Genomic Characterization of Environmental *Klebsiella pneumoniae* through Whole Genome Sequencing

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

Department of Mathematics and Natural Sciences
BRAC University
June 2024

Declaration

I hereby declare that:

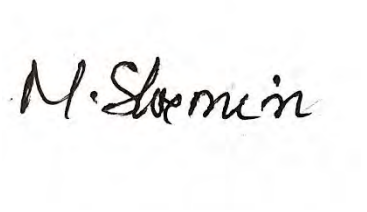
This thesis is my original work, completed as part of my degree at BRAC University.

It does not include material previously published or written by others, except where proper citations and references have been made.

It does not contain material accepted or submitted for any other degree or diploma at any university or institution.

All primary sources of assistance have been duly acknowledged.

Sincerely Yours,



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Approval

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Ethics Statement

I, Mehbooba Sharmin, a student of M.Sc. in the Department of Mathematics and Natural Sciences at BRAC University, hereby declare that the thesis titled "Genomic Characterization of Environmental *Klebsiella pneumoniae* through Whole Genome Sequencing" is an original and authentic record of my research work. This research was conducted under the joint supervision and guidance of Dr. Senjuti Saha, Deputy Executive Director of Child Health Research Foundation (CHRF), and Dr. Iftekhar Bin Naser, Associate Professor of Biotechnology in the Department of Mathematics and Natural Sciences at BRAC University, Dhaka, Bangladesh. I confirm that this work has not been submitted by me for any other degree.

Sincerely Yours,

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Abstract

Klebsiella pneumoniae poses a significant public health threat due to its high antibiotic resistance and its role in severe infections, especially in healthcare settings. Investigating environmental *K. pneumoniae* is crucial for understanding reservoirs and transmission of resistance and virulence genes. However, environmental data on *K. pneumoniae* is scarce in Bangladesh and globally.

This study aimed to characterize environmental *K. pneumoniae* using antimicrobial susceptibility testing (AST) and whole genome sequencing (WGS). From seven thanas across Dhaka City, 140 wastewater samples were collected. *Klebsiella spp.* were detected using biochemical tests and morphological characteristics. AST was then performed to determine their phenotypic resistance profiles against seven antibiotics. Based on the AST results and morphological characteristics, 14 isolates were selected for Sanger sequencing using a novel primer targeting the *rpoB* gene, designed to detect *K. pneumoniae*. From these, three confirmed *K. pneumoniae* isolates were selected for WGS to enable genomic characterization. A phylogenetic tree was generated using the WGS data in comparison with nine clinical isolates of *K. pneumoniae*.

The AST results for environmental *Klebsiella spp*. revealed high susceptibility to most antibiotics tested, unlike clinical isolates. However, whole genome sequencing identified resistance genes that were not consistent with the phenotypic AST results. The analysis also showed an absence of common virulence genes typically found in clinical isolates. The phylogenetic tree indicated that the three environmental *K. pneumoniae* isolates formed separate nodes compared to clinical isolates.

These findings highlight the unique genomic features of environmental *K. pneumoniae* compared to their clinical counterparts, emphasizing the importance of environmental monitoring to understand the dynamics of antibiotic resistance and virulence in this pathogen.

Keywords: *Klebsiella pneumoniae*, Antimicrobial Susceptibility Testing (AST), Whole Genome Sequencing (WGS), Sanger sequencing

Acknowledgement

My favorite Bollywood hero, Shahrukh Khan, once said in one of his movies, "If you want something with all your heart, the entire universe conspires to help you achieve it." This past year has been a testament to that quote for me. As a trainee at Child Health Research Foundation (CHRF), I aspired to learn, grow, and accomplish something truly my own. Indeed, the universe conspired to help me achieve it.

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1. Introduction

Klebsiella pneumoniae (K. pneumoniae) is a common gram-negative, encapsulated, non-motile bacterium that is ubiquitously found in nature including in plants, animals and humans and environmental sources such as water, soil etc. (Podschun *et al*., 2001). It is a member of the gram-negative ESKAPE pathogens that are declared as the most threatening members of the gram-negative clan as they confer the ability to be emerged as the most resistant members to all or most available antibiotics and are mainly nosocomial pathogens yet can infect healthy individuals as well which is known as community acquired disease (Wyres & Holt, 2018).

Figure 1.1**:** Electro microscopic picture of *Klebsiella pneumoniae*
Source: Centers for Disease Control and Prevention (CDC)

Only a few studies have been conducted on environmental *K. pneumoniae* in comparison to the many studies on isolates linked to clinical illnesses. According to some investigations, *K. pneumoniae* from the environment has many morphological and genetic traits similar to clinical isolates (Podschun & Ullmann, 1993), but other studies have found that the two groups differ in terms of virulence. Non-clinical habitats have been thought of as potential reservoirs for hyper-virulent and hyper-resistant *K. pneumoniae* due to the parallel evolution of the pathogen and putative acquisition of antimicrobial resistance determinants and virulence factors in healthcare

settings and the environment (Runcharoen et al., 2017), though there is conflicting evidence to support the potential clinical importance of non-clinical *K. pneumoniae*.

An experiment can be conducted to analyze the genetic relatedness, antimicrobial resistance, and virulence genes in *K. pneumoniae* from environmental water using whole genome sequencing. Through a comparison with isolates found in diverse clinical and non-clinical sources around the world, these genomes can be placed in a global perspective (Holt et al., 2015). The evolution and importance of environmental *K. pneumoniae* is poorly understood, despite increased information about the highly diverse population of *K. pneumoniae*. This analysis will help to fill the knowledge gaps related to environmental *K. pneumoniae.*

With advancements in sequencing technologies, there is a growing imperative to extend genomic investigations beyond clinical bacteria to include their environmental counterparts. This shift recognizes the need to comprehend the complete genomic landscape of bacteria like *K. pneumoniae,* considering their presence in diverse ecological niches, particularly wastewater. This approach aligns with the broader goal of utilizing technological progress to explore microbial genomics in diverse contexts, fostering a more comprehensive and integrated perspective in microbial research.

K. pneumoniae, recognized for its clinical significance, has increasingly been identified in wastewater, raising questions about its genomic attributes in this complex and dynamic environment. This study delves into the genetic makeup of environmental *K. pneumoniae* strains derived specifically from wastewater sources.

While wastewater is acknowledged as a potential reservoir for *K. pneumoniae*, a critical gap exists in our understanding of how the genomic characteristics of these strains influence their persistence, adaptation, and potential impact on public health. This research addresses this gap by conducting a comprehensive genomic analysis of *K. pneumoniae* strains isolated from wastewater.

Background of the study

At present, *K. pneumoniae* pneumonia stands as the predominant cause of pneumonia acquired in U.S. hospitals, contributing to 3% to 8% of total nosocomial bacterial infections (Ashurst & Dawson, 2024). It is declared as an urgent threat to human health by the World Health Organization (WHO), the US Centers for Disease Control and Prevention (CDC), and the Public Health England (PHE) (Aminul *et al*., 2021).

South and Southeast Asia are major hubs for antimicrobial-resistant (AMR) *K. pneumoniae*. In a recent study in Bangladesh, it was observed that majority of the isolated *K. pneumoniae* from clinical samples were multidrug resistant (MDR) which ignited the need for further surveillance and AMR profiling of isolates derived both from clinical and environmental sources to understand the actual scenario and to establish proper healthcare management (Aminul *et al*., 2021). Moreover, the intricate dynamics involved in the transfer of genes, plasmids, and bacteria responsible for acquiring and disseminating antimicrobial resistance (AMR) genes from environmental microbes to human-associated bacterial pathogens present a challenging landscape for accurate reconstruction. Despite the intricacies, noteworthy cases such as the mobilization of AMR genes, as seen with qnrA and *OXA-48*, originating from marine bacteria and traversing to *K. pneumoniae* and subsequently to other ESKAPE pathogens, serve as compelling illustrations. Although the precise routes of horizontal gene transfer (HGT) remain elusive, the prominence of *K. pneumoniae* in early clinical reports featuring emerging AMR genes emphasizes its pivotal role (Wyres & Holt, 2018). This underscores the significance of *K. pneumoniae* as a primary candidate for watchful surveillance, aimed at monitoring the introduction of novel AMR genes into populations of Gram-negative pathogens.

Objectives of this study

The overarching objectives of this dissertation are to elucidate the genomic diversity, assess potential virulence factors and antimicrobial resistance profiles of *K. pneumoniae* in wastewater collected from 7 Thanas across Dhaka city. The research questions guiding this inquiry include:

Objective 1: Detection of *Klebsiella pneumoniae* species complex in the wastewater.
Objective 2: Determination of AST and virulence factors of environmental *Klebsiella pneumoniae.*
Objective 3: Development of a genetic sequencing method to distinguish *Klebsiella pneumoniae* from other species of the complex.
Objective 4: Whole genome sequencing and comparison of environmental *Klebsiella pneumoniae* and clinical *Klebsiella pneumoniae.*

1.1 *Klebsiella pneumoniae*: The Pathogen

1.1.1 Historical background

K. pneumoniae was first found in the late 1800s by scientists Edwin Klebs and Friedrich Loeffler. They discovered it while studying infectious diseases and named it *Klebsiella* to honor Klebs. This bacterium was linked to respiratory infections, especially pneumonia, in its early observations. The finding happened during a time when scientists were learning a lot about bacteria and how they cause diseases. This period set the stage for understanding how infections work. So, the discovery of *K. pneumoniae* is a crucial part of the history of studying tiny living things that can affect human health. (Ashurst & Dawson, 2024)

1.1.2 Taxonomy

Taxonomy of *Klebsiella pneumoniae:*
(Dong et al., 2022)

Domain: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Enterobacterales
Family: Enterobacteriaceae
Genus: *Klebsiella*
Species: *pneumoniae*

1.1.3 *Klebsiella pneumoniae* species complex:

Members of the *Klebsiella pneumoniae* species complex (KpSC) were initially identified based on *gyrA* sequences and categorized as phylogroups of *K. pneumoniae*. Subsequent whole-genome sequencing (WGS) confirmed the distinction between species, revealing genome-wide average nucleotide identity of more than 3%, enough to establish new species. The identified species include *Klebsiella pneumoniae* (Kp1), *Klebsiella quasipneumoniae subsp. quasipneumoniae* (Kp2), *Klebsiella quasipneumoniae subsp. similipneumoniae* (Kp4), *Klebsiella variicola subsp.*

variicola (Kp3), *Klebsiella variicola subsp. tropica* (Kp5), *Klebsiella quasivariicola* (Kp6), and *Klebsiella africana* (Kp7).

The specific ecological niches of these species are not yet fully understood, but evidence suggests that *Klebsiella. variicola* is associated with plants, often carrying a nitrogen-fixing operon and cellulases absent in other species. All species have been found in the human gut, with *Klebsiella variicola subsp. tropica* being the exception. Notably, *Klebsiella variicola* and *Klebsiella quasipneumoniae* are common causes of nosocomial infections, occasionally leading to outbreaks. They can also acquire antimicrobial resistance genes from *K. pneumoniae* and have been implicated in community-acquired liver abscesses in humans and isolated from animals. (Wyres et al., 2020)

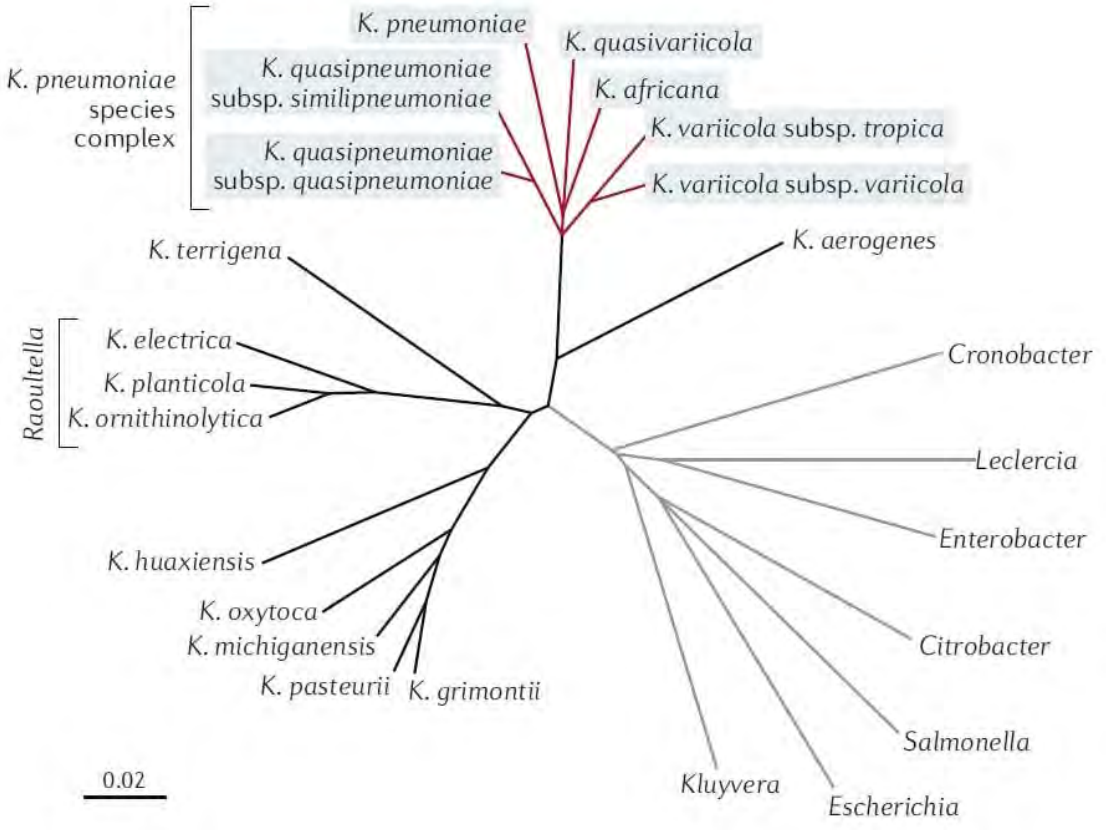

Figure 1.2: Taxonomic position of *Klebsiella pneumoniae*
(Wyres et al., 2020)

1.1.4 Commensalism to Pathogenicity:

The relationship between *K. pneumoniae* and human hosts is intricate and dynamic, as *K. pneumoniae* can function as a commensal, opportunistic pathogen, or pathogen. Commensal colonization in the gut and respiratory tract is widespread, with prevalence estimates varying by age group, geographic location, and recent healthcare exposure. Studies from the USA and Australia suggest gut colonization rates of approximately 4–6% in the community, rising to about 25% among individuals with recent healthcare exposure. Notably, higher community carriage rates for KpSC (ranging from 18 to 87%) have been reported for healthy adults in Korea, Japan, Singapore, Taiwan, and Malaysia. The duration of gut colonization is not fully understood but can extend beyond 12 months. The majority of global *K. pneumoniae* infections are opportunistic healthcare-associated infections (HAIs), often referred to as 'classical' *K. pneumoniae* infections. Of significant concern is the emergence of multidrug-resistant (MDR) and, especially, carbapenem-resistant *K. pneumoniae* (CRKp), with estimated mortality associated with CRKp HAI being higher compared to carbapenem-susceptible strains. Limited therapeutic options for MDR and CRKp infections have led to a resurgence in colistin use, despite rising resistance. Additionally, there is renewed interest in vaccines and other preventive measures against *K. pneumoniae*. Beyond healthcare settings, *K. pneumoniae* can act as a 'true' pathogen, causing severe community-acquired infections (CAIs) in otherwise healthy patients without the risk factors for HAIs (Wyres et al., 2020).

