Detection of Carbapenemase Gene from Multidrug-resistant *Pseudomonas aeruginosa* Isolated from Hospital Sewage Waste water and adjacent Community Tap Water

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

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CONTENT

DECLARATION

It is therefore proclaimed that_

- The thesis submitted is our unique work completed while completing the Bachelor's Degree at BRAC University. The thesis does not contain previously published content, nor is it produced by a third party, except when it is correctly cited through comprehensive and accurate referencing.

- The thesis contains no material that has been submitted or acknowledged for any other degree or diploma at a university or other institution.
- We have precisely identified all of the primary sources of assistance.

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APPROVAL

The thesis/ project titled "*Detection of Carbapenemase Gene from Multidrug-resistant Pseudomonas aeruginosa Isolated from Hospital Sewage Waste water and adjacent Community Tap Water*" submitted by_

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Of Batch Spring 2018 has been accepted as satisfactory in partial fulfillment of requirement for the degree of Bachelor of Science in Biotechnology on 03.10.2021.

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would not be the people we are today. We owe our accomplishment to our family's unconditional love and nourishment, and we aim to make them proud one day.

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LIST OF ABBREVIATIONS

- >= Equal or more than
- <= Equal or less than
- = More than
- **ATCC**= American type culture collection,
- ***bla***= Beta-lactamase gene
- **CFU**= Colony forming unit
- **CLSI**= Clinical and laboratory Standard Institute CTX-M= Active on cefotaxime
- **DNCC**=Dhaka North City Corporation
- **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 MgCl2= Magnesium chloride
- **MIC**= Minimum inhibitory concentration
- **OXA**= Oxacillinase
- **PCR**= Polymerase chain Reaction
- **SHV**=Sulfhydryl variable
- **TBE**= Tris-Borate-EDTA
- **TE**= Tris-EDTA
- **TEM**= Temoniera
- **V/v**= Volume/volume
- **VIM**= Verona integron-encoded metallo-β-lactamase W/v= Weight/volume
- **Zn**= Zinc

ABSTRACT

Introduction: Hospital wastewater is a prime breeding ground for antibiotic-resistant pathogenic microorganisms. The goal of our study is to explore the multi drug resistance pattern of *Pseudomonas aeruginos*a discovered in both hospital and community water to anticipate how our routine commutes are being exposed.

Methods: A total of 33 non-duplicate isolates of *Pseudomonas aeruginosa* were obtained from both the hospital and the surrounding community tap water. Initially, phenotype technique was used to identify multi-drug-resistant isolates. Antimicrobial susceptibility was detected by using the Kirby disk diffusion method. Each isolate was tested resistance to carbapenem, aminoglycosides, fluoroquinolones, lipopeptide, macrolides, beta-lactam combination agents, penicillin, and monobactam. The presence of the Metallo-beta-lactamase (MBLS) and Extended spectrum β-lactamase (ESBL) gene was determined using the traditional PCR method.

Result: Out of 33 isolates collected from hospital wastewater and community water show a comparable antimicrobial-susceptibility pattern against the stated antibiotic classes. Only 15 were selected for further antimicrobial-susceptibility test against the stated antibiotic classes. Isolates found resistant to Amikacin (26.6%), Amoxyclav (93.3%), Azithromycin (100%), Aztreonam (66.6%), Cefepime (20%), Ceftazidime (20%), Ceftriaxone (53.3%), Chloramphenicol (55.5%), Ciprofloxacin (33.3%), Colistin (26.6%), Erythromycin (93.3%), Gentamicin (26.6%), Imipenem (20%), Norfloxacin (40%), Piperacillin-tazobactam (26.6%), Polymyxin (13.3%) and Tetracycline (46.6%). On the contrary, 4 isolates obtained from both Hospital waste water and adjacent Community Tap water showed resistance to most of the mentioned antibiotics. Those isolates were tested positive for the presence of bla-NDM, NDM-1, bla-CTX-M, bla-OXA 48 and bla-TEM by PCR as, confirming the existence of a gene encoding extended spectrum beta-lactamase and MBL.

Conclusion: The result of this study provided insights into the high proportion of NDM-1, OXA-48, CTX-M and TEM producers concluding that Hospital wastes have an impact on the emergence of new diseases by transmitting multidrug-resistant microorganisms to nearby areas, exposing the general population to antibiotic resistance at an early stage and making them resistant to any treatments.

Keywords: *Pseudomonas aeruginosa*, multi-drug resistance, PCR, ESBLs, MBLs, Antibiotics.

1. INTRODUCTION

Pseudomonas aeruginosa (P. aeruginosa) is a common Gram-negative bacterium that causes nosocomial infections as well as fatal infections in immunocompromised people, such as cancer patients, people who have recently undergone surgery, people who have suffered from severe burns, or people who are HIV-positive (Michael John Gale, 2015; 2, 2015; A Gomila, 2018).The World Health Organization designated *P. aeruginosa* as a priority pathogen for research and development of new antibiotics in 2017 and acknowledged it as one of the most deadly bacteria (Organization, 2017). Due to *P. aeruginosa* flexibility and strong intrinsic drug resistance, common antimicrobial treatments such antibiotics typically demonstrate low efficiency, increasing mortality specially in immunosuppressed and burnt patients (Zheng Pang 1, 2019). The rise of Multidrug-Resistant (MDR) *P. aeruginosa* in hospital settings is most likely caused by the selective pressure imposed by incorrect antibiotic usage on the one hand, and the rising use of antibiotics on the other (Taipei, 2018).

As a gram-negative aerobic rod, *Pseudomonas aeruginosa* continues to be one of the most resilient causes of nosocomial infections. 10–11% of Nosocomial Infections are due to *P. aeruginosa*. This outcome is a result of the microorganism's resistance to numerous antimicrobials and disinfectants. It contributes to the growth of infections in the urinary, respiratory, and wound systems. It can lead to bacteremia, especially in hospitalized patients in the ICU or anesthesiology and resuscitation department who frequently have hemodynamic instability and respiratory insufficiency and need mechanical lung ventilation. The use of mechanical ventilation raises the risk of pseudomonad pneumonia significantly. *P. aeruginosa* possesses enzymes that are encoded on both chromosomes and plasmids. These enzymes are frequently used in conjunction with other resistance strategies, like lowering the permeability of the outer or cytoplasmic membrane. *P. aeruginosa* develops carbapenemases, which causes the bacteria to lose its sensitivity to the drug carbapenem and develop resistance to it. Additionally, cephalosporins, ureidopenicillins, and aminoglycosides no longer work on it. Quaternary disinfectants cannot effectively remove it. Hospital water, taps, shower heads, swimming pools, healing waters, and other surfaces can have pseudomonas nosocomial infections. It happens in sinks, humidifiers, anesthetic devices, inhalers, hand brushes, and other locations that have the right conditions, which includes a humid environment. Lubricating gels and disinfectants are tainted by pseudomonads (Labovská, 2021).

