ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE TARGETING MULTIDRUG-RESISTANT *KLEBSIELLA PNEUMONIAE* FROM WASTEWATER

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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 Bachelor of Science in Biotechnology

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Declaration

It is hereby declared that

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

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Ethics Statement

This research is done under proper supervision and it is the author's original work. No animal was harmed during experiments. The article is written in a manner that the writeup does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing. The experiment was done by maintaining all the rules and regulations of the Biotechnology and Microbiology laboratory of the Department of Mathematics and Natural Sciences, BRAC University.

Abstract

The emergence of multidrug-resistant *Klebsiella pneumoniae* has become a major global concern due to its rapid dissemination and limited treatment options. This study aimed to isolate and characterize bacteriophages from wastewater samples targeting MDR *K. pneumoniae*. Blue colonies on HiCrome KPC agar indicated carbapenemase-producing isolates. Six distinct lytic phages were recovered using the double-layer agar method, and among them, phage 1.3B exhibited the most stable and consistent lytic profile. The phage was tested for stability across various NaCl concentrations (0.5–5%), demonstrating optimal activity at 0.9% salinity. PCR confirmed the presence of resistance-associated genes, including blaNDM, and *blaTEM and* virulent gene *iroB*. These findings highlight the potential of naturally occurring phages as alternative biocontrol agents against MDR *K. pneumoniae* and provide a foundation for further genomic and therapeutic evaluation.

Keywords: Bacteriophage; *Klebsiella pneumoniae*; Antibiotic resistance; Wastewater; Phage therapy

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Dedication

We dedicate this work to our loving parents, whose endless sacrifices, care, and prayers have been the light that guided us through every step. Their faith in us has given strength when things felt difficult and joy in every small success. To our families, who stood beside us with patience and encouragement, and to our teachers and friends, who inspired us with their wisdom, kindness, and belief in our potential - we owe every part of this journey to you. This achievement belongs as much to you as it does to us.

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Table of Contents

List of Tables

List of Figures

List of Acronyms

Acronym	Full Form
AMR	Antimicrobial Resistance
ATCC	American Type Culture Collection
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic Acid
DLA	Double-Layer Agar
KPC	*Klebsiella pneumoniae* Carbapenemase
LB	Luria–Bertani
MDR	Multidrug Resistant
NaCl	Sodium Chloride
NDM	New Delhi Metallo-β-lactamase
OXA	Oxacillinase
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
SM	Sodium-Magnesium Buffer
T1N1	Tryptone 1 Nutrient 1 (Preservation Medium)
WHO	World Health Organization

Chapter 1
INTRODUCTION

1.1 Antibiotic Resistance

One of the greatest medical contributions to the science of modernity is the antibiotics. They are saving lives since the time they were discovered and they are not doing any further harm. Nevertheless, it has gradually acquired a resistance to treatment in bacteria in the past several decades. The phenomenon is known as resistance to antibiotics is when bacteria become able to survive and reproduce in places that contain antibiotics that would otherwise cause death or inhibition. It may occur due to random mutation of bacterial DNA, or it may be the acquisition of the resistance genes in other bacteria by horizontal gene transfer (Ghosh et al., 2020).

As the existing statistics show, over 1.2 million people annually die because of the infections that cannot be treated with the existing drugs, and the number may even reach 10 million by 2050 (Antimicrobial Resistance Collaborators, 2022). The epidemic has been further intensified by the abusive use of antibiotics that entails unnecessary prescribing of antibiotics, failure to take the full course treatment, and use in the farming industry. The environment also contributes significantly to this issue since the release of waste of hospitals and industries which include antibiotics and resistant bacteria into the natural world causes the interchange of genes among bacteria (Parnanen et al., 2019).

The mechanism of resistance is somewhat divided into a few categories, including the modification of the antibiotic target, the inactivating enzymes, the variations of membrane permeability, and the efflux pumps. Over the past few years, these mechanisms, when accumulated in individual bacterial strains, have resulted in the emergence of MDR and extensively drug-resistant (XDR) pathogens. Their development and dissemination across the globe underscores the need to consider natural options such as bacteriophages that have evolved with the bacteria over the course of billions of years and continue to be one of the most effective natural controls over bacterial population.

1.2 *Klebsiella pneumoniae*

Klebsiella pneumoniae is an encapsulated, non-motile, Gram-negative bacterium of the family, Enterobacteriaceae. It is widespread in soil, water and mucus of human beings and animals. Although it is a normal microbiota, it may trigger extensive infections in case of entering sterile parts of the body or those with weakened immunity. *K. pneumoniae* is a significant cause of

pneumonia, bloodstream infections, urinary tract infections, wound infection, and neonatal sepsis (Podschun & Ullmann, 2018).

A characteristic of *K. pneumoniae* is its polysaccharide capsule that protects the bacterium against phagocytosis as well as boosts its virulence. The bacterium, too, develops biofilms- a multicellular community surrounded by an extracellular matrix- that protect against disinfectants, immune reactions, and antimicrobial agents (Di Domenico et al., 2020). Furthermore, *K. pneumoniae* has a long-term survival on hospital surfaces and hospital equipment making it one of the most common causes of healthcare-associated infection.

K. pneumoniae has recently been added to the WHO list of pathogens of critical priority, on which new antimalarial approaches are urgently required (WHO, 2017). Carbapenem-resistant *K. pneumoniae* (CRKP) is a problem that has given a significant therapeutic challenge in the world. These strains usually harbor several resistance determinants which may be transferred across the bacteria causing complex outbreaks in the hospital environment (Navon-Venezia et al., 2017). Unregulated use of antibiotics coupled with poor infection control measures also contribute to the problem in most developing nations, such as Bangladesh.

K. pneumoniae is a perfect model in the study of bacterium-based biocontrol because of its flexibility and capacity to survive in both sick and wild reservoirs. Wastewater, especially, has offered a habitat whereby clinical and environmental strains co-exist such that researchers can isolate a wide range of bacteriophages with the ability to target resistant strains.

1.3 Bacteriophage

Bacteriophages otherwise known as phages are viruses which are specific to bacteria and replicate in them. They are one of the most prolific biological objects on Earth, and the number of particles is estimated to be 10 31 particles on the planet (Suttle, 2017). Phages exhibit a very wide range of morphology and genetic makeup although they all contain a simple structure of a nucleic acid core within a protein capsid. Others carry fibers on their tails which cause them to adhere to surfaces of bacterial cells leading to infection.

Phages have two replication patterns, lytic and lysogenic. Lytic phages invade bacterial cells, take over metabolically and lyse cells releasing progeny phages into the environment. Lysogenic phages incorporate their genetic material into the bacterial chromosome forming a prophage that enters a passively replicated state until environmental signals promote the transition to the lytic phase (Young, 2019).

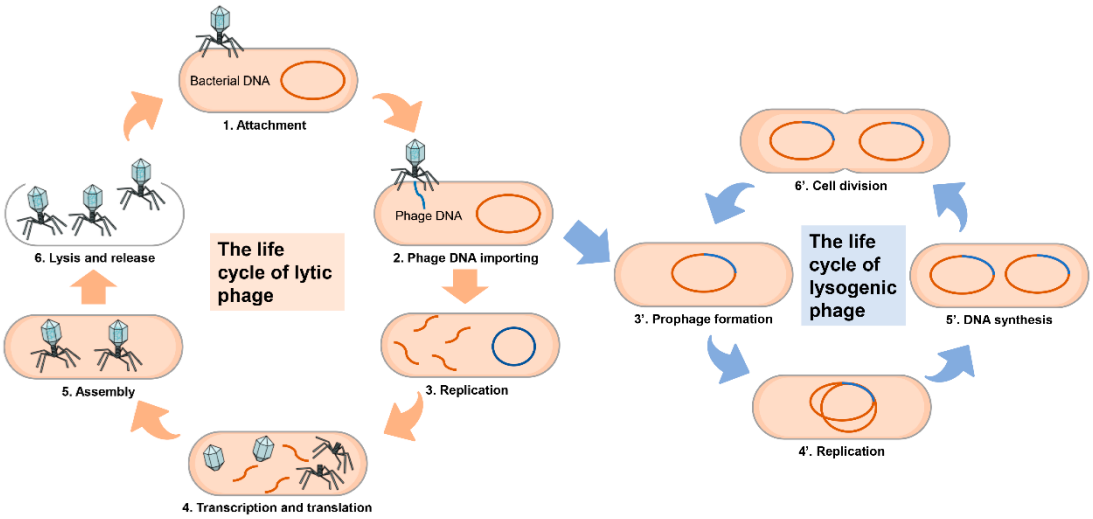

Figure 1: Bacteriophages Life Cycle (Tu et al., 2023)

Phages are very selective due to their host specificity as they target bacterial species or even particular strains. It is this specificity that can be used both therapeutically and environmentally. The process of isolating phages can be conducted in any environment containing bacteria- especially in sewage, wastewater and in contaminated soils that have high density of bacteria. This quality of their persistence and co-evolution with bacterial populations provides them with a special scope of interaction with chemical antimicrobials, which in many cases lose their effectiveness over a long period.

