# ANALYSING THE SYNERGISTIC EFFECT OF LYTIC BACTERIOPHAGE AND ANTIBIOTICS AGAINST MULTIDRUG RESISTANT *Klebsiella pneumoniae* CAUSING URINARY TRACT INFECTION

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**Dedicated to My Parents**

# Declaration

Date: ……..

I hereby declare that the thesis entitled "ANALYSING THE SYNERGISTIC EFFECT OF LYTIC BACTERIOPHAGE AND ANTIBIOTICS AGAINST MULTIDRUG RESISTANT *Klebsiella pneumoniae C*AUSING URINARY TRACT INFECTION" submitted to Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu for partial fulfillment of the requirement for the degree of M.Sc. in Biotechnology is a genuine work performed by me, Puja Dahal (T.U. Registration No:5-2-37-1630-2014) under the guidance and supervision of Asst Prof. Pragati Pradhan and Prof. Dr. Rajani Malla. No copies of this work have been published or presented previously anywhere or in any form.

……..

Signature of the candidate

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# ACRONYMS

AMR - Antimicrobial Resistance

AST- Antibiotic Susceptibility Testing

CDC-Center for Disease Control and Prevention

CDS- Coding Sequence

CLSI- Clinical Laboratory Standard Institute

CMDN- Center for Molecular Diagnosis Nepal

CRE-Carbapenem-Resistant Enterobacterales

CTAB- Cetyltrimethylammonium Bromide

DLAA-Double Layer Agar Assay

eDNA- Extracellular DNA

EPS -Extracellular Polymeric Substance

ESBL- Extended Spectrum Beta Lactamase

GNB -Gram-Negative Bacteria

I.P- Intraperitoneal

ICTV-International Committee on Taxonomy of Viruses

LB- Luria Bertani

MALDI-TOF -Matrix Assisted Laser Desorption Ionization – Time of flight

MBL- Metallobeta Lactamase

MDR- Multidrug Resistant

MHA-Muller Hinton Agar

MIC -Minimum Inhibitory Concentration

NCBI- National Center for Biotechnology Information

NIH-National Institutes of Health

ODc- Optical Density Cut Off

OD-Optical Density

ORF-Open Reading Frame

PAS -Phage Antibiotic Synergy

PDR-Pandrug Resistant

PRRs- Pattern Recognition Receptors

SDS-PAGE -Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SM buffer- Sodium Magnesium Buffer

STIDH -Sukraraj Tropical and Infectious Disease Hospital

Tm – Melting Temperature

TSA- Tryptic Soya Agar

TSB- Tryptic Soya Broth

UTI- Urinary Tract Infection

UV -Ultra-Violet

WHO- World Health Organization

XDR-Extensively Drug Resistant

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# ABSTRACT

**Introduction**: Antimicrobial resistance (AMR) is a growing worldwide phenomenon that affects both developed and developing countries. *Klebsiella pneumoniae* is considered to be one of the most concerning pathogen in today's era of antibiotic resistant and it has been categorized as ESKAPE pathogens alongside other extremely important multidrug resistant pathogens. Bacteriophage therapy recently is gaining momentum as a conceivable alternative towards all the resistant species. This study aims to explore efficacy of bacteriophage therapy against multidrug resistant (MDR) *Klebsiella pneumoniae* and use it in combination with antibiotic for the reduction of *Klebsiella pneumoniae* biofilm.

**Methodology**: Host bacterial strain was identified by biochemical test, mass spectrometry analysis and 16ssRNA sequencing. Antibiotic susceptibility test was performed by Kirby Bauer disc diffusion method and also confirmed by Vitek Compact System 2 analyzer. Bacteriophage isolation was done by Double Layer Agar Assay (DLAA) method. Burst size and latent period of the phage was determined by one step growth curve experiment. Phage stability was also analyzed against different temperature and pH range. Protein profiling of the phage was done by SDS PAGE. Whole genome sequencing was done using illumina sequencing. Bacteriophage was used to disrupt biofilm formed by *Klebsiella pneumoniae* by using microtiter dish method. The synergistic effect of phage and antibiotic in reducing biofilm was also analyzed.

**Results**: Lytic bacteriophage against *Klebsiella pneumoniae* was isolated. The latent period of the phage was found to be 20 minute and burst size was 119 virions per bacterium. The optimum temperature for the phage was 37˚C and optimum pH was 7. Three distinct bands of phage proteins were observed after performing SDS PAGE. Phage DNA size was determined to be larger than 10 Kb from agarose gel electrophoresis. Whole genome sequencing of phage revealed its size to be 45,288 nucleotides (45 Kb) in length with the GC content of 53.97%. The isolated phage showed the closest phylogenetic relation to *Klebsiella* phage myPSH1235. The phage effectively reduced biofilm formation and demonstrated a synergistic effect with antibiotics. Combining the phage with meropenem and ceftriaxone resulted in further biofilm reductions of 25.13% and 31.30%, respectively. Pretreatment with the bacteriophage was more effective than post-treatment, with an average bacterial load reduction of 75.75% after 24 hours. These results suggest significant potential for phage therapy in combating *Klebsiella pneumoniae* biofilms.

Keywords: Antibiotic resistance, Bacteriophage , Biofilm, Burst size, Phage therapy *Klebsiella pneumoniae*

# Chapter 1 INTRODUCTION

## 1.1 Background

Prior to the discovery of antibiotics, infective diseases were the leading causes of death worldwide. However, the breakthrough finding of penicillin by Alexander Fleming played a vital role in saving millions of lives (Yoneyama & Katsumata, 2006). Antibiotics have revolutionized medical practice by drastically reducing morbidity and death linked with bacterial infection. Infectious illnesses, however, continue to be the primary cause of mortality worldwide owing to antimicrobial resistance. Antimicrobial resistance (AMR) is a growing worldwide phenomenon that affects both developed and developing countries (Dhingra et al., 2020). Inappropriate prescribing practices, poor education related to patients, limited availability of diagnostic facilities, illicit selling of antimicrobial, few or not adequately operating drug regulatory systems, and usage of antimicrobials in non human use, like animals husbandry, are some of the factor contributing to the alarming rise in antimicrobial resistance (Ayukekbong et al., 2017).