According to the European Centre for Disease Prevention and Control (ECDC*), P. aeruginosa* is the fourth most common hospital pathogen in Europe, accounting for 9% of all hospital-based infections (P Ruiz-Garbajosa, 2017). Furthermore, the CDC discovered parallel findings in the United States, where P. aeruginosa infections occur about 7% of the time in hospital settings (Shelley S Magill 1 & Infe, 2014). A study conducted in Spain in 2016 discovered a 13% increase in the prevalence of this bacterium in hospital ICUs (Palomar M, 2014). P. aeruginosa is a representation of the bacterial resistance phenomenon, exhibiting all known enzymatic and genetic pathways of bacterial resistance (T Köhler 1 J. C., 1999). These characteristics are caused by the selective pressure of chromosomal gene alterations that result in efflux pump overexpression, oprD suppression or inactivation, and ampC hyperexpression (Livermore, 2002). P. aeruginosa can develop additional drug-resistance determinant by horizontally transferring mobile genetic elements that code for class B carbapenemases, often known as metallo-beta-lactamases or MBLs, which hydrolyze all -lactams except aztreonam (Anne Marie Queenan 1, 2007)

In addition to that, *Pseudomonas aeruginosa* is also a clinically significant organism that possesses inherent resistance to a number of antibiotics. A low level of outer membrane permeability and the synergy between widely selective drug efflux pumps lead to this inherent multidrug resistance. OprD loss, which primarily imparts a resistance to imipenem but also provides a low-grade resistance to meropenem, is principally responsible for the resistance to carbapenem antimicrobials (T Köhler 1 M. M.-H., 1999) (1, 1992). However, the multi-drug efflux mechanisms that mediate the resistance to quinolone, chloramphenicol, and many other antimicrobial drugs also contribute to the resistance to carbapenem. The strains that overexpress either the **MexAB-OprM** system or the **MexEF-OprN** system display carbapenem resistance by either pumping the drug out or by suppressing oprD transcription, respectively (T Köhler 1 M. M.-H., 1997) (H Maseda 1, 2000) (M M Ochs 1, 1999). On the other hand, imipenem, biapenems, and several -lactams became more effective against the NfxB mutants that expressed MexCE-OprJ. (Chemother, 1996). Chromosome-based AmpC -lactamase, in addition to OprD deletion or drug efflux pumps, is crucial for *P. aeruginosa* resistance to carbapenem (Nobuhisa Masuda, 1999)

P. aeruginosa generates a strong biofilm as a necessary tool for competing, thriving, and dominating in the polymicrobial environment, or to be more specific, as a critical survival strategy in both natural and artificial settings. This is why P. aeruginosa is regarded as a significant biofilm-forming pathogen in studies aimed at better understanding biofilm development and regulation. P. aeruginosa has a complex genome as well, and its pathogenic profile is connected with a wide range of virulence factors (Ruxana T. Sadikot, 2005; Ethan E

Mann 1, 2012). The capacity to form biofilms, in particular, gives bacteria a substantial advantage when infecting susceptible hosts and causing diseases such as VAP and CF lung infections. The extracellular polymeric substance (EPS) matrix-encased communities of sessile bacteria known as biofilms maintain homeostasis and stability in the face of extreme environmental circumstances, including those found within the human host (Hans-Curt Flemming, 2007) . Through a variety of adaptive processes, biofilms can shield bacteria from both host defenses and antibiotic treatment (David Lebeaux, 2014; Lewis, 2008; Ruxana T. Sadikot, 2005). A biofilm is a highly organized community of bacteria that is attached to an abiotic or biological surface and is enveloped in an extracellular matrix. More than 90% of the biofilm is made up of the EPS matrix, which is produced by the bacteria, which make up less than 10% of the biofilm biomass (Wingender, 2010). Polysaccharides, proteins, extracellular DNA (eDNA), and lipids make up the majority of the matrix (Qing Wei, 2013). The genetic make-up of *P. aeruginosa* isolates, environmental factors, and interactions between the two can all have an impact on biofilm production (Tolker-Nielsen, 2014). These variables consist of pH, temperature, nutritional concentrations, bacterial motility, intercellular communication, and host-derived variables (J.Plotkina, 2016) . Through the use of intercellular communication channels and resource sharing, the EPS matrix enables the microorganisms to work together as a cohesive community (Wozniak, 2016; Wingender, 2010) . Quorum sensing (QS), bis-(3′-5′) cyclic di guanosine monophosphate (c-di-GMP) signaling, and small RNA regulation are some of the mechanisms that control how biofilms form and spread (Mustafa Fazli, 2014). Quorum sensing (QS) is a bacterial communication system that enables the regulation of gene transcription and group activity of particular processes. There are two major interconnected QS systems in *P. aeruginosa* that depend on the signaling molecule acyl homoserine lactone (AHL): the Las system, which employs N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), and the Rhl system, which uses N-butanoyl homoserine lactone. *Pseudomonas* quinolone signal system and the integrated quorum sensing system (IQS) are two more QS systems that do not utilize AHL molecules and function as redundant auxiliary pathways (Zhang, 2015) . eDNA and the biosurfactant glycolipid rhamnolipid, which are crucial for the growth of mature biofilms, are produced under the direction of QS, which is why QS is required (Tolker-Nielsen, 2014; DAVID G. DAVIES, 1998)

P. aeruginosa can be acknowledged as one of the deadliest pathogens as the bacterium frequently results in multi-site infections, the most severe of which, bacteremia, with a death rate of 18–61% (Francesc Vidal, Josep Mensa, Manuel Almela, & al, 1996; H. S. M. Ammerlaan, 2013). Bearing all these in mind this study focuses on the antibiotic susceptibility patterns of *P. aeruginosa* clinical samples and their multidrug resistance pattern to identify

possible MR genes that accounts for ESBL production. Furthermore, this study focused on transmission of Multi-drug Resistant Isolates from hospital to adjacent communities ensuring mass population more resilient to antibiotic treatment as very little research is done in Bangladesh in this particular field. Also, the future goal of this study is aiming to compare the biofilm formation present in clinical and adjacent communities to ensure the transmission and the possible threats that comes along.

2. OBJECTIVES

Research Hypotheses

- Multi-drug Resistant Isolates are transmitted from Hospital waste water to Adjacent Communities ensuring mass population more resilient to antibiotic treatment.

General Objective

- To detect MBL and ESBL encoding gene bla-NDM, NDM-1, bla-CTX-M, bla-OXA 48 and bla-TEM among multidrug resistant *Pseudomonas aeruginosa.*

Specific Objectives

- To determine multi-drug resistance patterns of isolated *Pseudomonas aeruginosa.*
- To detect the carbapenamase coding gene by PCR among the multidrug resistant isolates.

Future Objectives

- Detection of biofilm formation among the multidrug resistant isolates to abode the presence of horizontal gene transfer during unfavorable environment and how its co-factors indirectly in the broader distribution of MDR of other gram-negative

3. Materials and Method

Bacterial Isolates:

The research has been conducted from June 2022 to December 2022, in the specialized research laboratory of MNS department (Mathematics and Natural science). To get a more precise finding each week we collected waste water samples from hospitals and their surrounding community area. The major focus has been National Institute of Cancer Research & Hospital (NICRH), DNCC Dedicated COVID-19 Hospital and Bangladesh Shishu Hospital. We have collected community samples from the tap water from the adjacent area of these three hospitals.

Identification of Species:

The collected samples were first distinguished in parts as of Hospital waste water and community water. For hospital waste waters samples were went through the process of serial dilution (10^-1 to 10^-7). After that, we would take direct raw sample as well as diluted sample in either 10^-1,10^-3,10^05 or 10^-2,10^-4,10^-6 manner. 100 microlitre of diluted sample were spread in Cetrimide agar which is a selective media for *Pseudomonas aeruginosa*. The plates were than left for overnight incubation at 37°C aerobically.

