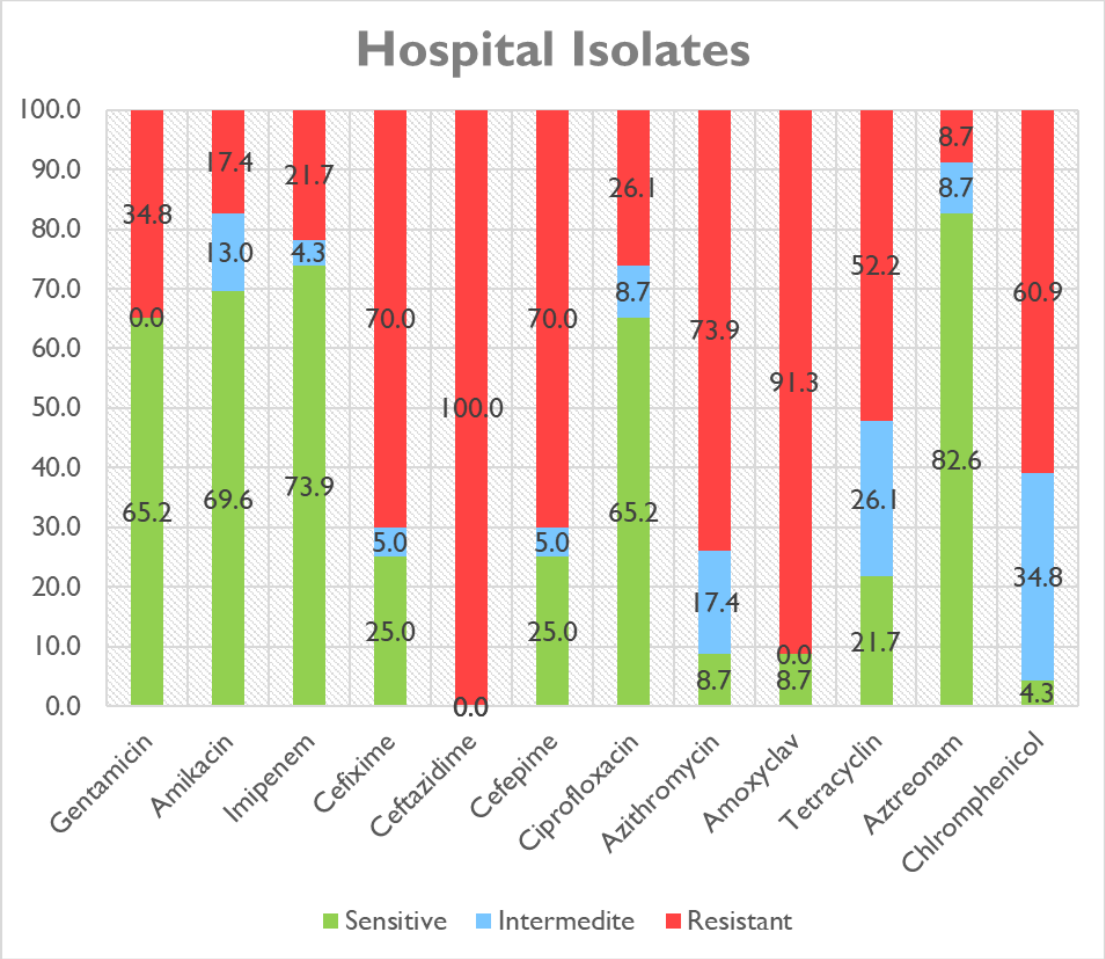


Figure 4: Percentage of Antibiotic Susceptibly Test of Hospital samples

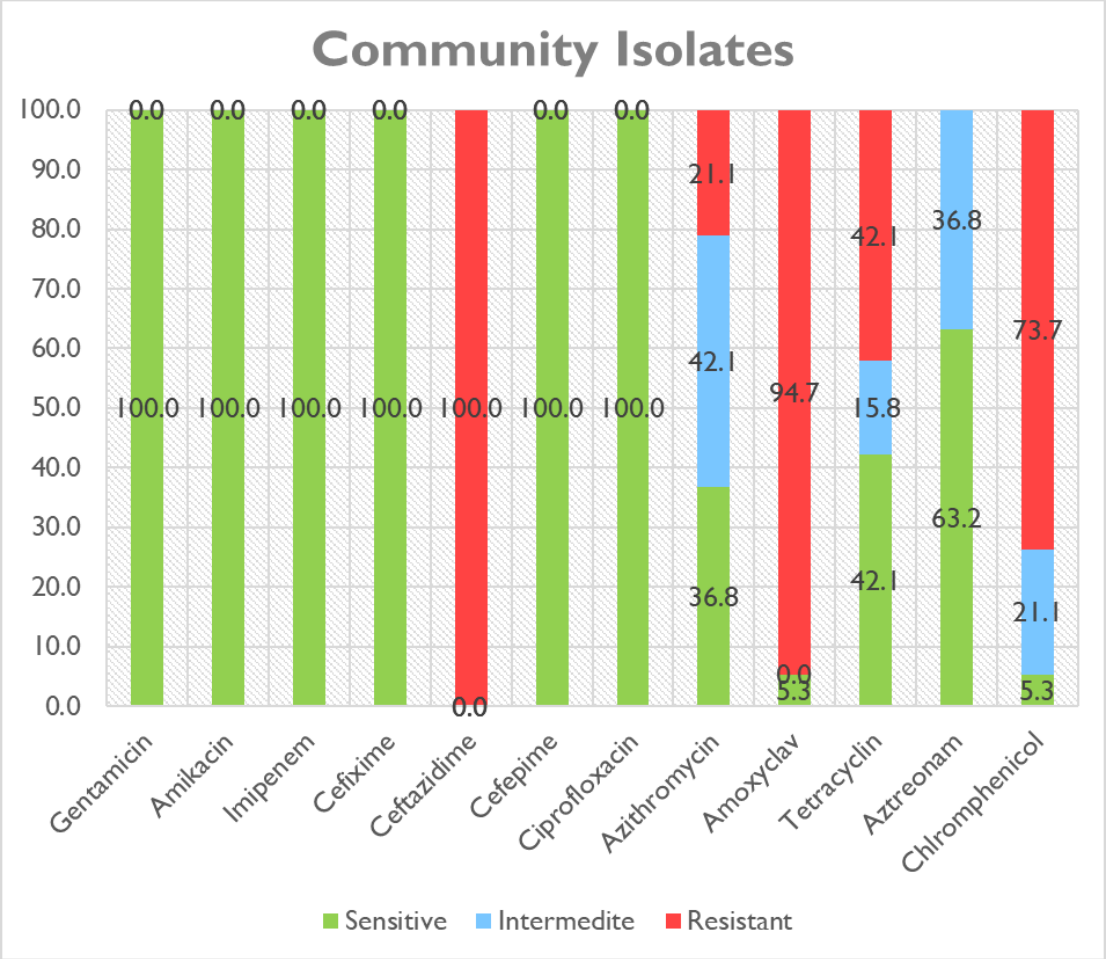


Figure 5: Percentage of Antibiotic Susceptibility Test of Community samples

Identification of MDR genes:

A total of 7 isolates were selected for further multi-drug resistant gene detection. All of them were from hospital wastewater. Because they showed more zones of intermediate or resistance to more than three antibiotic classes.

NDM- 1, *bla* TEM, *bla* VIM, *bla* IMP, *bla* CTX-M, *bla*KPC, *bla* SHV these 7 carbapenems and ESBL genes were examined in all of the selected 7 isolates. We found the presence of only 3 genes. Which were, *NDM- 1*, *bla* TEM, and *bla* VIM by using conventional PCR.

4 isolates were positive for *bla* TEM (57%), 3 isolates were positive for *NDM- 1* (42%), and only 1 isolate was positive for *bla* VIM (14%).

Also, the presence of both *NDM- 1*, and *bla* TEM genes was detected in 1 isolate, and the presence of *bla* TEM, and *bla* VIM genes was detected also in 1 isolate.

Table 1: Distribution of MDR gene among *Pseudomonas aeruginosa*-

Multi Drug resistant Gene names	Number of positive isolates	Percentage of positive isolates
NDM- 1	3	42%
bla $_{TEM}$	4	57%
bla $_{VIM}$	1	14%
NDM- 1+ *bla* $_{TEM}$	1	14%
bla $_{TEM}$ + *bla* $_{VIM}$	1	14%
NDM- 1 + *bla* $_{TEM}$ + *bla* $_{VIM}$	-	-
bla$_{IMP}$	-	-
bla$_{CTX-M}$	-	-
bla$_{KPC}$	-	-
bla$_{SHV}$	-	-