The rapid spread of "superbugs," which are microorganisms resistant to most antibiotics, has made drug-resistant infections a serious and alarming problem worldwide. The WHO have recognized that resistant to antimicrobial is among top three serious threats to public well-being. In fact, these drug-resistant infections now rank as the third leading cause of death, after only cardiovascular diseases (Salam et al., 2023).In a review conducted by Laxminarayan et al. in 2013, they illustrated devastating impact of antimicrobial resistance (AMR), with the US, CDC estimating approximate two million illness and 23,000 death per year due to antibiotics-resistance infections with the costs of around $55 billion annually. Similarly, in a review conducted in 2014 by Lord Jim O'Neil, a famous British economist, he estimated that about 700,000 people are dead every year all around the globe because of resistance to antimicrobial. He also predicted that by the year 2050 it may increase to 10 millions.

The wonderful age of antibiotic is coming to end as production of antibiotic could not keep up with the rising number of antibiotic-resistant microorganisms. Many leading institutions, including the Centers for Disease Control and Preservation (CDC), Infectious Diseases Society of America, World Economic Forum, and the World Health Organization (WHO) have announced resistance to antibiotics as a major worldwide public health issue. The WHO in its latest report of 2017 has clearly declared that there is worldwide shortage of antibiotics and therefore, search for an alternative is a matter of urgency (Aslam et al., 2018). Different alternatives such as vaccines, antibodies, pattern recognition receptors (PRRs), bacteriophages, peptides, phytochemicals, metals and

antimicrobial enzymes are being tested by scientist from different sectors (Mbarga Manga Joseph et al., 2022).

Among all the alternative options, bacteriophage has the longest history of being used as an antimicrobial agent. In fact bacteriophage was the only treatment option available before the development of antibiotic and has some promising result in treating bacterial infection. So, bacteriophage could be one of the most potent options to combat antimicrobial resistance as phages are the natural predators of the bacteria with the ability to kill even the MDR bacteria, destroy biofilm and are specific to certain species unlike antibiotics.

## 1.2 Bacteriophage

Bacteriophages, also known as phages, are a type of viruses that are known to attack and infect bacterial host. Bacteriophages are obligate bacterial parasitic viruses which require a host bacterium to reproduce. Bacteriophages were found by two scientists in two different years. One is by Frederick Twort, who was from England and was also medically trained bacteriologist in 1915. And other was during 1917 by Felix D'Herelle, microbiologist from Canada. The name "bacteriophage," which means "bacteria eater," was coined by D'Herelle (Duckworth, 1976).With an estimated $10^{32}$ bacteriophages on the globe, phages are the most prevalent 'life' forms and are 10 times more prevalent than the bacteria. They are commonly isolated from aquatic habitats, although they may be found everywhere bacteria lives (Hanlon, 2007).

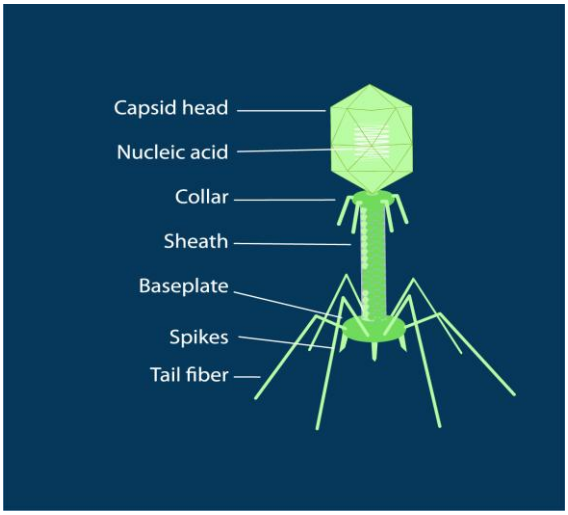



**Figure 1: Structure of Bacteriophage (Figure Adapted from Technology Networks)**

Structurally, bacteriophage consists of outer protein coat, nucleic acid (either RNA or DNA) and a tail having structures such as collar, sheath, base plate, tail fiber and pin. They comprise a central genetic material (nucleic acid) enclosed within a protein capsid.

This protein capsid protects the genetic material. The capsid or also called head consists of protomers which are the morphological subunits. Nucleic acid could either be RNA or DNA and can be either single stranded or be double stranded. The majority of phages also have a tail (formed of proteins) that allows them to binds to the host bacterium via the receptor found on the surface of host bacterium. Naming of the phages is usually done on the basis of groups, strains, or species of the host bacterium they infect as they are specific to limited host bacterium. Following example supports the above statement. The bacteriophages that attack *Escherichia coli* species are generally known as coliphages, such as T"even" phages T2, T4, and T6. The majority of phages belong to the tailed phage group (Order: Caudovirales), constituting 96% of all bacteriophages present in the Earth and also represents the oldest virus group to be known (Ackermann, 2003; Ackermann, 1998). Most phages typically exhibit lengths ranging from 22nm to 200nm. T4, the largest bacteriophage identified measures approximately 200nm in length and 8- to 100nm in width. Tailed phages having larger genome size i.e. more than 200 kbp are called as Jumbo bacteriophages (Yuan &Gao, 2017).