On the other hand, for community waste water filtration paper (45mm) was used. The samples were filtrated and the filtration paper was left in TSB for overnight enrichment in shaking incubator at 37°C.The following day the TSB enriched samples were processed for serial dilution just like the previous manner and diluted samples were spread in Cetrimide media and left for overnight incubation.

After overnight incubation in both hospital and community samples that were grown in the Cetrimide agar, UV glow is observed to find out specific strain of only *Pseudomonas aeruginosa.* The colonies that produced glow under UV are suspected and potential desired isolates. After that colonies were isolated by observing morphology.

Selected colonies were then streaked in Nutrient Agar media for obtaining successful single colonies. Single colonies were then further used for DNA extraction by boiling method (REF) which was required to perform PCR.PCR was performed to get the confirmation of selected colonies to be of *Pseudomonas aeruginosa.*

Polymerase Chain reaction (PCR) for detection of Pseudomonas aeruginosa:

DNA Extraction (Boiling Method)

- All the selected single colonies and 1 positive control were directly inoculated to 150µl of 1X TE buffer in micro-centrifuge tube.
- Secondly all the selected specimen including 1 positive control were boiled in water bath for 10-15 mins at 100 degrees Celsius.
- After the water bath, all the specimens were centrifuged for 5 mins at 13,000RPM
- Then the supernatants of all the specimen were individually separated from remaining pellet to another micro-centrifuge tubes.

Primer Design:

- **PA-SS-F** and **PA-SS-R were** used in the PCR amplification to ensure the specimen was specifically ***Pseudomonas aeruginosa.***

Primer	Primer Sequence	Target	Annealing Temp	Base pair
PA-SS-F	GGGGGATCTTCGGACCTCA	*P. aeruginosa*	58˚C	956
PA-SS-R	TCCTTAGAGTGCCCACCCG			

Preparation of Master Mix:

Master mix () ---------- 6.5 µl
Working forward Primer (**PA-SS-F**) ----------1 µl
Working Reverse Primer (**PA-SS-R**) ----------1 µl
Nuclease free water (NFW) ----------2.5µl

Total master mix stock---------- = 13 µl

- In each Eppendorf, we added 11µl master mix along with 2µl DNA template to make a total of 13µl reaction volume.

- These specimens were further assessed for PCR under following cycle.

Steps	Temperature	Time
Initial Denaturation	95˚C	2 mins
30 cycles	94˚C	20 sec
	58˚ C	20 sec
	72˚C	40 sec
Final extension	72 ˚C	1 min

After 1 hour 41 mins, PCR amplification was completed and the PCR product was run under gel electrophoresis for observing the desired bands.

Preparation of Gel Electrophoresis:

Gel composition:

- 1X TBE buffer =100ml (50X TBE =2ml + d.h20 =98ml)
- Agarose (1.5%) = 1.5 gm

{since the band are larger in size the agarose concentration is decreased, less concentration Agarose gels feature bigger pores, allowing for the separation of larger DNA fragments}

- 6µl of each specimen were put in well of the gel along with ladder, positive control and negative control.
- Which is further run under 120V for 60 mins.

Antimicrobial susceptibility test:

All isolates were tested for antimicrobial susceptibility on Mueller Hinton agar plates using the Kirby Bauer modified disk diffusion technique, and zones of inhibition were determined in accordance with CLSI standards (CLSI, 2010).The sensitivity patterns of the organisms were observed using antibiotic discs such as Amikacin (AK30) mm, Amikacin (AK30) mm, Azithromycin(AZM15) mm, Aztreonam (AT30), Cefepime (CPM30) mm, Ceftazidime (CAZ 30) mm, Ceftazidime (CAZ 30) mm, Chloramphenicol (C30), Ciprofloxacin (CIP5) mm, Colistin(CT10) mm, Erythromycin (E15) mm, Gentamicin(Gen10), Imipenem (IMP10), Meropenem (MRP), Norfloxacin (NX10), Piperacillin -tazobactam (PIT100/10), Polymyxin B (PB300), Tetracycline (TE30) mm, Vancomycin(VA30).

Preparation and Inoculation of test organisms:

A sterile wire loop was used to emulsify 3 ml of sterile normal saline with 3-5 well-isolated colonies of test organisms. The turbidity of the suspension was compared to the McFarland turbidity standard 0.5 by adding normal saline and inserting a printed card behind the test and standard inoculums under adequate lighting. This is how we made all of the test inoculums..

The bottom of the inoculum was then cleaned with a sterile swab stick. The swab stick was spun and rubbed against the edge of the tube above the level of the suspension to remove excess fluid. The Mueller Hinton agar plate was spun at roughly 60°C while the swab was applied to the plate's surface evenly in three directions. After that, the inoculating plate was left to dry for 3-5 minutes.

Antibiotic discs were spaced 25 mm apart on the inoculating plate and 15 mm from the plate's edge. After 30 minutes, the antibiotic discs were put, and the infected plates were incubated aerobically for 24 hours at 37°C.

Interpretation of zone of diameter:

After an overnight incubation, the test plates were tested to ensure that the organism was growing in confluence. The zone of inhibition was measured in millimeters with a ruler on the plate's bottom and assessed using the CLSI guidelines' standard chart. Based on the examined clear zone of inhibition surrounding the disc, test organisms were classed as resistant, moderate, or sensitive.

Polymerase Chain Reaction of for detecting ESBL producers:

Isolates that showed resistance towards all the selected antibiotics disk were further screened for ESBL producers to find MR genes. DNA extraction was proceed using the same protocol and followed by PCR and Gel Electrophoresis to get the confirmation final band under UV light.

Table of selected MR genes primer

Primer	Primer Sequence	Condition	Annealing Temp	Base pair
Bla CTX MF	5'-ACGCTGTTGTTAGGAAGTG-3'	94C = 3mins 94 C = 1 min 58 C= 30 sec 72 C = 1 min (35 cycles) 72 C= 10 mins	58˚C	857
Bla CTX MR	5' TTGAGGCTGGGTGAAGT 3'			

Primer	Primer Sequence	Condition	Annealing Temp	Base pair
Bla OXA 48 F	5'- GCTTGATCGCCCTCGATT -3'	95C = 5mins 94 C = 45 sec 55 C= 45 sec 72 C = 1 min (30 cycles) 72 C= 7 mins	55˚C	281
Bla OXA 48 R	5'-GATTTGCTCCGTGGCCGAAA -3'			

Primer	Primer Sequence	Condition	Annealing Temp	Base pair
Bla NDM F	5’-ACCGCCTGGACCGATGACCA-3’	94C = 10mins 94 C = 30 sec 52 C= 40 sec 72 C = 50 sec (30 cycles)	52˚C	264
Bla NDM R	5’-GCCAAAGTTGGGCGCGGTTG-3’	72 C= 5 mins		

Primer	Primer Sequence	Condition	Annealing Temp	Base pair
NDM F	5’GGTTTGGCGATCTGGTTTTC-3’	For lab PCR Default set up	58˚C	621
NDM R	5’-CGGAATGGCTCATCACGATC-3’			

Primer	Primer Sequence	Condition	Annealing Temp	Base pair
Bla TEM F	5’-AAAATTCTTGAAGACG -3’	95C = 3mins 95 C = 30 sec 51 C= 30 sec 72 C = 30 sec (30 cycles)	51˚C	500
Bla TEM R	5’-TTACCAATGCTTAATCA -3’	72 C= 1mins		

4. RESULTS

This investigation includes a total of 19 water samples that were obtained from the hospital and the neighborhood. 16 samples of tap water were taken from the nearby neighborhood of the stated hospitals, and 3 samples were obtained from the DNCC, Cancer, and Bangladesh Shishu Hospital sewage waste water, respectively.