Example of Gel Electrophoresis result of Multidrug resistant gene-

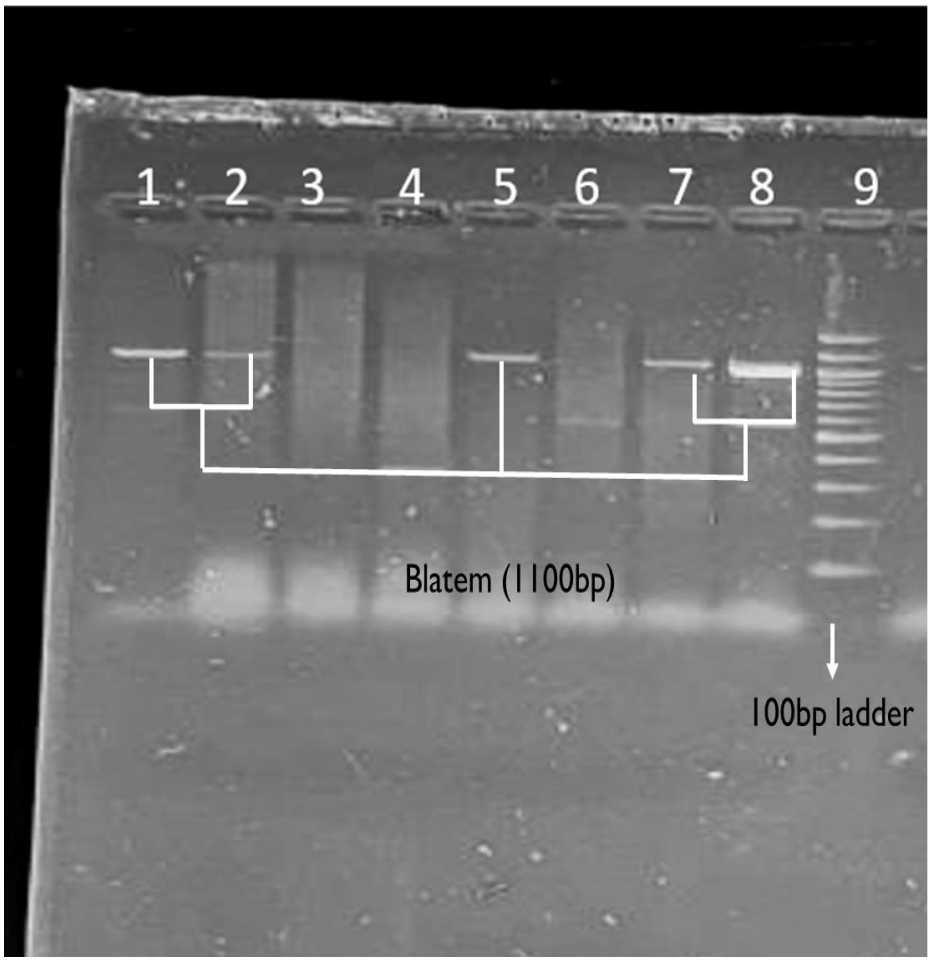


Figure: Gel Electrophoresis of *bla$_{tem}$* gene

- 1,2,5 and 7 numbers were the isolates selected from the hospital waste water sample.
- the 8 number was positive control.

Discussion:

Extended-Spectrum Beta-Lactamase and Carbapenems producing Gram-negative bacteria have been a very serious health concern in recent years and these are spreading worldwide among hospitals, communities, and the environment. (Dandachi et al., 2018). Physicians face the most important challenges during the treatment of infections which are caused due to Gram-negative pathogens for their emerging antibiotic resistance in the public healthcare sector. *Pseudomonas aeruginosa* is an opportunistic Gram-negative rod-shaped pathogen that has a leading role to cause infections and is multiple drug resistant, also has high morbidity and mortality rates. (Bassetti et al., 2018)

Antibiotic-Resistant emerge in *Pseudomonas aeruginosa* through intrinsic and extrinsic mechanisms. The extrinsic mechanism includes the adaption of resistant genes such as ESBLs and Carbapenems. (Pragasam et al., 2016). And these are Beta-lactamase resistant genes. The evolution and dissemination of beta-lactamase-resistant *Pseudomonas aeruginosa* is becoming a serious worldwide health issue and it is mostly studied for its rapid spreading. Beta lactamases are plasmid-encoded enzymes generated by ESBL by amino acid substitution. (Pfeifer et al., 2010).