1.1.5 Molecular Pathogenesis:

K. pneumoniae relies on a range of gene products to evade the host's innate immune mechanisms (Paczosa & Mecsas, 2016). The pathogenicity of *K. pneumoniae* is associated with various virulence factors, including four extensively studied categories: capsule, siderophores, lipopolysaccharide (LPS), and fimbriae. Additionally, there are other factors, such as outer membrane proteins (OMPs), porins, efflux pumps, and transporters, that contribute to its virulence, although they are not yet fully characterized. (Ramirez et al., 2014) (Davies et al., 2016)

The capsule of *K. pneumoniae* serves as a dense polysaccharide matrix, forming the outermost layer and evading the host's immune response by hindering phagocytosis, complement-mediated killing, and suppressing the expression of human beta-defensins (Paczosa & Mecsas, 2016).

Capsules, classified as strain-specific antigens (K antigens), exhibit biochemical complexity, with at least 78 identified capsular serotypes (Clegg & Murphy, 2016).

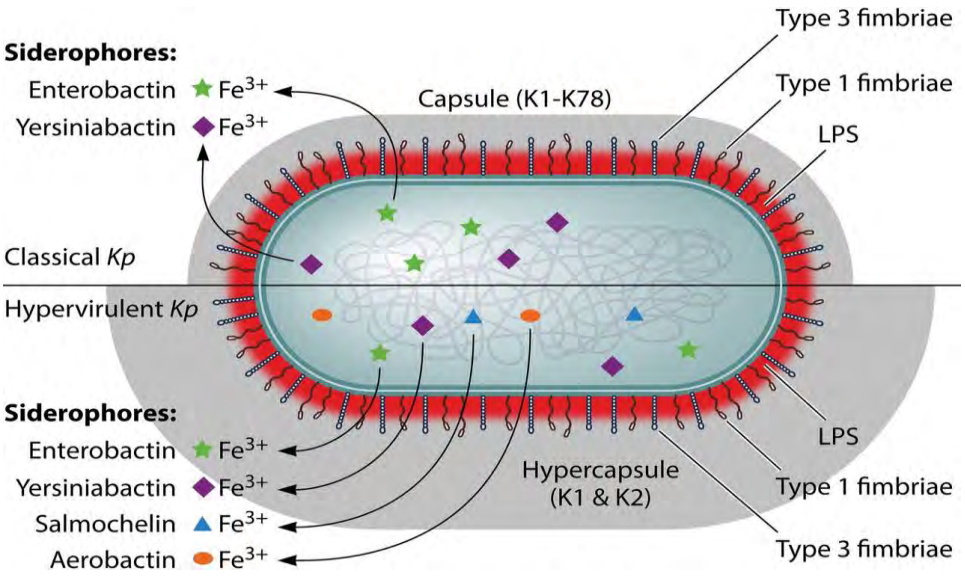

Figure 1.3: Well-characterized virulence factors in classical and hypervirulent *K. pneumoniae* (Paczosa & Mecsas, 2016)

The *cps gene cluster*, responsible for capsule production, encompasses various genes, including *wzi*, *wza*, *wzb*, *wzc*, *gnd*, *galF*, and *orf2*, along with strain-specific genes encoding distinct glycosyltransferases (Paczosa & Mecsas, 2016). Increased capsule production is linked to the mucoviscous phenotype of hypervirulent strains, regulated by genes such as *rmpA*, *rmpA2*, *rcsA*, and *rcsB* (Clegg & Murphy, 2016). Fimbriae, elongated surface appendages carrying adhesins, play a crucial role in biofilm formation and bacterial attachment to host and abiotic surfaces (Witkowska et al., 2005). Siderophores, including enterobactin, yersiniabactin, salmochelin, and aerobactin, facilitate iron acquisition, crucial for bacterial survival (Pal & Gokarn, n.d.). Lipopolysaccharides (LPSs), indispensable components of the outer membrane, protect against the host's complement system and play a crucial role in inflammation activation (Paczosa & Mecsas, 2016). Other virulence factors in *K. pneumoniae* include outer membrane proteins (OMPs), porins, efflux pumps, ABC iron transport systems, and operons involved in allantoin metabolism (Ratajczak, 2005).

1.1.6 Antimicrobial Resistance in *Klebsiella pneumoniae:*

The widespread utilization of antibiotics in clinical settings has led to a global crisis of antimicrobial resistance in modern medicine (*Antimicrobial Resistance*, n.d.). *K. pneumoniae*, categorized among the ESKAPE organisms *(Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*), represents a major nosocomial pathogen with a high resistance rate (Santajit & Indrawattana, 2016) (Navon-Venezia et al., 2017). Bacterial resistance mechanisms involve alterations in cell permeability, biofilm formation for mechanical protection, degradation or modification of antimicrobial agents, and target modification to prevent drug binding (Gorrie, 2018). Antimicrobial resistance can be intrinsic or acquired. Intrinsic resistance arises from inherent characteristics not acquired through exposure or horizontal gene transfer (Olivares Pacheco et al., 2013). Chromosomally encoded elements causing intrinsic resistance in *Klebsiella spp*. include different β-lactamases, such as *SHV* in *K. pneumoniae* (KpI), *OKP* in *K. quasipneumoniae* (KpII), and *LEN* in *K. variicola* (KpIII) (Bouza & Cercenado, 2002) . Additionally, *Klebsiella* strains can acquire drug resistance through mutations and horizontal gene transfer, primarily mediated by mechanisms like transduction, transformation, and conjugation (Barlow, 2009). Mobile genetic elements, including plasmids, integrative conjugative elements, integrons, insertion sequences, and transposons, play a crucial role in transferring acquired antimicrobial resistance genes among bacteria. More than 100 diverse acquired antimicrobial resistance genes have been identified in *K. pneumoniae*, contributing to resistance against various classes of antibiotics. The terms multidrug resistance (MDR), extensive drug resistance (XDR), and pandrug resistance (PDR) are used to categorize the non-susceptibility level of isolates to antimicrobial agents (Magiorakos et al., 2012). Carbapenemase production, particularly *KPC* (*K. pneumoniae* carbapenemase), is a significant clinical concern. Tigecycline and polymyxins are last-resort antibiotics for extensively drug-resistant, carbapenemase-producing *K. pneumoniae*, but resistance to these drugs has also been reported (Rodríguez-Baño et al., 2018). Tigecycline resistance is often mediated by mutations in chromosomal genes, while polymyxin resistance arises through mutations in two-component regulatory systems (Hentschke et al., 2010). The emergence of plasmid-borne *mcr-1* gene variants further contributes to colistin resistance (Kluytmans, 2017). Despite global reports of carbapenem resistance and other last-resort antibiotics in *K. pneumoniae*, Bangladesh lacks data to determine

the current state or burden of this resistance. Therefore, generating such data is crucial for understanding and addressing the issue locally.

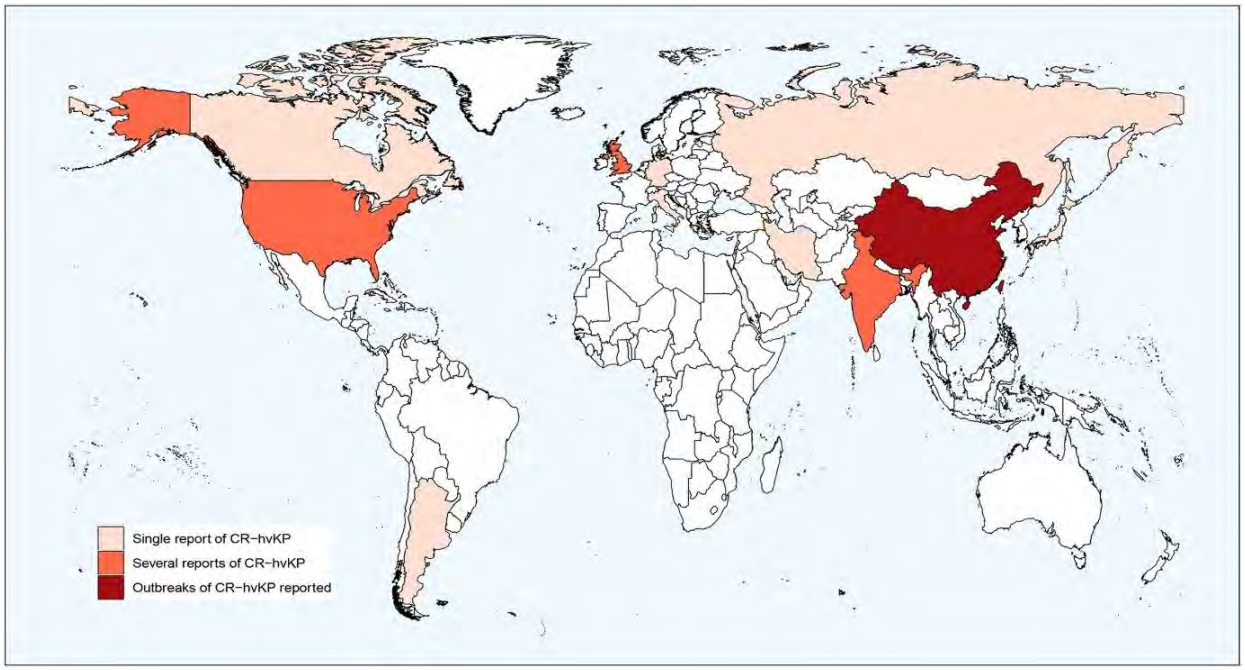

Figure 1.4: Global Burden of Carbapenem Resistant Hypervirulent *Klebsiella pneumoniae* (Lan et al., 2021)

1.1.7 Environmental Presence:

K. pneumoniae extends beyond healthcare settings and hospitals, establishing a ubiquitous presence in diverse environments. These encompass natural sources like soil and wastewater, as well as mucosal surfaces, the human and animal gut (Bagley, 1985). While environmental *K. pneumoniae* has received less scrutiny compared to clinical isolates, some studies indicate significant similarities in phenotypic and certain genetic features between the two groups, while others note distinctions in virulence characteristics. The co-evolution of *K. pneumoniae* in healthcare settings and the environment, marked by the potential acquisition of antimicrobial resistance and virulence factors, raises the possibility of non-clinical habitats serving as reservoirs for hyper-virulent and hyper-resistant strains. Despite inconclusive evidence regarding the clinical significance of non-clinical *K. pneumoniae,* recent research on meat-source isolates revealed differences in antibiotic resistance but similar virulence characteristics compared to those causing urinary tract infections in humans. These investigations contribute essential insights into the broad

ecological distribution of *K. pneumoniae,* emphasizing its adaptability beyond healthcare contexts. Recognizing its presence in non-clinical settings underscores the necessity for comprehensive surveillance and management strategies to mitigate potential health risks associated with this bacterium. The collective body of research plays a crucial role in enhancing our understanding of the intricate dynamics of *K. pneumoniae* across various environmental niches (Runcharoen et al., 2017).

1.1.8 Should Genomic Focus Shift from Clinics to Environments?

The shift from primarily clinical-focused studies to a broader exploration of environmental genomics marks a significant evolution in microbiological research. Traditionally, the emphasis has been on understanding bacteria within the context of human health and diseases. However, with advancements in technology and a growing recognition of the ecological impact of microbes, there's a rising interest in studying bacteria in their natural environments (Struve & Krogfelt, 2004).

This transition is driven by the realization that bacteria, like *K. pneumoniae,* exist not only within clinical settings but also in diverse ecological niches, including soil, water, and plants. Exploring the genomic characteristics of bacteria in these environments provides valuable insights into their adaptation strategies, evolutionary patterns, and potential roles in broader ecosystems.

By broadening the scope of environmental genomics, researchers aim to unravel the complexities of bacterial life beyond the confines of clinical contexts. This paradigm shift allows for a more comprehensive understanding of microbial diversity, ecological interactions, and the potential implications for human health and the environment.

In essence, transitioning from clinical-centric studies to environmental genomics represents a holistic approach that considers the broader ecological context of bacteria, recognizing their multifaceted roles in both clinical and environmental settings (Runcharoen et al., 2017) (Amirsoleimani et al., 2019) (Delgado-Blas et al., 2021).

1.1.9 Wastewater as a Distinct Ecological Niche:

Wastewater, often underestimated as a mere byproduct of human activities, stands as a distinct and intricate ecological niche teeming with microbial life. This complex environment serves as a dynamic arena where microorganisms engage in a constant interplay, adapting to a diverse range

of physicochemical conditions and selective pressures. From nutrient-rich substrates to the presence of antibiotics and human-associated microbes, wastewater encapsulates a unique ecosystem, offering valuable insights into the adaptability and evolution of microbial communities. Recognizing wastewater as a distinct ecological niche unveils its significance not only in environmental contexts but also in understanding microbial responses to various stressors and anthropogenic influences (Chahal et al., 2016) (Wu et al., 2019).