Table 4.1 and 4.2 demonstrates the distribution of Pseudomonas aeruginosa isolated from water sample from both hospital and their respective communities.

Among the isolated 33 *P. aeruginosa*, DNCC Hospital waste water contains 6 (18.1%) isolates, Cancer Hospital were of 5 isolates (15.15%) and Bangladesh Shishu Hospital also contained 5 isolates (15.15%).

Hospital Name	Species Name	Waste water (Number of isolates)	Waste water (Percentage %)
DNCC	*Pseudomonas aeruginosa*	6	18.1%
Cancer		5	15.15%
Bangladesh Shishu		5	15.15%
Total Isolates		16	48.4%

Among the isolated 33 *P. aeruginosa*, DNCC community water contains 14 (42.4%) isolates, Cancer Community has 2 isolates (6.06%) and Bangladesh Shishu community also contained 1 isolate (3.03%).

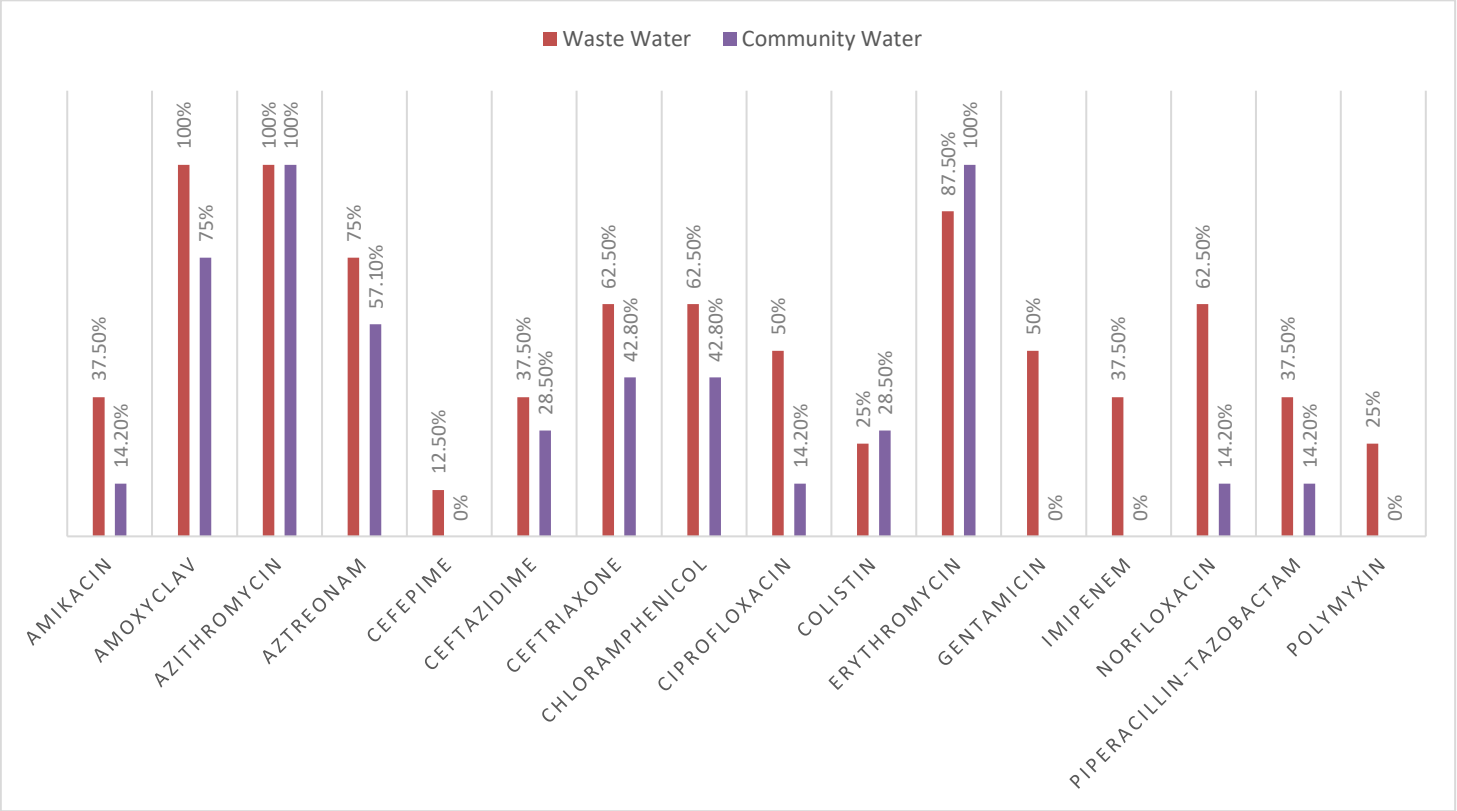
Hospital Name	Species Name	Community water (Number of isolates)	Community water (Percentage %)
DNCC	*Pseudomonas aeruginosa*	1	42.4%
Cancer		2	6.06%
Bangladesh Shishu		1	3.03%
Total Isolates		17	51.49%

Out of 33 isolates only 15 were selected for further antimicrobial-susceptibility test against the stated antibiotic classes. Isolates found resistant to Amikacin (26.6%), Amoxyclav (93.3%), Azithromycin (100%), Aztreonam (66.6%), Cefepime (20%), Ceftazidime (20%), Ceftriaxone (53.3%), Chloramphenicol (55.5%), Ciprofloxacin (33.3%), Colistin (26.6%), Erythromycin (93.3%), Gentamicin (26.6%), Imipenem (20%), Norfloxacin (40%), Piperacillin-tazobactam (26.6%), Polymyxin (13.3%) and Tetracycline (46.6%).

Table 4.3: Antimicrobial drug resistance pattern between Waste and community water.

Name of Antimicrobial Drugs	Waste Water Number of isolates, N=8	Community Water Number of isolates, N=7
Amikacin	37.5% (3)	14.2% (1)
Amoxyclav	100% (8)	75% (6)
Azithromycin	100% (8)	100% (8)
Aztreonam	75% (6)	57.1% (4)
Cefepime	12.5% (1)	0% (0)
Ceftazidime	37.5% (3)	28.5% (2)
Ceftriaxone	62.5% (5)	42.8% (3)
Chloramphenicol	62.5% (5)	42.8% (3)
Ciprofloxacin	50% (4)	14.2% (1)
Colistin	25% (2)	28.5% (2)
Erythromycin	87.5% (7)	100% (7)
Gentamicin	50% (4)	0% (0)
Imipenem	37.5 % (3)	0% (0)
Norfloxacin	62.5% (5)	14.2% (1)
Piperacillin-tazobactam	37.5% (3)	14.2% (1)
Polymyxin	25% (2)	0% (0)
Tetracycline	75% (6)	14.2% (1)

Table 4.4: Comparison of antibiotic resistance pattern of *Pseudomonas Aeruginosa* isolates collected from Water waste and Community Water:



