Characterizing the Co-Existence of Metallo-β-lactamase and Extended Spectrum Beta-Lactamase genes in *Escherichia coli* and *Klebsiella pneumonia* isolates in community wastewater samples of Dhaka, Bangladesh

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A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the degree of

Bachelor of Science

Microbiology

Mathematics and Natural Science

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing a degree at Brac University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material that has been accepted or submitted, for any other degree or diploma at a university or other institution.

4. We have acknowledged all main sources of help.

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Approval

The thesis titled "Characterizing the Co-Existence of Metallo-β-lactamase and Extended Spectrum Beta-Lactamase genes in *Escherichia coli* and *Klebsiella pneumonia* isolates in community wastewater samples of Dhaka, Bangladesh" submitted by

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of December 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology on 05/1/2023.

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Ethics Statement

In this project we have followed all the rules and avoided all the plagiarism. All the citations were included and added proper references at the last page. During the experiments and sample collection, the microbiological ethics were maintained properly.

Abstract/ Executive Summary

Gram-negative bacteria with Metallo Beta-Lactamase (MBL) and Extended-Spectrum Beta-Lactamase (ESBL) production are a new concern worldwide. And these are the most concerning topics as they are being antibiotic-resistant day by day which is becoming a world-alarming situation in medical therapies. Here the sample was collected from the community wastewater and the main concern was to find gram-negative bacteria *Escherichia coli* which are producing MBL and ESBL, MBL is a type of carbapenemase enzyme that displays the ability to inactivate all classes of β-lactam antibiotics, including the last-resort carbapenems, except for monobactams. ESBL enzymes, also most commonly found in *Enterobacteriaceae,* can inactivate cephalosporins and monobactams such as aztreonam.

In this project, the water sample was collected to isolate *E.coli* by using selective media with the addition of supplements after proper dilution. And antibiotic sensitivity tests were run throughout the project to identify the Multidrug Resistant (MDR) and then proceed with the PCR with multiple coexisting genes $bla_{\text{NDM-1}}$, bla_{IMP},bla_{SHV}, bla_{TEM}, and $bla_{\text{CTX-M}}$.

As a result, 110 isolates of *E.coli* were found after implying a proper way of spreading and streaking. 76 isolates were found to be resistant towards amoxicillin and 7 isolates were found to be resistant towards both gentamicin and meropenem where 30% of isolates of community wastewater exhibited multi-drug resistant properties. 48 isolates were confirmed for $bla_{\text{NDM-1}}$, bla_{IMP},bla_{SHV}, bla_{TEM}, and $bla_{\text{CTX-M}}$ and 7 isolates of *E.coli* displayed co-existence genes.

Keywords: Coexistence, Metallo beta-lactam (MBL), ESBL, MDR

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List of Acronyms

PCR	Polymerase Chain Reaction
OD	Optical Density
AST	Antimicrobial Susceptibility Testing
NDM	New Delhi Metallo Beta Lactamase
ESBL	Extended Spectrum Beta-Lactamase.
TNTC	Too Numerous to Count
TFTC	Too Few to Count
PBS	Phosphate Buffered Saline
TBE	Tris Borate EDTA
TAE	Tris Acetate EDTA
EMB	Eosin methylene blue
UV	Ultra Violet

Chapter 1

Introduction

1.1 Background

Worldwide the emergence of Extended Spectrum Beta-lactamase (ESBL) and Metallo-β-lactamase (MBL) producing Gram-negative organisms can be detected in a high percentage. Recent studies also show the possibilities and results of resistant pathogens including multiple drug resistance in the long run of the research (Qumar & Hassan, 2021). β-Lactam antibiotics have been widely used as therapeutic agents for the past 70 years, resulting in an abundance of β-lactam-inactivating β-lactamases. For example, the gene encoding the New Delhi Metallo-β-lactamase-1 ($bla_{\text{NDM-1}}$), leading to resistance to most β-lactam antibiotics, was identified earlier in 2006 and initially confined to the Indian subcontinent (Hu & Jhonug, 2013)(Kumar & Dutta, 2015). However, the gene became globally disseminated within fewer than 5 years of the first known occurrence, partly due to the travel of infected or colonized individuals to Europe, the Middle East, and North America. Thus, it is vital to monitor resistance as a function of time and location, with the goal of containing emerging resistance determinants in restricted geographical settings (Nordmann & Nass, 2011).

1.2 Objective

Antibiotics are used every day in our daily life to prevent diseases. On the other hand, the increased rate of antibiotic resistance is also observed frequently. It is essential to identify the ESBL and MBL gram-negative bacteria and the co-existing genes that are antibiotic resistant and this project was prepared to present and make aware of this highly alarming situation. The investigation characterizes the Co-Existence of Metallo-β-lactamase and Extended Spectrum Beta-Lactamase genes in *E.coli* isolates in community wastewater samples of Dhaka, Bangladesh**.** Some of the areas had been selected to collect samples and pick the isolates of *E.coli.* The main concern of the project is to identify the co-existence of the ESBL and MBL genes of the isolated pathogens such as $bla_{\text{NDM-1}}$, bla_{IMP},bla_{SHV}, bla_{TEM}, and $bla_{\text{CTX-M,}}$ etc which are

also multidrug resistant. The total isolates of *E.coli* from the different locations were 110(55.84%). From regions 1 to 4 *E.Coli* isolates confirmed were 31,31,35, and 13. The main objective of this project is to identify and characterize the co-existence of ESBL and MBL-resistant genes from the isolates that have been collected and successfully identified the presence of co-existing genes with the resistance of antibiotics.