Wastewater constitutes a dynamic ecosystem hosting a diverse array of microorganisms, encompassing bacteria, viruses, fungi, and protozoa, shaped by inputs from various sources. This microbial community thrives in a nutrient-rich environment, abundant in organic and inorganic substances, fostering microbial growth. The physicochemical conditions of wastewater undergo fluctuations, including variations in temperature, pH, and the presence of chemical compounds, to which microorganisms adapt, showcasing resilience to environmental stressors. Notably, wastewater often carries residues of antibiotics, pharmaceuticals, and chemicals, exerting selective pressures on microbial communities and influencing the prevalence of antibiotic-resistant strains, potentially leading to the development of multidrug resistance. Furthermore, inputs from domestic, industrial, and healthcare sources introduce human-associated microbes, including pathogenic bacteria of clinical significance, posing challenges for public health management. The high microbial density in wastewater creates opportunities for horizontal gene transfer, facilitating the exchange of genetic material, including antibiotic resistance genes, and contributing to the evolution of microbial communities (Chahal et al., 2016) (Wu et al., 2019) (Sun et al., 2023).

1.1.9.1 The role of *Klebsiella pneumoniae* in wastewater ecosystems:

Having explored the complex world of wastewater, filled with many different microorganisms and shaped by various environmental factors, our attention now turns to a particular organism– *K. pneumoniae.* Known for its involvement in human infections, this bacterium's presence in wastewater ecosystems invites us to understand its adaptive strategies, ecological importance, and potential consequences. Transitioning from the broader context of wastewater as a unique ecological space and its involvement in causing infections in humans with high drug – resistant pattern, we delve into a focused investigation of what *K. pneumoniae* in the wastewater looks like. Bacteria, such as *Klebsiella pneumoniae,* are exposed to diverse conditions in both infections and the environment, potentially resulting in the acquisition or loss of specific traits. Past studies,

focusing on the phenotypic and genotypic characterizations of these bacteria, particularly in the context of antibiotic resistance, have provided valuable insights. These investigations categorized isolates based on traits like resistance to tetracycline, meropenem, sulfonamides, and amoxicillin with clavulanic acid, as well as their capacity for forming biofilms and transferring antibiotic resistance genes.

Notably, the comparative analyses from these studies revealed that the origin of the isolates (clinical or environmental) did not dictate their grouping. Instead, specific traits, such as biofilm formation and the ability to transfer genes, were observed to be more frequent in clinical or wastewater isolates, indicating potential habitat adaptation. For instance, the capacity for conjugation with other bacteria or biofilm formation may confer advantages in different environments.

Genomic analyses conducted in the past identified the presence of genes associated with efflux, oxidative stress, and quorum sensing in all isolates. Importantly, certain virulence genes were linked to integrative and conjugative elements, suggesting potential acquisition paths associated with genetic lineages rather than isolation habitats.

Furthermore, these past studies investigated the prevalence of antibiotic-resistant Gram-negative rods (GNR), with a specific focus on *K. pneumoniae*. The majority of *K. pneumoniae* isolates exhibited resistance to various antibiotics, with a notable presence of carbapenemase genes. The studies also highlighted the occurrence of metal resistance genes flanked by insertion sequences, indicating potential mobilization (Wang et al., 2018).

In conclusion, the findings from these past studies underscore that the habitat of isolation, whether clinical or wastewater, did not distinctly influence the features of *K. pneumoniae* isolates. Instead, genetic lineage appeared to play a more crucial role in determining traits and resistance patterns. The presence of clinically significant pathogens in wastewater, as revealed by these past studies, emphasizes the importance of understanding the characteristics of bacteria like *K. pneumoniae* in environmental settings, contributing valuable insights to the broader issue of antimicrobial resistance.

1.1.9.2 *Klebsiella pneumoniae* in Bangladesh: Wastewater Insights

Having explored the role of *K. pneumoniae* globally, let's zoom in on Bangladesh. Here, we shift our focus to understand how *K. pneumoniae* behaves in the wastewater of this country. It's like

turning the spotlight onto what's happening specifically with *K. pneumoniae* in Bangladesh's water systems. Let's dive into this closer examination.

Globally, there is a scarcity of data on environmental *K. pneumoniae* compared to its clinical counterpart. However, in the specific context of Bangladesh, the available data on environmental *K. pneumoniae* is even more limited.

Globally, antibiotic resistance poses a significant threat, particularly in developing countries like Bangladesh (Hart & Kariuki, 1998). A study published in 2021 by (Rahman et al., 2021), focused on antibiotic-resistant bacteria in wastewater revealed noteworthy findings related to *K. pneumoniae* In Noakhali, Bangladesh. The study emphasized that untreated hospital wastewater is a substantial contributor to the emergence of multi-drug-resistant bacteria. *K. pneumoniae*, among other Gram-negative bacteria, exhibited resistance to various antibiotics, including β-lactams. The prevalence of resistance traits in *K. pneumoniae* highlighted its adaptability in wastewater environments. Additionally, the presence of antibiotic resistance genes, such as carbapenemase genes, underscored the potential for the spread of resistance. Moreover, *K. pneumoniae* displayed resistance to specific antibiotics like ampicillin, ciprofloxacin, and other classes. The genetic diversity within *K. pneumoniae* isolates in wastewater environments was evident, emphasizing the complexity of interactions in this ecosystem. The study's findings shed light on the ecological implications of *K. pneumoniae* in wastewater, emphasizing its role as a potential reservoir for pathogenic strains (Moges et al., 2014). The adaptability and persistence of clinically relevant traits in wastewater highlight the challenges associated with managing antibiotic resistance in environmental settings. These observations underscore the need for continued surveillance and a comprehensive understanding of *K. pneumoniae* dynamics in wastewater environments (Rabbani et al., 2017) (Rahman et al., 2021).

Another study where samples were taken from four sites, two from each Dhaka Medical College Hospital (DMCH) and Bangabandhu Sheikh Mujib Medical University (BSMMU). One site at each hospital directly received hospital and human waste (DMCH 1 and BSMMU 1), while the other site was situated away from the hospital or human waste disposal point (DMCH 2 and BSMMU 2). At each of the four sites, one to three separate sewage water samples were collected. In the conducted study on the escalating issue of drug resistance within Gram-negative organisms, particularly focusing on *K. pneumoniae*, substantial public health threats were revealed. The pivotal role of untreated hospital waste disposal in fostering antibiotic resistance among

environmental isolates was underscored, emphasizing the potential transition from environmental reservoirs to clinical manifestations in humans. Notably, resistance to 21 out of 25 tested antibiotics was observed in a *K. pneumoniae* isolate, indicating a significant reservoir for the transfer of resistant genes. Varying susceptibility patterns among *K. pneumoniae* isolates were observed, and a positive correlation between resistance development in associated sewage and hospital waste disposal was identified. Urgent preventive measures were emphasized to halt the disposal of antibiotic-containing waste into the environment, aiming to mitigate the development of multidrug resistance in hospital sewage. These findings are expected to serve as a foundational basis for further scientific investigations into the prevalence of emerging multidrug-resistant *K. pneumoniae* in environmental settings and its potential implications for public health (Rabbani et al., 2017).

The limited availability of clinical data on this specific pathogen in Bangladesh, coupled with the scarcity of environmental data, underscores the challenges in obtaining a comprehensive understanding. Moreover, existing studies conducted in Bangladesh often focus on specific areas, restricting the insights gained to localized contexts rather than providing a holistic view of the pathogen's prevalence and genomic characteristics across the entire country. While some studies have been conducted in localized areas, it's crucial to note that these often concentrate solely on clinical wastewater. To comprehensively understand the characteristics of environmental *K. pneumoniae*, it becomes imperative to broaden the scope of investigation. Exploring both affected and unaffected sources beyond clinical waste is essential for obtaining a detailed understanding of the pathogen's genomic profile in various environmental settings .

2. Methodology

Overview

This chapter outlines the methodological strategies utilized in this dissertation, encompassing comprehensive research design, meticulous sample selection and processing procedures, as well as the methodologies employed for data generation and subsequent analysis. The methodologies described here play a crucial role in fulfilling the objectives outlined in the research questions.

The 140 sewage water samples collected from 7 “thanas” across Dhaka city utilized in this study were derived from a separate project entitled "Bacteriophage and the Spread of Drug-Resistant *Salmonella* Typhi”. Individual colonies of *Klebsiella spp.* were isolated and identified using established methods such as culture and biochemical tests to ensure accuracy. Subsequently, these *Klebsiella spp.* strains underwent antibiotic susceptibility testing (AST) to assess their resistance patterns, shedding light on their potential impact on public health. A cost-effective approach was adopted, selecting representative antibiotics from each class for testing. Based on the results of the antibiotic tests, specific isolates with unique characteristics were carefully selected for further investigation, thus narrowing down the focus to key isolates requiring additional exploration. Furthermore, a novel primer was designed for Sanger sequencing to enable species-level identification of *K. pneumoniae,* which was followed by Sanger sequencing to confirm the identification. Confirmed *K. pneumoniae* underwent whole genome sequencing and computational analysis to explore genes, differences, and potential factors contributing to their morphological characteristics. The outcomes using this methodology will also generate data that will give an insight into both phenotypical characteristics of AST results and their corresponding genotypical characteristics of harboring AMR genes for environmental *K. pneumoniae.*

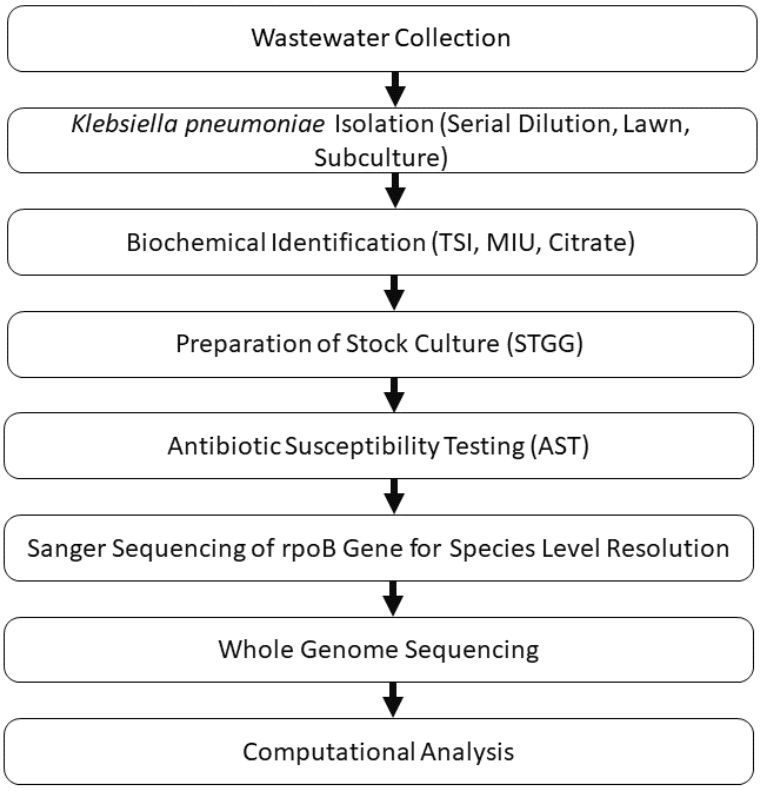

Figure 2.1: Flowchart of Methodological Procedures

2.1 Materials & Methods

2.1.1 Utilization of Previous Project Samples

The samples utilized in this study were collected as part of an ongoing environmental surveillance project conducted by the Child Health Research Foundation, focusing on bacteriophages and the spread of drug-resistant *Salmonella* Typhi. The overarching aim of this project is to investigate the influence of environmental niches on the evolution of antibiotic resistance in *S.* Typhi and to reduce the incidence of enteric disease-related deaths in children. Specifically, 140 sewage samples from 7 thanas across Dhaka city were analyzed for research purposes. The seven thanas are: Adabor, Kafrul, Pallabi, Mirpur, Mohammadpur, Sher-E-Bangla and Shah Ali.

