Environmental Isolation, Characterization & Antimicrobial Resistance Profiling of *Klebsiella pneumoniae* & *Acinetobacter baumannii* from community wastewater in Dhaka: A Comparative Analysis

By
Meer Zeshanul Wazid

ID: 22336023

Athoy Mannan

ID: 22126031

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School of Life Sciences,

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing our degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

Student's Full Name & Signature:

Meer Zeshanul Wazid
ID: 22336023
Department of Biotechnology,
School of Life Sciences, BRAC University

Athoy Mannan
ID: 22126031
Department of Microbiology,
School of Life Sciences, BRAC University

Approval

The thesis titled “Environmental Isolation, Characterization & Antimicrobial Resistance Profiling of *Klebsiella pneumoniae* & *Acinetobacter baumannii* from community wastewater in Dhaka: A Comparative Analysis” was submitted by

1. Meer Zeshanul Wazid (22336023)
2. Athoy Mannan (22126031)

The work of Spring, 2026 has been accepted as satisfactory in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology & Bachelor of Science in Microbiology on April 23, 2026.

Examining Committee:

Supervisor:
(Member)

_____
Dr. Fahim Kabir Monjurul Haque
Associate Professor
Department of Microbiology, School of Life Sciences
BRAC University

Departmental Head:
(Chair)

_____
Dr. Nadia Sultana Deen
Associate Professor and Chairperson
Department of Microbiology, School of Life Sciences
BRAC University

Departmental Head:
(Chair)

_____
Dr. Iftekhar Bin Naser
Associate Professor and Chairperson
Department of Biotechnology, School of Life Sciences
BRAC University

Abstract

Environmental antimicrobial resistance has become a growing global health concern, where community wastewater plays a critical role in the accumulation, persistence, and distribution of the resistant pathogens. This study focuses on investigating the environmental occurrence, phenotypic and molecular characterization, and antimicrobial resistance profiling of *Klebsiella pneumoniae* and *Acinetobacter baumannii* isolated from the community wastewater in multiple areas of Dhaka, Bangladesh, along with emphasizing the presence of potential co-existence of these organisms with associated resistance dynamics. The community wastewater samples were collected from different areas of Dhaka city and processed using culture-based isolation. Afterwards, the phenotypic identification and molecular characterization provided a validated species identification. Importantly, Antimicrobial susceptibility testing (AST) was performed to identify the resistance intensity and potential risk across these isolates. Moreover, 45 isolates of *Klebsiella pneumoniae* and 41 isolates of *Acinetobacter baumannii* were isolated and characterized from 32 samples of community wastewater, resulting in the highest co-dominance in the Mohammadpur area of Dhaka with 47.6% *Klebsiella pneumoniae* and 52.4% *Acinetobacter baumannii* prevalence. Besides, both organisms were frequently isolated with mixed colonies, indicating their co-existence across all sampling sites. Further, AST of mixed colonies exhibited an extreme resistance with 90% isolates with MAR index 1.0, and more the 90% isolates showed MAR index >0.2, indicating high antibiotic selective pressure in comparison to the resistance profile of *Klebsiella pneumoniae* and *Acinetobacter baumannii,* respectively. These findings highlight the Dhaka community wastewater as a high-risk AMR contamination site and co-existing pathogens promoting resistance dissemination and amplification via horizontal gene transfer and biofilm-mediated interactions, suggesting a critical need for integrated environmental surveillance and strategies to control the increasing antimicrobial resistance.

Keywords:

Environmental *Acinetobacter baumannii*; Environmental *Klebsiella pneumoniae*; Antibiotic resistance; Community Wastewater; Microbiology; Prevalence

Dedicated To

Our Parents and Friends

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List of Acronyms

AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
ESBL	Extended-spectrum beta-lactamases
PCR	Polymerase Chain Reaction
MAR	Multiple Antibiotic Resistance
MDR	Multidrug Resistance
XDR	Extensive Drug Resistance

Glossary

AST	Antibiotic susceptibility testing (AST) is the method to determine how bacterial strains are sensitive or resistant to various antibiotics, making use of techniques such as disk diffusion and tube dilution to classify bacteria as resistant, intermediately sensitive, or sensitive based on measured outcomes.
Lawn (or Lawning)	In Antimicrobial Susceptibility Testing (AST) or the Kirby-Bauer disk diffusion method, a lawn (or lawning) is defined as a uniform, confluent layer of bacterial growth covering the entire surface of an agar plate.
Mass spectrometry (MS)	Mass spectrometry (MS) in microbiology is an analytical technique used to identify microorganisms—bacteria, fungi, and viruses—by measuring the mass-to-charge ratio (m/z) of ionized molecules, primarily proteins and peptides derived from cellular components.
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is an analytical method that generates a distinctive proteomic spectrum (peptide mass fingerprint) to quickly and accurately identify and characterize microorganisms (bacteria, yeasts, and fungi).
MAR	Multiple Antibiotic Resistance (MAR), often known as Multi-Antibiotic Resistance, is a term used in microbiology. It describes the situation in which a microorganism (usually bacteria) shows resistance to three or more different types of

	antibiotics. The formula developed by Krumperman (1983) is used to compute it:
MDR	Multidrug resistance (MDR) in bacteria is the acquired non-susceptibility to at least one agent in three or more antimicrobial categories.
Prevalence	Prevalence is the total percentage of the population infected or affected by a certain disease or condition at a certain moment (point prevalence) or for a certain duration of time (period prevalence).
Simple Staining	Simple staining is defined as a staining procedure that uses a single dye to uniformly color all bacterial cells in a smear, increasing contrast to make microorganisms visible under a light microscope and enabling observation of basic morphological characteristics.
Spreading	Spreading (or the Spread Plate Technique) is a microbiological method in which a small, measured volume of a liquid microbial suspension is evenly distributed across the surface of a solidified agar medium in order to isolate, count, and enumerate viable aerobic and facultative aerobic microorganisms.
Streaking	Streaking is a basic mechanical laboratory method used in microbiology to separate a pure strain of a single species of microbe (often bacteria) from a mixed population. In order to create regions of decreasing cell density, a sample is dragged and dispersed across the surface of a solid agar medium in a

	Petri dish using a sterile instrument, usually an inoculating loop.
XDR	Extensively Drug-Resistant (XDR) in microbiology is a specialized category of antimicrobial resistance, typically characterized as attaining non-susceptibility to at least one agent in all but two or fewer antimicrobial categories.

Chapter 1

Introduction

Antimicrobial Resistance has become one of the most critical concerns for public health, creating barriers in effective treatment of infectious disease and increasing morbidity, mortality, and cost of treatment. Gram-negatives like *Klebsiella pneumoniae* and *Acinetobacter baumannii* have become prominent in their acquisition and dissemination of resistant strains, and promoting pathogenicity. Firstly, *Klebsiella pneumoniae* is commonly detected in community wastewater due to fecal contamination from human populations. Globally, it accounts for approximately 10–20% of hospital-acquired infections [1,2]. In wastewater studies, *Klebsiella pneumoniae* has been reported in 20–60% of samples, depending on location and detection methods, with many isolates carrying antimicrobial resistance genes [3,4]. Secondly, *Acinetobacter baumannii* is also frequently found in wastewater due to its ability to persist in harsh environments. It contributes to about 5–10% of hospital-acquired infections globally [5]. Environmental surveillance studies show that *Acinetobacter baumannii* is present in approximately 10–40% of wastewater samples, particularly in urban and hospital-influenced sewage systems [6,7]. Both pathogens are classified as critical priority organisms by the World Health Organization, and their presence in wastewater highlights its role as a reservoir for the spread of antimicrobial resistance [8].

Moreover, *Klebsiella pneumoniae* is frequently detected in environmental water sources in Bangladesh, reflecting its high carriage in both humans and the community environment. A study in Dhaka found that *Klebsiella pneumoniae* was present in 64% of household water samples and 85% of standing water samples in an urban community, with 16% of the water isolates showing multidrug-resistance and 14% exhibiting carbapenem resistance (high-priority antibiotic resistance) in water environments, which can include wastewater

streams (Dhaka) [1, 6, 9]. Although it was difficult to measure *Klebsiella pneumoniae* directly in public wastewater, broader environmental monitoring in Bangladesh has shown high levels of antibiotic resistance genes linked to *Klebsiella pneumoniae* in community wastewater samples. In a culture-independent analysis of wastewater from Dhaka and Cox's Bazar, 70 samples revealed that key antibiotic resistance genes (e.g., blaTEM, blaNDM, blaKPC) linked to *Klebsiella pneumoniae* were present in up to ~90% of samples, and co-occurrence of multiple resistance genes was observed in ~47% of samples, suggesting widespread dissemination of resistant bacteria in community wastewater discharges [10].

However, *Acinetobacter baumannii* has also been found in community wastewater discharges in Dhaka's Goranchatbari sub-catchment. Sampling of 28 water points showed that nearly all samples (27 out of 28) contained *Acinetobacter spp*. Additionally, 42.6% of representative isolates were ESBL-producing bacteria. This indicates substantial environmental prevalence and the potential for resistant Acinetobacter baumannii to enter wastewater systems from community sources [11]. Together, these data show that both *Klebsiella pneumoniae* and *Acinetobacter baumannii* are common in Bangladesh's community wastewater environments. They often carry important antimicrobial resistance genes and contribute to the environmental pool of resistant pathogens. These findings stress the need for wastewater surveillance to track the spread of resistant bacteria in urban water systems.

Klebsiella pneumoniae and *Acinetobacter baumannii* are two clinically important Gram-negative bacteria that are increasingly detected in community wastewater in Bangladesh. *Klebsiella pneumoniae* is a major cause of pneumonia, urinary tract infections, bloodstream infections, and wound infections [2]. Carbapenem-resistant strains (CRKP) are particularly concerning because they can cause treatment failure and high mortality, especially among hospitalized patients. *Acinetobacter baumannii*, on the other hand, is often associated with ventilator-associated pneumonia, wound infections, sepsis, and urinary tract

infections in critically ill patients [5]. The emergence of multidrug-resistant *Acinetobacter baumannii* (CRAB) has raised serious public health concerns due to limited therapeutic options.

The detection of these bacteria in community wastewater is alarming because it suggests the spread of resistant pathogens beyond hospital settings into the broader environment. Wastewater acts as a reservoir where these bacteria persist and exchange antimicrobial resistance genes with other microorganisms, increasing the risk of community-acquired infections [3,6]. People may come into contact with these pathogens through contaminated water, food, or recreational activities, creating a significant public health risk. Moreover, if untreated wastewater is used for irrigation, these pathogens can enter the food chain, impacting crops and livestock and spreading resistance genes further. The ability of *Klebsiella pneumoniae* and *Acinetobacter baumannii* to persist in the environment also disrupts microbial communities in water and soil ecosystems. This may change the ecological balance and increase the overall problem of antimicrobial resistance in the environment.

Hence, the presence of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in Bangladesh's community wastewater shows a dual risk. It directly threatens human health through resistant infections and indirectly affects the environment by acting as a reservoir and transmission route for antimicrobial resistance. These findings highlight the urgent need for effective wastewater management, environmental monitoring, and responsible use of antibiotics to prevent the further spread of these high-risk pathogens.

Besides, the study of community wastewater has gained attention because it represents a composite snapshot of the microorganisms and chemical contaminants present in human populations. Community wastewater, also called sewage, is domestic effluent from households, industrial discharge, hospital waste, stormwater runoff, and other urban or

community sources [3]. This mixture contains bacteria, viruses, fungi, antibiotic residues, and other pollutants that can collectively reflect the health status, hygiene practices, and antimicrobial resistance (AMR) burden of the surrounding population. Monitoring community wastewater is therefore a valuable tool for tracking the prevalence of pathogenic and resistant bacteria outside hospital settings, providing early warnings for emerging public health threats. Bacteria such as *Klebsiella pneumoniae* and *Acinetobacter baumannii* are particularly concerning in wastewater because they can persist in harsh environmental conditions and carry multiple antimicrobial resistance genes [5,6]. Once present in wastewater, these bacteria can spread through contaminated water bodies, irrigation systems, or recreational water use, and may eventually enter the human population via ingestion, skin contact, or inhalation of aerosols. For example, untreated or poorly treated wastewater can contaminate drinking water or crops irrigated with sewage, leading to community-acquired infections that are often resistant to conventional antibiotics. Additionally, wastewater provides an environment for horizontal gene transfer, where resistance genes can move between different bacterial species, amplifying the threat of multidrug-resistant pathogens in the environment.

Studying community wastewater is therefore critical because it allows researchers to assess the prevalence and spread of pathogenic bacteria in the general population, understand environmental reservoirs of resistance, and develop strategies for preventing outbreaks. Surveillance of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in wastewater not only informs public health interventions but also highlights the importance of wastewater treatment, hygiene improvement, and antimicrobial stewardship to reduce both human health risks and environmental impacts [3,6].

Importantly, Antibiotic Susceptibility Testing (AST) is essential to determine which antibiotics are effective against bacteria such as *Klebsiella pneumoniae* and *Acinetobacter*

baumannii. AST helps clinicians choose appropriate treatments and prevent the misuse of antibiotics, which can worsen resistance [2,5]. Multidrug-resistant (MDR) strains, resistant to three or more antibiotic classes, pose a major challenge because they limit treatment options and increase morbidity and mortality [6]. Globally, AST studies show that carbapenem-resistant *Klebsiella pneumoniae* can exceed 50%, and MDR *Acinetobacter baumannii* prevalence in ICUs often surpasses 60% [1]. In Bangladesh, clinical and wastewater isolates reveal similarly high resistance: *Klebsiella pneumoniae* shows widespread resistance to cephalosporins, fluoroquinolones, and carbapenems, while *Acinetobacter baumannii* frequently produces extended-spectrum β-lactamases (ESBLs) and is multidrug-resistant [9,11]. Comparing global and national data highlights that MDR bacteria are present not only in hospitals but also in community wastewater, emphasizing the importance of AST and MDR analysis for surveillance, treatment guidance, and public health interventions.

Despite the global rise in antimicrobial resistance and environmental surveillance of high-risk pathogens, research on *Klebsiella pneumoniae* and *Acinetobacter baumannii* in Bangladesh, particularly from community wastewater, remains very limited. To date, only a handful of studies have investigated these bacteria beyond clinical settings. One of the few published environmental studies isolated *Acinetobacter baumannii* from 31 water sites in Dhaka City, confirming 23 environmental isolates and showing antibiotic resistance patterns among them, but it also noted that this was the first such report and called for further work with larger sample sizes (32% of samples positive for *Acinetobacter baumannii*) [12]. Another recent study that focused on community wastewater from Dhaka and Cox's Bazar screened 70 wastewater samples for antibiotic resistance genes linked to clinically important bacteria, revealing a high frequency (77–90%) of resistance genes commonly associated with pathogens like *Klebsiella pneumoniae* (sampling data, n = 70) [13]. Additionally, while not

strictly wastewater-based, a community study in Dhaka reported *Klebsiella pneumoniae* presence in household and standing water samples (64% and 85%, respectively) and found multidrug resistance in 16% of water isolates, underscoring environmental reservoirs in the community [9]. Compared with the dozens to hundreds of global environmental studies on AMR bacteria, these only 2–3 published Bangladesh studies illustrate the knowledge gap in environmental monitoring of these pathogens in sewage and wastewater systems.

This gap is important because Bangladesh's dense urban populations and limited sanitation infrastructure may facilitate the dissemination of resistant bacteria from humans into the environment and back into communities. Without comprehensive, long-term surveillance and AST/MDR analyses of wastewater, public health officials lack crucial data for risk assessment and intervention planning. By expanding research on wastewater reservoirs of MDR *Klebsiella pneumoniae* and *Acinetobacter baumannii*, this study helps fill a critical gap, supports awareness among clinicians, policymakers, and the public, and emphasizes the need for improved wastewater management to reduce the societal burden of resistant infections.