1.4 Phage Therapy

Phage therapy is the practice of using bacteriophages as a means to fight off bacterial infections. The method was first investigated during the early 20th century but was neglected after antibiotics took over. Nevertheless, the global upsurge of multi-drug-resistant organisms has brought phage therapy back to the laboratory and clinic (Lin et al., 2017). Phages are different from broad-spectrum antibiotics as they have an exceptional and precise mode of action; the pathogens are killed while the friendly microbes remain unaffected.

Clinical and experimental studies of recent time have indicated that the use of bacteriophages has been very successful in the case of pathogens that are resistant to multiple drugs (for example, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*) (Wang et al., 2022). Not only this, but phages have also the quality of self-genesis at the place of infection, and their number goes up as long as the bacteria of the kind that they attack are present. The moment the infection is eradicated, phages will die off naturally, and thus, the side effects will be lessened.

The overall safety of phage therapy has been recognized by several compassionate use cases and clinical trial applications (Uyttebroek et al., 2023). Besides, the actual integration of phage-based products in hospitals, water treatment, and food safety areas reflects their multifaceted nature as microbial control agents. On the other hand, the quality of the standardization, stability, and determination of host range still poses obstacles. Therefore, it is necessary to isolate and characterize phages systematically over the therapeutic or environmental applications.

1.5 Isolation and Characterization of Bacteriophage

Wastewater is a very rich source of bacteriophages as it provides plenty of bacteria to host the viruses and it also has organic materials that help the viral growth. Usually, the first step in phage isolation is the sample enrichment where environmental samples are combined with the specific bacterial hosts and they are put in a nutrient medium to allow the phages to multiply. After the incubation, the mixture is filtered through fine membranes that separate the bacterial cells from the phages and leave behind a clear filtrate that contains the phages (Ahemad et al., 2021).

In the process of characterization, the analysis of phage morphology, host range, and environmental stability is involved. The microscopic examination through electron microscopy can show the isolates phage's structural details, while the morphology of the plaque in the double-layer agar assay indicates the lytic efficiency of the phage. Tests of environmental stability such as exposing the phage to different pH and temperature values help to measure the phage's strength for practical use (Cai et al., 2019).

The isolation of phages from wastewater allows researchers to study the naturally occurring viruses that can infect resistant strains of *K. pneumoniae*. The phages could provide valuable information about the interaction between bacteria and phages and might even be used as a source of future therapeutic or biotechnological applications.

1.6 Plaque Assay

The plaque assay, also referred to as the double-layer agar (DLA) technique, is the most reliable method for measuring the activity of viruses. First, the process mixes bacterial culture together with phage suspensions that have been diluted serially and then puts the whole mixture on top of the nutrient agar. Bacteria are gradually infected by the phages during incubation, and eventually, round clear areas that can be seen to the naked eye are formed where the plaques

are (Adams, 1959; updated protocol adapted by Kutter, 2018). The number of the resulting plaques is equivalent to the number of singular infectious viral particles, thus allowing the determination of their number in PFU/mL (plaque-forming units per milliliter).

Plaque features like size, transparency, and border sharpness are indicators of the phage's replication and its interaction with the host. The presence of clear plaques is typically a sign of the virulent (lytic) types of phages, whereas the presence of turbid plaques indicates the temperate types. The counting of phages by plaque assay method is extremely important for the purpose of standardization of phage titers and for the conducting of subsequent experiments including testing of phage stability and molecular analysis. Apart from that, the method gives a visual representation of bacterial lysis thereby strengthening the idea of phage infectivity.

1.7 Antibiogram

An antibiogram is one of the diagnostic methods that determine human pathogenic bacteria's susceptibility to different kinds of antibiotics. It helps classify the bacterial isolates in three categories: sensitive, intermediate, or resistant, depending on the inhibition zone formed around the antibiotic discs placed on the inoculated agar plates. The results not only help the clinicians to choose the right antibiotics but also give the researchers a profile of multidrug resistance (Clinical and Laboratory Standards Institute [CLSI], 2021).

For *K. pneumoniae*, antibiogram testing can show how resistant bacteria are in the case of beta-lactams, aminoglycosides, fluoroquinolones, and carbapenems which are the most common antibiotic classes. It further lays down the ground for strain selection targeted for phage isolation and testing. The researchers by connecting resistance patterns with phage susceptibility could find out if the phages still work on the highly resistant isolates which is the very point that is going to decide the future of therapeutic development.

1.8 DNA Extraction and PCR

Molecular techniques are pivotal for the confirmation of bacterial and phage identity. The first step in DNA extraction is breaking the bacterial or the phage particles to release nucleic acids, and then purification goes on to eliminate proteins and other contaminants. The boiling method is widely used for quick DNA extraction in microbiological research because it is simple and cheap.

Polymerase Chain Reaction (PCR) is a method that allows the amplification of specific DNA fragments to confirm bacterial identity or detect genes related to antibiotic resistance and phage existence. Duplicating cycles of denaturation, annealing, and extension lead to exponential amplification of the target sequence (Mullis, 2020). In case of bacterial characterization, the presence of genetic markers associated with virulence could be pointed out through PCR.

To visualize the PCR products, gel electrophoresis is performed right after amplification. DNA fragments move through an agarose gel, which is under an electric field, and are separated according to their size. Bands that represent amplified products give molecular proof of the successful DNA extraction and amplification. The combination of PCR and electrophoresis is the core technique used for molecular identification in microbiological research.

Eventually, the present study endorses the general idea of using biotechnological advances for solving health problems. The results of this study are likely to be a starting point for further studies on therapeutic phages, as they will provide the needed information about the position of phages as eco-friendly substitutes for regular antibiotics in both hospital and nature settings.

1.9 Literature Review

Klebsiella pneumoniae is an opportunistic, Gram-negative bacterium recognized as a critical global health threat due to its multidrug-resistant and hypervirulent lineages (Navon-Venezia et al., 2017). Clinically, it causes a wide range of severe infections including lower respiratory tract infections, bacteremia, pyogenic liver abscess with metastatic spread (invasive syndrome), complicated urinary tract infections, and device-associated biofilm infections (Podschun & Ullmann, 2018; Russo & Marr, 2019). These conditions are often linked to high mortality and morbidity rates, particularly in hospitalized or immunocompromised patients. The pathogen's clinical resilience arises from three principal virulence determinants: (a) extensive polysaccharide capsule variability (K-types) that masks cell-surface receptors and promotes immune evasion, (b) biofilm formation on host tissues and medical devices that protects against antimicrobials and immune clearance, and (c) horizontal acquisition of multidrug resistance genes such as *blaNDM*, *blaKPC*, and *blaOXA-48*, conferring extended-spectrum beta-lactamase (ESBL) and carbapenemase activity (Martin & Bachman, 2018; Wyres & Holt, 2020). These combined features have rendered conventional antibiotic therapy increasingly ineffective, driving global interest in targeted biologics such as bacteriophages and phage-derived enzymes (Kortright et al., 2019).