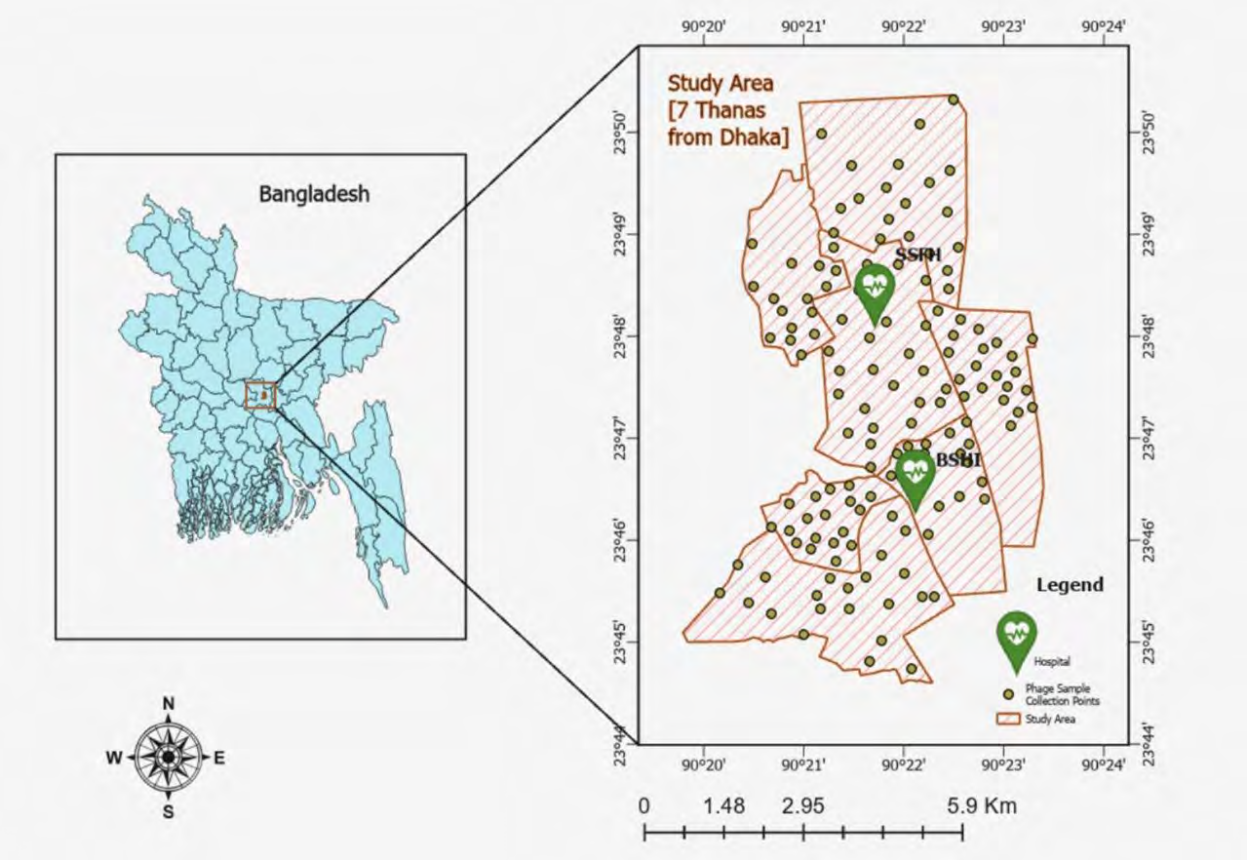

Figure 2.2: Sampling Points across Dhaka City

2.2 Sample Processing

2.2.1 Pretreatment before Lawning

Following the collection of samples, a centrifugation step was executed at 1000 rpm for 1 minute. This process effectively separated components within the samples, generating a supernatant. The supernatant, containing the desired elements, was carefully transferred to a fresh tube. Following centrifugation, a dilution series was meticulously prepared, extending up to 10^{-1}, 10^{-2} and 10^{-3}. This was achieved by combining 100µL of the centrifuged sample with 900µL of 0.9% sodium chloride solution (normal saline) in each dilution step. The purpose of this dilution series was to reduce the concentration of the sample, facilitating precise analyses and ensuring that subsequent procedures were conducted with appropriately calibrated samples.

2.2.2 Media Preparation (MacConkey Agar)

The MacConkey agar media utilized in this experiment were prepared in advance to ensure consistency and sterility. Precise quantities of MacConkey agar powder were measured and mixed with distilled water following established laboratory protocols. The resulting agar solution underwent thorough autoclaving to eliminate potential contaminants and achieve a sterile medium. Subsequently, the liquefied agar was carefully poured into sterile Petri dishes, creating solid agar plates that were left undisturbed to solidify. These pre-prepared MacConkey agar plates will undergo comprehensive quality control measures to validate their functionality and sterility before being distributed and made ready for subsequent experimental procedures. Quality control procedures were implemented to verify the functionality and sterility of the prepared MacConkey agar plates. ATCC strains of *Streptococcus pneumoniae, Haemophilus influenzae, Escherichia coli*, and *Staphylococcus aureus* were streaked onto separate MacConkey agar plates to assess the media's performance. Blank MacConkey agar plates were incubated overnight to check for any unintended microbial growth, ensuring the plates' integrity and absence of contaminants.

2.2.3 Inoculation of MacConkey Agar Plates (Lawning)

Using a sterile spreader, 100 µL from the 10^{-2} and 10^{-3} dilution of environmental samples was evenly spread over the surface of separate MacConkey agar plate, ensuring thorough coverage. The spreader was carefully dipped in ethanol after each inoculation to maintain sterility and prevent cross-contamination between samples. Plates were left undisturbed for a short period to allow absorption of the inoculum into the agar.

In a separate MacConkey agar plate, only saline was evenly spread using a sterile spreader to ensure the quality and sterility of the saline solution. Another control plate was dedicated to spreading only the spreader on the agar surface to verify the sterility of the spreading tool.

All plates, including inoculated samples and controls, were incubated overnight at the appropriate temperature to encourage microbial growth. After incubation, colonies on each plate were counted, and images were captured for documentation.

Three colonies with characteristics indicative of *K. pneumoniae* (e.g., mucoid appearance, pink color on MacConkey agar) were selected and subcultured onto fresh MacConkey agar plates for further analysis.

2.3 Identification

2.3.1 Observation

After incubation, the plates were carefully observed for single colonies displaying morphology resembling *K. pneumoniae,* including a mucoid appearance and characteristic pink color colonies on MacConkey agar. Colonies with clear *Klebsiella*-like morphology were chosen for further biochemical testing to confirm their identity. Moreover, the selected colonies for biochemical test were re-subcultured onto new agar plates so that stock culture could be prepared upon confirmation by biochemical test for future reference and analysis.

2.3.2 Media Preparation (Biochemical Test)

2.3.2.1 Triple Sugar Iron (TSI)

The dehydrated TSI powder (Oxoid CM0277, Hampshire, England) was suspended in distilled water according to manufacturer's instructions, specifically 13 gm in 200 ml for Oxoid product CM0277. After thorough mixing and boiling for 1 minute, the pH of the media was adjusted to 6.8-7.2. Next, 7 ml of media was dispensed into large screw cap tubes before autoclaving at 121°C for 15 minutes. Following autoclaving, the medium was allowed to solidify in a sloped position to yield a butt 25-30 mm deep and a slope 20-25 mm long. Plates were labeled with media details, and a sample was sent for QC testing, while the remainder was stored at 2 – 8ºC.

2.3.2.2 Motility Indole Urea (MIU)

The dehydrated powder (HIMEDIA M1076, Mumbai, India) was suspended in distilled water according to the manufacturer's instructions, specifically 3.6 gm in 190 ml for HIMEDIA product M1076. After thorough mixing and boiling for 1 minute, the pH of the media was adjusted to 6.6-7.0. A dry, tight cotton plug was inserted on the top of the flask, and the media was autoclaved at 121°C for 15 minutes. Following autoclaving, the media was transferred to a 50°C water bath. At 50°C, 10 ml of sterile 40% urea solution was aseptically added to 190 ml media and mixed gently to avoid forming air bubbles. Subsequently, 5 ml of media was dispensed into each tube (approx. 16×150 mm). The media was allowed to solidify in an upright position without any slope. Plates

were labeled with media details, and a sample was sent for QC testing, while the remainder was stored at 2 – 8ºC.

2.3.2.3 Citrate

The dehydrated powder (Oxoid CM0155, Hampshire, England) was suspended in distilled water according to the manufacturer's instructions, specifically 4.6 gm in 200 ml for Oxoid product CM0155. After thorough mixing and boiling for 1 minute, the pH of the media was adjusted to 6.8-7.2. Subsequently, 7 ml of media was dispensed into each large tube (approx. 16×150 mm) before autoclaving. The cap of the tube containing media was loosened, and autoclaving was performed at 121°C for 15 minutes. Following autoclaving, the medium solidified in an oblique position to prepare only a slant without a butt. Plates were labeled with media details, and a sample was sent for QC testing, while the remainder was stored at 2 – 8ºC.

2.3.3 TSI, MIU, Citrate Agar Test:

The TSI agar was stabbed and streaked with the bacterial culture using aseptic techniques. The agar tubes were incubated in an upright position. The MIU agar tube was inoculated with the bacterial culture using aseptic techniques. The agar tubes were incubated and observed the following day. The citrate agar tube was inoculated with the bacterial culture using aseptic techniques. The citrate agar tubes or plates were incubated. After incubation, observations were made for the following:

2.4 Stock Preparation:

Following confirmation of the isolates through biochemical testing, selected colonies exhibiting characteristics consistent with *Klebsiella spp.* were identified.

2.4.1 STGG Media preparation:

STGG (Skim Milk, Tryptone, Glucose, Glycerol) media was prepared by reconstituting skim milk powder, dissolving tryptone powder, glucose powder, and measuring glycerol. The preparation of the media involved mixing 2% skim milk powder, 3% Tryptic Soy Broth (TSB) broth, 10% glycerol, and 0.5% glucose. The skim milk powder was homogenized by placing the flask in a shaker for 15-20 minutes to ensure a homogeneous mixture before autoclaving the media at 121°C

for 10 minutes. After autoclaving, the vials were cooled, and the caps were sealed before storing them at -20 degrees Celsius. Quality control (QC) was conducted by plating the entire volume of one vial from each lot onto TSB broth with 5% sheep blood and incubating at 37˚C for 24 hours. Cryovials or suitable storage boxes were prepared for the stock culture. Each container was labeled with appropriate information, including isolate details. The boxes contained serial number and study name.

2.4.2 Inoculating onto STGG Medium:

Using aseptic techniques, the surface of the agar plate was swiped by a sterile swab, collecting bacterial material from the confirmed isolate colonies. Care was taken to cover the entire area where the colonies were present. The swab containing the collected bacterial material was then introduced into the STGG media solution. STGG medium, with its composition of skim milk, tryptone, glucose, and glycerol, served as a transport and storage medium for bacterial isolates. The prepared cryovials or containers were securely sealed and stored in a dedicated storage facility, such as a -80°C freezer, for long-term preservation.

2.5 Antibiotic Susceptibility Testing (AST)

2.5.1 Preparation of Bacterial Suspension:

Bacterial cultures stored in STGG (Skim Milk, Tryptone, Glucose, Glycerol) stock were streaked onto MacConkey agar plates. This step aimed to obtain isolated colonies for subsequent use in the antibiotic susceptibility test. The preparation of a bacterial suspension with a turbidity equivalent to 0.5 McFarland standard was initiated by selecting isolated bacterial colonies from the MacConkey agar plate culture. These colonies were aseptically transferred into a tube containing sterile saline solution, and the bacterial suspension was thoroughly mixed to achieve the target turbidity level. Verification of the turbidity was conducted using a spectrophotometer or visual comparison with a McFarland standard, ensuring the desired consistency.

2.5.2 Media preparation:

The dehydrated powder (Oxoid CM0337, Hampshire, England) was suspended in 1 liter of distilled water according to the manufacturer’s instructions, specifically 38 gm for Oxoid product

CM0337. After thorough mixing and boiling for 1 minute, the pH of the media was adjusted within the range of 7.2-7.6 at room temperature. Following autoclaving at 121°C for 15 minutes, the media was transferred to a 50°C water bath. At 50°C, 20 ml of media was dispensed onto a sterile petri dish, removing any bubbles formed. Plates were then left to solidify at room temperature in a leveled laminar flow. Each plate was labeled with media details, including name, date, and batch number. A sample was sent for quality assurance (QA) testing, while the remainder was stored at 2 – 8ºC.

2.5.3 Inoculation:

Using a sterile swab, the standardized bacterial suspension is evenly spread over the entire surface of the Mueller-Hinton agar plate. Care is taken to ensure uniform coverage, preventing concentration variations that may affect the test results. Sterile antibiotic disks, each impregnated with a specific antibiotic, were chosen based on the antibiotics being tested. In the Antibiotic Disk Placement step, a disc dispenser was employed to facilitate the placement of the selected antibiotic disks onto the previously inoculated agar surface. Seven different antibiotics were utilized in the testing. The antibiotic disks were gently dispensed onto the agar, ensuring a proper and consistent contact with the bacterial lawn. This method ensured a standardized and controlled application of the antibiotics, maintaining the accuracy and reproducibility of the Antibiotic Susceptibility Testing. The inoculated plates, containing antibiotic disks, were incubated overnight at 37°C, allowing the bacteria to proliferate.

2.5.4 Observation and Measurement:

Following incubation, the plates were observed for the appearance of zones of inhibition around the antibiotic disks. The diameter of these zones was measured using a calibrated ruler. The observed zones of inhibition were interpreted in accordance with established standards, signifying the susceptibility or resistance of the bacteria to the tested antibiotics. Detailed records were maintained, documenting the type and concentration of antibiotics used, along with the corresponding zones of inhibition for each bacterial isolate.

2.6 Sanger Sequencing Analysis of *rpoB* gene in *Klebsiella* spp. Isolates

2.6.1 Primer Design

A primer was designed targeting the variable region of the *rpoB* gene of *K. pneumoniae* to give species level resolution. The OligoAnalyzer tool provided by Integrated DNA Technologies (IDT) and The Primer3Plus online tool were utilized to design a novel primer of 21 base pairs that was designed in-house by CHRF. The designed primer was expected to produce an amplicon with a size of 940 base pairs (bp). The primer was validated by successfully amplifying the target sequence in control isolates. Additionally, the PCR conditions were optimized using gradient PCR to ensure robust and specific amplification.

2.6.2 Sample Preparation

Bacterial colonies were obtained by streaking the -80˚C stock culture onto MacConkey agar plates, a selective and differential medium. The plates were then incubated at 37˚C for overnight growth to allow for colony formation. To ensure purity and consistency, the culturing process was repeated from the first cultured plates. Subsequently, bacterial colonies were harvested by transferring them into 50µL of 1x TE buffer and subjected to thermal lysis by boiling at 95˚C for 15 minutes. Following thermal lysis, the suspension was centrifuged at 2000rpm to pellet cellular debris, and the resulting supernatant containing genomic DNA was carefully transferred to fresh tubes.

2.6.3 PCR Amplification

The targeted PCR amplification step involved amplifying specific regions of interest within the DNA template using the polymerase chain reaction (PCR) method. This process is pivotal for enriching the DNA fragments of interest before proceeding to downstream applications such as sequencing. All frozen reagents were thawed to room temperature before preparing a master mix in a 0.2mL PCR tube, ensuring thorough mixing through vortexing and centrifugation.

