Occurrence and characterization of pathogenic bacteria producing β-lactamase in biomedical waste water from hospitals in Dhaka city

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PhD Thesis

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Declaration

I hereby declare that the contents of this PhD dissertation is my original work and has not previously been submitted for a degree at this or any other university. To the best of my knowledge, it contains no previously published or written by other people except where due reference have been made.

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Signature of Candidate

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Certificate

This is to certify that the thesis entitled "Occurrence and characterization of pathogenic bacteria producing β-lactamase in biomedical wastewater from hospitals in Dhaka city " was carried out by Sultana Juhara Mannan, Registration number 107, and Session: 2021-2022 for the fulfillment of the degree of Doctor of Philosophy from the Department of Microbiology, University of Dhaka, Bangladesh. This original work was carried out under our supervision and the style and contents of the thesis have been approved and recommended for the award of PhD degree.

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List of Abbreviations

%	Percentage
1 kb	1 kilobase
Aac	aminoglycosides
ABC	ATP-binding cassette
AMR	Antimicrobial resistance
ARB	Antibiotic-resistant bacteria
ARGs	Antimicrobial resistance genes
Bla	β-lactams
CARD	Comprehensive Antibiotic Resistance Database
CDC	Centers for Disease Control and Prevention
CDS	Coding sequences
CGE	Center for Genomic Epidemiology
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CROs	Carbapenem-resistant organisms
Dfr	Trimethoprim
DFT	Density Functional Theory
DFT	Density functional theory
EARS-Net	European Antimicrobial Resistance Surveillance Network
ENA	European Nucleotide Archive
ESBL	Extended-spectrum β-lactamase
Fca	fluoroquinolones
Flo	Amphenicols
GNB	Gram-negative bacilli
HGT	Horizontal gene transfer
In	Integrons
IS	Insertion sequences
KPC	Klebsiella pneumoniae carbapenemase
LB	Luria-Bertani
MBLs	Metallo-β-lactamases
MDR	Multidrug resistance
MGEs	mobile genetic elements (MGEs)
MHA	Mueller-Hinton agar
MICs	Minimum inhibitory concentrations
MLST	Multi-locus sequence typing
MLST	Multilocus Sequence Typing
Mm	Mili meter
MRSA	Methicillin-resistant S. aureus
NCBI	National Center for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
NDM	New Delhi metallo-beta-lactamase
PBP	penicillin-binding proteins
PC	Positive control
PCR	Polymerase chain reaction
PDB	Protein Data Bank

QC	Quality control
QC	Quality control
QSAR	Quantitative structure-activity relationship
RC	Rolling circle
RFLP	Restriction fragment length polymorphism
RGI	Resistance Gene Identifier
SMR	Small multidrug resistance
Sul	sulfonamides
T6SS	Type VI secretion system
Tet	Tetracyclines
Tn	Transposons
UV	Ultraviolet
Van	glycopeptides
VFDB	Virulence Factor Database
WGS	Whole genome sequencing
WHO	World Health Organization
µL	Micro litre

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Abstract

Antimicrobial resistance (AMR), particularly among Gram-negative pathogens, presents a serious risk to environmental safety and public health worldwide. Hospitals are recognized as primary contributors to this crisis due to their release of high loads of antibiotics and resistant pathogens into waste water systems. This study focused on the detection, characterization, resistance pattern, and genomic analysis of extended-spectrum β-lactamase (ESBL) and carbapenemase-producing *Escherichia. coli* and *Pseudomonas aeruginosa* obtained from biomedical waste water collected from several major hospitals in Dhaka City, Bangladesh.

A total of 300 waste water samples were collected from hospital effluents and analyzed through standard microbiological, phenotypic, and molecular techniques. Out of 184 isolates, 42.7% (n=38) were *E. coli*, 25.9% (n=23) were *Acinetobacter baumannii*, 25.9% (n=23) were *P. aeruginosa* and 5.6% (n=5) were *Enterobacter cloacae*. Antibiotic susceptibility testing revealed that *E. coli* and *P. aeruginosa* exhibited multidrug resistance (MDR), with notably high resistance rates against ampicillin, cefuroxime and ceftriaxone. Alarming resistance to meropenem, a last-line antibiotic, was also observed.

Phenotypic confirmation of ESBL production using double-disc synergy method showed that 48.4% (n = 89) isolates were ESBL-positive. Carbapenemase activity was confirmed in isolates resistant to carbapenems. Multiplex polymerase chain reaction (PCR) indicated the existence of several key resistance genes: $bla_{\text{CTX-M}}$ (60%), bla_{TEM} (48%), bla_{SHV} (45%), and carbapenemase genes including $bla_{\text{NDM-1}}$ (26%), bla_{OXA} (22%), and bla_{KPC} (18%). Plasmid profiling confirmed that many of these genes were plasmid-borne, indicating the likelihood of horizontal gene transfer across bacterial species in the waste water environment.

In addition to phenotypic and molecular characterization, in silico docking analysis was employed to assess the binding affinities of clinically important β-lactam antibiotics (cefuroxime, ceftriaxone, and meropenem) against selected β-lactamase enzymes. Results indicated moderate binding affinities, with cefuroxime and ceftriaxone showing docking scores of –7.1 kcal/mol and –8.2 kcal/mol, respectively, against CTX-M-type enzymes. Modified derivatives with specific functional groups demonstrated enhanced binding, with docking energies improving up to –8.9 kcal/mol, suggesting that structural modification could potentially improve drug efficacy against resistant strains.

Whole genome sequencing (WGS) of selected MDR isolates revealed comprehensive resistomes containing not only ESBL and carbapenemase genes but also multiple virulence

factors and transferable genetic materials, such as integrons and transposons. Several isolates contained co-localized resistance determinants, including *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{NDM-1}}$, and sul1 on the same plasmid structures, reinforcing the threat of horizontal gene transfer. A comparative analysis between isolates from hospital effluents and other urban water sources revealed significant genomic similarity, confirming the environmental spread of antimicrobial resistance genes (ARGs).

The present study provides molecular insights into the structure-function relationship of antibiotics and β-lactamases, contributing to the larger field of drug redesign through computational approaches. Additionally, the findings emphasize the critical role that hospital waste water plays in the spread of multidrug-resistant bacteria and the pressing need for better waste management practices in densely populated urban centers.

This integrated study—combining environmental microbiology, molecular diagnostics, bioinformatics, and structural biology—advances our understanding of the AMR burden in biomedical waste and presents a multidimensional strategy for surveillance, mitigation, and future therapeutic development. The outcomes emphasize the importance of adopting One Health frameworks and implementing robust environmental monitoring systems to address the escalating AMR threat at the human–environment interface.

Chapter 1

Introduction

1.0 Introduction

The phenomenon of antimicrobial resistance represents a continuously developing challenge that spans across both human healthcare systems and veterinary medicine domains, as documented by the World Health Organization in its 2014 assessment. Recent epidemiological data reveal the devastating impact of bacterial resistance to antimicrobial agents, with direct causation linked to approximately 1.27 million deaths worldwide during 2019, while serving as a contributing factor in an additional 4.95 million fatalities, according to WHO's 2022 comprehensive analysis. The emergence of bacterial resistance to antimicrobial compounds occurs through genetic and phenotypic modifications within bacterial populations, resulting in diminished therapeutic efficacy of previously effective pharmaceutical interventions designed to treat infectious diseases. Due to this process of biological adaptation, antibiotic resistance has emerged as one of the most urgent health issues facing contemporary society in the twenty-first century.

A comprehensive analysis conducted under the auspices of the United Kingdom's governmental health authorities has projected alarming future scenarios regarding the trajectory of antimicrobial resistance. This authoritative investigation predicts that the global mortality burden attributable to drug-resistant bacterial infections could reach catastrophic proportions, potentially claiming as many as 10 million lives each year by the year 2050. Such forecasts underscore the urgent need for coordinated international solutions to address this growing threat to global health security (Murray et al., 2022). Healthcare institutions and municipal sewage systems have been extensively documented as primary reservoirs facilitating the emergence and environmental dissemination of drug-resistant bacterial pathogens. Medical facilities and metropolitan waste water infrastructure serve as collection points for effluents containing substantial concentrations of antimicrobial-resistant microorganisms, which subsequently flow into centralized sewage treatment operations throughout urban centers. The processed effluent from these treatment facilities functions as critical epidemiological focal points where environmental selective pressures exert profound influences on both susceptible and resistant microbial communities. These selective forces predominantly favor the survival and multiplication of drug-resistant bacterial variants due to the persistent presence of pharmaceutically active residues and antimicrobial compounds, which create inhospitable conditions for sensitive bacterial strains while simultaneously providing competitive advantages to resistant populations. During conventional waste water treatment procedures, substantial reductions in bacterial load are typically achieved through various physical,

chemical, and biological treatment mechanisms. Nevertheless, a considerable proportion of viable microbial cells, including numerous antibiotic-resistant bacterial species, successfully survive these treatment processes and subsequently contaminate adjacent freshwater bodies, rivers, and groundwater sources. This contamination pathway represents a critical route through which resistant bacterial strains infiltrate broader environmental ecosystems and potentially reach human populations through various exposure routes.

The proliferation and environmental distribution of antimicrobial resistance mechanisms pose a formidable challenge to contemporary infectious disease control strategies, necessitating comprehensive epidemiological monitoring programs. These surveillance activities are crucial for monitoring resistance patterns, detecting potential concerns, and executing suitable public health interventions to reduce the dissemination of resistant microorganisms. Medical waste generated by healthcare operations encompasses a wide range of materials, including expired pharmaceutical products, disposable medical equipment, biological specimens from laboratory analyses, and various byproducts from clinical and experimental research activities. The classification system for healthcare waste typically divides these materials into three distinct categories based on their potential hazard levels and disposal requirements. General healthcare waste, which accounts for approximately 85% of the total medical waste volume, primarily consists of non-hazardous materials that pose minimal risk to human health and environmental safety. Infectious waste materials constitute roughly ten percent of healthcare waste streams and include items contaminated with potentially pathogenic microorganisms that require specialized handling and disposal protocols. Chemical and radioactive waste materials account for the remaining five percent of healthcare waste and present unique environmental and occupational health challenges that require specialized treatment and disposal methods.

Within the infectious waste category, several subcategories of materials have been identified as presenting particularly high risks to public health and environmental safety. Sharp objects, including needles, scalpels, and broken glass containers, pose immediate risks of injury and potential pathogen transmission. Pathological waste materials derived from surgical procedures, autopsies, and tissue samples contain viable microorganisms and require specialized incineration or treatment protocols. Cytotoxic substances used in cancer treatment and chemotherapy procedures present significant health hazards due to their carcinogenic and mutagenic properties. Chemical waste streams from laboratory operations and diagnostic procedures often contain hazardous compounds requiring specialized neutralization and disposal methods. Pharmaceutical waste includes expired medications, unused drug

preparations, and contaminated packaging materials that can contribute to the development of antimicrobial resistance in environmental systems.

Radioactive materials from nuclear medicine procedures and research applications require extended storage periods and specialized disposal facilities to ensure radiation exposure remains within acceptable limits. These high-risk waste categories collectively account for between fifteen and twenty-five percent of total healthcare waste volumes, as documented in a 2019 comprehensive analysis of medical waste management practices by Padmanabhan and Barik. Metropolitan regions with high population densities, such as Dhaka City, encounter significant challenges in managing biomedical effluent due to the vast amounts of leavings produced by numerous healthcare facilities within limited geographical confines. The concentration of medical institutions, diagnostic laboratories, pharmaceutical manufacturing facilities, and research centers within urban environments creates unprecedented waste management challenges that strain existing infrastructure and disposal capabilities.

Hospital waste water effluents consistently demonstrate markedly elevated concentrations of various chemical and biological parameters that serve as indicators of environmental impact and treatment requirements. Biochemical oxygen demand assays, which determine the quantity of dissolved oxygen needed by aerobic microorganisms to break down organic matter in water samples, generally exhibit markedly elevated levels in hospital waste water relative to home sewage. Chemical oxygen demand values, which indicate the total oxygen required to oxidize both perishable and non-perishable biomolecules present within waste water, also exhibit significant rises in healthcare facility effluents.

Total organic carbon measurements, indicating the overall concentration of carbon-containing compounds dissolved or suspended in waste water, consistently exceed normal municipal waste water parameters. Nitrogen compounds present particular challenges in hospital waste water treatment due to elevated concentrations of ammonia nitrogen derived from biological waste materials and cleaning agents used in medical facilities. Organic nitrogen compounds originating from protein degradation and pharmaceutical metabolism contribute additional complexity to treatment requirements. Nitrite and nitrate compounds, formed through bacterial oxidation processes, create additional treatment challenges and may contribute to eutrophication in receiving water bodies. Total phosphorus concentrations in hospital waste water often exceed regulatory limits due to the presence of phosphate-containing cleaning agents, pharmaceutical compounds, and biological waste materials. Total solids measurements,

encompassing both dissolved and suspended particles present in waste water, typically demonstrate substantial elevation compared to residential waste water streams, requiring enhanced treatment capacity and specialized equipment for effective removal. (Emanuel, 2005; Young & Lipták, 2018).

In recent years, beta-lactam antibiotics—especially advanced-generation cephalosporins and carbapenems—combined with fluoroquinolones, have been key in treating infections triggered by Enterobacteriaceae. However, growing evidence across Europe highlights a rising trend in resistance to these critical drug classes, posing a significant challenge to effective clinical treatment. (Coque, 2008). In Gram-negative bacteria, the production of beta-lactamase enzymes plays a crucial role in the growing problem of antibiotic resistance, a trend exacerbated by the widespread use of antibiotics. A widely prevalent mechanism via which these bacteria develop resistance to beta-lactam antibiotics is through the acquisition of extended-spectrum beta-lactamases (ESBLs). These enzymes render most beta-lactam antibiotics ineffective, although carbapenems and cephamycins typically remain active. However, even these can be compromised when specific inhibitors, like clavulanic acid, are present. The genetic blueprints for this resistance can be passed down from one generation to the next or shared directly between different types of bacteria, allowing resistance to spread rapidly across diverse microbial populations. More than 300 distinct variants of extended-spectrum beta-lactamases (ESBLs) have been identified to date, with research suggesting they originated from a limited number of ancestral beta-lactamase forms, such as TEM-1, TEM-2, and SHV-1. Over time, genetic mutations in these original enzymes led to structural changes that expanded their activity, enabling them to break down advanced antibiotics, such as third-generation cephalosporins and aztreonam, which were previously effective against many bacterial infections. This evolutionary adaptability has significantly exacerbated the issue of treating antibiotic-resistant bacterial species (Smet et al., 2008). Certain members of the Enterobacteriaceae family produce extended-spectrum beta-lactamases (ESBLs) that are not derived from the well-known TEM or SHV enzyme lineages. Instead, they belong to other families, such as CTX-M and OXA types. Among these, CTX-M enzymes have become particularly dominant across Europe. These resistance genes likely originated from the chromosomal DNA of Kluyvera species, environmental bacteria that are not typically pathogenic. Over time, the genes encoding CTX-M enzymes were acquired by mobile genetic elements, particularly plasmids, which can easily transfer between bacteria. The capacity for horizontal transmission has allowed CTX-M-type beta-lactamases to emerge as one of the most

prevalent and extensively disseminated variants of ESBLs in clinical environments across the region (Korzeniewska & Harnisz, 2013). The emergence and dissemination of pathogenic bacteria that produce β-lactamase enzymes present a significant risk in hospitals and other healthcare settings. These enzymes enable bacteria to resist the effects of commonly prescribed antibiotics, particularly those in the β-lactam family, including penicillins. Among the most concerning are multidrug-resistant ESKAPE pathogens and *Escherichia coli*, which have increasingly shown resistance to cephalosporins—drugs often relied upon for treating severe infections. Such resistant infections predominantly affect individuals who are already critically ill or have weakened immune systems, making treatment even more difficult and outcomes potentially fatal. In Gram-negative bacteria, the main defense mechanism against antibiotic pressure involves the production of β-lactamase enzymes, which break down the antibiotic before it can act. The genes responsible for this resistance can be passed down from parent to daughter cells during reproduction, or they can move between unrelated bacterial species through horizontal gene transfer, accelerating the spread of resistance across diverse microbial populations. (Baquero et al., 2021).

Pseudomonas aeruginosa is a predominant cause of severe nosocomial infections in healthcare settings, especially among patients with compromised immune systems or in critical condition. It is often grouped with other high-priority pathogens, such as *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and various Enterobacter species—collectively recognized for their ability to develop resistance and cause difficult-to-treat infections (Pendleton et al., 2013). *P. aeruginosa* is especially concerning due to its frequent resistance to multiple classes of antimicrobial agents, including β-lactams, aminoglycosides, and fluoroquinolones, which are commonly used in clinical practice. The growing number of bacterial strains that no longer respond to carbapenems or advanced-generation cephalosporins—drugs often reserved as last-line treatments—has become a pressing global health concern (World Health Organization, 2021). Resistance in *P. aeruginosa* is primarily driven by two key mechanisms: the overproduction of chromosomally encoded AmpC β-lactamase and the synthesis of metallo-β-lactamases (MBLs), enzymes capable of degrading both cephalosporins and carbapenems, rendering these antibiotics ineffective. While traditionally not considered primary ESBL producers, an increasing number of *P. aeruginosa* isolates are now being identified that carry genes encoding extended-spectrum β-lactamases (ESBLs), further complicating treatment options. Alarmingly, co-expression of both bla_{ESBL} and bla_{MBL} genes in the same strain has been reported, indicating a convergence of resistance

mechanisms that severely limits therapeutic options (Marie & Karen, 2007). ESBLs are enzymes that inactivate a broad range of β-lactam antibiotics, including penicillins and extended-spectrum cephalosporins such as ceftriaxone, cefotaxime, and ceftazidime, as well as monobactams like aztreonam. These enzymes are particularly effective against oxyimino-β-lactams, a structural subclass of cephalosporins. Fortunately, most ESBLs remain susceptible to inhibition by agents such as clavulanic acid, sulbactam, and tazobactam, which are often combined with β-lactam antibiotics to restore their activity. Laboratory detection of ESBL-producing organisms typically relies on phenotypic tests that exploit the susceptibility of these enzymes to such inhibitors (Clinical and Laboratory Standards Institute, 2023).

Structurally, ESBLs are categorized into two of the four Ambler classes of β-lactamases—Class A and Class D. In P. aeruginosa, Class A enzymes include variants from the PER, GES, VEB, BEL, and PME families, while Class D enzymes, often referred to as extended-spectrum OXA-type β-lactamases (ES-OXAs), also contribute significantly to resistance (Poirel et al., 2010). Over 300 distinct ESBL subtypes have been documented, with the most prevalent being those encoded by $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and bla_{OXA} genes. However, an even greater threat arises from carbapenemases—another subgroup of β-lactamases capable of hydrolyzing carbapenems, which are among the most potent and broad-spectrum antibiotics available. Genes such as bla_{KPC} and $bla_{\text{NDM-1}}$ encode these carbapenemases and are increasingly detected in Enterobacteriaceae isolated from clinical settings worldwide.

E. coli and *K. pneumoniae* were among the first species found to carry plasmids harboring the $bla_{\text{NDM-1}}$ gene, but due to the mobility of these genetic elements, the resistance determinant has now spread to a wide range of Gram-negative bacteria (Kumarasamy et al., 2010a). The horizontal transfer of such plasmids facilitates the rapid dissemination of resistance across bacterial species, thereby accelerating the global spread of resistance. This trend has drastically reduced the effectiveness of many standard antibiotics, leaving clinicians with fewer viable treatment options and increasing the risk of untreatable infections. Comprehending the incidence and genetic attributes of β-lactamase-producing bacteria in hospital waste water is essential for monitoring and mitigating the spread of their resistant strains. Waste water from healthcare facilities often contains resistant bacteria shed by patients, making it a potential reservoir for environmental contamination and a hotspot for gene exchange among microbes (Rizzo et al., 2013). Monitoring such environments provides valuable insights into emerging resistance patterns and supports the development of effective containment strategies. Given that carbapenems are now considered last-resort antibiotics, the global rise of carbapenem resistance

poses one of the most serious threats to modern medicine. In response, this study aims to investigate the presence of ESBL-producing *E. coli* and *P. aeruginosa* in biomedical waste water collected from hospitals in Dhaka City. The isolates will be characterized through a comprehensive approach involving phenotypic screening for ESBL production, antibiotic susceptibility testing, multiplex PCR for resistance gene detection, plasmid profiling, and whole-genome sequencing (WGS) to understand their genetic makeup and transmission potential. In light of the declining efficacy of antibiotics such as meropenem, ampicillin, ceftriaxone, and cefuroxime, we also employ in silico modeling to explore how specific functional groups influence the binding affinity between antibiotics and their target sites. By analyzing molecular interactions, we aim to identify structural features that could be optimized to enhance drug efficacy. This research seeks to uncover strategies for improving the pharmacological properties of existing antibiotics, potentially restoring their utility against resistant strains.

Ultimately, this work underscores the urgent need for innovative approaches in antibiotic design and stewardship. By combining environmental surveillance with advanced molecular and computational techniques, we hope to contribute to the development of more effective treatments and help mitigate the growing crisis of antimicrobial resistance in both clinical and ecological settings.

1.1 Study objectives

i. To examine the prevalence and distribution of Gram-negative bacterial species, specifically *E. coli* and *P. aeruginosa*, in biomedical waste water samples collected from various hospitals within Dhaka City.

ii. To characterize the antibiotic susceptibility patterns of these bacterial isolates using established phenotypic methods, and to identify those exhibiting multidrug resistance (MDR) to understand the extent of antimicrobial resistance.

iii. To conduct targeted screening to determine the presence of extended-spectrum beta-lactamase (ESBL) producers and carbapenemase-producing multidrug-resistant isolates among the collected bacteria, thereby assessing critical resistance mechanisms prevalent in the hospital waste water environment.

iv. To apply in silico computational approaches to evaluate how different functional groups within antimicrobial compounds influence their binding affinities to bacterial targets, offering insights that could inform future drug design efforts.

v. To determine the genetic determinants underlying beta-lactam resistance through the detection of specific *bla* genes, as well as to identify plasmid-mediated carbapenemase resistance genes in selected bacterial isolates.

vi. To compare and analyze the genetic similarity or identity between bacterial strains isolated from biomedical waste water and those from other water sources throughout Dhaka City, exploring potential transmission pathways.

vii. To map the resistome profiles of the isolates by cataloging all antimicrobial resistance genes (ARGs) present, alongside associated virulence factors; this includes investigating the capacity of these bacteria to transfer resistance genes to other microbial populations horizontally.

1.2 The current scenario of antibiotics

Today, the pharmaceutical landscape devoted to antibiotic discovery has shifted dramatically from what it was just a few decades ago. Out of the twenty pharmaceutical giants actively involved in antibiotic research during the 1980s, only five remain engaged in this area, and most large, established companies have gradually stepped away from antibiotic innovation. This retreat has paved the way for smaller biotechnology firms and entrepreneurial start-ups to become the main drivers of new antibiotic development (Nicolaou & Rigol, 2018). Notably, the pipeline of fresh antibiotics remains quite limited, as highlighted in a World Health Organization report from 2017 (WHO, 2017).

Data compiled in 2018 paints a telling picture of this transformation. Among the 45 new antibiotic candidates undergoing clinical trials for approval in the United States, merely two medications were developed by large pharmaceutical companies; the overwhelming majority were being pursued by smaller and mid-sized companies, as well as academic or independent research laboratories (Pallasch TJ, 1986). The escalating challenge of bacterial resistance essentially propels this shift. While antibiotic resistance existed alongside early antibiotic use, the steady introduction of new antibiotics meant there was always another option when resistance emerged. Switching treatments was relatively seamless. However, this changed drastically in the 1980s, when the flow of groundbreaking antibiotics began to slow, stagnating innovation. Indeed, the final primary class of antibiotics entered the market in 1987, with

fluoroquinolones—some of the last broad-spectrum drugs—also developed in that decade (Durand et al., 2019). Since then, only a few new antibiotic categories have been introduced, and most have not met the challenge of combating today's level of antimicrobial resistance (Frieri M, 2017).

This lack of innovation brings not only scientific obstacles but also significant financial and regulatory burdens for drug developers. Creating antibiotics to outpace evolving resistant bacteria requires substantial investment and expertise—resources many companies are reluctant to risk, leading both big and small firms to reduce or halt antibiotic development altogether (Plackett, 2020). As a result, global health experts have warned that we may be nearing a "post-antibiotic era," where even once-manageable infections could become deadly once more.

Despite these alarming trends, there are reasons for cautious optimism. Recent years have witnessed a renewed focus on reinvigorating antibiotic discovery and diagnostic techniques, with increased investment from both public and private sectors. Collaborative efforts between universities and industry players have produced promising initiatives—such as CARB-X and ENABLE—which target the advancement of new antibiotics and rapid diagnostic technologies (Czaplewski et al., 2016). Furthermore, thorough scientific exploration is ongoing to develop suitable substitutes for conventional antibiotics, including antimicrobial peptides and bacteriophages (viruses that specifically infect and eliminate bacteria). While these alternatives are not yet widely implemented in medical practice and face practical limitations, they represent important complementary strategies in the battle against antimicrobial resistance. There is hope that, when combined with prudent antibiotic stewardship, these new approaches will help to secure the future of infectious disease treatment. In summary, although the antibiotic development landscape has undergone significant contraction and faces persistent hurdles, advancements in research, funding, and collaboration are opening new avenues to confront the ever-pressing threat of resistant bacteria.

1.3 Development of antibiotic resistance

Bacteria and other microorganisms are inherently dynamic organisms, constantly evolving in response to their surroundings. Their fundamental drive is to reproduce, survive, and spread as efficiently as possible. To accomplish this, microbes possess remarkable adaptability, allowing them to change genetically and behaviorally in response to environmental challenges (MacGowan & Macnaughton, 2017). When they encounter obstacles such as antibiotics or other

antimicrobial agents, bacteria can undergo genetic alterations that help them withstand these threats—a process that ultimately leads to resistance and continued survival, often against medical intervention (Munita & Arias, 2016)

The emergence of drug resistance is, in numerous respects, an inherent and inevitable aspect of a microorganism's life cycle. Microbial populations naturally diversify over time, and random genetic mutations may occasionally confer advantages that allow some individuals to thrive despite the presence of antimicrobial drugs. However, while this evolutionary process is inevitable, the dramatic rise of antibiotic resistance observed in recent decades is not solely due to natural selection. Rather, it results from a complex matrix of often preventable factors.

A variety of human-driven behaviors and systemic issues contribute significantly to the acceleration of antimicrobial resistance. Among these, the overuse and inappropriate administration of antibiotics play a leading role. Many antibiotics are prescribed unnecessarily or for illnesses that do not require them, such as viral infections. Misdiagnosis, lack of adherence to treatment guidelines, and the widespread tendency for self-medication contribute further to this problem (Chokshi et al., 2019; Mansour A Mahmoud, 2018; Sreeja M.K., 2017). Poor personal hygiene practices, inadequate infection control in healthcare settings, and the routine use of antibiotics in agriculture—both as growth enhancers and disease preventives in livestock—persist in fueling the escalation of resistant bacteria.

Ultimately, antimicrobial resistance arises from both unavoidable biological processes linked to microbial survival and from conditions shaped mainly by human actions, policies, and choices. By understanding the interplay between natural evolution and our own contributions to this pressing public health threat, we can better identify points of intervention and work towards strategies that protect the efficacy of antibiotics for generations to come.

During the process of bacterial replication, minor alterations in the genetic code can occur, often involving changes to just a few base pairs within the DNA sequence. These seemingly small genetic modifications, known as point mutations, can lead to the replacement of one or more amino acids in critical components of the bacterial cell, including enzymes, cell wall structures, or other vital cellular machinery. Additionally, mutations may affect regulatory genes or chromosomal regions that govern various bacterial functions. Collectively, these genetic shifts can give rise to novel strains of bacteria that possess enhanced resistance traits. As a result, the defense mechanisms developed by these microbes can significantly reduce or even nullify the

effectiveness of antibiotics that were previously reliable in inhibiting or killing them (Von Wintersdorff et al., 2016)

This adaptive process underscores the remarkable evolutionary flexibility of bacteria, allowing them to thrive in hostile environments where antimicrobial agents are present. Such genetic variability underscores why antibiotic resistance remains a persistent and growing challenge for modern medicine. Moreover, the emergence of resistant strains is not merely an isolated genetic event, but a dynamic evolutionary response in which bacteria continuously refine their defensive strategies. Understanding the molecular basis of these mutations is crucial for the development of next-generation antibiotics and effective therapeutic interventions that aim to circumvent or overcome these resistance mechanisms.

Bacterial strains that were once vulnerable to antibiotics can gain resistance by acquiring genetic material from other species or genera. This transfer of resistance is primarily facilitated by plasmids and various other mobile genetic elements, which serve as vehicles capable of moving antimicrobial resistance genes between different bacteria—often across distinct species or genus boundaries. Such horizontal gene transfer allows drug-resistant bacteria to effectively share copies of their resistance-conferring genes with previously non-resistant counterparts. As these recipient bacteria incorporate new genetic sequences into their genomes, they may develop resistance traits that render standard antibiotic treatments ineffective (Von Wintersdorff et al., 2016)

This mechanism of gene sharing is a significant factor in the rapid global spread of antibiotic resistance, contributing to the emergence of multidrug-resistant bacterial populations. The plasticity of bacterial genomes and the ease with which resistance elements circulate highlight the complexity of combating antimicrobial resistance. Understanding these processes is essential for devising strategies to interrupt gene transfer pathways and to develop therapeutics that can better withstand the evolving defenses of pathogenic bacteria. Overall, this genetic exchange exemplifies how microbial communities adapt collectively, posing ongoing challenges to public health and underscoring the need for targeted interventions.

Selective pressure refers to the specific environmental conditions that favor the survival and proliferation of organisms carrying distinctive genetic mutations or newly developed traits. In the context of antimicrobial therapy, this pressure plays a pivotal role: when microorganisms are exposed to antimicrobial agents, those lacking resistance genes are typically eliminated, whereas those possessing resistance traits survive the assault (Martínez et al., 2015) These

resistant survivors then multiply and gradually dominate the microbial ecosystem, effectively reshaping the community by becoming the most prevalent bacterial population (Uddin et al., 2021)

This process is a classic example of natural selection in action, driven by human interventions such as antibiotic use. As antimicrobial drugs exert pressure on microbial populations, only those best equipped to withstand the drugs' effects persist, leading to a rapid shift in the composition of bacterial communities. Over time, this dynamic accelerates the emergence and dissemination of resistant strains, posing significant challenges for infection control and public health. Understanding and mitigating selective pressure through prudent antimicrobial stewardship and alternative strategies are critical steps toward preserving the efficacy of current and future treatments.

1.4 Poor hospital waste management system

Hospitals serve as hubs where countless patients, healthcare workers, and visitors converge each day, each carrying their own distinct microbiomes along with bacteria that colonize their skin, clothing, and bodies. When infection control measures and sanitation protocols fall short or are inconsistently applied, these bacteria can easily spread throughout the healthcare environment. Such lapses create ideal conditions for the transmission of microbes, including those that are resistant to antibiotics. This dynamic underscore the critical need for stringent hygiene practices, rigorous cleaning processes, and comprehensive infection prevention strategies to curb the proliferation and dissemination of antimicrobial-resistant organisms within medical facilities.

By maintaining meticulous cleanliness and enforcing robust infection control protocols, hospitals can significantly reduce the risk of cross-contamination, protect vulnerable patients, and slow the advance of antimicrobial resistance, which remains a pressing challenge to global health.

1.5 Antibiotic working mechanisms

Antibiotics work by targeting specific processes in bacteria to stop them from growing or surviving. Their antibacterial effects generally fall into five main categories: interfering with cell wall building, disrupting protein or nucleic acid production, messing with metabolic processes, or damaging cell membranes (Kapoor et al., 2017).

1.5.1 Antibiotics that block cell wall formation

Bacterial cell walls are built from a tough material called peptidoglycan, which gives them structure and protection. Certain antibiotics, like beta-lactams (think penicillin, cephalosporins, and carbapenems) and glycopeptides such as vancomycin, stop this building process. Without a proper wall, the bacteria become vulnerable to bursting from internal pressure or their own breakdown enzymes. This makes these drugs bactericidal—they kill bacteria outright. The cool part is they're selective; animal cells don't have peptidoglycan, so they're safer for us.

1.5.2 Antibiotics that disrupt protein synthesis

Bacteria use ribosomes—tiny protein factories made of 30S and 50S subunits (measured in Svedberg units for their size)—to make the proteins they need to function. Some antibiotics target these: aminoglycosides and tetracyclines hit the 30S part, while macrolides, chloramphenicol, and oxazolidinones go after the 50S. By blocking protein creation, they halt bacterial growth.

1.5.3 Antibiotics that interfere with nucleic acid synthesis

These drugs target the creation of DNA or RNA in bacteria, preventing them from replicating genetic material or transcribing it into usable forms. This essentially stops bacteria from multiplying or functioning properly.

1.5.4 Disruptors of metabolic pathways

Some antibiotics act like imposters in bacterial metabolism, competing with normal enzymes. For example, sulfonamides and trimethoprim mess with the pathway that produces folic acid, a key nutrient bacteria need but can't get from their environment like we can. This starves them out and prevents growth.

1.5.5 Antibiotics That Damage Cell Membranes

A smaller group, like polymyxins (such as polymyxin B and E), works by punching holes in bacterial membranes. They're like detergents that latch onto and disrupt the outer layers, especially in Gram-negative bacteria, leading to leaks and cell death.

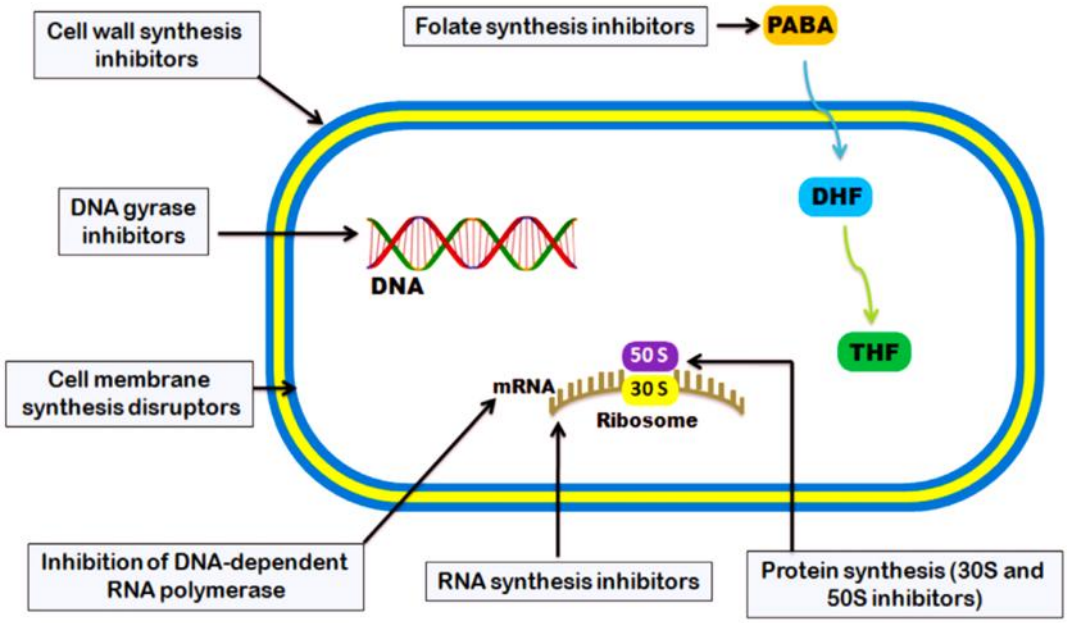


Figure 1-1 Antibiotic targets and mechanisms of drug resistance. Adopted from Healthcare 2023, 11, 1946 (Salam et al., 2023)

1.6 Resistome of Bacteria

Since the introduction of penicillin—the first antibiotic—these drugs have been indispensable in combating bacterial infections. Unlike many other medications, however, antibiotics face a unique and pressing challenge: the rapid onset and dissemination of antibiotic resistance among microorganisms. This phenomenon has been documented extensively in recent clinical reports, underscoring the formidable obstacles resistance presents to effective treatment (Liu et al., 2016; Yong et al., 2009a).

The origins of antibiotic resistance genes (ARGs) have shaped the evolving concept of the "resistome," which encompasses not only clinical resistance genes but also those found in the environment and those intrinsic to specific bacterial taxa. Additionally, it includes so-called proto-resistance genes, which serve as a reservoir of precursors that can evolve into fully functional resistance traits (Wright, 2007). As research on the resistome expanded, its definition has been refined to distinguish between different resistance types: intrinsic resistance, which is innate, vertically transmitted, and specific to particular taxa; silent or cryptic resistance, where functional resistance genes exist but remain unexpressed phenotypically; acquired resistance, which can be gained through vertical or horizontal gene transfer and is not limited by taxonomy; and proto-resistance, characterized by genes that currently confer little or no resistance until they undergo mutation (Perry et al., 2014).

Since the concept of the resistome gained prominence in 2006, it has provided profound insights into antimicrobial resistance (AMR) and its complexities (Kim & Cha, 2021). Among the key understandings to emerge are several crucial points: first, AMR is an ancient and pervasive trait present across diverse microbial communities; second, the resistome itself is remarkably intricate and possesses a vast diversity of resistance elements; third, natural environments harbor an environmental resistome that acts both as a source and as a reservoir for ARGs; fourth, the composition of microbial populations in these natural settings plays a decisive role in shaping the resistome's structure; fifth, human interventions and activities significantly impact the environmental resistome; sixth, presence of transferable genetic materials such as plasmids and transposons enable the horizontal spread of ARGs; and finally seventh, these antibiotic resistance genes are persistently swapped among humans, animals, and environmental niches.