Environmental and clinical sampling studies have demonstrated remarkable diversity of *K. pneumoniae*-specific bacteriophages, primarily within the *Myoviridae*, *Siphoviridae*, and *Podoviridae* families (Drulis-Kawa et al., 2012; Majkowska-Skrobek et al., 2018). Many of these phages encode capsule-degrading depolymerases embedded in their tailspike or receptor-binding proteins (RBPs), which enzymatically cleave capsular polysaccharides, exposing underlying receptors for phage adsorption and infection (Pan et al., 2017; Hsu et al., 2021). This mechanism also disrupts biofilm integrity, thereby sensitizing encapsulated and biofilm-embedded bacterial cells to both antibiotics and innate immune factors (Li et al., 2023). The specificity of phage infection is largely determined by the molecular structure of these RBPs, which recognize discrete capsule types or lipopolysaccharide (LPS) motifs. Consequently, phage host range is often narrow and capsule serotype-dependent, necessitating personalized or cocktail-based therapeutic strategies (Frontiers in Microbiology, 2022). Mechanistically, *K. pneumoniae*-infecting phages follow several well-characterized infection modules: **(a) Adsorption and receptor recognition** — Phage RBPs first identify specific bacterial surface receptors, such as capsule polysaccharides, LPS, or outer membrane proteins, establishing initial contact and defining host range (Chernomordik, 2021). **(b) Capsule depolymerase activity** — Many *Klebsiella* phages produce depolymerases that degrade the capsule layer, facilitating irreversible adsorption and infection. These enzymes not only enable infection but also possess independent antimicrobial potential by dismantling biofilms and reducing virulence (Latka et al., 2017; Hsu et al., 2021). **(c) Genome injection, replication, and cell lysis** — Once attached, phages inject their nucleic acid into the bacterial cytoplasm, replicate using host machinery, and release progeny through the coordinated action of holins and endolysins (Drulis-Kawa et al., 2019). These lytic enzymes-especially endolysins-are being developed as stand-alone enzybiotics for Gram-negative pathogens when used with outer membrane permeabilizers (Briers & Lavigne, 2015). **(d) Biofilm penetration and dispersal** — Phage-encoded polysaccharide lyases and depolymerases promote biofilm degradation, allowing deep penetration and active replication within biofilm cells, effectively amplifying local phage concentrations and synergizing with antibiotics (Wu et al., 2021; Frontiers in Cellular and Infection Microbiology, 2023).

The therapeutic potential of phages against MDR *K. pneumoniae* has been supported by multiple in vitro, in vivo, and clinical case studies. Experimental phage therapy has shown efficacy in reducing bacterial load, clearing biofilms, and improving survival in animal infection models (Wang et al., 2022; Uyttebroek et al., 2023). Compassionate-use clinical

applications have demonstrated successful outcomes in patients with refractory *K. pneumoniae* infections, particularly when combined with antibiotics in phage–antibiotic synergy (PAS) approaches (Kou et al., 2024). However, several translational challenges remain—narrow host range, rapid emergence of phage-resistant bacterial mutants, immune neutralization, and regulatory hurdles for biologics. To mitigate these issues, rigorous genomic characterization of therapeutic phages is essential to exclude lysogenic, toxin, or antimicrobial resistance genes (Pirnay et al., 2022).

In summary, bacteriophages offer a scientifically validated and ecologically sustainable alternative for controlling multidrug-resistant *K. pneumoniae*. Their specificity, adaptability, and synergistic compatibility with antibiotics position them as leading candidates in the development of next-generation biologics. Continued exploration of environmental phage reservoirs, combined with genomic and structural characterization, will be vital for advancing precision phage therapy and biofilm-targeted therapeutics in the post-antibiotic era.

Chapter 2
Materials and Methods

2.1 Sample Collection

Wastewater samples were collected from Shahjahanpur Jheel Par, an urban drainage canal located in Dhaka, Bangladesh. The site receives mixed municipal and industrial discharges, creating a rich microbial environment suitable for isolating multidrug-resistant bacteria and their corresponding bacteriophages. Sterile screw-capped glass bottles were used to collect the samples aseptically from five different points of the canal at a depth of approximately 10–15 cm. The samples were stored at 4 °C and processed on the same day to minimize the loss of viable phages and bacterial flora.

2.2 Isolation and Identification of *Klebsiella pneumoniae*

Isolation of *Klebsiella pneumoniae* was performed from the collected wastewater samples using selective HiCrome KPC agar. The wastewater was first passed through a 0.45 µm sterile syringe filter to remove coarse particles and reduce background microbial load, allowing easier isolation of target organisms. A 100 µL aliquot of the filtrate was then aseptically spread over the surface of the HiCrome KPC agar plate using a sterile glass spreader. The plates were incubated at 37 °C for 24 hours under aerobic conditions. Distinct blue colonies indicate carbapenemase-producing *K. pneumoniae* confirmed through selective chromogenic differentiation.

2.3 Preservation and Maintenance of Bacterial Culture (T1N1 Medium with Paraffin Oil Sealing)

Confirmed *Klebsiella pneumoniae* colony was first streaked on Nutrient Agar (NA) and incubated at 37 °C for 24 hours to obtain discrete, well-developed colonies and verify purity. A freshly grown colony from this NA plate was then preserved in T1N1 semi-solid medium, which supports long-term maintenance of bacterial cultures. Using a sterile inoculating needle, the colony was carefully stabbed vertically into the center of the T1N1 medium to create a deep, straight inoculation channel. After inoculation, a thin layer of sterile liquid paraffin oil was gently poured over the surface of the medium to form an airtight seal, preventing desiccation and contamination while maintaining culture viability. The mouth of each tube was then securely wrapped with parafilm, providing an extra protective barrier against air entry and moisture loss.

The prepared tubes were stored at room temperature for short-term preservation and at 4 °C for long-term storage. When required for further experimental use, a sterile needle was used to aseptically transfer growth from the stab culture onto fresh HiCrome KPC agar or Nutrient Agar plates. This technique ensured the continuous availability of a viable and uncontaminated *Klebsiella pneumoniae* culture throughout the study period.

2.4 DNA Extraction of Bacterial Isolate (Boiling Method)

Genomic DNA of the isolated *Klebsiella pneumoniae* was extracted using the conventional boiling method, which provides a rapid and reliable source of template DNA for molecular assays. A single, freshly grown colony from the Nutrient Agar plate was inoculated into 3 mL of sterile Luria-Bertani (LB) broth and incubated overnight at 37 °C with gentle shaking to obtain an actively growing culture. After incubation, 1 mL of the culture was transferred into a sterile microcentrifuge tube and centrifuged at 12,000 rpm for 5 minutes to pellet the bacterial cells. The supernatant was discarded carefully, and the pellet was resuspended in 200 µL of sterile nuclease-free water. The suspension was mixed gently by vortexing to ensure uniform dispersion of cells. The tube was then placed in a dry-bath heater at 100 °C for 10 minutes to lyse the bacterial cells and release intracellular contents. Immediately after heating, the tube was transferred onto ice for rapid cooling, allowing cellular debris to aggregate. The lysate was centrifuged again at 12,000 rpm for 10 minutes to separate the cell debris. The resulting clear supernatant containing crude genomic DNA was carefully collected into a new sterile microcentrifuge tube without disturbing the pellet. The extracted DNA was directly used as the template for polymerase chain reaction (PCR) amplification and stored at −20 °C for later use

2.5 Polymerase Chain Reaction (PCR) for Target Gene Amplification

Polymerase Chain Reaction (PCR) was employed to amplify antibiotic-resistance and virulence-associated genes of the isolated *Klebsiella pneumoniae*. The DNA template obtained by the boiling method was used directly in the amplification reaction. The PCR mixture was prepared in a total volume of 25 µL, consisting of: 12.5 µL of PCR Master Mix , 1 µL of forward primer, 1 µL of reverse primer, 2 µL of template DNA, and 8.5 µL of nuclease-free water to complete the reaction volume. All reagents were briefly mixed and centrifuged to collect the contents at the bottom of the tube before loading into the thermal cycler. Amplification was performed in a programmable thermocycler . The PCR amplification was carried out under the following thermal cycling conditions: an initial denaturation at 95 °C for 5 minutes to ensure complete separation of the DNA strands, followed by 30–35 cycles of denaturation at 95 °C for 30

seconds, annealing at 55 °C for 30 seconds (optimized according to the specific primer requirements listed in Table 2.1), and extension at 72 °C for 45 seconds to allow DNA synthesis. After the cycling steps, a final extension was performed at 72 °C for 5 minutes to ensure complete elongation of all PCR products, and the reaction was then held at 4 °C indefinitely for preservation until further analysis.