Table 2.1. Components and Volume for PCR Reaction Setup for Sanger Sequencing

Master mix Components	**Concentration**	**Volume per reaction(µL)**
HotFirepol Solis Biodyne Buffer	5X	5
rpoB Forward Primer	10µM (working solution)	1.25
rpoB Reverse Primer		1.25
DMSO		2
Molecular Grade Water		13.5
	Total	**23**

Subsequently, 23µL of the master mix was dispensed into each PCR reaction tube, followed by the addition of 2µL of the DNA template and secure closure of the tubes. The PCR tubes were then transferred to the thermocycler for thermal cycling according to the specified protocol. PCR amplification was conducted using a CFX96 Touch Real-Time PCR Detection System. under the following cycling conditions:

Table 2.2. PCR Conditions for Target Amplification in Sanger Sequencing

Steps	**Temperature**	**Time**	**Cycle**
Initial Denaturation	95	15mins	1
Denaturation	95˚C	30s	32
Annealing	64˚C	30s	
Extension	72	60s	
Final Extension	72˚C	5 mins	1

2.6.4 Gel Electrophoresis

In the Sanger sequencing workflow, following PCR amplification, gel electrophoresis was employed to confirm the presence of the desired PCR product. Gel electrophoresis separated DNA fragments based on size and charge, allowing for visualization and analysis. This step verified successful amplification of the target DNA sequence by observing a distinct band at the expected size. Additionally, non-specific amplification products, such as primer dimers, were detected as additional bands on the gel. By comparing the migration of PCR products to DNA size markers, the approximate size of amplified fragments was determined. Gel electrophoresis served as a quality control step, ensuring the integrity of PCR products before proceeding further. It was an essential component of the Sanger sequencing process, providing valuable information about amplification success and fragment size.

A 1% agarose gel was prepared by dissolving 0.3 grams of agarose powder in 30 mL of 1X TBE (Tris-borate-EDTA) buffer, supplemented with 2 µL SYBR Safe DNA gel stain. The molten agarose solution was cast, solidified, and placed in the gel electrophoresis chamber filled with 1X TBE buffer. 5 µL of PCR samples, already mixed with loading dye (PCR buffer contained loading dye), were loaded onto the gel alongside 3 µL of 1 KB Plus DNA ladder without additional loading dye. Gel electrophoresis was conducted at 100 volts and 400 milliamps for 30 minutes. Subsequently, DNA bands were visualized using a UV transilluminator.

2.6.5 PCR Product Purification

The purification step was conducted to eliminate unbound primers, dNTPs, and other contaminants from the PCR products using an enzymatic approach. Firstly, the contents of the ExoSAP-IT™ PCR Product Cleanup and PCR products were completely thawed. Subsequently, the tubes were briefly centrifuged for 2 to 3 seconds using a benchtop microcentrifuge to ensure the contents were settled at the bottom. Following this, the specified components were combined in a PCR tube to create the mixture required for enzymatic purification. The components were then gently mixed through pipetting. Finally, the prepared mixture was subjected to enzymatic purification within a thermocycler, following a programmed protocol. This meticulous procedure ensured the removal of unwanted residues, thereby enhancing the purity of the PCR products for subsequent analyses. Provided below are the details of the Master mix components and the PCR profile:

Table 2.3: Components and Volumes for PCR Product Purification Mix

Components	Volume per sample
ExoSAP-IT™ PCR Product Cleanup	2µL
PCR Product	5µL

Table 2.4: Thermal Cycling Conditions for PCR Product Purification

PCR Profile			
Steps	**Temperature**	**Time**	**Cycle**
1	37˚C	15mins	1
2	80˚C	15mins	1

2.6.6 QC and Quantification of Purified PCR Product

Before cycle sequencing, DNA was quantified to verify the presence of a sufficient amount. If necessary, dilution was performed, ensuring an appropriate quantity for the sequencing reaction. The adequacy of dilution depended on the initial concentration of the DNA sample, with adjustments made to achieve optimal concentrations for accurate sequencing. The purity and concentration of DNA were assessed and quantified on the NanoDrop One Spectrophotometer (Thermo Fisher Scientific, USA) by examining the entire absorption spectrum between 220-350nm. In preparation for sequencing, the quantification of DNA was initiated by placing 1µL of nuclease-free water on the pedestal, followed by the closure of the pedestal and blanking of the instrument. Subsequently, 1µL of purified DNA was placed on the pedestal, and after closing it, the purity and concentration were recorded. Upon completion of the experiment, the instrument was blanked once again. Dilution was conducted as required to achieve a final DNA concentration ranging between 50 to 100 ng per microliter, ensuring optimal conditions for sequencing.

2.6.7 Cycle Sequencing

During cycle sequencing, the polymerase adds fluorescently labeled ddNTPs, stopping DNA synthesis. This produces fragments of various lengths, each marked with a specific dye indicating its end base.

The contents of the BigDye™ Terminator v3.1 Cycle Sequencing Kit and the primers were completely thawed and stored on ice. Following this, the tubes were vortexed for 2 to 3 seconds, and then briefly centrifuged (2 to 3 seconds) with a benchtop microcentrifuge to ensure the collection of contents at the bottom of the tubes. It was noted to keep the reagents and sequencing reactions shielded from light exposure due to the propensity of fluorescent dyes to fade, which could potentially affect the sequencing analysis results. Subsequently, 8.25 µL of master mix was aliquoted into each PCR tube, followed by the addition of 0.75 µL of forward and reverse primers into distinct PCR tubes. It was emphasized that since Sanger sequencing typically utilizes a single primer in each reaction, the forward and reverse primers should be added into separate tubes. Finally, 1 µL of template DNA was added to the corresponding tube containing the master mix and primer. It was noted that the template should be added to both tubes containing forward and reverse primers.

Table 2.5: Components and Volumes for Cycle Sequencing Mix

Reagents	Volume (µL)
BigDye terminator 3.1 ready reaction mix	0.5
BigDye terminator v1.1 & v3.1 5x sequencing buffer	1.75
Water	6

Table 2.6: Thermal Cycling Conditions for Cycle Sequencing

Cycle Sequencing PCR Profile			
PCR Steps	**Temperature**	**Time**	**Cycle**
Initial Denaturation	96˚C	1min	1
Denaturation	96˚C	10s	30
Annealing	50˚C	5s	
Extension	60˚C	4mins	

2.6.8 Cycle Sequencing Product Purification

Cycle sequencing product purification involves removing excess primers and nucleotides from the sequencing reaction. This purification step ensures a clean template for downstream analysis, such as capillary electrophoresis.

The bottle of BigDye XTerminator™ beads was vortexed for 8 to 10 seconds before being mixed with the SAM solution. Following that, the SAM/BigDye XTerminator™ bead working solution was prepared. It was noted that due to the rapid settling of the BigDye XTerminator™ bead solution, it should be promptly added to the mixture, with additional vortexing performed as necessary for thorough mixing. After that, 55µL of bead mix was dispensed to each sample. The mixture was then vortexed at 2000rpm for 30 minutes. Finally, centrifugation was performed for 2 minutes to settle the beads.

2.6.9 Loading on SeqStudio Flex Series Genetic Analyzer, Thermofisher Scientific

In the sequencing reaction plate, 10µL of the forward reaction was loaded into one well, while 10µL of the reverse reaction from the same sample was added to another well. Each column

accommodated the addition of 4 samples, totaling 8 reactions. Throughout the process, it was ensured that all wells within a column were fully filled, leaving nonvacant. In instances where a blank well was identified among the columns, 10µL of Hi-Di™ Formamide was meticulously loaded into the empty wells to maintain consistency and optimize the sequencing procedure.
Upon the Genetic Analyzer run's completion, the raw sequence data from the respective samples were transferred and subjected to computational analysis using bioinformatics tools.

2.6.10 Analysis

Sequence trace files with the ".ab1" extension and corresponding FASTA files were imported and analyzed using SnapGene to assess signal quality. Poor quality regions at the beginning and end of sequences were trimmed. Forward and reverse sequences were merged using Merger, with the reverse sequence complemented via Nucleotide Massager for alignment. Optimal alignment and base call validation were manually inspected, ensuring accuracy in the contig files. These contig FASTA sequences were then submitted to NCBI BLAST for identification by comparing them against a database of known sequences.

2.7 Whole Genome Sequencing

2.7.1 Sample Selection for Whole Genome Sequencing

Three samples were selected for Whole Genome Sequencing based on specific criteria. All hypermucoid isolates exhibiting different resistance patterns were chosen. This selection included one isolate that demonstrated resistance to the highest number of antibiotics, one isolate that was resistant only to ampicillin, and the other one had an intermediate level of resistance, positioned between the least and most resistant isolates.

2.7.2 Genomic DNA Extraction for Whole Genome Sequencing

The selected isolates were taken from the STGG stock and cultured on MacConkey agar plates. Sufficient numbers of colonies were collected from the selective media and suspended into 800 µl of 1xPBS to reach a 0.5 McFarland standard. After thorough mixing, the suspension underwent centrifugation at 14000 rpm for 2 minutes, and the supernatant was subsequently discarded. In cases where the sample volume was less than 200 µl (such as blood plasma or other body fluids),

the volume was adjusted with Buffer GA to reach 200 µl. Proteinase K (20 µl) was added, and the mixture was vortexed and incubated at 56°C for 10 minutes. Following that, 6 µl of RNase A (100 mg/ml) was added, mixed by pulse-vortexing for 15 seconds, and incubated for 2 minutes at room temperature.

Buffer GB (200 µl) was then added to the sample, mixed by pulse-vortexing, and incubated at 70°C for 10 minutes. Subsequently, 200 µl of ethanol (96–100%) was added to the sample, mixed by pulse-vortexing, and applied to the spin column. After centrifugation at 12000 rpm for 30 seconds, Buffer GD (500 µl) was added to the spin column, followed by centrifugation at 12000 rpm for 30 seconds. Buffer PW (600 µl) was then added to the spin column and centrifuged at full speed (12000 rpm) for 30 seconds. This step was repeated, and the spin column was centrifuged at full speed for 1 minute to eliminate possible Buffer PW carryover. Finally, TE Buffer (50 µl) was added to the spin column, incubated at room temperature for 2-5 minutes, and centrifuged at 12000 rpm for 30 seconds. The samples were stored at -20°C.

2.7.3 Library Preparation of *Klebsiella pneumoniae* gDNA

This dissertation employs DNA sequencing methodology to uncover the genetic information harbored within *K. pneumoniae,* aligning with the research objectives. Next Generation Sequencing (NGS) will be conducted using the Illumina platform to analyze the genome of the organism and unravel the information that aligns with our research objectives.

2.7.3.1 DNA Quantification

Quantification before library preparation involves assessing the concentration of DNA or RNA in samples, ensuring sufficient genetic material for subsequent processing. This crucial step is essential to confirm the suitability of samples for downstream analysis and to determine the appropriate amount of material required for library construction.

In this dissertation research, genomic DNA (gDNA) was subjected to a methodical serial dilution process, resulting in a decrease in concentration by a factor of 100. The quantification of double-stranded DNA (dsDNA) was executed through the utilization of the dsDNA HS Qubit Assay.

To measure the concentrations of the 100-fold diluted samples, a working solution was carefully added to both standards and individual samples. Before any experiments were done, the reagents and dye were left to reach room temperature for optimal conditions for 30 minutes.

For the standards, 190 µL of the Qubit™ 1X dsDNA HS working solution was gently dispensed into two separate Qubit tubes, each designated for a specific standard. Next, 10 µL of each Qubit™ 1X dsDNA HS standard was added to the respective tube, followed by a quick vortexing for 2-3 seconds. Conversely, for each individual sample, 198 µL of the Qubit™ 1X dsDNA HS working solution was carefully pipetted into separate tubes, and 2 µL of the 100-fold diluted samples was added to each tube, followed by a brief vortexing step lasting 2-3 seconds. All the tubes were then incubated in darkness for a period of 2 minutes.

After the incubation period, the concentrations of all tubes, starting with the calibration standards, were assessed using the Qubit® Fluorometer. The measurements were performed within the High Sensitivity Range to ensure precise quantification of DNA concentrations across all samples and standards. If the Qubit® Fluorometer displayed a "Too Low" reading for any sample, a fresh working solution was prepared using a 10-fold dilution of the corresponding sample and subsequently measured once more. The concentrations of the diluted samples were then documented.

2.7.3.2 Normalization:

Normalization is the process of adjusting the concentrations of biological samples to a standardized level. It is needed to ensure that all samples contribute equally to downstream analyses, minimizing variability and enabling accurate comparisons between samples. After measuring the concentrations using the Qubit, all samples were normalized to achieve a final volume of 26 µL with approximately 52 ng of DNA, ensuring a consistent DNA content of approximately 52 ng per sample.

2.7.3.3 Fragmentation of gDNA

The preparation of DNA libraries for sequencing followed the protocol and utilized reagents from the NEBNext® Ultra™ II FS DNA Library Prep kit by New England Biolabs. In the library preparation process, fragmentation of DNA is an initial requirement. During the 37°C incubation step, fragmentation takes place, resulting in the generation of DNA fragments ranging in size from 300 to 700 base pairs.

To initiate fragmentation, a master mix was created by combining 7 µL of NEBNext Ultra II FS Reaction buffer with 2 µL of NEBNext Ultra II FS Enzyme mix. After thorough mixing, 9 µL

aliquots of the master mix were added to individual sample tubes. Each tube contained normalized DNA at a concentration of 52 ng in 26 µL.

Provided below are the details of the Master mix components and the PCR profile:

Table 2.7: Components and Volumes for gDNA Fragmentation

Reagents & Components	**Volume(µL)**
NEBNext Ultra II FS Reaction Buffer	7
NEBNext Ultra II FS Enzyme mix	2
Normalized DNA (~ 52ng)	26
Total	35

Table 2.**8**: Thermal Cycler Conditions for gDNA Fragmentation

PCR Profile		
Step	**Time**	**Temperature**
Fragmentation	7 min	37˚
Enzyme Deactivation	30 min	65˚
Hold	∞	4˚
Heated Lid: ON (105°C)		

2.7.3.4 Adaptor Ligation

The adaptor ligation step in library preparation involves the attachment of adaptors to fragmented DNA molecules. These adaptors contain sequences necessary for subsequent steps in the sequencing process, such as amplification and sequencing primer binding. This crucial step facilitates the identification and amplification of DNA fragments during sequencing, enabling comprehensive analysis of genetic material. First, The Illumina adaptors underwent a 100-fold pre-dilution for DNA input exceeding 5 ng.