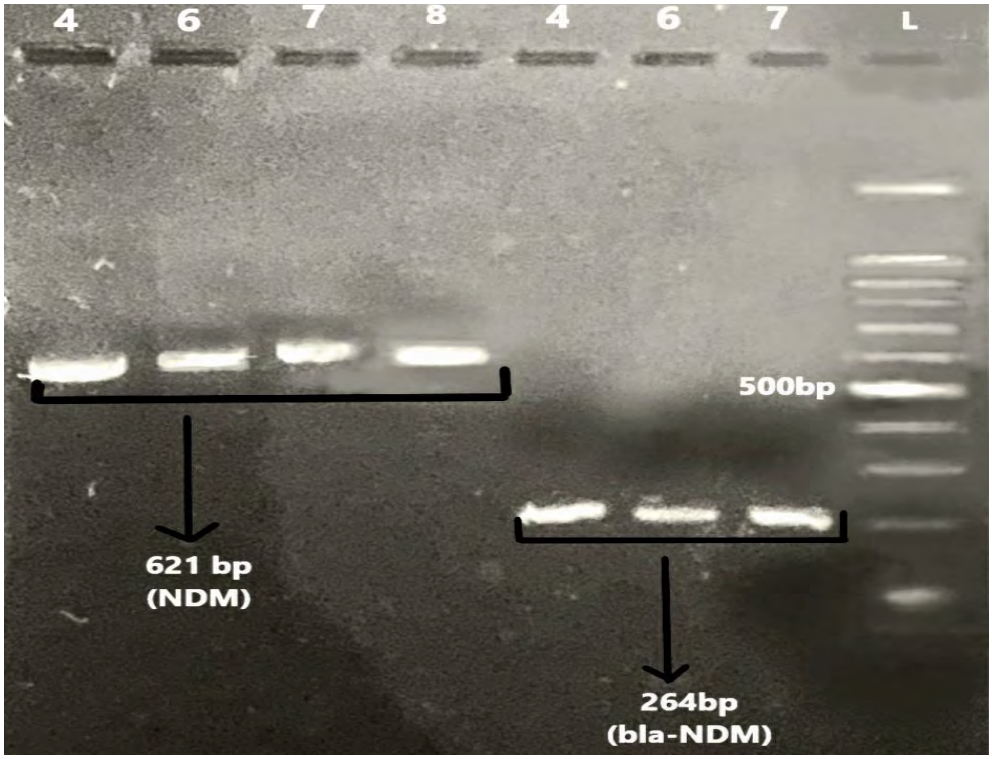
Eight isolates were ultimately chosen as carbapenem-resistant *Pseudomonas aeruginosa* on the basis of antibiotic susceptibility patterns. The presence of the bla-NDM, bla-OXA 48, TEM, CTX-M, and NDM genes was examined in all 8 isolates using traditional PCR.

4 *Pseudomonas aeruginosa* isolates (50%) were NDM positive by PCR out of eight putative carbapenem-resistant isolates. Three (3) of these four isolates were from hospital waste water, while one (1) came from community water.

Table 4.*5*: Distribution of *bla*-NDM, CTX-M, NDM-1, TEM and OXA 48 genes among suspected carbapenem-resistant *P. aeruginosa* isolates from both the Hospital waste and Community water.

Sample type	*Pseudomonas aeruginosa*	*Bla*-NDM Producer		CTX-M Producer		NDM-1 Producer		TEM Producer		OXA 48 Producer	
Hospital Waste Water	6	3	50%	2	33.3%	3	50%	2	33.3%	3	50%
Community water	2	-	-	-	-	1	50%	-	-	-	-
Total	8	3	37.5%	2	25%	4	50%	2	25%	3	37.5%

Figure 4.6: shows the gel electrophoresis result of both NDM (Left side) and *bla*-NDM (right side)- gene.

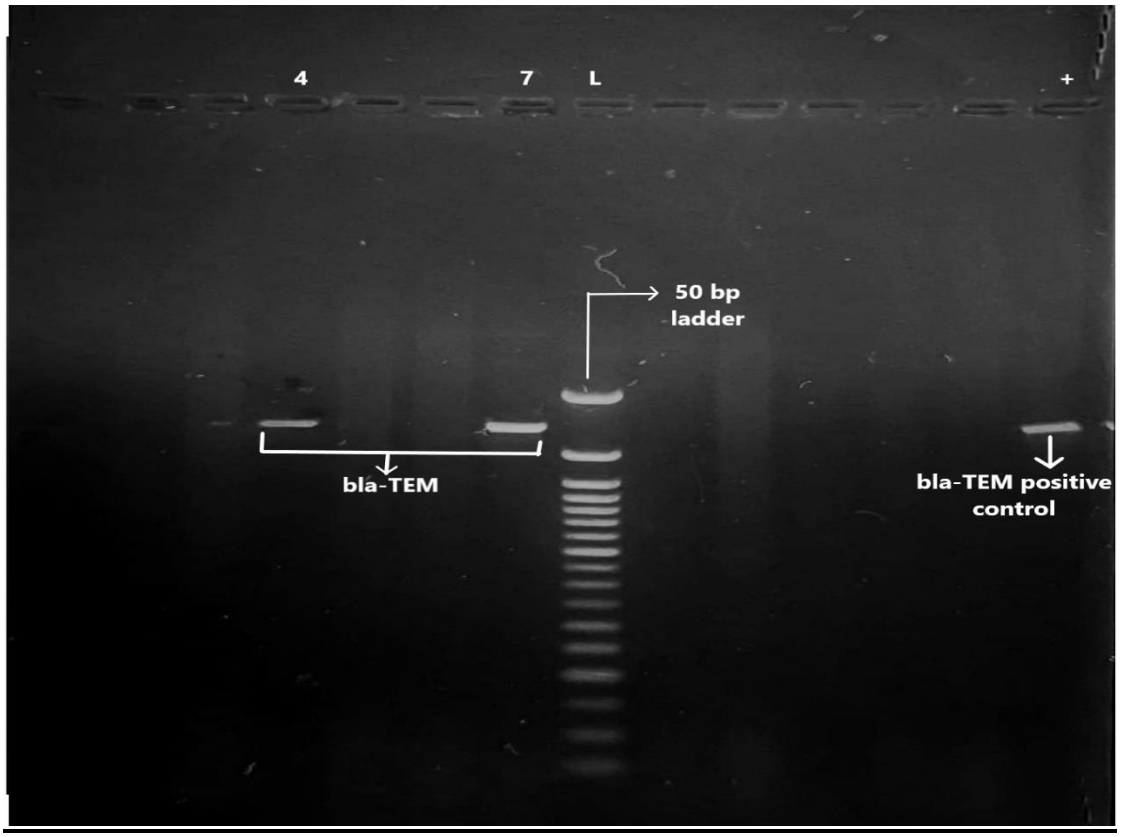


- **Lane 1-3** shows band at **621bp** of the respective ***isolates* 4,6,7(Hospital waste water), Lane 4** shows **isolate 8(Community water)** indicating the **presence of NDM gene.**

- In **Lane 5-7** shows the band at **264bp** of respective **isolates (4,6,7**) indicating the **presence of *Bla*-NDM gene.**
- **Lane 8** shows **100 bp DNA ladder**.