## 1.3 Classification of bacteriophage

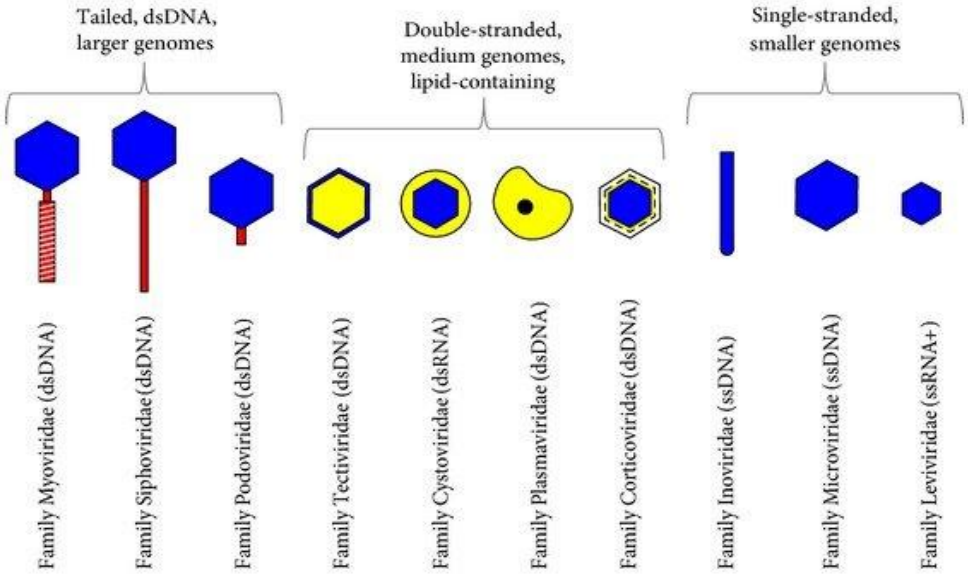



**Figure 2: Classification of Bacteriophages (Figure adapted from (Hyman & Abendon, 2011)**

Phages were classified according to their morphology even before the development of PCR or any other sequencing method. David Bradley was the first person to start classification scheme. He classified six fundamental morphological types, which include tailed phages with various types of tails (contractile, long non-contractile, and short), small isometric ssDNA viruses featuring fibrous or spiky surfaces, filamentous phages, and small ssRNA phages (Bardley, 1967). The most recent classification scheme for

bacteriophages is given by the International Committee on Taxonomy of Viruses (ICTV). The ICTV released first report in 1971 that consists of 6 bacteriophage “genera”: T-even phages, l, lipid phage PM2, the ϕ Xgroup, "filamentous phage", and "ribophage group". Currently, the ICTV recognizes a single order, 13 families, and 31 genera of phages (Calendar, 2006). Approximately 96% of known phages are categorized within the families Myoviridae, Podoviridae, and Siphoviridae under the order Caudovirales. The Caudovirales consists of tailed bacteriophages characterized by contractile, long and non contractile or short tails.

Generally, phages are classified based on their genome type and morphology of virion. Phages can possess single or double stranded either RNA or DNA. They can exhibit polyhedral or tailed particles, and some may be filamentous or pleomorphic in shape. Phages with larger genomes belong to the dsDNA phages and are classified within the viral order Caudovirales. In contrast, those with relatively smaller ssDNA genomes are categorized under the Microviridae family. Similarly, phages with smaller ssRNA genomes are part of the Leviviridae family (Elois et al., 2023).

## 1.4 Life cycle of bacteriophage

Phages are obligate parasites which depend on the cellular machinery of their host to replicate and reproduce. Understanding the interaction of bacteriophage with host bacterium is important to study their role. Phages have various lifecycles and are classified as lytic, lysogenic and pseudo-lysogenic based on their lifecycle (Zhang et al., 2022).The lytic lifecycle involves six main stages. It starts with the attachment of bacteriophage to the host bacterial surface. Soon after attachment, bacteriophage DNA is injected into bacterial cell. The phage takes over host metabolism to make more copies of its DNA and generate different proteins called as bacteriophage proteins. Bacteriophage capsid is assembled and packed together with genetic materials producing many phage progeny. Eventually, the lysis of host cell occur releasing progeny phage into the environment (Maciejewska et al., 2018).

Lysogenic lifecycle is an alternate life cycle of bacteriophage where host cell is not immediately ruptured. Instead, phage either merges with the genetic material of host or creates a distinct genetic entity forming a dormant state. These phage genome replicates in conjunction with host genome after integration. Upon stressed condition these phages eventually initiates their lytic lifecycles (Ofir & Sorek, 2018).

Pseudolysogeny is referred to as an interaction between a phage and a host cell where phage neither establish lysogeny nor a lytic response. Instead, the phage genome remains dormant within the host cell. These phenomena are believed to occur when the cell experience extreme starvation. However, when there is availability of nutrients to

the host cells, phage gains energy to enable gene expression and undergo either lytic or lysogenic life cycle (Ripp & Miller, 1997).

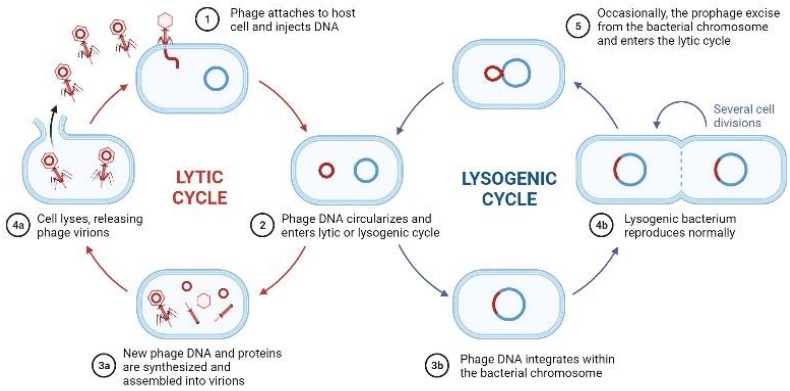



**Figure 3: Lytic and Lysogenic Lifecycle of Bacteriophage (Alsubhi, 2021)**

## 1.5 Research Hypothesis

The study aimed to evaluate the effectiveness of bacteriophages in synergistically reducing biofilms with antibiotics.

**Null Hypothesis (Ho):** Lytic bacteriophage is effective in reducing biofilm in synergism with antibiotic

**Alternative Hypothesis (H1):** Lytic bacteriophage is not effective in reducing biofilm in synergism with antibiotic.

## 1.6 Objectives

### 1.6.1 General objective

Analysis of the synergistic effect of lytic bacteriophage and antibiotics against multidrug resistant bacteria causing urinary tract infection.

### 1.6.2 Specific objectives

1. Isolation, screening and purification of lytic bacteriophage against *Klebsiella pneumoniae* from rivers of Kathmandu valley.
2. Determination of phage-host range using different types of multidrug-resistant bacteria
3. Protein profililing of phage protein by SDS-PAGE
4. Determination of growth characteristics and physiochemical properties of bacteriophage.
5. Analysing the phage genome through Whole genome sequencing
6. Applying the bacteriophage as antimicrobial agent for the disruption of the bioflim produced by *Klebsiella pneumoniae.*

## 1.7 Rationale of study

The rise of drug-resistant infections on a global scale has become a significant concern for the public health sector. Multi drug resistant pathogens are leading cause of death worldwide. *Klebsiella pneumoniae* is considered to be one of the most concerning pathogen in today's era of antibiotic resistant. The World Health Organization (WHO) and the Center for Disease and Preventions (CDC) have listed carbapenem-resistant enterobacterales (CRE) (including *Klebsiella pneumoniae)* as one of the most urgent and serious threats pathogen. Therefore, it's crucial to explore potential alternatives to combat this crisis of antimicrobial resistance.