In this Master mix preparation, the the pre-diluted adaptor sequences were excluded to prevent potential issues with dimer formation. Instead, the process of attaching adaptors to each DNA

fragment was initiated by creating a mix containing NEBNext Ultra II Ligation Master mix and NEBNext Ligation Enhancer. Following this, aliquots of this mix, along with a portion of the diluted adaptors, were added to each sample separately. Subsequently, the mixture was left to incubate at 20°C for 15 minutes without the lid on the thermocycler.

Table 2.**9**: Components and Volumes for Adapter Ligation

Reagents & Components	**Volume(µL)**	**Notations**
NEBNext Ultra II Ligation Master mix	30	Master Mix Preparation
NEBNext Ligation Enhancer	1	
DNA Fragments	35	Previous reaction
NEDNext Illumina Adaptor	2.5	Added Separately
Total	68.5	

Table 2.**10**: Thermal Cycler Conditions for gDNA Fragmentation

PCR profile		
Step	**Time**	**Temperature**
Fragmentation	15minutes	20˚C
Heated Lid: **Off**		

2.7.3.5 Clean-up of Adaptor-ligated DNA and Size Selection (0.9x wash)

The bead wash step in library preparation is significant as it removes unwanted contaminants and residual reagents from DNA samples, ensuring the purity and quality of the library. By selectively binding DNA fragments to magnetic beads and washing away impurities, bead wash enhances the efficiency and accuracy of downstream sequencing processes, ultimately leading to more reliable and reproducible results. In preparation for sequencing, library products were targeted to have a size of 300 bp to prevent gaps in the sequence data during analysis. To achieve this, the removal of unbound DNA, adaptors, and fragments less than 200 bp was carried out through a series of washes.

The bead wash process was initiated by allowing the AMPure XP beads to sit at room temperature for 30 minutes, followed by vigorous vortexing to ensure a homogenous mixture. A 0.9x ratio of beads to the total volume of the sample was used, and 80% ethanol was prepared for subsequent washes. Next, 61.65 µL of AMPure XP beads (0.9x) was added to the adaptor ligated products (~68.5 µL) and mixed thoroughly by pipetting. The mixture was then incubated for 5 minutes at room temperature and placed on a magnetic rack for 5 minutes to capture the beads. After the supernatant was carefully removed, 200uL of 80% ethanol was added to each sample on the magnetic rack, followed by incubation for 30 seconds and removal of the supernatant. This ethanol wash step was repeated once for a total of two washes to ensure thorough purification. Any residual ethanol was carefully removed with a p10 pipette tip. The beads were air-dried for 5-10 minutes on the magnetic rack with the lid open, taking care not to over-dry them. Once adequately dried, DNA was eluted from the beads into 17 µL of nuclease-free water. After thorough mixing and incubation for 2 minutes at room temperature, the solution was placed back on the magnetic rack until the solution became clear (~2 minutes). Finally, 15 µL of the supernatant was transferred to a clean nuclease-free PCR tube for further analysis. It was crucial to handle the beads carefully to ensure optimal DNA recovery and avoid over-drying, as indicated by the color change of the beads.

2.7.3.6 USER Digestion and Barcoding:

In this step, the USER enzyme was utilized to cleave the adaptor at the uracil region, generating a gap for the binding of index primers (barcodes). Subsequently, each sample was labeled with a distinct barcode during PCR enrichment to enable sample identification post-amplification.

To initiate USER End digestion, a master mix was prepared by combining 3 µL of USER Enzyme and 25 µL of NEBNext Ultra II Q5 master mix in a tube. After thorough mixing, 28 µL of the master mix was directly added to the adaptor-ligated washed tube. Additionally, 10 µL of pre-mixed forward and reverse Index primers (IDT i5/i7) were separately added to the mixture. The resulting reaction mixture, totaling 53 µL, was briefly vortexed.

Details of the Master mix components and PCR profile are provided below:

Table 2.**11**: Components and Volumes for User Digestion and Barcoding

Reagents & Components	**Volume(µL)**	**Notations**
USER Enzyme (Cat no. M5505L, 250 uL)	3	Master mix preparation
NEBNext Ultra II Q5 master mix	25	
Adaptor-ligated DNA	15	Previous step
IDT i5/i7 pre-mixed primers	10	**Added separately
Total	53	

Table 2.**12**: Thermal Cycler Conditions for User Digestion and Barcoding

PCR Profile	
Steps	**Cycle**
37°C for 15 minutes	1
98°C for 30 seconds	1
98°C for 10 seconds	14
65°C for 75 seconds	
65°C for 5 minutes	1
Hold at 4°C	∞
Heated Lid: ON (105˚C)	

2.7.3.7 Clean-up of Barcoded DNA and Size Selection (0.8x wash) :

This 0.8x SPRI bead cleanup process washes the barcodes and eliminates products that are less than 100 bp.

The bead wash process was initiated by allowing the AMPure XP beads to sit at room temperature for 30 minutes, followed by vigorous vortexing to ensure a homogenous mixture. A 0.8x ratio of beads to the total volume of the sample was used, and 80% ethanol was prepared for subsequent washes. Next, 42.4 µL of AMPure XP beads (0.8x) was added to the barcoded products (53 µL)

and mixed thoroughly by pipetting. The mixture was then incubated for 5 minutes at room temperature and placed on a magnetic rack for 5 minutes to capture the beads. After the supernatant was carefully removed, 200 µL of 80% ethanol was added to each sample on the magnetic rack, followed by incubation for 30 seconds and removal of the supernatant. This ethanol wash step was repeated once for a total of two washes to ensure thorough purification. Any residual ethanol was carefully removed with a p10 pipette tip. The beads were air-dried for 5-10 minutes on the magnetic rack with the lid open, taking care not to over-dry them. Once adequately dried, DNA was eluted from the beads into 44 µL of nuclease-free water. After thorough mixing and incubation for 2 minutes at room temperature, the solution was placed back on the magnetic rack until the solution became clear (~2 minutes). Finally, 40 µL of the supernatant was transferred to a clean nuclease-free PCR tube for further analysis. It was crucial to handle the beads carefully to ensure optimal DNA recovery and avoid over-drying, as indicated by the color change of the beads.

2.7.3.8 Clean-up of Barcoded DNA and Size Selection (0.75x wash)

This 0.75x SPRI bead cleanup process washes the barcodes and eliminates products that are less than 200 bp.

The bead wash process was initiated by allowing the AMPure XP beads to sit at room temperature for 30 minutes, followed by vigorous vortexing to ensure a homogenous mixture. A 0.75x ratio of beads to the total volume of the sample was used, and 80% ethanol was prepared for subsequent washes. Next, 30 µL of AMPure XP beads (0.75x) was added to the barcoded products (40 µL) and mixed thoroughly by pipetting. The mixture was then incubated for 5 minutes at room temperature and placed on a magnetic rack for 5 minutes to capture the beads. After the supernatant was carefully removed, 200 µL of 80% ethanol was added to each sample on the magnetic rack, followed by incubation for 30 seconds and removal of the supernatant. This ethanol wash step was repeated once for a total of two washes to ensure thorough purification. Any residual ethanol was carefully removed with a p10 pipette tip. The beads were air-dried for 5-10 minutes on the magnetic rack with the lid open, taking care not to over-dry them. Once adequately dried, DNA was eluted from the beads into 34 µL of nuclease-free water. After thorough mixing and incubation for 2 minutes at room temperature, the solution was placed back on the magnetic rack until the solution became clear (~2 minutes). Finally, 30 µL of the supernatant was transferred to a clean

nuclease-free PCR tube for further analysis. It was crucial to handle the beads carefully to ensure optimal DNA recovery and avoid over-drying, as indicated by the color change of the beads.

2.7.3.9 Loading on Illumina iSeq100

The quantification of each library was performed using the Qubit fluorometer. Subsequently, the individual libraries were merged into a single microcentrifuge tube and further diluted to reach a final concentration of 110 pM. Additionally, a 5% PhiX control at a concentration of 100 pM was prepared and added to the pooled libraries.

Following the recommended Illumina protocol for thawing and preparing the cartridge on the iSeq100 platform, 20 uL of the diluted libraries containing 5% PhiX were loaded into the loading well of the cartridge alongside the flow cell. The sequencing process was initiated as per the manufacturer's instructions.

Upon the completion of the iSeq100 run, the raw sequence data from the respective samples were transferred and subjected to computational analysis using bioinformatics tools.

2.7.3.10 Computational Analysis of *Klebsiella pneumoniae* genomic DNA

The bioinformatic section of this dissertation outlines a comprehensive methodology for computational analysis of biological raw data, offering valuable insights into *Klebsiella pneumoniae* genomics and diversity. It commences with preprocessing raw sequence data, involving quality control checks and removal of low-quality reads and adapter sequences through trimming by trimmomatic, followed by assembly of cleaned reads into full contig sequence files for respective samples using Unicycler (Wick et al., 2017). Subsequently, contig sequence files undergo protein annotation by Prokka (Seemann, 2014) and exploration of phylogenetic relationships among other known *K. pneumoniae* sequences by Snippy. Kleborate was utilized to conduct comprehensive analysis of *K. pneumoniae* sequences, providing insights into species, sequence types (ST), virulence genes, capsule typing, and antimicrobial resistance prediction (Lam et al., 2021). ResFinder was employed for the identification of antimicrobial resistance genes in the sequencing data and the prediction of phenotypes from genotypes (Florensa et al., 2022). It was utilized to identify antimicrobial resistance genes within *K. pneumoniae* sequences, aiding in the prediction of antimicrobial resistance phenotypes. Furthermore, the section highlights the utility of Conda, an open-source package management system and environment system widely

used in the bioinformatics community. Developed by Continuum Analytics, Conda simplifies package installation, runs, sharing, and updates across different computing platforms, enabling easy management of bioinformatics tools and creation of isolated environments with specific dependencies or packages

3 Result

Overview

This chapter of the dissertation presents the outcomes of the experimental investigations conducted to address this study's objectives. The results provide comprehensive information on environmental *Klebsiella pneumoniae* isolates from Dhaka City. 180 *Klebsiella spp.* Isolates underwent Antibiotic Susceptibility Test and their phenotypic resistant profiles were recorded. Three *Klebsiella pneumoniae* isolates were selected for whole genome sequencing based on their phenotypic, biochemical, and antibiotic resistance profiles ensuring a representative sample for genomic analysis. The findings in this section integrate laboratory experiments and subsequent bioinformatics analyses. The data generated at various stages of the study have been meticulously recorded, organized, and deeply analyzed to identify patterns and facilitate interpretation. Both Sanger sequencing and whole genome sequencing data underwent rigorous bioinformatics analysis to exclude background noise and enhance the resolution of relevant information. High-quality whole genome sequences were obtained, and assembly metrics and total contig length were assessed to confirm the accuracy and completeness of the genomes. Comprehensive genomic annotations identified coding sequences (CDS), other functional elements, with key genes related to virulence and antibiotic resistance. Bioinformatics analyses mapped and characterized antibiotic resistance genes, virulence factors, providing insights into the genetic mechanisms of resistance and pathogenicity. Comparative genomics and phylogenetic analyses explored the genetic diversity among the isolates, illustrating evolutionary relationships and highlighting conserved and variable genomic regions.

3.1 Area-wise Distribution and Biochemical Identification of Isolates

In this section, the distribution and biochemical identification of bacterial isolates from various areas are presented. Initially, subcultures were obtained from lawned plates. Upon examining these subcultures, selected isolates underwent biochemical testing. Following confirmation through these biochemical tests, *Klebsiella spp*. were identified and stocked, while other isolates were discarded. The table below summarizes the number of subcultures performed, the biochemical

tests conducted, and the identification of Klebsiella spp. along with other organisms derived from these subcultures.

Table 3.1: Area-wise Distribution and Biochemical Identification of Isolates

Area (Thana)	No. of Sample	Total sub-cultures	Total Tested (Biochemical)	No. of *Klebsiella spp.*	No. of *E. coli*	No. of *Enterobacter*
Adabor	18	54	50	20	11	19
Kafrul	21	63	50	23	23	4
Mirpur	25	75	61	44	12	5
Mohammadpur	22	66	55	33	17	5
Pallabi	20	60	36	20	10	6
Shah Ali	15	45	23	16	5	2
Sher-E-Bangla	19	57	35	24	6	5
Total	**140**	**420**	**310**	**180**	**84**	**46**

A total of 140 samples were collected from which 3 suspected *Klebsiella spp*. colonies per sample were subcultured, resulting in 420 subcultures. Out of these, 310 subcultures were selected for biochemical testing. The biochemical tests confirmed that 180 of these subcultures were *Klebsiella spp*., 84 were *Escherichia coli*, and 46 were *Enterobacter spp*. The 180 *Klebsiella spp*. isolates were stocked for further analysis.

3.2 Antibiotic Susceptibility Profile across Representative Classes

The stacked bar plot illustrates the antibiotic susceptibility profile across representative classes, depicting the percentage distribution of susceptibility, intermediate, and resistance categories for seven antibiotics. Each antibiotic represents a distinct class, providing insight into the effectiveness of treatment options against the tested bacterial strains. The Y-axis represents the percentage of isolates falling into each susceptibility category, while the X-axis displays the names of the antibiotics. The stacked bars visually convey the relative proportions of susceptibility, intermediate, and resistance within each antibiotic class, aiding in the interpretation of antibiotic resistance patterns.

