

Multi-Drug Resistance Patterns in Water *Escherichia coli*: Prevalence and Co-Occurrence Associations

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A thesis report submitted to the Department of Biotechnology in partial fulfilment of the
requirement for the degree of
Bachelor of Science in Biotechnology

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Declaration

It is hereby declared that

1. The thesis report submitted is our original work while completing my degree at BRAC University.
2. The report 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 report does not contain material 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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Ethics Statement

This research was conducted according to the ethical principles and environmental safety regulations surrounding microbiological research. Samples of water were taken and analysed from the area of Hatirjheel, Banasree, and BRAC University of Dhaka Metropolitan City. Since the study was not research involving human or animal subjects, there was no need to have an institutional review board.

All the sampling activities were conducted with minimal environmental pressure. During the collection of the samples, transportation, laboratory analysis and proper safety precautions were maintained to protect the safety of the researchers and the environment around them. No genetic manipulation or discharge of biological agents in natural environments was used in the study.

Data acquired were only used academically and in research. There was no fabrication or falsification of data. All the results and interpretations were presented in a transparent way. In general, the study followed the principles of scientific integrity, environmental responsibility and data protection.

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Table of Contents

Declaration.....	1
Approval.....	2
The thesis titled “Multi-Drug Resistance Patterns in Water Escherichia coli: Prevalence, Spectrum Analysis, and Co-resistance Associations” submitted by.....	3
Ethics Statement.....	4
Acknowledgement.....	5
Table of Contents.....	5
LIST OF FIGURES AND TABLES.....	6
LIST OF TABLES.....	7
List of Acronyms:.....	8
Abstract:.....	9
Chapter 1.....	10
1.1. Introduction:.....	10
1.2 Objective of this study:.....	15
Chapter 2.....	16
2.1 Methodology.....	16
2.1.1 Sample collection:.....	16
2.1.2 LB media preparation for filtration:.....	16
2.1.3 Sample Filtration and Serial Dilution:.....	17
2.1.4 EMB media preparation:.....	17
2.1.5 Bacterial inoculation methods used:.....	18
2.1.6 Isolation and Purification.....	18
2.1.7 Soft Agar culture and inoculation:.....	18
2.1.8 Glycerol Stock Preparation:.....	19
2.1.9 IMViC Biochemical Tests.....	19
2.1.10 MR-VP Broth Preparation and Procedure:.....	19
2.1.11 Preparation and Procedure, Indole Test:.....	20
2.1.12 Preparation and Procedure of Citrate Utilisation Test:.....	21
2.2. Polymerase Chain reaction (PCR).....	21
2.2.1 Colony PCR and PCR Mechanism:.....	21
2.2.2 Agarose Gel Electrophoresis:.....	22

2.2.3 Materials for Gel Electrophoresis:.....	22
2.2.4 Process of Gel electrophoresis:.....	22
2.2.5 DNA samples:.....	23
2.2.6 Loading and Running of the Gel:.....	23
2.2.7 Visualization of DNA bands:.....	24
2.3 Antibiotic Susceptibility Test (AST).....	24
2.3.1 Antibiotic Susceptibility Testing Overview and Principle.....	24
2.3.2 Preparation of Inoculum and McFarland Standardization:.....	25
2.3.3 Mueller-Hinton Agar Preparation.....	25
2.3.4 Inoculation of Mueller-Hinton Agar Plates:.....	27
2.3.5 Introduction of the Disc of the antibiotics:.....	27
2.3.6. List of Antibiotics:.....	28
2.3.7 Measurement of Zones of Inhibition.....	28
2.3.8 Zones of Inhibition Measurement and Interpretation.....	29
2.3 Parameters Used for Result Analysis:.....	30
Chapter 3.....	31
3. Result:.....	31
3.1 Statistical Analysis Results.....	31
3.1.1 PCA Clustering Analysis:.....	32
3.1.2 Narrow vs Broad Spectrum Analysis:.....	32
3.1.3 Co-Occurrence Network Analysis:.....	34
3.2 Result Interpretation:.....	37
3.2.1 Analysis of K-means Clustering.....	37
3.2.2 PCA:.....	39
3.2.3 Narrow vs Broad Spectrum:.....	41
3.2.4 Co-Occurrence Network:.....	43
3.2.5 Comparative Analysis of sample collection locations.....	45
3.3 Supplementary Findings:.....	45
3.3.1 Location based Findings:.....	45
3.3.2 IMViC Test results and interpretations:.....	49
3.3.3: Result of spreading based on visualisation.....	51
3.3.4 Streaking result based on visualization:.....	52
3.3.5 Results PCR Amplification/Gel Electrophoresis:.....	52

3.3.6 Multiplex/Dual-dye Fluorescence Gel:.....	53
3.3.7 Multi row agarose gel:.....	53
3.3.8 Single-Band PCR Products of High Yield:.....	54
Chapter 4.....	56
Discussion.....	56
4.1 Mechanistic Basis of Multi-Drug Resistance.....	56
4.1.1 Efflux Pump-Mediated Resistance.....	56
4.1.2 Beta-Lactamase Production.....	56
4.1.3 Environmental Selective Pressure.....	57
4.1.4 PCR and Gel Electrophoresis.....	57
4.2 MDR Prevalence and Antibiotic Resistance Spectrum.....	58
4.3 Evidence from Bangladesh.....	58
4.4 Patterns of Antibiotic Resistance Profiling and MDR.....	59
4.4.1 K-means Clustering.....	59
4.4.2 Narrow vs. broad spectrum.....	59
4.4.3 Co-occurrence Network.....	60
4.4.4 Inter-site Comparative Analysis.....	60
4.5 Environmental Selective Pressure and Ecological Amplification.....	61
4.6 Major Risk of Transmission: Water.....	61
4.7 Study Limitations.....	62
Chapter 5.....	62
Conclusion:.....	63
5.1 Future Directions.....	63
5.1.1 Whole-Genome Resistome Sequencing.....	63
5.1.2 Metagenomic Surveillance of the Environmental Resistome.....	64
5.1.3 Gene-Level Validation and Functional Studies.....	65
References.....	66

LIST OF FIGURES AND TABLES

Figure 1	Thermal Cycler Program Used for Colony PCR Amplification	25
Figure 2	MHA Plate after Antibiotic Susceptibility Tests (Kirby-Bauer)	30
Figure 3	Multi-Drug Resistance (MDR) Summary and Cluster Plot	36
Figure 4	Cluster Summary	41
Figure 5	Multi-Drug Resistance (MDR) Profile by Cluster.....	41
Figure 6	Principal Component Analysis (PCA) Biplot of K-Means Cluster Assignments (n = 150	43
Figure 7	Heatmap of the Narrow vs Broad Resistant Rate.....	44
Figure 8	Resistance Vs Sensitive Percentage	46
Figure 9	Co-Occurrence Network of Antibiotic Resistance (n = 150).....	47
Figure 10	IMViC Test: (MR) and VP test.....	52
Figure 11	IMVIC Test: Indole and Citrate.....	53
Figure 12	Spread Plate Inoculation of E. coli on EMB Media	54
Figure 13	Streaking of Different Bacterial Colonie.....	55
Figure 14	Dual-fluorescence gel showing colony PCR products with smearing and non-specific amplification.....	56
Figure 15	Gel showing variable colony PCR amplification with clear DNA ladder reference.....	57
Figure 16	Gel showing inconsistent colony PCR amplification with smear like bands. All the ladder loaded ran perfectly along with samples but showed not proper amplified bands...	58

LIST OF TABLES

Table 1	Composition of Mueller-Hinton Agar	30
Table 2	List of Antibiotics and Their Zones of Inhibition	32
Table 3	Descriptive Statistics: Inhibition Zone Values (mm)	36
Table 4	Spectrum-Level Summary: Average Resistance Percentages	37
Table 5	Statistical Tests: Chi-Square and Mann-Whitney Results	39
Table 6	Co-Occurrence Network: Edge List and Statistics.....	40
Table 7	Notable Co-Resistance Pairs	41
Table 8	Mean Zone Values by Site and Antibiotic (Threshold Values)	41
Table 9	K-Means Cluster Summary (n = 150 Isolates)	46
Table 10	Comparative Resistance Percentages Across 3 Sites.....	50
Table 11	Dates and Isolates Recovered from Hatirjheel Site (n = 50)	50
Table 12	Dates and Isolates Recovered from Banasree Site (n = 50)	53
Table 13	Dates and Isolates Recovered from BRAC Site (n = 50)	53

List of Acronyms:

Acronyms	Meaning
ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistance Gene
AMR	Antimicrobial Resistance
AST	Antibiotic Susceptibility Testing
DNA	Deoxyribonucleic Acid
DNTP	Deoxyribonucleotide Triphosphate
EMB agar	Eosin Methylene Blue
ESBL	Extended-Spectrum Beta-Lactamases
EtBr	Ethidium Bromide
HGT	Horizontal Gene Transfer Multidrug Resistant
MCT	Micro Centrifuge Tube
MDR	Multiple Drug Resistant
MHA	Mueller Hinton Agar
Nf water	Nuclease free water
PABA	Para-Aminobenzoic acid
PCR	Polymerase Chain Reaction
TAE Buffer	Tris-Acetate-EDTA buffer
TE Buffer	Tris-EDTA buffer
UV	Ultraviolet

Abstract:

Antimicrobial resistance (AMR) in the environmental water system is an emerging health issue of concern in the general population, especially in densely populated low and middle-income countries with poor wastewater management systems. This paper has

explored the prevalence, diversity, and patterns of co-resistance of multidrug-resistant (MDR) *Escherichia coli* in three ecologically different urban water bodies in Dhaka, Bangladesh-Hatirjheel, Banasree, and the BRAC University water body during a longitudinal surveillance period of April to December 2025. A total of 150 environmental *E. coli* isolates were confirmed by biochemical IMViC characterization and colony PCR. The Kirby-Bauer disk diffusion technique was used to measure phenotypic susceptibility using eight clinical-relevant agents that represented the six antibiotic classes: fluoroquinolones, phosphonic acids, tetracyclines, beta-lactams, cephalosporins, and macrolides. Breakpoint interpretation was done in accordance with CLSI thresholds. Statistical analyses including K-means clustering, Principal Component Analysis (PCA), Chi-square testing, Mann-Whitney U testing, and co-occurrence network analysis were conducted in R (version 4.3.1). The overall resistance burden was high: six out of eight antibiotics had resistance rates over 80% in all isolates. PCA indicated a strong spatial variation among sampling locations with the first two principal components having a cumulative (59%) total resistance variance. A statistically significant difference in the prevalence of resistance between broad-spectrum and narrow-spectrum antibiotics was seen at population level but not at isolate level (chi-square = 7.533, $p = .006$), which agrees with a generalized MDR phenotype. Co-occurrence network analysis revealed that there were two statistically significant pairwise co-resistance relationships; levofloxacin-azithromycin (0.473, $p = 0.002$), indicating possible cross-class resistance involving efflux pumps, and piperacillin-tazobactam-cefepime (0.452, $p < 0.00$). Inter-site comparison revealed that MDR was most prevalent in the BRAC University site followed by Banasree and finally, Hatirjheel. These results confirm the hypothesis that urban water bodies in Dhaka support high populations of MDR *E. coli* that is the result of multi-mechanistic resistance co-selection. The findings highlight the importance of the combination of environmental AMR surveillance, molecular characterization of resistance determinants, and evidence-based wastewater management policy in a One Health framework.

Keywords: AMR, *E. coli*, Multidrug Resistance, Co-resistance, Environmental Microbiology, Bangladesh, Urban water.

Chapter 1

1.1. Introduction:

Antimicrobial resistance (AMR) refers to the ability of microorganisms, which can be bacterial, viral, fungal, and parasites to survive and multiply in the presence of previously effective antimicrobial agents [1, 2]. After becoming susceptible to common treatment regimens, resistant organisms develop various biochemical and genetic responses and render the conventional antibiotics clinically ineffective, resulting in chronic infections, extended hospitalization, and high mortality rates [2]. The scale of this crisis is astounding: a landmark Global Burden of Disease analysis estimated that, in 2019, around 1.27 million deaths were directly related to bacterial AMR and almost 4.95 million to drug-resistant infections [3]. Predictions made in *The Lancet* also suggests that by 2050, AMR-related deaths may increase to ten million per year, to become the primary cause of premature mortality in the globe, even overtaking cancer [3, 4]. This crisis has been characterized by the spread of multidrug-resistant (MDR) organisms, which are defined as non-susceptibility to at least one agent in three or more antimicrobial classes, leading to extensive treatment failures and depleted treatment options in clinical environments [3]. To address this issue, global health agencies such as WHO, Centers for Disease Control and Prevention (CDC), and the Interagency Coordination Group on Antimicrobial Resistance (IACG) have unanimously made AMR a global health emergency, requiring urgent and coordinated, multisectoral response [1, 2].

Although the AMR has traditionally been viewed as an issue that occurs in a clinical/hospital setting, growing evidence now clearly indicates that it is much more than a healthcare facility issue, present in the larger environment [5, 6]. Antibiotic resistance genes (ARGs), resistant microorganisms, and some residual antimicrobial compounds have been found in soil, air, food, and in aquatic systems the most crucial of which is anthropogenic input [5]. This is an environmental component of AMR that is a very underestimated yet epidemiologically important axis of the resistance crisis. The understanding that the AMR and its genetic determinants are spread freely within the ecological realm has radically transformed the manner in which AMR should be researched, monitored, and controlled, calling upon a One Health framework that incorporates the human, animal, and environmental health paradigms [2, 6].

Aquatic systems have become one of the most critical storage spaces and dynamic channels of dissemination of AMR among the different environmental compartments [7, 8]. Water bodies can serve as an ecologically complex mixing zone where a variety of microbial communities with human, animal and environmental origins interact with antibiotic substances, heavy metals and resistance genes of various anthropogenic origins [8]. Agricultural runoff, sewage discharge, hospital effluent, pharmaceutical manufacturing waste, and industrial wastewater are still discharged into rivers, lakes, canals, ponds and estuaries, and lead to physicochemical conditions that are highly favourable to the survival, intensification, and spread of resistant organisms [8]. It is in these aquatic settings that microorganisms, antimicrobial substances, and mobile genetic elements interactively drive the acquisition, expression and transmission of resistance in taxonomically diverse bacterial populations [7, 8]. City water systems are some of the most dangerous hotspots of the environmental AMR environment. Although not high enough to cause direct bacterial death, these concentrations have long-lasting selective pressure that promotes the survival, growth, and proliferation of resistant strains compared to susceptible ones [8, 9]. At the same time, industrial effluents often carry heavy metals, biocides, and disinfectants that co-select resistance to antibiotics by having co-locating resistance genes or overlapping biochemical defence systems [10]. The cumulative impact of these numerous anthropogenic contributions implies that urban water bodies are essentially different and a great deal more resistance-facilitating than rural or ecologically clean water systems [8].

In addition to the direct process of selecting resistant organisms, urban aquatic environments are also actively involved in the genetic processes involved in transfer of resistance between microbial populations. These water systems contain an exceptionally large microbial biodiversity as they combine human, animal, and environmental sources of bacteria in a small spatial area [7]. The convergence of these microbes forms the best environment where horizontal gene transfer (HGT) the main vehicle through which ARGs spread through bacterial lineages, including non-pathogenic environmental bacteria and human pathogens [11, 19]. Resistance determinants coded on mobile genetic elements (MGEs) including plasmids, integrons, transposons, insertion sequences and genomic islands can be exchanged among phylogenetically divergent organisms at an astonishing pace and with great efficiency through conjugation, transformation, and transduction [12, 13]. The ecological design of city water, therefore, acts as a place of choice and a genetic exchange center and speeds up the

evolutionary process and transmission of AMR a thousand times faster than within a given species or ecological environment [8].

Escherichia coli, one of the species of bacteria that live in the environmental water system, holds a privileged place in the scientific and popular health concern. *E. coli*, a Gram-negative, facultatively anaerobic organism belonging to the family *Enterobacteriaceae*, is a normal commensal organism of the gastrointestinal tracts of warm-blooded animals, including humans, and its occurrence in environmental water is unanimously accepted as an accurate indicator of fecal contamination [7, 14]. The presence of *E. coli* in environmental water samples thus is a two-fold signal: indicative of fecal contamination and an approximate indication of the presence of enteric pathogens co-occurring, thus a priceless sentinel in the determination of the risk to the public health in water [16]. In addition to its usefulness as a contamination marker, *E. coli* has several characteristics that have rendered it the most common model organism in research of environmental AMR. Isolation and cultivation in environmental matrices It is technically simple to isolate and culture in environmental matrices with selective and differential media, its biochemical phenotypes are well catalogued, and its genome is one of the most well-characterised in microbiology. Most importantly, *E. coli* is firmly known to harbour clinically relevant resistance phenotypes, such as beta-lactam, fluoroquinolone, aminoglycoside, tetracycline, sulfonamide, and trimethoprim resistance [17]. Environmental strains have been reported to harbour ESBL-encoded genes most notably blaCTX-M, blaTEM and blaSHV that encode resistance to a broader spectrum of beta-lactam antibiotics including third-generation cephalosporins that are considered a backbone of empirical treatment of serious bacterial infections [18, 19]. ESBL-producing *E. coli* has been reported in environmental water samples worldwide, even in high-income countries with well-developed wastewater treatment systems, and is becoming a significant indicator of public health of clinical relevance circulating out of hospitals [20]. The simultaneous resistance to three or more antibiotic classes is a growing problem in environmental *E. coli* isolates, highlighting the clinical importance of environmental bacterial reservoirs [21].