In our study, we used eight categories of antibiotics 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 65 water samples from three different Hospitals and communities of those hospital areas of Dhaka, Bangladesh. From those samples, 43 isolates (27.95%) were confirmed as *Pseudomonas aeruginosa* and among them, 24 isolates (10.32%) were from Hospitals and 19 isolates (8.17%) were from community water samples. We got 13 confirmed isolates (54%) from Bangladesh Shishu Hospital, 7 confirmed isolates (29%) from National Cancer Hospital and lastly 4 confirmed isolates (17%) from Dedicated National Covid

Hospital, respectively we got 3 isolates (16%), 11 isolates (58%) and 5 isolates (26%) from the community water sample of those hospital areas. (Figures 1 and 2).

Antimicrobial drug-sensitive, resistant, and intermediate percentages of hospital wastewater and community water showed individually in Figures 4 and 5.

The graph showed that ceftazidime antibiotic was most resistant in both the hospital wastewater sample and the community sample. This antibiotic belongs to the cephalosporin group of antibiotics. The next most resistant antibiotic was amoxiclav and the antibiotic belongs to the Penicillin group.

A report published in Europe in 2016 showed that *Pseudomonas aeruginosa* was 33.9% resistant towards at least one of these (piperacillin ± tazobactam, fluoroquinolones, ceftazidime, aminoglycosides, and carbapenems) antibiotic groups. (European Centre for Disease Prevention and Control., 2017).

Also, a research conducted in India in 2021 showed that more than 50% of *Pseudomonas aeruginosa* clinical isolates in India are resistant to fluoroquinolones and third-generation cephalosporins, with a fearful 41.8% to 46.8% of strains showed carbapenem resistance. (Menon et al., 2021).

In our research, we observed that most of the community water isolates were sensitive toward different antibiotic classes. But there is a high chance that the multidrug-resistant isolates can be transmitted into community settings from hospital wastewater. The microbial load of the wastewater can affect the community water in many ways. (Asfaw, 2018). Also, we found 100% resistance of the Ceftazidime drug in both hospital isolates and community isolates. We can assume that this resistant strain may transmit through hospital water to community water. As some antibiotics were showing similar resistant patterns in both water isolates.

The hospital environment is susceptible to nosocomial infection. A report stated that in ICUs *Pseudomonas aeruginosa* is mostly responsible for nosocomial infection the rate is 13.2-22.6%, and caused all types of nosocomial infections in other patients the rate is 11%. (Driscoll et al., 2007) (Khan et al., 2015). A study conducted in 2015 reported that nosocomial pneumonia increased by 17%-30% over five years. (Khan et al., 2015). And this nosocomial pneumonia is most commonly caused by *Pseudomonas aeruginosa* in ICU patients. (Driscoll et al., 2007). These nosocomial isolates adapt to multidrug-resistant genes and for this, the treatment becomes very difficult. An example of 9-year surveillance conducted from 1994 to

2002 in a US hospital reported that 1%-16% increase in the number of nosocomial isolates of *Pseudomonas aeruginosa* that were multidrug resistant.

From antibiotic susceptibility results we found most of the isolates were resistant to particular drugs and we chose the 7 most multiple drug-resistant isolates among them, which we then tested to identify if they had ESBLs and carbapenems genes.

In our study of *Pseudomonas aeruginosa,* the presence of *bla* TEM was found among other ESBL genes. The percentage of *bla* TEM positive isolates was 57% among selected isolates. 4 of the 7 isolates were positive for this gene.

We also found *bla* VIM and *NDM*-type genes present in our isolates among other carbapenems genes. We observed only one positive isolate for the *bla* VIM gene, which was 14% of the total selected isolates, and 3 positive isolates for the *NDM- 1* gene, which was 42% from the total selected isolates.

Moreover, we observed both *bla* TEM and *NDM- 1* genes in 1 selected isolate. The isolate showed resistance towards aminoglycoside (Gentamicin, Amikacin), carbapenems (Imipenem), 4th generation cephalosporins (cefixime, ceftazidime), 4th generation cephalosporins (cefepime), fluoroquinolone (ciprofloxacin), Macrolides (Azithromycin), Penicillin (Amoxiclav, Tetracycline), Monobactam (Chloramphenicol).

Also, both *bla* TEM and *bla* VIM genes were found in 1 isolate. This isolate showed resistance towards aminoglycoside (Gentamicin, Amikacin), carbapenems (Imipenem), 4th generation cephalosporins (cefixime, ceftazidime), 4th generation cephalosporins (cefepime), fluoroquinolone (ciprofloxacin), Macrolides (Azithromycin), Penicillin (Amoxiclav) Monobactam (Chloramphenicol).