Chapter 2

Context

Bacteria, especially gram-negative bacteria, are showing increased resistance to the current antibiotics and drug development programs seem insufficient to provide therapeutic cover in the near future (Korzeniewska & Harnisz, 2013). The study aimed at evaluating the prevalence of extended-spectrum beta-lactamase (ESBLs) and Metallo-β-lactamase (MBL) genes in confirmed ESBL-producing Gram-negative (G-) bacteria (Coquel & Baquerol, 2008) (Kurokawa & Yagi, 1999). Extended-spectrum beta-lactamases (ESBLs) are enzymes or chemicals produced by germs like certain bacteria. These enzymes make bacterial infections harder to treat with antibiotics. An ESBL chemically breaks down and destroys its target antibiotic, making it useless against an infection. Infections resulting from Metallo-β-lactamase (MBL) and Extended-spectrum β-lactamase (ESBL) producing gram-negative bacteria can be extremely dangerous, as it confers resistance to a wide range of antibiotics (Chaudhary & Aggarwal, 2004). The coexistence of MBL and ESBL genes within these pathogens can further narrow down options for treatment, even with beta-lactams (β-lactams), which are the most widely used class of antibiotics (Ahmed & Hussain, 2021). Improperly treated wastewater, especially from hospital sources, raises the opportunity for the exchange of antibiotic resistance genes between such microorganisms while creating a possible risk of environmental spread that can eventually reach communities.

MBL is a type of carbapenemase enzyme that displays the ability to inactivate all classes of β-lactam antibiotics, including last-resort carbapenems, except for monobactams. The discovery of MBLs occurred through the detection of the bla_{IMP} gene in clinically isolated samples. A larger threat emerged with the later discovery of the bla_{NDM-1} gene that produced the New Delhi Metallo-β-lactamase-1 (NDM-1) enzyme, a novel β-lactamase that was usually found alongside other genes that rendered pathogens resistant to almost all antibiotics (Moellering & Jr.,2010). Most commonly found in *Klebsiella pneumonia* and *Escherichia coli,* the bla_{NDM-1} gene is easily transferable between *Enterobacteriaceae* (petit & Barani, 2013).

ESBL enzymes, also most commonly found in *Enterobacteriaceae,* can inactivate cephalosporins and monobactams such as aztreonam (Perez & Bonomo, 2019) (Rupp & Fey, 2003). Although ESBL organisms had originally been of either the bla_{TEM}, or bla_{SHV} types, $bla_{CTX-M,}$ type enzymes have now become the most commonly encountered in clinical scenarios (Yong & Toleman, 2009).

MBLs and ESBLs pose a threat to public health; thus, it is crucial to detect and prevent their environmental spread. NDM-1, originating from India, has already been disseminated globally, with a risk of high levels of transmission to neighboring countries, such as Bangladesh (Govind & Moodley, 2013). Previous studies on wastewater have already reported the presence of both MBL and ESBL-producing pathogens in the country, with greater prevalence reported near hospitals and communities close to healthcare centers (Moreira & Nunes, 2014).

The present study aims to detect the current prevalence and characterize the antibiotic resistances of ESBL and carbapenemase-producing *K.pneumoniae* and *E.coli* in wastewater of different community areas of Dhaka, Bangladesh. It also aims to confirm the molecular presence of MBL determinants, bla_{NDM-1}, bla_{IMP}, as well as ESBL genes, bla_{SHV}, bla_{TEM}, and bla_{CTX-M}, and map out their coexistence patterns within the resistant isolates (Senda & Arakawa, 1996).

Chapter 3

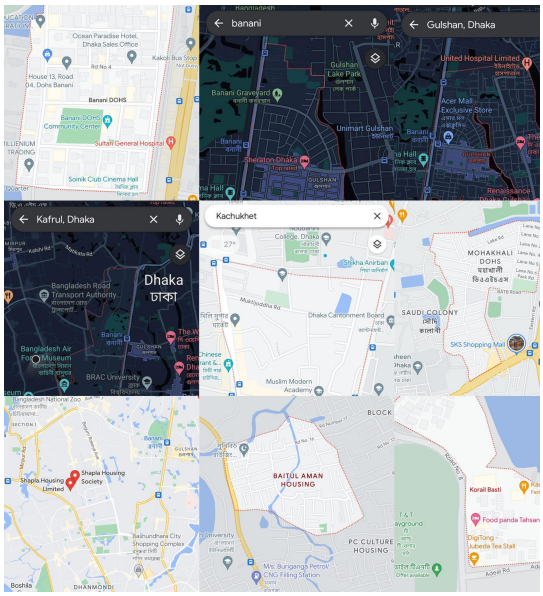
Methodology

3.1. Sample Collection and Processing

The samples were taken from open surface sewer drains and natural drainage canals linked to the city's rivers and lakes. This included regions chosen at random from different areas of Dhaka City locations. Banani DOHS, Banani Zone 1, Banani Zone 2 / Gulshan, Kafrul, Kochukhet, Mohakhali DOHS, Shapla Housing, Baitul Aman Housing, Korail Basti, Mohakhali, Niketon. (Figure _1). The locations that are closer together, are divided into cluster regions.