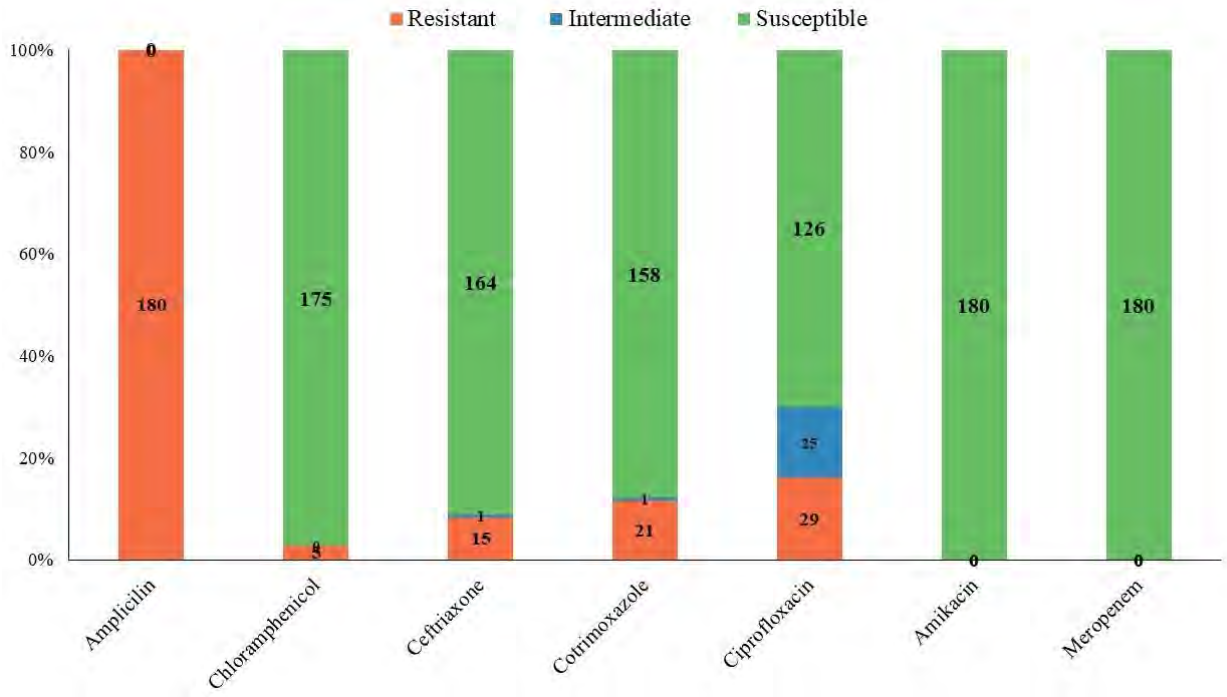

Figure 3.1: Antibiotic Susceptibility Profile across Representative Classes

The antibiotic susceptibility results, expressed as percentages, indicate that all isolates were resistant to ampicillin. For chloramphenicol, 2.78% of isolates showed resistance, while none displayed intermediate susceptibility, with 97.22% being susceptible. Regarding ceftriaxone, 8.33% were resistant, 0.56% showed intermediate susceptibility, and 91.11% were susceptible. Cotrimoxazole demonstrated resistance in 11.67% of isolates, with 0.56% showing intermediate susceptibility and 87.78% being susceptible. Ciprofloxacin revealed resistance in 16.11% of isolates, with 13.89% showing intermediate susceptibility and 69.44% being susceptible. In contrast, amikacin and meropenem showed no resistance or intermediate susceptibility, with 100% susceptibility observed for both antibiotics among the isolates tested.

3.3 Confirmation of Target Amplification and Sanger Sequencing Results

In this section, 14 isolates were meticulously chosen for Sanger sequencing analysis. Utilizing a novel primer specifically designed for this study, the isolates underwent amplification, aiming to yield a 940 bp product. The successful amplification of the desired product was visually confirmed through the gel image of the PCR products. A 1Kb Plus ladder was employed as a molecular weight marker to facilitate accurate

size determination of the amplified fragments. Notably, the presence of the expected 940 bp band across all isolates in the gel image provided preliminary validation of the primer's efficacy in target amplification.

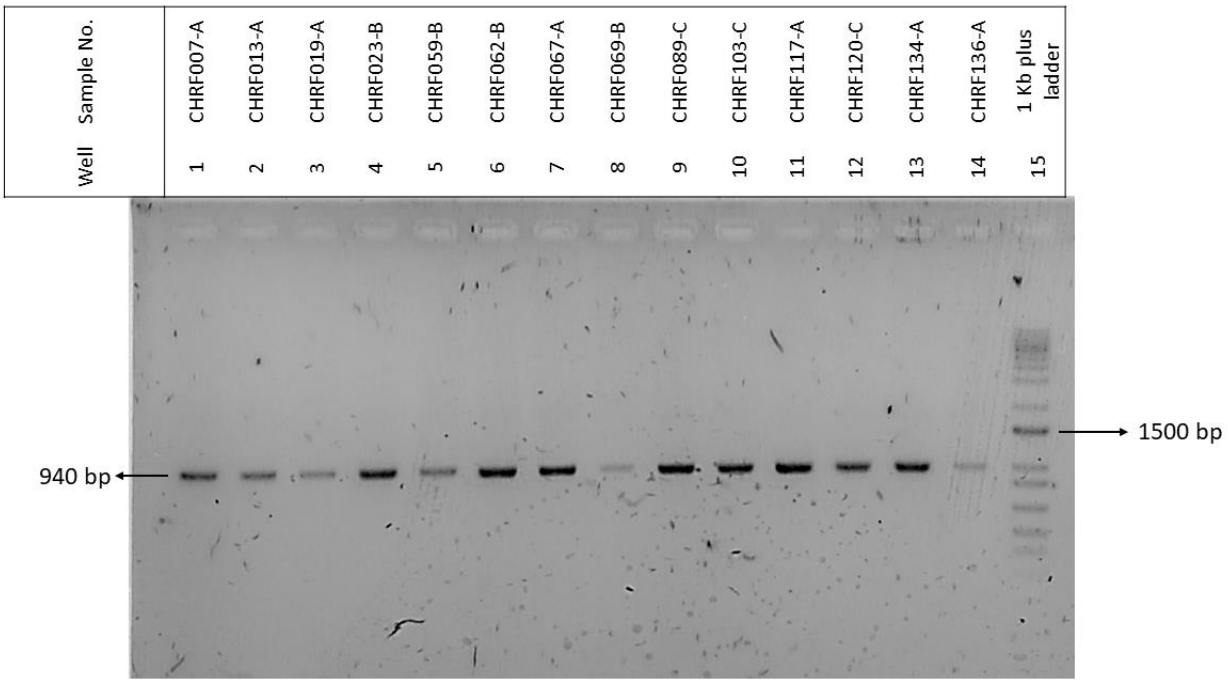

Figure 3.**2**: Gel Electrophoresis Confirmation of PCR Products Amplified from Selected Isolates

For Sanger sequencing, 2 isolates from each area were carefully selected. However, challenges were encountered in retrieving data from 3 isolates due to poor quality. Following bioinformatic refinement, BLAST analysis was conducted on the data generated by the rest of the isolates, revealing identification of 1 *Klebsiella quasipneumoniae* and 10 *Klebsiella pneumoniae*.

The lowest identity observed was 98.5 percent, indicating that the remaining isolates exhibited higher percentages of identity. Subsequently, 3 *K. pneumoniae* isolates were selected for whole genome sequencing based on their antibiotic resistance and unique traits such as hypermucoidity. These isolates were chosen to facilitate a comprehensive exploration of resistance and virulence mechanisms within *K. pneumoniae*.

Table 3.**2**: Summary of Sample Selection Criteria for Sanger Sequencing, BLAST Results, and Isolates Selected for Whole Genome Sequencing

Sample ID	Colony	AREA	Interpretation CLSI guideline 2020							Biochemical Characteristics		Sanger Results		
			AMP	C	CRO	SXT	CIP	AK	MEM	Urease	Mucoid	Organism detected	Global BLAST % identity	For WGS
CHRF007	A	Adabor	R	S	I	S	R	S	S	Positive	Positive	*Klebsiella pneumoniae*	99.88	Selected
CHRF013	A		R	S	R	R	R	S	S	Negative	Negative	*Klebsiella pneumoniae*	100	
CHRF019	A	Kafrul	R	S	R	R	R	S	S	Negative	Positive	*Klebsiella pneumoniae*	99.53	Selected
CHRF023	B		R	S	S	R	R	S	S	Negative	Negative	*Klebsiella pneumoniae*	99.65	
CHRF059	B	Mirpur	R	S	R	S	R	S	S	Positive	Negative	Poor Quality Data	-	
CHRF062	B		R	R	S	R	R	S	S	Negative	Negative	*Klebsiella pneumoniae*	100	
CHRF067	A	Mohammmadpur	R	R	S	R	R	S	S	Positive	Negative	*Klebsiella pneumoniae*	98.47	
CHRF069	A		R	S	R	S	R	S	S	Positive	Negative	Poor Quality Data	-	
CHRF089	C	Pallabi	R	S	S	S	S	S	S	Positive	Positive	*Klebsiella pneumoniae*	99.4	Selected
CHRF103	C		R	S	R	R	R	S	S	Negative	Negative	*Klebsiella pneumoniae*	100	
CHRF117	A	Shah Ali	R	R	S	R	S	S	S	Positive	Negative	*Klebsiella quasipneumoniae*	99.82	
CHRF120	C		R	S	S	R	R	S	S	Negative	Negative	*Klebsiella pneumoniae*	99.18	
CHRF134	A	Sher-E-Bangla	R	S	S	R	R	S	S	Negative	Negative	*Klebsiella pneumoniae*	98.73	
CHRF136	A		R	S	S	S	R	S	S	Positive	Negative	Poor Quality Data	-	

3.4 Whole Genome Sequencing Analysis and Characterization

In this section, an in-depth analysis of the genomic data obtained through whole genome sequencing will be conducted. Insights into crucial aspects such as genome size, coverage, sequence type, and the presence of resistance/virulence markers will be provided.

The following table offers a concise summary of essential metrics obtained from whole genome sequencing analysis. It presents key data points including genome coverage, total size, sequence type, and sub-lineage classification. This information serves as a foundational reference for understanding the genetic profiles and classification of the examined samples.

Table 3.**3**: Overview of Whole Genome Sequencing Data

Sample ID	Area	Species	Total size	Genome Coverage	Sequence Type	Sub-lineage
7A	Adabor	*Klebsiella pneumoniae*	5 445 654	32	ST14-1LV	14
19A	Kafrul	*Klebsiella pneumoniae*	5 155 129	32	ST2177	34
89C	Pallabi	*Klebsiella pneumoniae*	5 209 052	32	ST2850-1LV	New

The table summarizes the genomic analysis of three *K. pneumoniae* isolates collected from different areas in Dhaka city. Each isolate, originating from Adabor (7A), Kafrul (19A), and Pallabi (89C), exhibits unique sequence types and sub-lineages, highlighting the genetic diversity among the bacterial populations in these regions. The genome sizes retrieved, around 5.2 to 5.4 Mbp, are consistent with the typical genome size of *K. pneumoniae* at approximately ~5-6 Mbp (Wyres et al., 2020), indicating good quality genomic data. Notably, one isolate from Pallabi represents a new sub-lineage, suggesting the presence of novel genetic variants within the city's wastewater.

Table 3.4: Comparison of Phenotypic Antimicrobial Susceptibility Testing (AST) Results with Genotypic Resistance Gene Analysis

Sample ID	AST Result – Disc diffusion		Computational Results – Resistant Genes found	Computational Results – Antibiotics
7A	Ampilicin (AMP)	R	*blaSHV*-106, blaSHV-28, fosA6, OqxB, OqxA	Ampicillin, Chloramphenicol, Ciprofloxacin, Trimethoprim, Unknown Beta-lactam
	Chloramphenicol (C)	S		
	Ceftriaxone (CRO)	I		
	Co-Trimoxazole (SXT)	S		
	Ciprofloxacin (CIP)	R		
	Amikacin (AK)	S		
	Meropenem (MEM)	S		
19A	Ampilicin (AMP)	R	*blaSHV-26*, fosA6, OqxB, OqxA	Ampicillin, Chloramphenicol, Ciprofloxacin, Trimethoprim
	Chloramphenicol (C)	S		
	Ceftriaxone (CRO)	R		
	Co-Trimoxazole (SXT)	R		
	Ciprofloxacin (CIP)	R		
	Amikacin (AK)	S		
	Meropenem (MEM)	S		
89C	Ampilicin (AMP)	R	*blaSHV*-157, blaSHV-62, fosA6, OqxB, OqxA	Unknown Beta-lactam, Ampicillin, Chloramphenicol, Ciprofloxacin, Cephalothin, Trimethoprim
	Chloramphenicol (C)	S		
	Ceftriaxone (CRO)	S		
	Co-Trimoxazole (SXT)	S		
	Ciprofloxacin (CIP)	S		
	Amikacin (AK)	S		
	Meropenem (MEM)	S		

The table provides a comprehensive overview of the resistance gene profiling for the three *K. pneumoniae* isolates from different areas in Dhaka city, alongside a comparative analysis of their phenotypic and genotypic resistance characteristics. The isolates were subjected to antimicrobial susceptibility testing (AST) and whole-genome sequencing (WGS) to identify resistance genes, with genotypic resistance genes detected using the ResFinder tool. The antibiotics in red denotes that the gene have been found in the bacteria genome and have expressed while other genes regarding to antibiotic resistance are present but did not express.

Table 3.**5**: Observed Hypermucoidity and Absence of Hypermucoidity Genes in *Klebsiella pneumoniae* Isolates

Sample ID	Phenotype (Biochemical observation)	Bioinformatic Analysis
	Mucoid Morphology	**Mucoid genes**
7A	Mucoid	None
19A	Mucoid	None
89C	Mucoid	None

The table provides a comparison between the phenotypic and genotypic characteristics of the isolates. Although all isolates exhibited hypermucoid morphology phenotypically, none of the known hypermucoidity-associated genes were detected genotypically.