TEM-type enzymes are universally found among Enterobacteriaceae. (Weldhagen et al., 2003). This is the most abundant beta-lactamase gene among other ESBL genes. In our study, we found the majority of the isolates were *bla* TEM positive which was 57%. Similarly, we found a study reported in Sudan showed that the results on beta-lactamase genes in *Pseudomonas aeruginosa*, the most found gene was *bla* TEM which is 44.2% of total isolates. (Abdelrahman et al., 2020). Again, another study in Egypt showed that the TEM gene was found highly positive in *Pseudomonas aeruginosa* which was 50% among other organisms. (Mohamed et al., 2016). In our research, the percentage of positive isolates of the *bla* TEM gene was similar to other reports we found.

In previous years, many Metallo-Beta -lactamase genes were identified in *Pseudomonas aeruginosa*. The rate of mortality due to Metallo-Beta -lactamase producing *Pseudomonas aeruginosa* is 70%-90%. (Wang & Wang, 2020). *bla* VIM and NDM are Metallo-Beta -lactamase carbapenems genes. Bacteria containing VIM, and NDM enzymes are found in a variety of environmental, community, and hospital samples. (Boyd et al., 2020). *Pseudomonas aeruginosa* contains *bla* VIM integron in the chromosome. (Lauretti et al., 1999). Our study showed only one isolate was positive for the *bla* VIM gene. But a study reported that *bla* VIM gene can be found commonly in *Pseudomonas aeruginosa* within the Indian population. (Manohar et al., 2020). Also, *bla* VIM- type carbapenems-resistant *Pseudomonas aeruginosa* isolates are mostly found in the Gulf Cooperation Council countries (Saudi Arabia, Qatar, UAE, Kuwait, Bahrain, Oman). The percentage of resistant isolates is 39%. (Boyd et al., 2020).

In our study, we observed 42% of our isolates are positive for *NDM- 1* gene, which is much higher than the report studied from China (9.4%). (Wang & Wang, 2020). It is already reported that NDM producers become very popular among South Asian countries like India, Pakistan, and Bangladesh. Also, *NDM-1* eradicates the activities of penicillin, cephalosporin, and carbapenems. (Dortet et al., 2014). In our study, The NDM- 1 positive isolates were found resistant to those groups of antibiotics. The positive isolates are from hospital wastewater samples, so the presence of such gene-producing bacteria found in an environmental sample is so alarming for the public people who live in poor sanitation environments. The *NDM-1* gene-producing bacteria are also found in drinking water and environmental samples in India. (Dortet et al., 2014).

The antibiotics used on a daily basis for the treatment of *Pseudomonas aeruginosa* include aminoglycosides, cephalosporins, and carbapenems. (El Solh & Alhajhusain, 2009). But the expansion in resistance expressed to cephalosporins and imipenem is very alarming. (Hosu et al., 2021). A study conducted that 70% of the isolates are resistant to imipenem. (Aghamiri et al., 2014). Also, our present study showed most of the resistance towards cephalosporins (ceftazidime, cefepime). Cephalosporins are an important group of antibiotics as they have broad-spectrum activity and so used to treat many infections. The increase in resistance in the cephalosporins group may adversely affect their clinical efficacy. (Hosu et al., 2021).

There is a difference in the results of our study and other previous reports may be due to the geographic regions of the countries, the difference in samples, the difference in the usage of various antibiotics, and the difference in the treatment of antibiotics. (Aghamiri et al., 2014).

Monitoring and identification of these resistant genes among the microorganisms are very necessary because they can cause epidemics rapidly by increasing multiple drug-resistant infections as they are correlated to other resistomes. (Manohar et al., 2020).

Conclusion:

Pseudomonas aeruginosa isolates were collected from the selected hospitals and nearby community water, which was 27.95%. Among the isolates from hospital wastewater, we have found 10.32% isolates whereas from nearby community tap water isolates found 8.17%. Out of the selected 7 isolates for MDR gene identification NDM-1 was found in 42%, *bla* VIM in 14%, and *bla* TEM in 57% isolates. In the study, we have observed almost similar resistant patterns for both hospital wastewater and nearby community water in the Antibiotic susceptibility test. For instance, we have observed 100% ceftazidime resistance for both hospital waste and nearby community water. It has been claimed that Hospital wastewater is the reservoir of the antimicrobial-resistant organism and following that, we also found an Antimicrobial resistant gene on *Pseudomonas aeruginosa* from hospital wastewater. From our study, we assumed that community water has become resistant from the wastewater of the hospitals. 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, it is known that biofilm-producing bacteria show high antibiotic resistance than 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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