The paradigm shift of the paramount importance in AMR epidemiology has been demonstrated to be an environmental *E. coli* characterization: environmental isolates are not mere passive recipients or ecological mysteries, but active reservoirs of resistance genes with

direct paths to relevance in human clinical medicine [5]. The HGT can be associated with environmental *E. coli* as sources of resistance determinants, which are transferred to clinical *E. coli* and other *Enterobacteriaceae* by conjugative plasmid transfer, thus potentially increasing the resistance phenotype of human pathogens with no direct selection pressure by antibiotics in the clinical environment [19]. Empirical evidence of the transmission axis has been indeed provided by genomic studies in Bangladesh which show shared ARGs and clonal lineages of ESBL-producing *E. coli* between environmental, animal, and human sources [22]. The explanation of environmental *E. coli* resistance profiles is thus not only informative in the dynamics of ecological resistance in a local context, but also the vectors and pathways by which a resistance gene can be transmitted to a clinical organism.

The closely related, but conceptually distinct concept of MDR is the phenomenon of co-resistance simultaneous expression of resistance determinants to multiple, often structurally and mechanistically unrelated classes of antibiotics in a single bacterial isolate or within a single mobile genetic element [18]. The most common co-resistance occurs when several ARGs are co-localized on the same MGE, e.g., on a conjugative multidrug-resistant plasmid or class 1 integron containing a cassette array of resistance genes [9, 13]. Knowledge of co-resistance in environmental bacterial communities has significant scientific and community health implications. On the ecological level, co-resistance analysis sheds light on resistance evolution and persistence mechanisms by elucidating the mechanisms underlying resistance evolution and persistence in dynamic and complicated selective environments typical of urban water systems [15]. The observation of co-resistance patterns in environmental isolates at the clinical level offers an early warning of resistance gene combinations that are likely to develop or have already developed in clinical pathogens, enabling antibiotic stewardship efforts to be planned with co-selection dynamics in mind [9, 13]. In addition, a high level of co-resistance profiling of environmental *E. coli* can provide insight into the assembly and dissemination of multidrug resistance arrays in microbial communities a process directly pertinent to understanding the acquisition of additional resistance determinants in MDR phenotypes over time and under different anthropogenic pressures [18]. Although environmental AMR has become a widely known issue in the global health agenda, there is a lack of knowledge, especially in the low- and middle-income nations (LMICs) where water contamination is high, antibiotic prescription and usage are not controlled, and the AMR surveillance system is not well developed [2, 5]. This is a critical

challenge in Bangladesh. Being one of the most populated and quickly urbanizing nations globally, the problem of water pollution in Bangladesh is acute due to an insufficiently treated municipal sewage, pharmaceutical production wastes, hospital wastewater, and intensive agricultural and aquaculture practices [5, 22]. In Bangladesh, examination of urban and rural water bodies has reported disastrous amounts of environmental pollution by ESBL-producing and carbapenem-resistant *E. coli*, with as much as 98% of wastewater samples and a significant percentage of surface waters and drinking water sources reporting positive results of ESBL-producing *E. coli* in certain locations [5, 15, 19]. Despite these results, there are no systematic site-comparative investigations specifically focusing on the description of the prevalence of MDR, resistance patterns among various antibiotic classes and co-resistance patterns in urban aquatic environments in Bangladesh [15, 18].

To fill these gaps in evidence, the current study was developed to comparatively examine AMR among *E. coli* populations in urban aquatic environments in Bangladesh that are distinctly different in terms of anthropogenic pollution and human activity. The location of the studies was chosen to cover a range of contamination intensity: highly polluted urban waterways with direct releases of industrial effluents and poorly treated sewage, residential water channels with medium but stable human activity, to relatively less affected water bodies with lower anthropogenic pressure. It also indicates the results of studies done in Bangladesh that have shown that ESBL-producing *E. coli* concentrations and resistance gene burdens are much greater in urban than rural water systems, and in wastewater than in surface and drinking water systems [22].

The hypothesis of the study is that, the *E. coli* isolates present in urban aquatic environments in Bangladesh, with a higher load of anthropogenic pollution will show a significantly greater prevalence of multidrug resistance, a broader spectrum of resistance, and a higher rate of co-resistance when compared to the isolates that are present in less contaminated environments.

1.2 Objective of this study:

The specific objectives of this study were as follows-

The general objective was to isolate and phenotypically characterise *E. coli* of water samples at three urban water settings in Dhaka, Bangladesh. Also, to describe the phenotypes of antibiotic resistance of individual isolates against a mix of eight clinically relevant antimicrobial agents across drug classes. The research also aimed at identifying the distribution and prevalence of multidrug-resistant (MDR) phenotypes among the sites of sampling as well as the co-resistance patterns and determining statistically significant pairwise correlations between the resistance to different antibiotic classes. Lastly, the objective was to compare resistance profiles and prevalence of MDR across sites that represented various levels of anthropogenic impact.

The current study results are applicable to help seal a drastic AMR surveillance gap in urban Bangladeshi water settings [14, 23], as well as to apply directly to policymakers, practitioners in the public health field and water safety engineers who would like to identify evidence-based methods to keep AMR within the environment-human health interface [1, 8].

Chapter 2

2.1 Methodology

2.1.1 Sample collection:

The three urban water bodies in Dhaka where water samples were collected were Hatirjheel, Banasree, and BRAC University water bodies. The selection of these places was to represent various degrees of environmental factors and human activity, which have important effects in the distribution of bacteriophages and their bacterial hosts, such as *Escherichia coli*.

Hatirjheel was selected as a highly urbanized water system that receives industrial effluents, sewage input and runoff, and therefore it has a potential of being a hot spot of bacteriophage abundance and microbiological diversity. To measure microbiological contamination, which was primarily due to localized human actions and household trash, Banasree, a highly populated residential area with semi-stagnant waterways, was also taken into consideration. The BRAC University water body, in its turn, is going to be used as a reference point to compare with the more polluted areas as it is a less polluted and more controlled environment. Proper attention was given so that maximum results and microbes can be obtained [14].

2.1.2 LB media preparation for filtration:

The complex medium known as Luria-Bertani (LB) broth medium, which mostly contains tryptone, yeast extract, and NaCl, is known to encourage the structural development of subpopulations. It is often employed in biomolecular experiments to pre-culture strains so they can proliferate to fulfill the criteria. LB media has been shown to successfully enhance colony numbers. These findings suggested that the concentration of LB medium could be essential for promoting bacterial colony formation and isolating some advantageous bacterial strains. The cell heterogeneity within microbial communities and the gene expression patterns of microbial populations can be impacted by the gradual changes in pH and sugar availability

caused by *E. coli* cultured in LB medium [24]. LB media was used highly in our research works for filtration purposes as well as dilution.

2.1.3 Sample Filtration and Serial Dilution:

Sampling Frequency: Sample were collected 2 times every month for 6 months.

The water samples for the research were collected in the following areas; Hatirjheel, Banasree, and the BRAC water body. A single water sample was collected from each site. The location of sample collections varied a little. Samples collected were filtered with the assistance of funnel and Whatman filter paper to sterile Falcon tubes. Then, 1 mL of each filtered sample was aseptically transferred to 4 mL culture medium Falcon tubes of a larger size. This was carried out in all three sites of the sampling. The inoculation was incubated in falcon tubes and subsequently a shaking incubator (approximately 4 hours) was used to allow enrichment of the microbes. The samples were then incubated and then serial diluted. There were four microcentrifuge tubes and 900 μ L of LB medium was placed in each. The tube of Falcon was considered to be the stock (undiluted) sample since it was the culture that was enriched. Using this, 100 μ L was pipetted into the first tube with 900 μ L LB medium, to get a 10^{-1} dilution. A 100 μ L of the 10^{-1} dilution was then added to the second tube to make it 10^{-2} dilution. This dilution process was done in a stepwise fashion up to a final dilution of 10^{-4} . The same protocol was used to carry out serial dilutions, in every three samples. Aseptically, about 80 μ L of the sample was transferred to a plate of agar. The plates that were inoculated were incubated at 37°C to allow bacterial growth.

2.1.4 EMB media preparation:

The Eosin Methylene Blue (EMB) agar is a selective and differential media that is used in isolating and presumptive identification of gram-negative enteric bacteria, especially *E. coli*. Selectivity is endowed by the eosin Y and the methylene blue dyes, which prevent the growth of gram-positive organisms. Its differentiation mechanism takes advantage of lactose or sucrose fermentation: fermenting organisms that produce strong acids (e.g., *E. coli*) cause the dyes to precipitate and give a typical metallic green shininess on colonies, and non-fermenting or weakly fermenting organisms yield pink or no colony. This renders EMB

particularly useful when it is necessary to determine *E. coli* presumptively in environmental water samples, wherein a combination of gram-negative bacteria is anticipated. EMB powder was dissolved in distilled water, boiled and then autoclaved at 121°C for 90 minutes, cooled down to 45-50°C with gentle swirling to ensure the homogeneity of the dye and poured into sterile plates [7].

2.1.5 Bacterial inoculation methods used:

All inoculations were done in a laminar airflow cabinet under aseptic conditions using sterilized inoculating loops. Initial isolation of the colony was done by spread plating, and pure cultures were then obtained by streak plating to be used in downstream analysis.

2.1.6 Isolation and Purification

Undiluted stock was spread-plated (80 µL per plate) onto an agar in serial dilutions to a final 10^{-4} and labeled with sample identity, dilution factor and date. Plates were pre-warmed and dried before inoculation. Plates were placed without disturbance after spreading and inverted and allowed to incubate at 37°C after 20- 24 hours.

To be purified, individual colonies of the spread plates were sub-cultured using the zigzag streak plate technique on fresh agar. All streaking was done in a biosafety cabinet in a laminar flow. Inoculated plates were marked by sample identity, dilution factor, number of colonies, date and inverted and incubated at 37°C for 18-24 hours.

2.1.7 Soft Agar culture and inoculation:

Soft agar was made by adding agar and LB medium in the right proportions with the distilled water, which was autoclaved and aseptically transferred into sterile microcentrifuge tubes (~1 mL/tube). Tubes were kept in refrigeration until use. Three times of stabbing bacteria cultures in the soft agar using a sterile loop and storing at 4°C was used to inoculate the soft agar.

2.1.8 Glycerol Stock Preparation:

To preserve it in the long run, 700 μL of enriched culture was added to 300 μL of sterile glycerol in a microcentrifuge tube, which was covered with masking tape and aluminum foil and stored at -20°C .

2.1.9 IMViC Biochemical Tests

The IMViC series is a standardized set of four biochemical tests to differentiate members of the family *Enterobacteriaceae* and has particular uses in distinguishing between *E. coli* and morphologically and phenotypically similar coliforms such as *Enterobacter aerogenes* and *Klebsiella pneumoniae*. Both tests focus on different metabolic pathways and combining them will give a biochemical fingerprint enabling presumptive identification of the species. This is particularly significant in environmental microbiology, where various species of *Enterobacteriaceae* can be co-isolated in water samples, and the identity of species needs to be established before carrying out molecular analysis. *E. coli* has a typical ++ IMViC profile: indole positive (tryptophan degradation through tryptophanase encoded by *tnaA* gene), methyl red positive (mixed-acid fermentation with a sufficient amount of organic acids to lower pH to less than 4.4) [29], Voges-Proskauer negative (no butanediol fermentation pathway) [24], and citrate negative (no ability Provisional identification of isolates that matched this profile was done as *E. coli* [30, 31] and subjected to PCR confirmation. Isolates which were not consistent with this pattern were not further analyzed.

2.1.10 MR-VP Broth Preparation and Procedure:

Both the Methyl Red (MR) test and the Voges-Proskauer (VP) test, test the glucose fermentation pathways, but complement each other by identifying mutually exclusive end products, thus differentiating *Enterobacteriaceae*. The MR test identifies the mixed-acid fermentation pathway, where the organism like *E. coli* generates high amounts of stable organic acids (formic, acetic, lactic) that reduce the culture pH to 4.4 or lower, keeping the

methyl red indicator red [29, 30]. Conversely, the butanediol fermentation route is detected by the VP test, whereby acetylmethylcarbinol (acetylmethylcarbinol or acetyl) is the end product; acetylmethylcarbinol is oxidized to diacetyl in the presence of α -naphthol and KOH, which then reacts to form a red color [24, 30]. *E. coli* is usually MR-positive and VP-negative, and *Enterobacter aerogenes* is MR-negative and VP-positive.

Pure isolates were inoculated and left to incubate aerobically with 37°C at 24-36 hours. In the case of the Methyl Red test, five drops of 0.05% methyl red reagent were put into 2.5 mL of culture; red color persisted was a positive indicator of MR. In the case of the Vogesproskauer test, 0.6 mL of Barritts Reagent A (naphthol) was added to it; 0.2 mL of Reagent B (KOH) was then added; red color appeared on the surface in 30-60 minutes signifying VP-positive and yellow signified VP-negative [30].

2.1.11 Preparation and Procedure, Indole Test:

The indole test identifies the capacity of an organism to break down the amino acid tryptophan to indole, pyruvate, and ammonia through the enzyme tryptophanase, produced by the *tnaA* gene. One major characteristic that identifies *E. coli* is indole production, which is employed to differentiate it among other members of *Enterobacteriaceae* that do not have active tryptophanase (e.g. *Enterobacter spp.* and *Klebsiella spp.*). A test is performed on tryptone broth (a broth with high amounts of tryptophan) as the substrate and indole is identified by its reaction with the p-dimethylaminobenzaldehyde present in Kovacs reagent to form a red-coloured product, rosindole, which separates into the organic reagent layer at the surface of the broth [32]. A 5 mL of tryptone broth (peptone 0.5 g, NaCl 0.25 g in 50 mL distilled water) was dispensed into each tube and autoclaved at 121°C. Pure isolates were inoculated and allowed to grow aerobically at 37°C in 24-48 hours. After incubation, 5 drops of the Kovac's reagent were added to each tube. The presence of a red ring at the surface (*E. coli* expected result) was a positive result; the absence of a red ring was a negative result [32].

2.1.12 Preparation and Procedure of Citrate Utilisation Test:

The citrate utilization test identifies the ability of an organism to use citrate as its only source of carbohydrates and energy, a characteristic that needs the membrane transporter CitT and the enzyme citrate lyase. Bacteria that can use citrate like *Enterobacter aerogenes* and *Klebsiella pneumoniae* are able to metabolize citrate and at the same time use ammonium salts as the only source of nitrogen to generate alkaline by-products (carbonates and bicarbonates) which increase the pH of the medium and cause a change in the colour of the pH indicator bromothymol blue to *E. coli* does not have the required transporters to take in citrate in aerobic conditions and thus is citrate-negative, which means that the test is a dependable negative indicator to identify *E. coli* among the Enterobacteriaceae [31, 33]. Simmons Citrate Agar (0.729 g in 30 mL distilled water) was autoclaved at 121°C, allowed to cool to approximately 50°C and dispensed in 4 mL per vial as agar slants. The isolates were streak-inoculated and incubated (aerobically) at 37°C, 2448 hours. A color change of green to deep blue was a positive sign of citrate; no color change or pinpoint yellow colonies were the negative signs of citrate, the best possible outcome of *E. coli* [31].

2.2. Polymerase Chain reaction (PCR)

2.2.1 Colony PCR and PCR Mechanism:

Colony PCR was done to ensure the presence of bacterial DNA in isolated colonies. Since 16S rRNA is a universal bacterial marker, not an *E. coli*-specific gene, PCR amplification in 16S rRNA primers was a general bacterial confirmation step, which confirmed that isolated colonies were viable bacteria.

Name of Organism	Primers used	Primer Sequence	Size	Reference
<i>E.coli</i>	16S rRNA	F: 5'-GACCTCGGTTTAGTTCACAGA-3' R: 5'-CACACGCTGACGCTGACCA-3'	585 bp	[28]

Negative control: To ensure there was no contamination, 3 µL of nuclease-free water was used against DNA template and mixed with 10µL of PCR mixture (Total 13 µL).