Together, these insights highlight the complexity and interconnectedness of AMR, underscoring the need for integrated approaches that consider clinical, environmental, and ecological factors to effectively manage and mitigate the relentless spread of resistance.

1.7 Resistance of Gram-Negative Bacteria

The majority of bacteria listed by the World Health Organization (WHO) as critical threats are Gram-negative, mainly due to their distinctive structural features that inherently make them more resistant to antibiotics compared to Gram-positive bacteria. This distinctive design substantially impacts the worldwide burden of disease and mortality linked to these diseases (Gupta & Datta, 2019).

At the core of Gram-negative bacteria's formidable resistance lies their outer membrane, which serves as a protective barrier against various antibiotic classes, including β-lactams, quinolones, and colistins. For most antibiotics to exert their effects, they must penetrate this outer membrane to reach their intracellular targets. Hydrophilic antibiotics, such as β-lactams, typically navigate this barrier through specialized channels known as porins, whereas hydrophobic drugs may traverse it via diffusion pathways. Notably, some antibiotics, such as vancomycin, are structurally unable to cross the outer membrane altogether, as they cannot utilize either pathway (Exner et al., 2017).

Gram-negative bacteria can develop resistance by modifying their outer membrane; this may involve altering its hydrophobic properties or changing the structure and expression of porins, thereby reducing the uptake of antibiotics. The absence of this outer membrane in Gram-

positive bacteria partly explains their generally higher susceptibility to antibiotics (Breijyeh et al., 2020).

Beyond structural defenses, Gram-negative bacteria pose a significant threat to immunocompromised individuals, especially within healthcare settings. Nosocomial infections caused by Gram-negative bacilli (GNB) present some of the most daunting challenges for clinicians due to the pathogens' sophisticated resistance mechanisms. These mechanisms operate through both enzymatic and non-enzymatic pathways. Enzymatic resistance often involves bacteria producing enzymes that degrade antibiotics, such as β-lactamases and aminoglycoside-modifying enzymes. Non-enzymatic resistance can be mediated by elements like Qnr proteins, encoded by plasmid-borne genes, which confer resistance to fluoroquinolones among Enterobacteriaceae (Exner et al., 2017; Ruppé et al., 2015).

Alarmingly, resistance to critical antibiotics is increasingly widespread. For instance, resistance to third-generation cephalosporins in Enterobacteriaceae now exceeds 10%, while resistance to carbapenems ranges around 7%. The rapid spread of extended-spectrum β-lactamase (ESBL)–producing bacteria substantially drives these resistance rates. More specifically, carbapenem resistance rates in *K. pneumoniae* exceed 25%, while *P. aeruginosa* shows resistance rates between 20% and 40%, and Acinetobacter baumannii displays even higher rates, reaching 40% to 70% in intensive care unit (ICU) settings (Breijyeh et al., 2020; Ruppé et al., 2015).

This convergence of structural fortification and genetic adaptability underscores why Gram-negative bacteria remain among the most challenging pathogens in modern medicine. Continuous surveillance of resistance patterns, coupled with the development of novel therapeutic strategies, is essential to confront the escalating threat posed by these resilient microorganisms.

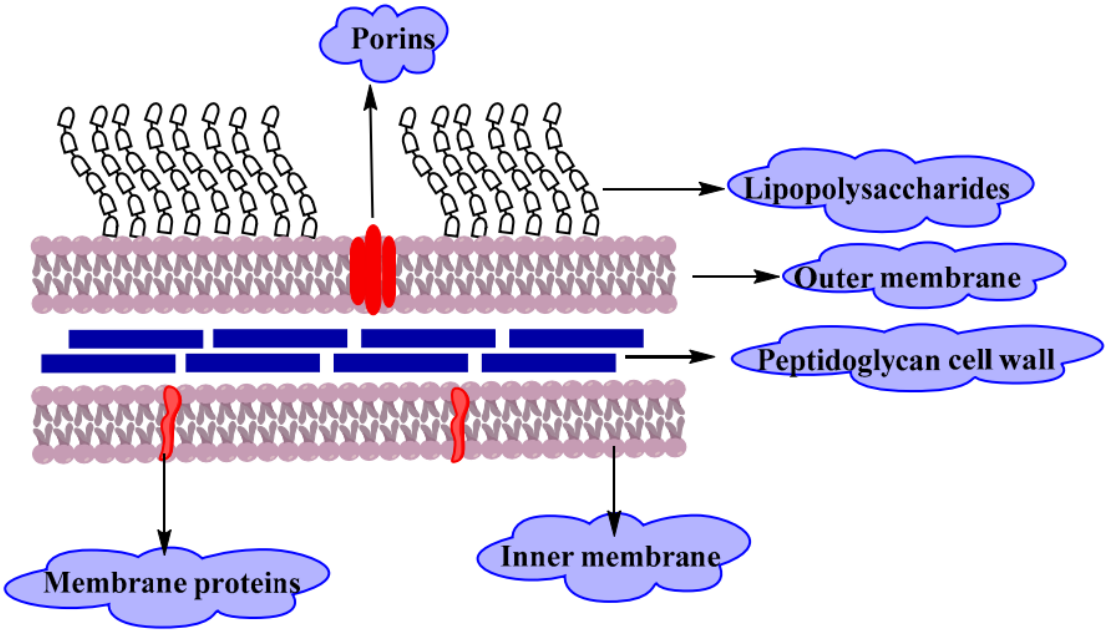


Figure 1-2 Bacterial cell envelope: structure and components

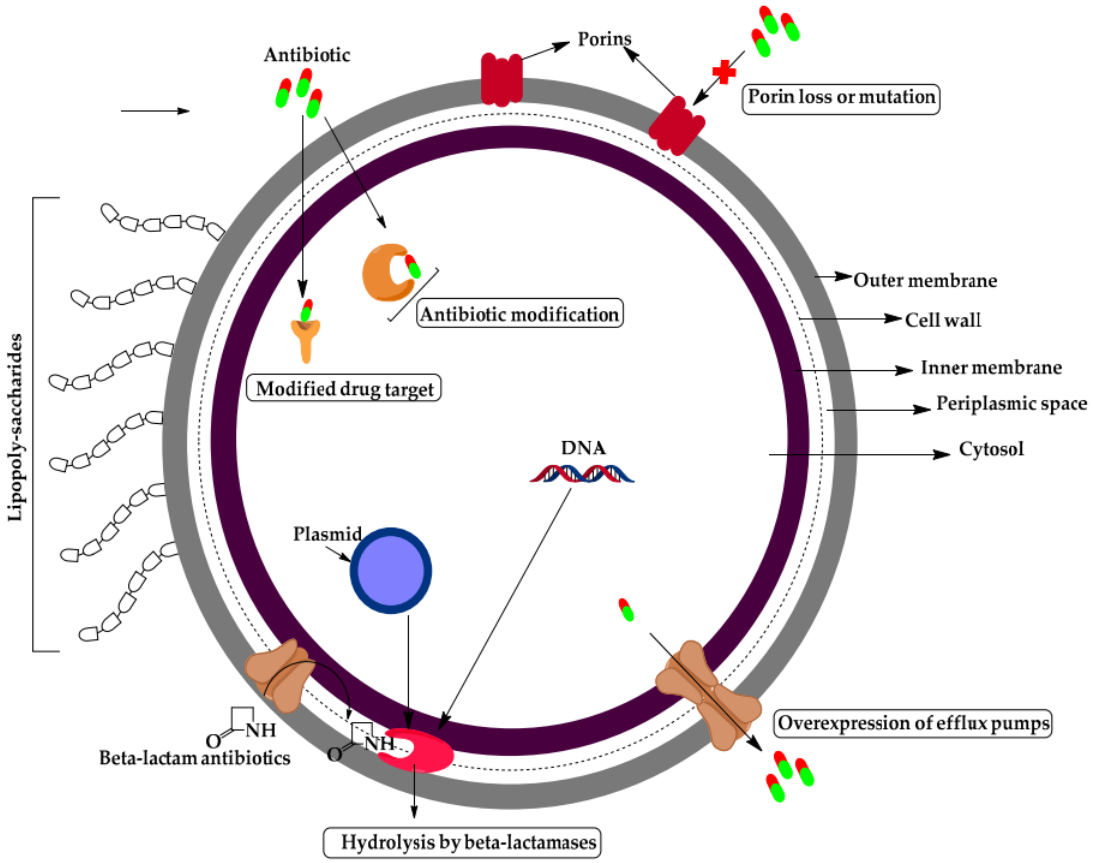


Figure 1-3 Mechanism of antibiotic resistance in Gram negative bacteria

1.8 Dissemination of resistance genes in waste water and sewage

Different classes of antibiotics tend to select for specific bacterial resistance genes, each conferring protection against their respective drugs. These antibiotic resistance genes (ARGs) can be broadly classified based on the antibiotic families they combat, including fluoroquinolones (fca), aminoglycosides (aac), tetracyclines (tet), sulfonamides (sul), β-lactams (*bla*), macrolides (erm), colistin (mcr), vancomycin (van), and multidrug resistance (mdr) genes. The behavior of both intracellular and extracellular ARGs, specifically their prevalence, persistence, and ability to transfer horizontally across bacterial populations, has been comprehensively discussed in studies such as those by Ali, Adam, Wright (2005) (Wright, 2005; Zarei-Baygi & Smith, 2021). A sweeping overview of the global resistome was provided by (Zhuang et al., 2021), who analyzed ARG-related literature drawn from PubMed over a thirty-year span from 1990 to 2020. Their work illustrated clear patterns in ARG distribution across various environments. In hospital settings, multidrug (e.g., mecA), glycopeptide (vanA, vanB), and β-lactamase (*bla*) genes were most commonly identified. Conversely, ARGs conferring resistance to sulfonamides (sul) and tetracyclines (tet) predominated in agricultural environments, waste water treatment plants, as well as in water bodies and soil.

Within six distinct environmental categories—namely farms, urban areas, waste water treatment plants, water sources, soil, and air—eight antibiotic resistance gene families consistently appeared among the top 50 ARG subtypes. These included β-lactams (*bla*), sulfonamides (sul), tetracyclines (tet), aminoglycosides (aad), multidrug resistance genes (mec), amphenicols (flo), trimethoprim (dfr), and glycopeptides (van). Among these, the multidrug resistance gene mecA was detected as the most prevalent globally, followed by the tetracycline resistance gene tetM, various β-lactamase genes, glycopeptide resistance determinants like vanA and vanB, and sulfonamide resistance genes sul1 and sul2.

Focusing on Asia, several specific ARGs have emerged as noteworthy contributors to antibiotic resistance dissemination. These include $bla_{\text{NDM-1}}$, $bla_{\text{CTX-M-15}}$, mecA, $bla_{\text{TEM-1}}$, sul1, vanA, $bla_{\text{KPC-2}}$, sul2, $bla_{\text{CTX-M-14}}$, and $bla_{\text{OXA-48}}$. Collectively, these genes are responsible for resistance against β-lactams, multiple drug classes, sulfonamides, and glycopeptide antibiotics, highlighting the widespread challenge ARGs pose beyond geographical borders.

When antibiotics enter various ecosystems—spanning human communities, healthcare facilities, and livestock production systems—they inadvertently select for antibiotic-resistant bacteria (ARB). Significant quantities of these resistant bacteria are often discharged into the

environment through pharmaceutical waste water and effluents from waste water treatment plants. In environmental reservoirs such as soil, surface waters, air, and groundwater, horizontal gene transfer occurs frequently between ARBs and opportunistic pathogens, fostering the development of multidrug resistance. This dynamic process is further complicated by the role of environmental ARBs, which can invade protozoa and insect hosts, thereby facilitating the movement of resistance genes up the food chain—from animals to human populations.

In light of these complex interactions, it is evident that the proliferation of ARGs is a global phenomenon, necessitating integrated surveillance approaches and coordinated interventions across clinical, agricultural, and environmental sectors to curb the expansion of antibiotic resistance.

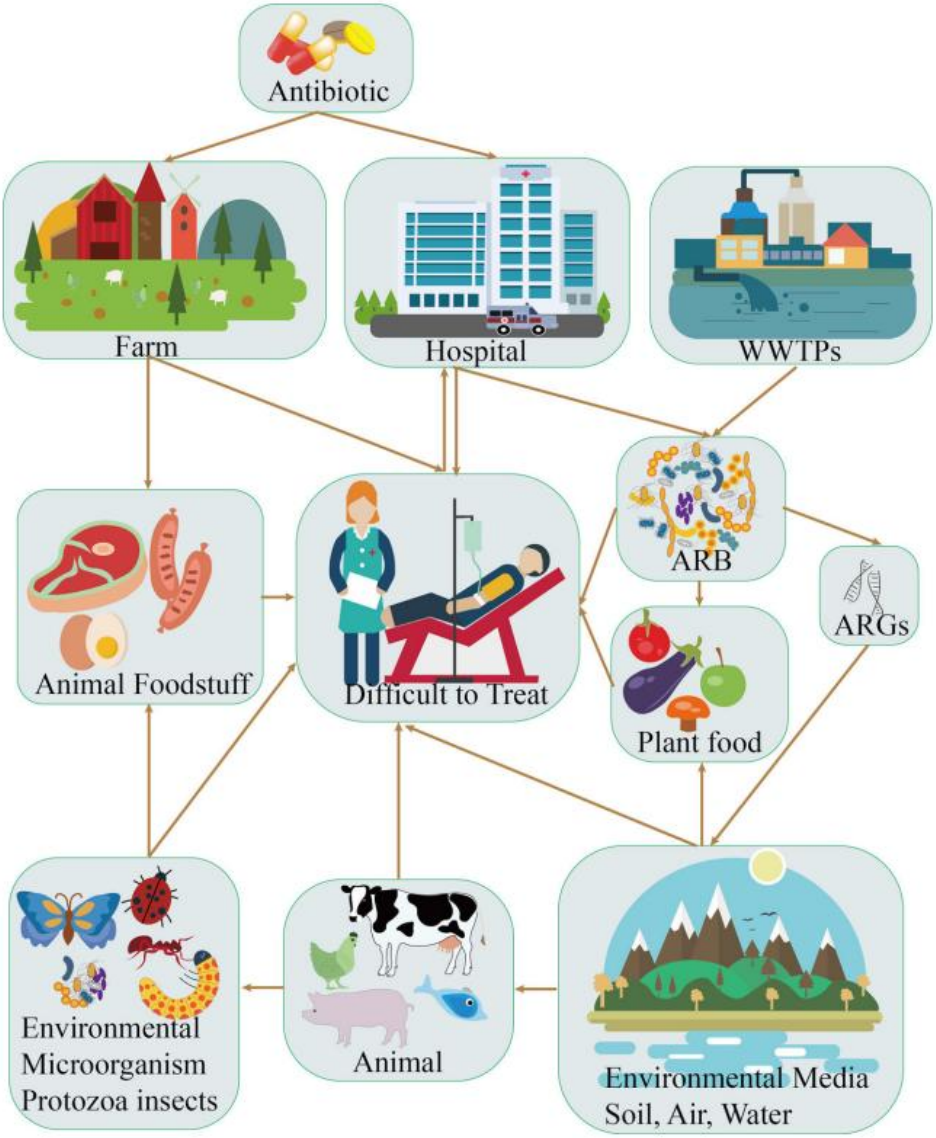


Figure 1-4 Route by which antibiotic resistance genes (ARGs) spread

1.9 Horizontal gene transfer (HGT)

A primary catalyst of bacterial evolution is the gaining of genetic material from external sources through a process known as horizontal gene transfer (HGT), which is vital role in the development and spread of antibiotic resistance. Bacteria traditionally employ three main strategies to incorporate foreign DNA: (i) transformation, where they take up free, naked DNA from their surroundings; (ii) transduction, which is mediated by bacteriophages (viruses that infect bacteria); and (iii) conjugation, often described as bacterial "mating," involving direct cell-to-cell contact for gene exchange.

Transformation is considered one of the simplest routes of HGT, yet only a select group of clinically significant bacteria possesses the natural ability to efficiently integrate naked DNA into their genomes to gain new traits, including resistance factors. On the other hand, conjugation is recognized as a highly efficient and frequent mechanism of horizontal gene transfer, particularly relevant in environments such as the human gastrointestinal tract—especially in patients undergoing antibiotic therapy—where bacteria exist in dense communities conducive to gene exchange. This mode of transfer also plays a prominent role in the acquisition of resistance traits within healthcare settings.

While direct chromosome-to-chromosome gene transfers between bacteria have been documented, conjugation more commonly relies on mobile genetic elements (MGEs) — such as plasmids, transposons, and integrative conjugative elements — which serve as vehicles for carrying and spreading critical genetic information, including antibiotic resistance genes. Among these genetic tools, integrons stand out as some of the most effective facilitators of resistance gene accumulation. Integrons function as specialized recombination platforms that capture and incorporate mobile gene cassettes into bacterial chromosomes, providing the necessary molecular machinery to ensure that newly acquired genes are not only inserted but also properly expressed.

By enabling bacteria to dynamically acquire, rearrange, and express diverse sets of resistance determinants, integrons represent a sophisticated mechanism that significantly accelerates bacterial adaptability. Their role underscores the complexity of bacterial genetic exchange systems and highlights why combating antibiotic resistance demands a comprehensive understanding of these molecular pathways.

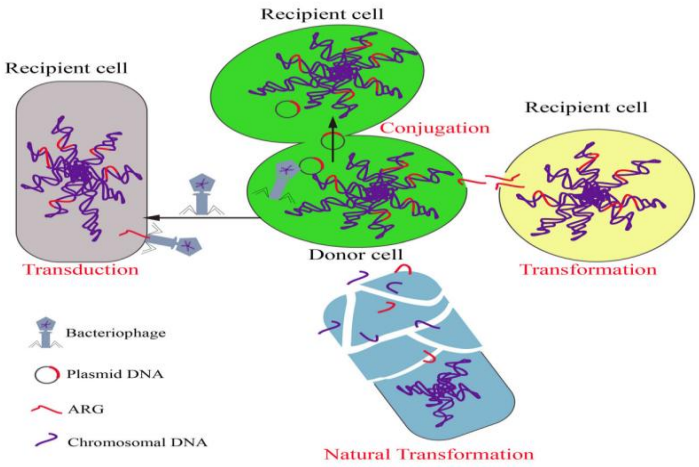


Figure 1-5 Horizontal transmission of genes for antibiotic resistance

1.10 Resistance Plasmids

Plasmids play a vital role as carriers of mobile genetic elements and associated antimicrobial resistance genes across both Gram-negative and Gram-positive bacteria. Their sizes can vary dramatically, ranging from less than one kilobase to several megabases. Despite differences in the bacterial strains they inhabit and the specific genes they carry, plasmids within incompatible groups share several core genetic components, including integrons, transposons, and insertion sequences.

Insertion sequences (IS), transposons (Tn), and integrons (In) are distinct DNA segments capable of relocating themselves or nearby resistance genes to new locations within the genome via a cut-and-paste mechanism. Typically, resistance plasmids harbor accessory regions composed of multiple resistance genes alongside these mobile elements, facilitating the capture and spread of antimicrobial resistance determinants.

The initiator protein, called Rep, binds to repeated DNA sequences also known as iterons at the origin of replication, triggering the start of the duplication process.

Plasmid replication begins at a specific site known as the origin of replication. This process often begins when an RNA transcript or, more commonly, an initiator protein—known as Rep and encoded by a rep gene on the plasmid—binds to adjacent repeated DNA sequences termed iterons. Circular plasmids replicate through one of three primary mechanisms:

i. Rolling circle (RC) replication, predominantly utilized by minuscule plasmids in both Gram-positive and Gram-negative species. Due to its efficiency in limiting plasmid size,

this replication mode often results in plasmids that are either cryptic or carry minimal genetic cargo, such as a single resistance gene.

ii. Initiator protein-driven localized unwinding of the double-stranded DNA near the replication origin, followed by replication primed by RNA transcripts.

iii. Strand displacement replication, characteristic of IncQ plasmids, in which both strands of DNA are simultaneously synthesized in opposite directions starting from the origin. IncQ plasmids are generally small in size.

Plasmids propagate their genetic material both vertically by passing to daughter cells during bacterial cell division and horizontally, transferring between unrelated bacterial cells. This dual mode of transmission underlies the powerful capacity of plasmids to disseminate antibiotic resistance genes within and across bacterial populations, substantially contributing to the spread of antimicrobial resistance.

By understanding the molecular biology of plasmids and their replication strategies, researchers and clinicians can gain a deeper understanding of how antibiotic resistance genes spread and explore potential avenues to combat this global health challenge.

1.11 β-lactamases encoded by the plasmids

Plasmid-encoded β-lactamases represent a significant and diverse group of enzymes, with over 50 different types identified among Gram-negative bacteria. These plasmid-mediated β-lactamases are notably prevalent, particularly in developing countries, where studies have found that between 30% and 80% of enterobacterial isolates produce these enzymes (Kesah C N F and Odugbemi T O, 2002). This widespread expression of β-lactamases greatly contributes to bacterial resistance, compromising the effectiveness of β-lactam antibiotics.

The clinical impact of plasmid-mediated β-lactamase production is vividly illustrated in pathogens such as *E. coli* and Neisseria species. For example, surveillance data from the National Reference Centre in Madrid and studies in Spain revealed that in 2010, approximately 23% of Neisseria gonorrhoeae isolates produced TEM-1 β-lactamases. This production renders these strains resistant to ampicillin, significantly complicating treatment options. The prevalence of β-lactamase production also varies geographically; in northern Spain, only about 3% of Pseudomonas aeruginosa isolates generated these enzymes, whereas in Catalonia, this figure climbed to nearly 28%.

More recently, the emergence of β-lactamase-producing Neisseria meningitidis strains has been documented, albeit infrequently. Notably, two such strains were identified in the Barcelona region, signaling the expanding scope of β-lactamase-associated resistance even in species previously regarded as less affected. Although these cases remain rare, they serve as a stark reminder of the evolving challenge that plasmid-mediated β-lactamase production poses to antimicrobial therapy.

Given the growing distribution and varied expression of these enzymes, continuous monitoring and robust antimicrobial stewardship are essential to manage and mitigate the spread of β-lactamase-mediated resistance in diverse bacterial populations globally.

1.11.1 Carbapenemases

In 1991, a distinctive carbapenemase enzyme was first identified in a strain of *P. aeruginosa* from Japan. This enzyme closely resembles broad-spectrum metallo-β-lactamases encoded chromosomally in certain bacteria, exhibiting the ability to hydrolyze a wide array of β-lactam antibiotics, including newer generations of cephalosporins and cephamycins. Notably, to date, this carbapenemase has only been found in a single *P. aeruginosa* isolate (Watanabe et al., 1991).

Concerning antibiotic susceptibility, the minimum inhibitory concentration (MIC) values for imipenem and meropenem against this strain are remarkably elevated—measured at 50 μg/ml and 100 μg/ml, respectively—indicating substantial resistance. Importantly, this carbapenemase is impervious to inhibition by the β-lactamase inhibitors currently available in clinical use, which presents an added therapeutic challenge.

The plasmid-mediated nature of this enzyme is particularly significant for two intertwined reasons: it confers broad-spectrum resistance to a wide range of β-lactam antibiotics, and it holds the potential for horizontal transfer among bacterial populations. This capability raises concerns about the enzyme's possible dissemination in the future, highlighting the urgent need for vigilant surveillance and innovative approaches to counteract the spread of such potent resistance mechanisms (Garau J, 1984).

1.11.2 β-Lactamases mediated by chromosomes

The genes which encode chromosome-mediated β-lactamase enzymes are located directly on the bacterial chromosome. These chromosome mediated β-lactamases are well documented in various enterobacterial species and are predominantly observed among Gram-negative bacteria. However, certain genera such as *Neisseria* and *Haemophilus* have not been shown to carry these enzymes, nor have species like *Pseudomonas*, *Moraxella*, *Bacteroides*, *Campylobacter*, *Acinetobacter*, *Legionella*, or *Pasteurella*.

Class 1 chromosomal β-lactamases generally exhibit clinically significant production only when an inducing agent—typically a β-lactam antibiotic—is present. In hospital environments, the induction of these enzymes becomes particularly important in bacterial genera such as *Pseudomonas, Citrobacter*, *Serratia*, and, *Enterobacter* which are common culprits behind nosocomial infections. The process of induction is dynamic: once the inducing β-lactam compound is removed or degraded through hydrolysis, the production of the β-lactamase enzyme usually returns to baseline levels.

Several bacteria, including *Enterobacter* species, *P. aeruginosa*, *Citrobacter freundii*, species of *Providencia*, *Proteus* species (indole-positive), *Morganella morganii*, and *Serratia marcescens*, are known to produce these inducible β-lactamases. Within bacterial populations, especially in *E. cloacae*, stable mutants that produce β-lactamase independently of induction—termed derepressed mutants—are frequently found and present a particular clinical challenge.

Clinical data, such as resistance emergence rates in patients receiving treatment with newer cephalosporins, further underscore the importance of these stably derepressed mutants, which allow bacteria to survive despite antibiotic pressure (Sanders C C and Sanders W E, 1988). Notably, a broad range of antibiotics—including most cephalosporins, cephamycins, monobactams, and extended-spectrum penicillins—are vulnerable to hydrolysis by class 1 β-lactamases. The use of these antibiotics has inadvertently selected for resistant bacterial populations through the expansion of these derepressed mutants.

It is also important to highlight that traditional β-lactamase inhibitors do not uniformly inhibit these inducible enzymes: clavulanic acid is generally ineffective against class 1 inducible β-lactamases, while tazobactam offers only limited inhibition against a subset of them. This

pharmacological limitation further complicates treatment strategies and calls for continued research into more effective inhibitors.

1.11.3 Plasmid-Mediated Extended Spectrum Beta-Lactamases (ESBLs)

A relatively recent and clinically important group of plasmid-mediated enzymes is the extended-spectrum β-lactamases (ESBLs). The first identification of oxyimino β-lactamase-type ESBLs occurred in 1983 in Frankfurt, Germany (Knothe H, 1983). Since their discovery, researchers have characterized nearly 40 different ESBL variants. Over the years, bacteria—particularly members of the Enterobacteriaceae family—have evolved genetic mechanisms that enable them to deactivate a broad spectrum of β-lactam antibiotics. Notably, ESBL production has been predominantly observed in *K. pneumoniae* and *E. coli* strains.

Most ESBL enzymes arise through mutations in well-known β-lactamases such as TEM-1, TEM-2, and SHV-1. These mutated enzymes not only hydrolyze advanced β-lactam antibiotics like second- and third-generation cephalosporins but also display the troubling ability to inactivate earlier β-lactamase inhibitors, including tazobactam, clavulanic acid, and sulbactam. Because of their capacity to target oxyimino-lactam antibiotics, these enzymes are collectively referred to as ESBLs (Garau J, 1984).

The emergence and spread of ESBL-producing bacteria pose a significant threat to antibiotic efficacy, complicating treatment protocols and underscoring the need for ongoing surveillance and development of novel therapeutic strategies.

1.11.4 SHV

Among the various extended-spectrum β-lactamases (ESBLs), SHV-type enzymes are often reported as more prevalent within clinical bacterial isolates (Paterson & Bonomo, 2005). The acronym SHV stands for “sulfhydryl variable,” a name that originated from early investigations into the enzyme's sensitivity to inhibitors. Initially, it was proposed that the inhibition of SHV β-lactamase activity by p-chloromercuribenzoate depended on the specific substrate used during testing, reflecting substrate-dependent variability in the enzyme’s response (Jacoby, 1997). However, subsequent experiments employing purified SHV enzymes did not confirm this substrate-related inhibition.

The kinetic properties and biochemical behavior of SHV-type β-lactamases have been extensively reviewed and summarized in the literature, providing comprehensive insights into their function and role in antibiotic resistance (Heritage, 1999; R.B., and K. B. Sykes, 1982).

Understanding these details is crucial, as SHV β-lactamases continue to contribute significantly to the evolving challenge of cephalosporin and penicillin resistance in clinical settings.

1.11.5 TEM

TEM-1 and TEM-2 serve as the foundational enzymes for the TEM family of extended-spectrum β-lactamases. The first discovery of TEM-1 dates back to 1965, originating from an *E. coli* isolate obtained from a patient named Temoniera in Athens, Greece—hence the name TEM (DATTA & KONTOMICHALOU, 1965). TEM-1 exhibits limited activity against extended-spectrum cephalosporins but is highly efficient in hydrolyzing ampicillin, outperforming its activity against other β-lactams such as carbenicillin, oxacillin, and cephalothin. Notably, its activity can be effectively inhibited by clavulanic acid, a β-lactamase inhibitor. Though TEM-2 shares a very similar hydrolytic profile with TEM-1, it differs in having a native promoter that drives higher gene expression, leading to increased enzyme production. This difference is also reflected in its isoelectric point, which is slightly greater at 5.6 compared to 5.4 for TEM-1. Furthermore, TEM-13 shares a hydrolytic capability akin to both TEM-1 and TEM-2, aligning it closely with its predecessors in this enzyme family (Jacoby & Medeiros, 1991).

These ancestral enzymes illustrate the evolutionary roots of many clinically important TEM variants, which have acquired mutations extending their spectrum of activity, thereby complicating treatment options for bacterial infections.

1.11.6 CTX-M and Toho β-Lactamases

The CTX-M family of β-lactamases derives its name from its potent ability to hydrolyze cefotaxime, a third-generation cephalosporin antibiotic. Bacteria producing CTX-M-type enzymes typically display minimum inhibitory concentrations (MICs) for cefotaxime that fall within the resistant range, often around 64 µg/ml. In contrast, the MICs for ceftazidime, another cephalosporin, generally remain in a range that appears susceptible (2 to 8 µg/ml). However, certain variants of CTX-M enzymes possess extended activity that enables them to break down ceftazidime effectively, driving resistance levels as high as 256 µg/ml in some cases (Stürenburg et al., 2004). The susceptibility profile for aztreonam, a monobactam antibiotic, varies depending on the bacterial context and the specific CTX-M enzyme expressed.

Of note, CTX-M β-lactamases also efficiently hydrolyze cefepime, a fourth-generation cephalosporin, resulting in elevated MICs that are often higher than those observed with other ESBL types (Yu, n.d.). When it comes to inhibiting CTX-M enzymes, tazobactam demonstrates

a remarkable potency, proving to be nearly ten times more effective than clavulanic acid in neutralizing their activity.

Closely related to these enzymes are Toho-1 and Toho-2 β-lactamases, named after Toho University School of Medicine Omori Hospital in Tokyo, where the Toho-1-producing *E. coli* was first isolated from a pediatric patient. Structurally, these Toho enzymes resemble CTX-M types and share a similar hydrolytic preference—they exhibit stronger activity against cefotaxime compared to ceftazidime, paralleling the behavior of most CTX-M β-lactamases (Paterson & Bonomo, 2005). Understanding the nuances of CTX-M and Toho β-lactamases is vital in clinical microbiology and antibiotic stewardship, as their varied enzymatic profiles directly impact therapeutic choices and outcomes in bacterial infections.

1.11.7 OXA

OXA-type β-lactamases derive their name from their ability to hydrolyze the antibiotic oxacillin. These enzymes are predominantly found in *P. aeruginosa* but have also been detected in a range of other Gram-negative bacterial species. Among them, OXA-1 is one of the more commonly encountered variants and has been identified in approximately 1 to 10% of *E. coli* isolates (Paterson & Bonomo, 2005). Unlike extended-spectrum β-lactamases (ESBLs), most OXA-type enzymes demonstrate only limited hydrolytic activity against extended-spectrum cephalosporins, which precludes their classification as ESBLs. Notably, OXA-10 exhibits modest ability to hydrolyze antibiotics such as cefotaxime, ceftriaxone, and aztreonam, leading to a measurable decrease in susceptibility in many bacterial strains.

This subtle enzymatic activity poses a clinical concern, as it can reduce the effectiveness of certain β-lactam antibiotics, complicating treatment strategies. Understanding the distribution and spectrum of OXA β-lactamases is crucial for guiding appropriate antimicrobial therapy and for monitoring emerging trends in resistance.

1.11.8 DHA

The *bla*$_{\text{DHA}}$ genes encode AmpC-type β-lactamases, enzymes that hydrolyze a broad range of β-lactam antibiotics, including penicillins and cephalosporins. These enzymes are predominantly produced by members of the Enterobacteriaceae family, with Enterobacter cloacae being one of the most common carriers. Clinically, DHA-type AmpC β-lactamases are commonly linked to resistance to third-generation cephalosporins, posing significant therapeutic challenges (Paterson & Bonomo, 2005).

Unlike extended-spectrum β-lactamases (ESBLs), DHA-type AmpC enzymes are notably more difficult to inhibit using traditional β-lactamase inhibitors such as clavulanic acid, which limits treatment options in clinical settings. Among these enzymes, DHA-1 is the most prevalent and has been frequently implicated in healthcare-associated infections, particularly in intensive care units. The presence of *bla*$_{DHA}$ genes within bacterial isolates significantly elevates their resistance profiles, often complicating antibiotic management strategies in vulnerable patient populations.

Although organisms producing DHA-type AmpC β-lactamases typically remain susceptible to carbapenems unless accompanied by additional resistance mechanisms, they consistently exhibit resistance to several cephalosporins like cefotaxime, ceftazidime, and ceftriaxone. This resistance spectrum underscores the importance of vigilant antimicrobial stewardship and precise diagnostic testing to guide effective therapy.

1.11.9 KPC (Klebsiella pneumoniae Carbapenemase)

The *bla*$_{KPC}$ gene encodes for Klebsiella pneumoniae carbapenemase (KPC), an enzyme that grants bacteria the ability to break down carbapenem antibiotics such as meropenem, imipenem, and ertapenem, which are often considered drugs of last resort. Bacteria producing KPC, particularly Klebsiella pneumoniae, frequently exhibit multidrug resistance, greatly complicating treatment efforts and posing serious challenges to healthcare providers (Nordmann & Poirel, 2002).

Originally identified in *K. pneumoniae* isolates from the United States during the early 1990s, the presence of *bla*$_{KPC}$ has since disseminated globally. This rapid expansion has been a significant factor behind severe hospital-acquired infections worldwide. KPC enzymes belong to the Class A β-lactamase family and possess an extensive substrate range, enabling them to hydrolyze a diverse range of β-lactam antibiotics, thereby undermining many frontline treatment options.

The alarming spread of KPC-producing bacterial strains has raised considerable concern within clinical settings, especially given the increasing detection of *bla*$_{KPC}$ genes among other problematic hospital pathogens such as *A. baumannii* and *P. aeruginosa*. This surge highlights the critical necessity for improved infection control protocols and the creation of novel therapeutic approaches to contain and manage these potent multidrug-resistant pathogens.

1.11.10 NDM (New Delhi Metallo-beta-lactamase)

The *bla*$_{NDM}$ gene encodes the New Delhi metallo-beta-lactamase enzyme, commonly known as NDM, which is a type of metallo-beta-lactamase that confers resistance to carbapenems, as well as a wide variety of other β-lactam antibiotics. This enzyme was first identified in 2008 in an *E. coli* isolate obtained from a patient in India and was subsequently named NDM-1 (Yong et al., 2009b). Representing a significant global public health threat, NDM can hydrolyze a broad range of β-lactam antibiotics, including penicillins, cephalosporins, and carbapenems, thereby severely limiting therapeutic options (Yong et al., 2009b).

NDM belongs to the Class B family of metallo-beta-lactamases, requiring zinc ions as cofactors to facilitate its enzymatic activity. Unlike many other β-lactamases, NDM is not inhibited by conventional β-lactamase inhibitors such as clavulanic acid, which further complicates treatment strategies.

Among bacterial species, *E. coli* and *K. pneumoniae* are the most frequently documented carriers of *bla*$_{NDM}$. Since its discovery, the gene has spread rapidly worldwide, with particularly high prevalence reported in regions such as South Asia, Europe, and North America. The swift dissemination of *bla*$_{NDM}$ underscores the escalating concerns surrounding carbapenem-resistant organisms (CROs), which pose challenges for managing multidrug-resistant (MDR) infections due to the limited number of effective antibiotics available (Bonomo & Szabo, 2006).

The gene's frequent localization on plasmids facilitates horizontal transfer among diverse bacterial species, thereby accelerating its spread within both hospital settings and the wider community. Effective containment of *bla*$_{NDM}$-positive infections hinges on rigorous infection control measures coupled with the urgent development of novel antibiotics or inhibitors capable of overcoming NDM-mediated resistance. Such multifaceted approaches are essential to combat the growing threat posed by *bla*$_{NDM}$-carrying pathogens to global health.

1.11.11 CMY (Cephalosporinase)

The *bla*$_{CMY}$ gene encodes CMY-type cephalosporinases, a subgroup of AmpC β-lactamases that confer resistance primarily to cephalosporins, with a notable impact on third-generation agents such as cefotaxime and ceftriaxone. These enzymes are commonly detected in members of the Enterobacteriaceae family, including *E. coli* and Salmonella species. *bla*$_{CMY}$-producing bacteria

are frequently implicated in healthcare-associated infections, underscoring their clinical importance in hospital environments.