Isolate 4,6,7 belongs from Hospital Waste water and confirms the presence of both bla-NDM and NDM-1 gene and on other hand, isolate 8 belongs from Community water which confirms only NDM gene.

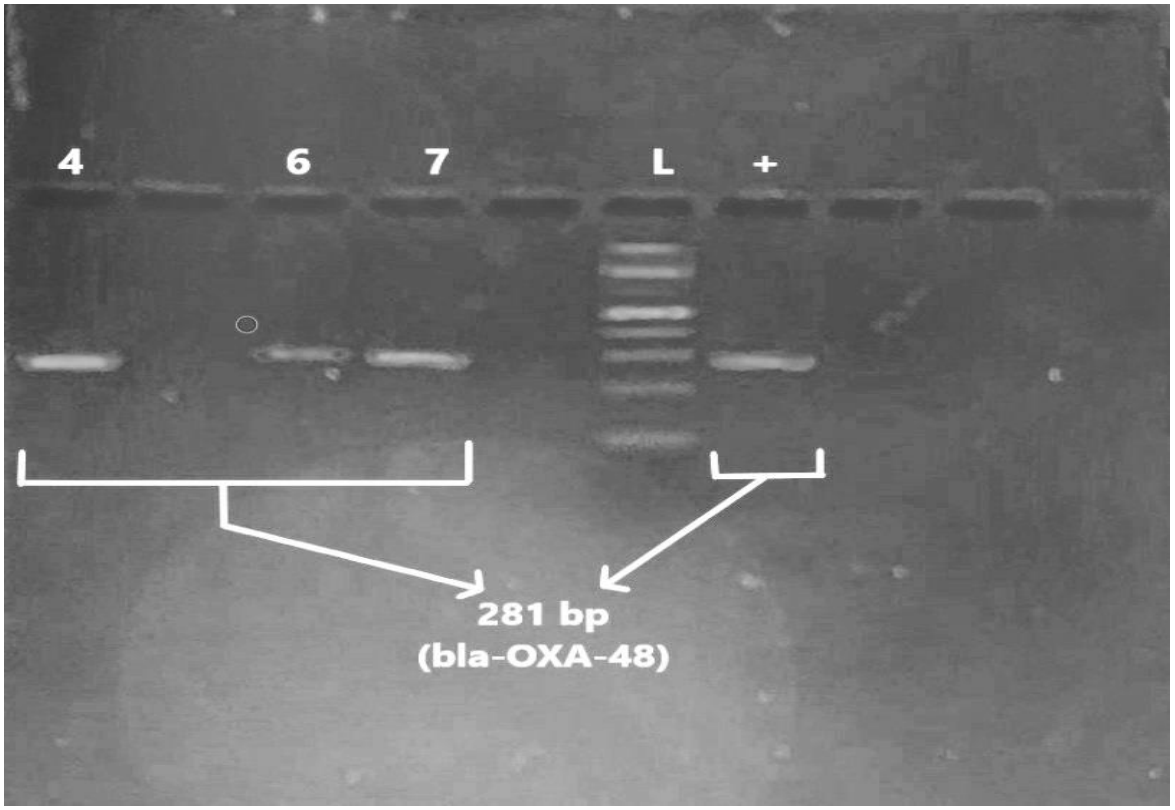
Figure 4.7: Gel electrophoresis of a 100 bp ladder and PCR product of bla-TEM gene



- **Lane 4 (isolate 4)** and **7 (isolate 7)** shows band at bp confirming the presence of **bla-TEM gene.**
- **Lane 8** shows **50 bp DNA ladder**.
- **Lane 13** consists of **negative control** (Reaction volume product without DNA template) and **Lane 14** consist of a **positive control.**

Isolate 4,7 belongs from Hospital Waste water and confirms the presence of bla-TEM gene.

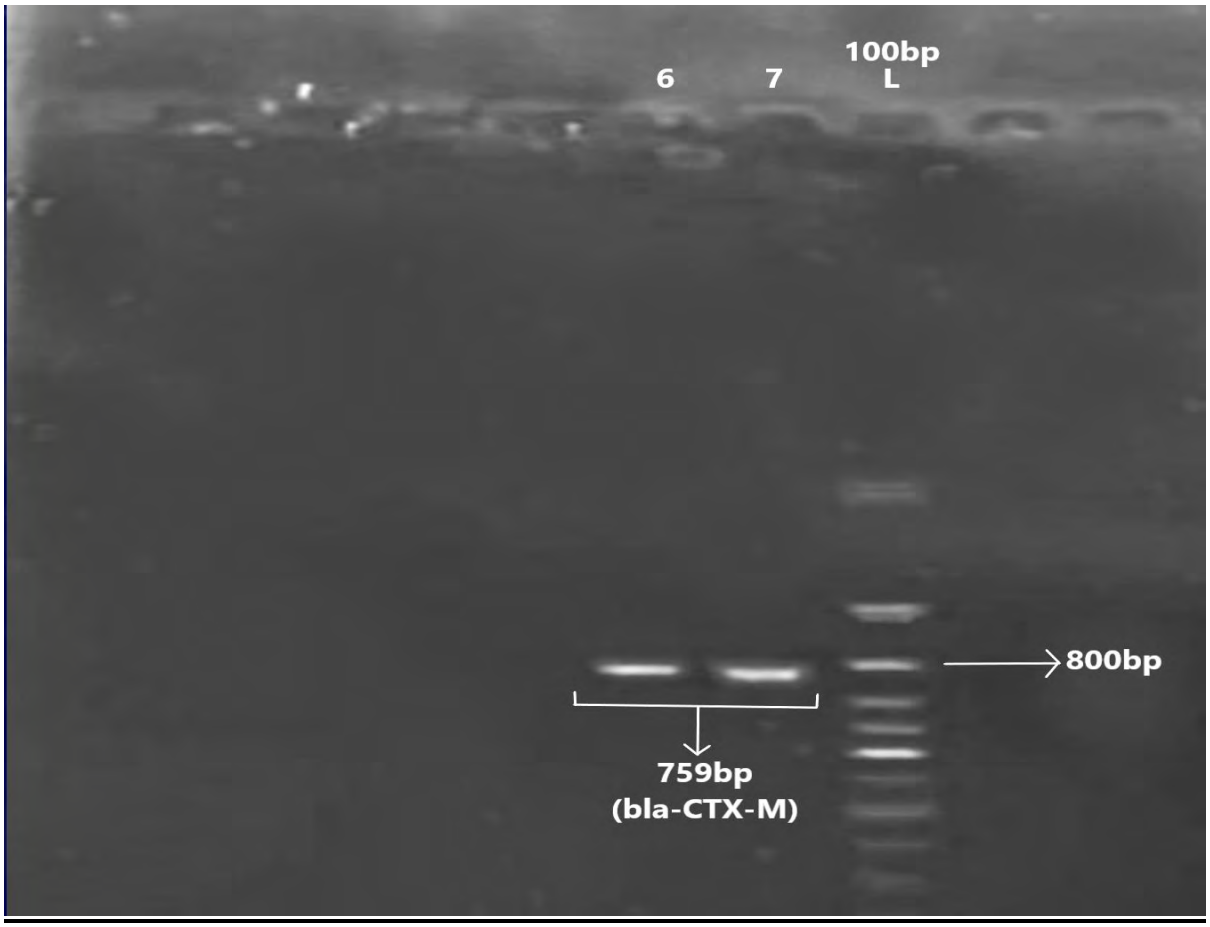
Figure 4.8: Gel electrophoresis of a 100 bp ladder and PCR product of bla-OXA 48 gene



- **Lane 1 (isolate 4)**, **Lane 3 (isolate 6)** and **Lane 4 (isolate 7)** shows band at **281 bp** confirming the presence of **OXA 48 gene**.
- **Lane 6** shows **100 bp DNA ladder**.
- **Lane 7** consists of **a positive control**.
- **Lane 8** consists **of negative control** (Reaction volume product without DNA template)

Isolate 4,6 and 7 belongs from Hospital Waste water and confirms the presence of bla-OXA 48 gene.