Therefore, this study focuses on the isolation and accurate identification of these bacterial species from community-derived wastewater samples and the determination of their prevalence within non-hospital wastewater environments. It further aims to evaluate the antibiotic susceptibility patterns of the isolates using standardized Antibiotic Susceptibility Testing (AST) methods and to identify and characterize multidrug-resistant (MDR) strains. In addition, the study seeks to assess the co-occurrence and distribution of multiple resistance genes within individual isolates and to conduct a comparative analysis of antimicrobial resistance profiles between *Klebsiella pneumoniae* and *Acinetobacter baumannii*. Finally, the study aims to evaluate the potential public health and environmental risks associated with the presence of resistant bacteria in community wastewater systems.

The study is based on the hypothesis that community wastewater, independent of direct hospital discharge, serves as a significant environmental reservoir for multidrug-resistant *Klebsiella pneumoniae* and *Acinetobacter baumannii*. It is hypothesized that a substantial proportion of isolates obtained from non-nosocomial wastewater sources will exhibit multidrug resistance and harbor multiple resistance determinants within individual isolates, indicating widespread dissemination of antimicrobial resistance at the community level. Furthermore, it is expected that measurable differences in antimicrobial resistance profiles will exist between the two bacterial species. Overall, the study hypothesizes that even in the absence of direct hospital influence, community wastewater contributes to the persistence and environmental circulation of antimicrobial resistance, posing significant risks to public health and ecological systems.

1.1 Objective & Aims

The primary objective was to identify and investigate the presence and antimicrobial resistance (AMR) profile of *Klebsiella pneumoniae* and *Acinetobacter baumannii* from community wastewater in Dhaka, Bangladesh, with a focus on the potential co-existence and their impact of the AMR burden and distribution.

Aims:

- To isolate and characterize *Klebsiella pneumoniae* and *Acinetobacter baumannii* from community wastewater using culture-based methods.
- To confirm species-level identification using molecular and proteomic approaches.
- To determine the antimicrobial resistance profiles against clinically relevant antibiotics.
- To assess the presence and impact of co-existence and analyze the areawise distribution and dominance.

Chapter 2

Literature Review

2.1 The Organism: *Acinetobacter baumannii*

2.1.1 Historical Background

Acinetobacter baumannii is a non-fermentative Gram-negative bacterium that has transitioned from being an environmental organism to one of the most important opportunistic nosocomial and environmental pathogens worldwide. Initially, members of the genus *Acinetobacter* were isolated from soil and water sources in the early 20th century and were considered relatively harmless environmental bacteria. However, with advancements in clinical microbiology and molecular identification techniques, *Acinetobacter baumannii* emerged as a distinct species within the *Acinetobacter calcoaceticus–baumannii* (ACB) complex and was increasingly recognized for its clinical importance in the late 20th and early 21st centuries [5, 14].

In recent years, *Acinetobacter baumannii* has been designated as a critical priority pathogen by the World Health Organization due to its alarming ability to acquire multidrug resistance and persist in both healthcare and environmental settings [15,16]. Environmental surveillance studies have further demonstrated its presence in wastewater systems, hospital effluents, and urban water bodies, indicating its dual role as both a clinical and environmental reservoir of antimicrobial resistance [17,18].

2.1.2 Taxonomy

Acinetobacter baumannii belongs to the domain Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, and family *Moraxellaceae*. It is part of the

Acinetobacter calcoaceticus–baumannii complex, which includes closely related species that are difficult to differentiate using conventional biochemical methods alone [19,20].

Modern taxonomic classification relies heavily on molecular techniques such as 16S rRNA gene sequencing, multilocus sequence typing (MLST), and whole genome sequencing, which have revealed extensive genetic diversity within clinical and environmental strains of *Acinetobacter baumannii* [21,22]. This genetic plasticity is one of the key reasons behind its adaptability to both hospital and wastewater environments.

2.1.3 Morphology, Physiological and Biochemical Characteristics

Acinetobacter baumannii is a strictly aerobic, non-motile, oxidase-negative, and catalase-positive coccobacillus. It appears as short rods or almost coccoid forms under the microscope and is typically arranged singly or in pairs. On culture media such as nutrient agar and MacConkey agar, it forms smooth, opaque, non-pigmented colonies and does not ferment lactose, a characteristic that aids in its differentiation from *Enterobacteriaceae* [5,14].

Physiologically, *Acinetobacter baumannii* exhibits remarkable metabolic versatility, enabling survival under nutrient-limited and desiccated conditions. It can persist on abiotic surfaces such as medical equipment, wastewater pipes, and hospital fomites for extended periods, sometimes ranging from days to weeks [23]. This survival ability is attributed to its robust stress response systems, including biofilm formation, outer membrane modifications, and efflux pump activity. Biochemically, the organism shows positive catalase activity and negative reactions for indole production, urease, and citrate utilization variability depending on strain origin. Its ability to form strong biofilms plays a critical role in environmental persistence and resistance to disinfectants and antibiotics [24].

2.1.4 Natural Habitat (Environmental and Wastewater Relevance)

Although historically considered a hospital-associated pathogen, *Acinetobacter baumannii* is now widely recognized as an environmental organism with strong adaptability to aquatic ecosystems. It has been isolated from soil, freshwater, sewage, and wastewater treatment plants, highlighting its ecological versatility [17]

Wastewater environments, particularly in densely populated urban regions such as Dhaka, provide ideal conditions for the survival and dissemination of *Acinetobacter baumannii*. These systems act as reservoirs where antibiotics, heavy metals, and organic waste create selective pressure that promotes the survival of resistant strains. Studies have demonstrated that wastewater effluents often contain multidrug-resistant *Acinetobacter baumannii*, suggesting that community wastewater plays a significant role in the environmental spread of antimicrobial resistance [25,26].

The ability of *Acinetobacter baumannii* to survive in wastewater is further enhanced by its biofilm-forming capacity, which allows it to attach to surfaces in sewage systems and resist environmental stressors such as chlorination and nutrient deprivation.

2.1.5 Clinical Significance and Community-Acquired Infections

Acinetobacter baumannii is primarily associated with healthcare-associated infections, particularly in intensive care units. It is a leading cause of ventilator-associated pneumonia, bloodstream infections, wound infections, urinary tract infections, and meningitis, especially in immunocompromised patients [21,27].

However, community-acquired infections have also been increasingly reported, particularly in tropical and developing regions. These infections often present as severe pneumonia and are associated with high mortality rates. Environmental reservoirs, including wastewater and

contaminated water sources, are believed to contribute to the emergence of community-acquired strains[28,29]

The increasing overlap between environmental and clinical strains highlights the importance of understanding *Acinetobacter baumannii* outside hospital settings, particularly in urban wastewater systems.

2.1.6 Pathogenicity and Virulence

The pathogenic success of *Acinetobacter baumannii* is attributed to multiple virulence mechanisms, including biofilm formation, outer membrane protein expression, lipopolysaccharide structure, iron acquisition systems, and efflux pump activity. Biofilm formation is particularly important, as it enhances adherence to surfaces and provides protection against antibiotics and host immune responses [18,24].

Additionally, *Acinetobacter baumannii* possesses a highly adaptable genome that facilitates horizontal gene transfer, allowing rapid acquisition of antibiotic resistance determinants. Efflux pumps such as AdeABC and OXA-type carbapenemases contribute significantly to resistance against multiple antibiotic classes, including carbapenems [30]. These virulence and resistance traits collectively make *Acinetobacter baumannii* a formidable pathogen in both clinical and environmental settings.

2.1.7 Epidemiology

The global epidemiology of *Acinetobacter baumannii* is characterized by its widespread distribution in healthcare facilities and increasing detection in environmental reservoirs. Carbapenem-resistant Acinetobacter baumannii (CRAB) has been reported globally and is associated with mortality rates ranging from 30% to 70% in severe infections [16, 31]

In low- and middle-income countries, including Bangladesh, limited wastewater treatment infrastructure and high antibiotic usage contribute to the environmental dissemination of resistant strains. Wastewater has increasingly been recognized as a key epidemiological link between community antibiotic use and environmental resistance gene propagation [25].

2.2 The Organism: *Klebsiella pneumoniae*

2.2.1 Historical Background

Klebsiella pneumoniae was first described in 1882 by Carl Friedländer as a causative agent of pneumonia. Since then, it has been recognized as a major opportunistic pathogen responsible for a wide spectrum of infections in both hospital and community settings. In recent decades, the emergence of hypervirulent and carbapenem-resistant strains has significantly increased its clinical and environmental importance [32,33].

Environmental studies have demonstrated that *Klebsiella pneumoniae* is not only restricted to human hosts but is also widely distributed in water, soil, and wastewater systems, where it contributes to the dissemination of antimicrobial resistance [34,35].

2.2.2 Taxonomy

Klebsiella pneumoniae belongs to the family *Enterobacteriaceae*, class Gammaproteobacteria. It is a Gram-negative, non-motile, encapsulated bacillus. Molecular analyses have identified several phylogenetic lineages within the species complex, including classical and hypervirulent strains, which differ in pathogenic potential and resistance profiles [36].

Whole genome sequencing has played a crucial role in understanding the evolution and global spread of multidrug-resistant *Klebsiella pneumoniae*, particularly carbapenem-resistant clones [37].

2.2.3 Morphology, Physiological and Biochemical Characteristics

Klebsiella pneumoniae is a Gram-negative, rod-shaped, non-motile bacterium characterized by a prominent polysaccharide capsule that gives colonies a mucoid appearance. It is a facultative anaerobe and a lactose fermenter, producing pink colonies on MacConkey agar [38].

Biochemically, it is catalase-positive, oxidase-negative, and capable of producing acid and gas from glucose fermentation. The capsule is a major virulence determinant that protects against phagocytosis and environmental stress.

2.2.4 Natural Habitat (Environmental and Wastewater Relevance)

Klebsiella pneumoniae is commonly found in soil, surface water, wastewater, and as part of the normal flora of the human gastrointestinal tract. Wastewater environments serve as important reservoirs where resistant strains can survive and exchange genetic material with other bacteria [39].

In urban wastewater systems, particularly in densely populated cities such as Dhaka, *Klebsiella pneumoniae* has been frequently isolated from sewage and hospital effluents. These environments facilitate the persistence and dissemination of carbapenem-resistant strains due to the constant exposure to antibiotics and disinfectants.

2.2.5 Clinical Significance and Community-Acquired Infections

Klebsiella pneumoniae is responsible for pneumonia, urinary tract infections, bloodstream infections, liver abscesses, and wound infections. It is a leading cause of hospital-acquired infections, particularly in intensive care settings [35].

Community-acquired hypervirulent strains have also emerged, causing severe invasive infections in otherwise healthy individuals. These strains often possess enhanced virulence factors such as increased capsule production and siderophore systems, leading to aggressive disease progression [40].

2.2.6 Pathogenicity and Virulence

The virulence of *Klebsiella pneumoniae* is primarily attributed to its polysaccharide capsule, lipopolysaccharide, fimbriae, and siderophore systems. These factors enable immune evasion, adherence to host tissues, and efficient iron acquisition.

In addition, plasmid-mediated resistance and virulence gene transfer contribute to the emergence of hypervirulent and multidrug-resistant strains. This convergence of virulence and resistance is particularly concerning in environmental reservoirs such as wastewater systems [41].

2.2.7 Epidemiology

The global spread of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has become a major public health concern. It is associated with high mortality rates and limited therapeutic options. Outbreaks have been reported worldwide, particularly in healthcare settings with inadequate infection control measures [37].

Environmental surveillance studies indicate increasing detection of *Klebsiella pneumoniae* in wastewater, suggesting that aquatic environments play a role in its epidemiology and resistance dissemination.

2.3 Co-existence of *Klebsiella pneumoniae* and *Acinetobacter baumannii*

2.3.1 Clinical Significance and Environmental Co-occurrence

The co-existence of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in environmental and clinical settings represents a significant public health challenge. Both organisms are frequently isolated from hospital wastewater, sewage systems, and intensive care units, where they contribute to polymicrobial infections and the spread of antimicrobial resistance [25,42]

In wastewater, these microorganisms thrive in similar conditions, facing the same challenges like antibiotics, heavy metals, and disinfectants. This shared environment helps them survive and develop resistance to these threats, making it easier for them to coexist and pass on their resistant traits.

2.3.2 Pathogenicity and Virulence in Co-existence

When present together, *Acinetobacter baumannii* and *Klebsiella pneumoniae* may contribute to increased disease severity due to synergistic effects of their virulence factors. Both organisms possess strong biofilm-forming capabilities, allowing them to persist on environmental surfaces and medical devices. Furthermore, wastewater environments provide opportunities for horizontal gene transfer, including plasmids carrying carbapenemase genes and other resistance determinants. This enhances the risk of emergence of multidrug-resistant strains that are difficult to treat and control [30,36]. The co-existence of these pathogens in urban wastewater systems, particularly in developing countries, underscores the importance of environmental surveillance in understanding the epidemiology of antimicrobial resistance.

Chapter 3

Methods & Materials

3.1 Sample collection & Dilution

This cross-sectional study investigated the prevalence and antimicrobial resistance burden of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in different community wastewater in Dhaka. A total of 32 Environmental samples were collected aseptically using sterile containers and transported to the laboratory under controlled conditions. All the samples were processed within 4-6 hours of collection, and data were recorded.

For microbial characterization and isolation, a 10-fold serial dilution (10^{-1}-10^{-5}) was performed using sterile saline (0.9% NaCl). This 10-fold dilution was done by transferring 1ml of the sample into 9ml of saline solution under aspiration conditions. Further, appropriate dilutions were selected for downstream culture-based analysis.

3.2 Culture-based Analysis

3.2.1 Sample Culture in differential & selective media

From the selected dilutions, 100µl diluted culture was inoculated onto differential and selective media, such as MacConkey Agar, Leeds Acinetobacter Agar, and KPC Agar, for isolation of targeted Gram-negative enteric bacteria. Two standard microbiological techniques were conducted to achieve the expected isolation. Firstly, the spread plate method was used, where the inoculum was evenly distributed over the agar surface using a sterile spreader to get isolated single colonies, and the single colonies were subcultured using the streaking method using a sterile loop to further validate the culture isolation and ensure pure

colony isolation. Notably, the culture plates were incubated aerobically at 37℃ for 18-24 hours. Then, the colony morphology was recorded

3.2.2 Biochemical Testing

To further validate the culture-selective isolation results, biochemical testing was performed, specifically for the mixed colonies. Firstly, simple staining was conducted by smearing a bacterial colony on a clean glass slide, heat-fixed and stained using crystal violet, followed by microscopic examination under a 100x objective with immersion oil to identify the cell morphology and structure. Secondly, two biochemical tests, i.e., the catalase and oxidase tests, were conducted. For the catalase test, a drop of 3% hydrogen peroxide was added to bacterial colonies on a clean glass slide, and immediate bubble formation indicates a positive result. Besides, the oxidase reagent, tetramethyl-p-phenylenediamine dihydrochloride, was added to the bacterial colony, and the development of a dark purple color within 30 seconds indicates a positive result.

3.3 Molecular characterization of bacterial isolates

3.3.1 Bacterial DNA Extraction

Bacterial isolates were subcultured on Nutrient Agar at 37 °C for 18-24 hours. Then, the culture was subjected to DNA extraction through a modified boiling extraction method. For DNA extraction, a loop full (1ml) of the overnight culture was transferred into a sterile microcentrifuge tube with 400µl TE buffer (10 mM Tris, 1 mM EDTA), followed by vortex it was centrifuged at 13,000RPM for 10 minutes at 25℃ to pellet the bacterial cells. The supernatant was discarded, and the pellet was resuspended in 400 µL of TE buffer and vortexed. Then, boiled at 98°C for 10-12 minutes to lyse the cells. Following boiling, the lysate was immediately cooled on ice for 10 minutes and centrifuged at 13,000RPM for 10

minutes at 25℃ to pellet cellular debris. The supernatant containing bacterial DNA was carefully transferred to a fresh microcentrifuge tube and stored at −20 °C until further use. The extracted DNA was used directly as the template for PCR without further quantification.