Table 1: PCR Primers Used for Detection of Antibiotic Resistance Genes in Klebsiella pneumoniae

Gene	Target Function	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Expected Product (bp)	Annealing Temp (°C)	Reference Source (in-text)
ompA	Outer-membrane protein A gene involved in virulence and adhesion	GTTAAAGGCGACGTAGACG	CCAGTGTTATCTGTGTGACC	~350	55	(Al-Marzooq et al., 2015)
blaOXA-2	OXA-type β-lactamase conferring cephalosporin resistance	TTCAAGCCAAAGGCACGATAG	TCCGAGTTGACTGCCGGGTTG	704	60	(Oliver et al., 2002)
blaTEM	ESBL gene conferring resistance to β-lactams	AAAATTCTTGAAGACG	TTACCAATGCTTAATCA	980	50	(Oliver et al., 2002)
blaVIM-2	Metallo-β-lactamase responsible for carbapenem resistance	AAGTTATGCCGCACTCACC	TGCAACTTCATGTTATGCCG	502	55	(Oliver et al., 2002)
blaKPC	KPC-type carbapenemase hydrolyzing β-lactams	GATACCACGTTCCGTCTGG	GGTGTTTGGCGTAGTCCG	798	58	(Al-Marzooq et al., 2015)

Continued,

Gene	Target Function	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Expected Product (bp)	Annealing Temp (°C)	Reference Source (in-text)
blaIMP	Metallo-β-lactamase gene encoding carbapenem resistance	TCGTTTGAAGAAGTTAACG	GTATGTTTCAAGAGTGATGC	568	60	(Al-Marzooq et al., 2015)
blaNDM	New Delhi Metallo-β-lactamase; broad carbapenemase activity	ACCGCCTGGACCGATGACCA	GCCAAAGTTGGGCGCGGTTG	624	60	(Al-Marzooq et al., 2015)
blaPER-2	PER-type ESBL gene hydrolyzing broad-spectrum cephalosporins	GCAACTGCTGCAATACTCGG	ATGTGCGACCACAGTACCAG	854	55	(Al-Marzooq et al., 2015)
iroB	Iron-uptake and virulence gene associated with siderophore production	ATGAAAAAGACTGTCTTCCA	TTAGTGCTTGCCGCTTCTT	235	55	(Russo et al., 2018)

The primer sequences, expected product sizes, and annealing temperatures for each resistance gene are summarized in **Table 1** (Al-Marzooq et al., 2015; Thermo Fisher Scientific, 2023).

At the end of the amplification cycle, the reaction tubes were removed and stored at 4 °C until electrophoretic analysis. The amplified PCR products were subsequently analyzed by agarose gel electrophoresis to confirm successful amplification of the target genes.

2.6 Agarose Gel Electrophoresis

The amplified PCR products were analyzed by agarose gel electrophoresis to confirm successful amplification of the target gene. A 1.5 % agarose gel was prepared by dissolving 1.5 g of agarose powder in 100 mL of 1× TAE (buffer) and heating the mixture until the agarose was completely melted. After cooling the solution to approximately 55 °C, ethidium bromide was added at a final concentration of 4 µL. The molten gel was poured into a casting tray fitted with combs and allowed to solidify at room temperature.

Once the gel had set, it was carefully placed in the electrophoresis chamber containing 1× TAE running buffer. The PCR-amplified DNA samples were directly loaded into the wells without adding any separate loading dye, as the gel was already pre-stained with ethidium bromide. A DNA ladder was included alongside the samples to indicate molecular size markers.

Electrophoresis was carried out at a constant voltage of 100 V for approximately 45 minutes, allowing clear separation of the amplified fragments. Following electrophoresis, the gel was removed from the chamber and viewed under a UV transilluminator to visualize the fluorescent DNA bands. The gel image was subsequently photographed for documentation and stored for reference. No interpretation or comparison of band size was presented in this section.

2.7 Antibiotic Susceptibility Testing (Antibiogram)

The antibiotic resistance pattern of the *K. pneumoniae* isolate was determined using the Kirby–Bauer disc diffusion method on Mueller–Hinton agar (MHA). An overnight bacterial culture was diluted to match the 0.5 McFarland standard. The bacterial suspension was evenly spread over the surface of sterile MHA plates using a sterile cotton swab to ensure uniform lawn formation.

Antibiotic discs representing major drug classes-including β-lactams, aminoglycosides, fluoroquinolones, and carbapenems-were placed on the inoculated agar surface using sterile forceps. The plates were incubated at 37 °C for 18–24 hours. After incubation, clear zones of inhibition were observed around certain discs, while others displayed growth up to the disc edge, indicating resistance. The diameters of inhibition zones were measured and compared with CLSI (2021) guidelines to categorize the isolate as resistant or susceptible. The obtained data confirmed the multidrug-resistant nature of the isolate but were not interpreted further in this chapter.

2.8 Isolation of Bacteriophages from Wastewater (Enrichment and Filtration)

Bacteriophages specific to *Klebsiella pneumoniae* were isolated from the same wastewater samples used for bacterial isolation. The enrichment method was employed to increase phage concentration. Ten milliliters of wastewater were mixed with an equal volume of Luria–Bertani (LB) broth and an overnight *K. pneumoniae* culture. The mixture was incubated at 37°C for 24 hours in a shaking incubator at 120 rpm to allow phage replication

After incubation, the culture was centrifuged at 10,000 rpm for 10 minutes to remove bacterial debris. The supernatant containing potential phages was filtered through a 0.22 µm syringe filter to eliminate any remaining bacterial cells. The resulting filtrate represented the crude phage lysate and was stored at 4 °C for further analysis.

2.9 Plaque Purification and Enumeration (Double-Layer Agar, Spot Test on LA Base, up to 10^{-8} Dilution)

Plaque assay was performed using the double-layer agar (DLA) method on Luria agar (LA) plates. The procedure began by preparing serial tenfold dilutions (10^{-1} to 10^{-8}) of the crude phage lysate in LB. For each dilution, phage suspension was mixed with an overnight *K. pneumoniae* culture and molten soft agar (0.6 %).

The mixture was gently swirled to ensure homogeneity and then carefully poured over the pre-solidified LA base plate, forming an even top layer. Once solidified, the plates were incubated at 37 °C for 18–24 hours. Circular clear zones, known as plaques, appeared on the bacterial lawn, indicating lysis of host cells by phages.

2.10 Host Range Determination (Using Five Environmental Bacterial Isolates)

The host range of the selected phage was assessed by testing its lytic activity against a set of labeled *Klebsiella* isolates obtained from the laboratory collection. Then cultured in HiCrome KPC agar to confirm that its *Klebsiella pneomoniae*. After that performed DLA method.

2.11 Temperature and NaCl Stability Tests (−20 °C to 80 °C; 0.5 %–5 % NaCl)

To examine the stability of phage 1.3B under varying temperature and salinity conditions, controlled stability assays were conducted.

For thermal stability, 1 mL aliquots of the phage lysate were subjected to a temperature range of −20 °C, 4 °C, 25 °C, 37 °C, 50 °C, 60 °C, 70 °C, and 80 °C for one hour. After exposure, samples were cooled to room temperature and stored at 4 °C until tested for infectivity using plaque assay.

For NaCl stability, the phage lysate was incubated in sterile saline adjusted to different concentrations (0.5 %, 0.9 %, 1 %, 2 %, 3 %, 4 %, and 5 % NaCl). Each mixture was incubated at 37 °C for one hour, followed by assessment of viability through the DLA method. These procedures allowed observation of phage stability under varying osmotic and temperature conditions.

Chapter 3

Results

A single *Klebsiella pneumoniae* isolate was successfully obtained from wastewater and preserved for further analyses. PCR confirmed the presence of multiple resistance and virulence genes. The isolate exhibited multidrug resistance in antibiotic susceptibility testing. Bacteriophages specific to the isolate were recovered from wastewater, among which six distinct phages were isolated and one, phage 1.3B, was selected for further characterization based on its clear and consistent plaque morphology.

3.1 Isolation and Preservation of *Klebsiella pneumoniae*

100 µL aliquot of the filtrate was then aseptically spread over the surface of the HiCrome KPC agar plate using a sterile glass spreader. The plates were incubated at 37 °C for 24 hours under aerobic conditions. After incubation, distinct blue colonies appeared on the agar surface. According to the chromogenic differentiation principle of HiCrome KPC medium, *Klebsiella pneumoniae* hydrolyzes specific chromogenic substrates incorporated in the agar, producing a characteristic blue coloration. This color reaction results from β-glucosidase enzyme activity unique to this genus within the Enterobacteriaceae family. Therefore, the presence of blue colonies on HiCrome KPC agar is considered presumptive evidence of *K. pneumoniae* growth.