It was ensured that sample collection points were unrelated to hospitals and were located within a 500-meter radius of no healthcare facilities. A minimum of 200ml of each sample was collected in sterile 500ml containers by lowering into the wastewater collection points.
To remove debris and obtain the supernatant, samples were centrifuged at 3000 rpm for 5 minutes.

A.



B.

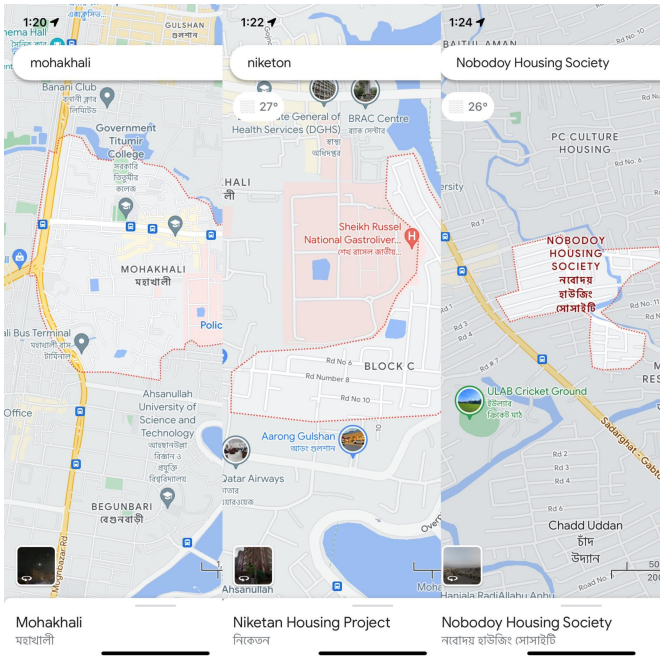


Figure_1: Maps of sample collection area

Sample no.	Area no.	Cluster	Area
1	1	2	Banani DOHS
2	2	2	Banani Zone 1
3	3	2	Banani Zone 2 / Gulshan
4	4	1	Kafrul
5	5	1	Kachukhet
6	6	1	Mohakhali DOHS
7	7	3	Shapla Housing
8	8	3	Baitul Aman Housing
9	1	1	Mohakhali DOHS
10	2	2	Banani Zone 1
11	2	2	Banani
12	1	2	Banani DOHS

13	5	1	Mohakhali dohs
14	4	1	Kafrul
15	9	4	Korail Basti
16	10	4	Mohakhali
17	3	2	Banani Zone 2 / Gulshan
18	11	4	Niketon
19	7	3	Shapla housing
20	12	3	Nobodoy housing
21	8	3	Baitul aman housing

Table_1: Area Wise Sample Collection

For the study, among 21 samples from 4 different cluster regions, a total of 110 colonies were isolated from *E. coli*. The table below shows that *E. coli* isolates were found in every area. (Table _1)

Area	**E.coli**
1	13
2	12
3	6
4	18
5	6
6	7
7	9
8	12
9	6
10	3
11	4
12	14
Total= 110	

Table_2: Distribution of *E. coli* across different sampling regions

3.2. Sample Processing and Bacterial Isolation

Dilution:

We used to perform log dilution, a tenfold dilution, meaning the concentration is decreased by a multiple of ten. To complete a tenfold dilution, the ratio must be 1:10. The 1 represents the amount of sample added. The 10 represents the total size of the final sample. For example, a sample size of 1 ml is added to 9 ml of diluent to equal a total of 10 ml. 10-fold dilution was performed 6 times on all the samples. (Microbiologics Dilution Guide)

Spread Plate:

Following dilution, the spread plate method was performed. The spread plate technique is the method of isolation and enumeration of microorganisms in a mixed culture and distributing them evenly. Samples were plated upon ESBL agar Chromogenic UTI Agar media, EMB Agar media, and MacConkey Agar Media.

ESBL Agar:

ESBL Agar is a chromogenic screening plate for the detection of Extended Spectrum β-Lactamase-producing organisms. Escherichia coli is one of the most common ESBL-producing pathogens.

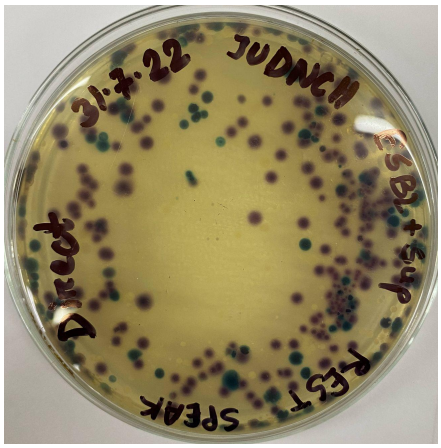


Figure_2: *E. coli* in ESBL media

Chromogenic UTI Agar media:

HiCrome UTI Agar is a differential medium recommended for presumptive identification and confirmation of microorganisms mainly causing urinary tract infections, and can also be used for testing water, food, environmental, and other clinical samples.