The dissemination of *bla*$_{CMY}$ is often facilitated by plasmids, which enable horizontal gene transfer among various bacterial species—this is particularly prevalent in regions experiencing high levels of antibiotic consumption. Organisms harboring *bla*$_{CMY}$ typically exhibit resistance to penicillins and cephalosporins, yet they generally remain susceptible to carbapenems, unless further resistance mechanisms, such as extended-spectrum β-lactamases (ESBLs) or carbapenemases, are concurrently present.

The global rise of *bla*$_{CMY}$-associated resistance spans multiple continents, with increasing occurrences documented in Asia, Europe, and North America (Rodríguez-Baño et al., 2008). To curb this expanding threat, it is imperative to implement robust surveillance strategies focused on AmpC-producing strains, alongside promoting judicious antimicrobial use in both clinical and veterinary settings. These procedures are essential for mitigating the advancement of resistance and maintaining the efficacy of vital antibiotics.

1.12 Different screening techniques of ESBLs

According to the interpretation guidelines established by the National Committee for Clinical Laboratory Standards (NCCLS), now known as the Clinical and Laboratory Standards Institute (CLSI), the presence of β-lactamase enzymes does not always elevate the minimum inhibitory concentrations (MICs) to levels that would categorize a bacterial isolate as resistant. While most extended-spectrum β-lactamases (ESBLs) confer resistance to one or more oxyimino-lactam antibiotics, the specific cephalosporin chosen for susceptibility testing profoundly influences the sensitivity and specificity of ESBL detection.

Several researchers have suggested that susceptibility tests involving cefpodoxime—whether by dilution or disk diffusion methods—demonstrate superior effectiveness in identifying ESBL producers compared to those using ceftazidime, cefotaxime, or ceftriaxone (Dongeun et al., 2003). Furthermore, the accuracy of various susceptibility testing platforms appears to differ in their capacity to detect cephalosporin resistance associated with ESBL-producing strains.

A notable example comes from Project ICARE (Intensive Care Antimicrobial Resistance Epidemiology), where (Steward et al., 2000) evaluated hospital laboratories' proficiency in recognizing antibiotic resistance mechanisms. In this assessment, only 35% of laboratories employing the Vitek system correctly identified ceftazidime and ceftriaxone resistance in a challenging Klebsiella pneumoniae ESBL strain. Contrastingly, all laboratories utilizing the

MicroScan system reported resistance in the same strain, highlighting significant variation among testing platforms.

The quest for reliable, accurate methods to detect ESBLs in clinical isolates remains ongoing. Current susceptibility testing approaches often suffer from limited sensitivity and specificity, posing challenges for the timely and precise identification of infections. Since ESBLs were first recognized, a multitude of testing techniques and protocols have emerged to improve detection rates. Detecting ESBL-mediated resistance is a complex challenge in numerous clinical microbiology laboratories, primarily due to the recent emergence and dissemination of novel community-acquired ESBL variants, which further complicate diagnostics.

Multiple phenotypic assays have been suggested for the identification and confirmation of ESBLs. Despite their utility, these tests are generally performed after the isolation of bacteria and completion of routine antibiotic susceptibility testing, limiting rapidity in clinical decision-making (Ben-Ami, 2006).

To screen for extended-spectrum β-lactamase (ESBL) production in Klebsiella, *E. coli*, and *Proteus mirabilis*, the Clinical and Laboratory Standards Institute (CLSI) recommends the use of disk diffusion susceptibility testing. This method allows laboratories to identify potential ESBL producers by observing specific antibiotic inhibition zone sizes that raise suspicion for ESBL activity. Several antibiotics can be employed for this initial screening, including cefpodoxime, ceftazidime, cefotaxime, aztreonam, and ceftriaxone. Utilizing multiple agents for screening improves the overall sensitivity, thereby increasing the likelihood of detecting ESBL formation.

When the zone diameters around any of these antibiotic disks fall below established thresholds, it warrants further investigation through phenotypic confirmatory tests to definitively establish ESBL production (Clinical and Laboratory Standard Institute (2009), 2009). Although cefpodoxime is not commonly used in inpatient treatment regimens, it serves as an effective screening antibiotic. Research has demonstrated that cefpodoxime disk diffusion testing effectively discriminates between ESBL-producing and non-producing strains of *E. coli* and *K. pneumoniae*.

Initially, the CLSI set the screening criterion for cefpodoxime at a zone diameter of 22 millimeters or less with a 10-µg disk. However, subsequent findings revealed that this cutoff lacked adequate specificity, particularly when applied to *E. coli* isolates. To improve selectivity, the CLSI revised the guideline, lowering the screening cutoff to a zone diameter of 17

millimeters or less. Isolates showing a zone diameter at or below this threshold should undergo confirmatory phenotypic testing to verify the presence of ESBL enzymes (Clinical and Laboratory Standard Institute (2009), 2009).

By refining these screening parameters and emphasizing confirmatory assays, clinical laboratories can more accurately identify ESBL-producing organisms, which is essential for appropriate antimicrobial stewardship and patient management.

The Clinical and Laboratory Standards Institute (CLSI) recommends dilution methods for screening *E. coli* and Klebsiella isolates that produce extended-spectrum β-lactamases (ESBLs). Specifically, the screening concentration for antibiotics such as ceftazidime, aztreonam, cefotaxime, and ceftriaxone is set at 1 µg/ml. When bacterial growth takes place at this concentration—meaning the minimum inhibitory concentration (MIC) for the cephalosporin is equal to or exceeds 2 µg/ml—the isolate is flagged as a potential ESBL producer and should undergo further phenotypic confirmation testing to verify this resistance mechanism (Clinical and Laboratory Standard Institute (2009), 2009).

Initially, isolates exhibiting a cefpodoxime MIC of 2 µg/ml or higher were also considered candidates for ESBL screening. However, a detailed study examining 59 *E. coli* strains with cefpodoxime MICs of 2 or 4 µg/ml found that none produced ESBL enzymes. Instead, the most common cause behind the decreased susceptibility to cefpodoxime was the alteration or loss of a key porin protein responsible for antibiotic uptake, often coupled with the expression of TEM-1 β-lactamase. Some isolates, while not producing TEM-1, still harbored modifications in their porin channels, occasionally alongside a minor upregulation of chromosomally encoded AmpC β-lactamase. Additionally, a few isolates were found to produce OXA-30 β-lactamase, adding another layer of resistance complexity (Oliver, 2002).

These findings highlight that reduced susceptibility to cefpodoxime may arise from multiple mechanisms beyond ESBL production, underscoring the importance of comprehensive testing and characterization of resistance determinants in clinical isolates to guide appropriate antimicrobial therapy.

1.13 Clinical microbiology techniques

The Clinical and Laboratory Standards Institute (CLSI) recommends using discs containing cefotaxime (30 µg) or ceftazidime (30 µg), both with and without clavulanate (10 µg), for the phenotypic confirmation of extended-spectrum β-lactamase (ESBL) production in *E. coli* and Klebsiella species. Before combination discs with clavulanic acid became widely available, it

was recommended to apply a clavulanic acid solution to the cephalosporin discs within one hour after placing them on agar plates, ensuring proper interaction for ESBL detection. The CLSI further recommends performing the disc diffusion test on Mueller-Hinton agar plates exhibiting confluent bacterial growth to optimize accuracy.

Phenotypic confirmation of ESBL presence is indicated when there is a difference of at least 5 millimeters between the inhibition zone diameters of the cephalosporin disc alone and the corresponding cephalosporin/clavulanate combination disc. This increase reflects the inhibitory effect of clavulanic acid on β-lactamase enzymes.

An alternative approach, known as the disc replacement technique, involves initially inoculating a Mueller-Hinton agar plate with the test organism, then placing three amoxicillin/clavulanate discs on the surface. After incubating at room temperature for one hour, these discs are carefully removed and replaced at the exact spots with discs containing cefotaxime, ceftazidime, and aztreonam. Simultaneously, control discs of the three antibiotics are placed on the plate at least 30 millimeters away from the replacement sites. A positive ESBL result is observed if the inhibition zone around the replacement discs exceeds that of the control discs by more than 5 millimeters.

Despite its effectiveness, the disc replacement method is less practical for high-throughput clinical microbiology laboratories due to the requirement for a second step—removing and substituting discs one hour after inoculation—which can be labor-intensive and time-consuming (Casals J, 1990).

Broth microdilution assays can utilize a range of antibiotic concentrations to perform phenotypic confirmatory testing for extended-spectrum β-lactamase (ESBL) production. Specifically, ceftazidime alone (ranging from 0.25 to 128 µg/ml), ceftazidime combined with clavulanic acid (0.25/4 to 128/4 µg/ml), cefotaxime alone (0.25 to 64 µg/ml), and cefotaxime with clavulanic acid (0.25/4 to 64/4 µg/ml) are commonly employed. It is crucial to include both ceftazidime and cefotaxime in these assays to ensure comprehensive detection. The broth microdilution method follows standardized protocols to accurately determine the minimum inhibitory concentrations (MICs). Phenotypic confirmation of ESBL production is achieved when the MIC of either cephalosporin decreases by at least threefold—or, in some criteria, by twofold or greater—upon addition of clavulanic acid compared to its MIC when tested alone. This significant reduction indicates the inhibitory effect of clavulanic acid on β-lactamase enzymes and confirms the presence of ESBL activity (Queenan AM, 2004).

Including both cephalosporins in the testing enhances the assay's sensitivity, accounting for variable enzyme activities and minimizing false negatives. These carefully calibrated procedures are essential for guiding effective antimicrobial therapy and improving patient outcomes.

A disc diffusion method was introduced by (Jarlier V, 1988) to identify extended-spectrum β-lactamase (ESBL) production by evaluating the interaction between beta-lactam antibiotics and β-lactamase inhibitors on agar plates. In this test, a 30 µg disc of cefotaxime and a 20 µg amoxicillin/10 µg clavulanate disc were placed 30 millimeters apart (measured center to center) on an agar surface inoculated with the test organism. The hallmark of ESBL presence was a "keyhole" effect—manifested as a noticeable distortion or extension of the cefotaxime inhibition zone edge toward the clavulanate disc, indicating synergy between the β-lactam antibiotic and the inhibitor.

Recognizing that the keyhole enhancement may not always appear with cefotaxime, additional 30 µg discs of ceftazidime, aztreonam, and ceftriaxone were similarly positioned 30 millimeters from the amoxicillin/clavulanate disc. These antibiotics, all containing the oxyanion group, frequently reveal synergy even when cefotaxime does not. The double-disc diffusion test demonstrates high diagnostic reliability, with reported sensitivities ranging between 79% and 97%, and specificities from 94% up to 100% when performed on bacterial strains genotypically confirmed as either ESBL producers or non-producers.

However, it has been documented that certain ESBL variants, such as those carrying TEM-12, SHV-2, and SHV-3 enzymes, may yield false-negative outcomes in this assay. To mitigate this, it is recommended that for isolates suspected of ESBL production but testing negative with the standard 30 mm disc spacing, the test be repeated with a narrower gap—around 20 millimeters center-to-center—to improve detection sensitivity (Perez F, 2007).

One of the principal advantages of this double-disc synergy test lies in its technical simplicity and cost-effectiveness, making it accessible in various laboratory settings. Nevertheless, interpretation demands careful judgment, as the degree of ESBL activity influences the test's sensitivity. Particularly at low expression levels of ESBLs, inhibition zones around cephalosporin and aztreonam discs may appear large and diffuse, potentially obscuring synergy detection. An example is Proteus mirabilis, which has been observed to present such subtle phenotypic effects, complicating ESBL identification.

In summary, while the double-disc diffusion test is a valuable tool for ESBL screening due to its ease and accuracy, it requires astute interpretation and occasional methodological adjustments to ensure reliable detection across diverse bacterial strains.

The three-dimensional test offers a phenotypic method to detect the inactivation of aztreonam or extended-spectrum cephalosporins by ESBLs, without specifically requiring evidence of β-lactamase inhibitor-mediated enzyme inhibition. In this procedure, a susceptible indicator organism to the β-lactam antibiotic is first cultured on a susceptibility agar plate using standard disk diffusion methodology. Then, either a small well or slit is created near the β-lactam antibiotic disc—typically about 7 to 8 millimeters from the disc edge in a spot form, or about 5 millimeters away along an outward radial slit in the agar. Into this well or slit, approximately 25 microliters of a turbid bacterial suspension—prepared to match a McFarland standard of 5 and presenting a milky appearance—is carefully applied. Following incubation at 37°C overnight, a positive result is indicated by a noticeable distortion or indentation in the inhibition zone around the β-lactam antibiotic disc, caused by the enzyme activity of the tested strain. This change manifests as a reduction in the zone's diameter, providing compelling phenotypic proof of ESBL-mediated hydrolysis of the antibiotic (Shahid M, 2007). This technique's strengths lie in its direct visualization of enzymatic effects on antibiotic activity, offering a valuable complement to other susceptibility and confirmatory tests, particularly in laboratories seeking practical yet reliable methods for detecting ESBLs.

1.14 Commercial detection techniques for ESBLs identification

1.14.1 E-test for ESBLs

AB Biodisk (Solna, Sweden) manufactures plastic strips impregnated with gradients of antibiotics designed for precise MIC testing. One end of these strips incorporates a gradient of ceftazidime, spanning concentrations from 0.5 to 32 µg/ml, while the opposite end contains a fixed amount of clavulanate (4 µg/ml) combined with varying ceftazidime concentrations. Similar strips are also commercially available for cefotaxime and combinations of cefotaxime with clavulanate. This testing approach boasts high phenotypic confirmatory accuracy for extended-spectrum β-lactamase (ESBL) detection, with reported sensitivities ranging from 87% to 100% and specificities between 95% and 100%. The test’s reliability hinges on comparing the minimum inhibitory concentrations (MICs) of the cephalosporin alone to those of its combination with clavulanate. In accordance with the manufacturer's specifications, a decrease of eightfold or more in MIC when clavulanate is added is indicative of ESBL production.

However, a technical challenge can arise due to the diffusion of clavulanic acid from one end of the strip, potentially distorting the inhibition zone near the cephalosporin-only gradient. This interference can complicate the precise determination of MIC for the cephalosporin alone. In such circumstances, many laboratory professionals advocate using separate conventional strips containing only ceftazidime or cefotaxime to more accurately measure the standalone MIC. Importantly, having both cefotaxime and ceftazidime strips available enhances the capacity to detect various ESBL variants, especially those like CTX-M-type enzymes that demonstrate a preferential ability to hydrolyze cefotaxime over other cephalosporins (Stürenburg E, 2004). This flexibility ensures a more thorough and sensitive assessment of resistance profiles, supporting optimal antimicrobial stewardship and clinical decision-making.

1.14.2 Automated ESBL testing

ESBL-producing bacteria are commonly detected using advanced automated systems designed for bacterial identification and antimicrobial susceptibility testing. The BD Phoenix System (Becton Dickinson Biosciences, Sparks, MD) evaluates bacterial growth responses to antibiotics such as ceftazidime, cefotaxime, ceftriaxone, and cefpodoxime, both alone and in combination with clavulanate, interpreting the results through its specialized expert software. Similarly, the Vitek 2 system (bioMerieux, Marcy L'Etoile, France) utilizes cards containing ceftazidime and cefotaxime individually and in combination with clavulanate to assess ESBL production. Meanwhile, the MicroScan WalkAway-96 System (Dade Behring, Inc., West Sacramento, CA) incorporates β-lactamase inhibitors alongside ceftazidime or cefotaxime within its testing panels. A comparative evaluation of these three semi-automated platforms against traditional phenotypic confirmatory assays — focusing on their ability to identify ESBL-producing Enterobacteriaceae such as Enterobacter spp., Citrobacter freundii, and Serratia marcescens — revealed that the Phoenix system exhibited the highest sensitivity at 99%. The Vitek 2 system followed this at 86% sensitivity and MicroScan at 84% (Rice LB, 2000). These findings underscore the superior detection performance of the Phoenix system in clinical settings. Although all three automated methods offer valuable tools for reliable and rapid ESBL identification, thereby facilitating timely and appropriate antimicrobial therapy, they also highlight the Phoenix system's advantages.

1.14.3 Molecular detection techniques of ESBLs

Early detection of β-lactamase genes, particularly those encoding TEM and SHV enzyme families, was accomplished using specific DNA probes. These probes played a crucial role in

studying the initial extended-spectrum β-lactamases (ESBLs). Among molecular approaches, the polymerase chain reaction (PCR) remains the most straightforward and widely applied technique for identifying the presence of β-lactamase genes, employing oligonucleotide primers targeted to specific gene sequences (Wu TL, 2001). However, a limitation of conventional PCR is its inability to differentiate among various TEM or SHV variants, as it merely confirms gene presence without resolving subtle genetic differences (Fluit AC, 2001).

Discerning between different ESBL variants—such as TEM-3 or SHV-2—and their respective non-ESBL parent enzymes like TEM-1, TEM-2, or SHV-1 necessitates more detailed analysis through DNA sequencing (Garza Ramos U, 2007). To circumvent the need for sequencing, researchers have developed several molecular methodologies that provide variant discrimination. For example, oligotyping employs oligonucleotide probes designed to hybridize stringently to sequences harboring characteristic point mutations, enabling differentiation between TEM-1 and TEM-2. This approach facilitated the identification of numerous novel TEM variants, though mutations responsible for broadening enzymatic substrate profiles may sometimes evade probe detection due to their subtle sequence changes (Queenan AM, 2004). Certain mutations also alter restriction enzyme recognition sites by creating or abolishing them, allowing PCR amplification of target β-lactamase gene segments followed by restriction fragment length polymorphism (RFLP) analysis to infer the presence of specific TEM or SHV-derived ESBLs. Another promising technique is PCR-single-strand conformation polymorphism (PCR-SSCP), which detects single nucleotide polymorphisms by changes in DNA strand mobility. This method has successfully identified point mutations within β-lactamase genes with encouraging accuracy (Wiegand I, 2007). Enhancing specificity further, the combination of PCR-SSCP with PCR-RFLP proves effective in distinguishing newer SHV variants. Additionally, the ligase chain reaction (LCR) offers high precision in detecting SHV genes by differentiating DNA sequences that vary by even a single nucleotide, making it a valuable tool distinct from conventional PCR and sequencing (Wiegand I, 2007). More recently, advancements include the continued progress in sequence-specific peptide nucleic acid (PNA)-based multiplex PCR assays, exemplified by accurate detection of *bla*_GES-2 genes. These PNA probes offer enhanced specificity and sensitivity compared to traditional methods, enabling the rapid and precise identification of clinically relevant β-lactamase variants without the need for laborious sequencing. Collectively, these molecular diagnostic tools provide a robust arsenal for detecting and characterizing β-lactamase enzymes, offering vital support for

clinical microbiology laboratories that strive to efficiently monitor the evolving landscape of antimicrobial resistance.

Technique type	Test	Advantages	Disadvantages	Reference(s)
Clinical microbiology	Standard NCCLS interpretive criteria	Easy to use, performed in every lab	ESBLs not always "resistant"	78, 111
	NCCLS ESBL confirmatory test	Easy to use and interpret	Sensitivity depends on choice of oxyimino-cephalosporin	111
	Double-disk approximation test	Easy to use, easy to interpret	Distance of disk placement for optimal sensitivity not standardized	76, 169, 71
	Three-dimensional test	Sensitive, easy to interpret	Not specific for ESBLS, labor intensive	169
	Etest ESBL strips	Easy to use	Not always easy to interpret, not as sensitive as double-disk test	183
	Vitek ESBL test	Easy to use, easy to interpret	Reduced sensitivity	149, 164
Molecular detection	DNA probes	Specific for gene family (e.g., TEM or SHV)	Labor intensive, cannot distinguish between ESBLs and non-ESBLs, cannot distinguish between variants of TEM or SHV	7, 55, 70
	PCR	Easy to perform, specific for gene family (e.g., TEM or SHV)	Cannot distinguish between ESBLs and non-ESBLs, cannot distinguish between variants of TEM or SHV	42, 90, 116
	Oligotyping	Detects specific TEM variants	Requires specific oligonucleotide probes, labor intensive, cannot detect new variants	117
	PCR-RFLP	Easy to perform, can detect specific nucleotide changes	Nucleotide changes must result in altered restriction site for detection	116
	PCR-SSCP	Can distinguish between a number of SHV variants	Requires special electrophoresis conditions	106, 107
	LCR	Can distinguish between a number of SHV variants	Requires a large number of oligonucleotide primers	80
	Nucleotide sequencing	The gold standard, can detect all variants	Labor intensive, can be technically challenging, can be difficult to interpret manual methods	25

Figure 1-6 A table representing the detection techniques of ESBL. Adopted from CLINICAL MICROBIOLOGY REVIEWS (A, 2001)

1.15 Prevalence of ESBLs in Gram-negative bacteria in biomedical waste

1.15.1 The global scenario of ESBLs

Waste water environments are increasingly recognized as critical reservoirs for pathogenic bacteria, particularly multidrug-resistant Enterobacteriaceae that produce extended-spectrum β-lactamases (ESBL-E). These bacteria pose significant risks to both human and animal health due to their ability to spread via diverse virulence factors and their robust antibiotic resistance profiles. The rising development of antibiotic resistance among Enterobacteriaceae has become a major public health concern worldwide (Azuma, 2020). When antibiotics enter waste water systems, they exert selective pressure on resident bacterial communities, thereby fostering the proliferation and dissemination of resistant strains.

A comprehensive systematic review and meta-analysis conducted by Zaatout et al. in 2021 evaluated the global prevalence of ESBL-producing Enterobacteriaceae across various geographic regions (Zaatout et al., 2021). Analyzing fifty-seven observational studies, the pooled prevalence of ESBL-E detected in waste water samples was approximately 24.8% (95%

confidence interval [CI], 19.28–30.77). Among the bacteria studied, *E. coli* exhibited the highest rate of ESBL production. Moreover, the *bla*$_{\text{CTX-M}}$ gene family emerged as the most dominant resistance determinant, featuring in 66.56% of ESBL-positive isolates. The meta-analysis highlighted stark regional differences: waste water samples from the Americas showed a notably higher combined prevalence of ESBL-E (39.91%, 95% CI, 21.82–59.51), with hospital and untreated waste water samples representing particularly significant reservoirs (33.98% and 27.36% prevalence respectively). Hospital waste water, in particular, stands out as a major hotspot for ESBL-E, a consequence likely linked to the intensive use of antibiotics within healthcare settings. Supporting this, (Kümmerer, 2009) reported that antibiotic concentrations in hospital effluents can be up to 100 times greater than those found in municipal waste waters. Such high antibiotic loads in hospital discharge create a highly selective environment conducive to the emergence and maintenance of ESBL-producing bacteria (Manel et al., 2018) (Duong et al., 2008). From a global perspective, Europe, which also leads in ESBL-E research, exhibits the lowest overall prevalence rates. Conversely, Asia and the Americas—especially South America, where the majority of studies originate from Brazil—show substantially elevated ESBL-E prevalence, with rates of approximately 39.9% and 33.95%, respectively. Meta-regression analyses conducted within the study confirmed a significant association between the presence of ESBL-E in waste water and studies originating from the Americas.

In many developing regions, inadequate treatment of hospital, municipal, and agricultural waste waters exacerbates the problem. Due to limited access to effective waste water treatment infrastructure, resistant bacteria such as ESBL-producing Enterobacteriaceae contaminate natural water bodies, thereby posing ongoing threats to public health and the environment (Behnam et al., 2020; WHO, 2018). Taken together, these findings underscore the urgent need for improved antibiotic stewardship in healthcare and agriculture, enhanced waste water treatment practices, and global surveillance strategies. Such measures are vital to curtail the environmental dissemination of multidrug-resistant bacteria and protect both human and ecological health from the escalating threat of antimicrobial resistance.

Subgroups	No. of studies	No. of ESBL	No. of *Enterobacteriaceae*	Pooled estimate (%) of ESBL	95% Confidence interval	Heterogeneity chi-squared (χ^2)	Heterogeneity test, I^2 (%)	Heterogeneity test, *p*-value
Continents								
Europe	18	1,112	15,700	10.19	5.73, 15.72	1,645.83	98.97	<0.001
Asia	18	830	2,433	33.95	24.05, 44.58	449.28	96.22	<0.001
Africa	13	388	1,285	29.31	17.86, 42.17	250.07	95.20	<0.001
America	8	288	812	39.91	21.82, 59.51	206.09	96.60	<0.001
Overall	57	2,618	20,230	24.81	19.28, 30.77	4,461.79	98.74	<0.001
Wastewater origin								
Hospital wastewater	17	743	2,344	33.98	23.82, 44.91	442.77	96.39	<0.001
Municipal wastewater	15	929	11,267	18.83	11.15, 27.88	1,410.51	99.01	<0.001
Wastewater from rivers	7	259	1,118	13.67	5.36, 24.64	96.29	93.77	<0.001
Hospital and municipal wastewaters and their receiving rivers	13	598	3,969	29.41	15.03, 46.19	1,123.05	98.93	<0.001
Wastewater from other sources (farms and slaughterhouses)	5	89	1,532	18.43	1.04, 48.62	273.15	98.54	<0.001
Overall	57	2,618	20,230	24.81	19.28, 30.77	4,461.79	98.74	<0.001
The use of treatment								
Treated wastewater	9	335	2,614	21.11	7.40, 39.17	649.12	98.77	<0.001
Untreated wastewater	23	1,229	6,350	27.36	19.12, 36.42	956.04	97.70	<0.001
Treated and untreated wastewater	25	1,054	11,266	23.93	15.87, 33.03	2,286.92	98.95	<0.001
Overall	57	2,618	20,230	24.81	19.28, 30.77	4,461.79	98.74	<0.001
Year of publication								
2020	11	200	1,201	19.32	10.31, 30.17	144.56	93.08	<0.001
2019	12	903	4,500	37.62	22.18, 54.43	766.42	98.56	<0.001
2018	9	598	5,401	33.93	14.01, 57.34	1,191.61	99.33	<0.001
2017	5	209	576	34.73	22.53, 47.96	28.30	85.86	<0.001
2016	7	155	1,210	15.51	7.02, 26.45	126.54	95.26	<0.001
2007–2015	13	553	7,342	15.08	7.17, 25.19	1,276.01	99.06	<0.001
Overall	57	2,618	20,230	24.81	19.28, 30.77	4,461.79	98.74	<0.001

Figure 1-7 Stratified pooled prevalence estimates of ESBL-producing Enterobacteriaceae in waste water. (Adopted from Journal of Water and Health Vol 19 No 5, 705) (Zaatout et al., 2021)

Subgroups	No. of studies	No. of ESBL	No. of *Enterobacteriaceae*	Pooled estimate (%) of ESBL	95% Confidence interval	Heterogeneity χ^2	Heterogeneity test, I^2 (%)	Heterogeneity test, *p*-value
***Enterobacteriaceae* species**								
E. coli	51	2,541	19,881	15.05	11.14, 19.41	2,902.78	98.28	<0.001
Klebsiella	29	1,222	5,732	9.66	5.83, 14.25	665.17	95.79	<0.001
Enterobacter	18	1,139	5,125	2.39	1.08, 4.09	149.25	88.61	<0.001
Citrobacter	8	714	1,846	2.69	0.50, 6.23	87.10	91.96	<0.001
Shigella	7	619	1,578	2.10	0.66, 4.19	28.58	79.01	<0.001
Proteus	6	323	900	3.80	1.61, 6.76	18.02	72.26	<0.001
Serratia	5	481	1,036	3.37	0.27, 8.89	48.36	91.73	<0.001
Other species	12	785	2,064	3.07	1.04, 5.96	169.26	93.50	<0.001
ESBL genes	**No. of studies**	**No. of ESBL**	**No. of *Enterobacteriaceae***	**Pooled estimate (%) of ESBL genes**	**95% Confidence interval**	**Heterogeneity χ^2**	**Heterogeneity test, I^2 (%)**	**Heterogeneity test, *p*-value**
$bla_{CTX\text{-}M}$	30	1,621	7,532	66.82%	52.57, 79.79	691.61	96.10	<0.001
bla_{TEM}	25	1,292	4,888	51.01%	35.52, 66.40	661.40	96.37	<0.001
bla_{SHV}	25	1,044	4,875	24.59%	15.42, 34.91	239.93	90.41	<0.001

Figure 1-8 Meta-analysis of ESBL-E at the species level and ESBL-E gene prevalence in waste waters (Adopted from Journal of Water and Health Vol 19 No 5, 705) (Zaatout et al., 2021)

1.15.2 South Asian developing countries' scenario

In the densely populated South Asian countries of Bangladesh, India, and Pakistan, antibiotic resistance has reached concerning levels in both human and animal populations. The rise of antimicrobial resistance (AMR) in these nations is largely attributed to several factors: easy access to antibiotics without prescriptions, common self-medication practices, incomplete courses of antibiotic treatment, excessive and unnecessary prescriptions by healthcare

providers, and indiscriminate use of antibiotics in the veterinary and agricultural sectors (Biswas et al., 2014).

A study conducted in a tertiary care hospital in Dhaka, Bangladesh, revealed that approximately 16% of patients—both those admitted indoors (~50%) and those in outpatient settings (13%)—were infected with extended-spectrum β-lactamase (ESBL)-producing bacteria. Among these isolates, the distribution included 15.75% *E. coli*, 14.01% Pseudomonas species, 36.84% Proteus species, 18.57% Klebsiella species, and 21.05% Acinetobacter species (Jobayer et al., 2017). Another related investigation found that about 34% of *E. coli* isolates associated with extraintestinal infections in patients produced ESBLs (Khan et al., 2018). Moreover, multidrug-resistant ESBL-producing *E. coli*, including the uropathogenic clone O25:H4, were reported to be widespread in Bangladesh, with most isolates carrying the $bla_{\text{CTX-M}}$ gene (Lina et al., 2014).

Further evidence of the extensive presence of ESBL-producing bacteria comes from studies in Rohingya refugee camps in Cox's Bazar, Bangladesh, where nearly 60% of *E. coli* strains in human fecal sludge were ESBL-positive, harboring a variety of genes such as $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$, $bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-25}}$, bla_{TEM}, and bla_{SHV} (Hossain et al., 2021). Alarmingly, stool samples from apparently healthy newborns in rural Bangladeshi communities showed that 74% of *E. coli* isolates were ESBL producers (Islam et al., 2019). One contributing factor to this dissemination in rural areas could be the common practice of disposing of infant feces in yards or nearby ditches, which potentially facilitates the spread of resistant bacteria to domestic and stray birds, as well as other animals (Hasan, 2012).

In India, ESBL-producing Enterobacteriaceae are estimated to constitute 70–90% of isolates, with CTX-M-15 β-lactamase being the predominant enzyme since it was first identified in Delhi in 2000 (Karim, 2001). Notably, from 2013 to 2019, the prevalence of ESBL-positive bacteria in animals increased sharply from 12% to 33% (Kumarasamy et al., 2010b). In northern India, the rate of ESBL occurrence in animals has been recorded as high as 26% (Kuralayanapalya et al., 2019).

Pakistan also reports widespread occurrences of ESBL producers in hospital settings, communities, and among animals across various regions. Among the genotypes identified, the $bla_{\text{CTX-M}}$ gene remains the most prevalent, while the bla_{TEM} and bla_{OXA} genes are less commonly encountered in clinical isolates (Abrar et al., 2019). Of particular concern is the detection of ESBL-producing *E. coli* in approximately 25.41% of milk samples from cattle

suffering from mastitis, highlighting potential public health risks associated with dairy production (Ali et al., 2017).

Together, these findings illustrate a pressing regional challenge where factors such as antibiotic misuse, environmental contamination, and close human-animal interaction synergize to drive the proliferation of multidrug-resistant bacterial strains. Tackling AMR in South Asia requires integrated efforts encompassing strict antibiotic stewardship, improved sanitation practices, community awareness, and enhanced surveillance both in human health and animal husbandry sectors.

	Country	ESBL	*Enterobacteriaceae*	Source	Prevalence	Reference
1	Bangladesh	$bla_{CTX-M-15}$	*E. coli*	Urine	80%	[83]
2	India	$bla_{CTX-M-15}$	*E. coli*	Skin and soft tissue	70%	[84]
3	India	$bla_{CTX-M-15}$	*E. coli*	Urine, pus, extra intestinal clinical samples	25%	[85]
4	Bangladesh	bla_{TEM}	*E. coli*	Urine	50%	[86]
5	Bangladesh	$bla_{CTX-M-15}$	*E. coli*	Rectal swabs	48.2%	[87]
		$bla_{CTX-M-1}$			11.1%	
		bla_{SHV-12}			11.1%	
		$bla_{CTX-M-14}$			7.4%	
		$bla_{CTX-M-27}$			7.4%	
		$bla_{CTX-M-9}$			3.7%	
		$bla_{CTX-M-14b}$			3.7%	
		bla_{SHV-28}			3.7%	
		bla_{TEM-12}			3.7%	
6	Bangladesh	$bla_{CTX-M-1}$	*E. coli*	Clinical specimens	33.9%	[88]
		$bla_{CTX-M-1}$	*K. pneumoniae*		51.4%	
7	Bangladesh	Non-specific	*E. coli*	Urine	25.84%	[81]
		Non-specific	*Klebsiella pneumoniae*		6.6%	
8	Bangladesh	bla_{TEM}	*E. coli*	Urine	22.7%	[89]
		bla_{CTX-M}			24.2%	
		bla_{SHV}			4.3%	
9	Bangladesh	Non-specific	*K. pneumoniae*	Tracheal swabs, sputum, wound swabs, pus, blood, urine	50%	[90]
		Non-specific	*K. oxytoca*		25%	
10	Bangladesh	$bla_{CTX-M-3}$	*Pseudomonas* spp.	Urine, swab, pus	78.0%	[91]
		$bla_{CTX-M-14}$			80.0%	
11	Bangladesh	bla_{TEM}	*E. coli*	Stool	41%	[62]
		$bla_{CTX-M-group-1}$			96%	
12	India	$bla_{CTX-M-15}$	*E. coli*	Urine	52%	[92]
		bla_{OXA-2}			8%	
13	India	Non-specific	*E. coli*	Pus	9.8%	[93]
				Urine	82.6%	
14	North-East India	bla_{CTX-M}	*E. coli*	Urine, sputum, vaginal discharge	54.34%	[94]
		bla_{TEM}			60.86	
		bla_{SHV}			63.04%	
15	South India	$bla_{CTX-M-15}$	*E. coli*	Urine, wound swab, sputum, pus, endotracheal secretions, bronchoalveolar lavage, bile fluid	90%	[95]
16	Bihar, India	bla_{TEM}	*E. coli*	Stool	51.8%	[96]
		bla_{SHV}			68%	
		bla_{CTX-M}			86.1%	
17	India	bla_{SHV}	*Pseudomonas aeruginosa*	Urine, blood, sputum, endotracheal aspirate	15.1%	[97]
		bla_{TEM}			57.1%	
18	Pakistan	$bla_{CTX-M-15}$	*E. coli*	Fecal samples	86.2%	[98]
19	North-West Pakistan	Non-specific	*P. aeruginosa*	Burn patients	35.85%	[99]
20	Lahore, Pakistan	bla_{CTX-M}	*E. coli, Klebsiella* spp., *Pseudomonas aeruginosa, Enterobacter* spp., *Acinetobacter* spp.	Urine, pus, wound swabs	76%	[100]
		bla_{TEM}			28%	
		bla_{SHV}			21%	
21	Faisalabad, Pakistan	$bla_{CTX-M-1}$	*E. coli*	Dog owners	59%	[101]
				Cat owners	73.9%	
				Veterinary professionals	80%	
22	Pakistan	bla_{CTX-M1}	*K. pneumoniae*	Hospital waste	71%	[102]
		bla_{TEM}			53%	
		bla_{SHV}			6%	
23	Lahore, Pakistan	$bla_{CTX-M-I}$	*E. coli*	Clinical specimens	72.1%	[103]
		$bla_{CTX-M-II}$			8.5%	
24	Peshawar, Pakistan	$bla_{CTX-M-15}$	*Pseudomonas aeruginosa*	Clinical specimens	19.71%	[104]
25	Lahore, Pakistan	Non-specific	*E. coli*	Healthy individuals	57.0%	[105]
				Patients	53.0%	
26	Faisalabad, Pakistan	bla_{CTXM-1}	*E. coli*	Urine	70%,	[106]
		bla_{TEM-1}			74.4%	
		$bla_{CTXM-15}$			49%	

Figure 1-9 Current status of ESBL as reported in Bangladesh, India, and Pakistan public health sectors from 2015 to 2023. Adopted from: Biomedicines 2023, 11, 2937 (Husna et al., 2023)

1.16 In-silico prediction of drugs and whole genome analysis

1.16.1 Binding affinity prediction with altered drug

In modern drug discovery, accurately predicting how strongly a molecule binds to or interacts with its biological target—be it a protein, enzyme, or receptor—is fundamental. This binding affinity is a key determinant of a drug's potency and effectiveness, as it determines the potency and specificity of the interaction between the therapeutic compound (ligand) and its intended target (protein). Being able to forecast binding affinity early in the drug development process enables researchers to refine lead candidates, enhancing their binding characteristics and prioritizing the most promising molecules for subsequent experimental evaluation. Precise binding affinity predictions play a crucial role not only in designing new therapeutic agents but also in tackling urgent health challenges such as antimicrobial resistance (AMR). Since the efficacy of antibiotics heavily relies on their ability to bind bacterial targets effectively, computational predictions guide the rational optimization of drugs to combat resistant pathogens.

A variety of computational and experimental approaches collectively contribute to this endeavor. Techniques such as molecular docking provide initial insights by estimating how a ligand fits into the binding site, while molecular dynamics simulations offer detailed views of the dynamic behavior of ligand-target complexes over time. Complementing these, machine learning algorithms have emerged as powerful tools, harnessing large datasets to predict binding affinities with increasing accuracy and speed. The integration of specialized software platforms and curated databases further enhances the reliability of these predictions, enabling high-throughput virtual screening and informed decision-making.