3.3.2 PCR Amplification for Species-Specific Confirmation

Molecular confirmation of bacterial isolates was conducted using polymerase chain reaction (PCR) with species-specific primers targeting genes unique to the suspected organisms (*Klebsiella pneumoniae* and *Acinetobacter baumannii*). Extracted bacterial DNA from each isolate was used as the template. The primer sequences used for molecular detection are listed in Table 1. Each PCR reaction was prepared in a total volume of 13 µL containing 6 µL PCR master mix (TakaraBio), 3 µL nuclease-free water, 2 µL template DNA, and 1 µL each of forward and reverse primers. PCR cycling conditions were optimized for each primer pair based on published protocols on Applied Biosystem (Thermo Fischer).

For *Klebsiella pneumoniae:*

Isolates identified as *Klebsiella pneumoniae* using culture-based analysis were selected for PCR confirmation of the identification of *Klebsiella pneumoniae* subsp. pneumoniae, using published specific primer pair for *Klebsiella pneumoniae* 16S-23S internal transcribed spacer gene: Pf: 5′- ATTTGAAGAGGTTGCAAACGAT-3′, and Pr1: 5′-TTCACTCTGAAGTTTTCTTGTGTTC -3′ (amplicon size: 130 bp). Cycling conditions were as follows: initial denaturation at 94°C for 10 min; 34 cycles of 94°C for 30 seconds, 57°C for 20 seconds, and 72°C for 20 seconds, followed by a final elongation at 72°C for 10 min and lid at 105°C. *Klebsiella pneumoniae* ATCC13883 was used as a positive control, and a reaction without template DNA served as the negative control.

For *Acinetobacter baumannii:*

Isolates identified as *Acinetobacter baumannii* using culture-based analysis were selected for PCR confirmation of the identification of *Acinetobacter baumannii*, using published specific primer pair for *Acinetobacter baumannii* blaOXA-51 gene: OXA-51-F: 5'-TAATGCTTTGATCGGCCTTG-3' and OXA-51-R: 5'- TGGATTGCACTTCATCTTGG-3' (amplicon size: 353 bp). Cycling conditions were as follows: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final elongation at 72°C for 10 min. *Acinetobacter baumannii* was used as a positive control, and a reaction without template DNA served as the negative control.

Table 1: Polymerase Chain Reaction (PCR) Conditions & Primers.

Organism	Primer pair	Primer's sequence (5'-3')	Target Gene	Amplicon Size	PCR Condition	Ref.
Klebsiella pneumonia	KP_Pf-F	5'-ATTTGAAGAGGTTGCAAACGAT -3'	16S-23S internal transcribed spacer gene	130bp	initial denaturation at 94°C for 10 min; 34 cycles of 94°C for 30 seconds, 57°C for 20 seconds, and 72°C for 20 seconds, followed by a final elongation at 72°C for 10 min	[44]
	KP_Pr1-R	5'-TTCACTCTGAAGTTTTCTTGTGTTC -3'				
Acinetobacter Baumannii	blaOXA-51 - F	5'-TAATGCTTTGATCGGCCTTG -3'	blaOXA-51 gene	353bp	Initial Denaturation at 94°C for 5 min; 30 cycles of 94°C 1 min, 55°C 1 min, and 72°C 1 min followed by final extension 72°C 10 min	[45]
	blaOXA-51 - R	5'-TGGATTGCACTTCATCTTGG -3'				

3.3.3 Visualization through Gel Electrophoresis

The PCR products were subjected to gel electrophoresis for visualization in a 1.4-1.7% w/v agarose gel in TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH of 8.0) containing 2-4μL nucleic acid stain, Ethidium bromide (EtBr). Electrophoresis was conducted using 100V for 40-45 minutes.

A 100bp DNA ladder was used as a molecular size reference for K.pneumoniae and a 1Kb DNA ladder was used for *Acinetobacter baumannii* for comparing the expected fragment lengths. Lastly, the gels were visualized using an Ultraviolet (UV) Transilluminator.

3.4 MALDI-TOF Mass Spectrometry Analysis

Matrix-Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is a proteomic identification technique that is rapidly used for bacterial species identification and is based on the generation of a characteristic protein-mass spectrum-based fingerprint, which is generated from abundant intercellular proteins like ribosomal proteins. Here, bacterial cells are combined with a chemical compound, commonly α-cyano-4-hydroxycinnamic acid (HCCA), to enhance the energy absorption and protect biomolecules during ionization. After laser irradiation, this chemical matrix absorbs energy and promotes desorption and ionization of bacterial proteins without major fragmentation. Then, the ionized proteins passed through an electric field, travelling through a vacuum tube. Importantly, the time-of-flight needed for ions to reach the detector is inversely proportional to the mass-to-charge ratio, and this allows the device to generate a mass spectrum. Subsequently, every bacterial species produces a distinct spectral pattern, which can be compared with the reference database using pattern-matching algorithms

The MALDI-TOF MS analysis followed a standard protocol where a suspected mixed colony was selected first from the culture plate, which was incubated for not more than 20hours, and a small amount of the colony was smeared on the MALDI target plate. Followed by mixing the smear with chemical matrix solution (HCCA) and allowing it to crystallize, the target plate was put into the device, where the laser irradiates and ionizes the sample-matrix complex. The ionized protein was then separated based on TOF and detected. The data was analysed by matching a reference database to assign organism identification with a confidence score.

3.5 Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing (AST) was performed using the Kirby-Bauer disk diffusion method on Mueller-Hinton Agar, followed by standard Clinical Laboratory Standards Institute guidelines (CLSI). Pure cultures of *Klebsiella pneumoniae* and *Acinetobacter baumannii*, confirmed using PCR, and mixed colonies (suspected co-existence) were used for AST. The antibiotics discs used were Chloramphenicol 30 (C30), Ceftazidime 30 (CAZ30), Piperacillin-Tazobactam 110 (TZP110), Amoxiclav 30 (AMC30/AK30), Cefepime 30 (CPM30 /FEP30), Azithromycin 15 (AZM 15), Levofloxacin 5 (LEV5), Ciprofloxacin 5 (CIP5), Nitrofurantoin 300 (F300), Colistin 10 (CT10), Gentamicin 10 (CN10).

Overnight cultures were used to make a 0.5 McFarland standard suspension, followed by lawning the bacterial suspension on MHA agar plates. Then the selected antibiotic discs were placed on the plates and incubated at 37°C for 18-24 hrs. The zone of inhibition was measured in millimeters and interpreted as susceptible, intermediate, and resistant, as seen in Table 2.

Table 2: List of antibiotics used in the study. *This table demonstrates the information and size of the inhibition zone, which determines the susceptibility, intermediate, and resistant status in accordance with the CLSI guidelines.*

Antibiotic class	Antibiotic	Abbreviation	Susceptible (S)	Intermediate (I)	Resistant (R)
Amphenicols	Chloramphenicol 30	C30	≥ 18mm	13-17mm	≤12mm
Beta-lactams	Ceftazidime 30	CAZ30	≥ 18mm	15-17mm	≤14mm
	Piperacillin-Tazobactam 110	TZP110	≥ 25 mm	21–24 mm	≤ 20 mm
	Amoxiclav 30	AMC30 / AK 30	≥17 mm	14–16 mm	≤13 mm
	Cefepime 30	CPM30 /FEP	≥18 mm	15-17mm	≤14 mm
Macrolides	Azithromycin 15	AZM 15	≥18 mm	14-17mm	≤13 mm
Fluoroquinolones	Levofloxacin 5	LEV5	≥17 mm	14–16 mm	≤13 mm
	Ciprofloxacin 5	CIP 5	≥21 mm	16-20mm	≤15 mm
Nitrofuran derivatives	Nitrofurantoin 300	F300	≥ 17 mm	15–16 mm	≤ 14 mm
Polymyxins	Colistin 10	CT10	-	11-17 mm	-
Aminoglycoside	Gentamicin 10	CN10	≥ 15 mm	13-14mm	≤ 12 mm

Consequently, the Multiple Antibiotic Resistance (MAR) index was calculated for isolates to investigate the level of antimicrobial resistance.

MAR = $\frac{a}{b}$; Here, a = number of antibiotics isolated that are resistant to, and b = total number of tested antibiotics.

MAR index ranges from 0 (susceptible) to 1(resistant). Isolates with MAR index ≥0.2 were considered to be isolated from a high-risk source with major antibiotic exposure. Groupwise distributions of the MAR index were visualized using line plots to compare the resistance pattern of *Klebsiella pneumoniae, Acinetobacter baumannii,* and mixed colonies. The statistical MAR index and distribution were conducted using mean and median MAR values for each group.

3.6 Contamination & Decontamination Strategy

All the procedures and methods were performed under aseptic conditions to prevent cross-contamination. All materials were used after sterilizing through autoclaving at 121°C for 2hours. Moreover, the work surfaces were disinfected with 70% ethanol. Besides, negative controls were used during PCR to identify contamination. DNA extraction, PCR setup, AST, and other lab work were performed inside a biosafety cabinet. Biohazardous waste was autoclaved at 121°C for 2hours prior to disposal.

3.7 Statistical analysis and visualization

All the data were analyzed using R programming. Descriptive statistics were used to measure and summarize the areawise bacterial prevalence, resistant profiles, and MAR index. Results were visualized using bar charts, heatmaps, and frequency distributions.

Chapter 4

Results

4.1 Study design and Integrated Analytical Framework

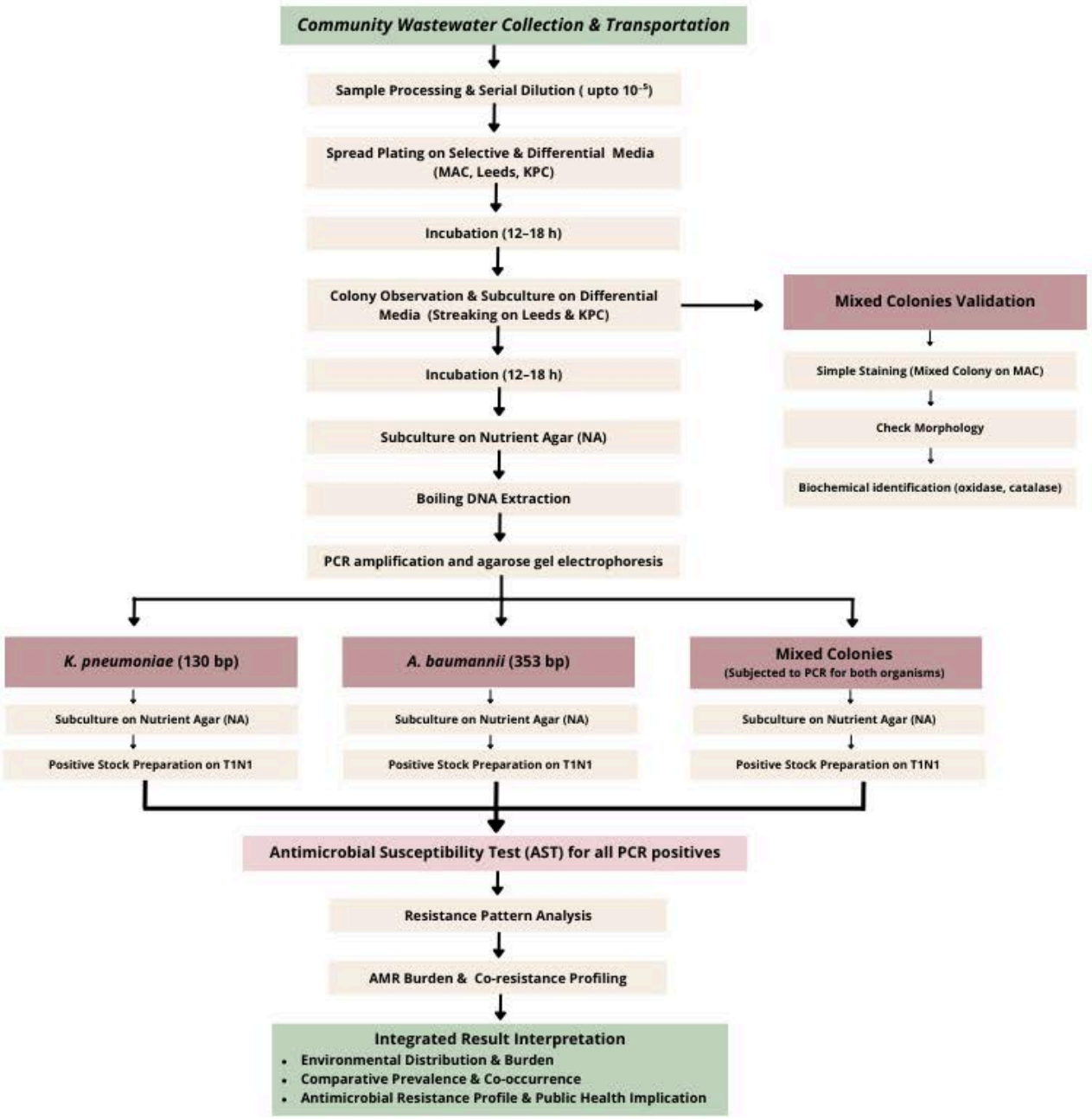


Figure 1: Schematic overview of the study design and workflow of the study. *This flowchart illustrates the workflow of culture-dependent Klebsiella pneumoniae &*

Acinetobacter baumannii comparative phenotypic and prevalence analysis, and the integrated findings interpreted the environmental distribution, burden, and AMR pattern in the Dhaka community wastewater.

This study demonstrated a comprehensive study design and workflow to identify the environmental presence, characterization, and antimicrobial resistance (AMR) profiles of *Klebsiella pneumoniae & Acinetobacter baumannii* isolated from community wastewater. A total of 32 samples were collected from multiple locations of metropolitan Dhaka city, followed by sample processing involving serial dilution, culturing on selective and differential media, and phenotypic isolation. Moreover, presumptive isolates were considered for molecular characterization by polymerase chain reaction (PCR), targeting species-specific gene fragments (130 bp for *Klebsiella pneumoniae* and 353 bp for *Acinetobacter baumannii*), followed by gel electrophoresis. Furthermore, the confirmed isolates were subcultured and preserved in T1N1 media, while the antimicrobial susceptibility testing (AST) was conducted to investigate the resistance profiles.

Consequently, the mixed colonies were examined through simple staining and biochemical assays, such as oxidase and catalase tests, to confirm the potential co-occurrence, co-existence, and morphological diversity. Therefore, this integrated analytical framework supported the synthesis of findings within spatial distribution, bacterial prevalence, and resistance patterns. These strategies provided a comparative analysis of organism-specific dominance, co-existence profile, and AMR burden in the multiple communities of metropolitan Dhaka city, facilitating the interpretation of environmental distribution and their potential role in increasing resistant pathogens across the community.