Each 13 μL PCR reaction contained 6.5 μL master mix (thermostable DNA polymerase, dNTPs, MgCl_2 , buffer), 1 μL each of forward and reverse primer, 2 μL nuclease-free water, and 2.5 μL of colony template. The cycling conditions were: 10 minutes of initial denaturation at 95°C; 35 cycles of 95°C 30 s, 58°C 30 s and 72°C 30 s; and 2 minutes of final extension of 72°C. Each run had a no-template negative control to identify contamination. To confirm the amplification, a positive control of known *E. coli* genomic DNA was used [25].



Figure 1: Thermal cycler program used for colony PCR amplification. The protocol included an initial denaturation at 95 °C for 10 min to lyse bacterial cells, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. The conditions enabled direct amplification of target DNA from bacterial colonies. (Picture taken from Brac University Molecular Lab.)

2.2.2 Agarose Gel Electrophoresis:

2.2.3 Materials for Gel Electrophoresis:

Agarose powder (1 g), 1X TAE (Tris-acetate-EDTA) buffer (100 mL), ethidium bromide (3 μL) was used as the following materials, DNA loading dye, plasmid DNA samples, a DNA ladder, a horizontal electrophoresis apparatus (Biometra), and a UV transilluminator [26].

2.2.4 Process of Gel electrophoresis:

Preparation of gel was done using Agarose gel. Agarose gel of 1% (w/v) was made to be used in the Biometra electrophoresis setup. A gram of agarose powder was measured then mixed with 100 mL of 1X TAE in the Erlenmeyer flask. The buffer electrophoresis medium chosen was TAE buffer (40 mm tris, 20 mm acetic acid and 0.1 mM EDTA in 1X context) that contained EDTA to forestall the activity of nucleases in the DNA [26]. The heating of the flask was done at a 30-second time, swirling every 30 seconds until the agarose was completely dissolved. Cooling of the solution was followed, to fit around 60°C then ethidium bromide was added. At the pre-pouring phase, ethidium bromide was added to the agarose solution kept on ice, which was added to the solution mix [26, 28]. Adding ethidium bromide in the gel and to the electrical current running buffer at the usual level does not impact the electrical flow at all [27]. Since ethidium bromide is a well-known mutagen, all the procedures were conducted wearing proper personal protective equipment that included gloves, protection of the eyes, and laboratory coat [26].

2.2.5 DNA samples:

The DNA samples were prepared taking 2.5 microliter of isolates and combining with 6.5 microliter of master mix, 1 microliter of forward, 1 microliter of reverse and 2 microliters of nuclease free water to get a 13 microliter Loading sample.

2.2.6 Loading and Running of the Gel:

After solidification, the gel comb was carefully inserted and the gel, in the casting tray, was placed in the electrophoresis tank with 1X TAE running buffer until completely submersible. The DNA samples were also loaded carefully into the respective wells with the help of the micropipette. The first lane contained a DNA marker (ladder) to determine the molecular weight and size of the plasmid bands. The apparatus was set up such that the electrodes were attached to a power source and the DNA moved on the negative electrode towards the positive electrode. The gel was subjected to a constant voltage of 80-120V and left to run in the gel lane before about 30-60 minutes. The gels are generally subjected to low, steady voltage that ensures small current flows, and asymmetric heating is minimized that may lead

to poor resolution and band artifact [27, 28]. The movement of the loading dye was tracked in the gel to monitor the progress of the migration.

2.2.7 Visualization of DNA bands:

The gel was rescued carefully and after the electrophoresis process, it was transferred to a UV transilluminator. The ethidium bromide-UV transilluminator system has a high detection sensitivity; UV rays with short wavelengths of 312 nm give intense excitation of the dye but at the expense of that wavelength changing the DNA strands as opposed to the longer wavelengths of UV rays [28, 34]. The fluorescent bands obtained were recorded with the help of a gel documentation system or photographed in UV light. The images were analyzed with image analysis software and band sizes estimated by comparing them to the co-migrating DNA ladder size [28, 34].

2.3 Antibiotic Susceptibility Test (AST)

2.3.1 Antibiotic Susceptibility Testing Overview and Principle

Antimicrobial susceptibility testing is a lab test done in order to determine the effective antimicrobial regimen to be used on individual patients. On the bigger scale, the testing helps in determining the treatment services by the hospitals, clinics, and national programs to manage and prevent infectious diseases. Antibiotic susceptibility testing (AST) helps prescribe a dose of the antibiotic and design a profile of empirical treatment of the effective treatment of the health of individual patients against fatal diseases, and, in such cases, fast diagnostics plays a crucial role in bacterial infection treatment [36]. This study used the Kirby-Bauer disk diffusion technique. One of the most common antimicrobial susceptibility tests and a commonly practiced one is the Kirby-Bauer disk diffusion method, and when carried out according to a typical procedure is reliable, and capable of giving a prediction of the clinical efficacy of the antibiotics which have been tested [37]. This technique is quite popular, as it is a simple and cost-effective protocol, which detects multiple targets at the

same time [35]. This follows the general principle of radial diffusion of the antibiotic in an impregnated paper disk into agar medium; the antibiotic impregnated disk is left to diffuse into the hardened agar, creating an inhibition zone when agar medium after overnight incubation, and the size of this inhibition zone is proportional to the concentration of the antibiotic to reach bacterial lawn [35].

2.3.2 Preparation of Inoculum and McFarland Standardization:

Bacterial colonies that were stored in soft agar were recovered with a sterilized inoculation loop. The visible bacterial growth was sampled using the loop, and the streak plate method was performed using the Eosin Methylene Blue (EMB) agar to isolate colonies. The incubation time of streaked plates was 18 hours after which colonies were well developed, and the rest of the procedure was done.

The inoculum diluent was a 0.9% (w/v) saline solution, which was made by dissolving 0.9 g of sodium chloride in 100 mL of distilled water. The solution was added to 3 mL glass vials, which were autoclaved to sterilize. The working bacterial suspension was prepared by inoculating a loopful of fresh overnight growth in the saline solution.

To adhere to the CLSI guidelines (2023), the turbidity of the bacterial suspension was adjusted to the 0.5 McFarland standard (resembling $1-2 \times 10^8$ colony-forming units (CFU)/mL) [38]. Turbidity was evaluated visually by comparing the suspension with the McFarland standard card in sufficient light with a white background having contrasted black lines. Suspensions exceeding the 0.5 McFarland turbidity were diluted with sterile saline until a desired turbidity was obtained. This standardization measure is very important to have a confluent bacterial lawn and reproducible zone diameters [38].

2.3.3 Mueller-Hinton Agar Preparation

The culture medium to be susceptibility tested on was Mueller-Hinton agar (MHA) as this is the standard culture medium recommended by Clinical and Laboratory Standards Institute

(CLSI) in disk diffusion susceptibility tests [37]. MHA was chosen because of its favorable physicochemical characteristics: it is a soft agar which facilitates better diffusion of antibiotics than most other media, making it give more accurate zones of inhibition. Also, para-aminobenzoic acid (PABA) and thymine/thymidine concentrations in MHA are kept at low concentrations to minimize the inactivation of sulfonamides and trimethoprim during susceptibility testing [37]. The nutrient sources in the medium are beef extract and acid hydrolysate of casein, and starch which is used in adsorbing toxic metabolites that bacteria may otherwise generate and interfere with the action of the antibiotics.

The right weight of MHA powder was measured and dissolved in the given amount of distilled water. The suspension was then warmed in a Bunsen flame and mixed stirred by keeping it constantly mixed until it dissolved completely, and the solution became clear. The flask was then left to cool down, wrapped in aluminum foil, labeled and autoclaved to sterilize it. The autoclaved medium was added in sterile Petri dishes (30 mL each in large plate) in a laminar airflow cabinet. The plates were left to solidify and dry at room temperature and labeled and kept refrigerated until usage. Before using, the pH of each batch of MHA was checked to be within the range of 7.2 to 7.4 at 25°C, as suggested by CLSI (2023) [37]. The 1L MH-agar media used in the lab has following composition showed in the table-

Ingredients	Quantity
Beef extract	2 g
Agar	17 g
Casein Hydrolysate	17.5 g
Distilled water	1000 ml
Starch	1.5 g

Table 1: Composition of MH agar medium showing ingredients and their respective quantities required for the preparation of 1L of culture medium.

Additionally, the pH was maintained at 7.3 ± 0.1 at 25°C . These nutrients, in the proper amount, provide a suitable environment for *E. coli* and many other bacteria to grow in the media.

2.3.4 Inoculation of Mueller-Hinton Agar Plates:

The sterile cotton swab was dipped into the standardized bacterial suspension, and the extra fluid was squeezed out by pressing the swab on the inside wall of the tube above the liquid. A swab was then used to inoculate the entire agar surface evenly on the surface of a dried MHA plate surface. This was done three times, turning the plate about 60° between sets of streaks, to cover all the surface and obtain a uniform and confluent bacterial lawn, as suggested by the Clinical and Laboratory Standards Institute (CLSI) [38]. The plates inoculated were left to dry 3-5 minutes at room temperatures and then antibiotic discs were applied.

2.3.5 Introduction of the Disc of the antibiotics:

Antibiotic disks of standard concentrations (as recommended in CLSI, 2023) were applied with sterile forceps on the agar plate that was inoculated [38]. A disk was pressed hard on the agar until the disk touched completely. Antimicrobial agents start to diffuse into the agar as soon as they come into contact with the disk, so disks were not repositioned after the first placing. Then disks were separated by a minimum of 24 mm (centre to centre) and a maximum of 12 disks were put on each 150 mm Mueller-Hinton plate to avoid zone overlap [35, 39].

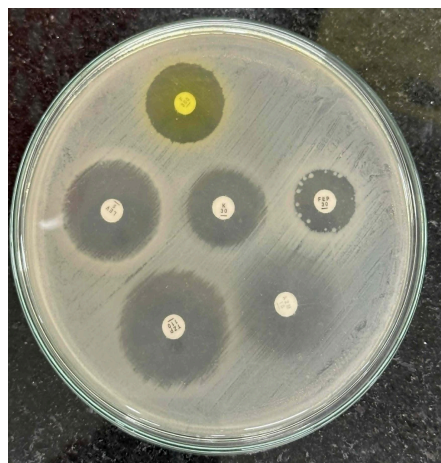


Figure 2: Mueller-Hinton agar (MHA) plate after 18-24 hours of incubation with antibiotic susceptibility test by using Kirby-Bauer disc diffusion technique. A monoculture of bacteria was created, and antibiotic impregnated discs were placed on it (n=1 representative plate). Sensibility of the isolate to certain antibiotics is represented by zones of inhibition around the discs with small or no zones indicating resistance.

2.3.6. List of Antibiotics:

The following antibiotics from various antimicrobial classes were chosen to carry out our Antibiotic Susceptibility Test as variables to determine the multidrug resistance patterns across the various isolates:

Antibiotic Group	Antibiotic Name	Code	Disk Concentration (µg)	Type of Species	Diameter of Zone Inhibition		
					Resistant (R) (mm)	Intermediate (I) (mm)	Susceptible (S) (mm)
Fluoroquinolone	Levofloxacin	Lev-5	5	Enterobacteriaceae	<1.3 mm	-	≥1.3 mm
Phosphonic acid	Fosfomycin	F-300	300	Enterobacteriaceae	<1.4 mm	-	≥1.4 mm
Tetracycline	Doxycycline	DO-30	30	Enterobacteriaceae	<1.2 mm	-	≥1.2 mm
Penicillin β-lactamase inhibitor	Piperacillin-Tazobactam	TZP-110	110	Enterobacteriaceae	<2.0 mm	-	≥2.0 mm
Cephalosporin – 4th gen	Cefepime	FEP-30	30	Enterobacteriaceae	<1.8 mm	-	≥1.8 mm
Macrolide	Azithromycin	AZM-15	15	Enterobacteriaceae	<1.3 mm	-	≥1.3 mm

Antibiotic Group	Antibiotic Name	Code	Disk Concentration (µg)	Type of Species	Diameter of Zone Inhibition		
					Resistant (R) (mm)	Intermediate (I) (mm)	Susceptible (S) (mm)
Aminoglycoside	Kanamycin	K-30	30	Enterobacteriaceae	<1.3 mm	-	≥1.3 mm
Penicillin	Piperacillin	PRL-100	100	Enterobacteriaceae	<1.4 mm	-	≥1.4 mm

Table 2: Antibiotics utilized in the study, along with their classifications, disk concentrations (µg), and associated zone of inhibition diameter breakpoints (mm) for *Enterobacteriaceae*, denoting resistant (R), intermediate (I), and susceptible (S) categories.

2.3.7 Measurement of Zones of Inhibition

The plates with inoculations were then inverted and allowed to incubate at 37°C (16-18 hours) according to CLSI (2023) recommendations [38]. During incubation, plates were not piled up to make the temperature distributed evenly. The incubation was done in an aerobic condition.

2.3.8 Zones of Inhibition Measurement and Interpretation

After incubation, circles of inhibition of each antibiotic disk were measured. Each petri plate was inverted, and placed a few centimetres above a black, non-reflective surface, and light reflected upon them. The size (in millimetres) of each complete inhibition zone- including the disk diameter was determined to the nearest whole millimeter by the naked eye with a calibrated ruler [38, 39]. The zone margin was considered as the point where no apparent growth of bacteria was observed.

The distance between the center of the disk and the edge of the zone was measured where the direct measurement was not possible and doubled. The disk was grown until the end of the disk was reached, which was 6 mm (disk diameter by itself). When dealing with strains of *Proteus spp.* that might swarm into perceived area of inhibition, only the particular outer

boundary of complete inhibition was documented, and the thin swarming film was ignored [36, 38]. In locations where discrete colonies were observed in an area of inhibition, the test was repeated using a pure culture; in the case of continued discrete colonies on repeated testing, the organism was considered resistant to the corresponding antibiotic. The CLSI breakpoint tables (CLSI M100, 2023 edition) were used to interpret the zone diameters. The results were put into one of the three categories of each antibiotic-organism-pairing:

Susceptible (S): Zone diameter is equal to or greater than the susceptible breakpoint, which means that the isolate is inhibited by the drug at the concentrations that are attainable on standard dosing schedules.

Intermediate (I): Zone diameter between the susceptible and resistant breakpoints. This type means that the isolate can be blocked by the drug at higher levels or in body areas where the drug concentrates physiologically and can be utilized as a buffer area to prevent small technical errors leading to significant category errors.

Resistant (R): Zone diameter of 10 mm or less, which means that the isolate is not suppressed by the drug at concentrations that can be acquired under the usual dosing schedules, and/or that resistance could potentially be developed.

Special attention was paid to the differentiation of zone diameters in the neighborhood of other susceptible and resistant breakpoint boundaries since small measurement errors can lead to big or very big classification errors. All the measurements of the zone diameter and gel images were systematically recorded, which would be later analyzed statistically [38, 39].

2.3 Parameters Used for Result Analysis:

All statistical analyses were conducted in R version 4.3.1 [40], implemented through the RStudio integrated development environment version 2023.09.0 [40]. Raw inhibition zone diameter measurements were first cleaned to remove non-numeric entries including untested plates denoted by "X", borderline results denoted by asterisks, and notations indicating overgrowth or double zones prior to statistical processing. In accordance with the binary

resistance classification framework outlined by Turnidge and Paterson (2007), resistance classification was carried out by comparing cleaned zone diameter values against antibiotic-specific breakpoint thresholds. Values at or above the defined threshold were coded as resistant (1), and values below the threshold were coded as sensitive (0). The per-antibiotic median value was used to predict missing values from untested antibiotic-isolate pairs before multivariate analysis, a commonly used method for addressing missing data in microbiological profiling research [41].

Chapter 3

3. Result:

3.1 Statistical Analysis Results

The amount of antibiotic resistance was found to be severely high in all 150 isolates with six out of eight antibiotics tested showing resistance above 80%. K-means clustering was used to separate isolates into a dominant high-resistance group (Cluster B, 93.3%, n=140) and a minor low-resistance group (Cluster A, 6.7%, n=10). Two statistically significant co-resistance pairs were obtained: Levofloxacin-Azithromycin (0.473, $p=0.002$) and Piperacillin-Tazobactam-Cefepime (0.452, $p < 0.001$) in line with the efflux pump-mediated

and beta-lactamase-mediated cross. The highest prevalence of MDR was at BRAC (86.0%), followed by Banasree (78.0%), and Hatirjheel (74.0%).