1.16.2 Structure prediction, modification and analysis

PubChem, maintained by the National Center for Biotechnology Information (NCBI), serves as an indispensable resource in modern drug discovery, providing an extensive repository of three-dimensional chemical structures used in pharmaceutical development. This comprehensive database not only provides detailed chemical structure information but also includes valuable bioactivity profiles and binding affinity data for a vast array of compounds. Researchers frequently use PubChem to retrieve chemical structures of potential drug candidates, which serve as foundational inputs for various computational studies, such as molecular docking and quantitative structure-activity relationship (QSAR) modeling. Moreover, PubChem's seamless integration with complementary databases, such as the Protein Data Bank (PDB), significantly

enhances its utility, granting scientists access to protein-ligand complex structures and facilitating the identification of relevant binding sites within target proteins. This interconnected network enables a holistic approach, making PubChem a pivotal starting point in the workflow of binding affinity prediction and rational drug design. Visualization tools such as Chem3D and PyMOL can then be employed to inspect these molecular structures, allowing users to delve into interaction details at the atomic level and better understand how candidate molecules might engage with their biological targets (Kim et al., 2016).

In the realm of chemical structure design and optimization, ChemDraw has become a staple tool among medicinal chemists and pharmaceutical researchers. Its intuitive interface simplifies the process of drawing complex molecular structures, analyzing their chemical properties, and generating three-dimensional models. ChemDraw is particularly valuable in binding affinity prediction workflows, as it empowers researchers to create analogues of lead compounds by systematically altering functional groups or substituting atoms to improve binding affinity or enhance pharmacokinetic properties potentially. Guided by insights gleaned from QSAR analyses or docking experiments, these virtual modifications can be rapidly constructed and evaluated, streamlining the iterative process of drug optimization. Once optimized molecular structures are prepared in ChemDraw, they can be exported seamlessly to specialized computational platforms for deeper analysis. One such advanced tool is Gaussian 16, a robust software package used for high-precision computational chemistry calculations. Through techniques such as Density Functional Theory (DFT) and ab initio quantum mechanical methods, Gaussian 16 enables quantitative predictions of binding affinities by assessing the electronic structure of drug molecules and their interactions with protein targets. Its capacity to handle both small molecules and large biomolecular complexes makes it highly suitable for multifaceted drug discovery projects.

By coupling Gaussian 16's quantum-level energy calculations with molecular docking and molecular dynamics simulations, researchers can obtain comprehensive insights into the energetics and stability of protein-ligand complexes. Calculations of parameters such as binding free energy or enthalpy are crucial in determining the strength and persistence of molecular interactions, thereby enhancing the predictability and reliability of binding affinity estimates. This integrative approach strengthens the drug design pipeline and accelerates the identification of promising therapeutic agents with optimal efficacy and specificity (Frisch et al., 2016). Together, these resources and computational tools form an integrated ecosystem that underpins contemporary drug discovery efforts, bridging chemical information management, molecular

design, and high-level computational modeling to tackle complex challenges such as antimicrobial resistance and beyond.

1.16.3 Molecular docking

Molecular docking stands out as a powerful computational technique widely employed in drug discovery to predict the most favorable orientation and binding strength between small molecules—often potential drugs—and their receptor proteins. In antimicrobial drug research, this method has proven indispensable for evaluating how candidate compounds interact with key bacterial proteins. By simulating the way antimicrobial agents bind to their bacterial targets, docking studies provide a significant understanding of specific binding mechanisms, interaction energies, and the potential efficacy of novel drug candidates, all before embarking on costly and time-consuming laboratory experiments.

The accessibility and convenience of obtaining high-resolution structural data of target proteins, combined with continuous advancements in docking algorithms and software platforms, have fueled the rise of molecular docking in the search for new antibiotics. Crucial to these studies is access to accurate three-dimensional representations of bacterial proteins, such as enzymes and structural components involved in essential biological processes. The Protein Data Bank (PDB) serves this role admirably by maintaining a comprehensive repository of experimentally determined protein structures. Researchers often utilize the RCSB PDB portal (Research Collaboratory for Structural Bioinformatics) to retrieve detailed PDB-format files, which can then be input into docking simulations to model ligand-target interactions precisely (Berman et al., 2000).

Virtual screening campaigns often target crucial bacterial proteins involved in antibiotic resistance or viability, including β-lactamases responsible for degrading antibiotics, DNA gyrase essential for DNA replication, and ribosomal proteins necessary for protein synthesis. By leveraging structural insights from the PDB, molecular docking enables the rapid identification and prioritization of promising antimicrobial compounds, thereby streamlining the drug development pipeline and accelerating the fight against antimicrobial resistance.

1.17 Docking tools for antimicrobial drug discovery

1.17.1 Discovery studio

Discovery Studio is a comprehensive software suite that integrates a wide range of molecular modeling, simulation, and docking analysis capabilities into a single platform. Widely used in

drug discovery, it facilitates virtual screening and the prediction of interactions between bacterial proteins and potential antimicrobial compounds. This versatility allows scientists to perform detailed binding affinity calculations, conduct molecular dynamics simulations, and execute protein-ligand docking studies with high precision.

The software incorporates multiple scoring functions designed to estimate the strength and stability of ligand binding to target proteins, providing crucial insights that help prioritize the most promising drug candidates for further development (Srinivasan et al., 2015). In the field of antimicrobial research, Discovery Studio has played a pivotal role in identifying novel inhibitors against critical bacterial enzymes such as β-lactamases, acetylcholinesterase (AChE), and DNA gyrase — targets intimately involved in antibiotic resistance and bacterial survival (Anwar et al., 2016). By simulating how antimicrobial agents bind to these specific biological targets, researchers can iteratively modify molecular structures to enhance binding affinity, aiming to overcome existing resistance mechanisms. This computational approach not only accelerates the early stages of drug design but also provides a valuable foundation for subsequent experimental validations, ultimately streamlining the path toward effective antimicrobial therapeutics. The integrated workflow and advanced analytical tools within Discovery Studio make it an indispensable asset for scientists striving to tackle the growing challenge of antimicrobial resistance by efficiently discovering and optimizing new drug candidates.

1.17.2 PyRx

PyRx is an open-source software platform widely used for virtual screening and molecular docking simulations, recognized especially for its seamless integration with established docking engines such as AutoDock Vina and AutoDock. This user-friendly tool has become increasingly popular in antimicrobial drug discovery, where accurately predicting the interactions between proteins and potential ligands is crucial. By enabling researchers to dock candidate antibacterial compounds within the active sites of bacterial proteins, including enzymes such as β-lactamase and DNA gyrase, PyRx facilitates the calculation of binding affinities, offering significant insights into the intensity and chemical nature of these molecular interactions. One of PyRx's strengths lies in its support for both rigid and flexible docking protocols. This flexibility enables a more nuanced and realistic modeling of how ligands interact with dynamic protein active sites, capturing the conformational changes that occur upon binding. Such sophisticated docking approaches enhance the predictive power of virtual screening campaigns aimed at targeting key bacterial enzymes involved in antimicrobial resistance.

PyRx has been effectively employed in screening libraries of antimicrobial agents against a variety of pathogenic bacteria, spanning both Gram-negative and Gram-positive species. Complementing its docking capabilities, PyRx includes intuitive visualization tools that, when used in conjunction with molecular viewers like PyMOL, enable scientists to examine protein-ligand complexes closely. This visualization helps pinpoint critical amino acid residues involved in binding, fostering a profound comprehension of the molecular determinants of drug efficacy. Moreover, the accessibility and open-source nature of PyRx make it an indispensable resource for researchers globally, streamlining the drug discovery process by combining computational efficiency with detailed structural analysis. This empowers scientists to accelerate the identification and optimization of promising antimicrobial candidates, addressing urgent challenges posed by resistant bacterial pathogens.

1.17.3 Bacterial protein and antimicrobial molecule docking applications

A key first step in discovering new antibiotics and developing treatment plans to combat antimicrobial resistance (AMR) is the docking of antimicrobial compounds to bacterial proteins. Among the most frequently targeted in bacterial docking research is beta-lactamase, an enzyme responsible for degrading beta-lactam antibiotics. Beta-lactamase inhibitors have been identified using docking studies as a means to restore the effectiveness of penicillin, cephalosporins, and carbapenems against resistant bacterial strains (Zhang et al., 2019). Likewise, DNA gyrase inhibitors—which target a vital enzyme involved in bacterial DNA replication—have been identified by docking research as potential antibacterial compounds (Molla et al., 2020). Apart from helping to discover new antibacterial chemicals, docking studies also provide insight into mechanisms of medication resistance. Docks can be used, for instance, to examine changes in bacterial proteins, such as beta-lactamases, and predict how they influence drug binding and resistance (Paterson & Bonomo, 2005). Docks can also be utilized to create multifunctional compounds that simultaneously block several bacterial targets, thereby providing a possible tool against multidrug-resistant bacteria. High-quality docking research relies on the combination of molecular docking tools with structural databases, such as RCSB PDB and PubChem. Accurate, experimentally confirmed 3D structures of bacterial proteins provided by the PDB form the basis for docking simulations. Using crystallographic or NMR data ensures that docking investigations are based on actual protein conformations, thereby enhancing the reliability of the predicted binding affinities (Berman et al., 2000). Molecular docking studies often utilize chemical libraries from PubChem to evaluate several

antimicrobial moieties that are subsequently docked to relevant protein targets with software such as PyRx and Discovery Studio.

1.18 Whole genome analysis of bacteria

1.18.1 Illumina NGS sequencing

Revolutionizing bacterial genomes, whole genome sequencing (WGS) offers high-resolution views of taxonomy, evolutionary history, virulence factors, and antibiotic resistance. Of the many sequencing systems available, Illumina is still the most often used for bacterial WGS because to its affordability, accuracy, and throughput. Typically, 150–300 base pairs (bp) in length, Illumina technologies such as MiSeq, NextSeq, and NovaSeq produce high-quality short reads, enabling multiplexing, which is crucial for high-throughput research (Quainoo et al., 2017). Using sequencing-by-synthesis chemistry, these devices image fluorescently labelled nucleotides integrated into DNA strands in real-time, hence enabling the high-fidelity identification of base sequences (Bentley et al., 2008). Although there are still restrictions in resolving repeated areas and structural variations, Illumina data's low error rate makes it the preferred choice for the initial assembly and variant detection.

1.18.2 Quality control, trimming, and assembly of the sequence

Accurate downstream studies depend on raw sequencing data passing rigorous quality control (QC). FastQC is often used to assess quality measures, including per-base sequence quality, duplication rates, and GC content (Andrews, 2010). Tools like Trimmomatic (Bolger et al., 2014) and Cutadapt (Martin, 2011) eliminate poor-quality bases and adapter sequences, hence greatly enhancing the quality of read alignments and assemblies.

Reads are assembled de novo or reference-guided into contiguous sequences (contigs) after QC and trimming. SPAdes (St. Petersburg genome assembler) is the preferred tool for de novo bacterial genome assembly. SPAdes is particularly suited for short genomes, as it employs a multi-k-mer technique (Bankevich et al., 2012). Unicycler combines all data types for hybrid assembly, including Illumina short reads and long reads (e.g., Oxford Nanopore or PacBio), thereby generating highly contiguous assemblies and resolving plasmids and repetitive components (Wick et al., 2017).

1.18.3 Identification of the isolates (KmerFinder, MLST)

Microbial genomics depends on correct species identification. Developed by the Center for Genomic Epidemiology, KmerFinder is a web-based and command-line application that finds

bacterial species by matching k-mer signatures of the query genome to those in a curated database (Larsen et al., 2014). Highly efficient, it provides species-level classification in minutes. Multi-locus sequence typing (MLST) is used for more granular typing. MLST enables exact strain-level resolution by examining allelic profiles from seven (or more) housekeeping genes. From assembled or raw reads, tools like mlst (created by Torsten Seemann) and SRST2 automate allele calling and sequence type assignment. Integrated with worldwide databases like PubMLST and BIGSdb, MLST data are vital for epidemiological monitoring (Maiden et al., 2013).

1.18.4 Annotation of the genome (Prokka)

Predicting gene architecture and attributing functional information to coding sequences defines genome annotation. A commonly used annotation tool for bacterial genomes, Prokka provides fast, consistent, high-quality annotations. Along with curated databases like UniProt and RefSeq, it combines gene prediction algorithms such Prodigal for coding sequences, Aragorn for tRNA, and RNAmmer/Barrnap for rRNA identification (Seemann, 2014). Prokka produces statistics, protein sequences, and annotated GenBank files, all of which are absolutely vital for functional profiling and downstream comparison genomics. Its speed and simplicity make it a standard tool in bacterial genome analysis processes.

1.18.5 Resistome analysis (CARD)

A main goal of WGS in clinical microbiology is to grasp the antibiotic resistance potential of bacterial isolates. Rigorously curated, the Comprehensive Antibiotic Resistance Database (CARD) is a tool for resistance gene identification and annotation (Alcock et al., 2020). CARD provides an ontology-based framework to analyze findings and stores resistance genes classified by mechanism—e.g., efflux, enzymatic degradation, target alteration. The main analysis tool of CARD, RGI (Resistance Gene Identifier), finds resistance factors with great accuracy using protein homology models, SNP-based detection, and curated cutoffs. RGI provides more sophisticated forecasts and wider resistance mechanism coverage than other tools as ResFinder (Zankari et al., 2012) and AMRFinderPlus (Feldgarden et al., 2019). Its frequent updates and connection with antibiotic susceptibility data make CARD very helpful in surveillance and clinical investigations, hence improving the predictive power for phenotypic resistance.

Chapter 2

Methods and materials

2.0 Methods and Materials

2.1 Collection of test sample

A cumulative 300 sewage samples were systematically obtained from the effluent discharge systems of both government and private hospitals throughout Dhaka City during two sampling phases: February to August 2022 and July to November 2023. Sampling took place on weekday mornings between 8:00 AM and 11:00 AM. Each sample was collected in a 15 mL sterile Falcon tube, properly labeled with the collection site information. To preserve sample integrity, all specimens were transported in ice-cooled containers and processed within two hours of their arrival at the laboratory.

2.2 Bacterial screening

Hospital-derived samples were inoculated onto nutrient agar plates (Hi-Media) supplemented with meropenem collected from Opsonin Pharmaceuticals Limited, Bangladesh, at concentrations of 2 µg/mL and 4 µg/mL. The plates were then incubated aerobically at 37°C for 24 to 48 hours. Colonies that exhibited growth in the presence of meropenem were subsequently sub-cultured onto fresh nutrient agar plates and preserved for further analysis.

2.3 Microbial identification of bacterial isolates using VITEK 2

VITEK 2 GN ID card system was implemented to conduct microbial identification. To generate morphologically distinct colonies, previously isolated colonies were cultured overnight on nutrient agar plates at 37°C. From each plate, one to three well-isolated colonies were selected and suspended in normal saline to achieve 0.5 McFarland standard turbidity. *Enterobacter hormaechei* (ATCC 700323) was used as the positive control.

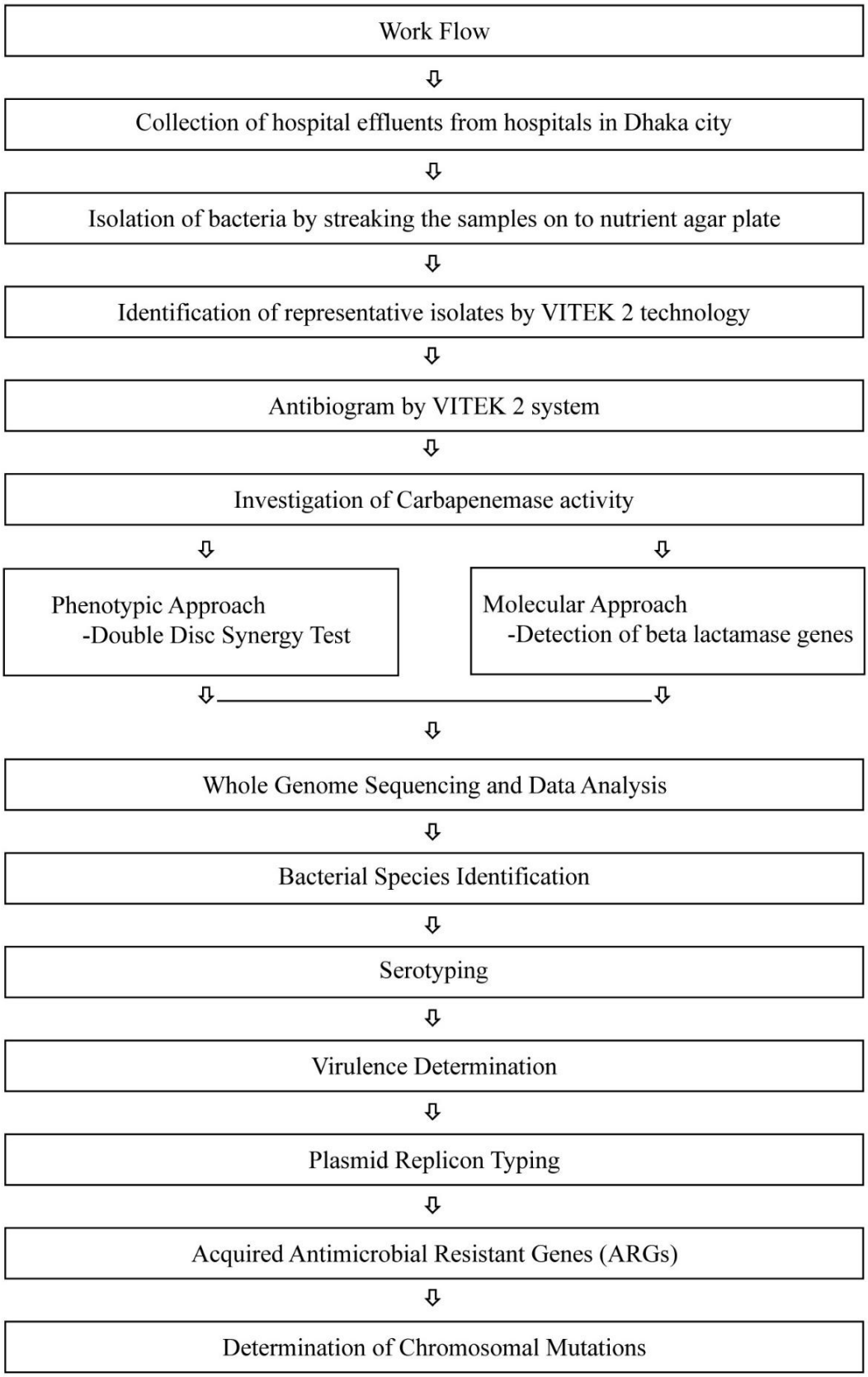


Figure 2-1 Complete design of the research work

2.4 Antibiotic susceptibility test

The antibiotic susceptibility of the isolates was assessed against 20 antimicrobial agents using the VITEK 2 system with AST N280 cards, following the Clinical and Laboratory Standards Institute (CLSI) guidelines and the manufacturer's instructions. *Enterobacter hormaechei* strain LBM 93.03.067 (ATCC 700323), known to be susceptible to all tested antibiotics, was used as the quality control strain. The antimicrobial panel (bioMérieux) comprised 20 agents:

1. Amikacin
2. Cotrimoxazole
3. Amoxicillin/Clavulanic Acid
4. Tigecycline
5. Ampicillin
6. Piperacillin
7. Ciprofloxacin
8. Nitrofurantoin
9. Cefuroxime Axetil
10. Nalidixic Acid
11. Cefuroxime
12. Meropenem
13. Ceftriaxone
14. Imipenem
15. Ceftazidime
16. Gentamicin
17. Cefepime
18. Ertapenem
19. Cefoperazone/Sulbactam
20. Colistin

Based on the minimum inhibitory concentrations, isolates were categorized as susceptible, intermediate, or resistant in accordance with CLSI criteria. Multidrug resistance (MDR) was defined as resistance to three or more classes of antibiotics.

2.5 Phenotypic detection of β-lactamases

To detect extended-spectrum beta-lactamase (ESBL) production, a double disc diffusion test was performed by inoculating Mueller-Hinton agar (MHA) plates with a bacterial suspension adjusted to a 0.5 McFarland turbidity standard, followed by placing discs containing ceftazidime (30 µg), cefotaxime (30 µg), and a combination of ceftazidime (30 µg) or cefotaxime (30 µg) with clavulanic acid (10 µg) at appropriate distances on the plates, which were then incubated at 37°C for 18–20 hours, with isolates identified as ESBL producers if the zone of inhibition around the combination disc was at least 5 mm larger than that around the

cephalosporin disc alone, indicating that clavulanic acid inhibited the ESBL enzyme and restored cephalosporin efficacy.

2.6 Identification and analysis of ESBL and carbapenemase genes

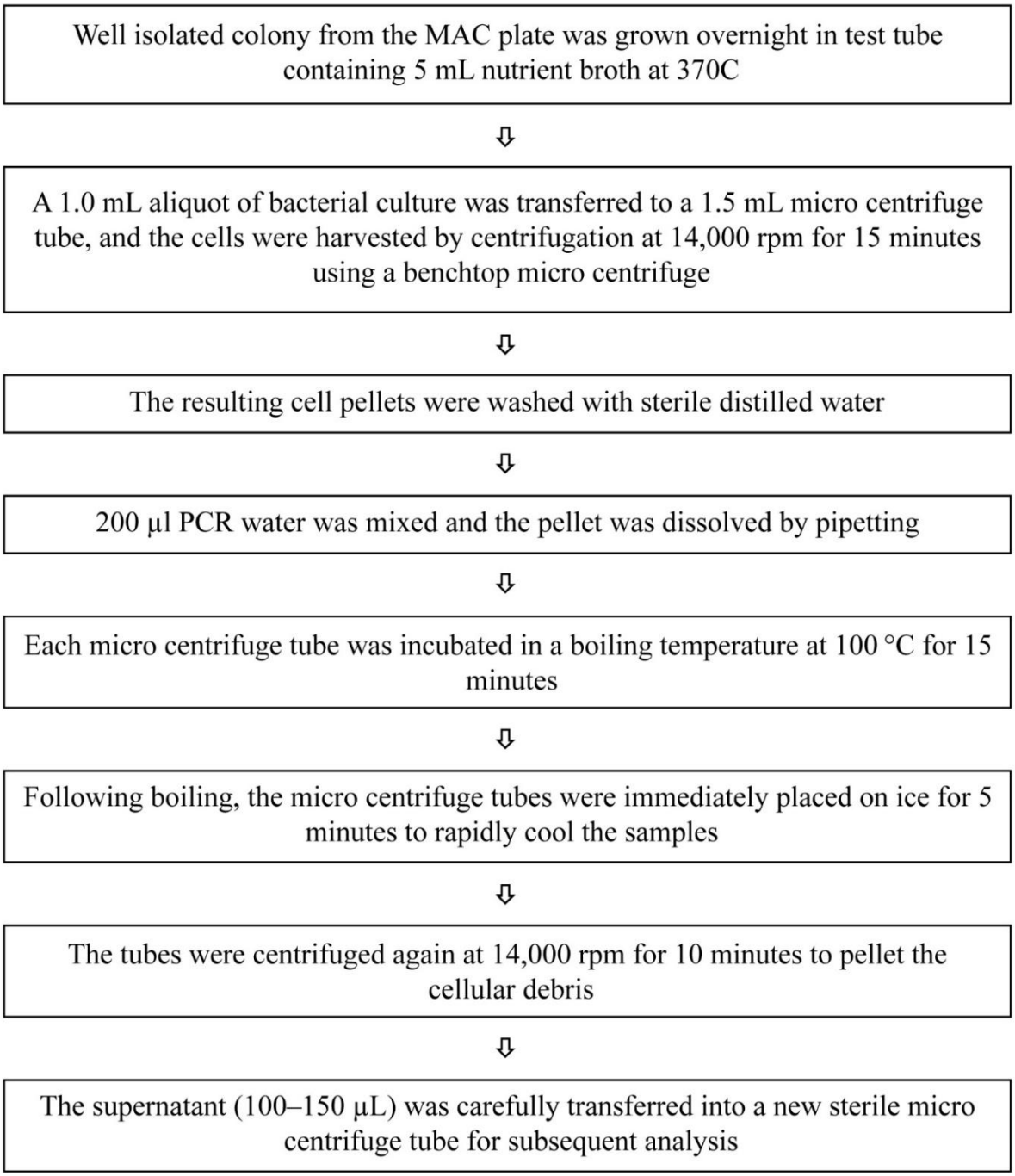
All β-lactamase-producing bacterial isolates were subjected to molecular screening for key resistance determinants associated with extended-spectrum β-lactamase (ESBL) and carbapenemase activity. The analysis focused on detecting the *bla*$_{CTX}$, *bla*$_{TEM}$, *bla*$_{OXA1}$, *bla*$_{SHV}$, *bla*$_{NDM-1}$, *bla*$_{KPC,}$ *bla*$_{DHA}$ and *bla*$_{CMY}$ gene families. Amplification was performed using a thermal cycler based on an established protocol, with slight modifications made to optimize the primer annealing temperatures. The ideal annealing temperature for each primer set (sourced from Macrogen Inc.) were established through a gradient PCR ranging from 55°C to 65°C.

2.6.1 Molecular identification of virulence gene

The isolates were grouped according to VITEK 2 results. One isolate was randomly selected from each group, and bacterial DNA was extracted using the boiling DNA method, followed by Polymerase Chain Reaction (PCR). DNA samples were amplified using a primer set, and gene amplicons of selected isolates were sent to ICDDR,B for genome sequencing.

2.6.2 DNA extraction

DNA extraction was carried out by boil DNA method using pure cultures of bacterial isolates.



2.6.3 Assessment of DNA concentration

The concentration of extracted DNA was quantified in nanograms per microliter (ng/µL) using a Nanodrop spectrophotometer (Thermo Scientific, USA). DNA purity was assessed by

calculating the absorbance ratio at 260 nm and 280 nm (OD260/OD280). A ratio of approximately 1.8 was considered indicative of high-purity DNA.

Table 1 PCR reaction mixture components

Reagents	Amount per Reaction
One Taq Quick-Load 2X Master Mix	12.5 µL
Forward Primer (10pM/µL)	1 µL
Reverse Primer (10pM/µL)	1 µL
Template DNA (50-80 ng/µL)	5 µL
Nuclease Free Water	5.5 µL
Total	25 µL

2.6.4 Identification of virulence genes using PCR

To evaluate the presence of different virulence factors in bacterial isolates, eight virulence genes (*bla*$_{DHA}$, *bla*$_{CMY}$, *bla*$_{SHV}$, *bla*$_{NDM-1}$, *bla*$_{KPC}$, *bla*$_{CTX-M}$, *bla*$_{TEM}$ and *bla*$_{OXA-1}$) were amplified with specific primer list as detailed in Table 2.

Table 2 Primers with their amplicon size used for the detection of virulence genes

Target genes	Primer	Sequences (5'-3')	Amplicon Length (base pair)	Annealing temperature (°C)	References
bla$_{NDM-1}$	*bla*$_{NDM-1}$-forward	AAT GGC TCA TCA CGA TCA TGC	220	60	Haller et al. (2018)
	bla$_{NDM-1}$-reverse	GGC CCG CTC AAG GTA TTT TAC			
bla$_{KPC}$	*bla*$_{KPC}$ - forward	ATGTCACTGTATC GCCGTCT	893	60	

Target genes	Primer	Sequences (5'-3')	Amplicon Length (base pair)	Annealing temperature (°C)	References
	bla$_{KPC}$ – reverse	TTTTCAGAGCCT TACTGC CC			Bratu et al. (2005)
bla$_{CTX-M}$	*bla*$_{CTX-M}$-forward	ATGTGCAGYACC AGTAARGTKATG GC	300	60	Birkett et al. (2007)
	bla$_{CTX-M}$ – reverse	ATCACKCGGRTC GCCXGG RAT			
bla$_{SHV}$	*bla*$_{SHV}$ - forward	GCCTGTGTATTAT CTCCCTGTTAGC	764	60	Soltan et al. (2013)
	bla$_{SHV}$ – reverse	CAGATAAATCAC CACAATGCGC			
bla$_{OXA-1}$	*bla*$_{OXA-1}$- forward	GGCACCAGATTC AACTTTCAAG	564	57.8	Ogutu et al. (2015)
	bla$_{OXA-1}$- reverse	GACCCCAAGTTT CCTGTAAGTG			
bla$_{TEM}$	*bla*$_{TEM}$ –forward	CATTTCCGTGTCG CCCTTATTC	800	60	Perez et al. (2007)
	bla$_{TEM}$ – reverse	CGTTCATCCATAG TTGCCTGAC			
bla$_{DHA}$	*bla* $_{DHA}$ – forward	AAC TTT CAC AGG TGT GCT GGGT	405	62	Perez et al. (2002)
	bla $_{DHA}$ – reverse	CCG TAC GCA TACTGGCTTTGC			

Target genes	Primer	Sequences (5'-3')	Amplicon Length (base pair)	Annealing temperature (°C)	References
*bla*CMY	*bla* CMY - forward	GTGGTGGATGCC AGCATCC	915	58	Hasman et al. (2005)
	bla CMY- reverse	GGTCGAGCCGGT CTTGTTGAA			

PCR amplification was performed using a Mastercycler Gradient thermal cycler (Eppendorf, Germany). The master mix was prepared according to the composition outlined in Table 2.1 and thoroughly mixed by gentle pipetting. Subsequently, 20 µL of the master mix was aliquoted into each PCR tube, followed by the addition of 5 µL of DNA template from each clinical isolate. The contents were homogenized by careful and repeated pipetting. The PCR tubes were then loaded into the thermal cycler, and the amplification was carried out under standardized cycling conditions. The entire PCR run lasted approximately 2 to 2.5 hours, after which the reaction was held at the final extension temperature for 10 to 15 minutes to ensure complete stabilization of the amplified products.

2.7 Analysis of virulence genes by agarose gel electrophoresis

PCR amplified products for the β-lactamase and the virulence genes were checked using 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) as intercalating agent. A 100bp DNA ladder was used to determine the approximate size of amplified product. To load the PCR products into the wells, 5 µL of each product was taken and mixed with 1 µL of 6x loading dye and then loaded in the wells. The gel was run in 70-80 volts until the first blue dye goes down. The gel was visualized under ultraviolet (UV) illumination following staining, and images were captured using a gel documentation system (Axygen, USA).

2.8 Preparation and optimization of ligands

The initial molecular structures of meropenem, ampicillin, ceftriaxone, and cefuroxime were retrieved from the comprehensive PubChem database. Using ChemDraw software, each of these compounds underwent targeted structural modifications by incorporating a benzene ring and a carboxyl (-COOH) functional group. Following this, the modified ligand structures were imported into Gaussian 16 to undergo rigorous structural optimization. This optimization was

carried out employing the hybrid B3LYP exchange-correlation functional together with the 6-311G(d) basis set, harnessing the power of density functional theory (DFT). To further improve computational precision, semi-core pseudopotentials were applied during the DFT calculations as recommended in prior studies (Obot et al., 2015; Ribeiro et al., 2015). Upon completion, the optimized molecular geometries were exported in PDB format, making them ready for downstream molecular docking analyses aimed at assessing their interaction with bacterial targets.

2.9 Preparation of protein structures and molecular docking analysis

Molecular docking analysis plays a pivotal role in predicting the effectiveness of drug molecules binding to their target proteins, providing insight into binding affinities that are critical for drug design (Muteeb et al., 2022b). In this study, docking simulations were conducted using structurally optimized ligands against the *E. coli* target protein (PDB ID: 1BTL). The three-dimensional protein structure was retrieved in PDB format from the RCSB Protein Data Bank and meticulously pre-processed using BIOVIA Discovery Studio to prepare it suitably for the docking experiments.

Following preparation, molecular docking was performed using the PyRx software platform. The search space for ligand interactions was carefully defined by setting the grid box center at coordinates X = −11.873884, Y = −24.3376, and Z = 19.0138, with the box dimensions spanning X = 29.7641, Y = 27.76183, and Z = 28.766 Å across the respective axes. This precise configuration ensured an optimal exploration area encompassing the protein's active site.

To thoroughly analyze and visualize the molecular interactions within the binding site, tools such as BIOVIA Discovery Studio Visualizer and ChimeraX were utilized, allowing detailed examination of critical amino acid residues involved in ligand engagement (Dallakyan & Olson, 2015; Ahmad et al., 2022). These combined methodologies facilitated a comprehensive understanding of ligand binding dynamics, which is instrumental in guiding further drug optimization against *E. coli* targets.

2.10 Plasmid profiling

The isolated carbapenem-resistant strains were cultured for plasmid extraction. In this case, the alkaline lysis method was followed. Three solutions were prepared (solution 1, solution II, and solution III).

2.10.1 Solution I preparation

Solution 1 was prepared by dissolving glucose, 1M Tris-HCl (pH 8.0), and 0.5M EDTA (pH 8.0) in distilled water (dH2O). Then the solution was autoclaved at 1150C for 10 minutes.

2.10.2 Solution II preparation

Solution II was prepared freshly in an autoclaved container. NaOH was mixed with 10% SDS dissolved in autoclaved dH2O.

2.10.3 Solution III preparation

For solution III preparation potassium acetate was mixed with glacial acetic acid and dissolved in dH2O. Then the solution was autoclaved at 1150C for 10 minutes.

2.10.4 TE buffer preparation

1M Tris-HCL (pH 8.0) and 0.5M EDTA (pH 8.0) were mixed with dH2O to prepare TE buffer.

2.10.5 Salt preparation (3M Sodium Acetate pH 5.2)

A solution of sodium acetate was prepared in dH_2O and its pH was adjusted to 5.2 by the gradual addition of glacial acetic acid. The final volume was brought up to 50 mL with dH_2O, followed by sterilization through autoclaving.

2.10.6 Plasmid extraction protocol:

i. A single, well-isolated colony from a freshly prepared culture plate was transferred into 5 mL of Luria-Bertani (LB) broth and incubated overnight at 37 °C under shaking conditions.
ii. An aliquot of 1.0 mL from the overnight bacterial culture was carefully pipetted into a sterile 1.5 mL micro centrifuge tube.
iii. The culture was subjected to centrifugation at 13,000 rpm for 5 minutes, and the resulting supernatant was completely removed.
iv. The resulting pellet was gently resuspended in 100 µL of Solution I.
v. Subsequently, 200 µL of Solution II was added, and the mixture was gently but thoroughly inverted to mix, followed by incubation on ice for 10 minutes.
vi. Following this, 150 µL of Solution III was introduced, mixed thoroughly by inverting the tube, and then centrifuged at 13,000 rpm for 10 minutes.
vii. The clarified supernatant was transferred to a fresh tube and pellet was discarded.
viii. To the supernatant, 40 µL of 3 M sodium acetate (pH 5.2) and 900 µL of ice-cold absolute ethanol were added and kept at -600C for 2 hours.

ix. The filtrate was further centrifuged for 5 minutes at 13000 rpm.
x. The supernatant was discarded and pellet was washed with 1 mL 70% ethanol two times
xi. The pellet was dried and resuspended in 30 µL TE buffer by vortexing.
xii. The resulting filtrate is the plasmid extract.

2.11 Separation of plasmid DNA by agarose gel electrophoresis

Plasmid DNA was isolated using horizontal electrophoresis on 1.2% agarose slab gels in TBE buffer at ambient temperature, applying 80 volts for 1.5 hours. Five microliters of plasmid DNA were combined with 1 µL of tracking dye and thereafter introduced into the individual well of the gel. The gel was stained with ethidium bromide for 20 minutes and subsequently de-stained with distilled water for 5 minutes. DNA bands were visualized and captured using a Gel Documentation System with a UV transilluminator. The dimensions of the unidentified plasmid DNA were determined by evaluating its mobility on an agarose gel and comparing it with the mobility of the 1kb DNA ladder (NEB, UK)

2.12 Conjugal plasmid transfer between the isolated bacterial strains

2.12.1 Preparation of competent cell

i. 100 µL Luria-Bertani (LB) broth was inoculated with 0.01 volume of a fresh overnight culture of the *E. coli* (dh5alpha) strain and grown at 37ºC to an OD_{600} of 0.5 to 0.6.
ii. Cells were chilled on ice for 10 to 15 min.
iii. They were harvested by centrifugation (750-1000x g, 12-15 min, 4º C).
iv. The supernatant was discarded. The pelleted fraction, i.e., the cells, was resuspended in 0.5 volume of ice-cold solution of 0.1M $CaCl_2$ and then kept on ice for 30 min.
v. Cells were harvested as before.
vi. The supernatant was decanted and discarded.
vii. The cell pellet was resuspended in 0.25 culture volume of ice-cold solution of 0.1M $CaCl_2$.
viii. Cells were preserved For storage, glycerol was added to attain a final concentration of 20%. The cells were divided into 50 µL aliquots, snap-frozen in liquid nitrogen, and then stored at -80 °C.

2.12.2 Transformation of plasmid

i. 50µl of competent cells were mixed with DNA solution (< 10 µl, 55-80 ng) that was previously isolated from the bacterial strains (*E.coli* PG1BP, DMIH, HCDL, NTIH and *Pseudomonas* B3ay, ID-16, ID-17, ID-24) and incubated on ice for 20 to 30 min.
ii. The cells were heat shocked at 42°C for 1 min and chilled immediately on ice for 2min.
iii. They were allowed to recover by the addition of 450 µl of LB and incubated for 45 min at 37 °C.
iv. The transformed cells were plated onto selective LB agar containing ampicillin.
v. Plates were incubated at 37°C for 16–18 hours, and transformation efficiencies were assessed thereafter.