4.2 Sample Collection Sites for Community Wastewater in Dhaka

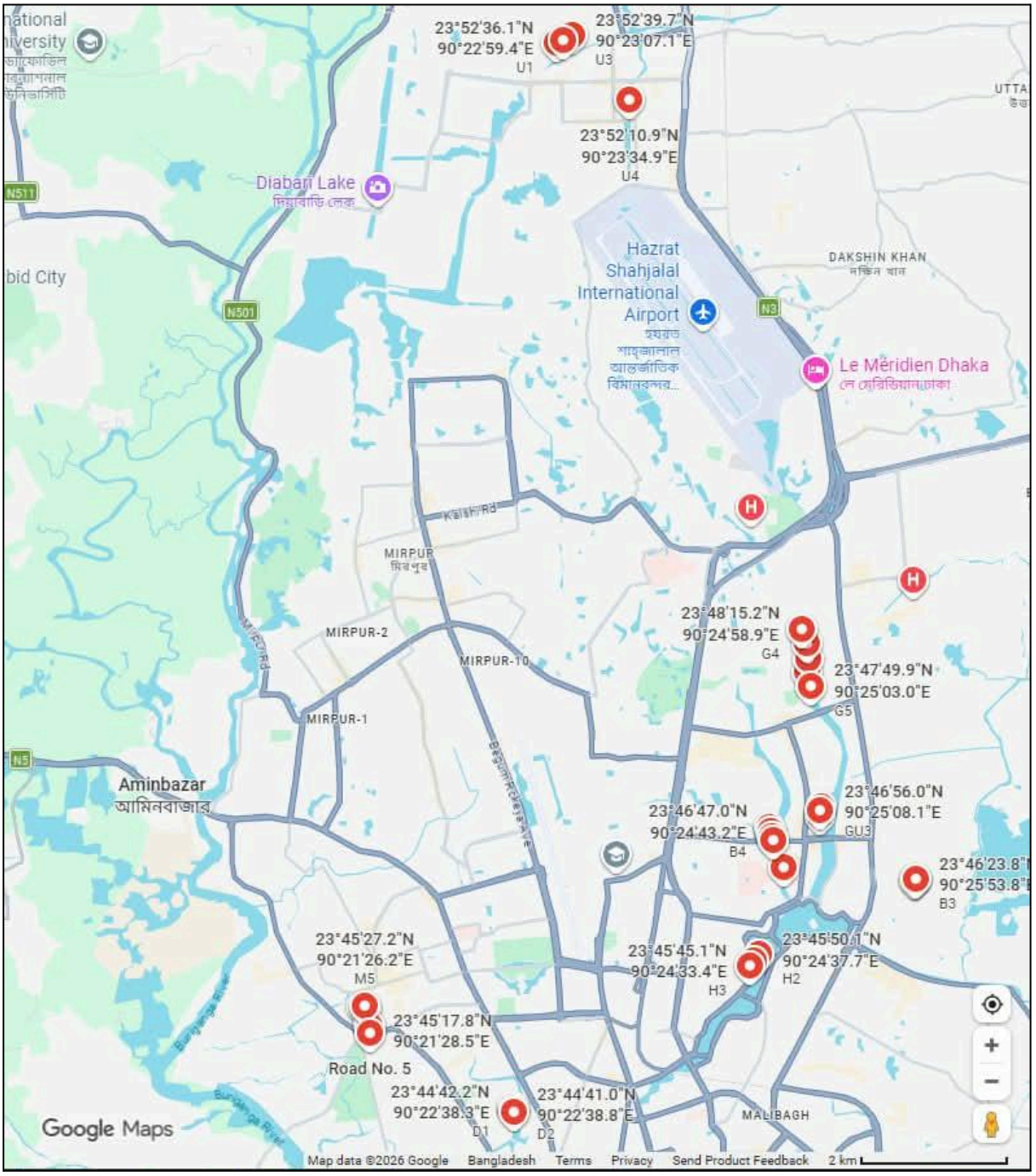


Figure 2: Sampling sites across Dhaka city. *This figure illustrates a Map showing the sites of sample collection which was examined through this study.*

The objective was to determine the presence of *Klebsiella pneumoniae* and *Acinetobacter baumannii* from community wastewater in Dhaka. For this, 32 sample of community

wastewater was collected from 8 areas of Dhaka, i.e. Hatirjheel, Gudaraghat, Mohammadpur, Badda, Gulshan, Banani, Uttara, and Dhanmondi. Figure 2 shows the exact location of sample collection sites along with longitude and latitude of the exact site.

Table 3: Distribution of Sampling sites & collection across Dhaka city. *This table demonstrates the number of samples collected from each sampling site and isolates retrieved from samples of each area.*

Area	Total Samples	Total Suspected Isolates
Hatirjheel	3	33
Gudaraghat	3	27
Mohammadpur	6	71
Badda	3	40
Dhanmondi	3	34
Uttara	4	33
Gulshan	6	32
Banani	4	52
TOTAL	**32**	**322**

From table 3, the number of sample collections from each area was observed among which the highest number of samples were collected from Mohammadpur and Gulshan. Whereas, 322 isolates were cultured and retrieved to facilitate the study from all 32 samples with highest isolate from Mohammadpur samples.

4.3 Culture-based Identification & Phenotypic Analysis

4.3.1 Culture-based Isolation & Identification

32 samples were cultured on three selective media, first through the spread-plate method. Here, primary isolation on MacConkey agar showed a heterogeneous morphology across the community wastewater samples, specifically in specimens indicating mixed bacterial population. Besides, the isolation on KPC chromogenic agar and leeds acinetobacter agar differentiates between two specimens, as these two media are selective for the specimens.

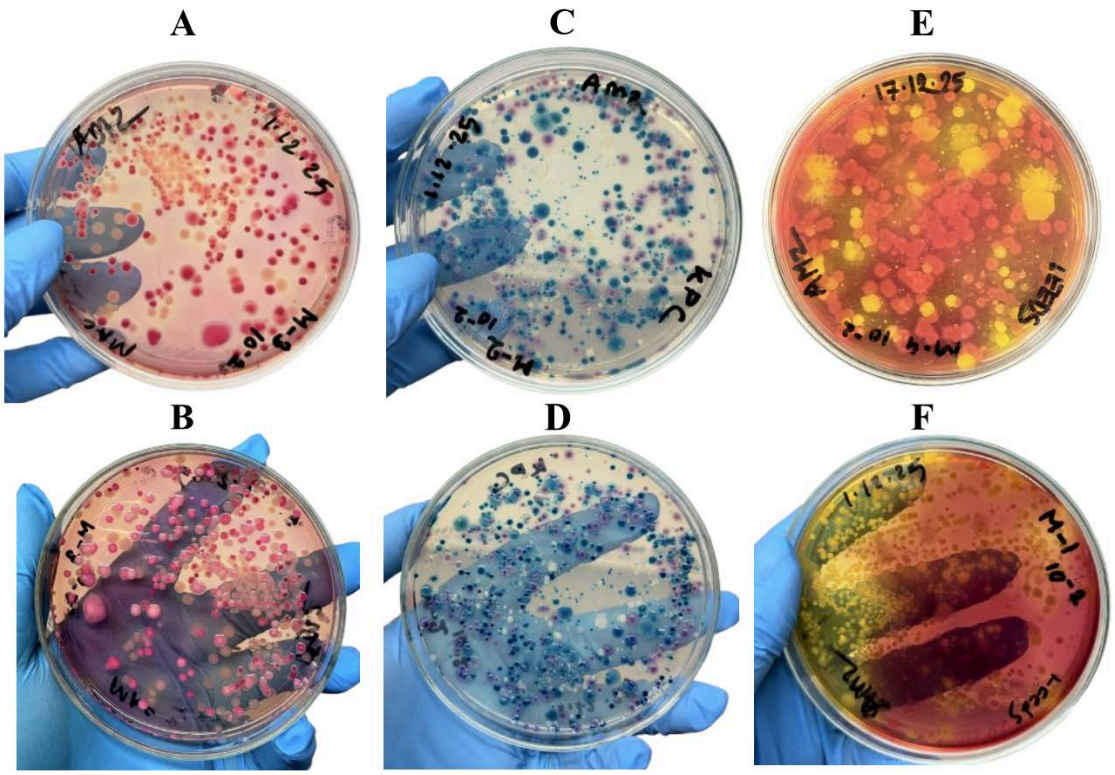


Figure 3: Spreading-based Culture Results in MacConkey Agar, KPC Chromogenic Agar, and Leeds Acinetobacter Agar. *This figure illustrates the colony morphology of suspected Klebsiella pneumoniae and Acinetobacter baumannii.* ***A, B)*** *These two figures show the colony growth in MacConkey media, where Klebsiella pneumoniae gives a bright pink colony, Acinetobacter baumannii gives a pale pink colony, and mixed colonies indicating potential co-existence were also observed.* ***C, D)*** *These figures show the colony growth in*

KPC Chromogenic Agar, which is selective for Klebsiella pneumoniae and gives turquoise-blue colonies for it. ***E, F)*** *This figure shows the colony growth in Leeds Acinetobacter Agar, which is selective for Acinetobacter baumannii and gives pink colonies for it.*

From figure 3A-3B, distinct colony morphology was observed consistently, characterized by large, mucoid, bright pink colonies in MAC agar, suggesting lactose-fermenting *Klebsiella pneumoniae,* while small, pale pink to colorless colonies in MAC agar were indicative of non-lactose fermenter *Acinetobacter baumannii.* Additionally, the co-occurrence of the morphological characteristics in MAC agar suggests potential co-existence in the community wastewater samples. Supportingly, in the selective KPC chromogenic agar media, Klebsiella pneumoniae gives a distinct turquoise-blue color in colonies (Figure 3C- 3D). Furthermore, the Leeds Acinetobacter agar media, which is selective for Acinetobacter baumannii, gives a characteristic pink colony (Figure 3E-3F).

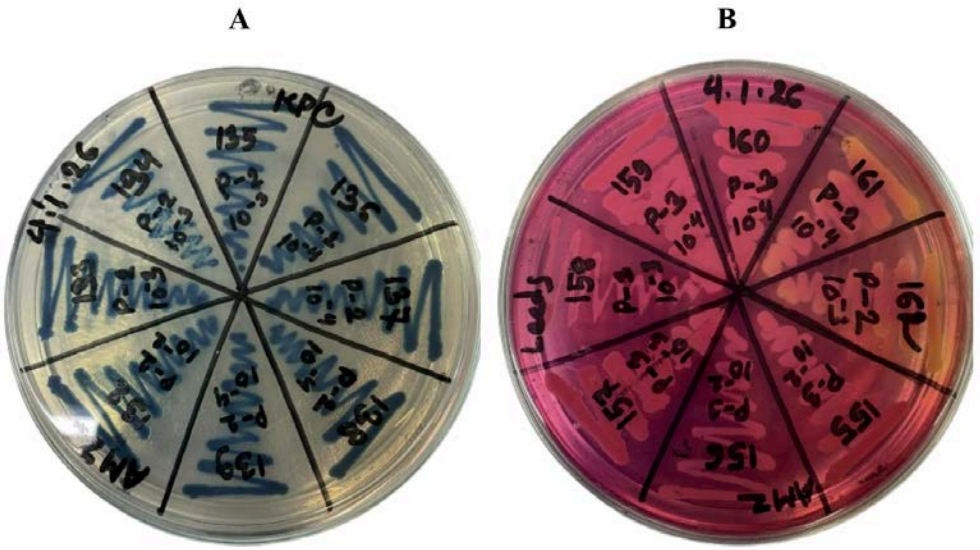


Figure 4: Streaking-based Confirmation of the Spreading Culture Results. *This figure illustrates the streaking results, which further validate the spreading-based results. A) This figure shows the subcultured colonies from MacConkey and KPC agar spread-plates, validating the presence of Klebsiella pneumoniae, showing turquoise blue colonies. B) This*

figure shows the subcultured colonies from MacConkey and Leeds agar spread-plates, validating the presence of Acinetobacter baumannii, showing pink colonies.

To ensure species-level differentiation and stronger validation, isolates from spread-plates were sub-cultured onto the KPC chromogenic Agar and Leeds Acinetobacter Agar through the streaking method. As seen in Figure 4A, *Klebsiella pneumoniae* producing turquoise-blue colonies in KPC agar indicates particular enzymatic cleavage of chromogenic substrates, which are associated with carbapenemase-synthesizing *Enterobacteriaceae*. This agar media shows high specificity for presumptive detection of *Klebsiella pneumoniae*. Similarly, Leeds media gives pink colonies for *Acinetobacter baumannii*, along with inhibiting the growth of competing Gram-negative organisms, making it more selective for the specimen and enabling selective enrichment (Figure 4B). Finally, this highlights the morphological and selective culture-based analysis, which were further supported by biochemical analysis.

4.3.2 Biochemical Assay-based Analysis

To validate the presumptive results of culture-based analysis, phenotypic identification was conducted using standard biochemical tests. This mainly focuses on the characteristic enzymatic activities relevant for gram-negative bacteria differentiation.

Figure 5: Catalase test of mixed colonies. *(A) Exemplary outcome from the catalase test depicting Sample A showed no bubbles, signifying a catalase-negative result and the absence of catalase enzyme production. (B) A representative result from the catalase test showing control Sample B shows significant bubbling following the addition of hydrogen peroxide, confirming catalase positive.*

Consequently, Both organisms showed catalase-positive reactions through rapid bubble formation due to exposure to Hydrogen Peroxide and ultimately confirmed the presence of catalase enzyme, which is a common feature of aerobic and facultative anaerobic bacteria (Figure 5B).

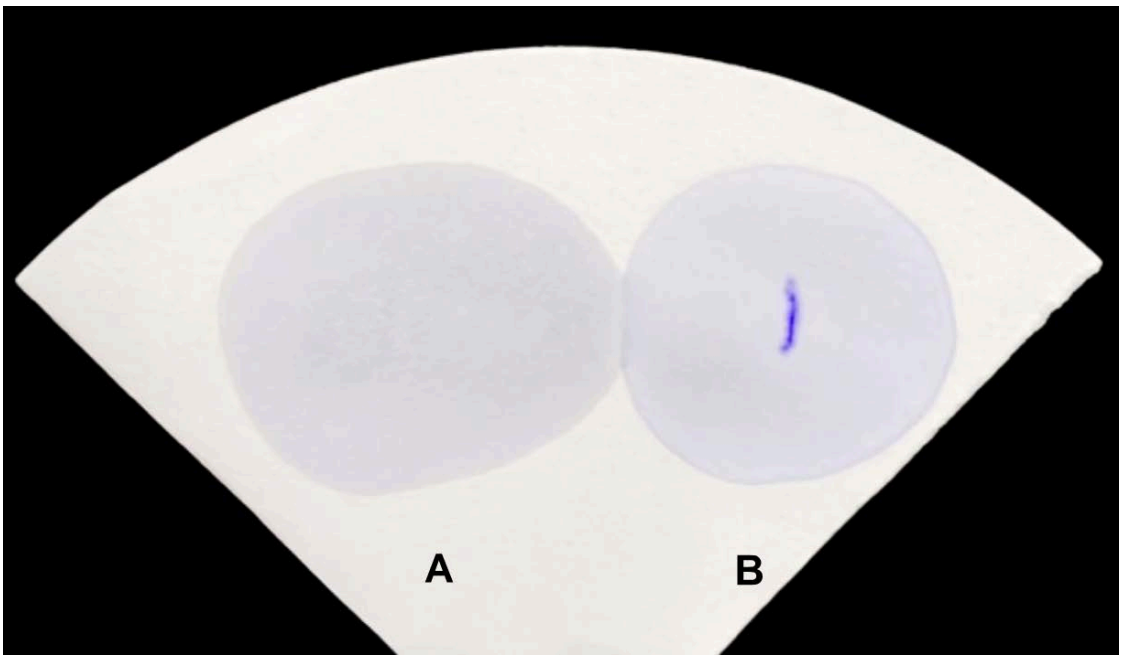


Figure 6: Oxidase test of Mixed colonies. *(A) A representative result from the oxidase test of Sample A exhibited no color change in the oxidase test, indicating an oxidase-negative result and the absence of cytochrome-c-oxidase enzymes. (B) Exemplary outcome from the Sample B displayed an instant purple color in the oxidase test, signifying an oxidase-positive result and the production of cytochrome-c-oxidase enzymes.*

Notably, both organisms as well showed an oxidase-negative result as it belongs to the oxidase-negative gram-negative group, and this mostly excludes oxidase-positive non-fermenters like *Pseudomonas spp.* (Figure 6A). Though both the biochemical assay results are identical, this was valuable for the exclusion of non-target organisms and confirmation of broad taxonomic groups. For mixed colonies, this test suggests that the mixed population was composed of some oxidase group, highlighting the likelihood of co-existence instead of contamination with oxidase-positive species and validating the composition of mixed isolates.