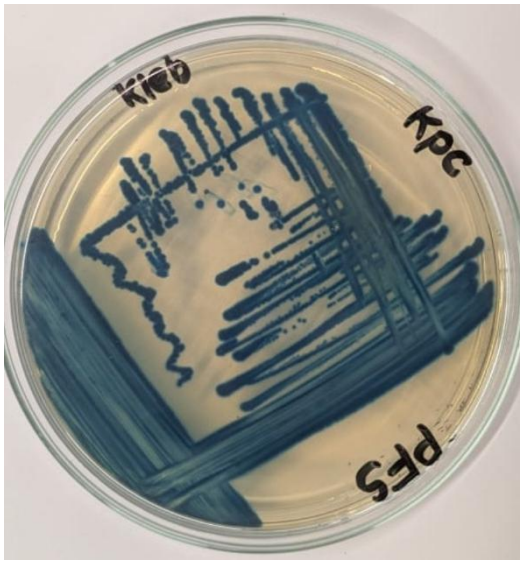

Figure 2: Streak plate showing blue colonies of Klebsiella pneumoniae on HiCrome KPC agar.

A single blue colony was selected, streaked for purity, and subcultured on Nutrient Agar. The isolate was preserved in T1N1 stab medium, covered with paraffin oil, and the tube cap sealed with parafilm for long-term maintenance.

3.2 PCR Amplification of Resistance and Virulence Genes

Species-specific amplification was first performed to confirm the identity of the isolate, yielding a distinct 130 bp band. PCR amplification of nine target genes was subsequently conducted. Positive amplification was obtained for blaNDM (~624 bp), blaTEM (~980 bp), and iroB (~235 bp). No visible bands were detected for blaOXA-2, blaVIM, blaIMP, or blaPER under identical conditions.

All amplified products were visualized on a 1.5 % agarose gel stained with ethidium bromide.

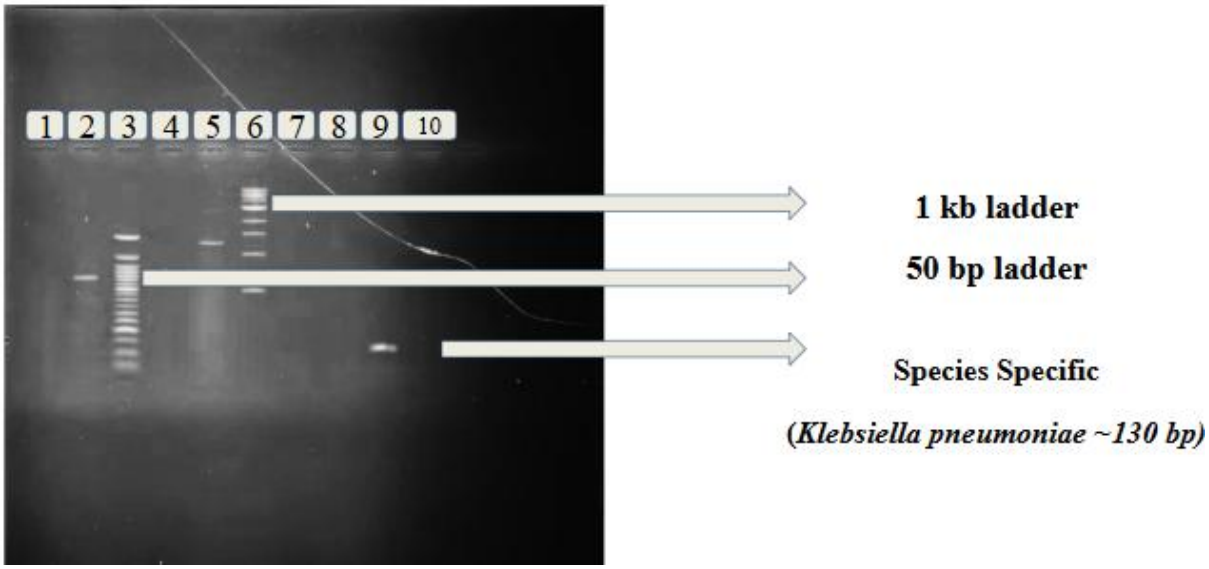

Figure 3: PCR of Klebsiella Species-specific pneumoniae

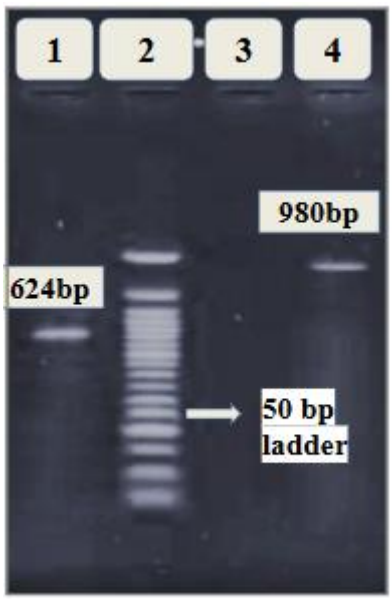

Figure 4: : PCR amplification of blaNDM (Lane 1 ~624 bp) and blaTEM (Lane 4 ~980 bp)

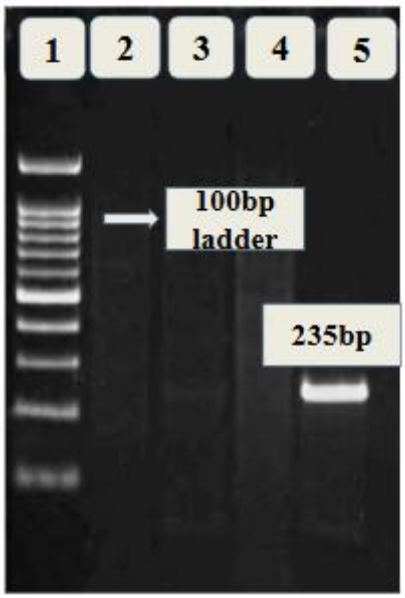

Figure 5: PCR amplification of virulent gene iroB (~235bP)

3.3 Antibiotic Susceptibility Pattern

Antibiotic susceptibility testing of the *Klebsiella pneumoniae* isolate revealed variable resistance to different antibiotic classes. Out of the tested agents, the isolate was resistant to most β-lactams and cephalosporins, while retaining susceptibility to several quinolones, tetracyclines, and carbapenems.

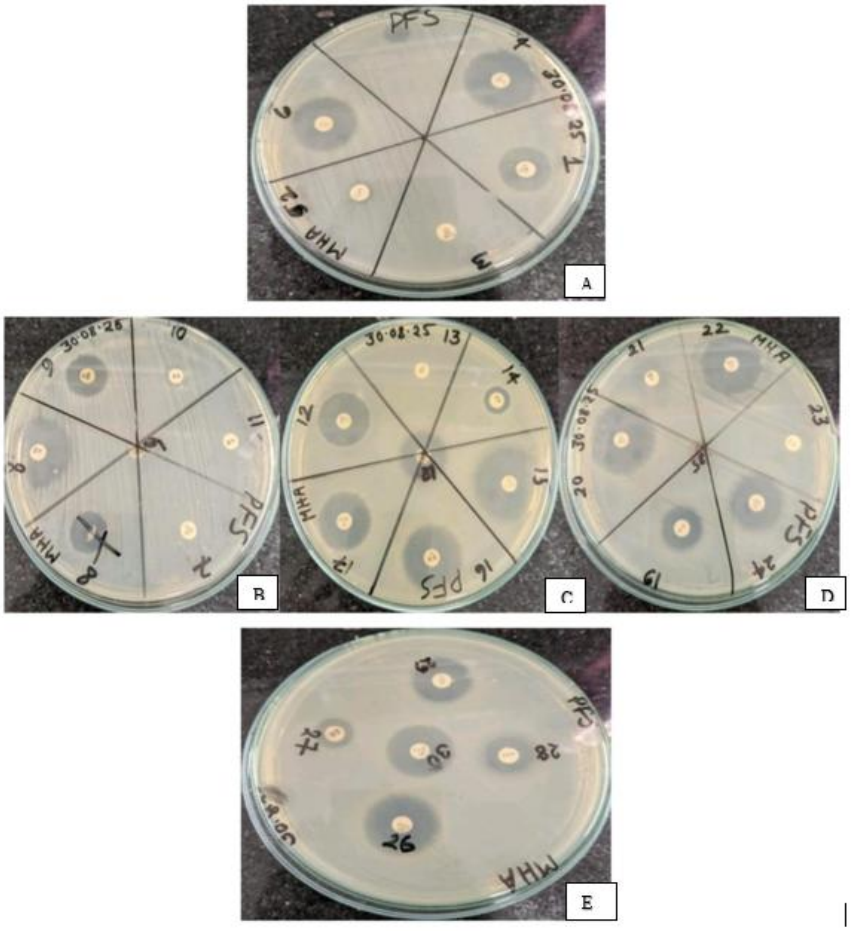

Figure 6: Antibiotic susceptibility testing of Klebsiella pneumoniae using multiple antibiotic discs on Mueller–Hinton agar (A-E).