2.13 Preparation of genomic DNA and whole genome analysis

Genomic DNA was extracted from four *E. coli* strains—DMIH, HCDL, NTIH, and PG1BP—as well as four *Pseudomonas* strains—B3ay, ID-16, ID-17, and ID-24—cultured on MacConkey agar following overnight incubation at 37°C. The extractions were performed using the Promega DNA isolation kit (Promega, Madison, WI) according to the manufacturer's instructions. DNA concentrations were then measured using a Nanodrop One spectrophotometer (ThermoFisher Scientific, USA) to ensure sample quality and quantity for downstream applications.

Whole-genome sequencing (WGS) was conducted at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), employing the Illumina DNA Prep protocol (Illumina, USA). In this process, genomic DNA underwent tagmentation using Bead-Linked Transposomes (BLT), which facilitated the attachment of adapter sequences necessary for constructing the sequencing library. Post-tagmentation cleanup was followed by a limited-cycle PCR amplification step, where index adapters were added to enable multiplexed cluster generation.

The resulting amplified libraries were purified through a double-sided bead purification approach to enrich for appropriate fragment sizes and remove contaminants. Subsequently, libraries were pooled and quantified using the Qubit fluorometric assay with double-stranded DNA-specific fluorescent dye to optimize cluster density before sequencing.

Sequencing was performed on the Illumina NextSeq 2000 platform (Illumina, USA), producing high-throughput data that were subjected to rigorous quality control assessment using the

FastQC toolkit. Low-quality reads and adapter sequences were trimmed employing Trimmomatic software to ensure accuracy and reliability (Jia et al., 2024; Kyung et al., 2023).

For genome assembly, the filtered high-quality reads were processed with SPAdes version 4.0.1 using the "careful correction" option, designed to reduce mismatches and improve assembly integrity. K-mer sizes were selected automatically by the software based on data characteristics (Lofgren et al., 2022). To promote transparency and data sharing, the raw sequencing reads generated from this study were deposited in the Sequence Read Archive (SRA), accessible through the European Nucleotide Archive (ENA).

2.14 Data analysis

Bacterial species identification, serotyping, virulence determination, plasmid replicon typing, detection of antimicrobial resistance genes (ARGs), and chromosomal mutations in the bacterial chromosome were identified using bioinformatics tools available at the Center for Genomic Epidemiology (CGE) web server (http://www.genomicepidemiology.org/services/). All the heatmaps generated display the diversity of ARGs, including predicted phenotypic resistance, the preponderance of different virulence genes, and the presence of plasmid replicon types.

Chapter 3

Result

3.0 Result

3.1 Screening of bacterial isolates using meropenem

A total of 184 bacterial isolates were successfully obtained through selective culturing in the presence of meropenem. This antibiotic-based selection was designed to enrich for meropenem-resistant bacteria, ensuring that only those capable of surviving under the antibiotic pressure were able to grow. The isolated colonies were then subjected to further analysis to identify potential mechanisms of resistance and their relevance in the context of antimicrobial resistance studies (Figure 3-1).

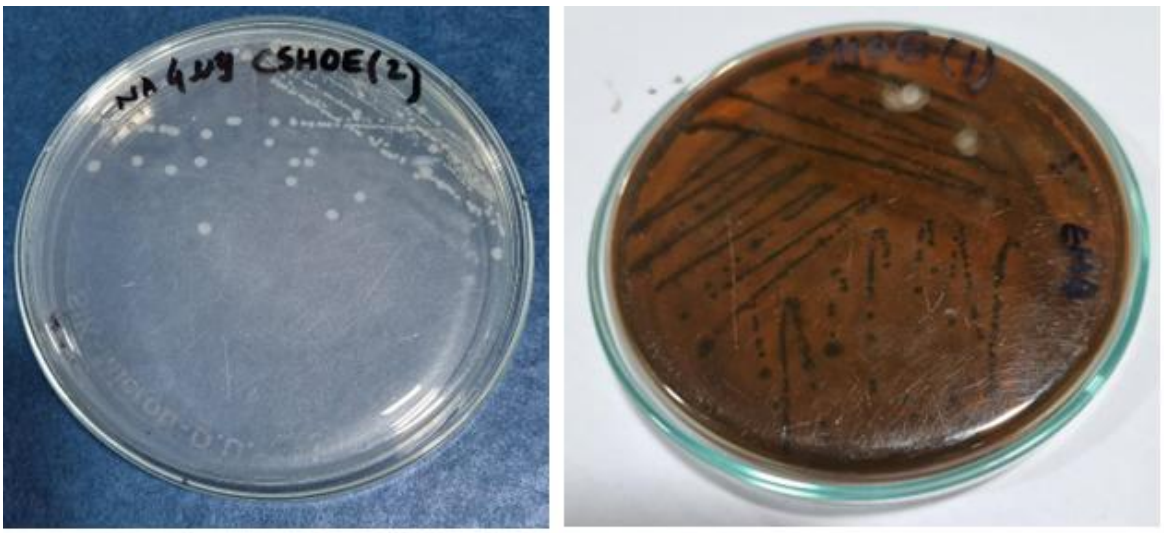


Figure 3-1 Two plates showing the pure culture obtained from the sample. The sample was first screened with the meropenem. Then it was subjected to pure culture via taking different single colonies

3.2 Growth on nutrient agar

The isolated bacterial colonies were subsequently sub-cultured on nutrient agar medium to ensure the robustness of their growth and to maintain pure cultures for further analyses. This step was crucial for assessing the general viability of the isolates in a non-selective environment, providing a baseline for comparison with future experiments (Figure 3-2)

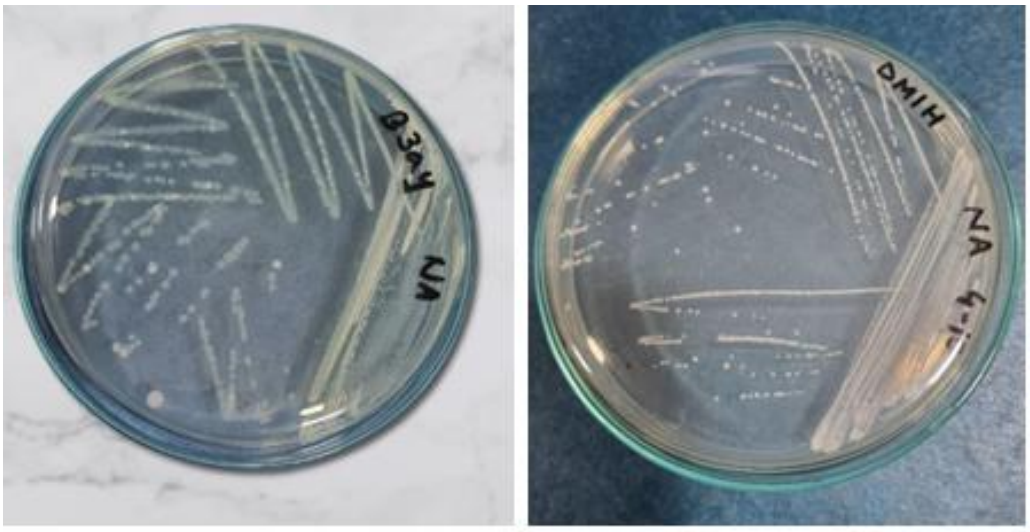


Figure 3-2 Two plates showing the pure culture on nutrient agar plate. This step was done to ensure robust growth and maintenance of the pure cultures and get more concise result on the downstream steps ahead

3.3 Identification using VITEK 2

The identification of bacterial isolates was performed using the VITEK 2 system, which offers automated and accurate identification of microbial species. Among the 184 bacterial isolates, 48.4% (n = 89) were identified as beta-lactamase producers, indicating resistance to beta-lactam antibiotics. Among the identified isolates, the most frequently occurring species in hospital effluent samples were *E. coli* (42.7%, n = 38), *A. baumannii* (25.9%, n = 23), *P. aeruginosa* (25.9%, n = 23), and *E. cloacae* (5.6%, n = 5) (Figure 3-3). These findings highlight the dominance of opportunistic and antibiotic-resistant pathogens in hospital waste water, underscoring the public health risks associated with such environments.

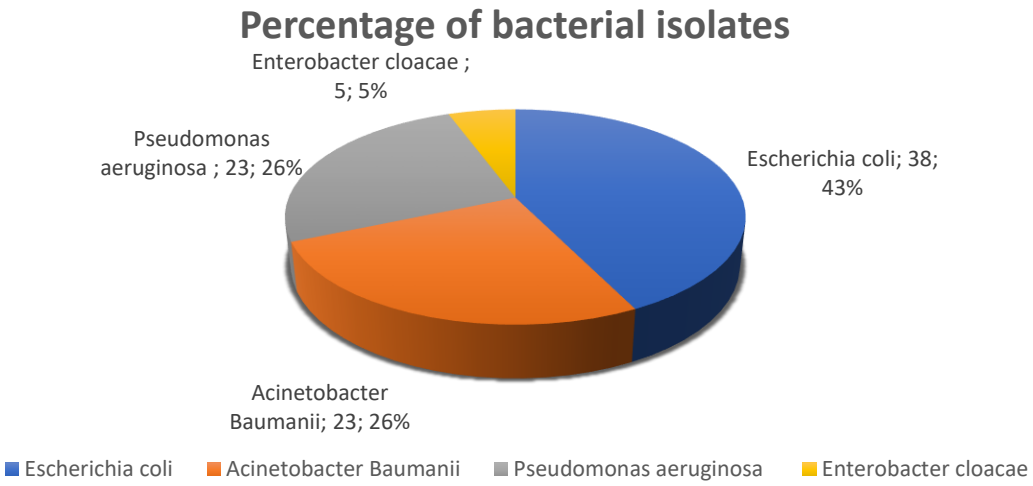


Figure 3-3 Percentage of bacterial isolates identified by the VITEK 2 system. Out of 184 isolates 42.7% were E. coli, 25.9% were A. baumannii, 25.9% were P. aeruginosa and 5.6% were E. cloacae

3.4 Antibiogram of prevalent species

3.4.1 Antibiogram of *E. coli*

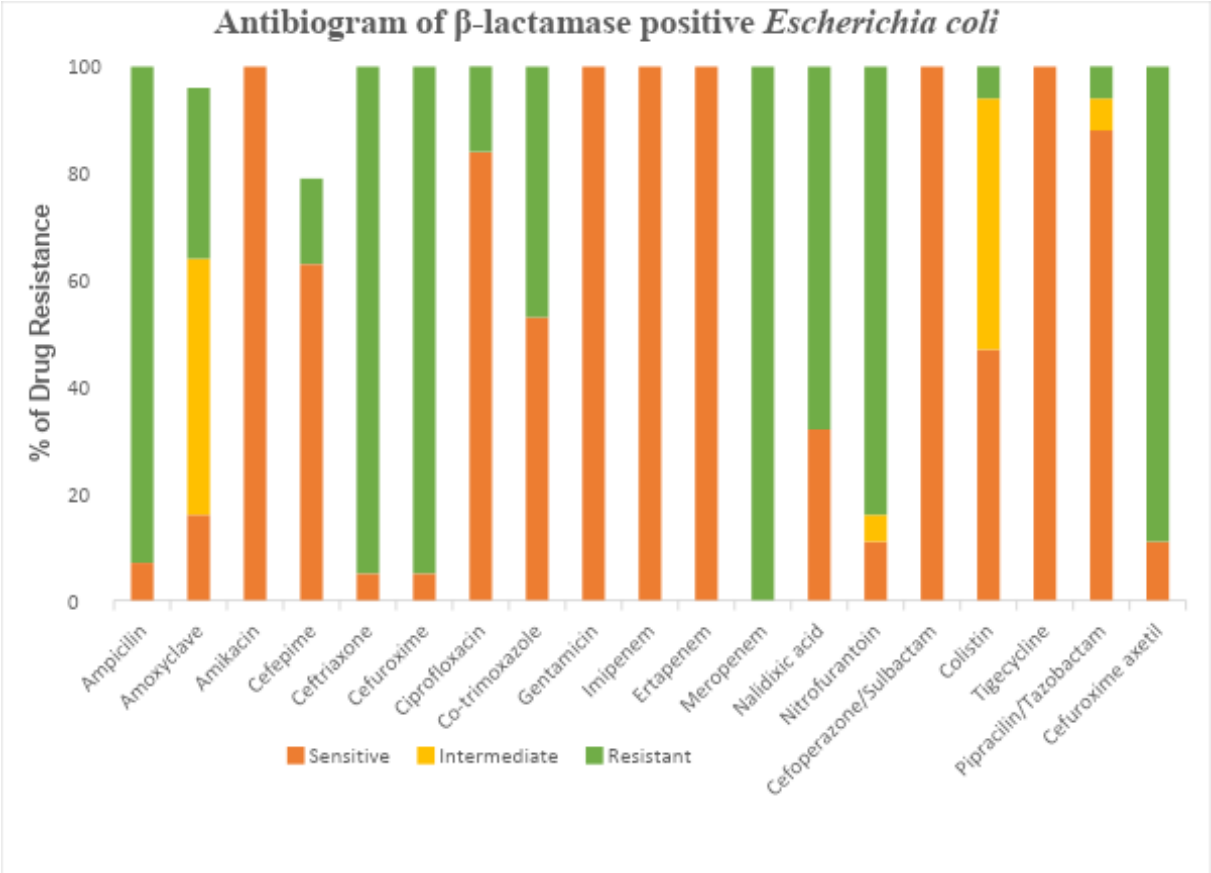


Figure 3-4 Antibiogram result of beta-lactamase positive E. coli

The antibiogram for β-lactamase positive *E. coli* isolates indicates high levels of resistance to several key antibiotic classes. As observed in the chart (Figure 3-4), *E. coli* demonstrates nearly 100% resistance to β-lactam antibiotics, including ampicillin, amoxicillin, and amoxicillin/clavulanic acid, reflecting the effectiveness of β-lactamase enzymes in degrading these drugs. Furthermore, significant resistance was noted for third-generation cephalosporins, such as ceftriaxone and ceftazidime, highlighting the challenges these enzymes pose to treating infections with standard β-lactam drugs.

Intermediate levels of resistance were observed with some antibiotics, such as ciprofloxacin and gentamicin, indicating partial efficacy in combating these isolates. On the other hand, a few antibiotics like tigecycline and colistin retain their effectiveness, as shown by a higher percentage of isolates that remain sensitive. This suggests that although multidrug resistance is widespread among the isolates, some last-resort antibiotics could still provide therapeutic value.

3.4.2 Resistance pattern of *A. baumannii*

The antibiotic susceptibility profiles of the *A. baumanii* isolates revealed significant levels of resistance. Meropenem exhibited the highest resistance rate among the tested antibiotics, with ampicillin showing a comparable level of resistance, as 87% of the *A. baumannii* isolates were resistant to it. Remarkably, all *A. baumannii* isolates exhibited multidrug resistance (MDR), defined by resistance to antibiotics from at least three different classes, indicating a severe resistance phenotype within the bacterial population (Figure 3-5).

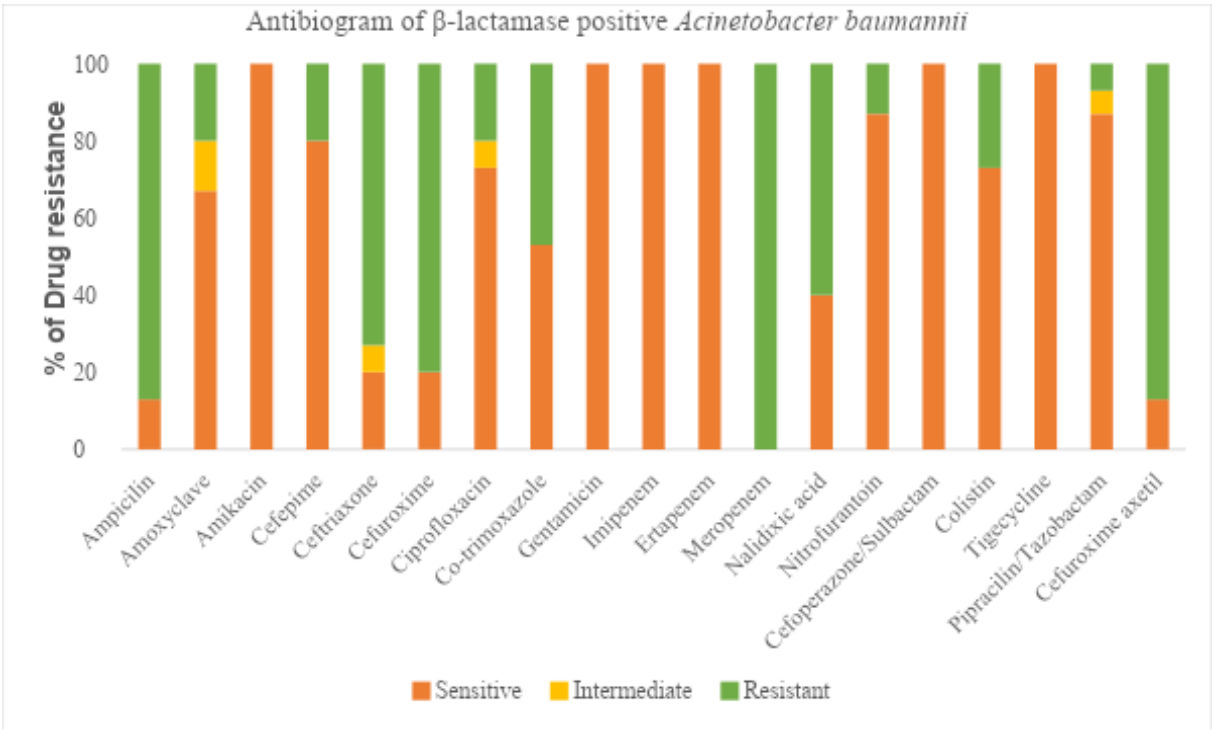


Figure 3-5 Antibiogram result of beta-lactamase producing A. baumannii

3.4.3 Antibiotic susceptibility profile of *P. aeruginosa*

Resistance to meropenem in *P. aeruginosa* was accompanied by reduced susceptibility to antibiotics from three distinct classes. Ampicillin showed the greatest resistance, with 94% of isolates resistant. Resistance was observed in 80% of isolates for ceftriaxone, cefuroxime, and cefuroxime axetil, and in 53% for nalidixic acid. (Figure 3-6). These data highlight the strong resistance capacity of *P. aeruginosa* strains, particularly against beta-lactam antibiotics, which poses a significant challenge for treatment.

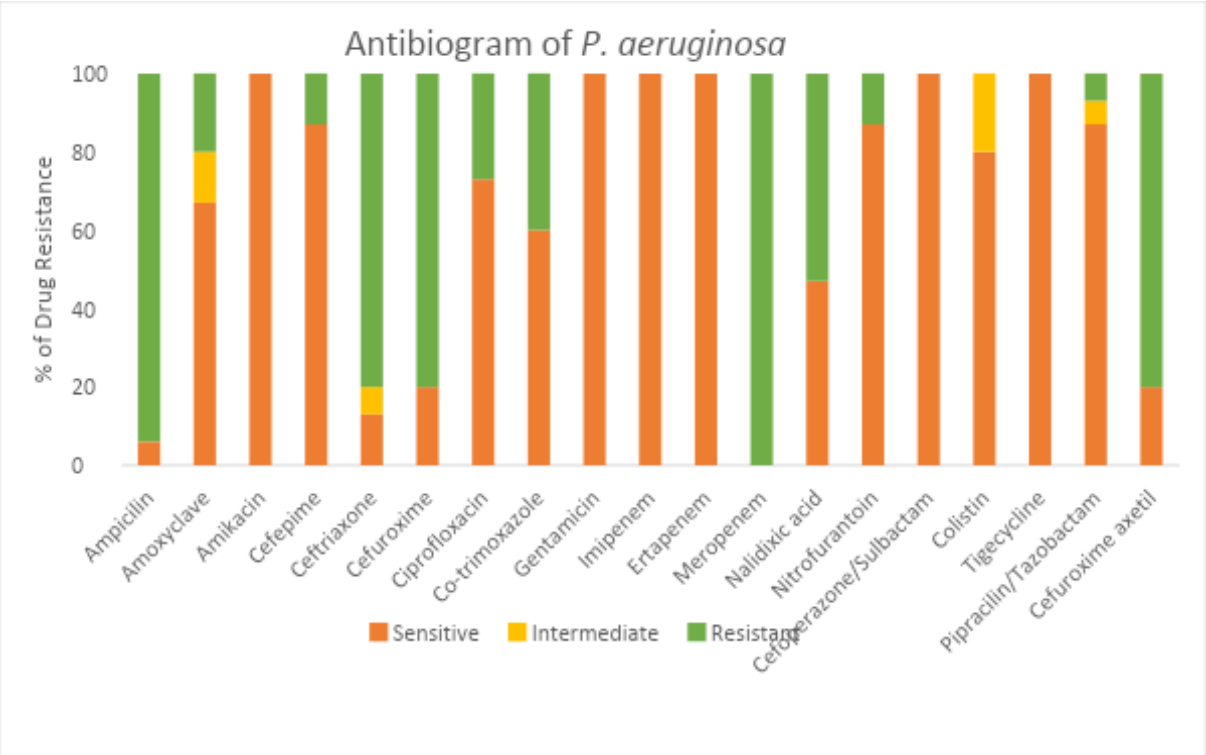


Figure 3-6 Antibiogram result of beta-lactamase positive P. aeruginosa

3.4.4 Antibiotic resistance profile of *E. cloacae*

All five *E. cloacae* isolates showed complete resistance to ceftriaxone, meropenem, and cefuroxime axetil in disk diffusion tests, underscoring the critical issue of carbapenem resistance in this species. In addition, 80% of the *E. cloacae* isolates exhibited resistance to ampicillin, while 60% were resistant to nalidixic acid and cefuroxime. Resistance to nitrofurantoin and cotrimoxazole was observed in only 40% of the isolates, and the lowest rates of resistance, at 20%, were recorded for ciprofloxacin and amoxiclav. All isolates (100%) exhibited multidrug resistance, confirming the widespread presence of MDR *E. cloacae* in the sampled population (Figure 3-7). These findings illustrate the alarming prevalence of resistance across multiple antibiotic classes, necessitating immediate attention to combat the stretch of these resistant microbes.

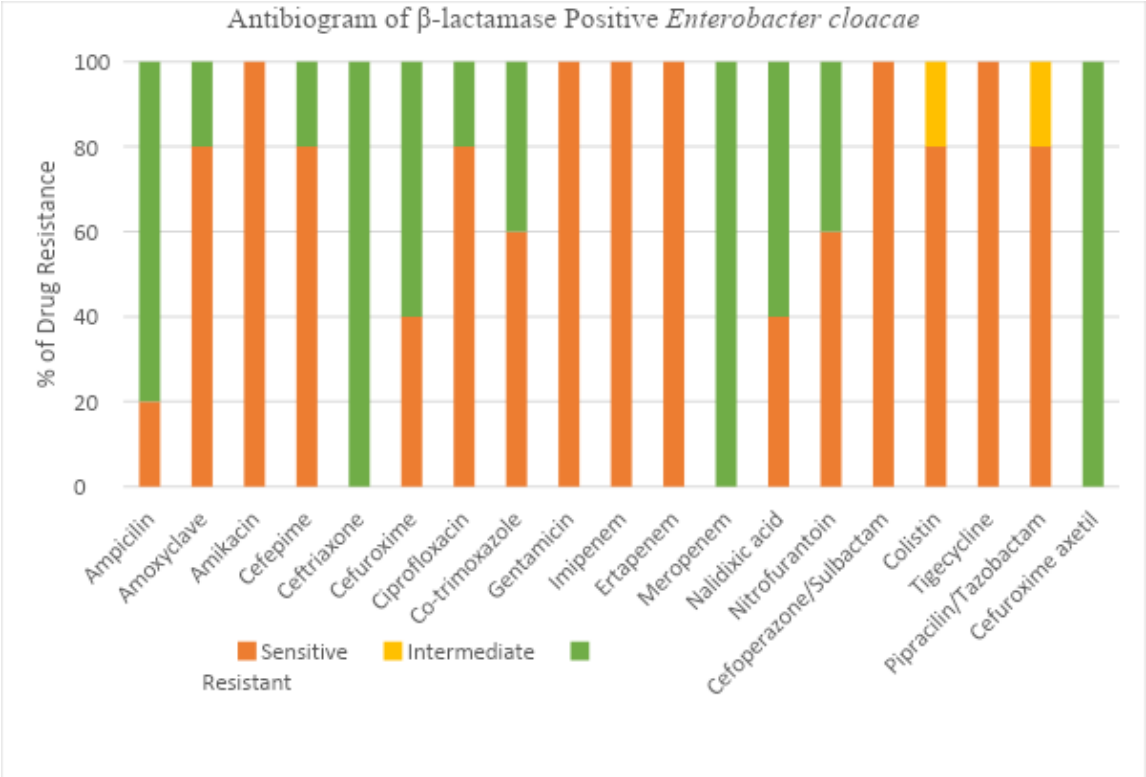


Figure 3-7 Antimicrobial resistance profile of β-lactamase-producing E. cloacae

3.5 Phenotypic and genotypic detection of β-lactamases

A double disc diffusion test was conducted to identify beta-lactamase-producing bacteria, with results shown in Table 3. The test revealed both positive and negative reactions for beta-lactamase production among the bacterial species tested. *P. aeruginosa* showed consistent beta-lactamase positivity across multiple samples, with inhibition zones ranging from 6 to 9 mm (Figure 3-8). *P. putida* samples, however, displayed negative results for beta-lactamase production, with inhibition zones ranging from 2.8 to 4.5 mm. Similarly, *P. mendocina* was negative for beta-lactamase, producing an inhibition zone of 4.2 mm. *A. baumannii* complex and *Vibrio mimicus* both demonstrated positive beta-lactamase production, with inhibition zones of 5.5 mm and 5.2 mm, respectively. In contrast, *Aeromonas sorbia* samples were beta-lactamase negative with a consistent 3.5 mm inhibition zone. *E. coli* samples all tested positive for beta-lactamase production, with inhibition zones between 6 to 6.4 mm.

Table 3 Phenotypic detection of β-lactamases

ID	Species	Beta lactamase	Diameter (mm)
B3ay	*Pseudomonas aeruginosa*	Positive	7.1
ID-16	*Pseudomonas aeruginosa*	Positive	8.5

ID	Species	Beta lactamase	Diameter (mm)
ID-17	*Pseudomonas aeruginosa*	Positive	6
ID-24	*Pseudomonas aeruginosa*	Positive	9
CSHOE(3)	*Pseudomonas putida*	Negative	3.2
NTOE(3)	*Pseudomonas putida*	Negative	2.8
CSHOE(2)	*Pseudomonas putida*	Negative	4.5
PGIH(1)	*Pseudomonas putida*	Negative	3.8
DMOE	*Pseudomonas mendocina*	Negative	4.2
NTOE(1)	*Acinetobacter baumannii complex*	Positive	5.5
NTOE(2)	*Vibrio mimicus*	Positive	5.2
CSHOE(1)	*Aeromonas sorbia*	Negative	3.5
CSHIH(3)	*Aeromonas sorbia*	Negative	3.5
DMIH	*E. coli*	Positive	6.2
PG1bp	*E. coli*	Positive	6
NTIH	*E. coli*	Positive	6.2
HCDL	*E. coli*	Positive	6.4

Figure 3-8 Representative image of the double disc diffusion assay conducted for detecting the beta-lactamase activity of the isolates.

A detailed molecular analysis was performed on the β-lactamase-positive isolates to determine the genetic factors responsible for extended-spectrum β-lactamase (ESBL) production. Out of the 89 isolates examined, the most frequently detected carbapenemase gene was *bla*$_{\text{NDM-1}}$, present in all isolates (100%), followed by *bla*$_{\text{OXA}}$, identified in 41.2% of the samples. Additionally, other β-lactamase genes such as *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{KPC}}$ were detected, each represented in 5.9% of the isolates. Notably, the *bla*$_{\text{TEM}}$ β-lactamase gene was absent in all isolates, indicating a significant alteration in the distribution of β-lactamase genes among the studied strains.

The prevalence of *bla*$_{\text{NDM-1}}$ was particularly notable among both Enterobacteriaceae members, including *E. coli* and *E. cloacae*, as well as non-Enterobacteriaceae species such as *P. aeruginosa* and *A. baumannii*. Conversely, the presence of *bla*$_{\text{KPC}}$ was predominantly associated with *A. baumannii*, while *bla*$_{\text{OXA}}$ was primarily detected in *P. aeruginosa*. The occurrence of *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{SHV}}$ genes was relatively limited among the identified species, suggesting that these bacterial populations employ varying mechanisms of resistance (Figure 3-9).

The gel electrophoresis results further confirmed the presence of the targeted β-lactamase genes. Lane L showed the presence of the molecular weight standard (Gene Ruler 100 bp DNA Ladder), while the positive control (PC) exhibited a distinct and well-defined amplification band. Distinct PCR product bands of approximately 220 bp were observed in lanes 1 to 16 and lane 18, whereas no amplification was detected in the negative control (NC) lane, confirming the specificity of the PCR reaction (Figure 3-10). Prominent PCR products of approximately 893 bp were detected in lanes 12, 17, and 21, as illustrated in Figure 3.6.3. In Figure 3.6.4, distinct 764 bp amplicons were observed in lanes 10 and 13, while lane NC functioned as the negative control. Furthermore, the electrophoretic gel image demonstrated consistent amplification, with the positive control (PC) and samples in lanes 15, 17, 20, 24, 26, and 32 displaying distinct bands corresponding to a 564 bp PCR product (Figure 3-13).

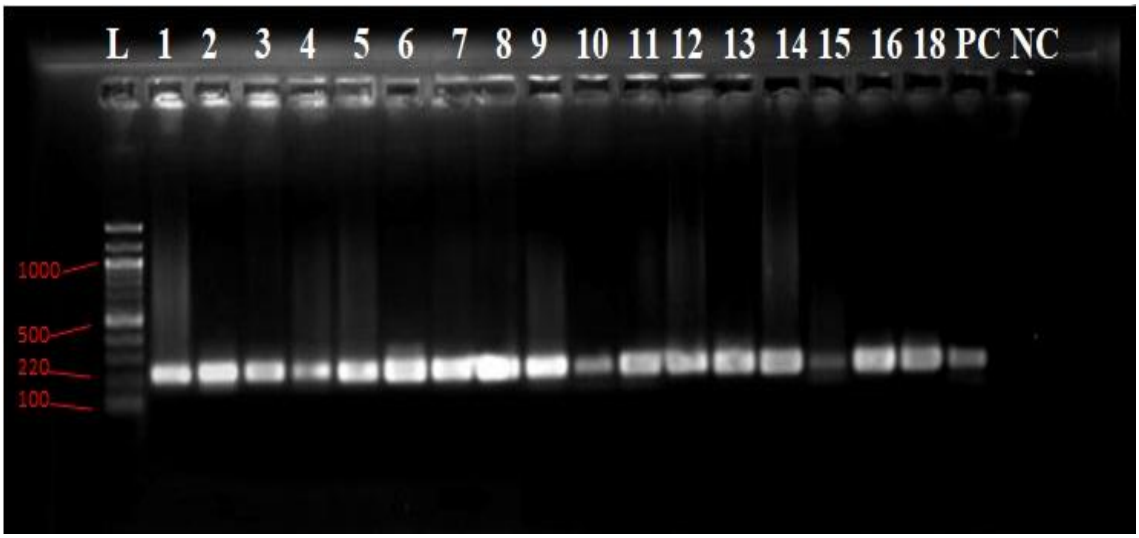


Figure 3-9 Agarose gel electrophoresis image of PCR amplification products for the bla_{NDM-1} gene. The gel shows a molecular weight marker (Gene Ruler 100 bp DNA ladder) in lane L, and a clear amplification band is visible in the positive control (PC). Distinct PCR products of approximately 220bp are observed in lanes 1 through 16 and lane 18. No amplification band is detected in the negative control (NC) lane, indicating the specificity and reliability of the PCR assay.

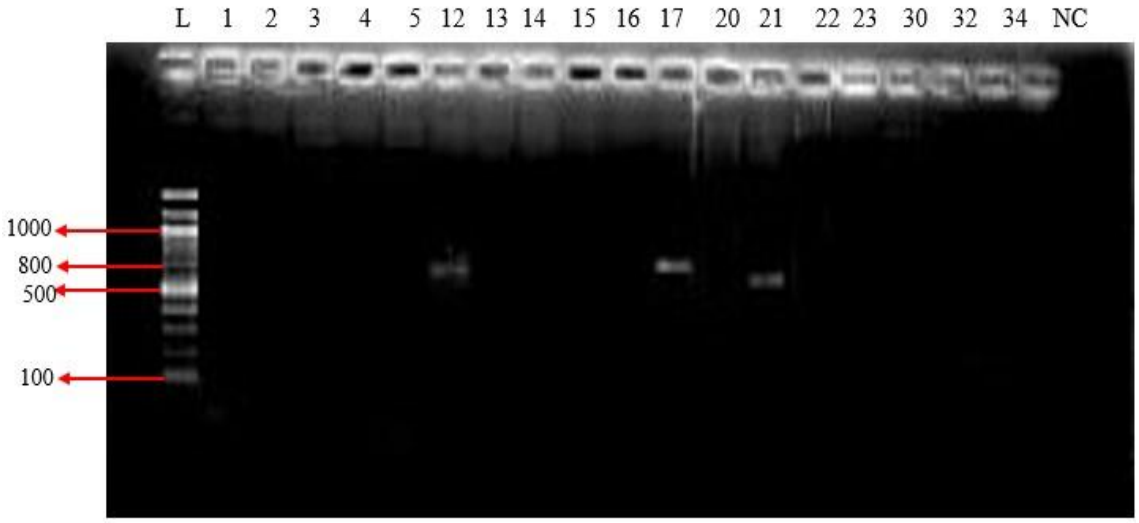


Figure 3-10 Agarose gel electrophoresis image of PCR amplification products targeting the bla_{KPC} gene. As shown in Figure 3.6.2, lane L contains the molecular weight marker (Gene Ruler 100 bp DNA ladder). Prominent PCR bands of approximately 893 bp are observed in lanes 12, 17, and 21, indicating successful amplification of the target gene.

Figure 3-11 Agarose gel electrophoresis image displaying the PCR amplification products of the bla_{CTX-M} gene. The L lane contains the molecular weight marker (Gene Ruler 100 bp DNA ladder). Lanes numbered 12 exhibit distinct bands corresponding to approximately 300 bp, indicating successful amplification of the target gene. The NC lane represents the negative control, which shows no amplification.

Figure 3-12 The agarose gel electrophoresis image illustrates the PCR amplification products of the bla_{SHV} gene. Lanes 10 and 13 display clear bands at approximately 764 bp, indicating successful amplification of the target fragment. The lane labeled NC− serves as the negative control and shows no detectable amplification.

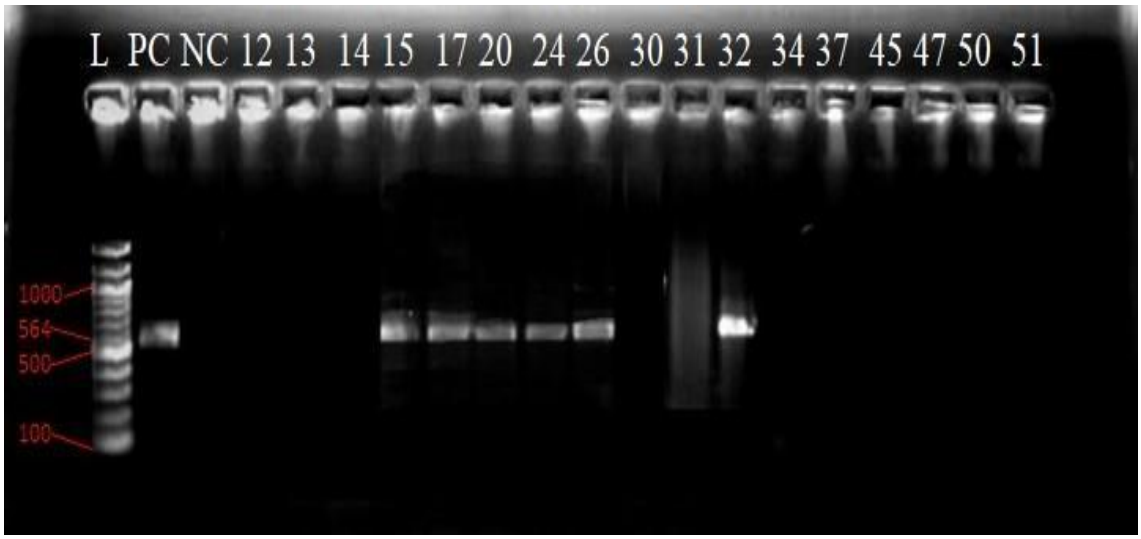


Figure 3-13 The agarose gel electrophoresis image represents the PCR amplification products of the bla_{OXA-1} gene. Lane L contains the molecular weight marker (Gene Ruler 100 bp DNA ladder). The positive control (PC), along with PCR products in lanes 15, 17, 20, 24, 26, and 32, displays distinct bands at approximately 564 bp, confirming successful amplification of the target gene fragment.