Overall, the outcomes from differential media, colony morphology, and biochemical assays demonstrated a multiple-level phenotypic identification approach. The biochemical test

confirmed the basic taxonomic grouping, which indicated the presence of the concerned organisms, while selective media primarily differentiated on the species level. Importantly, observation of mixed colony morphology on MAC agar, followed by selective growth on KPC and leeds media, suggested the presence of potential co-existence of the two organisms, which was further examined through molecular characterization and MALDI-TOF analysis.

4.2.3 Microscopic Characterization by Simple Staining

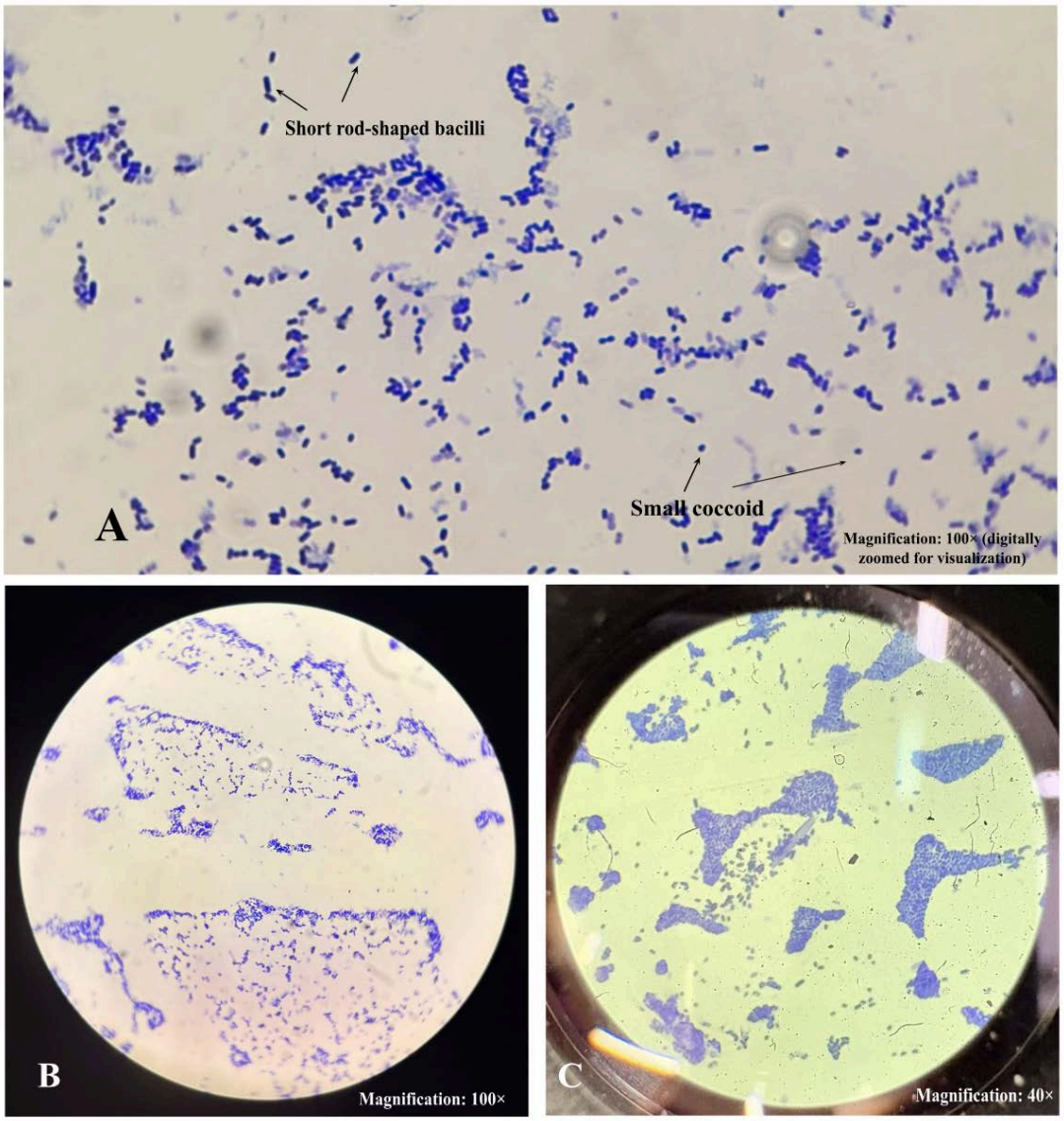


Figure 7: Microscopic View of Simple Staining Results of Mixed Colonies. *This figure demonstrates the co-presence of two different bacterial morphologies, that is, short rod-shaped bacilli, indicating Klebsiella pneumoniae, and small coccid to short coccobacilli*

structure, indicating Acinetobacter baumannii. A) This microscopic representation demonstrates the 100x magnified image which was further digitally zoomed to clearly visualize the morphology of the mixed colonies. B) This figure illustrates the 100x magnified image of a mixed colony. C) This image reflects the microscopic view at 40x magnification of the mixed colony, These findings suggest the potential co-existence of these two bacterial species identified from mixed colonies of MacConkey Agar.

Microscopic characterization of mixed colonies from MAC agar was conducted through simple staining to observe the cellular morphology and confirm the phenotypic identification. The stained bacterial smear showed a co-occurrence of two distinct bacterial morphology, that is, short rod-shaped bacilli, which are characteristic of *Klebsiella pneumoniae* and this straight rods were often found single or in clusters, and small coccoid to short cocco-bacillary forms which are morphological property of *Acinetobacter baumannii* and this are mostly observed as rounded and sometimes as slightly elongated cells (Figure 7A). The observation of bacillary and coccoid morphologies in the same bacterial colony smear strongly suggests the presence of a mixed bacterial population. Though the simple staining method lacks species-level specificity, the presence of two distinct morphologies supports the culture-based differentiation and aligns with the hypothesis of mixed colonization.

4.4 Molecular & Proteomic Characterization of Bacterial Isolates

4.4.1 PCR Amplification and Gel Visualization

To validate the culture-based phenotypic analysis, molecular confirmation of the isolates was conducted through PCR to target *Klebsiella pneumoniae* and *Acinetobacter baumannii* species.

Amplification of *Klebsiella pneumoniae* was performed using species-specific primers, KP_Pf-F and KP_Pr1-R, targeting 16S-23S internal transcribed spacer gene, which produced an expected amplicon band size of 130bp, as seen in Table 1. In the gel image of Figure 8A, the bands supporting the target fragment were observed in lanes 4-17, and positive and negative controls were used in lanes 2 and 3, respectively, to ensure accuracy and specificity of the amplification. Besides, Lane 1 visualized the 100bp ladder, which facilitated the detection of band size.

Similarly, *Acinetobacter baumannii* isolates were confirmed through targeting the blaOXA-51 gene using primer pairs blaOXA-51-F and blaOXA-51-R (Table 1). The expected band size was observed at 353bp, and lanes 4-17 showed a distinct band at the expected size determined by the reference DNA ladder of 1Kb size at lane 1 (Figure 8B). Moreover, lane 2 and 3, containing positive and negative controls, respectively, supported the accuracy of the amplification results.

Hence, these results confirmed the presence of both target organisms in the community wastewater sample, where 41 isolates of *Acinetobacter baumannii* and 45 isolates of *Klebsiella pneumoniae* were confirmed through PCR amplification within 32 samples.

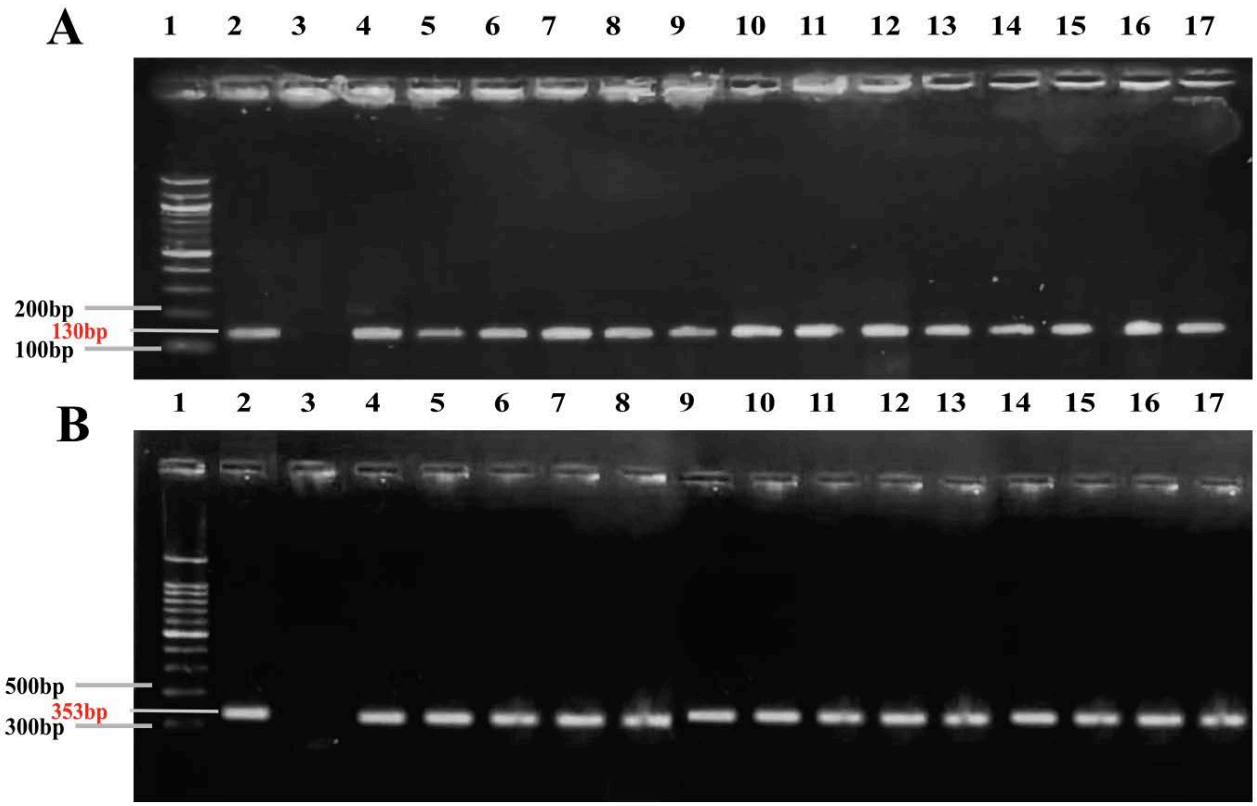


Figure 8: Gel visualization of *Klebsiella pneumoniae* and *Acinetobacter baumannii* species identification via Polymerase Chain Reaction (PCR). *A) This gel image demonstrates the PCR results for Klebsiella pneumoniae positive isolates at 130bp. B) This gel image demonstrates the PCR results for Acinetobacter baumannii positive isolates at 353bp. Here, Lane 1 shows a 100bp DNA Ladder in A and a 1KB DNA Ladder in B, Lane 2 and 3 show positive and negative control, respectively. And, Lane 4-17 shows the results of isolates from different samples.*

Though the mixed colonies showed strong evidence of co-existence of *Klebsiella pneumoniae* and *Acinetobacter baumannii*, molecular confirmation through PCR showed positive results only for one organism instead of showing positive results for both organisms. So, PCR amplification was ineffective in validating the co-existence of the specimens. As a result, these mixed colonies were subjected to MALDI-TOF MS analysis to investigate the organisms present in the mixed colonies.

4.4.2 MALDI-TOF MS Analysis

Although PCR amplification is highly specific for targeted organisms, it is inefficient to detect multiple organisms present within the same isolate. In this regard, MALDI-TOF MS provides a wide range of unbiased proteometric approaches, identifying unknown bacterial species in the sample. So, this approach was expected to validate the suspected mixed cultures identified through culture-based phenotypic analysis.

Result overview table--continued from previous page

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A11 (-) (C)	D1 (standard)	no peaks found	0.00	no peaks found	0.00
A12 (+) (B)	N2 (standard)	Klebsiella pneumoniae	1.74	No Organism Identification Possible	1.66

Table 4: Results of the MALDI-TOF Analysis. *This table shows the tabular results from MALDI-TOF analysis conducted from National Institute of Biotechnology (NIB), Bangladesh, showing that among the two mixed colonies with suspected co-existence one colony showed no organism identified and another showed match with Klebsiella pneumoniae. This results highlights the unidentified colony was not a pure culture with a single bacterial presence.*

From Table 3, the results of MALDI-TOF MS showed two isolates suspected of potential co-existence, subjected to this analysis gave an interpretation of “no organism identification possible” & “no peaks found” with a score value of 0.00. This result indicates the presence of a mixed or impure culture that might have generated overlapping or inconsistent protein spectra which could not be matched with any single reference protein spectra profile in the database. Because in this approach, pure bacterial culture under controlled conditions produces a clear and detectable spectral fingerprint. On the other hand, multiple species

present in one sample can overlap the protein patterns and lead to an undetectable or unrecognizable spectral pattern, giving an inconclusive result.