Bacteriophage, among all the alternative options, holds the longest history of being utilized as an antimicrobial agent. In fact, before the development of antibiotics, bacteriophage was the sole treatment option available and showed promising results in treating bacterial infections. In earlier times, the utilization of phages as antimicrobials was restricted because of insufficient understanding and the absence of sophisticated instruments for studying them further. However, with advancements in technology like whole genome sequencing and electron microscopy, previous limitations can now be revisited, allowing for easier investigation into the diversity of bacteriophages. Additionally, this technology facilitates the examination and identification of harmful or toxic genes within the genome before their application.

In the context of Nepal, the significant increase in antibiotic resistance has emerged as a major public health issue. However, there has been minimal effort directed towards investigating alternative treatments to antibiotics and only a limited amount of research have been published on Bacteriophages. In this study, we aim to isolate and characterize lytic bacteriophages targeting *Klebsiella pneumoniae*. The study also evaluated the synergistic effects of phages and antibiotics in reducing biofilm formation.

# Chapter 2 LITERATURE REVIEW

## 2.1 Phage antibiotic synergism

Bacteria can develop variety of mechanisms to protect themselves from the phage attack. This poses a challenge for the continuity and effectiveness of phage therapy. Various methods have been explored to overcome this problem including combining different phages with each other or with antimicrobials to create synergistic effects (Pires et al., 2017). The combination of phages with antibiotics to produce synergistic effects has been gaining significant attention. Rather than completely swapping antibiotic with bacteriophages, researchers came with the idea of using both bacteriophages and antibiotics together as it might increase the effectiveness of both than using either independently. The joint approach may offer several advantages, some of which are, enhanced bacterial suppression, improved penetration into biofilms, and a decreased potential for bacteria to develop resistance to rather phages or antibiotics (Li et al., 2021).

During the early 20$^{th}$ century, phages were frequently utilized alongside sulphonamides. The synergistic effect of combining phages with sulphonamides was first demonstrated by Zaytzeff-Jern et al. in 1941. Their research demonstrated using of phages with sulphanilamide and sulphapyridine effectively suppressed the growth of both *Escherichia coli* and *Staphylococcus aureus* in vivo ( Zaytzeff-Jern et al.,1941). This early discovery laid the foundation for exploring the potential of combining phages and antibiotics as a strategy to combat bacterial infection more efficiently. Recently, researcher have described about a phenomenon known as phage antibiotic synergy( PAS) in *Pseudomonas aeruginosa* involving β-lactam and quinolone antibiotics. The phenomenon showed that the lytic activity of phages was amplified when used in combination with sub-inhibitory concentration of these antibiotics (Comeau et al., 2007). Next study performed by Verma et al. aimed to observe the efficacy of ciprofloxacin and bacteriophage treatment in both immature and mature *Klebsiella pneumoniae* bioflims. The results indicated that when the phage and antibiotics were used in combination, there was a slightly greater eradication of bofilms (Verma et al., 2010). The research conducted by Coulter et al. investigated the effects of combination therapy using T4 phage and tobramycin on biofilms of both *Escherichia coli and Pseudomonas aeruginosa*. In *E. coli*, there was over 99% reduction in antibiotic resistance and 39% reduction in phage resistance, while in *P.aeruginosa*, there was a 60% reduction in antibiotic resistance and 99% reduction in phage resistance. The study highlighted the effectiveness of the combination therapy in reducing resistance in biofilms of two bacteria (Coulter et al., 2014). When Bedi et al. conducted their study in

2009, they found that the combined use of Amoxicilin and specific bacteriophage resulted in a significantly greater destruction of the biofilm structure of *Klebsiella pneumoniae* (Bedi et al., 2009).In a case study involving an old 63-year female patient having a persistant UTI, it was found that the use of sulfamethoxazole-timethoprim with the cocktails of different bacteriophage was effective in preventing development of any new phage resistant mutants and it was also successfully in curing urinary tract infection in patient. This suggests that combining non active antibiotics with bacteriophages could be promising approach for personalized phage therapy (Bao et al., 2020). Another case study involves a thirty years old bombing victim experiencing a pandrug-resistant Klebsiella pneumoniae infection. The patient received treatment with a combination of a phage, colistin and meropenem, followed by ceftazidimem/avibactum which leads to notable enhancements in the patient's wound healing and overall health. Additionally, laboratory tests demonstrated the remarkable effectiveness of the combination of bacteriophage and antibiotic against the patient's *Klebsiella pneumoniae* strain in different conditions, such as in vitro, 7-day mature bioflims and suspensions (Eskenazi et al., 2022).

## 2.2 Multidrug resistant *Klebsiella pneumoniae*

*Klebsiella pneumoniae* is a gram negative bacterium having rod shape and belonging to genus *Klebsiella* and Enterobacteriaceae family. *Klebsiella* is ubiquitous in nature i.e. they are found everywhere in nature. They are mostly found in mouth, skin and gut as normal flora but are also found in natural environment (Wu & Li, 2015). *Klebsiella pneumoniae* was first described by Carl Friedlander in 1882 as an encapsulated bacillus. He isolated *Klebsiella* bacterium from the patient's lungs that died with pneumonia. The bacteria were first known as Friedlander's bacillus while the name *Klebsiella* was given only in 1886. It is an opportunistic pathogen that is responsible for causing community acquired as well as nosocomial infections, including the infection of urinary tract, respiratory, wounds and soft tissue (Herridge et al., 2019).