3.6 Binding affinity calculation of *E. coli* with the most resistant antibiotics

3.6.1 Ligand generation and structural optimization

The molecular structures of meropenem, ampicillin, ceftriaxone, and cefuroxime were initially sourced from the PubChem database. Structural modifications were carried out using ChemDraw, wherein a benzene ring and a carboxyl (-COOH) group were incorporated, as illustrated in the corresponding figure (Figure 3-14). The modified ligands were subsequently imported into Gaussian 16 for geometry optimization. The optimization process employed the hybrid B3LYP exchange-correlation functional, combined with the 6-311G(d) basis set. Additionally, semi-core pseudopotentials were incorporated within the density functional theory (DFT) framework. The optimized structures were then exported in PDB format for use in molecular docking studies.

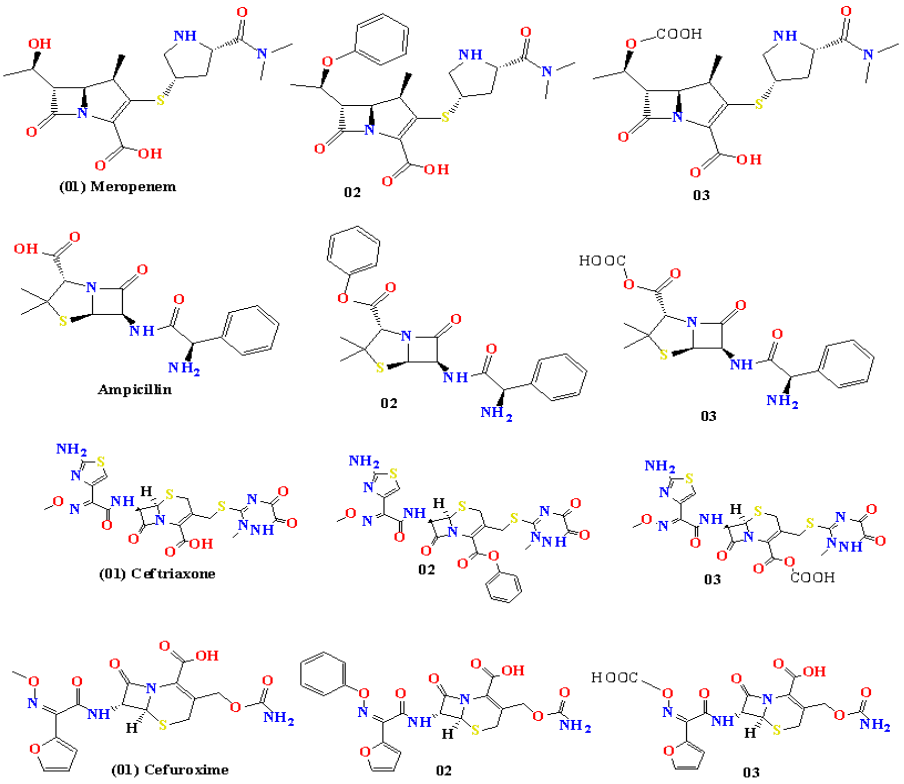


Figure 3-14 Structural representations of the modified compounds

3.6.2 Protein processing and computational docking studies

In this study, molecular docking analysis was employed as a key computational approach to predict the binding affinities between modified drug ligands and a specific target protein from *E. coli* (PDB ID: 1BTL) (Figure 3-15). The protein's three-dimensional structure was obtained from RCSB Protein Data Bank in (PDB format) and subsequently pre-processed using BIOVIA's Discovery Studio to ensure optimal readiness for the docking technique.

Following, docking simulation studies were carried out using the PyRx software, where the search space for ligand binding was precisely defined by a grid box centered at coordinates X = -11.873884, Y = -24.3376, and Z = 19.0138, with dimensions extending 29.7641 Å, 27.76183 Å, and 28.766 Å along the respective axes. This meticulous setup optimized the exploration of potential binding conformations within the active site region.

Post-docking, the interactions between the ligands and the protein's active site residues were visualized and thoroughly examined using both BIOVIA Discovery Studio Visualizer and ChimeraX. These tools facilitated detailed three-dimensional inspection of the binding modes,

allowing for an in-depth understanding of molecular interactions critical to the ligand's affinity and specificity for the bacterial target.

This integrative docking workflow provided comprehensive insights into how structural modifications of the ligands influence their binding characteristics, which is essential for guiding subsequent drug optimization efforts against *E. coli*.

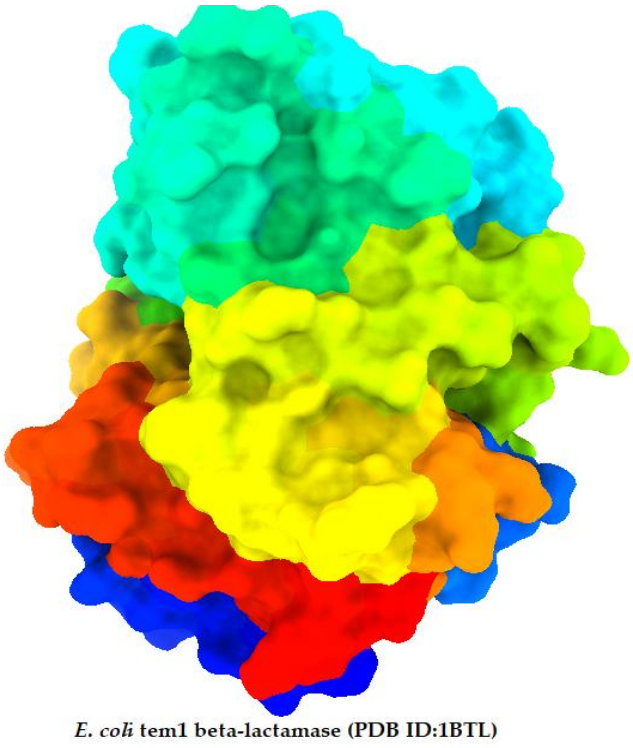


Figure 3-15 Protein model of E. coli Tem1

3.6.3 Docking stimulations targeting E. coli

A computer-aided docking investigation was performed to evaluate the binding affinities and interactions at the active sites of various drug-protein complexes targeting *E. coli*. The study concentrated on four widely used antibiotics—Meropenem, Ampicillin, Ceftriaxone, and Cefuroxime—which have exhibited notably high resistance rates in E. coli populations, with reported resistance percentages of 100%, 80–93%, 73–100%, and 80–95%, respectively. To address this challenge, these antibiotics underwent targeted structural modifications aimed at enhancing their therapeutic effectiveness. Subsequent molecular docking analyses were performed to assess the impact of these chemical alterations on their binding affinities with bacterial protein targets.

The docking outcomes revealed considerable improvements in binding strength following the modifications. Meropenem initially exhibited an interaction energy of –7.5 kcal/mol, which increased to –8.3 kcal/mol post-modification, indicating a stronger and more favorable binding interaction. Ampicillin exhibited a modest yet significant enhancement, with its binding energy shifting from –7.9 kcal/mol to –8.0 kcal/mol following structural changes. Ceftriaxone's binding affinity also improved, with a change from –8.2 kcal/mol to –8.5 kcal/mol. Most strikingly, Cefuroxime demonstrated the greatest affinity increase, wherein its binding energy markedly improved from –7.1 kcal/mol to –8.9 kcal/mol following change (see Table 4).

These results highlight the considerable potential of strategic structural changes to enhance the binding interactions of these medicines with their bacterial targets. Such enhancements in molecular affinity provide a promising avenue toward overcoming existing antimicrobial resistance in *E. coli*, offering insights into the development of more effective therapeutic agents.

Table 4 Binding interactions of ligands with target proteins

***E. coli* tem1 beta-lactamase (PDB ID:1BTL)**							
Binding energy Kcal/mol		Binding energy Kcal/mol		Binding energy Kcal/mol		Binding energy kcal/mol	
Meropenem	-7.5	Ampicillin	-7.9	Ceftriaxone	-8.2	Cefuroxime	-7.1
Derivatives 02	-8.3	Derivatives 02	-8.2	Derivatives 02	-7.7	Derivatives 02	-8.9
Derivatives 03	-8.0	Derivatives 03	-8.6	Derivatives 03	-8.5	Derivatives 03	-8.1

3.6.4 Analysis of active site residues and identification of the binding pocket

An examination of the active site residues within the binding pocket was conducted to elucidate the probable interaction processes and identify the individual amino acids involved in the formation of the drug–protein complex. As illustrated in Figure 3-16, the unmodified form of meropenem interacted with only five active amino acid residues. However, following the introduction of functional groups, there was a notable increase in the number of interacting residues. This enhancement contributed to the formation of stronger interactions between *E. coli* and the modified meropenem derivatives.

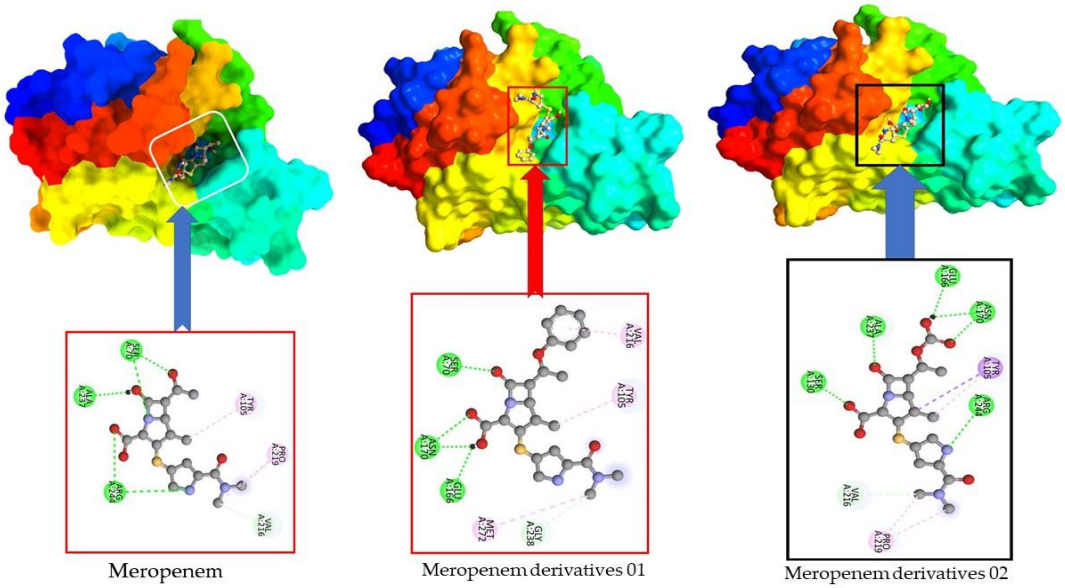


Figure 3-16 Active site interactions and key amino acid residues involved in the binding of meropenem and its derivatives

This observation emphasizes the dynamic behavior of drug–protein interactions, particularly the role of functional group modifications in altering binding characteristics. The observed increase in interacting amino acid residues likely contributes to the improved binding affinity of meropenem derivatives, potentially enhancing their efficacy and specificity against *E. coli* infections. These results highlight the significance of structural modifications in optimizing ligand–target interactions.

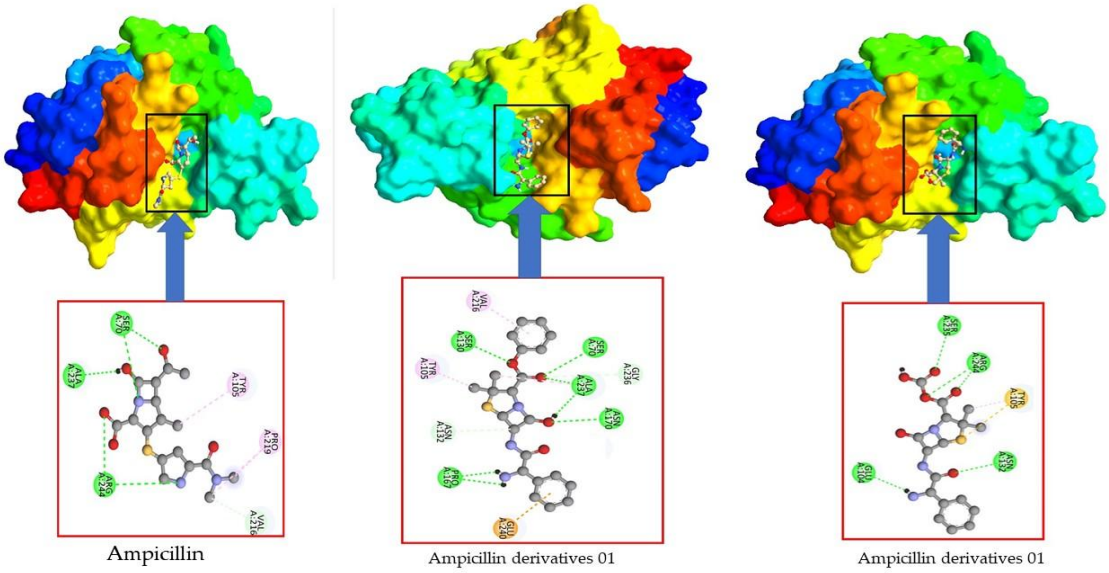


Figure 3-17 Characterization of active sites and interacting amino acid residues for ampicillin and its derivatives

The analysis further demonstrated that the native form of ampicillin interacted with six active amino acid residues within its binding area. Following the introduction of a benzene ring in derivative 02, the number of interacting residues increased substantially to eleven, indicating an improvement in the antibiotic's binding affinity toward its target. Conversely, the incorporation of additional functional groups in derivative 03 resulted in a reduction of active residues, with only five observed interactions (as illustrated in Figure 3-17).

These findings highlight the structural flexibility of ampicillin and its derivatives in engaging with target proteins. Variations in the interacting amino acid residues resulting from chemical modifications may substantially influence binding affinity, thereby affecting the overall therapeutic efficacy of the antibiotic.

In the case of ceftriaxone and its products, the unmodified ceftriaxone molecule interacts with seven active amino acid residues. However, upon incorporation of a benzene ring and a carboxylic acid (–COOH) functional group, the binding site is broadened to involve ten residues, as seen in derivatives 02 and 09 (Figure 3-18)

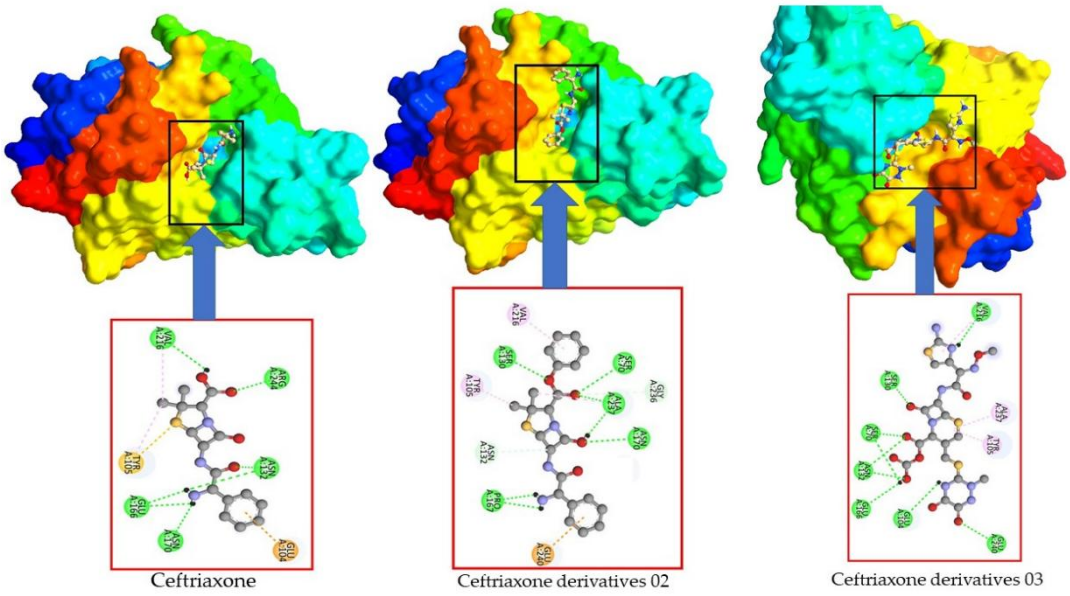


Figure 3-18 Binding site profiling and amino acid interactions of ceftriaxone derivatives

The study further revealed that the natural form of ampicillin engaged with six active amino acid residues in its binding region. After the incorporation of a benzene ring in derivative 02, the quantity of interacting residues significantly rose to eleven, signifying an enhancement in the antibiotic's binding affinity for its target. In contrast, the addition of extra functional groups in derivative 03 led to a decrease in active residues, with merely five interactions noted (as depicted in Figure 3-17).

These findings underscore the structural adaptability of ampicillin and its derivatives in interacting with target proteins. Chemical changes in the interactions between amino acid residues may significantly alter binding affinity, thereby impacting the overall therapeutic efficacy of the antibiotic.

The unaltered ceftriaxone molecule engages with seven active amino acid residues. Upon the insertion of a benzene ring and a carboxylic acid (–COOH) functional group, the binding site expands to encompass ten residues, as illustrated in derivatives 02 and 09 (Figure 3-18).

In the analysis of cefuroxime and its derivatives, a notable pattern was observed in the number of interacting amino acid residues. The parent cefuroxime molecule engages with eleven active site residues. Derivative 02 maintained this interaction profile, also involving eleven residues. However, derivative 03 exhibited a slight reduction in binding interactions, involving ten amino acid residues within the active site (as illustrated in Figure 3-19).

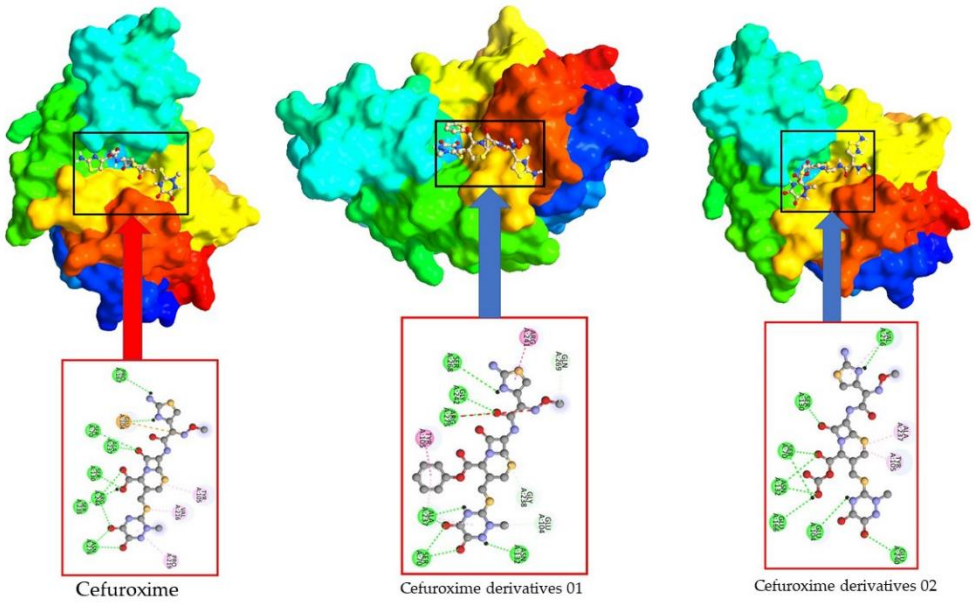


Figure 3-19 Profiling of amino acid residues involved in the active site of cefuroxime and its derivatives

3.7 Plasmid profiling and plasmid shifting

3.7.1 Plasmid profiling

Plasmid profiling was conducted to assess the presence and size of plasmids in bacterial isolates exhibiting varying levels of resistance, as determined by phenotypic and genotypic tests. Electrophoresis was performed using a 1 kb DNA ladder as a molecular weight marker, providing an accurate estimation of plasmid sizes. A positive control, consisting of *E. coli*

carrying a known plasmid of approximately 4 kb, was included in the first lane to serve as a reference.

The plasmid profiles of the most resistant isolates revealed the consistent presence of a single plasmid band, ~4 kb in size. The position of these bands aligns closely with the positive control, indicating that these isolates harbor plasmids of a similar size. This observation strongly suggests a potential role of the ~4 kb plasmid in conferring resistance, as it is present in isolates exhibiting robust resistance phenotypes (Figure 3-20).

Interestingly, the plasmid profiles of less resistant bacterial isolates varied. Several of these isolates did not display bands in the region typically associated with plasmids (~4 kb), suggesting either the absence of a plasmid or the presence of plasmids below the gel's detection threshold. These isolates were selected for further analysis, including transformation experiments, to explore the potential acquisition of resistance through plasmid uptake.

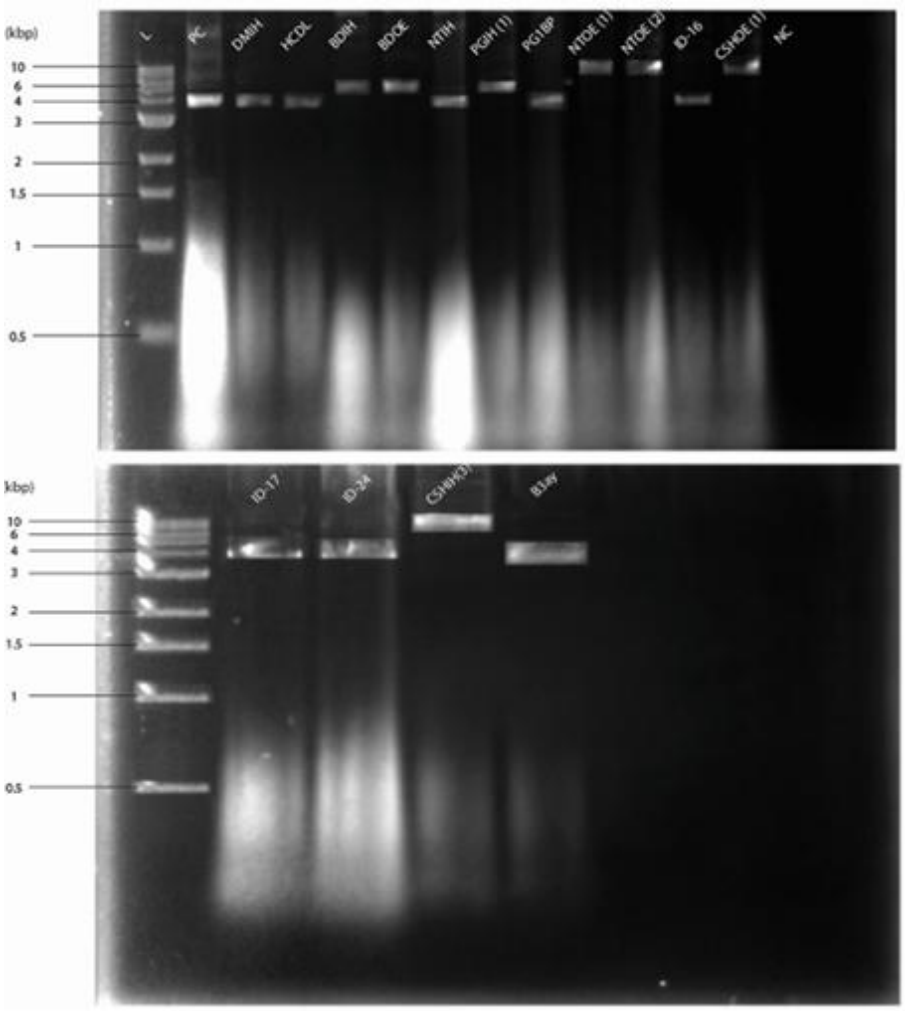


Figure 3-20 Plasmid profiling of the 15 isolates that were found to have most resistant genes in phenotypic and genotypic test

3.7.2 Transformation of the bacteria

Following the transformation process, the transformed isolates were subjected to a screening procedure to confirm the successful uptake of the plasmid. The screening was performed on Luria-Bertani (LB) agar plates supplemented with meropenem, which served as a selection marker. Meropenem was chosen due to the presence of meropenem resistance genes on the plasmid of interest.

Transformed bacteria were plated onto LB agar containing 2 µg/ml of meropenem. Growth on these plates indicated the successful acquisition of the plasmid, as only bacteria harboring the plasmid-encoded meropenem resistance genes were capable of surviving and proliferating under these conditions. In contrast, non-transformed bacteria, lacking the resistance genes, were unable to grow in the presence of meropenem (Figure 3-21).

This screening process effectively demonstrated the functional transfer of resistance genes via plasmid uptake, highlighting the role of horizontal gene transfer in spreading antimicrobial resistance.

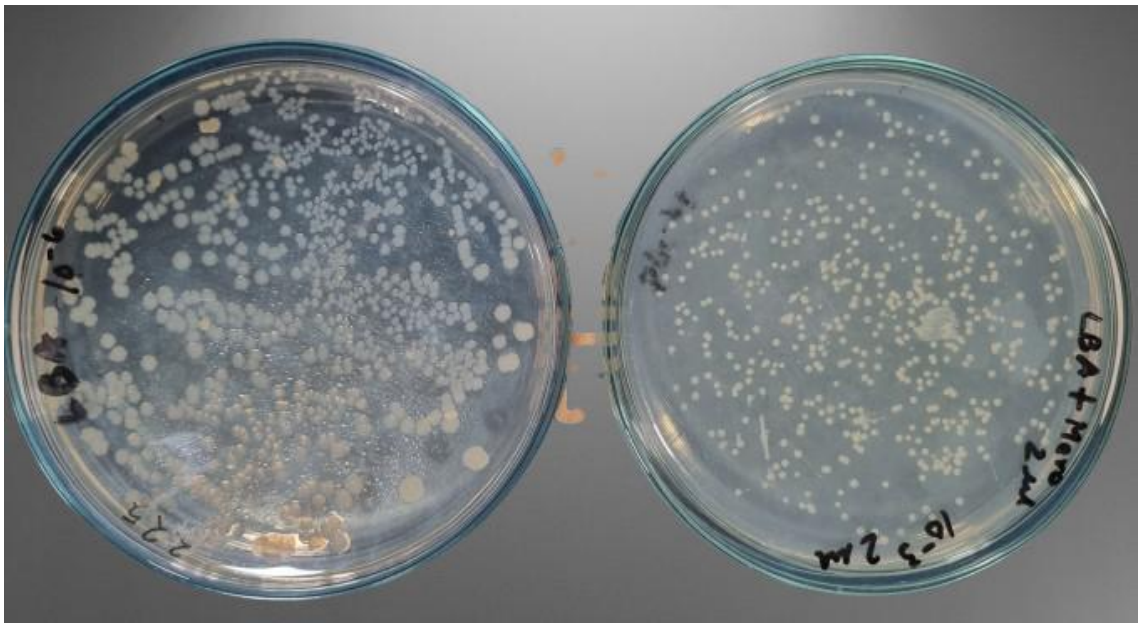


Figure 3-21 Transformed bacteria using LB agar media and meropenem

3.7.3 Plasmid shifting visualization through gel electrophoresis

To confirm the successful transfer of the plasmid into previously non-resistant bacteria, plasmids were extracted from the transformed isolates and analyzed using gel electrophoresis. The results showed a distinct band at approximately 4 kb, consistent with the size of the plasmid originally identified in the highly resistant donor bacteria.

The appearance of this band in the transformed isolates, which initially lacked any detectable plasmids in this size range, provides compelling evidence of horizontal plasmid transfer. This finding indicates that the plasmid encoding resistance genes was successfully introduced into and maintained by the recipient bacteria (Figure 3-22).

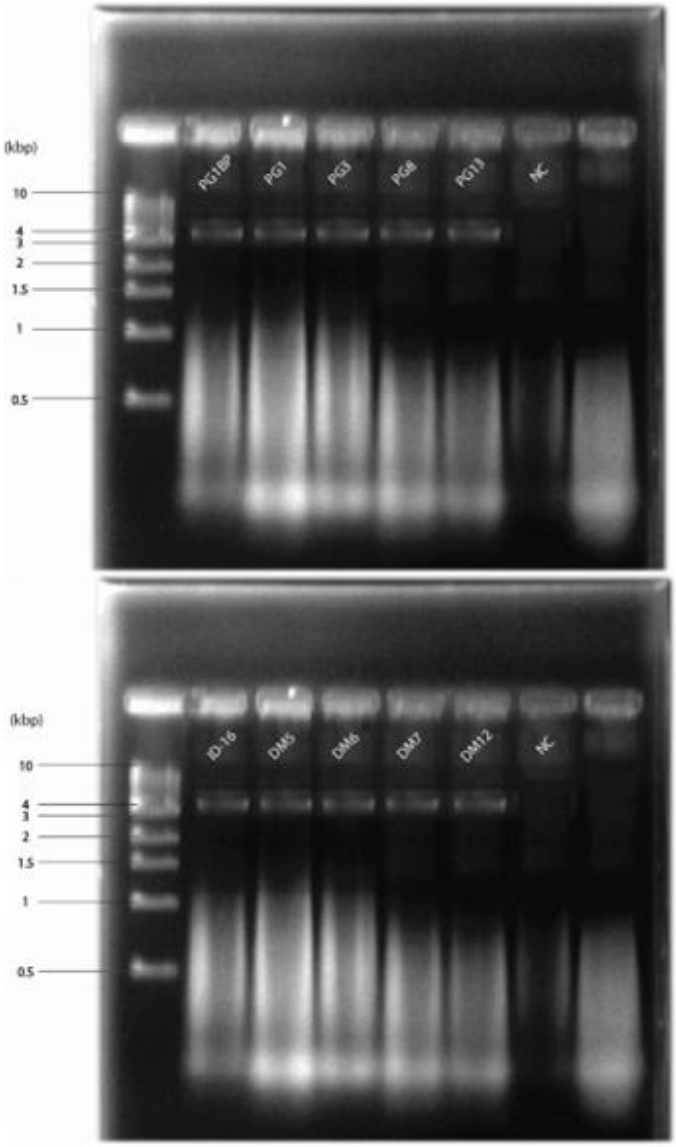


Figure 3-22 Gel electrophoresis results of the transformed bacteria showing bands at particular position of E. coli and Pseudomonas isolates

3.8 Whole genome analysis

3.8.1 Whole genome sequencing and analysis

To further elucidate the resistance mechanisms of the selected bacteria, whole-genome sequencing (WGS) was performed on eight isolates using the Illumina next-generation sequencing platform. The high-resolution sequencing data allowed for a comprehensive analysis of the genetic determinants underlying their resistance phenotypes.

Preliminary analysis of the genome sequences revealed that four of the isolates were presumptively identified as *E. coli*, designated as DMIH, HCDL, NTIH, and PG1BP. The remaining four isolates were identified as *Pseudomonas* species, designated as B3ay, ID16, ID17, and ID24.

3.8.2 Quality control, trimming, assembly and scaffolding of raw sequences

The whole-genome sequencing of the eight bacterial isolates was performed using the Illumina NovaSeq 6000 platform, generating high-quality raw reads in FASTQ format. A series of quality control (QC) steps were conducted to ensure the reliability of the sequencing data and optimize downstream analyses.

The raw reads were subjected to trimming to remove adapter sequences and low-quality bases. Reads with a Phred quality score below 30 were excluded to enhance the overall accuracy of the data. Additional QC parameters, such as per-base sequence quality, per-tile sequence quality, per-sequence quality scores, per-sequence GC content, per-base N content, sequence length distribution, and sequence duplication levels, were assessed, all yielding satisfactory results.

Following trimming, the high-quality reads were assembled to generate genome sequences in FASTA format. Since incomplete or erroneous genome assemblies can introduce misassembled regions, gaps, and errors, scaffolding was employed to further refine the assemblies. Scaffolding not only improved the accuracy and quality of the genome assembly but also reduced the number of contigs, enhancing the contiguity of the genomic data.

3.8.3 Identification of the isolates

The identification of eight bacterial isolates was conducted using two complementary genomic analysis tools, *KmerFinder* and Multilocus Sequence Typing (MLST). *KmerFinder* identified the isolates based on unique k-mer patterns in their genomic sequences, while MLST validated these results by analyzing sequence variations in a set of conserved housekeeping genes. The analysis revealed that four isolates (DMIH, HCDL, NTIH, and PG1BP) were consistently

identified as *E. coli* by both methods, confirming their classification. Among the other isolates, B3ay was identified as *Pseudomonas otitidis*, whereas ID-16, ID-17, and ID-24 were classified as *P. aeruginosa* (Table 5).

Table 5 Identification of the isolates using KmerFinder and MLST

Isolates	Identification	
	KmerFinder	MLST
DMIH	*E. coli*	*E. coli*
HCDL	*E. coli*	*E. coli*
NTIH	*E. coli*	*E. coli*
PG1BP	*E. coli*	*E. coli*
B3ay	*Pseudomonas otitidis*	*Pseudomonas otitidis*
ID-16	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
ID-17	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
ID-24	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*

3.8.4 Genome annotation and mapping of *E. coli* genomes

The genome annotation of four *E. coli* isolates (DMIH, HCDL, NTIH, and PG1BP) was performed using Prokka, yielding comprehensive insights into their genomic features. The number of contigs varied among the isolates, with DMIH having the highest number (284) and NTIH the lowest (139). The total genome size ranged from 4,985,284 bases in NTIH to 5,442,080 bases in HCDL. The predicted coding sequences (CDS) were also consistent with the genome sizes, with HCDL containing the highest number of CDS (5074) and NTIH the least (4675). The rRNA and tRNA counts varied slightly, with HCDL possessing 8 rRNAs and 85 tRNAs, while NTIH had 10 rRNAs and 82 tRNAs. Additionally, all isolates had a single tmRNA, and the number of repeat regions ranged from 2 to 3. These annotation results provide a foundational understanding of the genomic architecture of these isolates, supporting further functional and comparative genomic analysis (Table 6).

Table 6 Genome annotation results by prokka of E. coli isolates

Character	DMIH	HCDL	NTIH	PG1BP
Contigs	284	144	139	176
bases:	5265278	5442080	4985284	5171706
CDS	5016	5074	4675	4872
rRNA	4	8	10	9
tRNA	49	85	82	86
tmRNA	1	1	1	1
repeat_	2	2	2	3

3.8.5 Resistome analysis of *E. coli* isolates

The antibiotic resistance gene analysis via CARD (Comprehensive Antibiotic Resistance Database) of four *E. coli* genomes—DMIH, HCDL, NTIH, and PG1BP—reveals multiple antibiotic resistance genes and mechanisms across the strains. The analysis identifies significant resistance determinants for various antibiotic classes, including peptide antibiotics, macrolides, tetracyclines, aminoglycosides, cephalosporins, and nitroimidazoles. Each genome displays genes associated with antibiotic efflux mechanisms, such as ATP-binding cassette (ABC) transporters and small multidrug resistance (SMR) pumps, which contribute to the expulsion of antibiotics from bacterial cells, thereby reducing drug efficacy. Genes encoding proteins like msbA and mdfA found consistently across the genomes, are linked to resistance through efflux pump mechanisms, particularly against tetracycline and other broad-spectrum antibiotics. Several genes exhibit a high sequence similarity to known resistance determinants from other species, like *K. pneumoniae* efflux pump genes (KpnE and KpnF), suggesting potential horizontal gene transfer events (Table 7) (Figure 3-23).

The VFDB analysis of the *E. coli* strains DMIH, HCDL, NTIH, and PG1BP reveals several critical virulence genes that enhance these strains' pathogenicity by promoting adhesion, immune evasion, and nutrient acquisition. Notably, the gene espX5, identified in HCDL, is associated with the Type III secretion system, which is instrumental in manipulating host cell processes. This gene enables bacteria to adhere to host cells and evade immune detection, thus facilitating persistent infections. Another essential gene, entA, found in NTIH and PG1BP,

plays a central role in iron acquisition by producing siderophores, a vital adaptation in iron-limited host environments. Iron uptake is crucial for bacterial growth and colonization, allowing these strains to establish infections effectively (Table 8).