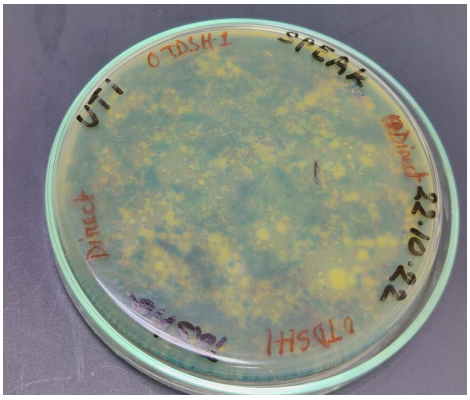


Figure_3: *E. coli* in UTI media

Eosin Methylene Blue (EMB) Agar:

Eosin Methylene Blue (EMB) agar is a differential microbiological medium, which slightly inhibits the growth of Gram-positive bacteria and provides a color indicator distinguishing between organisms that ferment lactose and those that do not.

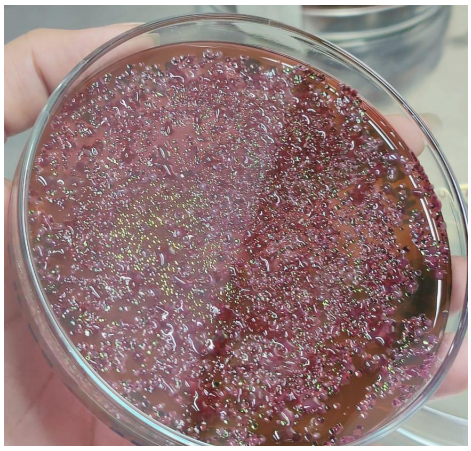


Figure_4: *E. coli* in EMB media

MacConkey agar:

MacConkey agar is a selective and differential media used for the isolation and differentiation of non-fastidious gram-negative rods, particularly members of the family *Enterobacteriaceae* and the genus *Pseudomonas*. (Allen, 2005)

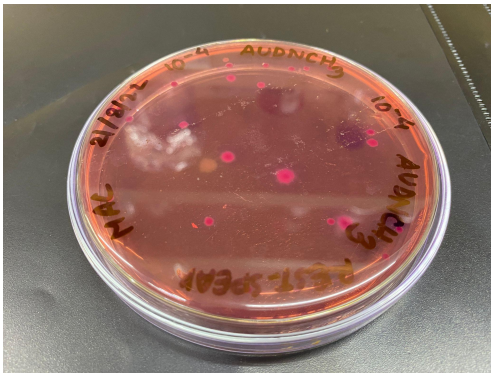


Figure_5: *E. coli* in EMB media

All of the plates were incubated at 37°C for 18-24 hours to allow colonies to grow. A colony count was performed and recorded. Species The colony's morphological features were observed on selective media for identification. For example, because chromogenic substrates are present in the UTI agar media, *E. coli* produces purple-magenta colonies. Similarly, on EMB agar media, *E. coli* produces purple colonies with a black center and a green metallic sheen and finally, on MacConkey agar, *E. coli* growth was visible as pink to red colonies. On ESBL agar, *E.coli* grows in either pink or purple colonies.

Streak Plate Method:

Isolates were then streaked upon Nutrient Agar plates for further analysis. Streaking is a technique used in microbiology for the isolation of single colonies of microorganisms, either from a mixed species or from the same species. It can be described as a rapid qualitative isolation technique.

3.3. Extraction of DNA from the Resistant spp.

DNA extraction was carried out for all suspected *E.Coli* isolates. Because of its simplicity, cost-effectiveness, and short handling time, the 'Boiling method' was used to extract the resistant bacterial DNA. The resistant bacterial species were grown overnight in LB broth. A series of centrifugation and washing steps were performed before boiling at 95°C for 15 minutes. Afterward, the tubes were immediately cooled on ice for 10 minutes. The supernatant containing DNA was collected and stored at -20°C.

PCR Amplification and Nucleotide Sequencing:

Extracted DNAs were further examined by conventional PCR gene amplification and gel electrophoresis. For confirmation of E.Coli, the primer "ECO" was used.

ECO- **F- GACCTCGGTTTAGTTCACAGA**

R- CACACGCTGACGCTGACCA

3.4. Antibiotic sensitivity Test

An antibiotic sensitivity test is used to help find the best treatment for a bacterial infection. It helps to determine the antibiotic susceptibility for each antibiotic disk. All antibiotics have their own different range of susceptibility determined by CLSI (https://drive.google.com/file/d/1q3BEFeuetj2Zjqr7_JCcQkn_M2K8Fq84/view?usp=share_link). Through the antibiogram susceptibility range, we can identify the resistant pathogens and go through further processes. (Jorgensen & Ferraro, 2009)

Among the isolates, *E. coli* were chosen for detailed antibiogram analysis using Kirby-Bauer disc diffusion susceptibility testing with Amoxicillin-clavulanate, Cefepime, Azithromycin, Ciprofloxacin, Piperacillin/Tazobactam, Imipenem, Amikacin, Colistin, Norfloxacin, Meropenem, and Gentamicin standard disks. The test organisms were lawn cultured onto a Mueller-Hinton agar plate. After incubating the MHA plates for 18-24 hours at 37°C, the resistance profiles were compared using CLSI standards (2021), and the data were eventually saved. (Hudzicki, 2009)