*Klebsiella pneumoniae* is considered to be one of the most concerning pathogen in today's era of antibiotic resistant and it has been categorized as an ESKAPE organism alongside other extremely important multidrug resistant pathogens (Navon-Venezia et al., 2017). The global rate of drug resistance in *Klebsiella pneumoniae* has escalated to 70% and the fatality rate associated with infections caused by this bacterium ranges from 40% to 70%. Over the past few years, the emergence of multidrug resistant (MDR*) Klebsiella pneumoniae* and Carbapenem Resistant *Klebsiella pneumoniae (CRKP)* are considered as a significant public health concern on a global scale (Li et al., 2022). The important organizations like that of WHO and also CDC have listed carbapenem-resistant enterobacterales (CRE) as one of the most urgent and serious threats pathogens

(WHO,2017 and CDC,2019). The CDC states that carbapenem-resistant enterobacterales are of major concern especially for the hospitalized patient with the estimated cases 13,100 and death cases 1,100 in the year 2017 (CDC,2019).

The one of the most common CRE, *Klebsiella pneumoniae* has contributed to a notable rise in global disease cases. The emergence of carbapenem producing *Klebsiella pneumoniae* has established them as significant nosocomial pathogens linked with various sever infection with high mortality rate (Karaiskos et al., 2021). One of the recent comprehensive analyses showed that colonization of carbapenem resistant *Klebsiella pneumoniae* (CRKP) varies globally, spanning from 0.13% to 22% with a total prevalence of 5.43%. Similarly, presence of CRKP colonization varies from 2% to 73% with an average prevalence of 22.3% (Tesfa et al., 2022). In context of Nepal, *Klebsiella pneumoniae* is one of the most frequently isolated clinical specimens after *E.coli* (Pyakurel et al., 2021).

## 2.3 Metallobeta lactamase producing bacteria

Metallo-β-lactamases (MBLs) are becoming a major cause of resistance in Enterobacteriaceae. These enzymes were discovered in the 1960s, but it wasn't until the 1990s that they gained attention due to an increased number of outbreaks related to transmissible MBL genes in Gram-negative bacteria (GNB) (Logan & Bonomo, 2016). Metallo-β-lactamases can be found encoded in bacterial species in two ways: they may be part of the chromosomal framework in certain bacteria (referred to as resident metallo-β-lactamases), or they may be encoded by external genes acquired through horizontal gene transfer (known as acquired Metallo betalactamase). There are minimum 9 distinct types of acquired metallo betalactamase) that are identified, among which IMP-type, VIM-type, SPM-type, and NDM-type enzymes hold more significance (Cornaglia et al., 2011) . The NDM-1 enzyme, a recent addition to acquired metallo-β-lactamases, firstly identified in *Klebsiella pneumoniae* in year 2008 from individual who had received healthcare in India and was returning to Sweden. Since then, numerous finding of this organism have emerged in various parts all around the world, and they now are among one of the most found MBL gene globally (Cornaglia et al., 2011).

## 2.4 Urinary tract infection and *Klebsiella pneumoniae*

Urinary tract infections (UTIs) are common bacterial infections which occur due to entry of different bacteria to the urethra often via the skin or rectum which then infect the urinary tract and cause its infection (CDC, 2021). Urinary tract infection (UTIs) lead to more than eight million visits to doctor’s office, trips of about 1.5 million to the emergency rooms, also about 3 lakh admissions in hospitals annually only in the US.

They are the second most common infection and are the most prevalent urological condition in the country, costing over $3.5 billion each year (Tabibian et al., 2008).

These infections constitute 12.9% of infections associated with healthcare system and 23% intensive care units (ICUs) infections, with about 70% attributed to catheter associated infections (CAUTIs). The catheters are believed to provide an optimal space where bacterial colonization and biofilm formation can occur, due to which there is significant increase in the risk of infection and compromising treatment effectiveness (Liu et al., 2020). Organisms such as *E. coli, Proteus mirabilis, P. aeruginosa, K.pneumoniae,* and *Streptococcus faecalis* are most commonly responsible for catheter-associated UTIs (Mittal et al., 2009). *Klebsiella pneumoniae* (KP), which are the normal flora of the intestinal tract, are frequently associated with cross-transmission UTIs. Numerous research have identified *Klebsiella pneumoniae* as the second most isolated pathogen in UTIs among Enterobacteriaceae, following *Escherichia coli*(Liu et al., 2020). *Klebsiella pneumoniae* is a considered significant pathogen responsible for various infections, including infection of bloodstream and also pneumonia, particularly affecting neonates and patients in ICU (Ding et al., 2021).

## 2.5 Bacteriophage History

The discovery of bacteriophage has always been the subject of discussions and disagreements, especially regarding the attribution of credit for their initial discovery. In 1896, British bacteriologist Ernest Hankin observed a significant antibacterial effect against *Vibrio cholera* due to waters of the two holy rivers found in India which was Ganges and Jamuna. Hankin proposed that an unidentified particle, which was heat sensitive and was capable of, passing through fine porcelain filter, was the reason for the phenomenon and contributed in limiting the outbreaks of cholera (Sulakvelidze et al., 2001). In 1915, about twenty years after the initial discovery, Frederick Twort, a bacteriologist from England explained a glassy transformation which he observed while working with micrococcus cultures. He could not state that the glassy transformation was due to virus. He concluded that phenomenon was caused by an infectious agent which was filterable, and had the ability to both multiply and kill the bacteria. However, it wasn't until March 26, 1921, five years, three months, and twenty-two days later, that Twort's paper gained recognition. The discovery or rediscovery of bacteriophage by Felix d'Herelle, a French-Canadian microbiologist is often associated with a severe outbreak of hemorrhagic dysentery among French troops in July-August 1915. However, d'Herelle in 1910 has initially observed the bacteriophage phenomenon while studying ways to control a locust epidemic in Mexico. Following the outbreak, he investigated the illness and conducted experiments using bacteria-free filtrate of patient's feces sample. He mcombined both and incubated these filtrates with strains of *Shigella*. He inoculated

some portion of the mixture in his experimental animal and spread a portion on agar medium. It was on these agar cultures that d'Herelle noticed small, clear areas which he named taches, later known as plaques. In 1917, d'Herelle presented his findings at a scientific meeting and also proposed that the phenomenon was due to virus which was capable of killing bacteria. He coined the term "bacteriophage" in 1916, deriving it from Greek words for "bacteria" and "to eat/devour", suggesting that phages consume bacteria (Duckworth, 1976 and Sulakvelidze et al., 2001).