Table 7 Notable antibiotic resistance efflux pumps and gene cluster found via analysis on CARD of E. coli isolates

Sample	Notable antibiotic resistance gene efflux pumps and clusters
DMIH	AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, EmrE, EmrKY-TolC, FloR family, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtEF-TolC, MdtL, MdtM, QacE, SugE, Tet(A), TolC/OpmH, MarA, MarB, MarR
HCDL	AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, EmrKY-TolC, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtEF-TolC, MdtL, MdtM, QacE, SugE, Tet(A), TolC/OpmH,MarA, MarB, MarR
NTIH	AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, EmrKY-TolC, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtEF-TolC, MdtL, MdtM, QacE, SugE, Tet(A), TolC/OpmH,MarA, MarB, MarR
PG1BP	AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, EmrE, EmrKY-TolC, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtEF-TolC, MdtL, MdtM, QacE, SugE, Tet(A), TolC/OpmH,MarA, MarB, MarR

Table 8 Notable virulence genes found via analysis on VFDB of E. coli isolates

Sample	Notable virulence gene
DMIH	faeJ, papB, papI, papG, papF, papE, papK, papJ, papD, papC, papH, aslA, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR, espR4, espX5, espX4, csgB, astA, entA, entB, entE, entC, fepB, entS, fepD, fepG, fepC, entF, fes, fepA, entD, espR1, espX1, gspC, gspD, gspE, gspF, gspG, gspH, gspI, gspJ, gspK, gspL, gspM, kpsM, espL1, ompA, kpsD, iutA, iucD, iucC, iucB, iucA, csgF, csgG
HCDL	espX5, entA, entB, entE, entC, fepB, entS, fepD, fepG, fepC, entF, fes, fepA, entD, espY4, fimB, fimE, fimA, fimI, fimC, fimD, fimF, fimG, fimH, aslA, espL1, csgB, csgD, csgF, csgG, papX, papG, papF, papK, papJ, papD, espY3,

Sample	Notable virulence gene
	ompA, ybtS, ybtX, ybtQ, ybtP, ybtA, irp2, irp1, ybtU, ybtT, ybtE, fyuA, fdeC, ykgK/ecpR, yagZ/ecpA, yagY/ecpB, yagX/ecpC, yagW/ecpD, yagV/ecpE, chuS, shuA, shuT, chuW, shuX, chuY, chuU, chuV, espX4, gspM, gspL, gspK, gspJ, gspI, gspH, gspG, gspF, gspE, gspD, gspC, papI, papB, papH, papC, espY1, espX1, espL4, iutA, iucD, iucC, iucB, iucA
NTIH	entA, entB, entE, entC, fepB, entS, fepD, fepG, fepC, entF, fes, fepA, entD, gspC, gspD, gspE, gspF, gspG, gspH, gspI, gspJ, gspK, gspL, gspM, aslA, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR, fdeC, ompA, espL4, espX4, espL1, csgB, csgD, csgF, csgG, espR1, espX5, iutA, iucD, iucC, iucB, iucA, papI, papB, papH, papC, papD, papJ, papK, papF, papG, papX, espR4, ybtS, ybtX, ybtQ, ybtP, ybtA, irp2, irp1, ybtU, ybtT, ybtE, fyuA, espY1, espX1
PG1BP	entA, entB, entE, entC, fepB, entS, fepD, fepG, fepC, entF, fes, fepA, entD, csgB, csgD, csgF, csgG, espL1, astA, fimB, fimE, fimA, fimI, fimC, fimD, fimF, fimG, fimH, espX5, espX4, ompA, espX1, espR1, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR, fdeC, gspC, gspD, gspE, gspF, gspG, gspH, gspI, gspJ, gspK, gspL, gspM

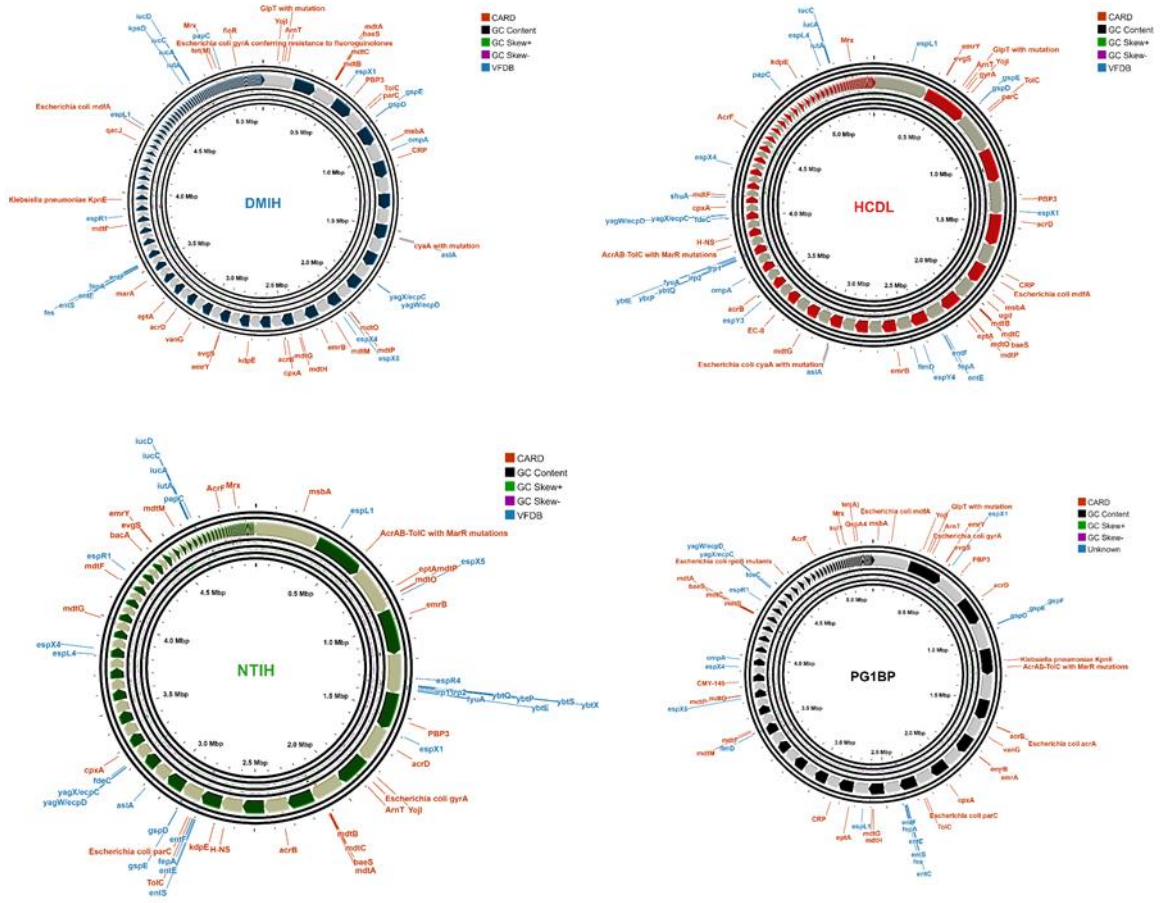


Figure 3-23 Mapping of resistome and virulence factors of E.coli isolates

3.8.6 Plasmid analysis of E. coli isolates

The plasmid analysis of clinical isolates DMIH, HCDL, NTIH, and PG1BP revealed distinct profiles of antibiotic resistance genes. DMIH contained TEM-1, sul2, and APH(3')-Ia, conferring resistance to β-lactams, sulfonamides, and aminoglycosides. HCDL harbored the CTX-M beta-lactamase gene, which allows resistance to cephalosporins, highlighting a significant threat to treatment efficacy. NTIH exhibited resistance genes CTX-M-15, OXA-1, and AAC(6')-Ib-cr5, targeting cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones. PG1BP, however, displayed the most extensive resistance profile with genes like CMY-145, dfrA12, aadA2, qacEdelta1, sul1, BRP(MBL), NDM-5, rmtB, TEM-1, Mrx, mphA, ErmB, and tet(A), covering multiple antibiotic classes, including carbapenems.

The virulence gene analysis identified notable differences in pathogenic potential. DMIH and NTIH both contained genes for the aerobactin siderophore system (iutA, iucD, iucC, iucB, and iucA), crucial for iron acquisition in host environments. Additionally, DMIH carried the astA

gene, which encodes an enterotoxin linked to gastrointestinal symptoms. In contrast, HCDL exhibited the pap operon (papI, papB, papH, papC, papX, papG, papF, papK, papJ, papD), known for its role in adherence to host cells, particularly in urinary tract infections. PG1BP, notably, lacked detectable virulence genes, suggesting most of it virulence gene comes from genomic DNA (Table 9) (Figure 3-24).

Table 9 Result of plasmid analysis of E. coli isolates

Sample	Antibiotic resistance genes	Virulence genes
DMIH	TEM-1, sul2, APH(3')-Ia	iutA, iucD, iucC, iucB, iucA, astA
HCDL	CTX-M beta-lactamase	papI, papB, papH, papC, papX, papG, papF, papK, papJ, papD
NTIH	CTX-M-15, OXA-1, AAC(6')-Ib-cr5	iutA, iucD, iucC, iucB, iucA
PG1BP	CMY-145, dfrA12, aadA2, qacEdelta1, sul1, BRP(MBL), NDM-5, rmtB, TEM-1, Mrx, mphA, ErmB, tet(A)	N/A

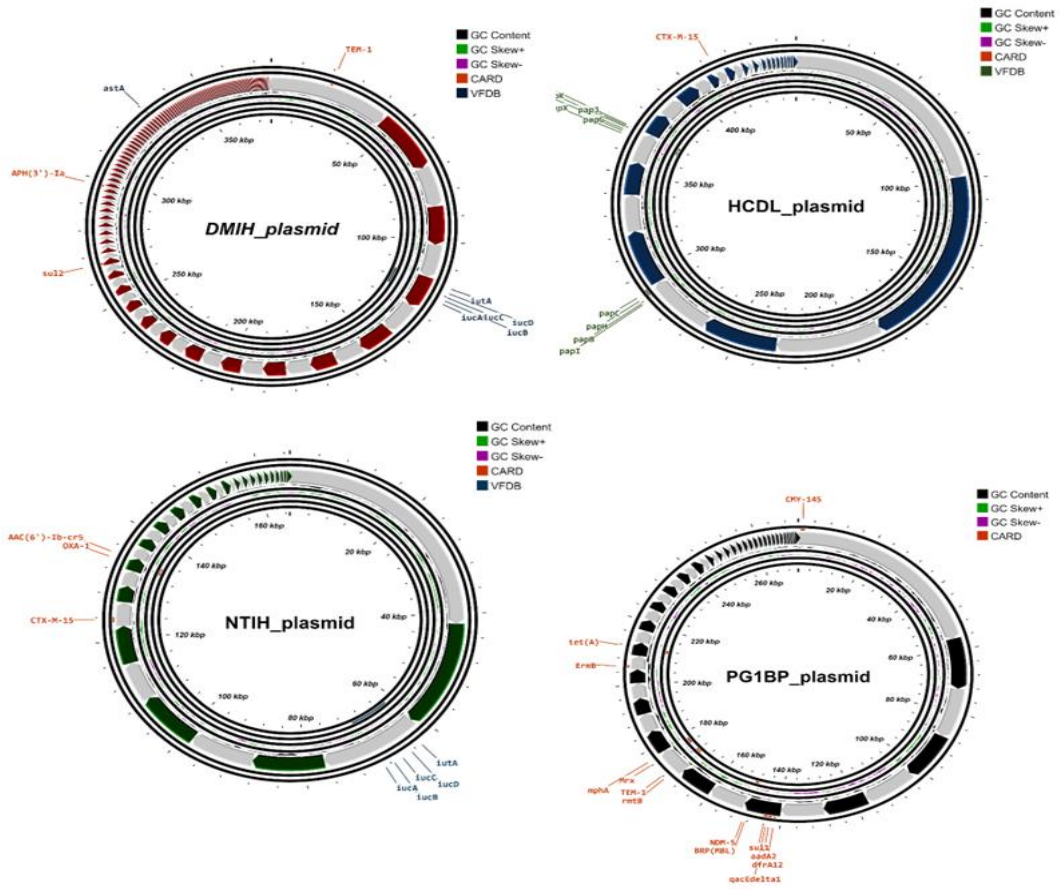


Figure 3-24 Genomic mapping of the plasmid of E. coli isolates

3.8.7 Comparative analysis of *E. coli* isolates

A comparative examination of multidrug-resistant bacteria in waste water adjacent to hospitals in Bangladesh is crucial due to the public health threats they pose. Hospital waste water often harbors elevated levels of antibiotics and pathogens, creating conditions that enable bacteria to rapidly acquire and disseminate resistance genes, leading to the development of multidrug-resistant strains. This resistance may spread to communal water systems and agricultural regions, presenting significant risks to human health and the environment. Through comparative analysis, researchers can identify common resistance genes and understand resistance patterns, facilitating the development of targeted intervention methods to curb the spread of these infections. This investigation provides policymakers with essential data, enabling them to implement more stringent laws on waste management and antibiotic use to mitigate the spread of resistant microorganisms within the population.

For the comparative analysis of the *E. coli* isolates, both codon-based and nucleotide identity metrics were utilized to determine the genetic similarities and evolutionary relationships. The study involved the selection of three comparative groups: nine genomes of *E. coli* from Bangladesh, ten genomes from other regions of Asia (excluding Bangladesh), and seven genomes from other *Escherichia* species, which served as outgroups. In total, 26 genomes were included in the analysis and compared against the four newly sequenced isolates. This comprehensive approach facilitated a strong comparison, emphasizing both intra-species and inter-species differences while offering insights into the genetic relationships and possible regional adaptations of the examined isolates.

3.8.7.1 Phylogenetic tree of *E. coli* isolates

The phylogenetic tree established on codon analysis provides a comprehensive visualization of the evolutionary relationships among various bacterial isolates, particularly focusing on *Escherichia* species. This analysis highlights the genetic closeness and evolutionary divergence within the dataset. Isolates PG1BP and DMIH exhibit close genetic proximity, forming a distinct subgroup within the tree. Similarly, isolates HCDL and NTIH cluster closely, suggesting a shared evolutionary lineage. All four of these isolates collectively form a clade alongside isolates retrieved from the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), which were predominantly sourced from human feces. This clade underscores a potential linkage to human hosts and the environmental conditions in Bangladesh, highlighting a geographical and ecological context to their evolutionary trajectory. The presence of distinct outgroups in the tree provides robust validation for its construction, as these

outgroups form separate, well-defined branches, confirming the evolutionary divergence of unrelated species from the primary clades of interest and reinforcing the credibility of the phylogenetic relationships depicted (Figure 3-25).

The clustering of the studied isolates with Bangladeshi strains may indicate shared genetic determinants and adaptations specific to their origin, particularly in relation to host-associated environments. These observations suggest that isolates DMIH, HCDL, NTIH, and PG1BP, which show genetic proximity to Bangladeshi human fecal isolates, may share virulence factors or traits relevant to epidemiological studies. Additionally, the inclusion of diverse Escherichia species, such as *Escherichia albertii* and *Escherichia fergusonii*, further illustrates the genetic diversity and distinct evolutionary pathways within the genus, as reflected in the separation of these species into individual lineages. The observed evolutionary closeness among some isolates may also provide valuable insights into their genetic composition, potential pathogenicity, and their adaptation to specific hosts or environmental conditions.

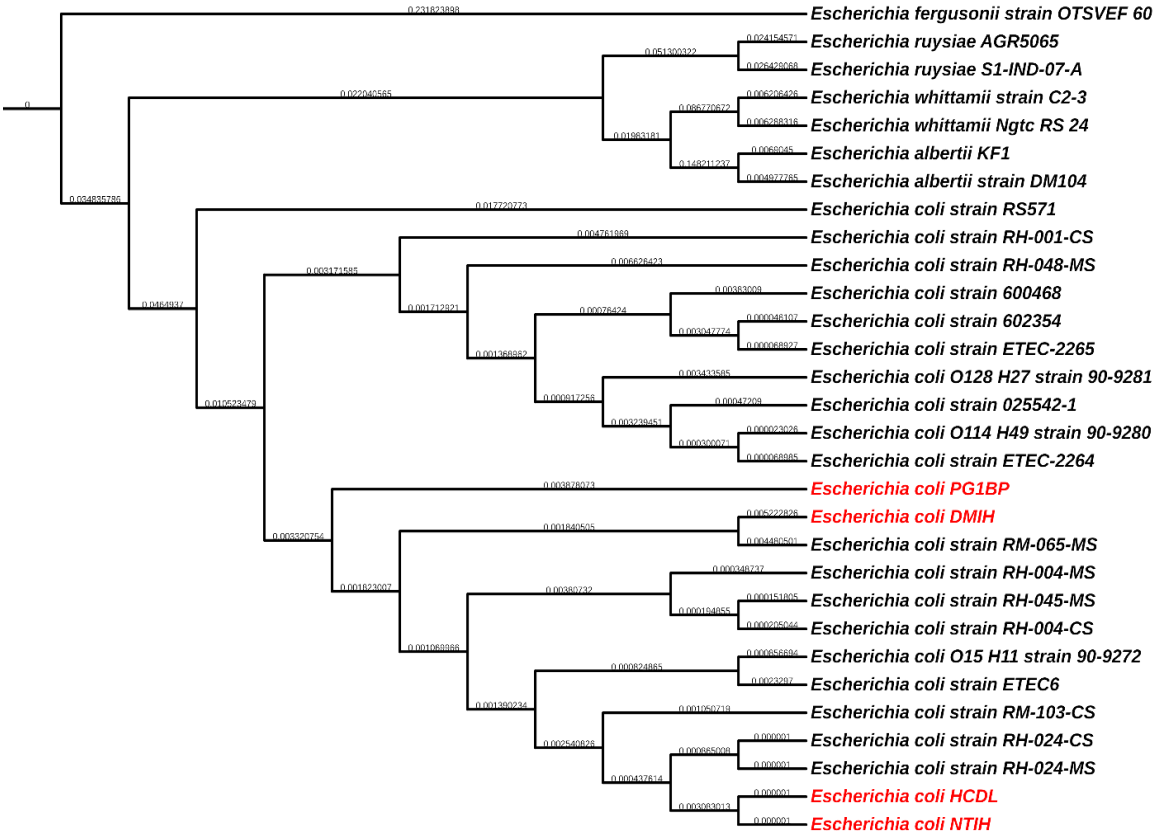


Figure 3-25 Phylogenetic tree of E. coli isolates

3.8.7.2 Average nucleotide identity analysis of *E. coli* isolates

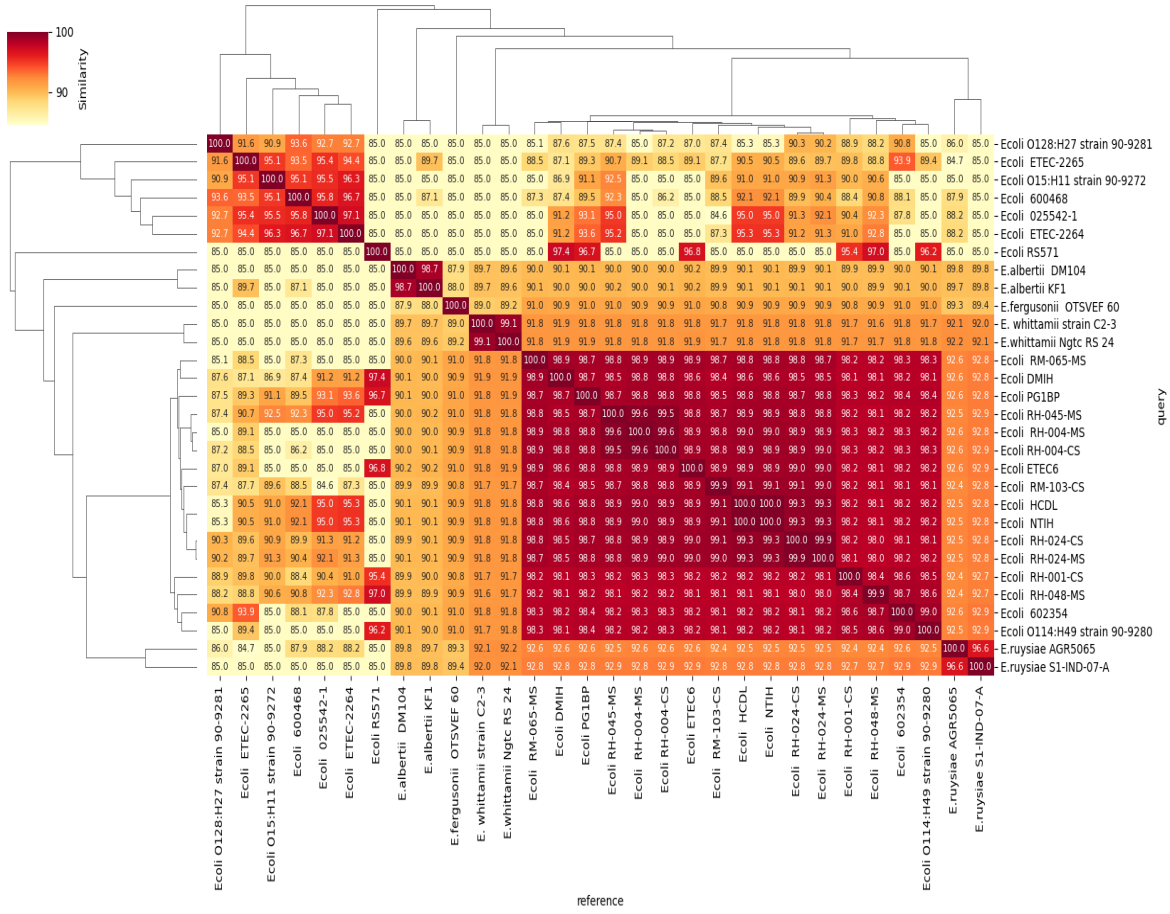


Figure 3-26 Heatmap based on ANI of E. coli isolates

The tree constructed using average nucleotide identity further supports the comparison of genetic similarities and relatedness among the isolates. The results reveal that DMIH and PG1BP demonstrate a close genetic relationship, as do NTIH and HCDL, reflecting a pattern of clustering based on genetic proximity. Moreover, the nucleotide identity of all the isolates aligns closely with that of isolates predominantly found in Bangladesh, suggesting a significant evolutionary connection or shared genetic determinants with isolates from this region. Interestingly, when comparing the isolates to other *E. coli* serotypes, the genetic dissimilarities are more pronounced than when compared to other *Escherichia* species, further emphasizing the unique characteristics of the Bangladeshi isolates (Figure 3-26).

The phylogenetic relationships also highlight the formation of three distinct evolutionary groups: the outgroup, consisting of *E. coli* strains unrelated to those found in Bangladesh; other

strains of *E. coli* found in Bangladesh; and a separate clade comprising isolates retrieved from Bangladesh. This division underscores the evolutionary divergence within the *E. coli* species and suggests that environmental or geographical factors in Bangladesh may play a significant role in shaping the genetic makeup of these isolates. The clustering of Bangladeshi isolates as a cohesive group, distinct from other global strains, further validates the genetic uniqueness and evolutionary trajectory of these organisms.

3.8.8 Genome annotation and mapping of *Pseudomonas* genomes

The assembly report of the four *Pseudomonas* isolates—B3ay, ID-16, ID-17, and ID-24 revealed notable differences in their genomic characteristics. The number of contigs varied significantly, with B3ay exhibiting the lowest count at 37, suggesting a more contiguous and complete genome assembly. In contrast, ID-24 showed the highest count at 112, indicating a more fragmented assembly. ID-16 and ID-17 display intermediate contig numbers at 75 and 65, respectively, reflecting variations in sequencing or assembly processes. When comparing the total bases, ID-24 has the largest genome, with 6,835,783 bases, followed closely by ID-17 with 6,777,677 bases and ID-16 with 6,749,922 bases. In contrast, B3ay has the smallest genome, at 6,165,903 bases. This suggests that B3ay may have a smaller genome size relative to the other isolates, which are relatively similar in size.

The number of coding sequences (CDS) follows a similar trend, with B3ay having the fewest CDS at 5,560, while ID-16, ID-17, and ID-24 have higher counts at 6,221, 6,247, and 6,246, respectively. The presence of ribosomal RNA (rRNA) genes is consistent across most isolates, with B3ay, ID-17, and ID-24 containing six rRNA genes, whereas ID-16 has three, indicating a possible variation in rRNA gene content or assembly completeness. The transfer RNA (tRNA) gene counts are highest in B3ay at 68, while ID-17 and ID-24 show slightly lower numbers at 67 and 65, and ID-16 has the fewest at 58. All isolates contain one tmRNA gene, suggesting consistency in this feature. Additionally, the presence of repeat sequences varies, with B3ay lacking repeats entirely, while ID-16 and ID-17 have two repeats each, and ID-24 has three (Table 10).

Table 10 Genome annotation results by prokka of Pseudomonas isolates

Character	B3ay	ID-16	ID-17	ID-24
Contigs	37	75	65	112
bases:	6165903	6749922	6777677	6835783
CDS	5560	6221	6247	6246

Character	B3ay	ID-16	ID-17	ID-24
rRNA	6	3	6	6
tRNA	68	58	67	65
tmRNA	1	1	1	1
repeat_	0	2	2	3

3.8.9 Resistome analysis of *Pseudomonas* isolates

The resistome analysis of the four *Pseudomonas* isolates—B3ay, ID-16, ID-17, and ID-24—revealed significant differences in their antibiotic resistance strategies, particularly involving efflux pump systems and enzymatic antibiotic inactivation. The B3ay isolate primarily depends on several well-characterized efflux pumps, such as EmrAB-OMF, MexAB-OprM, and MexCD-OprJ. These molecular transporters actively expel diverse antibiotics from the bacterial cell, lowering intracellular concentrations and thereby contributing to its resistance. Interestingly, no antibiotic-modifying enzymes were detected in B3ay, suggesting that efflux-based mechanisms predominantly drive its defense against antimicrobials.

In contrast, isolates ID-16 and ID-17 exhibit a multifaceted resistance approach, combining efflux pump activity with enzymatic degradation of antibiotics. Both share a largely overlapping complement of efflux pumps, including EmrAB-OMF, MexAB-OprM, and MexEF-OprN, which are central contributors to multidrug resistance phenotypes. Alongside these, they harbor aminoglycoside-modifying enzymes such as APH(3')-II and APH(3')-XV, as well as beta-lactamases from the OXA-50 and PDC families, which neutralize beta-lactam antibiotics. These combined mechanisms afford these isolates a robust and versatile resistance profile, effective against multiple classes of antibiotics. The striking similarity in their resistomes points toward a close genetic or evolutionary relationship, likely shaped by shared environmental exposures or selective antibiotic pressures.

Among the four, ID-24 stands out for possessing the most varied and extensive resistome. Beyond the efflux pumps common to the other isolates, ID-24 also encodes additional transporters, such as the CmlA family, which mediates resistance to chloramphenicol, and Tet(G), which confers protection against tetracycline antibiotics. Complementing these efflux systems is an expansive array of antibiotic inactivation enzymes, including diverse variants of the AAC(3') and APH(3') families. Moreover, ID-24 carries members of the NDM family of

metallo-beta-lactamases—known for conferring formidable resistance to carbapenems—as well as the VEB family of extended-spectrum beta-lactamases. This combination enables ID-24 to resist a broad spectrum of antibiotic classes, spanning aminoglycosides, beta-lactams, carbapenems, chloramphenicol, and tetracyclines, reflecting its evolutionary adaptation to complex and varied selective environments (see Table 11).

Together, these findings illustrate the heterogeneity in resistance mechanisms across *Pseudomonas* isolates and highlight how diverse genetic repertoires equip bacteria to survive under antimicrobial pressure, informing strategies for combating multidrug-resistant infections.

Table 11 Result of resistome analysis of Pseudomonas isolates

Sample name	Notable resistance gene efflux pumps and gene cluster	Antibiotic inactivation enzyme
B3ay	EmrAB-OMF, EmrAB-TolC, MacA, MacB, MdtABC-OMF, MdtABC-TolC, MexAB-OprM, MexCD-OprJ, MexCD-OprJ system, MexEF-OprN, MexEF-OprN system, MexJK-OprM/OpmH, MexVW-OprM, TolC/OpmH, TriABC-OpmH	N/A
ID-16	EmrAB-OMF, EmrAB-TolC, MacA, MacB, MdtABC-OMF, MdtABC-TolC, MexAB-OprM, MexCD-OprJ, MexCD-OprJ system, MexEF-OprN, MexEF-OprN system, MexJK-OprM/OpmH, MexPQ-OpmE, MexPQ-OpmE system, MexVW-OprM, MexXY-OMP, TolC/OpmH, TriABC-OpmH	APH(3')-II/APH(3')-XV, CatB family, OXA-50 family, PDC family
ID-17	EmrAB-OMF, EmrAB-TolC, MacA, MacB, MdtABC-OMF, MdtABC-TolC, MexAB-OprM, MexCD-OprJ, MexCD-OprJ system, MexEF-OprN, MexEF-OprN system, MexJK-OprM/OpmH, MexPQ-OpmE, MexPQ-OpmE system, MexVW-OprM, MexXY-OMP, TolC/OpmH, TriABC-OpmH	APH(3')-II/APH(3')-XV, CatB family, OXA-50 family, PDC family

Sample name	Notable resistance gene efflux pumps and gene cluster	Antibiotic inactivation enzyme
ID-24	CmlA family, EmrAB-OMF, EmrAB-TolC, FloR family, MacA, MacB, MdtABC-OMF, MdtABC-TolC, MexAB-OprM, MexCD-OprJ, MexCD-OprJ system, MexEF-OprN, MexEF-OprN system, MexHI-OpmD, MexHI-OpmD system, MexJK-OprM/OpmH, MexPQ-OpmE, MexPQ-OpmE system, MexVW-OprM, MexXY-OMP, QacE, Tet(G), TolC/OpmH, TriABC-OpmH	AAC(3)-I, AAC(3)-II,III,IV,VI,VIII,IX,X, APH(3')-II/APH(3')-XV, APH(3')-III/APH(3')-IV/APH(3')-VI/APH(3')-VII, CatB family, NDM family, OXA-50 family, PDC family, VEB family

The analysis of virulence gene profiles among the four *Pseudomonas* isolates—B3ay, ID-16, ID-17, and ID-24—reveals notable diversity, reflecting varying degrees of pathogenicity and environmental adaptation.

Isolate B3ay exhibits a comparatively modest assortment of virulence genes. It carries key genes involved in alginate biosynthesis, such as algC, algR, and algB, which contribute to biofilm formation and help the bacterium evade host immune responses. Moreover, the genes linked with Type VI secretion system (T6SS), including icmF1/tssM1 and dotU1, enable B3ay to compete with neighboring bacteria and interact effectively with host cells. The presence of pili-related genes, such as pilT, pilU, and pilI, further supports its motility and ability to adhere to host tissues. Regulatory genes, particularly algU, facilitate adaptability in changing environments. Taken together, these elements suggest that while B3ay possesses fundamental virulence factors, its overall pathogenic potential is relatively subdued compared to its counterparts.

In contrast, ID-16 exhibits a significantly more extensive repertoire of virulence genes. This includes a broad set of genes that govern motility—pilJ, pilK, fliA, fliC, fliD—as well as those involved in adhesion and robust biofilm production such as algC, algU, mucA, mucB, and mucC. Its arsenal contains several T6SS components (hcp1, vgrG1a, vgrG1b) and Type III

secretion system (T3SS) genes (pscC, pscD, pscF), underscoring a heightened capacity for interacting with and manipulating host cells. Additionally, the presence of toxin-related genes, such as exoY and exoT, suggests a potent ability to disrupt host cellular mechanisms. Complementing these features, siderophore biosynthesis genes including pvdM and pvdF enable efficient iron acquisition, a critical aspect of bacterial survival and virulence. Collectively, this gene ensemble highlights ID-16's aggressive pathogenic potential coupled with sophisticated adaptive strategies.

Similarly, the virulence profile of isolate ID-17 closely aligns with that of ID-16. It shares many key virulence determinants, such as T6SS genes (hcp1, vgrG1a), T3SS components (pscC, pscF), as well as toxin-related genes (exoY, exoT). Biofilm-related genes including algC and algU, along with a range of pili and flagella-associated genes (pilJ, pilK, fliA, fliC, fliD), equip ID-17 with strong capabilities in motility, adhesion, immune system evasion, and host cell interference. This similarity suggests comparable virulence and adaptability to ID-16, marking both as potentially formidable pathogens.

Among all isolates, ID-24 possesses the most comprehensive and diverse virulence gene collection. Beyond sharing important genes with ID-16 and ID-17—such as hcp1, vgrG1a, exoT, and algU—ID-24 harbors unique virulence factors including cmlA and floR, which contribute to resistance against host defense molecules. The presence of rhlA, rhlB, and rhlC genes is particularly notable, as these are fundamental to the biosynthesis of rhamnolipids, surfactant molecules pivotal for virulence and biofilm architecture. Moreover, ID-24 carries multiple siderophore-associated genes (pvdM, pvdN, pchA, pchE), enhancing its ability to scavenge iron and thrive in hostile environments. This extensive virulence gene profile suggests ID-24 is the most adaptable and potentially pathogenic isolate within the group.

Overall, the range of virulence gene profiles among the isolates paints a spectrum of pathogenic potential—from the relatively rudimentary virulence machinery of B3ay to the complex and robust systems present in ID-16, ID-17, and most notably, ID-24. This gradient reflects differing capabilities in host interaction, immune evasion, and environmental fitness (see Table 12 and Figure 3-27).

Table 12 Result of virulence gene analysis of Pseudomonas isolates

Sample Name	Virulence Gene
B3ay	algC, algR, pilT, pilU, pilG, pilH, pilI, pilJ, icmF1/tssM1, dotU1, hsiJ1, hsiB1/vipA, hsiC1/vipB, hcp1, hsiF1, hsiG1, clpV1, vgrG1a, vgrG1b, pvdL, mbtH-like, pvdS, xcpA/pilD, pilR, algU, pvdM, pvdN, pvdO, pvdH, pvdA, fliA, fleN, flhA, fliR, fliQ, fliP, fliN, fliM, fliI, fliG, fliF, fliE, fleQ, flgI, flgH, flgG, flgF, flgC, xcpT, xcpS, xcpR, xcpQ, algW, algA, algF, algI, alg8, algD, waaF, waaC, waaG, waaP, waaA, motA, algB
ID-16	chpE, chpD, chpC, chpB, chpA, pilK, pilJ, pilI, pilH, pilG, pilU, pilT, algB, algU, mucA, mucB, mucC, mucD, phzM, mucE, pvdF, pvdO, pvdN, pvdM, pvdP, pvdA, pvdQ, exoY, algC, motB, motA, waaA, waaP, waaG, waaC, waaF, pilQ, pilP, pilO, pilN, pilM, algP/algR3, algQ, algR, algZ, pilS, pilR, fimT, fimU, pilV, pilW, pilX, pilY1, pilY2, pilE, ptxR, pvcD, pvcC, pvcB, pvcA, pchA, pchB, pchC, pchD, pchR, pchE, pchF, pchG, pchH, pchI, fptA, phzS, vgrG1a, clpV1, hsiH1, hsiG1, hsiF1, hsiE1, hcp1, hsiC1/vipB, hsiB1/vipA, hsiA1, fha1, lip1, hsiJ1, dotU1, icmF1/tssM1, tagF/pppB, pppA, ppkA, tagT, tagS, tagR, tagQ, phzH, exoT, pvdS, pvdG, pvdL, pvdH, mbtH-like, mucP, algA, algF, algJ, algI, algL, algX, algG, algE, algK, alg44, alg8, algD, motY, tse3, rhlA, rhlB, rhlI, flgN, flgM, flgA, tse2, xcpZ, xcpY, xcpX, xcpW, xcpV, xcpU, xcpT, xcpS, xcpR, xcpP, xcpQ, fimV, phzA1, lasA, tse1, pscL, pscK, pscJ, pscI, pscH, pscG, pscF, pscE, pscD, pscC, pscB, exsD, exsA, exsB, exsE, exsC, popD, popB, pcrH, pcrV, pcrG, pcrR, pcrD, pcr4, pcr3, pcr2, pcr1, popN, pscN, pscO, pscP, pscQ, pscR, pscS, pscT, pscU, phzG1, phzF1, phzE1, phzD1, phzC1, lasB, pilF, exoS, plcH, flgB, flgC, flgD, flgE, flgF, flgG, flgH, flgI, flgJ, flgK, fleQ, fleS, fleR, fliE, fliF, fliG, fliH, fliI, fliJ, rhlC, toxA, algW, pilB, xcpA/pilD, motD, motC, fliA, fleN, flhF, flhA, flhB, fliR, fliQ, fliP, fliO, fliN, fliM, fliL, fliK, lasI, aprA
ID-17	motD, motC, fliA, fleN, flhF, flhA, flhB, fliR, fliQ, fliP, fliO, fliN, fliM, fliL, fliK, lasI, aprA, algB, mucD, mucC, mucB, mucA, algU, exoY, pilS, pilR, fimT, fimU, pilV, pilW, pilX, pilY1, pilY2, pilE, mucE, phzM, phzA1, phzB1, pvdQ, pvdA, pvdP, pvdM, pvdN, pvdO, pvdF, motB, motA, waaA, waaP, waaG, waaC, waaF, pilQ, pilP, pilO, pilN, pilM, algP/algR3, algQ, algR, algZ, algC, ptxR, pvcD, pvcC, pvcB, pvcA, mbtH-like, pvdH, pvdL, pvdG, pvdS, phzS, fptA, pchI, pchH, pchG, pchF, pchE, pchR, pchD, pchC, pchB, pchA, vgrG1a, clpV1, hsiH1, hsiG1, hsiF1, hsiE1, hcp1, hsiC1/vipB, hsiB1/vipA, hsiA1, fha1, lip1, hsiJ1, dotU1, icmF1/tssM1,