Figure 4.9: Gel electrophoresis of a 100 bp ladder and PCR product of bla-CTX-M



Lane 6 (isolate 6), and Lane 7 (isolate 7) shows band at **759bp** confirming the presence of **bla-CTX-M gene.**

Lane 8 shows **100 bp DNA ladder.**

Isolate 6,7 belongs from Hospital Waste water and confirms the presence of bla-CTX-M gene.

DISCUSSION

The spread of carbapenem-hydrolyzing β-lactamases in Enterobacteriaceae especially in *Pseudomonas aeruginosa* is becoming a major public health issue. *Pseudomonas aeruginosa* is a ubiquitous aerobe that is present in water, soil. *Pseudomonas aeruginosa* causes nosocomial infection and has a tendency to acquire antibiotic resistance due to the presence of β-lactamases.

According to study by Nordmann and Poirel (2012), beta-lactamases are plasmid-encoded enzymes that easily transmit between enterobacterial species while navigating other gram-negative bacteria that are resistant to any class of antibiotics. They also hydrolyze nearly all -lactams resulting in isolates that are multidrug resistant. Our research focuses on the route by which bacteria go from hospital waste water to the nearby community's tap water. According to recent research, it was apparent that the number of pathogenic strains in both community and nosocomial sources remained nearly consistent over the 18-month study period. As 25–40 different strains of *P. aeruginosa* were found in each quarter in samples taken from nosocomial sources, it was determined that the pathogen was evenly distributed throughout each hospital unit with slight increases (2012) Sahu et al. The majority of Multi-Drug Resistant (MDR) isolates may have made their way to the general population by resisting antibiotic treatment because hospitals are a hotspot for amassing microbes and exposed antibiotics.

In our study, total of 19 water samples collected from hospital and surrounding localities were included in this study. 3 samples were obtained from the DNCC, Cancer and Bangladesh Shishu Hospital sewage waste water respectively and 16 samples were Tap water collected from the adjacent community of the mentioned hospitals. Among the thirty-three (33) isolated *P. aeruginosa*, DNCC Hospital waste water contains 6 (18.1%) isolates, Cancer Hospital were of 5 isolates (15.15%) and Bangladesh Shishu Hospital also contained 5 isolates (15.15%). (Table 1.1) Along with DNCC community water contains 14 (42.4%) isolates, Cancer Community has 2 isolates (6.06%) and Bangladesh Shishu community also contained 1 isolate (3.03%). (Table 4.2)

Thirty three confirmed *P. aeruginosa* isolates (both the Hospital Waste water and community water) were found resistant to Amikacin (26.6%), Amoxyclav (93.3%), Azithromycin (100%), Aztreonam (66.6%), Cefepime (20%), Ceftazidime (20%), Ceftriaxone (53.3%), Chloramphenicol (55.5%), Ciprofloxacin (33.3%), Colistin (26.6%), Erythromycin (93.3%), Gentamicin (26.6%), Imipenem (20%), Norfloxacin (40%), Piperacillin-tazobactam (26.6%), Polymyxin (13.3%) and Tetracycline (46.6%) imipenem and 36.20% resistant to meropenem (Table 4.3) This increasing trend of resistance may be due to selective pressure on carbapenems as a result of increased use and an increase in carbapenemase production along with intrinsic resistance by *P. aeruginosa*. On the basis of similarity in antimicrobial susceptibility pattern, we further selected 8 isolates both from Hospital waste water and community water as suspected carbapenem resistant isolates. According to new research conducted in two English hospitals showed that, multidrug-resistant *Pseudomonas aeruginosa* (MDR-P) expressing metallo-beta-lactamase is an emerging source which reports of hospital outbreaks of P. aeruginosa associated to environmental contamination, including tap water. (Breathnach et al., 2012).

Our study observed 4 isolates (50%) NDM-1 producer, 3 isolates (37.5%) *bla*-OXA 48 producer, 3 isolates (37.5%) *bla*-NDM producer, 2 isolates (25%) CTX-M and 2 isolates (25%) TEM producer among the 8 carbapenem-resistant P. aeruginosa isolates. Thus, navigating the presence of ESBL (extended spectrum beta-lactamases) and MBL (metallo-beta lactamases). **(Table 4.4)**

A diversified group of enzymes known as metallo-beta- carbapenemase catalyze the hydrolysis of a wide variety of beta-lactam medications, including carbapenems. The discovery that the enzyme processes vary depending on whether one or two zinc ions are bound in the active site, which in turn depends on the subclass of -lactamase, is indicative of this variety. One of the class B metallo-beta-lactamases is the NDM-1 enzyme. NDM-1, a carbapenemase beta-lactamase produced by the blaNDM-1 gene, hydrolyzes and inactivates various carbapenem drugs. In the study by Walsh et al, the blaNDM-1 gene was not found in a single species but

in many unrelated species and its spread in the environment, at least in the Indian subcontinent) size of the reservoir—the Indian subcontinent has >1.4 billion persons (2011).

In our study, we found that four isolates (both from hospital wastewater and community water) produce 50% of the NDM-1 enzyme, and of those, three isolates (all from hospital wastewater) also produce 37.5% of the bla-NDM enzyme, indicating the presence of the bla-NDM gene class, which also transcribes the NDM-1 enzyme. Distinguishingly, 1 out of 4 showed only the presence of NDM-1 enzyme only and not the whole bla-NDM gene class. New study included that the blaNDM-1 gene is not associated with a single clone but rather with nonclonally related isolates and species and has been identified mostly in *E. coli* and *K. pneumoniae* and to a lesser extent in other enterobacterial species. (Nordmann et al., 2011). As bla-NDM gene is an interchange of plasmid, intergons and cassette among the enterobacteria, it so might occur to have intersections of the gene being missed out. On that note, we can predict that this community driven isolate obtained from this study perhaps codes for NDM-1 enzyme gene only rather than the whole class of bla-NDM gene pool. MBL exhibits a wide spectrum of resistance to all beta lactams including the carbapenems (imipenem and meropenem) (Bassetti et al., 2018). Moreover, all 3 hospital waste water isolates show complete resistance to all beta-lactam class such as Amikacin, Amoxyclav, Azithromycin, Aztreonam Cefepime, Ceftazidime, Ceftriaxone, Ciprofloxacin, Colistin, Erythromycin, Gentamicin, Imipenem, Norfloxacin, Piperacillin -tazobactam, Tetracycline including carbapenem. Excluding the community water isolate which expresses intermediate result against to carbapenem even though expressing positive for NDM-1 gene. A multidrug-resistant E. coli strain with the NDM-1 gene was also described in a study by Poirel et al. (2010). This strain was only susceptible to tetracycline, Fosfomycin, and colistin and was resistant to all beta-lactams (including carbapenems), all aminoglycosides, fluoroquinolones, nitrofurantoin, and sulfonamides. This might be as a result of metallo-beta-lactamases having high carbapenemase activity and being resistant to -lactamase inhibitors like clavulanic acid (Cornaglia et al., 2011; Walsh et al., 2005).