## 2.6 Phage therapy and its history

Phage therapy, using bacteriophage for treatment of bacterial infection, has been present for over hundred years. Bacteriohage therapy was known to humans since early $20^{th}$ century. It was first mentioned by Frederick Twort in 1915, and then later by Felix d'Herelle who isolated bacteriophages from the feces of dysentery patients in 1917 while working at Pasteur Institute in Paris (Brives & Pourraz, 2020). Shortly after the discovery of bacteriophage, d'Herelle was the first to use bacteriophage therapeutically in 1919. Along with several interns working in the Hospital des Enfants-Malades in Paris, d'Herelle ingested Shigella bacteriophage cocktail to assess its safety. Encouraged by the results, he administered a single dose of the cocktail to a little boy of 12 year who was suffering from severe dysentery. The boy fully recovered within few days with just single dose of phage therapy (Aswani & Shukla, 2021). The first therapeutic utilization of bacteriophage in United States was in 1922 by Davison (DAVISON, 1922). Furthermore, phage therapy was also used to treat German and Japanese armies during World War II (Summers, 2012).

d'Herelle in his book discussed the establishment of two industrial centers in India in 1931. These centers were dedicated to producing bacteriophages specially targeted against cholera. Additionally, d'Herelle's laboratory which was present in Paris also developed 5 different bacteriophage preparations to combat different infection related to bacteria. These preparations were known as Bacte-coli-phage, bacte-rhino-phage, Bacte-inteti-phage, Bacte-pyo-phage and bacte-staphy-phage(Chanishvili, 2012 and Sulakvelidze et al., 2001). The Oswaldo Cruz Institute in 1924, in Rio de Janeiro, Brazil, began the manufacturing of bacteriophages with the aim of addressing dysentery within Latin American nations(Chanishvili, 2012). During the 1940s, the Eli Lilly Company developed and manufactured 7 bacteriophage products intended for usage by humans. These preparations were specifically designed to combat bacterial pathogens such as *Staphylococci*, *Streptococci*, *Escherichia coli*, and other related strains. Successful bacteriophage treatment trials have been reported in various cases, including septicemia, UTI, a surgical infection, a skin infections, peritonitis, otolaryngology

infections, and also colitis caused by *Salmonella and Shigella* (El-Shibiny & El-Sahhar, 2017).

The concept of using phages for therapeutic to combat bacterial infections faced significant controversy and lacked widespread acceptance among both general public and the medical community. Initial research faced criticism due to inadequate control measures and inconsistent outcomes (Wittebole et al., 2013). As a result, after the development of antibiotic in 1940s, bacteriophage therapies were largely dismissed by the Western medicine. However, former Soviet Union and Eastern Europe continued using phage therapy to cure infections related to antibiotic resistance which were caused by a range of infectious bacteria, including *Staphylococcus, Pseudomonas, Klebsiella,* and *E.coli* (Lin et al., 2017).

With the emergence of MDR (Multidrug Resistant), XDR( Extensively Drug Resistant), and PDR (Pandrug Resistant) bacteria, and also because of limited possibilities of developing any new antibiotics, a renewed interest in bacteriophage therapy is seen all-around the world among scientific community. Bacteriophage therapy is now considered as an alternative approach in order to combat wide range of infection caused by different antibiotic resistant bacteria (Taati Moghadam et al., 2020).

## 2.7 Revitalization of Phage therapy

Antibiotics have brought great advancements in medicine and saved many lives, marking a significant turning point in history of medicine. However, the rise in resistant strains has weakened their effectiveness (Davies & Davies, 2010). The current rate at which resistance to antibiotics is emerging far exceeds the rate of discovery and development of new antibiotics, posing a substantial and urgent global public health challenge. Studies showed that by the year 2050, the toll of antimicrobial resistance could result in the loss of over 10 million lives annually (O neil, 2014).

This growing problem of antibiotics resistance has revitalized attention in bacteriophage therapy. The bacteriophage therapy presents many advantages over antibiotics, which includes, precise targeting of specific bacteria (which allows for the preventions of the body's natural bacterial flora), ability to multiply within bacterial host after administration, pose ability to adapt and modify themselves in order to effectively infect and lyses evolving bacteria (including the strains that have developed resistant), and the capability to penetrate preformed bioflims (Rehman et al., 2019).

In recent studies using animal models, researchers have examined the use of phage therapy as a potential treatment for various pathogens that hold clinical importance. A study in mice induced with contact burn wound infections caused by the bacterium *Klebseilla pneumoniae* , showed that a single injection of phage was effective to reduced

mortality with a 73.33% overall survival rate (Kumari, 2010). Another study demonstrated that administration of bacteriophage orally to mice challenged with all gut-derived sepsis from *P. aeruginosa* saved 66.7% from dying, in comparision to 0% in the control group (Watanabe et al., 2007). Next research performed in hamster model when infected with *Clostridium difficile*, just a single dose of bacteriophage given at the time of *C.difficile* administration effectively prevented infection. And administering phage treatments after infection rescued 11 out of 12 mice, while the control group, which received both *C.difficile* and clindamycin, experienced mortality within 96hours (Ramesh et al., 1999). In another study, it was demonstrated that a single intraperitoneal(i.p) injection of phage was sufficient in saving mice infected with vancomycin-resistant *E. faecium* . And the treatment was seen effective even when administered up to 5hours after the infection occurred (Biswas et al., 2002). An experiment conducted by Hung et al. involving an intragastric model of *Klebsiella pneumoniae* infection showed that administration of bacteriophage, either orally or intraperitoneally protected mice from infection and minimized liver damage (Hung et al., 2011). Another study conducted by Cao,Wnag et al in 2015 revealed that the intranasal administration of bacteriophage effectively prevented lethal pneumonia caused by *Klebsiella pneumoniae* lung infection. Additionally, in a sublethal pneumonia model, mice treated with the phage showed a reduced *Klebsiella pneumoniae* level found in their lungs when it was compared with the control group that is the group which was not treated with bacteriophage (Cao et al., 2015).