Antibiotic	Disc Potency (µg)	Diameter Zone (mm)		
		R	I	S
Gentamicin	10	≤12	13-14	≥15
Amoxycillin-clavulanate	20/10	≤13	14-17	≥18
Piperacillin/Tazobactam	100/10	≤17	18-20	≥21
Imipenem	10	≤19	20-22	≥23
Meropenem	10	≤19	20-22	≥23
Ciprofloxacin	5	≤21	22-25	≥26
Amikacin	30	≤14	15-16	≥17
Cefepime	30	≤18	19-24	≥25
Azithromycin	15	≤12	-	≥13

Table_3: zone diameters for antibiotics used for *E.coli* isolates according to CLSI(2020)

Antibiotic Susceptibility Profiles (Group, Class)

Antibiotic susceptibility testing of 110 isolates were done using different antibiotics. The selected antibiotics for the experiment are divided according to their group and classes.
(Table_4 & 5)

Antibiotic Group	A	B	C	O	U
	Gentamycin	Piperacillin	Meropenem	Erythromycin	Norfloxaci
	Amoxicillin	Cefepime	Imipenem	Azithromycin	
		Ciprofloxacin		Colistin	
Antibiotic Name		Amikacin			

Table_4: Group division of Antibiotics

Antibiotic	Class
Amoxicillin-clavulanate	beta-lactam
Cefepime	cephalosporin
Azithromycin	macrolide
Ciprofloxacin	fluoroquinolones
Piperacillin/Tazobactam	beta-lactam
Imipenem	beta-lactam
Amikacin	aminoglycoside
Colistin	polymyxin
Norfloxacin	fluoroquinolones
Meropenem	beta-lactam
Gentamicin	aminoglycoside

Table_5: Class division of antibiotics

3.5. Phenotypic Selection of Carbapenemase and ESBL producers:

Resistance to imipenem and meropenem antibiotics, as shown in Table_1, was used to detect carbapenemase production. The resistant isolates were chosen for further investigation. Isolates were grown on HiChrome ESBL agar which contained supplements to help distinguish ESBL-producing organisms. Isolates that grew were suspected of producing ESBL enzymes. Double disc synergy testing confirmed this phenotype.

3.6. PCR Amplification and Nucleotide Sequencing (Gene Resistance):

All suspected carbapenemase and ESBL-producing isolates' extracted DNAs were further examined by conventional PCR gene amplification and gel electrophoresis. For MBL encoding genes, $bla_{\text{NDM-1}}$, and bla_{IMP} as well as ESBL encoding genes, bla_{SHV}, bla_{TEM}, and $bla_{\text{CTX-M}}$. The primers used in the study are listed in Table_6.

Gene	Primer Sequence	Product Size	Reference
bla_{NDM-1}	F 5'- GGTTTGGCGATCTGGTTTTC-3' R 5'- CGGAATGGCTCATCACGATC-3'	621	
bla_{IMP}	F 5′- GAAGGCGTTTATGTTCATAC-3′ R 5′- GTATGTTTCAAGAGTGATGC-3′	587	C
bla_{SHV}	F 5'-TACCATGAGCGATAACAGCG-3' R 5'-GATTTGCTGATTTCGCTCGG-3'	450	A, B
bla_{TEM}	F 5′-ATCAGCAATAAACCAGC-3′ R 5′-CCCCGAAGAACGTTTTC-3′	516	
bla_{CTX-M}	F 5′-ACGCTGTTGTTAGGAAGTG-3′ R 5′-TTGAGGCTGGGTGAAGT-3′	857	D

Table_6: primers used for gene amplification in the study

The PCR reaction was carried out in a final volume of 13 microliters, which contained 2 microliters of DNA template, 2.5 microliters of nuclease-free water, 4 microliters of primers (2M each), and 6.5 microliters of EmeraldAmp® GT PCR Master Mix (2X). Thermocycler was used for PCR amplification. The amplification program was configured in accordance with the references. PCR product aliquots electrophoresis in 2% (w/v) agarose gel. The electrophoresis was run at a constant voltage of 110V for 60 minutes using 1 Tris-acetic acid and EDTA (TAE) buffer stained with EtBr. UV light was used to visualize the DNA bands, and images were captured and saved. (K. & Arakawa , 1996)

Chapter 4

Results

4.1 Incubation on selective media

HiChrome ESBL agar

The colony morphology of *E. coli* in ESBL (supplement) media is moist and circular elevated purple colored. (figure_2)

HiChrome UTI agar

The colony morphology of *E. coli* in UTI media is moist and circular elevated purple colored. (figure_3)

EMB agar

The colony morphology of *E. coli* in EMB media is green sheen. (figure_4)