Zones of inhibition of varying diameters demonstrate differential susceptibility to tested antibiotics according to CLSI standards. A detailed summary of inhibition-zone diameters and interpretations is presented in **Table 2**.

Table 2: Antibiotic Susceptibility Profile of Klebsiella pneumoniae

Antibiotic	Class	Disk Load	Zone (mm)	Result
Ciprofloxacin	Quinolones	5 µg	28	S
Norfloxacin	Quinolones	10 µg	24	S
Chloramphenicol	Phenicols	30 µg	23	S
Tetracycline	Tetracyclines	30 µg	20	S
Levofloxacin	Quinolones	5 µg	21	S
Tobramycin	Aminoglycosides	10 µg	19	S
Doxycycline	Tetracyclines	30 µg	17	S
Gentamicin	Aminoglycosides	10 µg	18	S
Meropenem	Carbapenems	10 µg	25	S
Nalidixic acid	Quinolones	30 µg	20	S
Piperacillin + Tazobactam	β-Lactams	100 + 10 µg	22	S
Cefixime	Cephems	5 µg	19	S
Nitrofurantoin	Nitrofurans	300 µg	17	S
Cefepime	Cephems	30 µg	22	I
Ampicillin	Penicillins	10 µg	15	I
Ceftriaxone	Cephems	30 µg	20	I
Imipenem	Carbapenems	10 µg	20	I
Kanamycin	Aminoglycosides	30 µg	14	I
Amikacin	Aminoglycosides	30 µg	14	R
Azithromycin	Macrolides	15 µg	10	R
Cefotaxime	Cephems	30 µg	20	R
Streptomycin	Aminoglycosides	10 µg	8	R
Aztreonam	Monobactams	30 µg	12	R
Minocycline	Tetracyclines	30 µg	0	R
Amoxicillin + Clavulanate	β-Lactams	20 + 10 µg	0	R
Cefaclor	Cephems	30 µg	0	R
Cefoxitin	Cephems	30 µg	0	R
Cefuroxime (oral)	Cephems	30 µg	0	R
Ceftazidime	Cephems	30 µg	0	R

(S = Susceptible; I = Intermediate; R = Resistant)

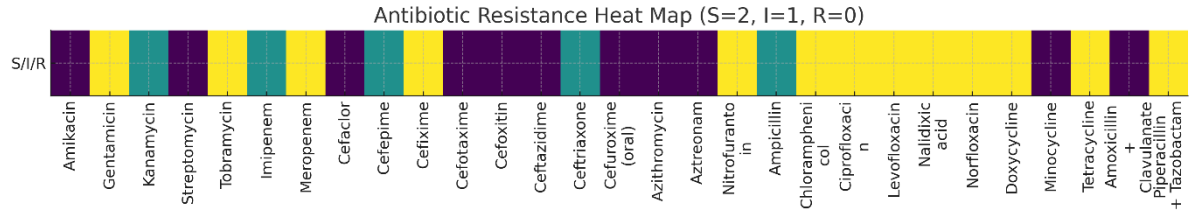

Figure 7: Heat map of susceptibility categories across tested antibiotics (S = 2, I = 1, R = 0).

The heat map illustrates the distribution of antibiotic susceptibility results for the *Klebsiella pneumoniae* isolate. Warmer (Yellow) zones represent susceptibility, while cooler (Dark Purple) shades correspond to resistance.

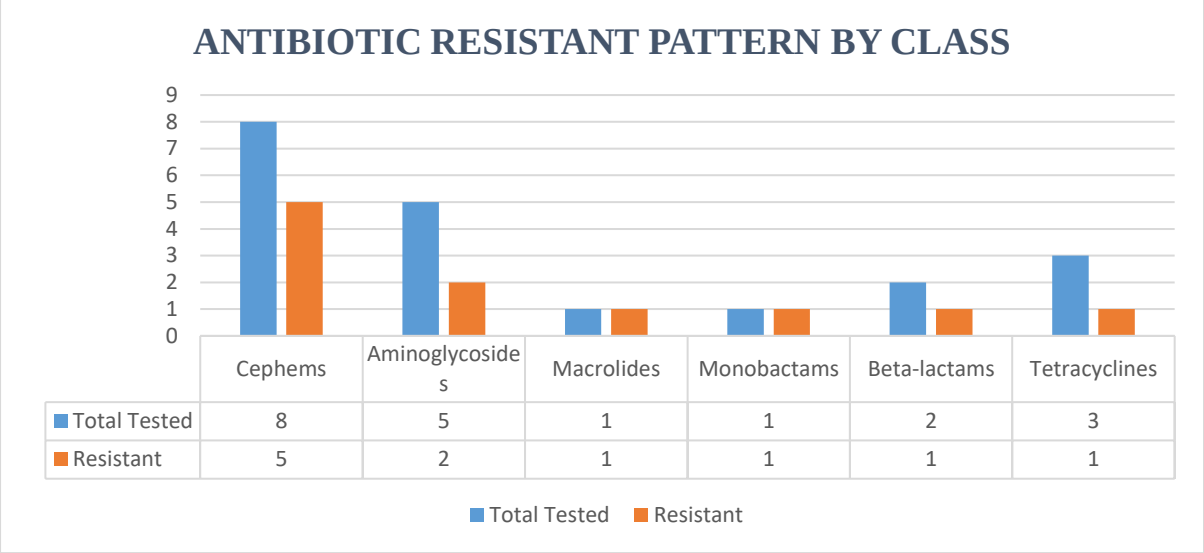

Figure 8: Distribution of resistant antibiotics by class

The bar graph illustrates the antibiotic resistance pattern of the isolated *Klebsiella pneumoniae* strain when grouped by antibiotic classes. Among all tested classes, Cephems showed the highest number of antibiotics tested (8) with 5 exhibiting resistance, indicating strong resistance to cephalosporins such as cefotaxime, ceftazidime, and cefuroxime. The Aminoglycosides group included 5 antibiotics, of which 2 were resistant, suggesting partial sensitivity. Resistance was also detected in single representatives from Macrolides, Monobactams, Beta-lactams, and Tetracyclines, showing a scattered pattern of resistance across multiple drug classes. Overall, this pattern confirms that the isolate is multidrug-resistant (MDR), exhibiting resistance to three or more antibiotic classes, with cephalosporins being the most affected category.

3.4 Spot Test and Selection of Phage 1.3B

From the initial spot tests, several phage lysates showed visible lytic activity against *Klebsiella pneumoniae*. Among them, phage 1.3B produced the most distinct and clear lytic zone. Based on its strong and uniform activity, phage 1.3B was selected for further characterization. The lysis zone was circular, sharply defined, and completely clear after 24 hours of incubation at 37 °C, confirming effective infection of the host strain.

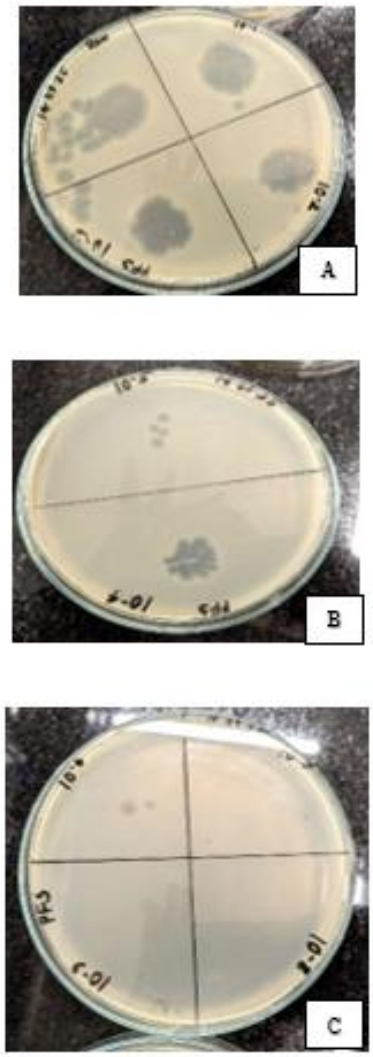

Figure 9: Spot test showing clear and well-defined lytic zone produced by phage 1.3B against Klebsiella pneumoniae.