DESCRIPTIVE STATISTICS - Inhibition Zone Values (mm)									
Antibiotic	Threshold (mm)	n Tested	n Resistant	n Sensitive	% Resistant	Mean	Median	SD	Range
Lev-5 (R<1.3)	1.3	90	79	11	87.8	2.45	2.65	0.941	0.0–4.0
F-300 (R<1.4)	1.4	129	123	6	95.3	2.012	2	0.331	1.0–2.7
DO-30 (R<1.2)	1.2	44	25	19	56.8	1.35	1.2	0.639	0.0–2.3
TZP-110 (R<2.0)	2	97	93	4	95.9	2.631	2.7	0.455	0.0–3.4
FEP-30 (R<1.8)	1.8	88	82	6	93.2	2.85	3.2	0.851	0.0–4.0
AZM-15 (R<1.3)	1.3	75	54	21	72	1.528	1.5	0.831	0.0–2.9
K-30 (R<1.3)	1.3	66	60	6	90.9	1.873	2	0.581	0.0–2.6
PRL-100 (R<1.4)	1.4	22	18	4	81.8	2.105	2.45	0.928	0.0–3.1

Table 3: Descriptive statistical analysis of inhibition zone diameters (mm) of the selected antibiotics with threshold values, number of isolates tested, resistant and sensitive isolates, percentage of resistance and summary statistics (mean, median, standard deviation and range).

3.1.1 PCA Clustering Analysis:

The highest silhouette score (0.509) amongst the candidates ($k = 2$ to $k = 6$) was used to select k-means clustering ($k = 2$). The predominant MDR group (93.3 percent, $n = 140$) was cluster B, which exhibited resistance to majority of the antibiotics tested. The resistance was lower in Cluster A (6.7%, $n = 10$), especially to Levofloxacin and Azithromycin. This separation was verified by PCA, where 37.5% of the total resistance variance was explained by PC1, and 21.5% by PC2 (total: 59%). A global, cross-class resistance mechanism (likely

AcrAB-TolC efflux pump overexpression or presence of multidrug resistance plasmids) was implied by all eight antibiotics loading positively and uniformly on PC1. The variability that was captured by PC2, was specific to Doxycycline (DO-30) and Piperacillin (PRL-100), indicating that the selective pressure was independent between agricultural and veterinary antibiotics.

3.1.2 Narrow vs Broad Spectrum Analysis:

Key finding: At the population level, broad-spectrum antibiotics showed significantly more prevalent resistance (85.8%) than narrow-spectrum antibiotics (81.6%), confirmed by Chi-square test ($\chi^2 = 7.533$, $df = 1$, $p = 0.006$). However, there was no significant difference at the individual isolate level (Mann-Whitney $U = 8796.5$, $p = 0.156$), which shows that an isolate that has gained MDR is resistant to both the spectrum categories at similar rates. These are characteristics of broad-acting mechanisms like- efflux pumps and extended-spectrum beta-lactamases.

The Chi-square test revealed a statistically significant difference in resistance rates between the two groups ($\chi^2 = 7.533$, degrees of freedom = 1, $p = 0.006$), indicating that resistance was significantly more prevalent among broad-spectrum antibiotics at the population level. Broad Spectrum Averages at **85.8%** resistance and Narrow Spectrum Averages at **81.6%** resistance.

SPECTRUM-LEVEL SUMMARY					
Spectrum	# Antibiotics	Total Tested	Total Resistant	Total Sensitive	Avg Resistance %
Broad	5	448	402	46	85.8%
Narrow	3	163	132	31	81.6%

Table 4: Broad- and narrow-spectrum antibiotics comparative summary with the list of the number of antibiotics per group, the total number of isolates tested, the number of resistant and sensitive isolates, and the average percentage of resistance per group [Data Visualized in: MS Excel]

Mann-Whitney tests reveal that at individual isolate level, no significant difference in per-plate resistance percentages between the two spectrum categories ($U = 8796.5$, $p = 0.156$) leading to a conclusion that individual isolates show resistance to both narrow vs broad

spectrum antibiotics in contrast to a Multidrug Resistant Phenotype. Following table presents the data found from the statistical measurement of the narrow vs broad spectrum analysis:

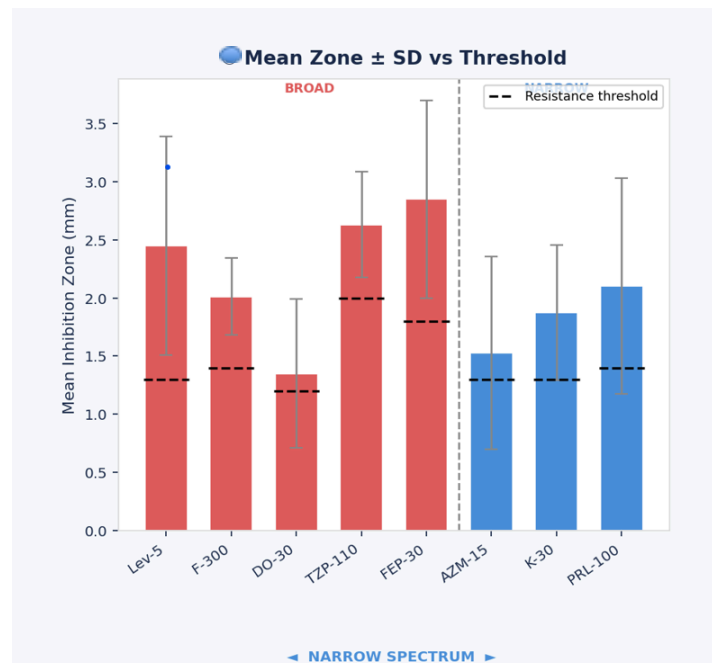


Figure 3: Multi-drug resistance (MDR) summary and cluster profile of $n = 150$ environmental *Escherichia coli* isolates, clustered by K-means cluster allocation. The left panel (tabular summary) shows the number of antibiotic classes resisted by the isolate per cluster with MDR characterization as not being susceptible to one or more agents in three or more antimicrobial categories [41]. The bar chart on the right panel shows that the x-axis indicates the number of antibiotics that are simultaneously resisted each plate (0 to 8) and the y-axis shows the number of plates in each category. The visualized two overlapping distributions are the Cluster A (red; $n = 10$; low-resistance profile) and Cluster B (blue; $n = 140$; multi-drug-resistant profile). The significant eventual far-rightward movement of the Cluster B distribution, as compared to Cluster A, is due to the resistance breadth accrued by the prevalent MDR phenotype. The lack of plates of Cluster B at zero antibiotics resisted and the concentration of Cluster A plates at the bottom of the distribution are also supportive of the biological distinction between the two clusters revealed by the K-means algorithm [40].

From the above table, if we run a chi square test and a Mann-Whitney test, we get the following outcome:

STATISTICAL TESTS

Test	Method	Statistic	degrees of freedom	p-value	Significance
Chi-square	Resistance counts (Broad vs Narrow)	Chi*2=7.5334	df=1	0.0061	Significant
Mann-Whitney U	Per-plate % resistance	U=8796	-	0.1563	Not significant

Table 5: A summary of statistical test findings, including degrees of freedom, p-values, Chi-square and Mann-Whitney U test statistics, and corresponding significance interpretations, comparing antibiotic resistance between broad-spectrum and narrow-spectrum groups.

3.1.3 Co-Occurrence Network Analysis:

Key finding: Two statistically significant pairwise co-resistance relationships were identified among the 150 isolates. Levofloxacin–Azithromycin showed the strongest cross-class co-resistance ($\phi = 0.473$, $p = 0.002$), suggesting efflux pump-mediated cross-resistance between a fluoroquinolone and a macrolide. Piperacillin-Tazobactam–Cefepime was the second significant pair ($\phi = 0.452$, $p < 0.001$), consistent with ESBL-mediated beta-lactam cross-resistance. All other antibiotic pairs showed weak or no significant associations.

Using Chi-Square test for pairs of antibiotics that had statistically significant outcome, the following outcomes were found:

1. Levofloxacin (LEV) and Azithromycin (AZM) had the strongest association, ($\phi = 0.473$, $p = 0.002$) leading to conclusion that isolates that resisted LEV will be more likely to resist AZM as well. This meant that there was a cross resistance between a narrow and a broad-spectrum antibiotic which tells us that there was real variation in the data, something beyond prediction or probability.
2. Piperacillin-Tazobactam and Cefepime were the second most significant pair ($\phi = 0.452$, $p < 0.001$) suggesting that a near universal resistance to these two offers little variation in the data and does not provide any selective link between the two.

Antibiotic A	Antibiotic B	Co-resistant (n)	Only A Resist	Only B Resist	Both Sensitive	n Valid	Phi Coefficient	Jaccard Index	Fisher p-value	Association
F-300	TZP-110	72	4	6	0	82	-0.0636	0.878	1	Weak/None

Lev-5	F-300	70	1	9	1	81	0.1821	0.875	0.233	Moderate Positive
TZP-110	FEP-30	66	3	0	2	71	0.6186	0.9565	0.004	Strong Positive
F-300	FEP-30	65	6	6	0	77	-0.0845	0.8442	1	Weak/None
F-300	K-30	58	6	1	0	65	-0.0399	0.8923	1	Weak/None
F-300	AZM-15	46	19	1	1	67	0.0772	0.697	0.511 1	Weak/None
Lev-5	TZP-110	43	2	9	1	55	0.0944	0.7963	0.459 1	Weak/None
Lev-5	FEP-30	43	4	7	1	55	0.0489	0.7963	0.559 1	Weak/None
TZP-110	AZM-15	42	18	1	2	63	0.1677	0.6885	0.234 4	Moderate Positive
Lev-5	K-30	38	3	2	0	43	-0.0605	0.8837	1	Weak/None
FEP-30	AZM-15	35	17	1	2	55	0.1623	0.6604	0.271 6	Moderate Positive
TZP-110	K-30	32	3	2	0	37	-0.071	0.8649	1	Weak/None
Lev-5	AZM-15	29	8	1	7	45	0.5343	0.7632	0.000 9	Strong Positive
FEP-30	K-30	26	4	4	0	34	-0.1333	0.7647	1	Weak/None
AZM-15	K-30	21	2	9	1	33	0.0209	0.6562	1	Weak/None
F-300	DO-30	20	14	1	2	37	0.1404	0.5714	0.567 6	Weak/None
DO-30	TZP-110	20	1	17	1	39	0.0179	0.5263	1	Weak/None
DO-30	FEP-30	18	1	16	0	35	-0.1574	0.5143	1	Moderate Negative
F-300	PRL-100	18	4	0	0	22	0	0.8182	1	Weak/None
Lev-5	PRL-100	16	1	1	0	18	-0.0588	0.8889	1	Weak/None
Lev-5	DO-30	16	5	2	4	27	0.378	0.6957	0.135 8	Strong Positive
K-30	PRL-100	16	1	1	1	19	0.4412	0.8889	0.204 7	Strong Positive
DO-30	AZM-15	14	4	9	5	32	0.1489	0.5185	0.453 3	Weak/None
DO-30	K-30	3	1	2	0	6	-0.3162	0.5	1	Strong Negative
FEP-30	PRL-100	2	1	0	0	3	0	0.6667	1	Weak/None

Table 6: Antibiotic pairwise co-occurrence analysis that displays the number of isolates that are resistant and sensitive, statistical measures such as the Jaccard index and Phi coefficient, Fisher's exact test p-values, and the interpretation of association strength that follows.

NOTABLE CO-RESISTANCE PAIRS				
Pair	Co-resistant	Phi	Jaccard	Interpretation
TZP-110 & FEP-30	66	0.6186	0.9565	Strong positive co-resistance- these isolates tend to be resistant to both TZP-110 and FEP-30 together

Lev-5 & AZM-15	29	0.5343	0.7632	Strong positive co-resistance- these isolates tend to be resistant to both Lev-5 and AZM-15 together
K-30 & PRL-100	16	0.4412	0.8889	Strong positive co-resistance- these isolates tend to be resistant to both K-30 and PRL-100 together
Lev-5 & DO-30	16	0.378	0.6957	Strong positive co-resistance- these isolates tend to be resistant to both Lev-5 and DO-30 together
DO-30 & K-30	3	-0.316 2	0.5	Negative association- resistance to DO-30 tends to NOT co-occur with K-30

Table 7: Significant antibiotic co-resistance pairs that show co-occurrence counts, Phi coefficients, Jaccard indices, and an explanation of the degree of correlation between antibiotic pairs.

Comparative Analysis of locations for sample collection:

Antibiotic	Threshold (R<)	Hatirjheel Mean	Banasree Mean	BRAC Mean	Lowest Mean Site	Highest Mean Site
Lev-5	<1.3	2.33	2.41	2.59	Hatirjheel	BRAC
F-300	<1.4	1.98	2.03	2.05	Hatirjheel	BRAC
DO-30	<1.2	1.28	1.5	1.3	Hatirjheel	Banasree
TZP-110	<2.0	2.64	2.57	2.66	Banasree	BRAC
FEP-30	<1.8	2.9	2.69	2.94	Banasree	BRAC
AZM-15	<1.3	1.4	1.66	1.53	Hatirjheel	Banasree
K-30	<1.3	1.9	1.88	1.82	BRAC	Hatirjheel
PRL-100	<1.4	2.32	1.71	2.42	Banasree	BRAC

Table 8: Mean inhibition zone values (mm) for each antibiotic are compared across various sample collection sites, along with resistance threshold values and the locations with the lowest and highest mean values.

Key takeaway: When combined, the four statistical tests lead to a unified viewpoint: the isolates of all the three urban water bodies are dominated by a generalised MDR phenotype that can be explained by the co-selection of multi-mechanistic resistance and site-specific outliers in MDR breadth and prevalence indicate that residential and peri-institutional settings (BRAC and Banasree) have resistance burdens equal or greater than Hatirjheel.

3.2 Result Interpretation:

3.2.1 Analysis of K-means Clustering

Instead of theorizing the optimal number of clusters (k) to use in the K-means algorithm, the silhouette coefficient was used as the main model selection rule to reach these numbers empirically [44]. Silhouette coefficient of a particular isolate is defined as $s = (b - a) / \max(a, b)$ where a is the mean intra cluster distance (average dissimilarity of the isolate and all the other isolates in the cluster to which the isolate belongs) and b is the mean nearest cluster distance (mean dissimilarity of the isolate and all the other isolates in the next closest cluster) [44]. The coefficient lies between -1 and +1: values close to +1 suggest the isolate fits well in its own cluster and is a poor fit within its neighbor clusters, values close to 0 suggest that the isolate is at a cluster boundary, and values less than 0 suggest that the isolate is very likely to be misassigned [44]. The average silhouette score of all isolates thus gives a unique, interpretable measure of the overall quality of a clustering solution- the extent to which the proposed partition results in internally cohesive and externally separated groups [44].

Candidate solutions were evaluated to k = 2 up to k = 6 with each solution linking n start = 10 random restarts to reduce the chances of being trapped in a local optimum as opposed to all time global optimum [44]. The resulting silhouette scores were as follows: k = 2 (0.509), k = 3 (0.354), k = 4 (0.270), k = 5 (0.314), k = 6 (0.308). The k=2 solution with the best score, 0.509, was chosen as it was regarded as a good to reasonable structure. The decreasing scores at k ≥ 3 point to the fact that more clusters are merely sub-dividing the large high-resistance population along lines of continuous variation, not necessarily bringing out biologically significant phenotypic differences [40]. This behavior is typical of data, where there is one phenotypically extreme population- in this case, the MDR Cluster B - where K-means can only form more clusters by simply splitting the majority population across axes of continuous instead of categorical variation [40]. This selection of k = 2 is then motivated on three different counts: silence being maximized; is consistent with the known binary structure of the resistance data as defined by the narrow vs. broad spectrum analysis (which showed that MDR phenotype is indiscriminate between the two classification on the isolate condition); and results in clusters with recognizable biological interpretations between a minority of low-resistance condition subpopulation and a majority MDR phenotype; a distinction of direct clinical and ecological relevance [41].

CLUSTER SUMMARY								
Cluster	n Plates	% Total	Lev-5 Mean	F-300 Mean	DO-30 Mean	TZP-110 Mean	FEP-30 Mean	AZM-15 Mean
Cluster A	10	6.7%	0.35	1.789	1.04	2.25	2.371	0.275
Cluster B	140	93.3%	2.712	2.029	1.39	2.675	2.891	1.678

Figure 4: K-means clustering of n = 150 isolates based on mean inhibition zone diameters (mm). Two clusters were identified: Cluster A (6.7%) and Cluster B (93.3%). Cluster B showed higher mean inhibition zones, indicating greater antibiotic susceptibility compared to Cluster A.

Cluster A- 6.7% of the total plates fall under this cluster, and these isolates behave differently than the others; It could reflect a less resistant strain or plates where the bacteria had not yet developed full resistance to Fluoroquinolones and Macrolides.