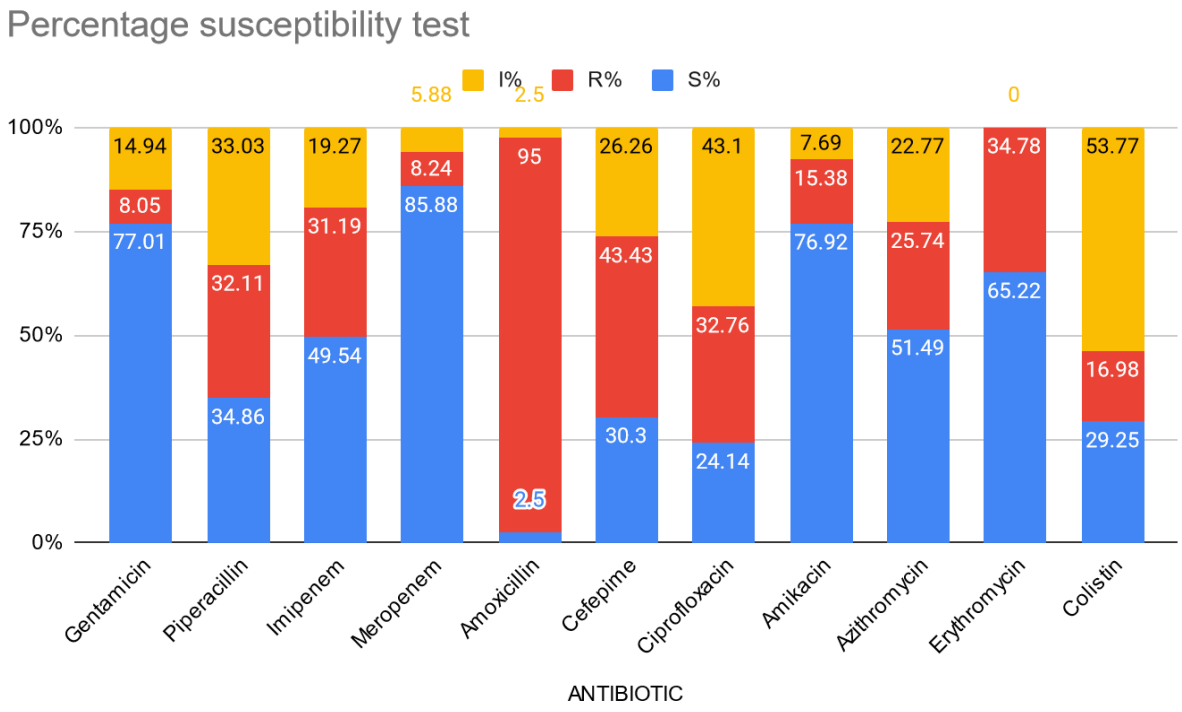
MacConkey

The colony morphology of *E. coli* in MacConkey media is dry and donut-shaped pink colored. (figure_5)

4.2. Antimicrobial Susceptibility Profiles (Percentage)

The tested isolates were classified as sensitive (S), intermediate (I), and resistant (R) based on inhibition zone measurements in accordance with Clinical Laboratory Standards Institute (CLSI).

From the graph, the highest resistance was observed towards amoxicillin (95%) and the least resistance was observed towards gentamicin (8.05%). Two carbapenems of the beta-lactam class were tested for *E.coli* and displayed a higher antibiotic resistance capacity towards imipenem (31.19%) compared to meropenem (8.24%), which had the highest number of isolates that were sensitive (85.88%). (Graph_1)

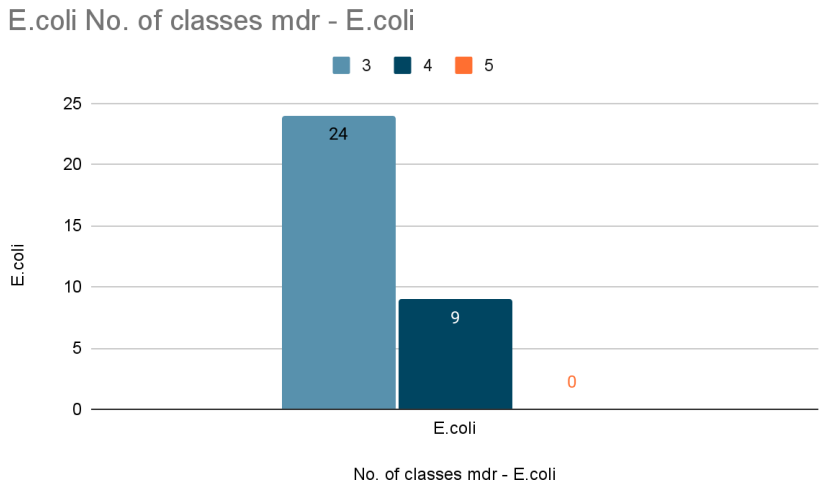


Graph_1: Percentage of resistant, susceptible, and intermediate *E. coli* isolates for each antibiotic tested. Gentamicin resistance occurred in 8.05% of *E.coli* isolates. Piperacillin resistance occurred in 32.11% of *E.coli* isolates. Imipenem resistance occurred in 31.19% of *E.coli* isolates. Meropenem resistance occurred in 8.24% of *E.coli* isolates. Amoxicillin resistance occurred in 95% of *E.coli* isolates. Cefepime resistance occurred in 43.43% of *E.coli* isolates. Ciprofloxacin resistance occurred in 32.76% of *E.coli* isolates. Amikacin resistance occurred in 15.38% of *E.coli* isolates. Azithromycin resistance occurred in 25.74% of *E.coli* isolates. Erythromycin resistance occurred in 34.78% of *E.coli* isolates. Colistin resistance occurred in 16.98% of *E.coli* isolates.