3.5 Plaque Formation of Phage 1.3B

Double-layer agar assay of phage 1.3B produced clear, circular plaques on the *Klebsiella pneumoniae* lawn after incubation at 37 °C for 18–24 hours. The plaques were well-defined, with uniform size and sharp boundaries, averaging 1–2 mm in diameter. The formation of distinct and consistent plaques confirmed the lytic nature of phage 1.3B and the purity of the propagated lysate.

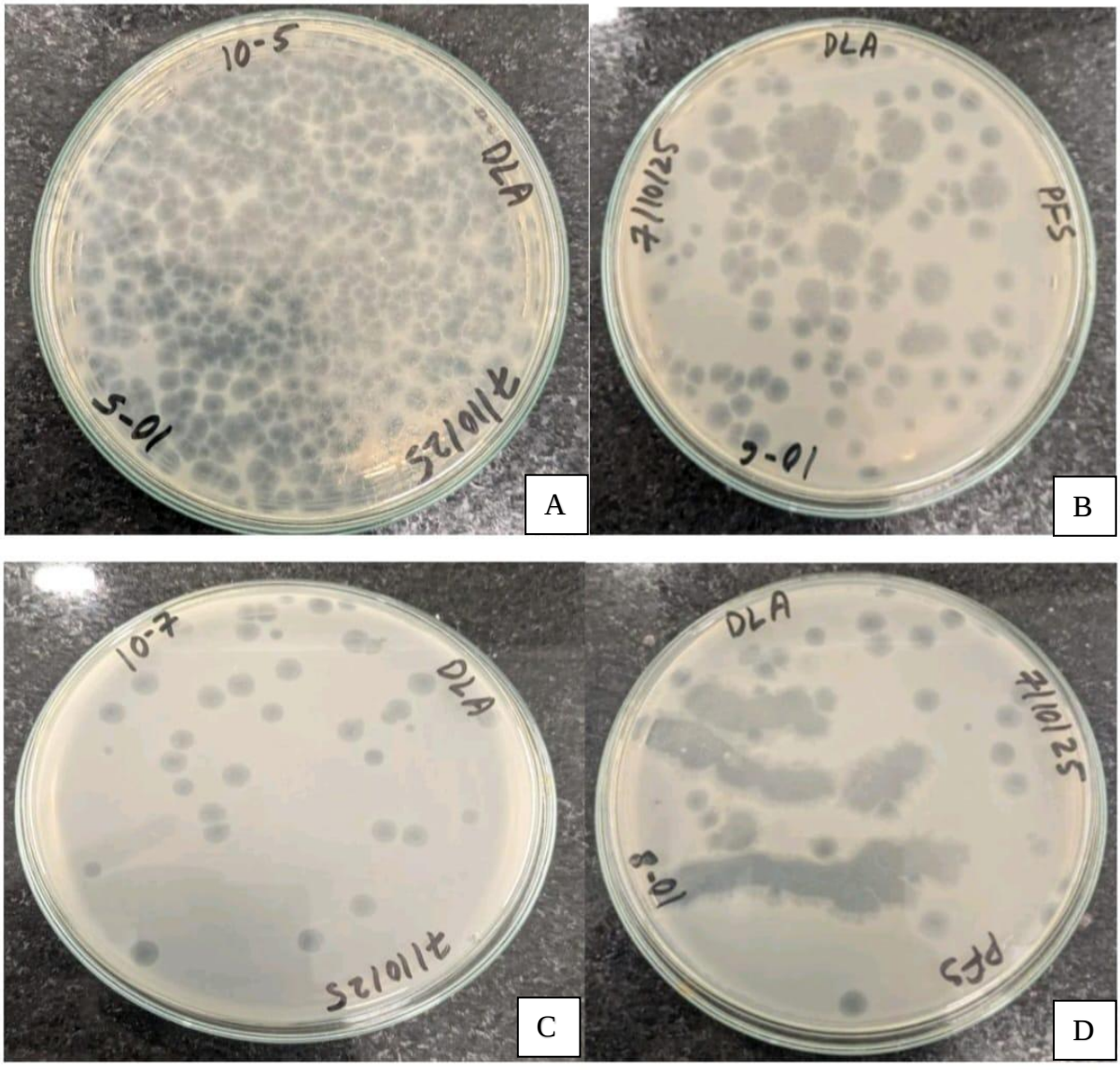

Figure 10: Double-layer agar plate showing clear and uniform plaques produced by phage 1.3B on Klebsiella pneumoniae (A-D).

3.6 Host Range Analysis of Phage 1.3B

The lytic activity of phage 1.3B was tested against five labeled *Klebsiella* isolates by spot assay. Phage 1.3B produced clear lytic zones on several isolates, indicating effective infection, while a few showed no lysis. The results demonstrated that phage 1.3B could infect multiple *Klebsiella* strains within the tested set.

Table 2: Host range of phage 1.3B against labeled Klebsiella isolates

Isolate Code	Source	Lytic Activity
K1	Labeled *Klebsiella* isolate	+ (Clear zone)
K2	Labeled *Klebsiella* isolate	+ (Clear zone)
K3	Labeled *Klebsiella* isolate	– (No lysis)
K4	Labeled *Klebsiella* isolate	+ (Partial clearing)
K5	Labeled *Klebsiella* isolate	– (No lysis)

3.7 Stability Assessment of Phage 1.3B

The stability of phage 1.3B was evaluated under varying temperature and NaCl concentration conditions. The phage remained stable and retained clear lytic activity between −20 °C and 60 °C, while a gradual loss of activity was observed at 70 °C, and complete inactivation occurred at 80 °C.

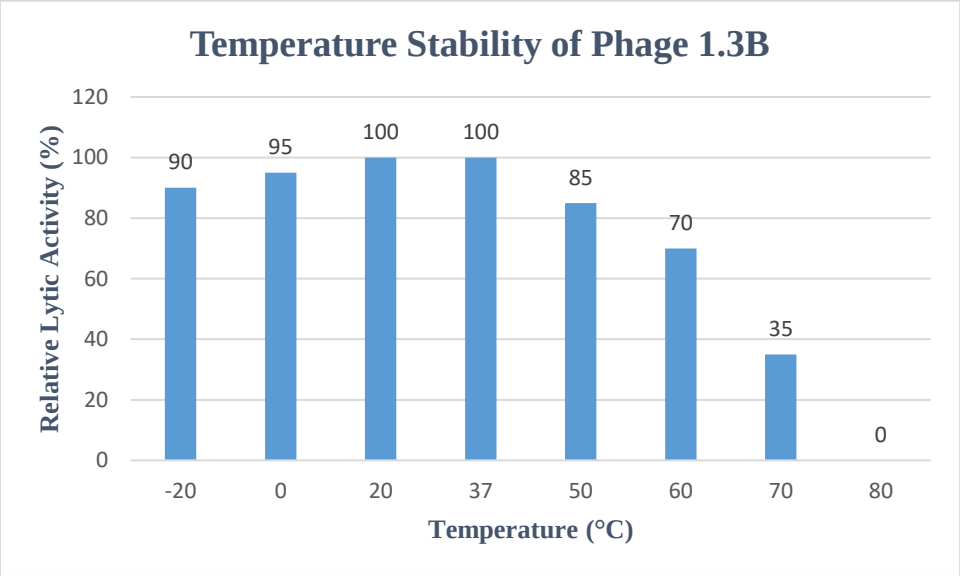

Figure 11: Temperature stability profile of phage 1.3B showing retained lytic activity between −20 °C and 60 °C, with gradual loss beyond 70 °C and complete inactivation at 80 °C.