4.5 Areawise Bacterial Prevalence & Dominance Analysis

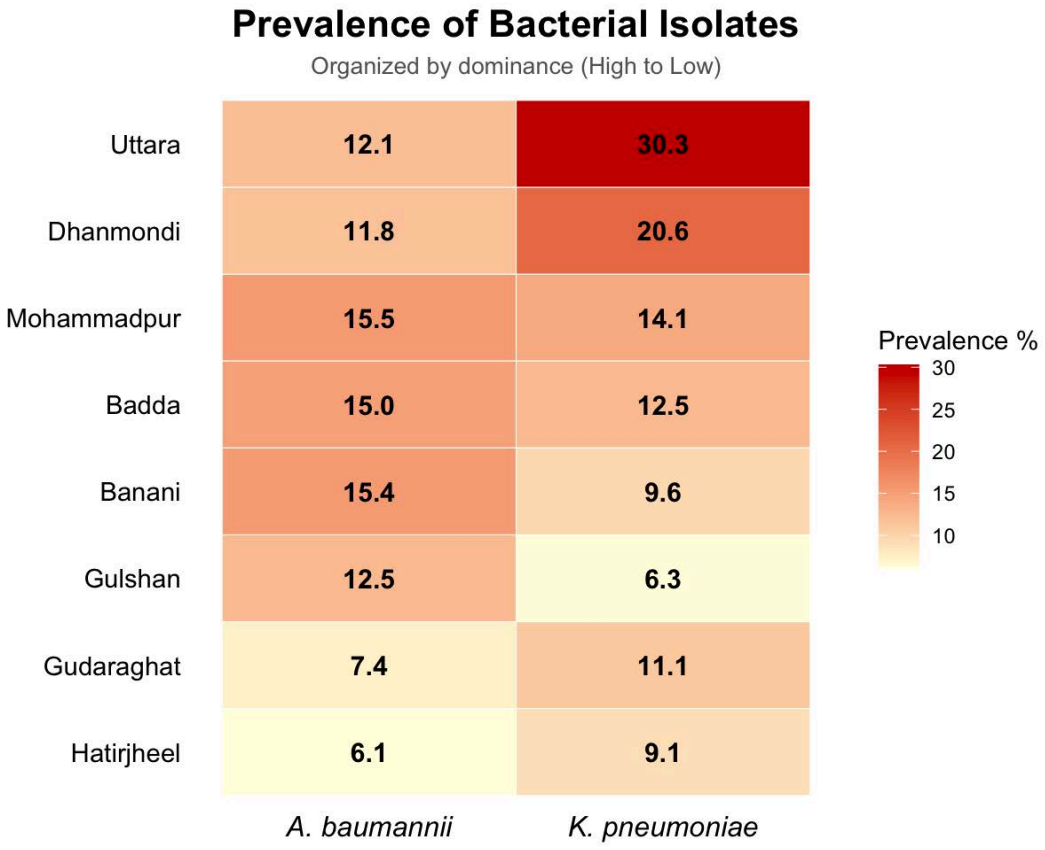


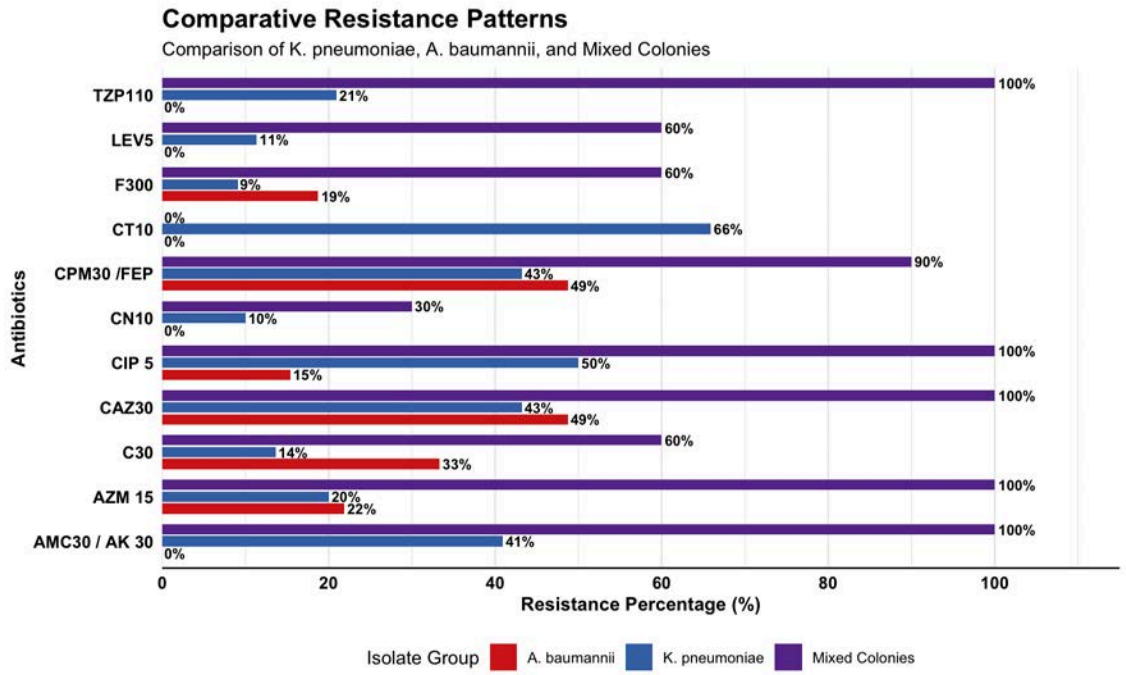
Figure 9: Prevalence Heatmap of Bacterial isolates across different communities of Dhaka city. *This heatmap shows spatial distribution via heatmap highlighting the non-uniform bacterial prevalence and burden across different sampling sites, where Klebsiella pneumoniae was seen as the most prevalent and dominant species, with the highest prevalence in Uttara among other tested areas of Dhaka. Acinetobacter baumannii showed the highest dominance in Mohammadpur with 15.5% prevalence.*

The areawise heatmap and distribution revealed a clear variation in the prevalence and dominance of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in different community

wastewater sites in Dhaka city. A total of 32 samples were collected, in which 45 isolates were *K.pneumoniae* and 41 isolates were *Acinetobacter baumannii*, corresponding to 52.3% of total isolates being *Klebsiella pneumoniae* and 47.7% were *Acinetobacter baumannii*. Furthermore, Mohammadpur showed 47.6% *Klebsiella pneumoniae* and 52.4% *Acinetobacter baumannii*, representing the highest overall isolated prevalence and showing a balanced co-dominance for both organisms. While Uttara shows 71.4% *Klebsiella pneumoniae* and 28.6% of *Acinetobacter baumannii* with a strong *Klebsiella pneumoniae* dominance, Banani showed 38.5% *Klebsiella pneumoniae* and 61.5% *Acinetobacter baumannii* with a clear *Acinetobacter baumannii* dominance (Figure 9, Supplementary Table S4). The heatmap exhibits that *Klebsiella pneumoniae* is a common organism that is present in all the locations with relatively stable distribution, whereas *Acinetobacter baumannii* demonstrates a more variable and location-specific dominance, like Banani and Gulshan. Certain areas, like Mohammadpur, show co-dominance, highlighting the favorable conditions for the simultaneous persistence of both organisms.

4.6 Antimicrobial Resistance (AMR) Burden & Co-Resistance Profiling

While environmental isolation and molecular characterization identified the presence of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in a concerning range, the antimicrobial susceptibility testing (AST) was conducted using the Kirby-Bauer Disk Diffusion Method to all the PCR confirmed isolates of this two organisms, along with mixed colonies, to investigate the AMR burden to assess the complexities concerning with the diseases and treatment associated with this organisms (Supplementary figure S1). The comparative resistance pattern was illustrated in Figure 8, with organism-specific patterns demonstrated in Supplementary figures S2-S4.

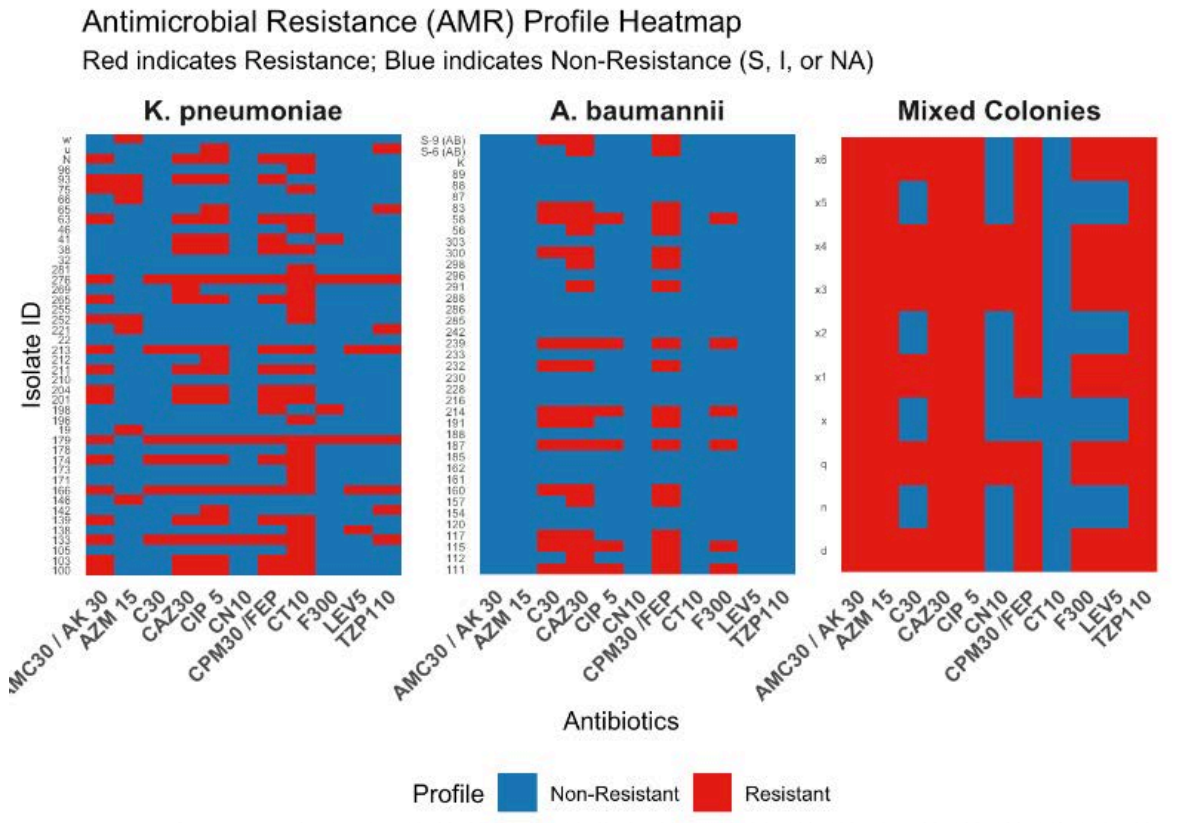


Isolate number of *K. pneumoniae* (n= 45): 19, 22, 32, 38, 41, 46, 63, 65, 66, 75, 93, 96, 100, 103, 105, 133, 138, 139, 142, 146, 166, 171, 173, 174, 178, 179, 196, 198, 201, 204, 210, 211, 212, 213, 221, 252, 255, 265, 269, 276, 281, N, U, W.
Isolate number of *A. baumannii* (n= 41): 6, 9, 56, 58, 83, 87, 88, 89, 111, 112, 115, 117, 120, 154, 157, 160, 161, 162, 185, 187, 188, 191, 214, 216, 228, 230, 232, 233, 239, 242, 285, 286, 288, 291, 296, 298, 300, 303, K.
Isolate number of *mixed colonies* (n= 10): d, n, q, x, x1, x2, x3, x4, x5, x6

Figure 10: Comparative Representation of Antimicrobial Resistance Pattern of *Klebsiella pneumoniae, Acinetobacter baumannii* & Mixed Colonies. *This bar chart illustrates the resistance percentage in the x-axis against 11 antibiotics in the y-axis for positive isolates of Klebsiella pneumoniae (n=45), Acinetobacter baumannii (n=41) & mixed colonies (n=10) from the community wastewater of Dhaka. The antibiotics used are Chloramphenicol 30 (C30), Ceftazidime 30 (CAZ30), Piperacillin-Tazobactam 110 (TZP110), Amoxiclav 30 (AMC30 / AK 30), Cefepime 30 (CPM30 /FEP30), Azithromycin 15 (AZM 15), Levofloxacin 5 (LEV5), Ciprofloxacin 5 (CIP 5), Nitrofurantoin 300 (F300), Colistin 10 (CT10), Gentamicin 10 (CN10).*

Among the tested antibiotics, *Acinetobacter baumannii* showed a higher resistance pattern compared to *Klebsiella pneumoniae*. For instance, as seen in figure 10, resistance to β-lactam antibiotics, class like ceftazidime and amixiclav was consistently observed in *Acinetobacter baumannii* with approximately 60-80% resistance, while *Klebsiella pneumoniae* showed 30-50% resistance to this class. Likewise, *Acinetobacter baumannii* showed a higher resistance (around 60%) to piperacillin-tazobactam compared to *Klebsiella pneumoniae* (around 40%). Besides, *Klebsiella pneumoniae* showed a moderate resistance to chloramphenicol with a resistance of 30-40%, yet *Acinetobacter baumannii* exhibited a higher resistance (around 50-60%).

Notably, the mixed colonies, which are suspected of co-existence, showed the highest resistance pattern, with resistance percentage up to even 100% for certain antibiotics like Levofloxacin 5. Notably, levofloxacin showed around 60% resistance in mixed colonies, whereas only 11% resistance in *Klebsiella pneumoniae* and negligible resistance in *Acinetobacter baumannii.* However, these isolates showed higher resistance to β-lactam and other classes, exceeding 70-90% resistance (Figure 10, Supplementary Figure S2-S4). This pattern suggests that mixed microbial populations might be overlapping or synergistic resistance determinants, which might be influenced by environmental stress and horizontal gene transfer, indicating co-existence.



Isolate number of *K. pneumoniae* (n= 45): 19, 22, 32, 38, 41, 46, 63, 65, 66, 75, 93, 96, 100, 103, 105, 133, 138, 139, 142, 146, 166, 171, 173, 174, 178, 179, 196, 198, 201, 204, 210, 211, 212, 213, 221, 252, 255, 265, 269, 276, 281, N, U, W.
Isolate number of *A. baumannii* (n= 41): 6, 9, 56, 58, 83, 87, 88, 89, 111, 112, 115, 117, 120, 154, 157, 160, 161, 162, 185, 187, 188, 191, 214, 216, 228, 230, 232, 233, 239, 242, 285, 286, 288, 291, 296, 298, 300, 303, K.
Isolate number of *mixed colonies* (n= 10): d, n, q, x, x1, x2, x3, x4, x5, x6

Figure 11: Heatmaps of Antimicrobial Resistance Pattern of *Klebsiella pneumoniae, Acinetobacter baumannii* & Mixed Colonies. *This heatmap illustrates the antibiotics' names in the x-axis against isolate IDs in the y-axis for isolates of Klebsiella pneumoniae, Acinetobacter baumannii & mixed colonies from the community wastewater of Dhaka. Here, the red-colored region indicates resistance to that antibiotic, and the blue-colored region demonstrates non-resistance to that antibiotic.*

The heatmap, as observed in Figure 11, supported the comparative analysis of AMR pattern, showing approximately 45% of the *Klebsiella pneumoniae* aligned with the criteria of MDR, indicating resistance towards multiple antibiotic classes, with the highest resistance observed against colistin at 65.9%, 50% resistance to ciprofloxacin and cefepime at 43.2%. Besides, moderate resistance towards amoxicillin-clavulanic acid/ amikacin at 40.9%, and others

showed a lower resistance level. In contrast, *Acinetobacter baumannii* exhibited a cephalosporin-associated MDR with high resistance towards ceftazidime and cefepime at 48.7%, while chloramphenicol at 33.3%. Also, other critical antibiotics showed 100% susceptibility to this organism, suggesting a narrower resistance spectrum to *Klebsiella pneumoniae*.

However, the mixed colonies reflected a robust resistance burden towards multiple antibiotic classes, with 100% universal resistance against Ceftazidime, piperacillin-tazobactam, amoxicillin-clavulanate/amikacin, azithromycin, and ciprofloxacin. Besides, high resistance was also observed for cefepime at 90% and chloramphenicol at 60%.