4.3. Multidrug-Resistant

Multidrug-resistant organisms are bacteria that can no longer be controlled or killed by some medications because they have developed a resistance to those antibiotics. To determine the presence of multi-drug resistance in *E. coli* isolates, their resistance to more than one individual

antibiotic and antibiotic classes were grouped together. (Graph_2 & 3). Being resistant to 3 or more antimicrobial classes has been defined as Multi-Drug Resistant used by a majority of studies (Magiorakos et al., 2012). According to this reference, 30% of *E.coli* isolate from the pie chart displayed MDR properties.



Graph_2: The percentage of *E. coli* resistant to different numbers of antibiotic classes. 24 *E. coli* isolates are resistant to 3 antimicrobial classes and 9 *E. coli* isolates are resistant to 4 antimicrobial classes.

The table below shows the number and percentage of *E.coli* resistant to different classes of antibiotics. Here the resistance percentage of the beta-lactam class is the highest whereas the polymyxin class is the lowest.

Resistance to classes		
Antibiotic Class	No. of *E.coli*	%
Beta-Lactam	96	87.16
Cephalosporin	48	43.43
Fluoroquinolone	30	27.38
Aminoglycoside	35	32.04
Macrolide	38	34.78
Polymyxin	19	16.98
Total *E. coli* isolates=110		

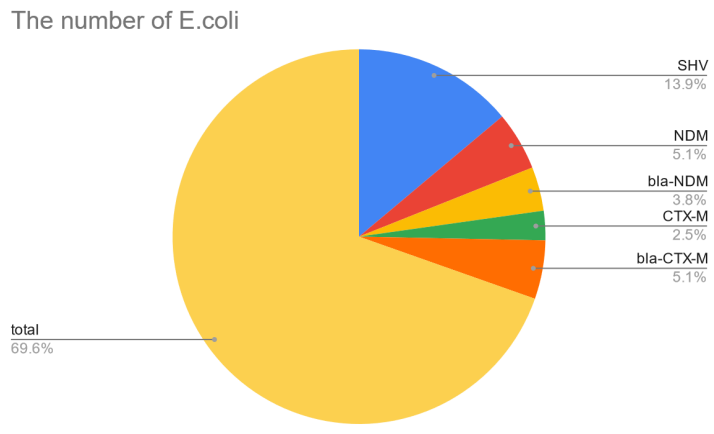
Table_7: Distribution of isolates resistant to different classes of antibiotics.

4.4 Resistant Gene Profile

8 different primers were used to check the resistant gene pattern. Among these, *E.coli* isolates could not be confirmed from bla-IMP, bla-TEM, and bla-Vim. Sample 1-3, 4-8 was identified as batch 1,2, and sample 9-16, 19-21 were identified as batch 3,4. 18 *E.coli* isolates of batch 1,2 and 4 *E.coli* isolates of batch 3,4 were confirmed for SHV. 6 isolates of batch 1,2 and 2 isolates of batch 3,4 were confirmed for NDM. 6 isolates of batch 1,2 were confirmed for bla-NDM. 4 isolates of batch 3,4 were confirmed for CTX M. 8 isolates of batch 1,2 were confirmed for bla-CTX M.

Resistant Gene Profile	
Primer name	The number of E.coli
SHV	22
NDM	8
bla-NDM	6
CTX-M	4
bla-CTX-M	8
bla-IMP	0
bla-TEM	0
bla-VIM-2	0
total	110

Table_8: *E. coli* isolates from resistant genes. Among 48 isolates of *E. coli,* only 48 *E. coli* isolates were confirmed for 8 primers.



Graph_3: *E.coli* isolates from resistant genes.

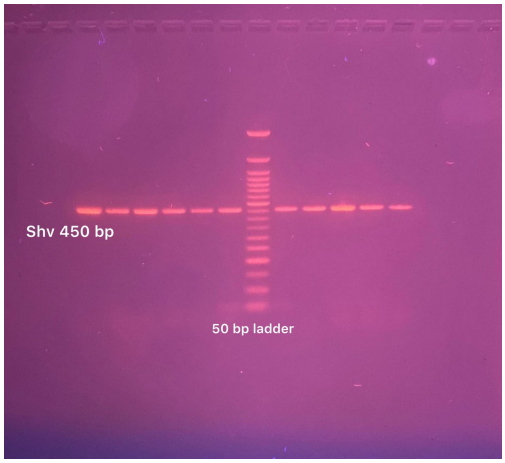


Figure_6: PCR products with SHV primer. 50 bp ladder was used. Bands that fall within the 621 bp area are considered positive.

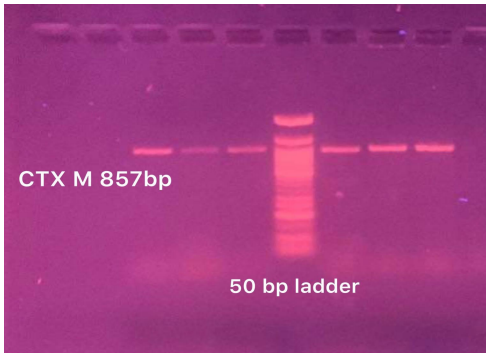


Figure_7: PCR products with CTX-M primer. 50 bp ladder was used. Bands that fall within the 857 bp area are considered positive.