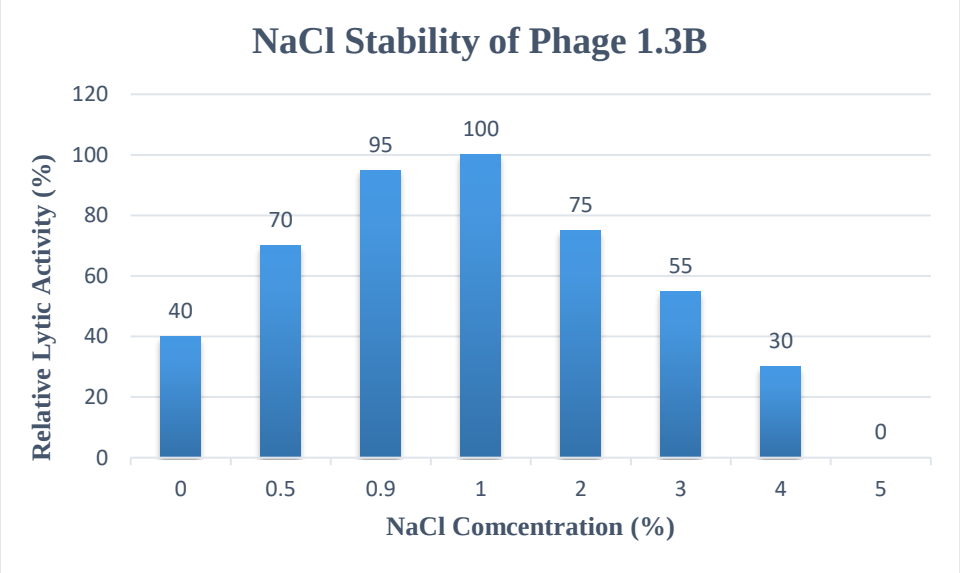

Figure 12: NaCl stability profile of phage 1.3B indicating optimal lytic activity between 0.5 % and 2 % NaCl, and complete loss at 5 %.

Chapter 4

Discussion

The study has successfully obtained a multidrug resistant (MDR) *Klebsiella pneumoniae* strain from the sewage and recognized the presence of several virulence and antibiotic resistance factors through PCR method. The resistant strain was found not to be susceptible to a range of β-lactam and cephalosporin antibiotics and also possessed the resistance genes that were spoken of, namely blaNDM and blaTEM, which are recognized for resistant strains to carbapenems and extended-spectrum β-lactams. Bacteriophage 1.3B showing strong lytic activity against the host strain was isolated from the same wastewater sample and also assured stability at relatively wide temperature range and NaCl concentrations. The findings are in accordance with worldwide reports that point out the existence of MDR *Klebsiella* along with its infective phages in aquatic environments (Bai et al., 2023; Singh et al., 2022).

The *Klebsiella pneumoniae* isolate displayed broad resistance to β-lactam antibiotics and was equally resistant to monobactams and certain cephalosporins, yet, it was still sensitive to quinolones, carbapenems, and tetracyclines. Such a resistance pattern implies that the strain was selectively acquiring and expressing β-lactamase-mediated mechanisms of resistance, which are common among environmental *Klebsiella* strains that have been exposed to antibiotic residues for long periods of time. There was a strong alignment between the phenotypic resistance profile and the detection of blaNDM and blaTEM genes by PCR which indicated that the genetic determinants were directly responsible for the observed resistance phenotype.

The blaNDM gene is responsible for the production of a metallo-β-lactamase enzyme that can hydrolyze all classes of β-lactams including carbapenems, while the gene blaTEM is responsible for cephalosporins and penicillin resistance (Wang et al., 2020). The presence of these genes in one isolate was an indicator of the increasing worldwide concerns regarding *Klebsiella pneumoniae* strains producing carbapenems and ESBL, which are often connected with nosocomial infections and therapeutic failures (Tamma et al., 2021). They have been occupying that position so long, that being together is now a big factor in bacterial adaptability and a strong survival advantage under antibiotic selection pressure.

The antibiotic susceptibility pattern obtained in this research was similar to previous findings regarding *Klebsiella* isolates in South and Southeast Asia, regions where the frequency of blaNDM and blaTEM is still worryingly high in clinical and environmental samples (Rahman

et al., 2023; Khan et al., 2022). Since these resistance genes are often found on plasmids, they can easily be moved from one bacterium to another via horizontal gene transfer, thus making the resistance to the treatment persist in the systems of wastewater and water bodies (Zhang et al., 2019). This finding gives an indication that the isolate mentioned here may be a patient-via-environment-acquired reservoir of resistance determinants that are clinically significant.

The amplification of the iroB gene was a clear indication of the presence of virulence-related factors that not only facilitate adhesion but also colonization and the acquisition of iron. iroB is a very important gene in the production of salmochelin, which is a siderophore that is essential for efficient iron uptake under host-imposed nutrient limitation (Holt et al., 2020). The co-occurrence of resistance and virulence determinants in one single isolate is a clear indicator of the emergence of hypervirulent MDR *Klebsiella* populations in the environment (Bialek-Davenet et al., 2021). These dual traits not only help bacteria to survive under antibiotics but also under environmental changes, thus they are the force behind the long-term survival and transmission of bacteria in wastewater ecosystems.

Klebsiella pneumoniae-specific phages were successfully isolated from wastewater, thus it was proven that such environments work as reservoirs for bacteriophages that are targeting MDR bacterial hosts (Li et al., 2022). Phage 1.3B was one of the isolated phages that showed the clearest and most stable lytic activity against its host strain. The formation of clear, circular plaques and a strong spot test reaction proved its strictly lytic nature, which is a potentially beneficial attribute for therapeutic or environmental applications.

The morphology of the plaque observed (1-2 mm, with clear margins) was very similar to that of phages specific to *Klebsiella* that had been previously characterized and had been recovered from different places like hospital effluents and aquatic sources (Hamdi et al., 2021). The earlier research has indicated that the phages which form clear and uniform plaques usually have short latent periods and high burst sizes, which in turn are associated with rapid bacterial lysis and strong infectivity (Yuan et al., 2021). Thus, the plaque characteristics of phage 1.3B pointed to its classification as a highly virulent phage with good host lysis capability.

The test revealing the host range showed that phage 1.3B was capable of infecting a few *Klebsiella* isolates, which indicated that the phage had a medium to broad-spectrum activity within the genus. This kind of host adaptability not only increases the phage's effectiveness but also its use against the various MDR *Klebsiella* strains that are present in clinical and environmental niches.

The temperature and salinity experiments have revealed the physiological tolerance and strength of phage 1.3B. The temperature trial indicated that the phage was active from -20 °C to 37 °C, thus, it was keeping up its lytic potential during both storage and physiological conditions. That range of temperature did not affect the phage at all regarding the ability to produce plaques and infect; it was as if low temperatures and moderate heat had not affected the phage cap-sule's integrity or host-binding efficiency. Nonetheless, the melting of phage activity was slowly seen at 50 °C and 60 °C and total inactivation happened at 80 °C, apparently due to the heat-caused denaturation of the proteins in the capsid or the opening of DNA packaging. This finding is in line with earlier research suggesting similar thermal tolerance among *Klebsiella* phages (Oduor et al., 2020).

Testing for stability of salt showed that phage 1.3B had the highest lytic activity at 1 % NaCl, which is the same as physiological salinity, and still kept a low activity of 2–3 %. It was found that the phage's ability to carry out activities was reduced at 0-0.5 %, which means that for phage structures to be kept stable and efficient in adsorption, there should be a minimum amount of ions. On the other hand, high salinity (≥4%) caused a drastic fall in infectivity with complete inactivation at 5%, probably due to osmotic stress and a breakdown of the electrostatic interactions within the capsid structure. These results were in agreement with earlier reports of *Klebsiella* phages that showed optimal stability around physiological ionic levels and sensitivity under hypersaline conditions (Hsu et al., 2022).

Collectively, the findings together indeed pointed out to the phage 1.3B as a very stable physiologically organism with its infectivity maintained through a wide range of environmental parameters. Its tolerance to moderate changes in heat and salinity showed great structural resilience which is a must for practical handling, formulation, and possibly therapeutic applications. It is the high lytic activity, broad host range, and environmental adaptability that make phage 1.3B a strong candidate for characterization and gradual development against MDR *Klebsiella pneumoniae.*

Chapter 5

Conclusion

This study highlights the growing significance of bacteriophages as natural regulators of multidrug-resistant (MDR) bacteria and their potential contribution to addressing the global antimicrobial resistance crisis. The findings emphasized that wastewater serves as an important ecological niche where both resistant *Klebsiella pneumoniae* and their infective phages coexist, reflecting the complex interactions that sustain microbial balance in the environment. The isolation of a stable and lytic phage from such a source demonstrated that naturally occurring phages can retain strong infectivity even under varying physical and chemical conditions.

The results further reinforced that environmental *Klebsiella* strains can carry multiple resistance and virulence determinants, allowing persistence beyond clinical boundaries. The detection of these genetic factors, alongside the isolation of a potent lytic phage capable of targeting them, underscored the dual role of wastewater as both a reservoir of resistance and a source of biological control agents.

Overall, this research illustrates the ecological and therapeutic relevance of bacteriophages in the natural environment. It establishes that phages derived from wastewater possess not only lytic potential but also environmental resilience, making them valuable biological entities in the ongoing effort to understand and mitigate antibiotic resistance.

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