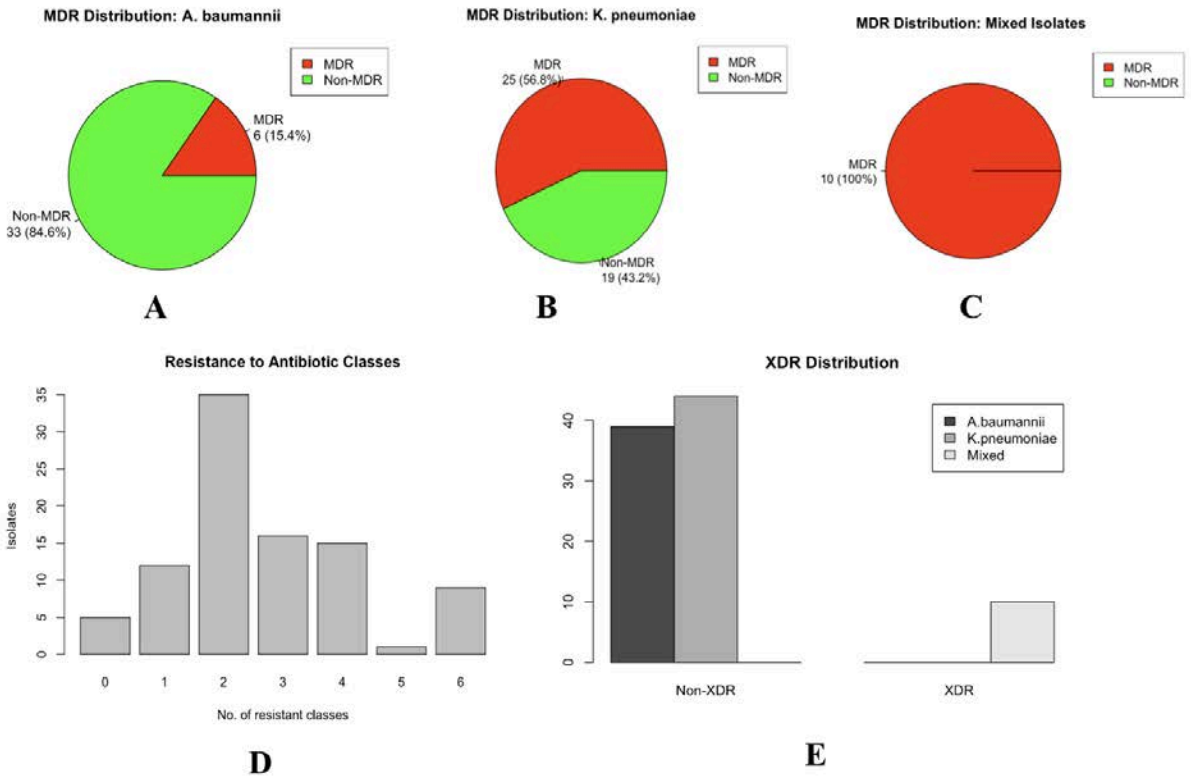


Figure 12: Distribution of MDR and XDR & Resistance to antibiotic classes of environmental A. baumanni, Klebsiella pneumoniae, and mixed isolates. *A, B, C) These pie charts illustrate the comparative distribution of Multidrug resistance (MDR) patterns*

across the two organisms and their mixed colonies. Here, green represents the non-MDR class and red indicates the MDR class. D) The bar chart demonstrates the resistance to antibiotic classes for these organisms and mixed colonies. E) The bar chart shows the XDR distribution between these organisms. Here, dark grey = Acinetobacter baumannii, moderate grey = Klebsiella pneumoniae, and light grey = mixed colony.

Overall, the statistical analysis highlights *Acinetobacter baumannii* exhibited a higher resistance within maximum tested antibiotic classes, and *Klebsiella pneumoniae* showed a moderate but increasing resistance specifically towards β-lactams, while the mixed colonies exhibited significantly elevated and heterogeneous resistance patterns. From Figure 12D, the 11 tested antibiotics under 7 different classes were observed to investigate the resistance pattern between the varying number of these classes. A total of 41 isolates showed resistance to 3 or more antibiotic classes, highlighting the prevalence of MDR. Besides, the majority of isolates (35 isolates) were observed to be resistant to 2 antibiotic classes. Notably, 9 isolates showed resistance to 6 antibiotic classes, indicating an extreme resistance pattern. Only 5 isolates were not resistant to any of the classes.

And based on standard definitions, multidrug-resistant (MDR) isolates, where isolates are resistant to ≥1 agent in ≥3 antibiotic classes, were highly observed in both *Acinetobacter baumannii* (15.4%) and *Klebsiella pneumoniae* (56.8%), with a higher proportion in *Klebsiella pneumoniae*. However, mixed colonies exhibited 100% MDR pattern (Figure 12A-12C). Furthermore, mixed isolates indicated an extensively drug-resistant (XDR) pattern, i.e., resistant to ≥1 agent in all classes but ≤2 categories are still susceptible, showing increased resistance with multiple antibiotic categories, but individually these two organisms did not exhibit any XDR trends (Figure 12C). These results indicate the community wastewater is a concerning site for resistance amplification and potential interspecies interactions, imposing a concern over public health.

4.7 Multiple Antibiotic Resistance (MAR) Analysis

MAR index highlights the estimate of selective stress due to antibiotic use in community wastewater. Notably, MAR ≤ 0.2 indicates isolates with a low antibiotic exposure and MAR > 0.2 suggests a higher risk of contamination and higher antibiotic selective pressure. The MAR index (a/b) & Percentage of MAR for *Klebsiella pneumoniae, Acinetobacter baumannii* and mixed colonies were measured with certain factors, like number of Antibiotics Resistant to (a), Frequency of MAR isolates (n), No. Antibiotics tested (b), as shown in supplementary tables S1-S3 respectively.

Table 5: Distribution of MAR index. *This table compares how resistance is distributed across Klebsiella pneumoniae, Acinetobacter baumannii, and mixed colonies. Here, nearly all isolates in mixed colonies are highly resistant, Acinetobacter baumannii shows a very tight distribution with almost all isolates between 0.5-0.8, and Klebsiella pneumoniae shows a bimodal pattern with high resistant peaks at 1 and 0,5.*

MAR Index	***Klebsiella pneumoniae*** **(Frequency / %)** **[n=45]**	***Acinetobacter baumannii*** **(Frequency / %)** **[n=41]**	***Mixed Isolates*** **(Frequency / %)** **[n=10]**
1	11 (25%)	0 (0%)	9 (90%)
0.9	0 (0%)	0 (0%)	1 (10%)
0.8	2 (5%)	6 (15.38%)	0 (0%)
0.7	4 (9%)	20 (51.28%)	0 (0%)
0.6	1 (2%)	6 (15.38%)	0 (0%)
0.5	10 (23%)	7 (17.95%)	0 (0%)
0.4	6 (14%)	0 (0%)	0 (0%)

0.3	1 (2%)	0 (0%)	0 (0%)
0.2	2 (5%)	0 (0%)	0 (0%)
0.1	5 (11%)	0 (0%)	0 (0%)
0	2 (5%)	0 (0%)	0 (0%)

The MAR index measured the significant variation of antimicrobial selective pressure (Table 4). Mixed colony isolates showed a higher intensity of resistance to all 11 antibiotics tested, within 90% of isolates having a MAR index of 1.0, whereas the rest 10% reflected a MAR index of 0.9.

However, *Acinetobacter baumannii* isolates exhibited a lower MAR index compared to mixed colonies, with 51.28% isolates clustered at a MAR index of 0.7, showing resistance to seven antibiotics, 15.38% showed 0.8 and 0.6 MAR index, and 17.95% showed 0.5 MAR index, which highlights a consistently higher but variable resistant pattern.

Besides, *Klebsiella pneumoniae* showed a distinct bimodal resistance profile with 25% of isolates having a MAR index of 1.0 and 23% of isolates having a MAR index of 0.56. A lower peak was also observed at the MAR index of 0.4 (14%), 0.78 (9%), 0.89 (5%), and 0.1 (11%). Moreover, 5% isolates demonstrated a MAR index of 0.0, suggesting susceptibility towards the antibiotics tested.

Finally, 100% of *Acinetobacter baumannii* and mixed isolates, and 90% of *Klebsiella pneumoniae* showed MAR index more than 0.2, highlighting these isolates from community wastewater with high antibiotic selectivity stress, which suggests it as a potential source of multidrug-resistant bacteria.

Chapter 5

Discussion

This study demonstrated that community wastewater in Dhaka has a high burden of multidrug-resistant *Klebsiella pneumoniae* and *Acinetobacter baumannii*, along with clear regional variation and potential co-existence. The phenotypic, molecular, and proteomic analyses confirmed the identification of both organisms. Besides, the antimicrobial susceptibility testing demonstrated the increased resistance to multiple antibiotic classes. Notably, mixed colonies showed the highest resistance intensity, having a MAR index of approximately 1.0, highlighting selective pressure. Furthermore, the areawise analysis identified the localized bacterial dominance and co-occurrence, which highlights wastewater as a fundamental reservoir for AMR emergence and spread.

Moreover, The results from the study were validated by multiple analytical approaches, where each methodological step indicated the reliability of the findings. Firstly, the culture-based isolation on selective and differential media like MacConkey, KPC, and Leeds agar gave a consistent and accurate morphological characterization of *Klebsiella pneumoniae* and *Acinetobacter baumannii*. The colony morphology observed within the media confirms the accuracy of the phenotypic identification. Secondly, the catalase and oxidase tests did not differentiate between the two organisms but their inclusion in the study was significant for excluding the non-target bacteria and highlighting the taxonomic consistency. These tests reduced the risk of misinterpretation in the complex environmental samples where multiple Gram-negative organisms potentially co-exists. Moreover, the microscopic analysis showed the co-existing bacilli and cocco-bacilli morphology, demonstrating a visual confirmation of the mixed colony with presumptive co-existence. This observation supported the

culture-based evidence and strengthened the presumption of potential co-occurrence in the same samples.

Furthermore, the phenotypic identification was further validated using PCR amplification and MALDI-TOF MS, which are highly specific at the species level. The combination of phenotypic, molecular and proteomic methods strengthens the validation with minimizing the chance of false identification. Finally, the pairwise dominance & bacterial prevalence is justified through the difference in local environment and anthropogenic factors like wastewater composition, population density, and proximity to healthcare or industrial discharge points. These factors contribute to the dominance and localized selection of specific bacterial species. showing regional heterogeneity.

Subsequently, AST results from wastewater justified with the observed environmental context and the wastewater was known to accumulate antibiotic residues, and resistant bacteria promoting the emergence and persistence of resistant strains. The higher resistance level observed, especially in mixed colonies are consistent with the environmental-associated selective pressure. Besides, the MAR index distribution supports the interpretation as the MAR value > 0.2 in isolates indicates high risk contamination source and confirms that the site of sample collected is highly exposed to antimicrobial agents. The extreme MAR values in mixed isolates indicates resistance accumulation due to microbial interaction or shared resistance mechanisms.

Most importantly, the primary aim of the study was to assess the environmental presence, identification and antimicrobial resistance profiles of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in community wastewater of Dhaka city. The particular focus was on their co-existence and potential role in resistance induction. Overall the findings of this study highly supports the initial research objective and goals highlighting the community

wastewater play role in promoting high antimicrobial resistance and potential microbial infection apart from serving as a natural habitat for the two organisms.

Additionally, the AMR profiles demonstrated a consistent and notable deviation from the global and national case reports, highlighting the distinct selective pressure observed in the community wastewater in Dhaka. Wastewater studies globally suggest these treatment systems serve as AMR promoters and spread through this wastewater. Evidently, MDR and even colistin-resistant *Klebsiella pneumoniae* were recorded in treated wastewater effluents [46]. Besides, clinical and community wastewater isolates were often demonstrated with similar resistance patterns, suggesting an environmental persistence of clinically relevant strains [47]. This strongly aligns with our findings, along with a novel distinguishing feature, which is the extreme resistance pattern in mixed colonies, where 100% resistance was observed for multiple antibiotics and a MAR index of 1.0 in 90% of the isolates. This level of resistance was a rare finding in the global scenario, where maximum reports focused on a single species, indicating possible synergistic resistance mechanisms, increased horizontal gene transfer, and biofilm-mediated protection in the wastewater environment.

Besides, *Klebsiella pneumoniae* exhibited resistance to third generation cephalosporins with 43-48% and fluoroquinolones with approximately 50% resistance in this study, which is consistent with the global studies. Here, the resistance to these antibiotic classes was widespread and influenced disease burden. Besides, resistance to cephalosporins and carbapenems was observed in a large portion of *Klebsiella pneumoniae* and promotes *Klebsiella pneumoniae* associated mortality, particularly in South Asia and underdeveloped countries. Conversely, a major difference was observed in colistin resistance (approx. 65.9%), which is drastically higher in comparison to global studies, with typically 5%. Initial studies demonstrate such resistance as an uncommon and emerging threat. This indicates unregulated antibiotic usage in humans and other sectors in developing regions, selective pressure from

antibiotic presence in wastewater, and spread of resistance promoting elements like plasmids with carbapenemase and colistin resistant genes.

On the other hand, initial studies revealed *Acinetobacter baumannii* as a major pathogen with a high resistance to carbapenems (around 33-79%) and β-lactam inhibitors (around 66%) [48]. Further, Carbapenem resistance in many regions, particularly in hospital environments, was observed to be exceeding 80-90% [49]. Comparatively, this study exhibits moderate to high resistance to cephalosporins and fluoroquinolones and relatively lower resistance to aminoglycosides. This variation is indicative of environmental isolates being exposed to lower but continuous antibiotic concentrations and heterogeneous wastewater composition. Overall, these global reports suggest that *Acinetobacter baumannii* rapidly becomes resistant by carbapenemase production, efflux methods, and biofilm formation.

Interestingly, a novel finding of this study was the extreme resistance profiles in mixed colonies, where 90% isolates were resistant to multiple antibiotics. These resistance levels are rare in global studies, as most studies have focused on single-species isolates. The higher resistance observed in this study compared to global studies can be attributed to many factors, such as high antibiotic usage with a lack of antimicrobial handling, and extensive use of antibiotics in the agriculture sector.

Consequently, the investigation into potential co-existence through assessing the mixed colonies significantly provided insights into the polymicrobial interaction which are often neglected in single-isolate analysis. The co-existence of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in the same environmental condition is concerning due to their opportunistic and Multidrug resistance (MDR) potential associated with major health concerns and infections. From a microbiological perspective, this co-existence in community wastewater promotes horizontal gene transfer alongside exchange of plasmids, integrons, and

transposons, which carry the antimicrobial resistance genes. These interactions enhance emergence of XDR and PDR strains, as evidenced from this study, supported by an extremely high MAR index and maximum resistance patterns [50-52],. In addition, both species have the ability to form biofilms, which influences bacterial survival under environmental stress and increases the efficiency of genetic material exchange. In these biofilm communities, resistance genes can be rapidly spread to other bacteria in the environment, amplifying the overall resistance pattern [50].

This co-existence of pathogenic bacteria can be involved with increased disease severity & complexity, prolonged hospitalization and limited therapeutic strategies, leading to synergistic pathogenicity where one organism will promote virulence or antibiotic tolerance of the other. Additionally, the detection of a highly resistant mixed population entails that these community wastewater environments not only accumulate resistant organisms but also promotes evolution and dissemination of resistance under selective pressure. The release of this bacteria into natural water bodies can disrupt the microbial ecosystem and have an impact on the environment and public health [53-54]. Thus the focus on mixed colonies and potential co-existence is critical to assess the antimicrobial resistance dynamics and emergence of high risk pathogenic strains.

4.1 Limitation & Research Gaps

The study assesses the prevalence of the environmental existence and antimicrobial resistance trends of *Klebsiella pneumoniae* and *Acinetobacter baumannii* and presence of their potential co-existence. But this study could not fully validate the co-existence of the bacterial species due to methodological constraints. Mixed colonies imposed a major challenge in distinguishing the species individually. Besides, the laboratory experiment procedures were limited by standard laboratory conditions, which were not able to fully mimic the natural

environment of the organisms, affecting the bacterial growth and interaction approach. Another major constraint was the limitation in laboratory material, like primer availability, which imposed potential bias in molecular assays and limited the comprehensive analysis of species identification. Additionally, MALDI-TOF MS analysis failed to generate a definitive result for the mixed colony, and Budget constraints prevented the use of more sensitive techniques like single-cell sequencing, which could provide more accurate and reliable results and identify the co-existing species. Lastly, the inefficiency in isolating and characterizing the mixed bacterial colonies remains a gap in studying the dynamics and potential synergistic pathogenicity of the co-existing bacteria in the environment

4.2 Future Prospects

Despite these limitations and research gaps, this study provides a major framework to further analysis. The future studies should involve advanced high-throughput techniques to isolate and characterize mixed bacterial isolates accurately. Single-cell sorting methods like Fluorescence-activated cell sorting (FACS), Microfluidics-based separation or laser capture microdissection might isolate the individual bacterial cells from the mixed population. Besides, combining these methods with single-cell sequencing or metagenomic analysis can give a more precise identification of co-existing species. Additionally, assessing the sampling strategies with different wastewater sources and seasonal variation could improve the detection of these two bacterial species. Also, integration of phenotypic and genomic characterization to investigate the virulence factors and survival strategies of these bacteria can strengthen future research. Use of techniques like whole genome sequencing (WGS), and other bioinformatics pipelines can give a more advanced strain identification and distinguish between the strains. Furthermore, expanded AMR profiling with molecular detection of resistant genes can efficiently promote tracking of the spread of resistance in community

wastewater [55-57]. Lastly, these future studies can promote interventions to reduce the dissemination of AMR in urban cities like Dhaka.