The next study involving an old patient of 57 year affected from multi-site colonization by multidrug resistant (MDR) strain of *Klebsiella pneumoniae*(Kp) achieved successful resolution after undergoing a 3 week course of bacteriophage therapy administered orally and intra-rectally (Corbellino et al., 2019). Another case study showed a 58 year old patient with renal transplant with the problem of frequent or recurrent urinary tract infection (UTI) due to an EBL-positive *Klebsiella pneumoniae* strain was treated successfully using meropenem and bacteriophage combination when there was multiple failed attempts with meropenem therapy alone (Kuipers et al., 2019). Similarly, bacteriophage therapy was proved successful in treating a *Pseudomonas aeruginosa* multidrug resistant infection in a 26-year-old cystic fibrosis patient (Law et al., 2019).

## 2.8 Advantages of Bacteriophage therapy

One of the main advantage of bacteriophage is they are highly specific in nature. They kill the specific pathogen without disturbing the normal flora (Domingo-Calap & Delgado-Martínez, 2018).Furthermore the administration of bacteriophage is easier. Unlike antibiotics they do not need repeated administration as they have the capability of self multiplication at the site where infection have occur (Principi et al., 2019).

Another notable characteristic of bacteriophage is their ability to co evolve alongside their host. This can be used to combat potential resistant mutants. Viruses, including phages, are known to have higher mutation rates compared to bacteria (Stern & Sorek, 2010). Furthermore, the emergence of bacteriophage resistance developed due to use of only one bacteriophage could be improved by using bacteriophage cocktails (combination of different types of bacteriophages) or by the combination of phage and antibiotics (Golkar et al., 2014). Phages have short replication time and can be produced in large number in their specific hosts. They can also be produced quickly with low production cost (Domingo-Calap & Delgado-Martínez, 2018). Bacteriophage have also shown the ability to destroy biofilms either by lysing individual layers of bacteria or through the production of enzymes that degrade the exopolymer matrix of bioflims (Loc-Carrillo & Abedon, 2011).

## 2.9 Bacterial Biofilm

Biofilm is commonly described as an aggregate of microorganism that adhere to a surface be it living or nonliving. Within biofilm, microorganisms are found in a complex matrix of extracellular polymeric substance (EPS) being embedded within them, which is synthesized by the microorganism itself (Hall-Stoodley et al., 2012). The biofilm matrix primarily consists of water, comprising up to 97% of its composition, This water phase contains important components such as soluble polysaccharides, proteins, and Extracellular DNA (eDNA) and insoluble elements including amyloids, cellulose, and various appendages like fimbriae, pilli, and flagella (Flemming et al., 2016).

Anthony van Leeuenhoek (1632- 1723), a Dutch scientist from Delft, first observed and documented biofilms using his primitive microscope (Høiby, 2017). Bacteria in biofilm use different strategies to escape host defense mechanism either by remaining dormant and or by hiding to the host immune system. Bacteria adapt by altering their metabolism, gene expression, and protein production in response to limited oxygen and nutrients (Vestby et al., 2020). Biofilm forming bacteria poses a significant risk for persistent infections, particularly in implant recipients and are considered one of the serious public health issues due to high mortality rates worldwide (Kumar et al., 2023). Common biofilm producing bacteria are *Klebsiella pneumoniae*, *Staphylococcus aureus, Escherichia coli, Acinetobacter baumannii, Peudomonas aeruginosa* and so on. Biofilm formation is linked to 65% of microbial infection and 80% of chronic infections by the National Institutes of Health (NIH) (Folliero et al., 2021 and Jamal et al., 2018).

## 2.10 Whole Genome Sequencing

The development of DNA sequencing techniques by Frederick Sanger and Allan Maxam / Walter Gilbert in the 1970s have revolutionized the field of molecular biology and have a profound impact on various areas of research, including biology and medicine(Linnarsson, 2010). The first ever genome to be sequenced was of the bacteriophage PhiX174, which was of 5,375 nucleotides. The sequencing was achieved using simple plus-minus method (Sanger et al., 1977). The next significant achievement was human genome project. The project of human genome initiated in 1990 with only one objective that is sequencing and also identifying base pairs in human genome, with the aim of discovering the genetic causes of diseases and ultimately developing treatments. This project was also recognized as megaproject as human genome consists of approximately 3.1 base pairs (Moraes & Góes, 2016).

The sequencing method proposed by Sanger is considered as the first generation sequencing. Biologist primarily relied on Sanger and Maxam-Gilbert sequencing techonogies before the development of new sequencing technologies which revolutionized genome exploration and analysis. The latest sequencing technology, also known as second-generation or next-generation sequencing represents a notable advancement over Sanger sequencing. It is typically classified into three main techniques: Roche/454 pyrosequencing (introduced in 2005), Illumina/Solexa sequencing by polymerase synthesis (introduced in 2006), and ABI/SOLiD sequencing by ligase (introduced in 2007) (Qiang-long et al., 2014). These sequencing techniques offer rapid speed and high throughput, enabling genome sequencing projects that used to take years with the Sanger technique to be accomplished within weeks (Ansorge, 2009).

## 2.11 Illumina Sequencing

Illumina sequencing also known as next generation sequencing is a powerful and widely used method for determining the nucleotide sequence of DNA or RNA molecules. The illumina sequencer employs sequencing by synthesis technology and provides an unparalled quality of short reads (Klumpp et al., 2012). MiSeq, a next-generation sequencing instrument developed by Illumina, is among one of the tiniest benchtop sequencers that is available. It facilitates generation of cluster onboard, an amplification process, sequencing of genomic DNA, and comprehensive analysis of data. This includes base calling, alignment, and variant calling, all conducted seamlessly within a single run. MiSeq has emerged as a cost-effective and widely used platform which yields upto 15Gb output in as little as 4 hours, providing rapid turnaround times, with up to 25 million single reads and 50 millions paired –ends reads(Ravi et al., 2018).

# Chapter 3 MATERIALS AND METHODS

## 3.1 Sample collection and culture

The urinary bacterial samples for the experiment were obtained from Sukraraj Tropical and Infectious Disease Hospital (STIDH) and stored aseptically at 4˚C. Thus collected bacterial samples were firstly subculture on Macconkey agar to distinguish Gram negative bacteria from Gram positive bacteria and then it was further sub cultured on nutrient agar. The bacterial strains were confirmed as respective strains by Gram staining and few confirmatory tests including biochemical tests.