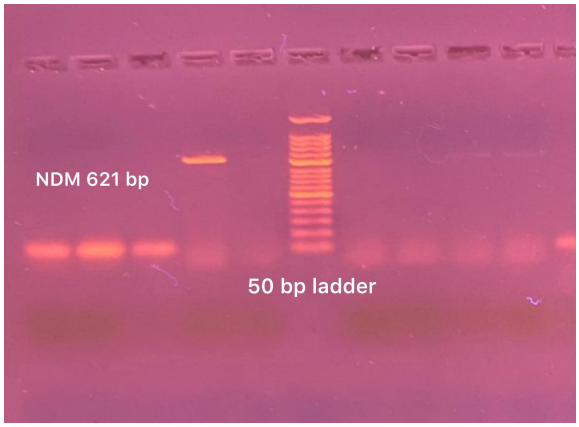


Figure_8: PCR products with NDM primer. 50 bp ladder was used. Bands that fall within the 621 bp area are considered positive.

4.5. Co-Existence of Genes

Among 110 isolates of *E.coli*, only 7 isolates showed the co-existence of genes. As 9 different primers were used in the experiment, 4 *E.coli* isolates showed co-existence in 2 types of genes and 2 *E.coli* isolates showed co-existence in 3 types of genes. 4 isolates from batch 1,2 showed co-existence for *bla-NDM* and *NDM*. 2 isolates from batch 1,2 showed co-existence for *bla-NDM, NDM,bla-CTX M, and bla-SHV.* 1 isolate from batch 3,4 showed co-existence for *NDM, CTX-M.*

Gene Type	Identified Gene	Co-existence
2	NDM+bla-NDM	3
	NDM+CTX-M	1
	blaCTX-M+NDM	1
3	NDM+bla-NDM+blaCTX-M	1
	bla-NDM+blaCTX-M+bla-SHV	1

Table_9: Co-existence of genes

Chapter 5

Discussion

Carbapenems are considered the last line of drugs against gram-positive and gram-negative organisms as they have the strongest effectiveness and the broadest spectrum of activity against bacteria. (Murugan & Sinha, 2019). Due to the rising resistance to cephalosporin antibiotics in *E. coli,* carbapenem usage has increased which led to carbapenem resistance. The emergence of extended-spectrum lactamases (ESBL) is largely to blame for this cephalosporin resistance. Carbapenem resistance to *E.coli* has become a serious threat to the human healthcare system.

A new problem in the management of bacterial infections is the formation of New Delhi Metallo-lactamase-1 (NDM-1) which confers resistance to carbapenem drugs (Karthikeyan et al., 2010). To monitor the possible source of the spread of the organisms in Dhaka, Bangladesh, community wastewater samples were collected and went for checking the presence of *E. coli.* After isolating the organism, the antibiogram susceptibility test was done to check the percentage of resistance, susceptibility, and intermediate. This results in the understanding of multidrug resistance. Wastewater contributes significantly to the spread of antibiotic-resistant bacteria in the environment by containing a variety of microbial pathogens. The scientific community has made numerous mentions of how wastewater treatment facilities contribute to the spread of antimicrobial resistance and multidrug resistance. From the example of the study (Magiorakos & Srinivasan, 2012), despite the fact that the drugs are different, two *E. coli* isolates, one resistant to trimethoprim-sulfamethoxazole, cefazolin, and ciprofloxacin and the other to ertapenem, gentamicin, and tigecycline, will both be classified as MDR. These definitions do not provide a further classification of MDR bacteria's resistance depending on the agents to which they are resistant. In our study, two *E.coli* isolates, one resistant to imipenem, amoxicillin, amikacin and erythromycin and the other to gentamicin, meropenem, and azithromycin. PCR was performed with resistant genes, SHV occurred in 33,3%, bla-SHV occurred in 12.5%, CTX-M occurred in 8.3%, bla-CTX-M occurred in 16.7%, NDM occurred in 16.7%, bla-NDM occured in 12.5%, bla-IMP, bla-TEM, bla-VIM2. Then confirmed *E. coli* isolates were checked for the coexistence genes. This result shows the possession of a threat of the expansion of microbial populations with high resistance through environmental reservoirs.

Chapter 6

Conclusion

This study has investigated the current situation of carbapenem resistance in the community wastewater isolates of *E.coli* and demonstrated that the resistance is brought on by beta-lactamase. *E.coli* found in the water are a significant source of antimicrobial resistance. The worldwide expansion of carbapenemase-encoding genes that cause transferable carbapenem resistance has significantly reduced the number of available treatments. The exceptional efficacy of antibiotics to treat bacterial infections has resulted in their overuse of them in human healthcare.

This study was carried out to determine the prevalence of carbapenem-resistant genes like SHV, bla-SHV, NDM, bla-NDM, CTX-M, bla-CTX-M, bla-IMP, bla-TEM, bla-VIM2. With the prevalence of multidrug resistance rising and carbapenem-resistant *E. coli* linked to hospital-acquired illnesses, antimicrobial resistance surveillance will continue to be a top priority. To minimize or completely prevent the spread to natural ecosystems, proper wastewater treatment for both hospital and community wastewater must be ensured. The wastewater must be treated using cutting-edge methods before being released into the environment, such as UV and ozone treatments, which have been shown to considerably slow the spread of antibiotic-resistant bacteria and antibiotic-resistant genes. (Azuma & Hayashi, 2022)

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