Cluster B- 93.3% of the total plates fall under this cluster marking it as a “High Resistance” group. The isolates are Multi Drug Resistant or MDR as they have a high resistance rate against most of the antibiotics used.

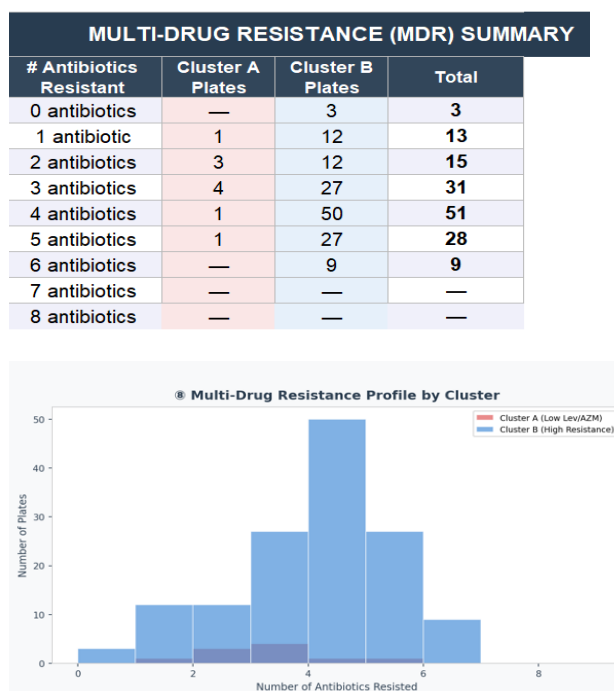


Figure 5: K-means cluster assignments of 150 environmental *Escherichia coli* isolates profiled on eight antibiotic variables as Principal Component Analysis (PCA) biplot. The horizontal axis and (PC1) accounts for 37.4% of the overall variance in the dataset of the inhibition zone diameter and is the global profile of multi-drug resistance: isolates on the right of the graph (high PC1 scores) have consistently large zone diameters in all eight antibiotics, indicating the presence of a susceptible phenotype, whereas those on the left (low

PC1 scores) have uniformly smaller zone diameters PC1 positively loaded all eight antibiotic variables at the same time, and the first axis of variation in this dataset therefore is not resistance to a single drug but a coordinated, cross-class decline in susceptibility that is likely either due to a broad-spectrum efflux pump overexpression or multi-resistance plasmid conservation [45]. The vertical axis (PC2) describes 21.5 percent of the total variance and captures most of the independent change of ATPs in the inhibition zone diameter of Doxycycline (DO-30) and Piperacillin (PRL-100) - compounds that have had different historical profiles of selective pressure due to agricultural and veterinary use of antibiotics [41]. PC2 is perpendicular to PC1 and as a result, the variability of tetracycline and penicillin resistance largely does not depend on the global gradient of MDR represented by PC1. The cumulative percentage of variance of PC1 and PC2 equals 59.0. Cluster A: These are low-resistance profile (n = 10; represented by red points); Cluster B: These represent multi-drug resistant profile (n = 140; represented by blue points). The outer boundaries of any cluster in the PCA space are represented by convex hull boundaries (dashed lines). The × characters represent the centres of clusters. The strategic spatial distinction between clusters on the PC1 axis proves that the K-means algorithm detected two phenotypically different and biologically consistent resistance profiles [41, 44].

3.2.2 PCA:

Principal Component Analysis (PCA) was performed to the median-derived inhibition zone diameter matrix, to visualize the K-means solution and understand the main axes of resistance variation [44]. Every principal component contains a weighted sum of the eight antibiotic variables, where loading coefficients show the nature of direction and strength of contribution of each antibiotic to that component.

PC1 (37.4% of variance) was loaded in a positive direction and with a very generally equal strength across all eight antibiotics at the same time. This loading pattern implies that PC1 does define a global susceptibility gradient: isolates at the high-PC1 end of the biplot have large inhibition zone diameters in all eight antibiotics (i.e., are broadly susceptible), whereas isolates at the low-PC1 end have uniformly small inhibition zone diameters (i.e., are broadly resistant). Biologically, it is an incompatibility between this pan-antibiotic loading pattern and independent drug-by-drug resistance acquisition, which would give rise to divergent

loading magnitudes, and part of the expected signature of a systemic cross-class resistance mechanism, most likely constitutive AcrAB-TolC efflux pump overexpression [45] or multi-resistance plasmid acquisition [47]. Cluster B is concentrated at the low-PC1 end of the biplot confirming that their resistance is not drug-specific but spread throughout the entire panel of antibiotics, the phenotypic effect of a resistance infrastructure acting at the general levels, and not to any particular agent.

An orthogonal and independent dimension of variability was measured in PC2 (21.5% of variance) and was highly loaded by Doxycycline (DO-30) and Piperacillin (PRL-100). This PC1 independence is also mechanistically possible: tetracycline resistance in *E. coli* is normally mediated by tetA/tetB efflux genes carried on narrow-host range plasmids that can be obtained without other large ESBL plasmids, or downregulation of repression of AcrAB-TolC, that drives the PC1 gradient [40]. Likewise, global efflux pump, independent of chromosomal penicillinase expression (AmpC) can be upregulated. PC2 probably thus indicates that historical selective pressure on a different genetic substrate existed on agricultural and veterinary tetracycline, as opposed to the global MDR determinants.

The striking distinctness of Cluster A and Cluster B on the PC1 axis supports the view that the PCA biplot is not just a statistical visualisation but an effector representation- the two clusters appear different in the occurrence of or lack of global/cross-class resistance infrastructure likely mediated by AcrAB-TolC overexpression and the presence of multi-drug resistance plasmids [44, 45, 46].

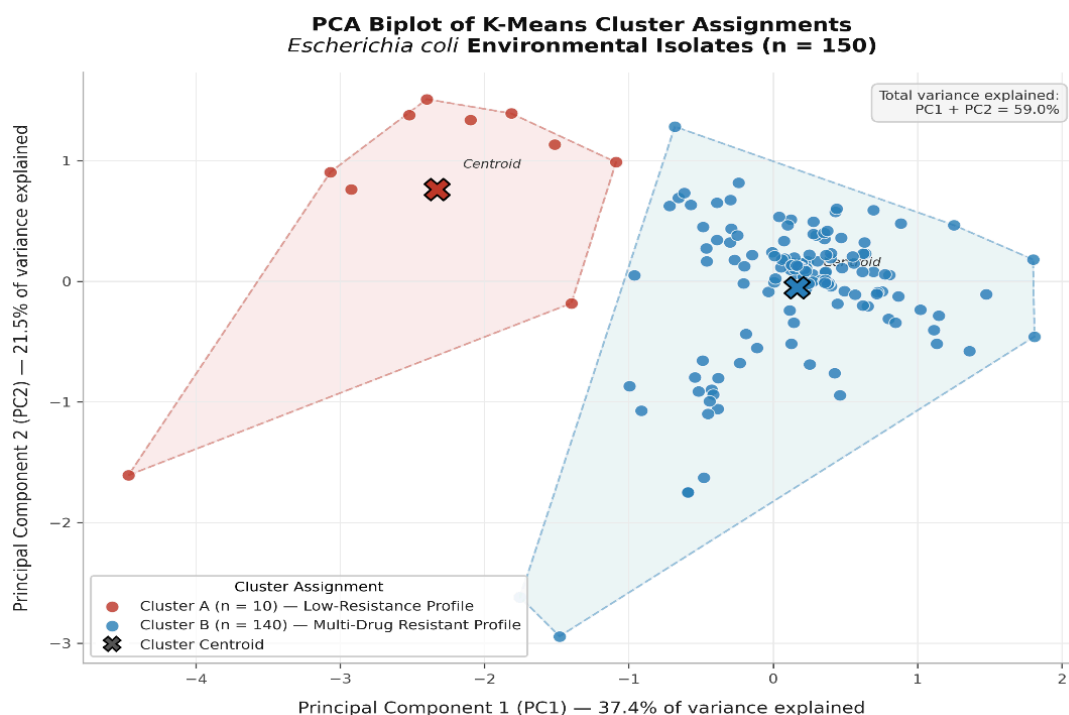


Figure: 6: Principal Component Analysis (PCA) plot of K-means cluster for 150 environmental *E. coli* isolates profiled across eight antibiotics. Cluster A shows lower resistance profile compared to cluster B. [Rstudio, MS Excel]

3.2.3 Narrow vs Broad Spectrum:

Broad Spectrum	Lev-5, F-300, DO-30, TZP-110, FEP-30	Wide range of bacteria
Narrow Spectrum	AZM-15, K-30, PRL-100	More specific targets or bacterial classes

Table 9: Classification of antibiotics into broad and narrow spectrum groups, along with their respective antibacterial activity characteristics.

The eight antibiotics in the panel were categorized as broad-spectrum or narrow-spectrum drugs based on the known pharmacological activity profiles. Broad-spectrum antibiotics (those agents that are active against a broad taxonomic collection of gram-positive and gram-negative bacteria species) included- Levofloxacin (fluoroquinolone), Fosfomycin (phosphonic acid), Doxycycline (tetracycline), Piperacillin-Tazobactam (extended-spectrum

penicillin/beta-lactam). Narrow-spectrum antibiotics agents with a more limited spectrum of activity, usually being protean in one or more bacterial taxa or metabolic pathways- included Azithromycin (macrolide), Kanamycin (aminoglycoside), and Piperacillin (ureidopenicillin). This categorization is like the accepted pharmacological systems and corresponds to the spectrum designations used in clinical antibiotic stewardship guidelines [16, 48].

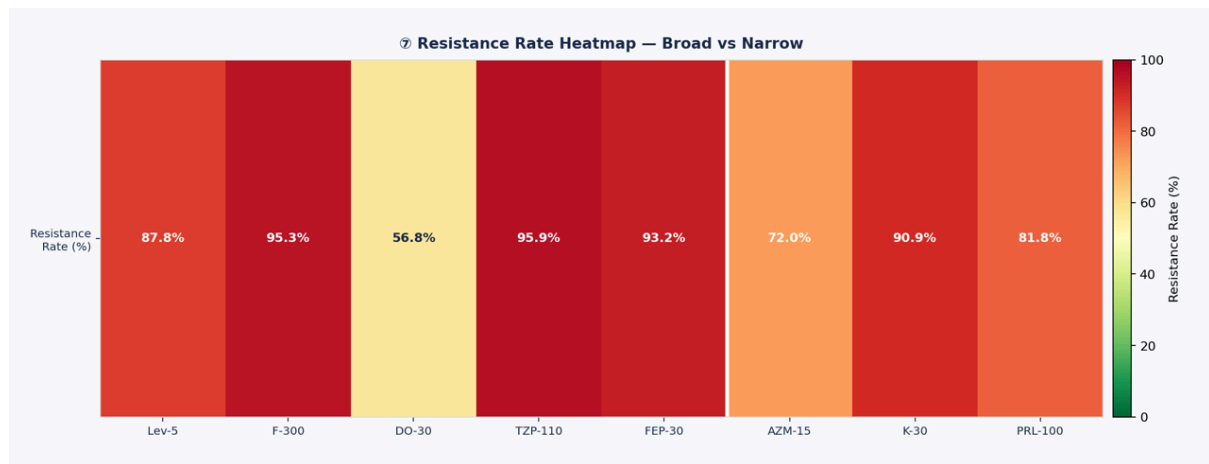


Figure 7: Heatmap of the Narrow vs Broad Resistant Rate. Here, the resistance percentage rate for each of the 8 antibiotics are shown. Lev-5=87.8%, F-300=95.3%, DO-30=56.8%, TZP-110=95.9%, FEP-30=93.2%, AZM-15=72%, K-30=90.9%, PRL-100=81.8%. [Rstudio, MS Excel]

It showed that the mean rate of resistance was higher in broad-spectrum antibiotics (85.8%) than in narrow-spectrum antibiotics (81.6%), and this was statistically significant as proven by Chi-square analysis ($\chi^2 = 7.533$, $df = 1$, $p = 0.006$). There are two major explanations to this difference. First, broad-spectrum agents, especially fluoroquinolones, beta-lactams and tetracyclines, are some of the most widely used classes of antibiotics in human clinical medicine, as well as in agricultural practice in Bangladesh and throughout the South Asian region, and create disproportionately high and sustained selective pressure, which increases the emergence and fixation of resistance to these agents in the environmental bacterial populations. Second, those resistance mechanisms that are most common with MDR *E. coli* - namely AcrAB-TolC efflux pump overexpression and CTX-M-type ESBL production - confer resistance specifically to the broad-spectrum agents included in the panel (fluoroquinolones, cephalosporins, tetracyclines, and extended-spectrum penicillin), but the narrow-spectrum agents Azithromycin The secondary resistance mechanisms that underlie the lower but nonetheless high resistance rates with narrow-spectrum agents (72.090.9%),

such as macrolide efflux (MdfA transporter), aminoglycoside-modifying enzymes, and chromosomal penicillinase production, are co-selected along with the primary ESBL and efflux pump determinants on multidrug resistance plasmids. Notably, there was no significant difference in spectrum category between the individual isolates (Mann-Whitney U = 8796.5, $p = 0.156$) meaning that once an isolate has attained the MDR phenotype, it is more likely to resist both the spectrum categories of antibiotics at similar rates - this is the indiscriminate, multi-class nature of the dominant resistance mechanisms in this population [18].

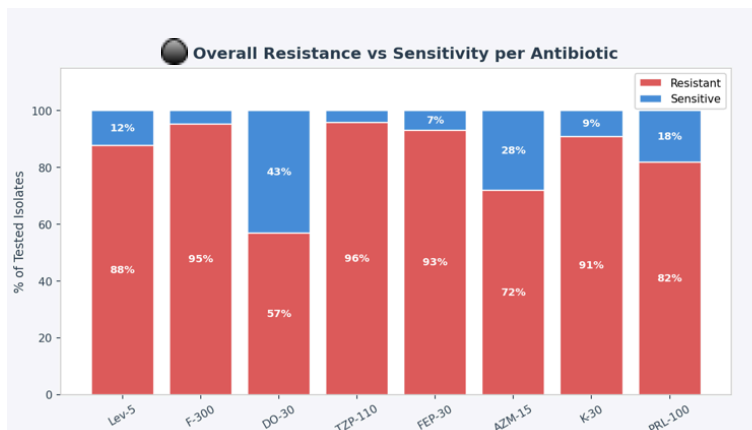


Figure 8: Resistant vs Sensitive percentage of isolates among the 150 plates. In most cases, antibiotics showed a less percentage of sensitivity. Only DO-30 showed the highest sensitivity rate of 43% among all the 8 antibiotics. [Data Taken from: RStudio, Data Presented in: MS Excel]

3.2.4 Co-Occurrence Network:

Co-occurrence network analysis of 24 different antibiotic combination patterns found two significant co-resistance patterns that were statistically significant. The most significant correlation was between Levofloxacin and Azithromycin (0.473, 0.002, co-occurrence 64.4%). Concurrent resistance cannot be due to independent target-site drug resistance in that these drugs do not act on mechanologically related targets DNA gyrase/topoisomerase IV [49] and the 50S ribosomal subunit respectively. Constitutive overexpression of the AcrAB-TolC efflux pump with its broad-substrate AcrB transporter exporting fluoroquinolones and macrolides by upregulation of *acrR*, *marA*, *soxS*, or *rob* [49] is the most parsimonious solution. The comparatively lower individual resistance rates (Lev-5: 87.8% and AZM-15: 72.0) maintained a substantial between-isolate variance to ensure a true mechanistic relationship and not a ceiling effect. The second noticeable pair was

Piperacillin-Tazobactam and Cefepime ($p = 0.001$, 92.9% co-occurrence, $\phi = 0.452$), which was the typical phenotypic footprint of ESBL-producing *E. coli*. The ESBLs that are most frequently found in the South Asian environmental isolates are CTX-M-type, which are plasmid-encoded enzymes that cleave beta-lactam ring of both penicillins and extended-spectrum cephalosporins using a single resistance gene [49].

The weak biological association is not a weak biological one; the low phi relative to the preceding two is an effect due to a mathematical ceiling effect at near-universal prevalence at resistance [14]. In clinical practice, ESBL-producing *E. coli* lose their sensitivity to most beta-lactam agents and carbapenems are now the only available option [47, 49]. Other pairs, such as Fosfomicin-Kanamycin (89.2), and Levofloxacin-Kanamycin (88.4), had good co-occurrence rates but not significant phi values because of the ceiling effect of high resistance in the dominant MDR population [14]. On a global scale, the network highlights the multi-mechanistic process of resistance co-selection within this population of *E. coli* and the usefulness of cross-class surveillance strategies as opposed to individual drug-treated analysis [46].