Plasmids with the blaNDM-1 gene are diverse and can carry a large number of resistance genes linked to other carbapenemase genes (oxacillinase-48 [OXA-48] types, VIM types), ESBL genes, aminoglycoside resistance genes (16S RNA methylases), macrolide resistance genes (esterase), rifampin (rifampin-modifying enzymes), and sulfamethoxazo (Nordmann et al., 2011). Hence, resulting those 3 isolates to be 37.5% bla-OXA 48 producer, 2 isolates being 25%)CTX-M producer and 2 isolates 25%TEM producer. all of them are derived from hospital waste water.

The first identified OXA-48 producer was from a *K. pneumoniae* strain isolated in Turkey in 2003 (Poirel et al., 2004). A point mutant analog of OXA-48, with similar carbapenemase activity, has been identified in strains from India or of Indian origin. OXA-48 are peculiar because they weakly hydrolyze carbapenems and broad-spectrum cephalosporins, such as ceftazidime, and aztreonam (Castanheira et al., 2011). According to a recent study, *K. pneumoniae* and *E. coli* are the two enterobacterial species that manufacture the most OXA-48, and both organisms typically exhibit higher levels of carbapenem resistance (Nordmann et al., 2011). Concluding, that a horizontal transfer of mobile genetic material occurred to the Hospital waste water isolates. Thus resulting, resistance to the ceftazidime (cephalosporin Class), cefepime (Cepham Class) and Aztreonam (Monobactam class) through hydrolysis.

Extended spectrum β-lactamases (ESBLs) are enzymes encoded by mutation of a class of genes such as TEM, SHV, OXA and CTX etc. in gram negative Enterobacteriaceae. The report was focusing on the multidrug resistance in *Escherichia coli* and *Klebsiella pneumoniae* producing extended spectrum β-lactamases (ESBLs) encoded from mutated TEM and SHV genes (Karim et al., 2017b). ESBL Enterobacteria types of enzymes such as TEM, SHV and CTX-M were identified in *Pseudomonas aeruginosa*, likely followed through horizontal gene transfer. This ESBL enzyme were resistant to third class of cephalosporins but not to carbapenem (Bassetti et al., 2018b). In research from Oberoi, the ESBL producing organisms also express the Amp β-lactamases and they may be co-transferred with the plasmids, thus mediating the fluoroquinolone and the aminoglycoside resistance (2013). In our study 2 isolates obtained

from hospital waste water shows the presence of CTX-M and TEM producer along with resistance to the broader spectrum of cephalosporin. Concluding that Hospital waste water shows more prevalence of ESBL (especially class A and D), MBL resulting the isolate to resistance to most of the antibiotic classes. Use of non-prescription and inappropriate antibiotics may have contributed to the emergence of this novel resistance mechanism in this subcontinent (Mamun et al., 2006). There are currently few effective therapeutic alternatives available for the treatment of severe infections due to the widespread development of medication resistance. The NDM-1 producer has been found in tap and ambient water in New Delhi, according to studies by Walsh et al (2011). Our study was conducted to link the transmission of resistant isolate to the community as hospital is a vessel for achieving resistant due to the constant antibiotic used by millions of patients for treatment purpose. Constant exposure to antibiotics (through intakes of patients) and deprivation of nutrient in a competitive atmosphere (sewage water) might led to Horizontal gene transfer in order to survive. As, the NDM-producer we observed, are both of Hospital sewage water and community water which shares familiar pattern resistance toward specific antibiotic classes it draws to the conclusion of how mass those residing around the hospital area are being affected and easily exposed to multi-drug resistant gram-negative bacteria including *Pseudomonas aeruginosa*.

CONCLUSION

The majority (51.49%) of the *P. aeruginosa* isolates came from community tap water, whereas 48.4% came from hospital waste water. Of the 8 selective MDR isolates, bla-NDM is found in 37.5%, bla-CTX-M in 25%, bla-NDM-1 in 50%, and bla-OXA 48 in 37.5% of the carbapenem-resistant isolates. Hospital and community water isolates both produced NDM-1 rather than ESBL and displayed more comparable resistance patterns. A verified metallo-beta-lactamase and extend spectrum-beta-lactamase-containing isolate displayed substantial levels of antimicrobial resistance to all -lactam drugs. Hospital wastewater and community samples both include a high percentage of multi-drug resistant isolates. Concerns about the possible spread of the multi-Drug gene throughout bacterial populations are warranted, and the findings of this study emphasize even more how dangerous germs might enter our homes through the tap water. The rapid spread of these pathogens could be prevented by early diagnosis of this resistance mechanism, rigorous antimicrobial regulations, and infection control measures.

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APPENDIX 1

Preparation of Media

Cetrimide Media Preparation:

- Cetrimide agar base: 44 grams.
- Distilled water: 990 ml.
- Agar base :6gm
- Glycerol :10ml
- P^H: 6.8
- Media, agar base and glycerol were dissolved in distilled water. P^H adjusted at 7.2. Autoclaved at 121°C for 15minutes under 15 Lbs. pressure, cooled down at 50°C, poured into sterile Petri dishes.

Tryptic soy broth Preparation:

- Suspend of dehydrated media =30 g
- Purified filtered water? Distilled water= 1000ml
- Sterilize at 121°C for 15 minutes. Cool to 45- 50°C and poured into falcon tubes or Eppendorf tubes according to necessity.

Muller-Hinton agar Preparation:

Dehydrated MH base: 38 grams.

- Distilled water: 1000 ml.
- PH: 4± .02 is adjusted according per necessity.
- MH base is dissolved in distilled water. Autoclaved at 121°C for 15 minutes under 15 Lbs. pressure, cooled down at 50°C, poured into sterile Petri dishes.

APPENDIX 2

BUFFERS AND REAGENTS

Name of the Buffer or reagents	Component	Amount (g/L)
1 M Tris-HCL	Tris	**121.14**
	HCL	**As required to adjust the P^H**
	Final P^H	**8.00**
0.5 M EDTA	EDTA	**186.00**
	NAOH	**As required to adjust the P^H**
	P^H	**8.00**
1X TE Buffer	1 M Tris-HCL	**10.00**
	0.5 M EDTA	**2.00**
	P^H	**8.00**
1X TBE Buffer	Tris base	**10.80**
	Boric acid	**5.50**
	0.5 M EDTA	**4.00**
	P^H	**8.00**

APPENDIX 3

LIST OF EQUIPMENT

INSTRUMENT	MANUFACTURER
Weighing Machine	Adam Equipment, UK
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator	Model: WIS-20R Daihan Scientific Companies, Korea
UV Transilluminator	Model: MD-20 Wealtec Corp, USA
-20°C Freezer	Siemens, Germany
Magnetic Stirrer	Model: JSHS-180 JSR, Korea
Vortex Machine	VWR International

Microwave Oven	Model: MH6548SR LG, China
P^H Meter	P^H ep Tester Hanna Instruments, Romania
Eppendorf	Germany, Ireland
Refrigerator	(40C) Model: 0636 Samsung