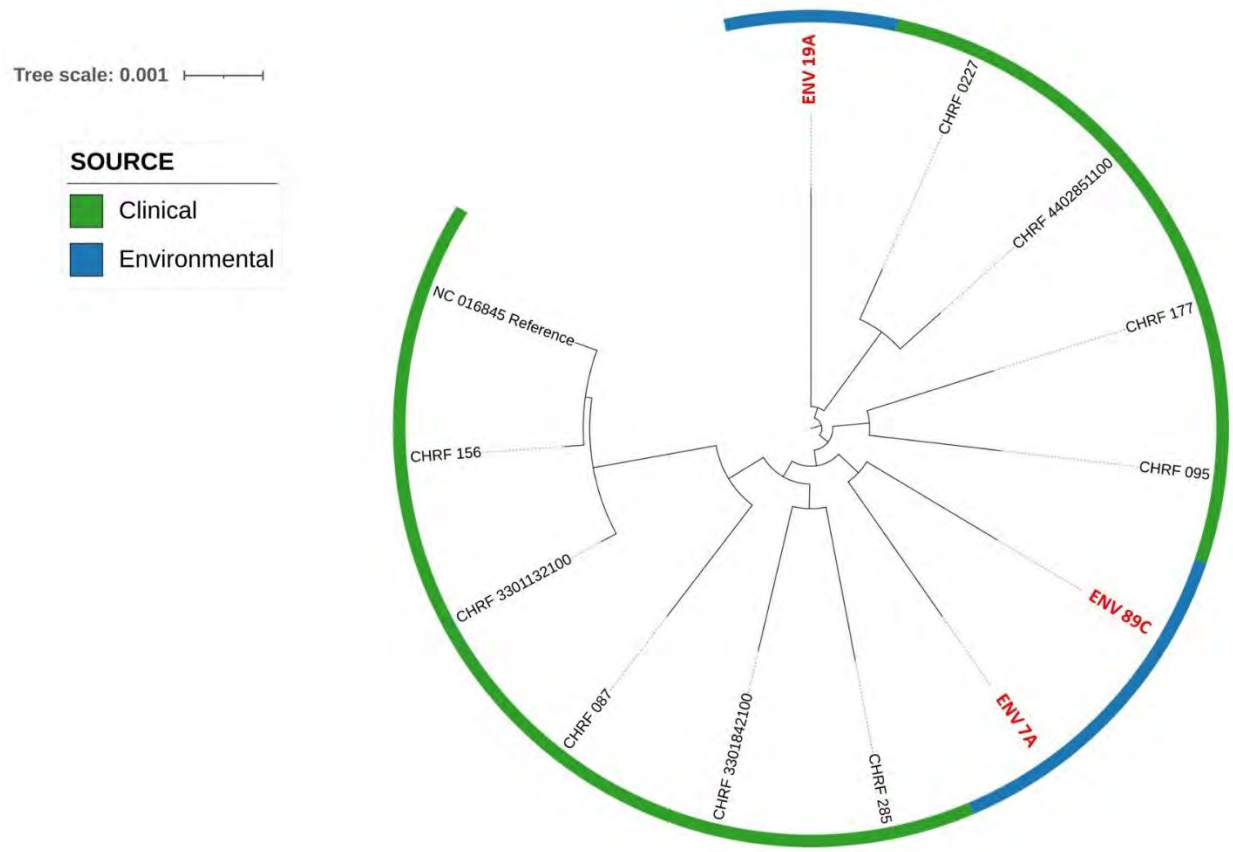

Figure 3.**3**: Phylogenetic Analysis of Environmental *Klebsiella pneumoniae* isolates.
The bold mark IDs highlighted in red are sample sequences that were generated from this study.

The phylogenetic tree illustrates the evolutionary relationships among 3 environmental *Klebsiella pneumoniae* isolates and 9 clinical *Klebsiella pneumoniae* isolates that were previously sequenced at CHRF. Also, 1 clinical reference sequence obtained from NCBI has been included for comparative purposes. This tree provides insights into the genetic similarities and differences between environmental and clinical strains of *Klebsiella pneumoniae*.

4 Discussion

This dissertation represents a comprehensive investigation into the genomic diversity, assessment of virulence factors, and profiling of genotypic and phenotypic antimicrobial resistance in *Klebsiella pneumoniae* isolated from wastewater samples collected from 7 thanas across Dhaka city. With a substantial number of samples collected and isolates obtained for the analysis of phenotypic antibiotic resistance pattern and corresponding genomic analysis, significant insights were generated about the unknown aspects of environmental *K. pneumoniae*. The phenotypic and genotypic diversity of the wastewater-derived isolates has been successfully assessed by comparing them with clinical samples. Furthermore, an in-depth evaluation of potential virulent factors present in these isolates has been conducted. Additionally, crucial insights into the resistance patterns of *K. pneumoniae* in this context have been provided through the evaluation of antimicrobial resistance profiles. Moreover, the presence of antimicrobial resistance genes and their evolutionary relationships with clinical and environmental samples have been explored through whole-genome sequencing conducted on 3 selected isolates. Through the integration of genomic data, our study aims to unravel the intricate dynamics of *K. pneumoniae* within this environmental niche, contributing to a deeper understanding of bacterial ecology and antimicrobial resistance dissemination.

A total of 140 wastewater samples were collected for the study. From each sample, three suspected *Klebsiella spp.* colonies were isolated, resulting in a preliminary collection of 420 colonies. After subculturing and observing morphological characteristics, 310 of these colonies were selected for biochemical testing. Through this testing, 180 *Klebsiella spp.* isolates were confirmed. These 180 *Klebsiella spp.* isolates subsequently underwent antimicrobial susceptibility testing (AST) for seven different antibiotics.

The data analysis from this study unveils a notable trend of low resistance levels among environmental isolates. Out of the 180 isolates examined, only 22 exhibited resistance against two or more antibiotics, accounting for 12.22% of the samples. It is noteworthy that all isolates displayed resistance against ampicillin, a finding consistent with the intrinsic resistance of the *Klebsiella spp.* to penicillins, serving as a control measure in this study (Li et al., 2023). The antibiotics to which the isolates demonstrated the highest resistance rates were ceftriaxone, cotrimoxazole, ciprofloxacin, and chloramphenicol. This observation may be attributed to the

widespread usage of these antibiotics for managing a broad spectrum of bacterial infections (Biswas, Roy, Tajmim, et al., 2014). In Bangladesh, where antibiotic misuse without proper prescription is prevalent, these antibiotics are often familiar household names compared to meropenem and amikacin, typically reserved for severe infections or as last resort treatments (Biswas, Roy, Manik, et al., 2014). Given that our wastewater samples were devoid of hospital sewage, it's plausible that the limited exposure to these last resort antibiotics contributed to the higher susceptibility rates observed. Environmental bacteria are likely subjected to selective pressure from commonly used antibiotics, fostering the development and dissemination of resistance mechanisms. Various factors such as pollution, antibiotic residues, and human activities can intensify this selective pressure, further prolonging the cycle of antibiotic resistance. Cross-resistance may also play a role in the heightened resistance observed against certain antibiotics belonging to the same class or sharing similar mechanisms of action, such as chloramphenicol and cotrimoxazole. Bacteria that evolve resistance to one antibiotic often exhibit low susceptibility to others with analogous modes of action (Christaki et al., 2020). This underscores the relationship between antibiotic usage, environmental factors, and bacterial adaptation in shaping resistance profiles among environmental bacteria. Also, a potential limitation of the study was identified in that while it suggested a variety of *Klebsiella pneumoniae* existed in the environment, the most dominant strains in the environment might have differed from those that caused infections in humans. It was plausible that some environmental strains were resistant to antibiotics but were not captured in this study due to their lower prevalence. Additionally, not every suspected *K. pneumoniae* colony from every sample was tested, which might have led to the underrepresentation of less common resistant strains.

In laboratory settings, accurate identification of *Klebsiella pneumoniae* poses a significant challenge due to limitations in current diagnostic methods (He et al., 2016). Traditional serological tests are unable to differentiate between different members of the *Klebsiella pneumoniae* Species complex, leading to misidentification and potential misinterpretation of clinical samples. Furthermore, existing PCR techniques lack specificity and sensitivity in distinguishing *Klebsiella pneumoniae* strains reliably. To address these limitations, this study designed and tested a novel primer for Sanger sequencing, aiming to improve the accuracy of *Klebsiella pneumoniae* identification. Although initial results from the primer design and sequence analysis appeared promising, it's essential to recognize the limitations of the study. The primer was tested on a small

sample size of only 14 samples, which may not adequately represent the genetic diversity present within *Klebsiella pneumoniae* populations. Additionally, while BLAST analysis and Sanger sequencing of *rpoB* genes suggested the primer's effectiveness in identifying *Klebsiella pneumoniae*, further validation through large-scale testing is necessary to confirm its efficacy across a broader range of strains and clinical samples.

The genomic analysis of three isolates derived from distinct geographic regions, each exhibiting varying patterns of antibiotic resistance, showed similarity in their genetic profiles. Despite originating from different areas, all isolates shared a common class of resistance genes. Among these, the presence of *bla*$_{SHV}$ genes (including *bla*$_{SHV}$-106, *bla*$_{SHV}$-28, *bla*$_{SHV}$-26, *bla*$_{SHV}$-157, and *bla*$_{SHV}$-62) was notable, conferring resistance against beta-lactam antibiotics such as penicillins and cephalosporins. Additionally, the fosA6 and fosA genes were identified, encoding enzymes responsible for modifying or degrading fosfomycin, a commonly used antibiotic for treating urinary tract infections. Furthermore, the presence of OqxB and OqxA genes, components of the oqxAB operon, provided resistance against a range of antibiotics, including chloramphenicol, quinolones (e.g., nalidixic acid and ciprofloxacin), and trimethoprim (a component of cotrimoxazole). However, despite the consistent genomic profile across the isolates, the phenotypic resistance patterns did not align with the genetic findings. Specifically, one of the isolates, 89C, exhibited susceptibility to all antibiotics except ampicillin in AST, contrasting with the expected resistance profile based on the detected resistance genes. Notably, the utilization of ResFinder tool for resistance gene detection may introduce biases, as it relies on a database of well-characterized antimicrobial resistance genes, potentially overlooking novel resistance determinants present in the environment (Florensa et al., 2022). Additionally, factors such as regulatory elements and mutations arising within bacterial populations may contribute to divergent phenotypic outcomes despite consistent genotypic profiles. This divergence between genotypic predictions and phenotypic observations underscores the need for further research to elucidate the mechanisms driving antibiotic resistance and to develop more comprehensive and accurate methodologies for predicting antibiotic susceptibility. Addressing these challenges is critical for informing antibiotic treatment strategies and combating the growing threat of antimicrobial resistance in clinical settings.

The virulence factors of the selected *Klebsiella pneumoniae* isolates were assessed using Kleborate, focusing on traditional virulence genes typically found in pathogenic strains (Lam et

al., 2021). The virulence score, associated with gene clusters for siderophores such as Yersiniabactin, Colibactin, Aerobactin, and Salmochelin, indicated that these clusters were mostly absent, resulting in no significant virulence marking. Furthermore, the identified K-locus and O-locus types were non-virulent, and one isolate had an unknown K-locus type, making its association with virulence indeterminate due to a lack of existing data. Despite these findings, it was notable that all isolates subjected to whole genome sequencing (WGS) exhibited hypermucoidity on culture plates, verified by the string test, which is typically associated with hypervirulent *K. pneumoniae*. Surprisingly, the common genes linked to the hypermucoid phenotype were absent in these isolates. This discrepancy suggests that the characterization of hypervirulent or hypermucoid *K. pneumoniae* strains is predominantly based on clinical isolate data. The tools used for genomic comparison rely on well-characterized databases, which can introduce biases.

Given the scarcity of global environmental data on *K. pneumoniae*, particularly in Bangladesh, it is plausible that other genes, beyond the traditional ones, contribute to the mucoid characteristics observed. Additionally, the association between the hypermucoid phenotype and virulence might not always hold true for environmental bacteria. This discrepancy indicates that environmental strains of *K. pneumoniae* may possess different genetic determinants responsible for their mucoid phenotype, which are not typically identified in clinical isolates. To comprehensively understand the divergence between these environmental isolates and their clinical counterparts, further investigation is essential. This includes exploring the underlying genetic factors responsible for the observed phenotypic differences and elucidating the environmental influences contributing to these variations. Such insights are crucial for advancing our knowledge of *K. pneumoniae* pathogenicity and the potential public health implications of these environmental strains. This research underscores the need for broader genomic databases that include environmental isolates to provide a more complete understanding of the genetic diversity and pathogenic potential of *K. pneumoniae*.

The phylogenetic analysis of 13 *Klebsiella pneumoniae* samples, including 9 clinical isolates, 1 NCBI reference sequence, and 3 environmental samples from wastewater, reveals key insights into their genetic relationships and transmission dynamics. Environmental sample 19A branches directly from the root, indicating it as the most genetically distinct sample, highlighting significant genetic diversity within environmental *K. pneumoniae*. This underscores the necessity for diverse

source monitoring to comprehensively understand the pathogen's evolution and potential reservoirs. Moreover, environmental samples 7A and 89C, forming a monophyletic group with clinical isolates, suggest potential cross-environmental transmission, necessitating integrated surveillance to effectively mitigate transmission. Clinical isolates form distinct clusters, indicating common sources or similar evolutionary processes. Understanding these relationships aids in identifying outbreak sources and implementing targeted infection control measures. Additionally, the close relationship between the NCBI reference sequence and a clinical sequence provides a benchmark for comparing clinical isolates, aiding in understanding their proximity to known strains. Overall, this analysis emphasizes the need for integrated surveillance and targeted control strategies to manage *Klebsiella pneumoniae* effectively, while advocating further studies on specific sources and transmission pathways.

5 Conclusion

In conclusion, this study represents a significant step forward in our understanding of the characteristics of *Klebsiella pneumoniae* in wastewater environments, providing valuable insights into genomic diversity, virulence factors and antimicrobial resistance. The study highlights the complex interplay between environmental factors, bacterial adaptation, and antimicrobial usage in shaping resistance patterns and genetic profiles. Moreover, it underscores the importance of integrated surveillance and continued research efforts to address the challenges posed by antimicrobial resistance in both clinical and environmental settings.

Looking ahead, future research should focus on longitudinal studies, exploration of alternative detection methods, deeper investigation of virulence factors, and collaboration across disciplines to develop effective strategies for surveillance and control of *K. pneumoniae* infections and antimicrobial resistance. By addressing these challenges and building upon the findings of this dissertation, we can advance our understanding of bacterial ecology, improve patient outcomes, and mitigate the public health threat posed by antimicrobial resistance.

6 References

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