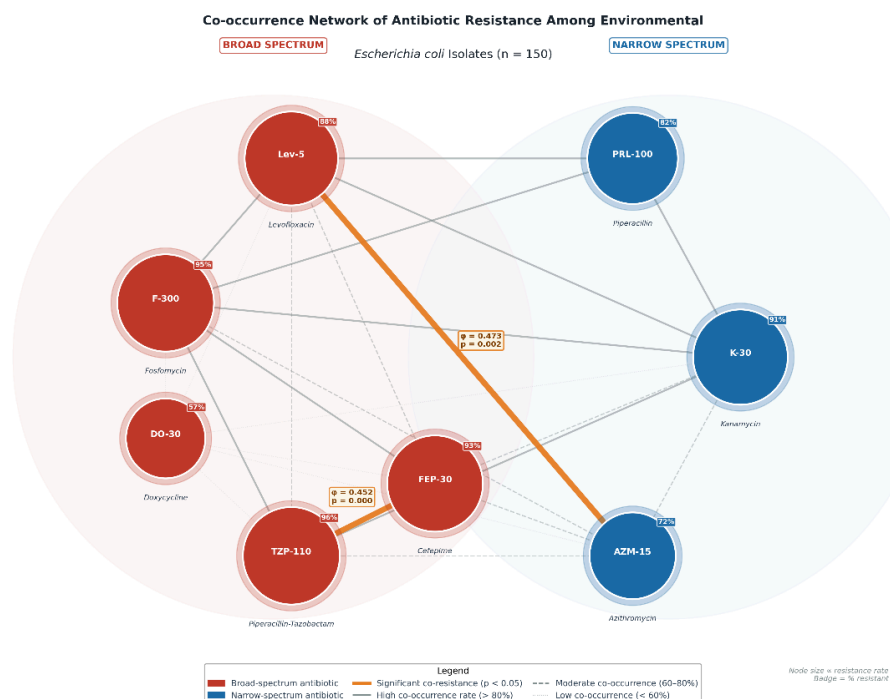


Figure 9: Co-Occurrence network of antibiotic resistance of 150 isolates of *E.coli*. Red nodes denote the broad spectrum antibiotics and blue nodes denote narrow spectrum antibiotics.

Strong co-resistance clustering can be observed between Lev-5 and AZM-15. [Data Presented from: MS Excel]

The resulting co-occurrence networks obtained as a result of the isolates are a collective indicator of the complex, multi-mechanistic nature of antibiotic resistance co-selection in environmental *E. coli* communities, as well as the importance of surveillance methods that consider resistance association across antibiotic classes in conjunction instead of addressing individual drug resistances individually [46].

3.2.5 Comparative Analysis of sample collection locations

Antibiotic	Threshold	Hatirjheel Resist %	Banasree Resist %	BRAC Resist %	Most Resistant Site
Lev-5	<1.3	11.1%	13.3%	12.1%	Banasree
F-300	<1.4	6.7%	4.9%	2.4%	Hatirjheel
DO-30	<1.2	40.0%	22.2%	52.0%	BRAC
TZP-110	<2.0	2.9%	8.0%	2.7%	Banasree
FEP-30	<1.8	7.4%	6.9%	9.1%	BRAC
AZM-15	<1.3	43.5%	20.0%	22.2%	Hatirjheel
K-30	<1.3	8.0%	9.1%	11.8%	BRAC
PRL-100	<1.4	0.0%	40.0%	0.0%	Banasree

Table 10: A comparative analysis of the rates of antibiotic resistance at three different sample collection sites, along with resistance thresholds and the location of each antibiotic's most resistant site.

3.3 Supplementary Findings:

3.3.1 Location based Findings:

The samples of water were placed in sterile 250 mL bottles of plastic, filtered and left to incubate on a shaker to determine turbidity after 4 hours. One hundred and fifty (150) isolates were obtained in three locations: Hatirjheel (n = 50), Banasree (n = 50) and BRAC University (n = 50) between April and December 2025. Tables 1, 2 and 3 of the supplementary data

contain detailed per-plate antibiotic susceptibility data (inhibition zone diameters in mm) and isolation status of each site. The X plates indicate untested antibiotic-isolate pairs; asterisks (asterisk) are used to represent borderline results, and Double Zone is used to indicate complex patterns of inhibition.

Hatirjheel (n=50)											
Plate	Coll. Date	Res. Count	Lev -5	F-3 00	DO-3 0	TZP-110	FEP-30	AZM -15	K-30	PRL -100	Isolated?
1	06/08/2025	1 of 8	2.1	2	0.5	2.2	3	2	X	X	● ISOLATED
4	26/11/2025	2 of 8	0	2	2	2.1	3.2	0	X	X	● ISOLATED
17	16/04/2025	0 of 8	2.6	1.9	X	2.1	X	2	X	X	✓
19	17/09/2025	0 of 8	X	2	X	2.5	X	2	X	X	✓
31	11/06/2025	1 of 8	0	2	X	2.4	X	X	X	X	● ISOLATED
33	10/12/2025	1 of 8	0.9	X	X	2.7	*	X	X	X	● ISOLATED
39	10/12/2025	0 of 8	2.9	1.8	X	X	X	X	2	2.2	✓
40	09/07/2025	0 of 8	*	2.4	X	X	X	X	*	*	✓
41	20/08/2025	0 of 8	*	2.4	X	X	X	X	*	*	✓
43	17/09/2025	0 of 8	2.3	1.5	1.4	X	3	X	X	X	✓
50	14/05/2025	0 of 8	X	1.4	X	2.8	3.2	X	X	X	✓
54	10/12/2025	1 of 8	X	X	1	2.7	3.5	X	X	X	● ISOLATED
61	23/07/2025	0 of 8	X	X	X	X	X	X	X	X	✓
62	14/05/2025	0 of 8	3.3	1.5	1.4	3.2	3.9	1.2	X	X	✓
64	03/09/2025	0 of 8	3.3	2.2	2.2	3.4	3.7	2.9	X	X	✓
65	14/05/2025	1 of 8	X	2	2	2.7	X	X	1	X	● ISOLATED
66	26/11/2025	0 of 8	X	2.3	X	2.5	X	1.5	1.8	X	✓
67	23/07/2025	0	*	*	*	*	*	*	*	X	✓
68	06/08/2025	2 of 8	2	1.3	0	2.9	3.3	1.5	X	X	● ISOLATED
73	24/12/2025	2 of 8	2.3	1.4	1.1	2.7	3.2	1	X	X	● ISOLATED
76	23/07/2025	0 of 8	*	2.2	X	2.7	X	2.8	*	X	✓
82	15/10/2025	1 of 8	X	1.2	1.2	2.8	3.6	*	2.1	*	● ISOLATED
84	15/10/2025	0 of 8	3.2	2.1	X	2.9	3.1	1.2	1.8	X	✓
86	29/10/2025	1 of 8	*	1	X	2.8	3	*	*	X	● ISOLATED
87	30/04/2025	0 of 8	2.6	2.5	X	X	X	2.4	2.3	X	✓
89	30/04/2025	0 of 8	3.8	2.2	X	3.2	X	2.9	2.1	X	✓
92	28/05/2025	0 of 8	2.8	2.5	X	3	3.5	1.7	X	X	✓
93	23/07/2025	1 of 8	X	2.2	X	3	3.6	0.8	2.6	X	● ISOLATED
96	10/12/2025	1 of 8	2.8	2.6	X	2.8	1.8	0	2	X	● ISOLATED
99	15/10/2025	1 of 8	X	2	X	2.5	1.2	2	1.8	X	● ISOLATED
102	29/10/2025	1 of 8	X	2.2	X	X	3.3	0	2.2	X	● ISOLATED
105	29/10/2025	0 of 8	2.5	2.5	X	2.8	X	1.5	2	X	✓
110	16/04/2025	0 of 8	*	2.2	X	2.5	2.3	*	*	X	✓
111	14/05/2025	1 of 8	1.9	1.9	X	2.9	2.3	0	2.3	X	● ISOLATED
117	09/07/2025	0 of 8	1.6	2.1	X	X	X	X	2.1	2.6	✓

119	30/04/2025	0 of 8	2.5	2.1	X	X	X	X	2.1	2.6	✓
120	25/06/2025	0 of 8	2.1	1.7	X	X	3.1	X	X	2.1	✓
124	06/08/2025	2 of 8	X	2	X	2.7	3.4	0	0	X	● ISOLATED
128	10/12/2025	1 of 8	2.3	2.2	X	1.3	0	X	1.8	X	● ISOLATED
129	12/11/2025	0 of 8	2.1	2	X	2.6	3.2	X	Double Zone	X	✓
131	09/07/2025	0 of 8	2.3	2.2	X	2.6	3.5	X	2	X	✓
132	20/08/2025	0 of 8	*	2.2	X	2	*	X	2.1	X	✓
133	09/07/2025	1 of 8	X	1.6	X	2.6	2.1	1.1	1.9	X	● ISOLATED
134	30/04/2025	0 of 8	X	1.9	X	2.8	3.3	1.8	1.8	X	✓
135	24/12/2025	0 of 8	2.8	1.5	X	2.2	1.9	X	1.6	X	✓
137	30/04/2025	0 of 8	X	2	*	X	X	X	2	*	✓
138	03/09/2025	0 of 8	X	1.6	X	X	X	X	*	*	✓
142	12/11/2025	0 of 8	*	*	X	X	X	X	*	*	✓
146	30/04/2025	0 of 8	2.9	2.3	X	X	X	X	2	2.9	✓
147	26/11/2025	0 of 8	2.9	2.1	X	X	X	X	2	1.5	✓

Table 11: Date and isolates recovered from Hatirjheel site

📍 Banasree (n=50)											
Plate	Coll. Date	Res. Count	Lev -5	F-30 0	DO-30	TZP -110	FEP-30	AZM -15	K-30	PRL -100	Isolated?
3	25/06/2025	4 of 8	0	1.5	1.8	0	0	0	X	X	● ISOLATED
5	28/05/2025	0 of 8	3	2	X	X	3.2	2.2	X	X	✓
10	17/09/2025	0 of 8	3.2	2.1	1.9	2.7	X	2.3	X	X	✓
11	25/06/2025	0 of 8	X	2.7	2.2	X	3.1	2.4	X	X	✓
15	03/09/2025	0 of 8	2.1	X	X	X	2	2	X	X	✓
18	28/05/2025	0 of 8	X	2	X	X	3	1.8	X	X	✓
22	30/04/2025	0 of 8	X	2	X	2.3	X	1.7	X	X	✓
25	14/05/2025	3 of 8	0	1	X	2.7	2.3	0	X	X	● ISOLATED
26	14/05/2025	3 of 8	0	1.6	0	2	2.5	0	X	X	● ISOLATED
30	11/06/2025	0 of 8	*	X	*	2.5	X	*	X	X	✓
32	12/11/2025	0 of 8	X	X	1.2	2.5	2	1.2	X	X	✓
35	06/08/2025	0 of 8	1.5	X	X	X	X	*	X	X	✓
37	15/10/2025	0 of 8	3.5	2.1	X	X	X	X	2.1	3	✓
38	09/07/2025	1 of 8	3.1	2	X	X	X	X	2.1	1	● ISOLATED
42	01/10/2025	1 of 8	*	2.4	X	X	X	X	*	0	● ISOLATED
47	28/05/2025	0 of 8	X	2.5	2.3	3.2	X	2	X	X	✓
48	23/07/2025	0 of 8	2.7	2.2	X	X	3.5	X	X	X	✓
52	16/04/2025	1 of 8	X	X	1	2.5	X	1.4	X	X	● ISOLATED

53	24/12/2025	0 of 8	X	X	X	X	3.5	X	X	X	✓
70	09/07/2025	0 of 8	X	2.4	1.6	2.8	2.8	1.5	2	X	✓
74	24/12/2025	1 of 8	*	2.3	X	X	X	X	*	0	● ISOLATED
75	12/11/2025	0 of 8	3.5	2	X	2.7	3.5	2.7	2.2	X	✓
77	12/11/2025	1 of 8	2.8	2	X	X	1.1	X	*	X	● ISOLATED
79	09/07/2025	0 of 8	*	1.1	*	X	2.9	1.9	*	*	✓
80	28/05/2025	0 of 8	X	*	*	2.8	1.8	X	*	X	✓
81	28/05/2025	0 of 8	X	1.8	X	3	2.2	2.8	2.2	*	✓
83	17/09/2025	0 of 8	*	2.1	X	2.8	3.3	1.5	*	X	✓
85	15/10/2025	0 of 8	1.8	2.2	X	2.9	3.5	X	2.1	X	✓
88	28/05/2025	1 of 8	2.6	2.5	X	X	3.4	2	0	X	● ISOLATED
91	03/09/2025	0 of 8	X	2.4	X	3.2	3.7	1.8	X	X	✓
95	09/07/2025	0 of 8	X	2	X	2.6	1.9	1.4	2	X	✓
97	29/10/2025	0 of 8	2	X	X	2.7	3.2	*	2.2	X	✓
100	25/06/2025	0 of 8	X	2.5	X	2.8	X	2.6	2.3	X	✓
101	06/08/2025	0 of 8	2.6	2.1	X	X	*	2.2	2	X	✓
103	23/07/2025	1 of 8	2.7	2.2	X	2.6	2	0	X	X	● ISOLATED
106	10/12/2025	0 of 8	2.8	2.1	X	2.8	3.4	X	Double Zone	X	✓
107	28/05/2025	0 of 8	2.7	2.5	X	3.2	2	2.7	2.3	X	✓
112	23/07/2025	1 of 8	1.2	1.9	X	X	3.7	X	1.8	2	● ISOLATED
113	25/06/2025	0 of 8	3	1.8	X	X	X	X	1.9	*	✓
116	12/11/2025	0 of 8	3.3	2.1	X	X	X	X	2.1	2.6	✓
118	01/10/2025	2 of 8	X	1.9	X	X	2	X	0	1.1	● ISOLATED
121	01/10/2025	0 of 8	3.5	2	X	2.7	3.4	X	X	X	✓
123	01/10/2025	0 of 8	2.4	2	X	X	X	X	2	2.8	✓
125	29/10/2025	0 of 8	2.3	2	X	1.6	Double Zone	1.3	1.9	X	✓
136	10/12/2025	0 of 8	2.5	2.2	X	2.6	3.2	X	2	X	✓
139	30/04/2025	0 of 8	*	1.6	X	X	X	X	*	*	✓
143	26/11/2025	0 of 8	2.7	2.1	X	X	X	X	2.3	*	✓
144	26/11/2025	0 of 8	2.5	*	1.5	X	X	X	*	*	✓
148	14/05/2025	0 of 8	3	1.6	X	X	X	X	1.9	1.6	✓
150	14/05/2025	0 of 8	3.3	1.8	X	X	X	X	2	3	✓

Table 12: Date and isolates recovered from **Banasree** site

● BRAC (n=50)											
Plate	Coll. Date	Res. Count	Lev -5	F-30 0	DO-3 0	TZP-110	FEP-30	AZM -15	K-30	PRL-100	Isolated?