4.3 Concluding Remarks

This study demonstrates the dominance and antimicrobial resistance of Klebsiella pneumoniae and Acinetobacter baumannii in community wastewater in Dhaka, promoting public health risk. Community wastewater was observed to be a significant reservoir for *Klebsiella pneumoniae* and *Acinetobacter baumannii,* and the increased resistance in mixed colonies showed enhanced AMR dynamics in the same environmental condition. The findings exhibit wastewater promoting the emergence and spread of high-risk resistant pathogens alongside the need for environmental AMR surveillance and a targeted intervention approach. This research highlights the challenges and complexities of identifying co-existing bacterial species in mixed colonies under standard laboratory conditions.

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Appendix A.

Supplementary Tables:

Supplementary Table S1: Multiple Antibiotic Resistance (MAR) Index of each *Klebsiella pneumoniae* isolate obtained from the community wastewater.

No of Antibiotics Resistant to (a)	Frequency of MAR isolates (n = 44)	No. Antibiotics tested (b)	MAR index (a/b)	Percentage of MAR
9	11	9	1	25
8	2	9	0.89	5
7	4	9	0.78	9
6	1	9	0.67	2
5	10	9	0.56	23
4	6	10	0.4	14
3	1	10	0.3	2
2	2	10	0.2	5
1	5	10	0.1	11
0	2	9	0	5

Supplementary Table S2: Multiple Antibiotic Resistance (MAR) Index of each *Acinetobacter baumannii* isolate obtained from the community wastewater.

No of Antibiotics Resistant to (a)	Frequency of MAR isolates (n = 44)	No. Antibiotics tested (b)	MAR index (a/b)	Percentage of MAR
10	0	10	1	0
9	0	10	0.9	0
8	6	10	0.8	15.38
7	20	10	0.7	51.28
6	6	10	0.6	15.38
5	7	10	0.5	17.95
4	0	10	0.4	0
3	0	10	0.3	0
2	0	10	0.2	0
1	0	10	0.1	0
0	0	10	0	0

Supplementary Table S3: Multiple Antibiotic Resistance (MAR) Index of each mixed colony obtained from the community wastewater.

No of Antibiotics Resistant to (a)	Frequency of MAR isolates (n = 44)	No. Antibiotics tested (b)	MAR index (a/b)	Percentage of MAR
10	9	10	1	90
9	1	10	0.9	10
8	0	10	0.8	0
7	0	10	0.7	0

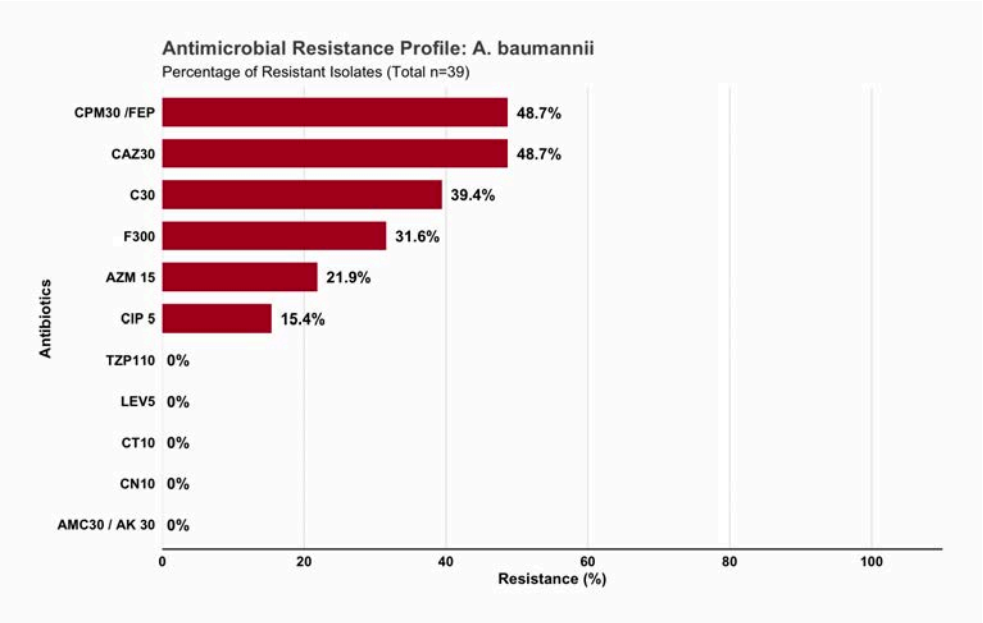
6	0	10	0.6	0
5	0	10	0.5	0
4	0	10	0.4	0
3	0	10	0.3	0
2	0	10	0.2	0
1	0	10	0.1	0
0	0	10	0	0

Supplementary Table S4: Tabular Representation of Areawise Bacterial Prevalence. *This table demonstrates the presence, distribution, and prevalence of Klebsiella pneumoniae and Acinetobacter baumannii in different areas of Dhaka city.*

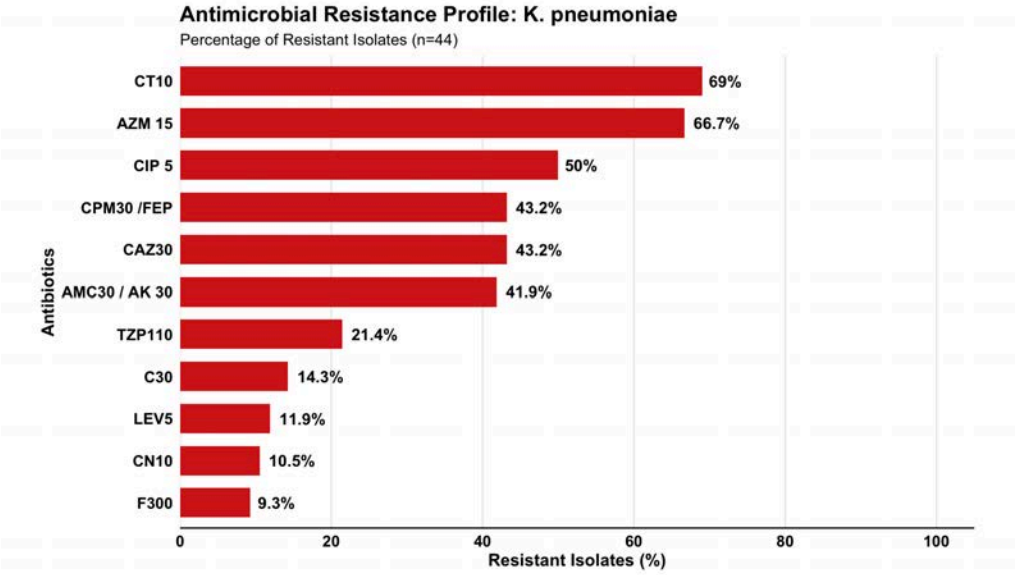
Area	**Total Samples**	**Total Isolates**	***Klebsiella pneumoniae* (+)**	***Acinetobacter baumannii* (+)**	**Average *Klebsiella pneumoniae* per sample**	**Average *Acinetobacter baumannii* per sample**	**Percentage of *Klebsiella pneumoniae***	**Percentage of *Acinetobacter baumannii***
Hatirjheel	3	33	3	2	1	0.67	9.1	6.1
Gudaraghat	3	27	3	2	1	0.67	11.1	7.4
Mohammadpur	6	71	10	11	1.67	1.83	14.1	15.5
Badda	3	40	5	6	1.67	2	12.5	15
Dhanmondi	3	34	7	4	2.33	1.33	20.6	11.8
Uttara	4	33	10	4	2.5	1	30.3	12.1
Gulshan	6	32	2	4	0.33	0.67	6.3	12.5
Banani	4	52	5	8	1.25	2	9.6	15.4
TOTAL	**32**	**322**	**45**	**41**	-			

Supplementary Figures:

Supplementary Figure S1: Results of the Kirby-Bauer Disk Diffusion Susceptibility Testing. *This figure shows multiple results of different Klecbsiella pneumoniae, Acinetobacter baumannii, and mixed colony isolates, which were subjected to Kirby-Bauer Disk Diffusion Susceptibility Testing to analyze the resistance profiles and identify the AMR burden.*

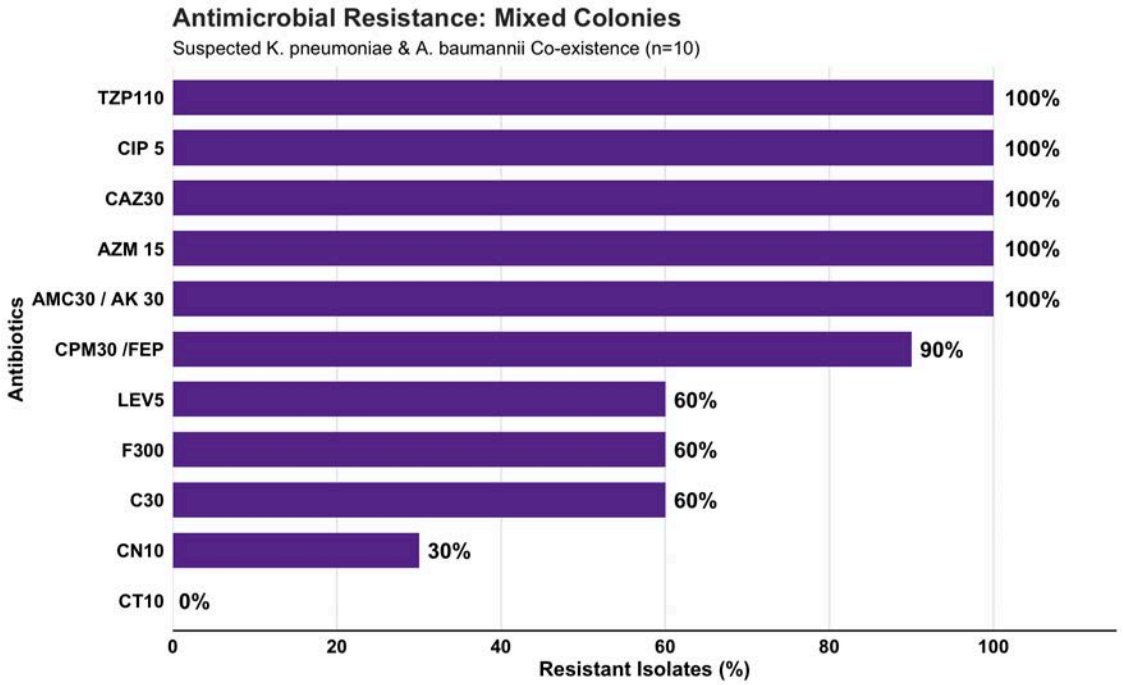


Supplementary Figure S2: Graphical Representation of the Antimicrobial Resistance Profile of *Acinetobacter baumannii*. This graph illustrates the resistance profiles and identifies the AMR burden. Here, the resistance percentages are shown in the x-axis, and the y-axis shows the antibiotics used.

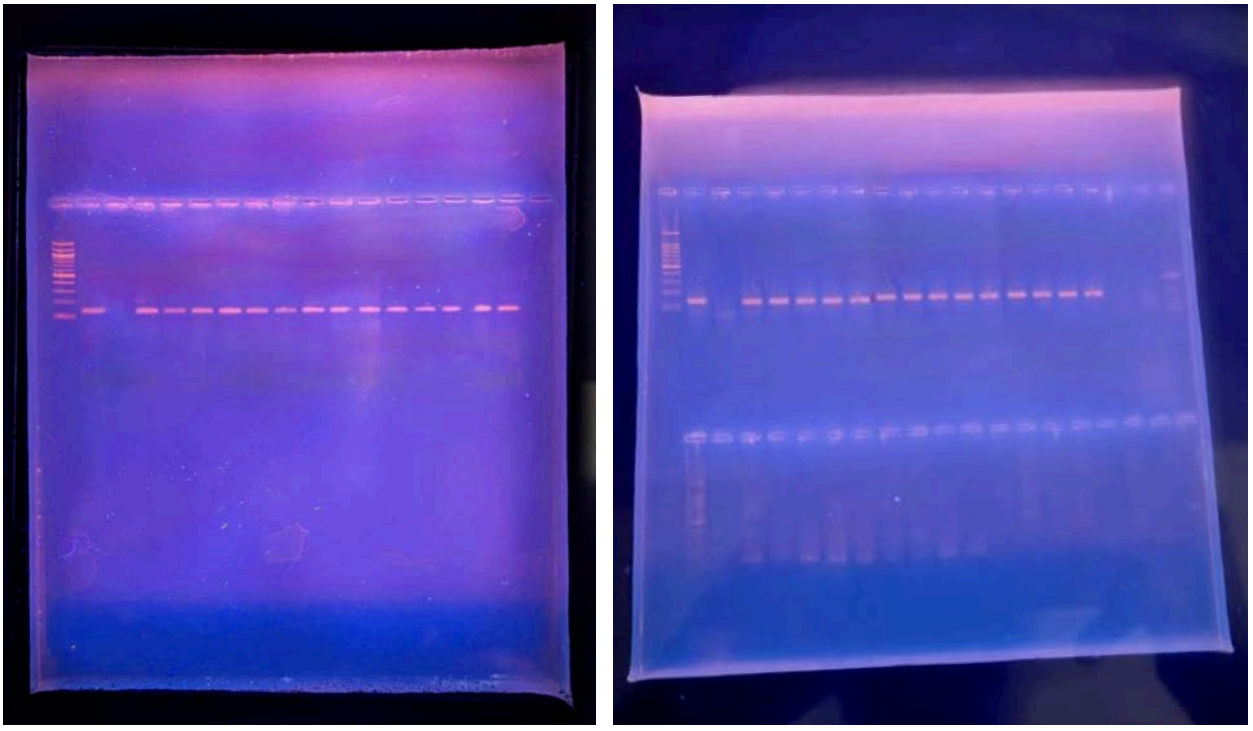


Supplementary Figure S3: Graphical Representation of the Antimicrobial Resistance Profile of *Klebsiella pneumoniae*. This graph illustrates the resistance profiles and identifies

the AMR burden. Here, the resistance percentages are shown in the x-axis, and the y-axis shows the antibiotics used.



Supplementary Figure S4: Graphical Representation of the Antimicrobial Resistance Profile of Mixed Colonies. *This graph illustrates the resistance profiles and identifies the AMR burden caused by the suspected co-existence of these two bacterial species in community wastewater. Here, the resistance percentages are shown on the x-axis, and the y-axis shows the antibiotics used.*



A B

Supplementary Figure S5: Raw Gel visualization of *Klebsiella pneumoniae* and *Acinetobacter baumannii* species identification via Polymerase Chain Reaction (PCR). *A) This gel image demonstrates the PCR results for Klebsiella pneumoniae positive isolates at 130bp. B) This gel image demonstrates the PCR results for Acinetobacter baumannii positive isolates at 353bp. Here, Lane 1 shows a 100bp DNA Ladder in A and a 1KB DNA Ladder in B, Lane 2 and 3 show positive and negative control, respectively. And, Lane 4-17 shows the results of isolates from different samples.*