Sample Name	Virulence Gene
	tagF/pppB, pppA, ppkA, tagT, tagS, tagR, tagQ, phzH, exoT, tse2, phzB1, phzA1, lasA, tse1, pscL, pscK, pscJ, pscI, pscH, pscG, pscF, pscE, pscD, pscC, pscB, exsD, exsA, exsB, exsE, exsC, popD, popB, pcrH, pcrV, pcrG, pcrR, pcrD, pcr4, pcr3, pcr2, pcr1, popN, pscN, pscO, pscP, pscQ, pscR, pscS, pscT, pscU, phzG1, phzF1, phzE1, phzD1, phzC1, mucP, algA, algF, algJ, algI, algL, algX, algG, algE, algK, alg44, alg8, algD, motY, tse3, rhlA, rhlB, rhlI, flgN, flgM, flgA, fimV, xcpQ, xcpP, xcpR, xcpS, xcpT, xcpU, xcpV, xcpW, xcpX, xcpY, xcpZ, exoS, pilF, lasB, xcpA/pilD, pilB, algW, pilT, pilU, pilG, pilH, pilI, pilJ, pilK, chpA, chpB, chpC, chpD, chpE, plcH, flgB, flgC, flgD, flgE, flgF, flgG, flgH, flgI, flgJ, flgK, fleQ, fleS, fleR, fliE, fliF, fliG, fliH, fliI, fliJ, rhlC, toxA
ID-24	vgrG1b, vgrG1a, clpV1, hsiH1, hsiG1, hsiF1, hsiE1, hcp1, hsiC1/vipB, hsiB1/vipA, hsiA1, fha1, lip1, hsiJ1, dotU1, icmF1/tssM1, tagF/pppB, pppA, ppkA, tagT, tagS, tagR, tagQ, phzH, exoT, pvdQ, pvdA, pvdP, pvdM, pvdN, pvdO, pvdF, mbtH-like, pvdH, pvdL, pvdG, pvdS, pilE, pilY2, pilY1, pilX, pilW, pilV, fimU, fimT, pilR, pilS, algB, pilT, pilU, pilG, pilH, pilI, pilJ, pilK, chpA, chpB, chpC, chpD, chpE, motD, motC, fliA, fleN, flhF, flhA, flhB, fliR, fliQ, fliP, fliO, fliN, fliM, fliL, fliK, lasI, exoU, flgB, flgC, flgD, flgE, flgF, flgG, flgH, flgI, flgJ, flgK, phzB1, phzA1, phzM, mucE, pilF, lasB, mucP, algA, algF, algJ, algI, algL, algX, algG, algE, algK, alg44, alg8, algD, motY, rhlI, rhlB, rhlA, tse3, algC, algZ, algR, algQ, algP/algR3, pvcA, pvcB, pvcC, pvcD, ptxR, flgN, flgM, flgA, xcpZ, xcpY, xcpX, xcpW, xcpV, xcpU, xcpT, xcpS, xcpR, xcpP, xcpQ, fimV, toxA, rhlC, fliJ, fliI, fliH, fliG, fliF, fliE, fleR, fleS, fleQ, pchA, pchB, pchC, pchD, pchR, pchE, pilM, pilN, pilO, pilP, pilQ, waaF, waaC, waaG, waaP, waaA, motA, motB, phzS, fptA, pchI, pchH, pchG, pchF, xcpA/pilD, pilB, algW, plcH, mucD, mucC, mucB, mucA, algU, tse2, phzC1, pscU, pscT, pscS, pscR, pscQ, pscP, pscO, pscN, popN, pcr1, pcr2, pcr3, pcr4, pcrD, pcrR, pcrG, pcrV, pcrH, popB, popD, exsC, exsE, exsB, exsA, exsD, pscB, pscC, pscD, pscE, pscF, pscG, pscH, pscI, pscJ, pscK, pscL, tse1, lasA, phzA1, phzB1, aprA, phzG1, exoY

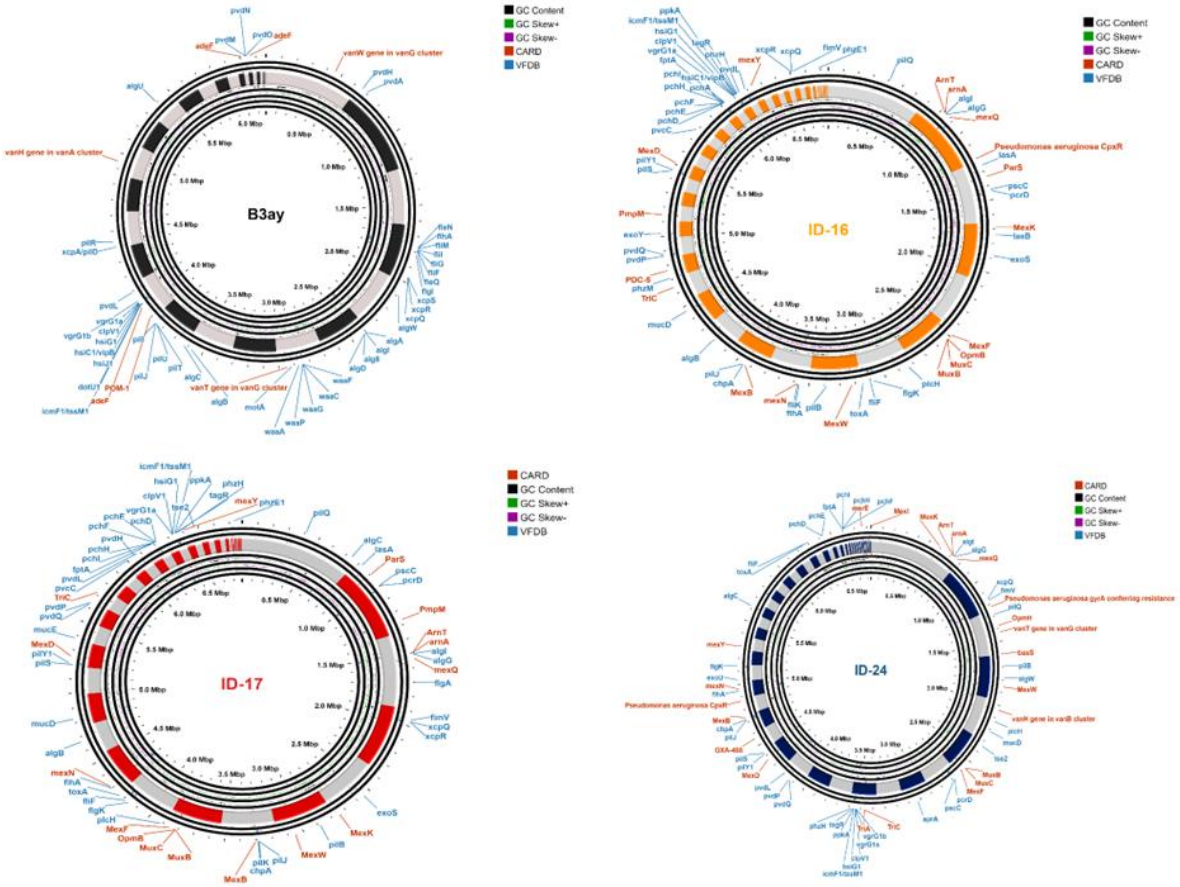


Figure 3-27 Mapping of the resistome and virulence factor of Pseudomonas isolates

3.8.10 Plasmid analysis of *Pseudomonas* isolates

Plasmid analysis of four *Pseudomonas* isolates—designated B3ay, Id-16, ID-17, and ID-24—revealed significant genetic diversity concerning resistance and virulence determinants. Notably, isolate B3ay carried the resistance gene *PDM-1* alongside virulence-related genes *algC*, *algR*, and *algB*, which are known to contribute to biofilm development and evasion of host immune responses. This genetic configuration suggests an enhanced potential for pathogenicity in this isolate. Conversely, isolates Id-16 and ID-17 did not display any detectable plasmid-borne resistance or virulence genes, implying a possible lack of plasmid-mediated traits that aid in adaptability or survival. In contrast, isolate ID-24 was found to possess a broad spectrum of resistance genes, including *APH(3')-VIa*, *VEB-9*, *cmlA5*, *AAC(3)-Ic*, *ANT(2'')-Ia*, *dfrA1*, *qacEdelta1*, and *sul1*, which collectively confer multidrug resistance. Interestingly, this isolate lacked identifiable virulence genes, suggesting a potential evolutionary trade-off where increased antimicrobial resistance occurs at the expense of virulence factors (see Table 13 and Figure 3-28).

Table 13 Result of plasmid analysis of Pseudomonas isolates

Sample name	Resistance gene	Virulent gene
B3ay	PDM-1	algC, algR, algB
ID-16	N/A	N/A
ID-17	N/A	N/A
ID-24	APH(3')-VIa, VEB-9, cmlA5, AAC(3)-Ic, ANT(2'')-Ia, dfrA1, qacEdelta1, sul1	N/A

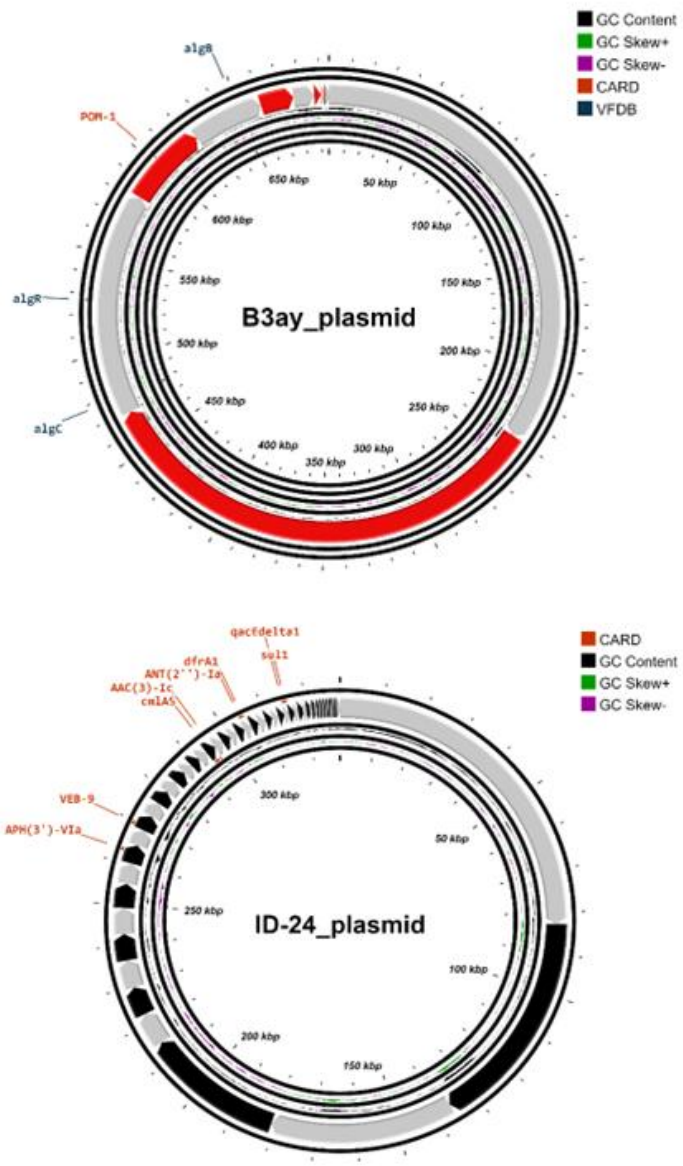


Figure 3-28 Mapping of the resistome and virulence factor of Pseudomonas sp. plasmid

3.8.11 Comparative analysis of *Pseudomonas* isolates

For the comparative genetic analysis of *Pseudomonas* isolates, both codon-based metrics and nucleotide identity measures were utilized to thoroughly assess genetic similarities and unravel evolutionary relationships. The study encompassed a wide-ranging collection of isolates, including one *Pseudomonas putida*, two *Pseudomonas fluorescens*, five *Pseudomonas otitidis*, and a total of ten isolates originating from Bangladesh. In addition to these, four newly sequenced isolates were integrated with twelve others sourced from neighboring regions across Asia. Altogether, this comprehensive dataset comprised 30 genomes, establishing a robust framework for in-depth comparative analysis.

This methodical approach enabled the identification of both intra-species and inter-species variations, offering nuanced perceptions into the genetic correlation among the isolates as well as illuminating possible regional adaptations within the *Pseudomonas* genus. By incorporating isolates from diverse geographic locations and various species, the study ensured a broad and detailed evaluation of evolutionary dynamics and genetic diversity, enhancing our understanding of how these bacteria have evolved and adapted in different ecological niches.

3.8.11.1 Phylogenetic analysis of the *Pseudomonas* isolates

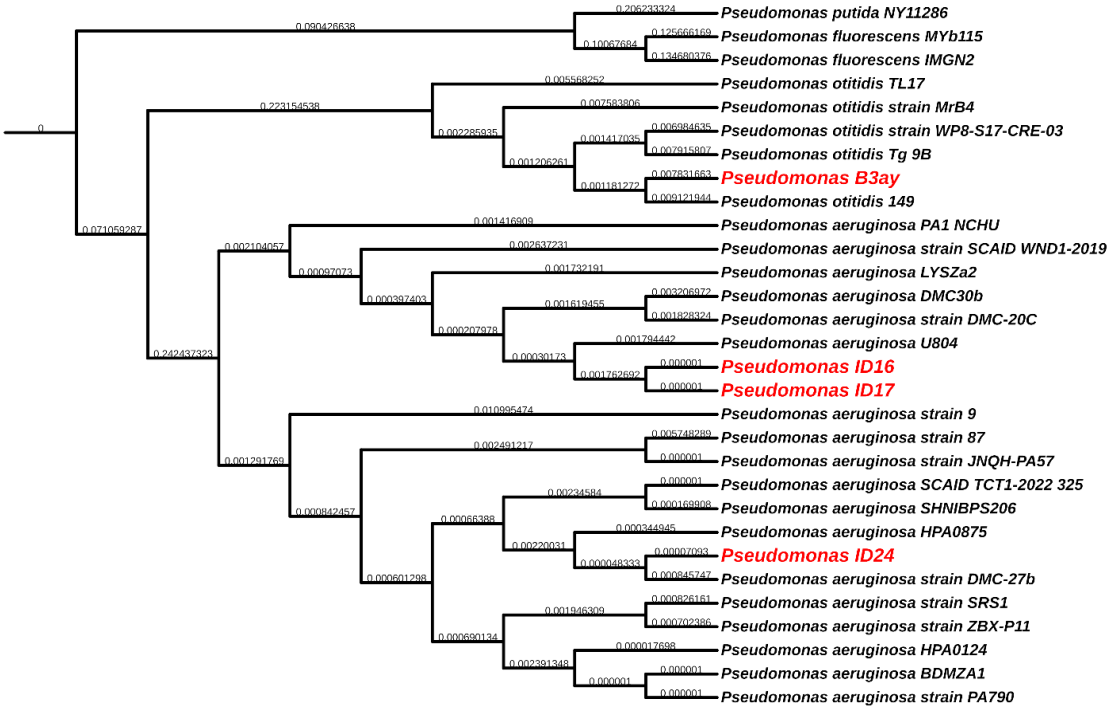


Figure 3-29 Phylogenetic tree of the Pseudomonas isolates

The phylogenetic analysis of the *Pseudomonas* isolates presents several notable insights into their evolutionary relationships. The isolate B3ay distinctly clusters within the *Pseudomonas otitidis* lineage, underscoring its close genetic affiliation with this species—a feature that distinguishes it from the other four newly sequenced samples. This clear placement affirms the uniqueness of *Pseudomonas otitidis* as a separate evolutionary branch, distinct from its congeners.

Meanwhile, isolates ID-16 and ID-17 form a tightly associated clade, reflecting their considerable genetic similarity. This cluster also encompasses other isolates from Bangladesh, including DMC-30b and DMC-20c, as well as certain strains from broader Asian contexts. Such grouping suggests a shared ancestral lineage and hints at regional evolutionary pressures, possibly driven by localized environmental conditions or host interactions that have shaped adaptation in this geographic area.

On the other hand, isolate ID-24 occupies a separate clade within the *Pseudomonas aeruginosa* cluster, which includes the Bangladeshi isolate DMC-27b and additional Asian strains. This suggests its genetic congruence with a subgroup of *P. aeruginosa* that may be specialized or evolved to thrive under specific regional environmental conditions.

Notably, the phylogenetic tree reveals a well-defined distinction of *Pseudomonas putida* and *Pseudomonas fluorescens*, which stand as clear outgroups, highlighting their evolutionary divergence from the main P. otitidis and P. aeruginosa clades. This separation reaffirms the genetic distances and supports their classification as distinct evolutionary lineages within the genus.

Overall, the tree's topology portrays a landscape where Bangladeshi isolates exhibit strong genetic cohesion within their regional clusters, yet simultaneously display a broad diversity in their evolutionary paths. The distinct branching patterns of species such as P. putida and P. fluorescens further validate the phylogenetic distances illustrated. The segregation of the four sequenced isolates into discrete groups accentuates their genetic heterogeneity, likely reflective of varied ecological roles or adaptive strategies across species and locales.

These comprehensive findings not only highlight the expansive genetic diversity within Pseudomonas species but also emphasize the pivotal role geographic and environmental factors play in shaping their evolutionary trajectories and potential functional specializations (see Figure 3-29).

3.8.12 Average nucleotide identity analysis of the *Pseudomonas* isolates

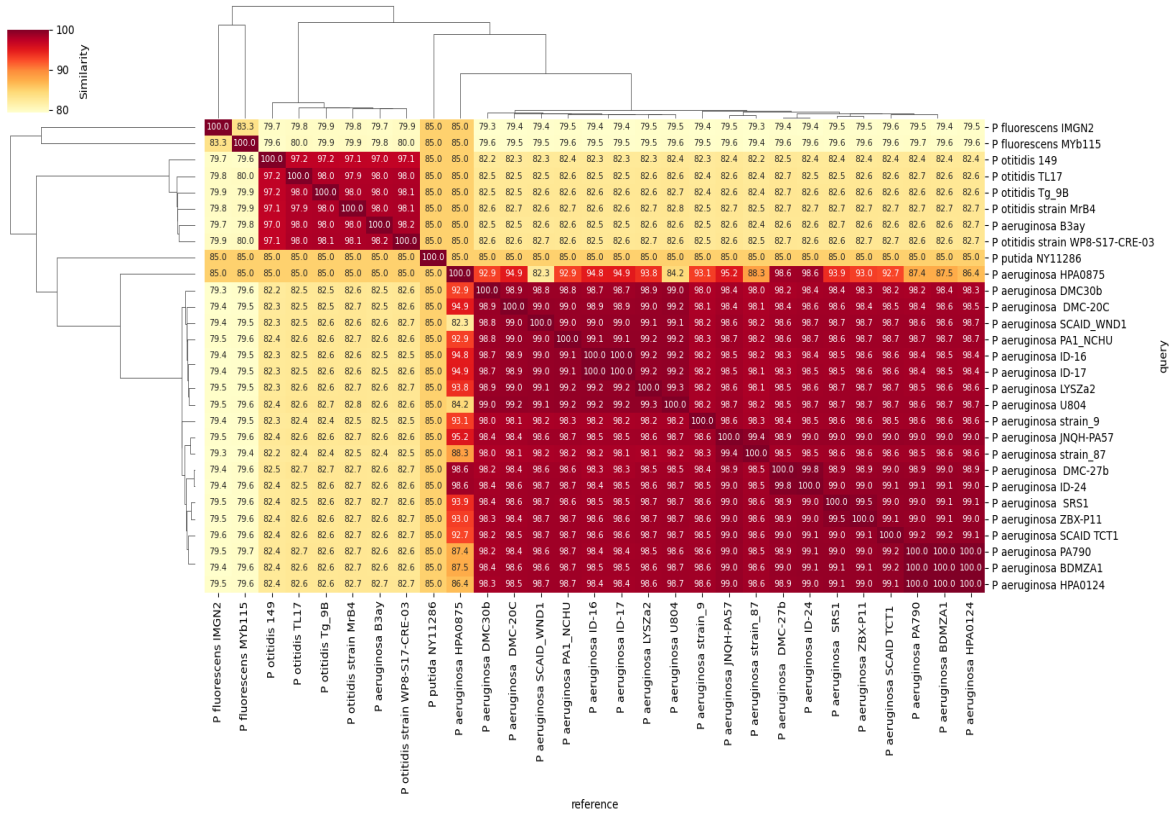


Figure 3-30 Heatmap based on ANI of the Pseudomonas isolates

The Average Nucleotide Identity (ANI) analysis presents a comprehensive matrix that illustrates the genetic relatedness among the isolates examined in this study. The resulting ANI heatmap reveals distinct clustering patterns based on sequence similarity, providing a clear visualization of the genetic relationships across the bacterial strains.

Specifically, the matrix confirms that *P. otitidis* B3ay exhibits the highest ANI scores when compared with other *P. otitidis* strains, including TL17, 19B, and WP8-S17-CRE-03. This strong sequence concordance clearly indicates a close genetic alignment within this subgroup, a fact further reinforced by the clustering patterns demonstrated in the heatmap visualization. In contrast, the *Pseudomonas* aeruginosa isolates—namely ID-16, ID-17, and ID-24—show elevated ANI values relative to various *P. aeruginosa* strains such as DMC-30b and DMC-20c, as well as additional isolates from Bangladesh and broader Asia. These data confirm their shared evolutionary heritage.

More detailed examination shows that ID-16 and ID-17 possess particularly high ANI scores with each other and with Bangladeshi isolates like DMC-30b and DMC-20c, underscoring their close genetic kinship and substantiating their grouping within a common clade on the constructed phylogenetic tree. Similarly, ID-24 aligns more closely with *P. aeruginosa* strains such as DMC-27b—also endemic to Bangladesh—and other Asian isolates, supporting its classification within a distinct subgroup of *P. aeruginosa*.

The analysis also highlights pronounced genetic distinctions between species. For example, *P. putida* and *P. fluorescens* exhibit markedly lower ANI values when compared to *P. aeruginosa* and *P. otitidis*, emphasizing their evolutionary divergence and distinct phylogenetic trajectories. These observations correspond well with the phylogenetic tree's depiction of clear separations among these species.

In summary, the ANI analysis not only quantitatively measures genetic similarity and divergence but also effectively corroborates the phylogenetic findings presented. It delineates firm intra-species clustering within *P. aeruginosa* and *P. otitidis*, while also revealing significant inter-species variation. These insights contribute valuable knowledge regarding the genetic diversity and potential adaptive variation within regional isolates studied (refer to Figure 3-30).

Chapter 4

Discussion

4.0 Discussion

Antimicrobial resistance (AMR) poses a significant threat to public health, worldwide, potentially undermining the effectiveness of numerous vital medical interventions. The World Health Organization (WHO) has projected that by the year 2050, AMR could cause up to 10 million deaths annually, imposing a staggering economic toll of $100 trillion on the global economy, should no preventive measures be implemented. Within this growing concern, hospital waste water stands out as a critical but often underestimated element in the complex ecosystem of AMR. It acts as a reservoir, amplifier, and transmitter of resistant microorganisms and their genetic resistance determinants (Le et al., 2016; Mehanni et al., 2023).

In this research, we concentrated on effluents originating from six major hospitals in Dhaka, Bangladesh, aiming to provide an extensive analysis of the occurrence, genetic mechanisms, and environmental spread of multidrug-resistant (MDR) Gram-negative bacteria. When examined in light of the most recent global findings, our results highlight deeply concerning patterns of antimicrobial resistance that require immediate action from medical professionals, policymakers, and the scientific community alike. The isolation of 184 meropenem-resistant bacteria, including significant strains such as *E. coli, A. baumannii, P. aeruginosa*, and *E. cloacae*, underscores that this issue is not confined to local environmental settings; it is part of a global crisis manifesting in hospital waste water worldwide (Siri, Bumyut, et al., 2024; Siri, Sresung, et al., 2024).

The discovery of multidrug-resistant phenotypes in over 80% of isolates from the hospital waste water of Dhaka mirrors findings from numerous studies spanning South Asia and beyond. A large-scale analysis of hospital waste water in Singapore revealed similar distributions of resistant bacteria, particularly in areas like clinical isolation wards, where high concentrations of antibiotic-resistant bacteria (ARB) were observed (Le et al., 2016). Meta-analyses conducted in Ethiopia have reported a significant prevalence of MDR bacteria, with 65.26% of hospital isolates showing resistance, with hospital effluents exhibiting even higher rates of 81.5% compared to non-hospital environments (Tilahun et al., 2025). This global consistency indicates that hospitals worldwide are under immense selective pressures, promoting the persistence and emergence of MDR bacteria.

Among the most frequently isolated pathogens in our study, *E. coli* accounted for 35% of the isolates. This finding is in line with global surveillance data, which often shows *E. coli* as one of the predominant species in hospital waste water resistomes, comprising between 30-40% of

MDR isolates (Le et al., 2016). Additionally, the prevalence of *P. aeruginosa* (25% in our study) was consistent with similar reports from Asian hospitals, underscoring the role of this opportunistic pathogen in the environmental persistence of resistance mechanisms (Mehanni et al., 2023; Sayem et al., 2025).

A deeper comparison with recent findings from South Asia brings certain notable patterns into focus. The 2016 study by Le et al. from Singapore hospitals reported resistance patterns that largely overlap with ours but displayed variations in gene distributions, where bla_{KPC} was more prevalent in Enterobacteriaceae, while bla_{NDM} was found in various species, including *Pseudomonas* and *Acinetobacter* (Le et al., 2016). This contrasts with our research, where $bla_{\text{NDM-1}}$ was universally present among carbapenem-resistant isolates, illustrating regional discrepancies in carbapenemase epidemiology.

The identification of *A. baumannii* in 20% of our isolates requires special attention, given its emergence as a leading cause of hospital-acquired infections globally. Systematic reviews indicate that NDM-1-producing *A. baumannii* has a high prevalence rate in Asia, recorded at 64.7% (Bright Esegbuyota et al., 2022). Our study further contributes to this troubling trend and highlights the environmental persistence of *A. baumannii* resistance.

In contrast to some studies that report seasonal fluctuations in resistance prevalence, our dataset, gathered over a year, demonstrated a remarkably consistent rate of MDR bacteria across all seasons. This stability stands in contrast to findings from temperate regions where seasonal patterns in antibiotic use are often correlated with changes in resistance rates (Chakraborty et al., 2024). The lack of such fluctuations in our study is likely influenced by Bangladesh's tropical climate, continuous high antibiotic consumption in hospitals, and the relatively stable healthcare practices throughout the year.

The resistance profiles observed in our study for β-lactam antibiotics were particularly troubling. More than 95% of *E. coli* isolates were resistant to ampicillin, with 88% showing resistance to amoxicillin/clavulanate, and 82% resistant to third-generation cephalosporins. These resistance rates are alarmingly higher than those typically seen in developed nations but align with recent findings from other South Asian countries. The Ethiopian meta-analysis documented resistance rates of 95.8% to cefotaxime and 93% to amoxicillin/clavulanate among ESBL-producing isolates (Tilahun et al., 2025), while Egyptian studies reported similarly high resistance levels to β-lactam/β-lactamase inhibitor combinations (Elshamy et al., 2020).

Our study also revealed concerning rates of carbapenem resistance, with 75% of *E. coli* isolates exhibiting resistance to meropenem. Carbapenems are considered last-resort antibiotics, and their widespread resistance is particularly alarming. Recent systematic reviews from Africa report carbapenemase gene prevalence in hospital waste water reaching 23.8% (Hamidou et al., 2025), with our findings suggesting that hospitals in South Asia may be experiencing even more significant resistance to these critical antibiotics.

In terms of fluoroquinolone resistance, our study observed resistance rates exceeding 80% for both ciprofloxacin and levofloxacin. This rate is consistent with global trends but exceeds the levels typically found in clinical isolates. A similar phenomenon was observed in Singapore, where environmental isolates exhibited higher resistance rates than their clinical counterparts (Le et al., 2016). The presence of plasmid-mediated quinolone resistance genes, including qnrS and qnrB, in 92.3% of isolates from Egypt further supports the widespread nature of quinolone resistance mechanisms, consistent with our findings (Elshamy et al., 2020).

Our molecular analyses highlighted the widespread presence of ESBL genes, particularly $bla_{\text{CTX-M}}$, which was found in 65% of ESBL-producing isolates. This trend aligns with global patterns, where CTX-M enzymes have largely replaced TEM and SHV variants in many regions (Elshamy et al., 2020; Le et al., 2016). Similar findings were observed in Singapore, where CTX-M genes were prevalent among hospital waste water isolates, alongside significant levels of bla_{SHV} and bla_{TEM} genes (Le et al., 2016).

The universal detection of $bla_{\text{NDM-1}}$ in our carbapenem-resistant isolates stands out as the most striking result of our study. This 100% prevalence rate is notably higher than those reported in most clinical studies and reflects patterns observed in environmental surveys from South Asia. A study in Vietnam documented NDM-1 in 47 CRE strains, but environmental studies have consistently shown higher NDM-1 rates than clinical reports (Bose et al., 2022; Bright Esegbuyota et al., 2022; H. H. Tran et al., 2015).

Plasmid profiling showed that resistance genes were often associated with mobile genetic elements. Plasmids of approximately 4 kb were found in highly resistant isolates, and the successful transformation of these plasmids into susceptible *E. coli* strains demonstrates the potential for horizontal gene transfer (HGT) in hospital waste water environments. Recent studies have supported this finding, confirming that fluctuating antibiotic levels in sewage contribute significantly to the horizontal transfer of resistance genes (Brown et al., 2024). The

detection of class 1 integrons in 19.3% of *E. coli* isolates provides further evidence of the role of mobile elements in the spread of resistance genes.

Our whole genome sequencing (WGS) analysis of selected isolates provided valuable insights into the resistomes of the bacterial strains, revealing a diverse array of resistance mechanisms spanning multiple antibiotic classes. The co-existence of β-lactamase genes alongside resistance to aminoglycosides, fluoroquinolones, and sulfonamides reflects the multifactorial nature of resistance in hospital waste water. This finding is in agreement with recent genomic studies, which have demonstrated that hospital-derived strains typically carry a broader spectrum of resistance genes compared to community-acquired isolates (Mehanni et al., 2023; Sayem et al., 2025). This underscores the unique selective pressures present in healthcare environments, which favor the emergence and persistence of such multidrug-resistant pathogens.

Moreover, our sequencing data revealed the presence of virulence factors in addition to antimicrobial resistance genes (ARGs). This raises significant concerns regarding the potential pathogenicity of these environmental isolates. Contrary to the traditional belief that antibiotic resistance comes at the expense of bacterial fitness, emerging research suggests that some resistant bacteria may actually possess enhanced survival traits in hospital settings, possibly due to the co-selection of resistance and virulence traits (Sayem et al., 2025). This complex relationship between resistance and virulence could pose a dual threat to public health, increasing both infection severity and treatment failure rates.

A critical environmental concern highlighted by our study is the persistence of MDR bacteria and ARGs in hospital waste water, even after passing through conventional waste water treatment systems. Several studies have documented the failure of traditional treatment methods in completely eradicating antibiotic-resistant bacteria and resistance genes (Hamidou et al., 2025; Zhao et al., 2025). This finding is consistent with our own research, which demonstrated the persistence of both MDR bacteria and their associated ARGs in the waste water effluent from the studied hospitals.

The study by Zhao et al. (2025) reinforces these concerns, showing that sludge water, a byproduct of waste water treatment, serves as a major pathway for the transmission of ARGs into the broader environment. Their research found that specific ARG subtypes were more abundant in sludge water than in the original hospital waste water (Zhao et al., 2025). This

highlights the potential for ARGs to spread far beyond hospital boundaries, contaminating agricultural systems, water sources, and even food supplies, thereby contributing to the environmental burden of antimicrobial resistance.

Recent systematic reviews from Africa have highlighted the insufficient capacity of conventional waste water treatment technologies to completely eliminate carbapenemase genes from hospital effluents (Hamidou et al., 2025). Similarly, our study found that despite treatment, significant levels of antibiotic-resistant bacteria remained in hospital waste water. This suggests that standard waste water treatment practices are insufficient for managing the growing challenge of antimicrobial resistance, particularly in high-risk environments such as hospitals.

Furthermore, the persistence of these resistant infections is enhanced by their capacity to form biofilms and demonstrate resilience to environmental stimuli. Recent studies have shown that biofilm formation enables resistant bacteria to survive under hostile environmental conditions, further exacerbating the spread of AMR (Mehanni et al., 2023). These biofilms may serve as reservoirs of resistance, potentially contaminating surrounding ecosystems through various transmission routes, including recreational water, irrigation systems, and even through contact with food.

The One Health concept, which underscores the interconnection between human, animal, and environmental health, is particularly relevant in addressing the challenge of antimicrobial resistance. Recent studies from Southeast Asia have identified water as a critical medium for the spread of AMR, with hospital waste water leaking antibiotics and resistant pathogens into the broader environment (Lambraki et al., 2023). This finding supports the notion that antimicrobial resistance is not confined to clinical settings but instead extends into environmental and agricultural systems, potentially affecting human populations through indirect exposure via food and water.

In particular, the reuse of untreated hospital waste water in agriculture has been identified as a significant route through which antibiotics and ARGs are transferred to the environment. The subsequent contamination of water supplies and soil with resistant organisms poses a serious public health risk, emphasizing the need for integrated management strategies that consider all aspects of the One Health framework (Lambraki et al., 2023).

The widespread presence of MDR bacteria in hospital waste water highlights the urgent need to strengthen infection prevention and control (IPC) measures within healthcare facilities. Several studies have demonstrated that the implementation of comprehensive IPC programs

can significantly reduce the environmental contamination with resistant bacteria (Mehanni et al., 2023; Tilahun et al., 2025). Such measures, when combined with rigorous surveillance of hospital waste water, can play a critical role in curbing the spread of antimicrobial resistance.

The World Economic Forum's recent report on AMR in Asia stressed the importance of improving pharmaceutical regulations and addressing the widespread issue of counterfeit antimicrobials, which contribute to the escalation of resistance rates (Targeted Action and Financing the Fight Against Antimicrobial Resistance in Asia May 2025 In Collaboration with Centre for Impact Investing and Practices and Philanthropy Asia Alliance, 2025). This, in conjunction with effective IPC programs, would help mitigate the environmental spread of resistant pathogens.

Given the limitations of conventional waste water treatment technologies in removing ARGs and resistant bacteria, there is a pressing need to explore and implement advanced treatment methods. Recent research has shown that processes such as advanced oxidation, membrane bioreactors, and UV disinfection are more effective at reducing the abundance of ARGs in waste water (Evoung Chandja et al., 2024). However, it is crucial to recognize that in regions with high population densities, such as South Asia, the challenge of managing untreated waste water remains significant, and these advanced technologies must be adapted to local contexts.

The study by Chakraborty et al. (2024) highlights the specific risks faced by South Asian countries, where the high population density and limited resources for waste water treatment could result in health risks due to antimicrobial resistance (Chakraborty et al., 2024). Therefore, there is an urgent need for region-specific solutions that prioritize the implementation of advanced waste water treatment methods.

Regular surveillance of hospital waste water for MDR bacteria and ARGs is crucial for identifying emerging resistance threats and preventing their spread into the broader environment. By integrating environmental surveillance data with clinical AMR monitoring, health authorities can develop more comprehensive strategies to address the growing problem of antimicrobial resistance (Lan et al., 2025; Mehanni et al., 2023). Advances in molecular diagnostics, such as metagenomic sequencing, offer promising tools for monitoring the resistome in hospital waste water, enabling early detection and intervention to curb resistance development and transmission (Zhao et al., 2025).

Although our study offers valuable insights into the role of hospital waste water in the spread of AMR, there are several limitations inherent in the methodology. The use of culture-based

techniques may underestimate the full diversity and abundance of ARGs, as recent studies have highlighted the importance of culture-independent approaches in detecting resistant microorganisms (N. Hasan et al., 2024; Lan et al., 2025). Incorporating these approaches, such as metagenomic sequencing and PCR-based detection of ARGs, would provide a more comprehensive understanding of the resistome in hospital effluents.

Furthermore, recent research by Mehanni et al. (2023) has shown that extracellular DNA carrying antibiotic resistance genes in hospital effluents poses a significant environmental hazard (Mehanni et al., 2023). Future studies should therefore consider investigating both culturable and non-culturable bacteria, as well as the role of extracellular DNA in the persistence and spread of AMR.

Further studies are required to assess the functional significance of the resistance genes detected in our study. While genetic analysis provides valuable information about the presence of ARGs, the actual expression of these genes in environmental settings needs to be evaluated through phenotypic validation and expression studies. Moreover, the development of quantitative risk assessment models that consider factors such as gene abundance, mobility, and exposure pathways is essential for better estimating the public health impact of environmental AMR (Evoung Chandja et al., 2024).

Our Study (Dhaka)	South Asia Average	Global Range	Clinical vs Environmental	References
MDR prevalence	>80%	78-87%	22-95%	(Le et al., 2016; Siri, Bumyut, et al., 2024; Tilahun et al., 2025)
***bla*$_{NDM-1}$ detection**	100% (CR isolates)	65-85%	15-90%	(Bright Esegbuyota et al., 2022; Tilahun et al., 2025, & W. H. F. Tran, 2015)
ESBL prevalence	75% (E. coli)	70-80%	45-85%	(Elshamy et al., 2020; Sayem et al., 2025; Tilahun et al., 2025)
Plasmid mobility	High (confirmed)	High	Variable	(Bright Esegbuyota et al., 2022; Brown et al., 2024; Mehanni et al., 2023)
Species diversity	4 major species	3-5 species	2-8 species	(Le et al., 2016; Mehanni et al., 2023; Sayem et al., 2025)

Chapter 5

Conclusion

5.0 Conclusion

Our study provides robust evidence that hospital waste water in Dhaka acts as a critical reservoir and disseminator of multidrug-resistant bacteria and their associated resistance genes. The detection of 184 meropenem-resistant isolates, predominantly *E. coli, A. baumannii, P. aeruginosa, and E. cloacae*, reflects both local and global concerns regarding hospital effluents as a source of antimicrobial resistance.

The global identification of $bla_{\text{NDM-1}}$ in carbapenem-resistant isolates, along with the extensive prevalence of ESBL and carbapenemase genes, highlights the significance of mobile genetic factors in the environmental transmission of resistance. The successful plasmid transfer tests validate the potential for horizontal gene transfer in hospital waste water, hence exacerbating the dissemination of resistance.

Our findings align with international research, underscoring the need for collaborative initiatives to address the environmental and public health implications of antimicrobial resistance in hospital waste water. Future research should focus on developing effective intervention techniques, enhancing understanding of resistance gene movement, and refining surveillance systems to address the growing issue of antimicrobial resistance and maintain the efficacy of critical antibiotics.

Chapter 6

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6.0 References

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