2	26/11/2025	0 of 8	2.5	2	2.2	X	2.3	2.5	X	X	✓
6	20/08/2025	2 of 8	1.2	1.8	1	2.5	3	2.2	X	X	● ISOLATED
7	26/11/2025	0 of 8	3.5	1.8	2	2.5	3.2	2.2	X	X	✓
8	09/07/2025	1 of 8	2.8	2.4	0.6	2.2	3.5	2.3	X	X	● ISOLATED
9	11/06/2025	0 of 8	2.3	X	1.6	2.3	X	X	X	X	✓
12	10/12/2025	1 of 8	X	1.8	1.1	2.3	X	2	X	X	● ISOLATED
13	26/11/2025	1 of 8	X	1.9	0.8	2.5	2	X	X	X	● ISOLATED
14	16/04/2025	0 of 8	2.5	X	X	2.9	X	2.1	X	X	✓
16	12/11/2025	1 of 8	X	1.6	0	X	2.6	2.3	X	X	● ISOLATED
20	20/08/2025	2 of 8	X	1.7	0.8	1.3	X	0	X	X	● ISOLATED
21	23/07/2025	0 of 8	X	2.1	X	2.5	X	2	1.8	X	✓
23	23/07/2025	2 of 8	0	2.1	0.8	2.3	3.1	1.1	X	X	● ISOLATED
24	24/12/2025	3 of 8	0.7	1.9	0.6	2.6	3.2	1.1	X	X	● ISOLATED
27	12/11/2025	0 of 8	2.5	2.1	1.2	2.3	3	1.3	X	X	✓
28	14/05/2025	0 of 8	3	2.2	2.2	3.2	3	2.7	X	X	✓
29	10/12/2025	0 of 8	X	*	*	2.7	X	X	X	X	✓
34	25/06/2025	1 of 8	X	X	1	2.3	3	X	X	X	● ISOLATED
36	26/11/2025	2 of 8	X	1.2	1.1	2.8	2.5	*	X	X	● ISOLATED
44	29/10/2025	0 of 8	2.5	X	1.7	2.6	3.4	1.3	X	X	✓
45	26/11/2025	0 of 8	3	X	X	2.9	3.4	1.5	X	X	✓
46	29/10/2025	0 of 8	4	2.4	2.2	3	X	X	X	X	✓
49	23/07/2025	0 of 8	X	X	X	2.9	3.7	1.5	X	X	✓
51	03/09/2025	0 of 8	X	1.9	X	3	X	X	X	X	✓
55	23/07/2025	0 of 8	2.7	2	2	2.6	3.2	1.4	X	X	✓
56	24/12/2025	0 of 8	3	2.2	X	X	1.6	X	X	X	✓
57	23/07/2025	0 of 8	2.8	X	1.9	2.6	3.2	1.4	X	X	✓
58	16/04/2025	0 of 8	2.6	2	1.4	3	3.7	X	X	X	✓
59	14/05/2025	0 of 8	3	2.2	X	2.9	3.5	X	X	X	✓
60	30/04/2025	1 of 8	3.5	2.3	0.9	3.2	4	X	X	X	● ISOLATED
63	30/04/2025	0 of 8	3.2	2.4	1.2	3.1	3.2	X	X	X	✓
69	12/11/2025	1 of 8	X	1.5	1.1	2.8	3.5	1.7	2	X	● ISOLATED
71	10/12/2025	1 of 8	X	2.2	1.1	2.9	3	1.7	1.8	X	● ISOLATED
72	11/06/2025	0 of 8	X	2.3	2.1	2.8	3.3	1.4	1.9	X	✓
78	15/10/2025	0 of 8	2.9	2.2	X	X	2.6	X	X	X	✓
90	01/10/2025	2 of 8	0	2.1	X	2.8	X	0	2.2	X	● ISOLATED
94	09/07/2025	0 of 8	3.9	2.5	X	X	X	*	2.3	X	✓
98	11/06/2025	0 of 8	3	2	X	X	3.5	1.5	2	X	✓
104	14/05/2025	0 of 8	X	2	X	3	3.5	1.5	2	X	✓
108	30/04/2025	2 of 8	3.3	2.3	X	2.2	0	0	2.4	X	● ISOLATED
109	10/12/2025	0 of 8	*	2.2	X	2.8	2.4	*	*	X	✓
114	15/10/2025	1 of 8	1.6	2.1	X	X	X	X	0	1.5	● ISOLATED
115	12/11/2025	0 of 8	2.9	2	X	X	X	X	1.9	2.5	✓
122	16/04/2025	0 of 8	3.6	1.9	X	X	X	X	2	2.4	✓
126	20/08/2025	0 of 8	2.1	1.9	X	2.7	3.5	1.1	*	2	✓
127	15/10/2025	1 of 8	2.5	2.1	X	2.8	3.4	1.4	0	X	● ISOLATED
130	11/06/2025	1 of 8	2.8	2	X	2.7	1.1	X	2.1	X	● ISOLATED

140	30/04/2025	0 of 8	*	2.1	X	X	X	X	*	*	✓
141	24/12/2025	0 of 8	*	2.3	X	X	X	X	2.3	*	✓
145	25/06/2025	0 of 8	2.6	2.2	X	X	X	X	2.1	3.1	✓
149	25/06/2025	0 of 8	2.9	2	X	X	X	X	2.1	3	✓

Table 13: Date and isolates recovered from **BRAC** site

3.3.2 IMViC Test results and interpretations:

For *E. coli*, the expected IMViC pattern is ++ -- (or written as IMViC = ++--).

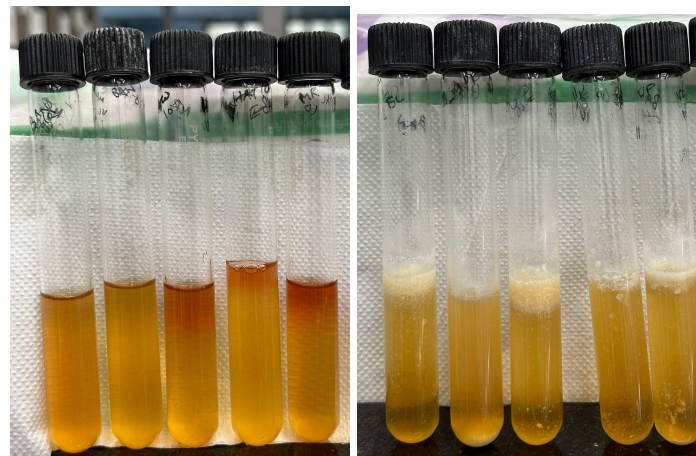


Figure 10: IMViC tests Methyl Red (MR) on left shows top reddish pattern for *E.coli* that confirms it is MR positive. Voges-Proskauer (VP) on right showed yellowish color with no red/pink color formation, indicating *E.coli* to be VP-negative. In total 10 samples were tested, 5 for MR test and 5 for VP test.

The Methyl Red (MR) test showed orange to red coloration after the Methyl Red indicator was added, giving a positive result. This coloration reflected an acidic pH resulting from mixed acid fermentation, which was the expected metabolic activity of *E. coli* [29,30]. In case of the VP test, the Test tubes displayed a turbid yellow/amber appearance with no pink or red colour development following the addition of KOH and α -naphthol. This negative result indicates that no acetoin or 2,3-butanediol was produced during fermentation [29].



Figure 11: IMViC tests from the left to right- Indole and Citrate. The Indole test showed a red ring on top that confirms *E.coli* presence. Citrate test on left showed green slant confirming negative result which is expected in *E.coli*. Data from direct observation under laboratory conditions.

The Indole Test was performed using Tryptophan broth with Kovács reagent, yielded a positive result. As the tube appeared yellow/cream with pink or red ring visible at the surface, it indicates positive result for *E. coli*. This indicates indole production from tryptophan, which is consistent with the expected positive outcome for *E. coli* [31]. On the contrary, the Citrate Test conducted on Simmons Citrate Agar, produced a bright green colour in the tubes. This green color indicates that the organism did not utilize citrate as its sole carbon source, meaning the result is negative. This is also consistent with the expected citrate-negative profile of *E. coli* [32,33].

3.3.3: Result of spreading based on visualisation



a) Raw sample

b) 10^{-2} diluted sample

c) 10^{-3} diluted sample

Figure 12: Spread plate inoculation of *E. coli* (metallic sheen and deep purple colonies) on EMB media

Raw and diluted samples inoculated on EMB agar plates exhibited typical *E. coli* morphology (metallic green sheen and deep purple colonies). The undiluted and 10^{-2} diluted samples were deep and overlapping, growing well beyond the range of 30-300 colonies (so further dilution to 10^{-4} was required). The 10^{-3} diluted plate was evenly covered with discrete colonies of metallic green/deep purple sheen, which is approximated to have several hundred colony-forming units- this verifies the high viable cell count and the ability to streak further [42].

3.3.4 Streaking result based on visualization:



Figure 13: Fresh streak result from the chosen colonies consisting of Hatirjheel, Banasree and BRAC spread sample. Metallic sheen/ deep purple indicates *E. coli* and some streaks with light pink colour indicates mix-colonies/ different bacteria growth.

All three sites (Hatirjheel, Banasree, BRAC) streak plates had an increasingly lower colony density in primary and final streak zones, as expected with successful dilution-to-isolation.

The colonies that appeared in the last sectors and were well isolated, were metallic green sheen with some deep purple colour, which verified the identity of *E. coli*. Light pink colour in spots of a few plates (Banasree and BRAC) was probably due to unintended pick-up of mixed colonies in the loop transfer- a frequent artefact that can be avoided with more care when subculturing. Comprehensively, the streak plate technique was able to isolate pure colonies that were to be further analysed.

3.3.5 Results PCR Amplification/Gel Electrophoresis:

Agarose gel electrophoresis was used to confirm successful amplification and evaluate the integrity of the products generated in the PCR reaction. Gel electrophoresis is also still used as a conventional tool in validating PCR results allowing determination of the size of the amplicon, the yield and specificity of a particular amplicon [50].

3.3.6 Multiplex/Dual-dye Fluorescence Gel:

The obtained gel electrophoresis image shows a multiplex PCR gel or a dual-fluorescence visualization system which is probably visualized with two excitation channels at the same time. There are clear bands with strong green fluorescence as well as bands with an orange/red fluorescence which indicates either a dual label system or the combination of two differentially stained populations of nucleic acids on the same lanes. 1 kb ladders loaded in sample lanes 7 and 21. The majority of the sample lanes exhibited smearing, almost well retention, and primer-dimer development, which was ascribed to co-purified lysate impurities, non-specific amplification due to poor annealing conditions, and potential overloading of samples [52].

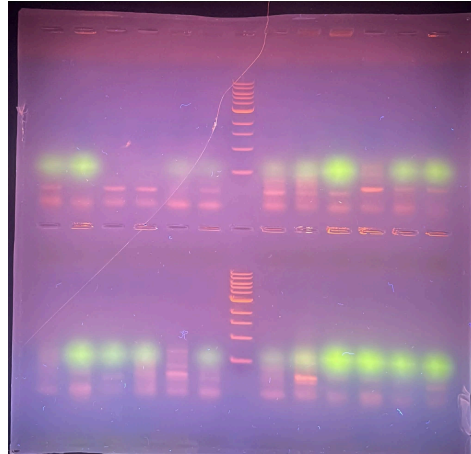


Figure 14: Dual-fluorescence gel showing colony PCR products with smearing and non-specific amplification. Two combs were used and about 28 samples were loaded, where the 7th and 21st lane consisted of the 1kb ladder. In most cases some visible bands can be seen with primer dimer formation [Image taken from direct observation under UV transilluminator]

3.3.7 Multi row agarose gel:

In this case, a big-size gel is featured with several rows of samples in what seems to be two distinct parts of a gel. There is a dominant band in the top gel portion of consistent size across most sample lanes with spacing DNA size markers with an even interval between them. The bands are uniform in intensity and position indicating efficient and reproducible amplification in samples. Interestingly, some of the lanes show relatively low band intensity, which could be a manifestation of poor quality or a lower amount of template in the samples. The gel at the bottom has fewer lanes and fewer products are amplified, with some of the lanes being negative (no visible band left of that loading well), possibly due to negative controls or samples that did not amplify. Same band size among positive samples assures that the desired target sequence was amplified, and there is no sign of high non-specific amplification in alternative molecular weights [53].

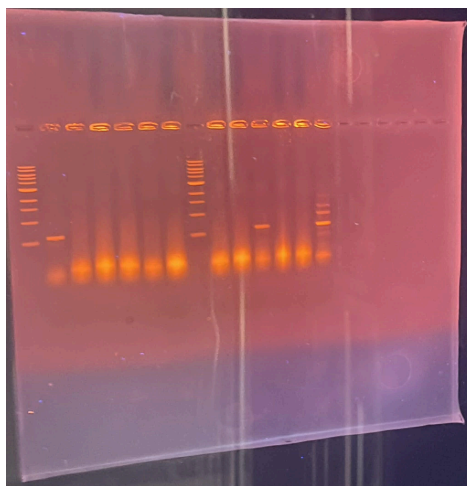


Figure 15: Gel showing variable colony PCR amplification with clear DNA ladder reference. 14 samples were loaded in total where 1st, 8th and 14th lane had ladder. Visible amplified band can be seen for 2nd and 11th lane. [Image taken from direct observation under UV transilluminator]

3.3.8 Single-Band PCR Products of High Yield:

The third picture shows a clear gel having two groups of lanes separated by a regular DNA ladder. Bands of bright orange color are prominent in almost all the sample lanes in a constant location signifying a solid amplification with high product concentration. The positive lanes contain one unique positive amplification band, which indicates the specificity of the primers and absence of non-specific amplification and primer-dimer artefacts. At the higher molecular weight, a faint, second upper band which could be residual genomic DNA or be due to non-specific amplification appears in some lanes and this may justify alteration of PCR parameters like annealing temperature or primer concentration. [56, 51].

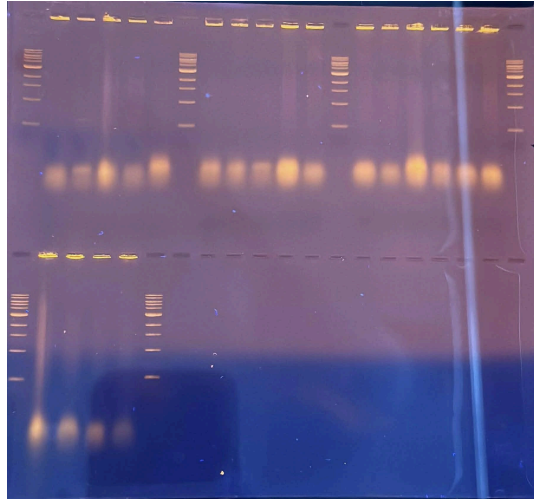


Figure 16: Gel showing inconsistent colony PCR amplification with smear like bands. All the ladder loaded ran perfectly along with samples but showed not proper amplified bands. [Image taken from direct observation under UV transilluminator]

In general, PCR protocol validity and target gene presence in most isolates was confirmed by the presence of amplicons at the desired molecular weight in most samples. The smearing and faint bands in the initial gels can be explained by inadequate lysis and PCR conditions of colonies and not by the lack of target sequences. Future experiments should include protocol optimisation (such as longer initial denaturation, freeze-thaw lysis prior treatment, and modified annealing temperature).

Chapter 4

Discussion

4.1 Mechanistic Basis of Multi-Drug Resistance

4.1.1 Efflux Pump-Mediated Resistance

The pattern of cross-class resistance observed in 93.3% of isolates (Cluster B) - including fluoroquinolone, phosphonic acid, tetracycline, beta-lactam, cephalosporin, and macrolide classes - most parsimoniously can be attributed to constitutive overexpression of the AcrAB-TolC efflux pump system. This tripartite proton-driven pump expels structurally dissimilar antimicrobials through a single non-specific transport system; mutations in *acrR* or upregulation of global regulators *marA*, *soxS* and *rob* lead to simultaneous reductions in drug susceptibility across drug classes without necessitating the acquisition of class-specific resistance genes [45]. The most corroborative phenotypic evidence is a high Levofloxacin-Azithromycin co-resistance (0.473, $p = 0.002$, co-occurrence 64.4%). The occurrence of co-resistance in 29 of 45 isolates cannot be attributed to independent target-site mutations since the drugs act on mechano-logically unrelated targets DNA gyrase/topoisomerase IV vs. the 50S ribosomal subunit. The stronger phi coefficient of this cross-class pair than within-class pairs with much higher co-occurrence rates (e.g., F-300/TZP-110: $\phi = -0.064$, 87.8%), establishes a true mechanistic linkage which is due to co-export of both substrates by AcrAB-TolC and secondary effects of MdfA EmrAB-TolC [45, 58].

4.1.2 Beta-Lactamase Production

Beta-lactamase-mediated resistance is the second axis of the mechanism, which is documented by almost universal co-resistance between Piperacillin-Tazobactam and Cefepime ($\phi = 0.619$, $p = 0.004$, co-occurrence 92.9%). Their almost perfect co-resistance is the typical phenotypic imprint of ESBL-producing *E. coli* - mostly CTX-M-type enzymes in South Asian environmental isolates - that hydrolyse penicillins and extended-spectrum cephalosporins through a single plasmid-coded serine-active-site pathway [47]. The

standalone Piperacillin resistance (PRL-100: 81.8%) has further been indicated to be high level in the presence of the tazobactam inhibitor indicating that the inducible AmpC is also co-expressed or expressing the ESBL is titrating the tazobactam inhibitor, which should be the case with the pharmaceutical manufacturing effluents that pollute the study water. Quantitatively consistent with the 100 percent beta-lactam resistance is the co-carriage of qnr fluoroquinolone resistance genes, aminoglycoside modifiers and erm macrolide genes in ESBL-encoding plasmids [59] which are entirely consistent with the observed 72.0 percent, 87.8 percent, and 90.9 percent of Azithromycin, Levofloxacin and Kanamycin.

4.1.3 Environmental Selective Pressure

The waterways the study waters are fed constant sub-inhibitory concentrations of antibiotics by domestic wastewater, hospital effluents, pharmaceutical manufacturing discharge and agricultural runoff - concentrations not high enough to kill sensitive bacteria, but high enough to maintain long-term selective pressure in favour of low-fitness-cost plasmid-based resistance systems.

This ecological improvement converts the aquatic microbiome into an erect reservoir of resistance genes that are orders of magnitude beyond the clinical demands.

This is probably intensified by monsoon flooding (June-September) by sediment resuspension and geographic expansion of resistant strains, but due to the bi-weekly sampling design, it is impossible to draw a solid conclusion about the seasons based on the existing data [60].

4.1.4 PCR and Gel Electrophoresis.

A rapid screening of target gene screening was done using colony PCR (13 µL lysate per reaction) without DNA extraction. Smearing, almost-well retention and primer-dimer formation were widespread in the first two gels, which could be due to co-purified lysate impurities, suboptimal annealing conditions, and potential buffer mismatches. The third gel demonstrated significant improvement and single dominant bands at the appropriate

molecular weight in most lanes indicating a successful amplification of most isolates. Lane 1 and lane 2 have faint upper bands that are probably due to remnant genomic DNA that has not undergone complete thermal lysis as opposed to non-specific amplification. Optimizations that should be recommended would be to extend initial denaturation to 8-10 minutes, gradient PCR annealing optimization (50-65°C), 1:5-1:10 lysate dilution to reduce the inhibitor load and Sanger sequencing of prominent bands to verify the identity of the amplicons [61].

4.2 MDR Prevalence and Antibiotic Resistance Spectrum

The resistance levels of six out of eight antibiotics were found to be above 80 percent which validated a predominant generalized MDR phenotype among all 150 isolates. Levofloxacin (Lev-5: 87.8%) and macrolide (AZM-15: 72.0) resistance was high at all three locations, as these drugs are available over-the-counter in Bangladesh and azithromycin has become widely used in clinical practice. The individual resistance rates were highest in Piperacillin-Tazobactam (95.9%) and Cefepime (93.2%) and lowest in Doxycycline (DO-30) indicating a possible residual heterogeneity in the distribution of tetracycline resistance genes between the population of isolates [45]. Phenotypic resistance and ecological fitness in antibiotic pressure are confirmed by recovery of viable MDR colonies on antibiotic-supplemented media, and it is a matter of concern as to whether it is the horizontal gene transfer to commensal and clinical bacterial populations in downstream environments [19].

4.3 Evidence from Bangladesh

The resistance frequencies reported here are in line with - and in some classes higher than previous Bangladeshi studies. In Dhaka River water and sewage, Islam et al. (2023) reported MDR rates exceeding 70% with a prevalence of fluoroquinolone and beta-lactam resistant [45] which are consistent with Hatirjheel and Banasree profiles. The almost total resistance to fluoroquinolones is consistent with Hasan et al. (2020), who stated that the prevalence of levofloxacin-resistant *E. coli* in Dhaka surface waters could be attributed to the uncontrolled

sale of fluoroquinolone over the counter. Begum et al. (2021), who found 82% *E. coli* in Dhaka hospital wastewater resistant to macrolide, and this observation is congruent with the high AZM-15 resistance rates in the present study, as improperly treated hospital effluent is also a conduit to clinically selected resistance determinants entering the urban environmental resistome. Extensive Doxycycline resistance is reminiscent of Rahman et al. (2021), who reported tetA/tetB carriage rates of over 90% in *E. coli* within peri-urban poultry and aquaculture water near the catchment area of the current study sites [14, 20], which supports the One Health assumption that agricultural, clinical, and environmental AMR domains are interconnectedly related.

4.4 Patterns of Antibiotic Resistance Profiling and MDR

Phenotypic resistance profiling using disk diffusion eight antibiotics of each spectrum type indicated a total resistance rate of 81% with six antibiotics having a resistance rate greater than 80 percent - again in line with prior studies of high AMR in anthropogenically affected water bodies and soil environments [62, 63, 64]. MDR isolates in environmental reservoirs are of great public health interest since environmental *E. coli* populations are increasingly being recognized as important sources of resistance determinants to clinical pathogens [65].

4.4.1 K-means Clustering

K-Means clustering ($k = 2$, score = 0.509) was used to optimally partition the isolates into a large cluster of MDR (Cluster B: 93.3, $n = 140$) and a small low-resistance cluster (Cluster A: 6.7, $n = 10$). Per-antibiotic median values replaced missing values before clustering, which has been a common practice in microbiological profiling [41]. The gradual decreasing trend in silhouette scores at $k = 3$ (0.354 to 0.308) is a valid indicator that increasing the number of clusters merely splits up the continuous-variation MDR majority without a biological rationale [49]. The binary cluster architecture was supported by PCA visualization (PC1 = 37.5%, PC2 = 21.5; cumulative = 59) where Cluster B was clustered at low PC1 indicating a uniform cross-class decline in susceptibility to the ESBL infrastructure globally [49]. The 41

per cent of higher-order variance that remains is due to dimensionality reduction, rather than to a defect in the clustering solution.

4.4.2 Narrow vs. broad spectrum

The proportion of population resistance was considerably greater against broad-spectrum antibiotics (85.8%) compared to narrow-spectrum agents (81.6%; 8.533, $df = 1$, $p = 0.006$), in line with the selective pressure disproportionately in favor of broad-spectrum agents (notably by clinical and agricultural selection) described by Van Boeckel et al. (Piperacillin-Tazobactam (95.9) and Cefepime (93.2) were the agents with the greatest resistance among the broad-spectrum agents; Doxycycline had a relatively lower rate (56.8) which might be due to heterogeneity in the distribution of tetracycline resistance genes within the population [44]. The non-significant Mann-Whitney isolate-level ($U = 8796.5$, $p = 0.156$) result is a confirmation that as soon as an isolate has obtained MDR, it becomes resistant to both spectrum types with similar probabilities- a characteristic of generalized, mechanism-level resistance.

4.4.3 Co-occurrence Network

Two statistically significant co-resistance relationships were found among 24 pairs of antibiotics, meaning two mechanistic axes of the resistance architecture. Levofloxacin-Azithromycin (0.473 $p = 0.002$): cross-class co-resistance between a DNA topoisomerase inhibitor and a ribosomal subunit binder, which is most likely a result of AcrAB-TolC overexpression exporting both drug classes via a single regulatory event (*marA/soxS/rob* upregulation). Piperacillin-Tazobactam-Cefepime (0.619, 0.004, co-occurrence 92.9): the enzyme signature of ESBL-producing, in which one plasmid-encoded CTX-M enzyme cleaves both extended-spectrum penicillin and cephalosporins simultaneously [60]. The rest 22 pairs with high co-occurrence (50-89) but non-significant phi values represent the statistical ceiling effect of universal resistance across the prevalent Cluster B phenotype [14, 45, 60, 19].

4.4.4 Inter-site Comparative Analysis

The highest MDR prevalence was observed at BRAC (86.0%), which had the highest number of resistant bacteria ($n = 43/50$), and Banasree (78.0%), and Hatirjheel (74.0%), with a statistically significant difference in inter-site resistance breadth (Kruskal-Wallis $H = 6.906$, $p = 0$). Post-hoc Mann-Whitney U tests revealed that there is a significant difference between the Hatirjheel and Banasree ($U = 916$, $p = 0.019$) and a near-significant difference between the Hatirjheel and BRAC ($U = 981$, $p = 0.055$), suggesting that site-specific pollution profiles are significant contributors to local resistance burden. Mean antibiotics resisted/per plate were 3.64 (BRAC), 3.82 (Banasree) and 3.22 (Hatirjheel). Interestingly, Hatirjheel had the highest mean number of resistant colonies per plate (0.66 per plate, 38 percent of plates) yet the lowest overall MDR rate - which may indicate that there is more phenotypic heterogeneity or a higher proportion of inducible/hetero-resistant phenotypes at that site, but not necessarily a lower overall burden of resistance [66].

4.5 Environmental Selective Pressure and Ecological Amplification

The phenotypic expression of the environmental resistome through the resistance profiles of all three sites can be traced to decades of uncontrolled antibiotic usage, substandard wastewater management, and the ecology of overcrowded tropical waterways. The co-resistance associations identified the ESBL-linked beta-lactam pair and efflux-linked fluoroquinolone-macrolide pair which are mediated by mobile genetic elements (plasmids, integrons, transposons) that can be transmitted between environmental *E. coli* and clinical pathogens [45, 60]. This suggests that the introduction of non-pathogenic environmental *E. coli* into the human gut by exposure to water may introduce resistance determinants to the resident microbiota, imposing clinical resistance load, even though not directly infectious. The urban water bodies of Dhaka are thus quite dynamic platforms of exchange of resistance genes with direct implication to treatment options that can be offered to clinicians in

Bangladesh. To tackle this resistome, wastewater treatment engineering, antibiotic stewardship, and environmental monitoring should all work together in a One Health approach, as advocated by WHO, FAO, and UNEP in their combined AMR action plans [16, 67].

4.6 Major Risk of Transmission: Water

The overall prevalence rates of MDR *E. coli* in all three locations (74.0, 78.0 and 86.0) have direct implications on the population of Dhaka. Water is both a primary infection pathway, a carriage of resistance determinants across geographic borders, and a selective environment, in which MDR populations have endured and flourished [9, 10]. Since Dhaka has been known to have deficiencies in coverage of wastewater treatment and dependence on surface water to supply large populations in domestic and agricultural activities, the reported MDR *E. coli* levels are a risk at the population level. The main exposure pathways among humans are direct-water exposure through contaminated water, transmission via the food chain through the irrigation of crops and vegetables, and occupational exposure among farmers and fishermen, which are the most significant exposure pathways in terms of epidemiological importance, although they are disproportionately under-emphasized in policy (with respect to their importance) [60, 64, 10]. ESBL-based co-resistance (TZP-110-FEP-30: co-resistance 92.9%): any organism contracted in such waterways would most likely be resistant to most common empirical treatment options based on beta-lactams, in combination with the fluoroquinolone-macrolide co-resistance via efflux, patients exposed to water sources would be subjected to multiple.

4.7 Study Limitations

There are two major restrictions, which are worth mentioning. To begin with, the application of only phenotypic disc diffusion in the absence of whole-genome sequencing (WGS) or the specific resistance gene PCR implies that the underlying mechanisms of resistance, be it efflux or enzymatic, chromosomal or plasmid-borne cannot be definitively established. Characterization (blaCTX-M, qnr, tetA/B, erm), clonal ancestry (e.g., the pandemic ST131 clone), and plasmid transferability have not been characterized, restricting the mechanistic inferences to the phenotypic. Second, having 50 isolates per site, the subgroup analysis within

seasons, classes of antibiotics or isolation status have broad confidence intervals and small statistical power to identify moderate effect sizes. The incomplete coverage of antibiotic testing - large numbers of plates with a X or indeterminate result (X) - also decreased the effective sample size of some antibiotic-specific tests, and the bi-weekly sampling design, although covering both pre-monsoon and monsoon seasons, did not provide enough per-season replication to definitively make seasonal conclusions.

Chapter 5

Conclusion:

This study confirms that MDR *E. coli* is a population-level norm in Dhaka's urban waterways, with 93.3% of isolates falling within a high-resistance cluster and resistance rates exceeding 80% for six of eight antimicrobials tested [51, 68]. Statistically significant co-resistance between levofloxacin and azithromycin ($\phi = 0.473$, $p = 0.002$) and between piperacillin-tazobactam and cefepime ($\phi = 0.452$, $p < 0.05$) provides mechanistic evidence of concurrent efflux pump-mediated cross-resistance and probable ESBL co-production [52]. Significant inter-site variation ($H = 6.906$, $p = 0.032$) confirms that this burden is not location-specific-residential and peri-institutional waterways alike represent surveillance priorities [8].

The primary limitation remains the absence of WGS and targeted resistance gene PCR, precluding definitive identification of ESBL genotypes, efflux pump genes, and pandemic clonal lineages such as ST131 [27]. In a megacity of twenty million where daily geography is inseparable from resistance-laden water bodies, a coordinated One Health response spanning wastewater management, antibiotic stewardship, and molecular diagnostic capacity is a public health necessity, not merely a research agenda [16].

5.1 Future Directions

The mentioned limitations are, at the same time, the most fruitful directions of research in the future. The results of this research provide an empirical phenotypic baseline on the prevalence of MDR *E. coli* in the urban waterways of Dhaka and produce a number of high priority mechanistic and epidemiological hypotheses that need to be rigorously tested by implementing molecular and metagenomic methods.

5.1.1 Whole-Genome Resistome Sequencing

Applying whole-genome sequencing to archived isolates from the current study group would be the most immediate significant methodological extension of this work. Resistance genes, their genetic contexts (chromosomal versus plasmid-borne, within integrons or transposons), and their phylogenetic origins could all be definitively identified through WGS-based resistome analysis, which is the thorough cataloging of all resistance-conferring genomic elements within a bacterial isolate [44]. For the antibiotic resistance profiles observed in this study, the most clinically significant targets for sequencing-based characterisation would include the full complement of beta-lactamase genes (*bla*CTX-M, *bla*TEM, *bla*SHV, *bla*OXA); fluoroquinolone resistance determinants (*qnr*A, *qnr*B, *qnr*S, *aac*(6')-Ib-cr, plasmid-mediated quinolone resistance [PMQR]); macrolide resistance genes (*erm*A, *erm*B, *erm*C, *mph*A); and tetracycline resistance determinants (*tet*A, *tet*B, *tet*M). MLST-based lineage assignment would further identify whether the MDR strains recovered belong to globally disseminated high-risk clonal lineages such as sequence type 131 (ST131) the pandemic fluoroquinolone- and ESBL resistant *E. coli* clone whose environmental reservoir status in South Asian waterways has not yet been fully characterized [44].

Long-read sequencing platforms, particularly Oxford Nanopore Technology (ONT), offer an additional advantage for environmental AMR studies in that they enable complete circularization and characterization of plasmid replicons a capability critical for establishing the transferability and incompatibility group classification of resistance-carrying plasmids. Given the plasmid-mediated co-resistance hypotheses generated by the current phenotypic data, long-read sequencing of selected high-resistance isolates would be a particularly valuable investment for future iterations of this research programme.

5.1.2 Metagenomic Surveillance of the Environmental Resistome

Beyond the culture-based, isolate-centred approach employed in this study, shotgun metagenomic sequencing of water and sediment samples from the study sites would provide a fundamentally different and complementary perspective on the environmental resistome [9]. Culture-based methods are inherently limited to the viable and culturable fraction of the microbial community estimated to represent as little as 1-10% of environmental bacterial diversity and therefore cannot capture the full breadth of resistance genes present in the environmental DNA pool, including those carried by non-culturable or viable-but-non-culturable (VBNC) organisms, free extracellular DNA on mobile genetic elements, and bacteriophage-associated resistance gene transfer agents.

Metagenomic methodologies that are culture-independent, analyzed with specially designed resistome databases like the Comprehensive Antibiotic Resistance Database (CARD), the Resistance Gene Identifier (RGI), and ResFinder would allow quantitative mapping of the entire ARG (antibiotic resistance gene) repertoire in the water matrix spanning all antibiotic classes, and not just those present in the disc diffusion panel used. Repeated metagenomic sampling of the monsoon and dry seasons would additionally allow evaluation of the seasonal dynamics of resistance gene abundance and diversity a dimension that the bi-weekly sampling timeline of the present research was intended to help characterize phenotypically, but could not fully be resolved due to sample size limitations. Linear combination of 16S rRNA amplicon sequencing data with ARG abundance data would further enable exploration of the ecological interdependence between microbial community make up and resistome affluence, such as which bacterial taxa are primary resistance gene reservoirs in any specific water body.

5.1.3 Gene-Level Validation and Functional Studies

To close the research gap between the phenotypic data produced during this study and molecular mechanism, a feasible intermediate solution, where targeted gene-level validation is possible via quantitative PCR (qPCR) would not necessitate the investment and infrastructure that WGS would. Multi-drug-resistance gene Multiplex qPCR assays of the

most clinically relevant families of resistance genes specifically CTX-M-group ESBLs, tetA/tetB, ermB and qnrS could be used on the individual strains as well as on the water DNA extracts, allowing high-throughput detection of resistance genes without requiring their culture [69]. By comparing the results of qPCR with the results of the phenotypic disc diffusion data of this study, one would determine the gene-phenotype agreement in this environmental *E. coli* population, which would offer an empirical basis to the mechanistic inferences made in Section 4.8.1.

The transferability of the resistance determinants by conjugation the mechanism that this study hypothesizes is the main driver of the spread of environmental resistance would directly be tested by conjugation tests with the representative MDR isolates of this study used as donor strains and standard laboratory *E. coli* recipient strains (such as the J53 or K-12 strains). Successful transconjugant transfer of the plasmid-based co-resistance hypothesis with the multiple resistance phenotypes would provide functional validation of the epidemiological relevance of the aquatic *E. coli* population as a repository of transferrable resistance to clinically important organisms. Judging of the rates of conjugation under conditions to model the physicochemical conditions of the waters of study such as high temperature, changing levels of pH and presence of subinhibitory levels of antibiotics would further clarify the ecological conditions which are best favoring in situ resistance transfer.

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