**Table 1: List of collected bacterial samples and their codes**

| S.N | Bacteria | Bacterial Code |
|---|---|---|
| **1.** | *Klebsiella Pneumoniae* | 6697 |
| **2.** | *Klebsiella Pneumoniae* | 5615 |
| **3.** | *Escherichia coli* | 2778 |

## 3.2 Matrix Assisted Laser Desorption Ionization- Time of flight (MALDI-TOF) Analysis Assay

For further bacterial identification, MALDI-TOF mass spectrometry was done. For this, bacteria were sent National Public Health Laboratory (NPHL). Identification of bacteria using MALDI-TOF mass spectrometry, begin with isolating a single, pure colony of the bacteria on a culture plate. This colony is then picked and placed on a MALDI-TOF mass spectrometry plate, which has defined spots. A thin layer of matix, like a mixture that cotain α-cyano-4-hydroxy-cinnamic acid, acetonitrile, and trifluoroacetic acid, was applied over the organism. After drying for about 5 minutes, different spots on the plate are used for identification, calibration standards, and checking for reagent contamination. The MALDI-TOF mass spectrometer's chamber is filled with the plate, and the process is automated. The mass spectrum was created for the test isolates then it was compared to the reference spectra that are already present in a database The software evaluates how the similarity between the spectrum of test isolates with the spectra that are known and found in the system, providing a ranking or score, also provides a list of potential organisms, and lastly the level of identification confidence. This is done in a way similar to a Google search, eliminating the need for the technologist to interpret the mass spectrum formally. Multiple isolates can be tested simultaneously, and the analysis turnaround time is about three minutes per isolation.

Comparing this technology to traditional biochemical-based automated instruments, MALDI-TOF is considered more environmentally friendly ("green") because it requires fewer disposable items and reagents, and the plates can be reused (Patel, 2013).

## 3.3 Antibiotic Susceptibility Test

As the collected samples were clinical samples, antibiotic susceptibility testing was employed to confirm that the strain were multidrug resistant. The test was done with the help of Kirby-Bauer disc diffusion technique. Antibiotic belonging to different generations and various classes were used. To begin, a bacterial culture in its logarithmic growth phase was prepared, adjusting its turbidity to match a 0.5 MacFarland standard. This culture was then evenly spread across the Muller Hinton agar plates using cotton swab which are sterile and then are allowed to air dry. After drying of the plate, with the help of sterile forceps, discs of antibiotics was placed carefully on the agar surface maintain a distance between the different discs. The agar plates were then overnight incubated at 37°C. After overnight incubation, the zones where bacterial growth was inhibited by the antibiotics were measured using a calibrated ruler.

**Table 2: Lists of Antibiotic used**

| SN | Antibiotics used | Short Form | Concn of discs (mcg) |
|---|---|---|---|
| 1 | Ciprofloxacin | CIP | 5 mcg |
| 2 | Gentamicin | GEN | 10 mcg |
| 3 | Ceftazidime | CAZ | 14 mcg |
| 4 | Cotrimoxazole | COT | 25 mcg |
| 5 | Amoxicillin-Clavulanic acid | AMC | 10/10 mcg |
| 6 | Meropenem | MEM | 10 mcg |
| 7 | Tigecycline | TGC | 15 mcg |
| 8 | Doxycycline | DOX | 30 mcg |
| 9 | Cefepime | CFM | 300 mcg |
| 10 | Cefatrizine | CFS | 75/30 mcg |
| 11 | Imipenem | IPM | 10 mcg |
| 12 | Nitrofurantoin | NIT | 300 mcg |

| | | | |
|---|---|---|---|
| 13 | Nalidixic acid | NA | 10 mcg |
| 14 | Piperacillin | PIP | 100/10 mcg |

## 3.4 Detection of Extended Spectrum beta lactamase (ESBL) and Metallobeta lactamase (MBL) production

For the possible ESBL production, the bacteria *Klebsiella pneumoniae* was firstly screened using ceftazidime (30ug)). As per the CLSI guidelines, isolates demonstrating reduced susceptibility to either ceftazidime (with a zone of inhibition ≤22 mm) or cefotaxime (with a zone of inhibition ≤27 mm) are classified as potential ESBL producers. To confirm ESBL production, combined disc assay using ceftazidime(30ug) and ceftazidime/clavulanic acid(30/10ug),discs was used. The zone of inhibition observed with ceftazidime was compared to those observed with ceftazidime/clavulanic acid. A positive confirmation of ESBL production was determined if there was an increase in zone of diameter of ≥5mm in the presence of clavulanic acid.

**Table 3: Extended Spectrum beta lactamase detection**

| SN | Antibiotics used | Code | Concn of discs(mcg) |
|---|---|---|---|
| 1 | Ceftazidime +Ceftazidime/Clavulanic acid | CAZ/CAC | 30 mcg+30/10 mcg |

The isolate displaying resistance to imipenem was further examined to confirm the production of metallobeta- lactamase(MBLs) using a combined disc assay. This assay involved the use of both imipenem and imipenem/ethylenetetracetate(EDTA). The zone of inhibition of imipenem was compared with the zone of inhibition of imipenem/ethylenediaminetetracetate (EDTA). A positive confirmation of MBL production was determined if the zone of inhibition with the imipenem in combination with EDTA disc was found to be greater or equal to 7mm than the imipenen disc alone.

**Table 4:Metallobeta lactamase detection**

| SN | Antibiotics used | Code | Concn of discs (mcg) |
|---|---|---|---|
| 1 | Imipenem/ Imipenem +EDTA | IMI/IMD | 10mcg+(10-750) mcg |

## 3.5 Antibiotic Susceptibility Test Using Vitek Compact System 2

Further confirmation of Antibiotic Susceptibility test was done using Vitek Compact System. Bacteria were sent to Siddhi Poly Path Lab. The Vitek2 compact system from bioMierieux is an extensively automated setup utilizing minute plastic reagent cards, roughly the size of a credit card, each containing 64 wells and minute amounts of test media and antibiotics. During a short incubation period, bacterial growth is continuously monitored by the Vitek2 through turbidimetry. Depending on the setup, the instrument can handle 30-240 tests simultaneously. The susceptibility card permits testing of common, rapidly proliferating gram-negative and gram-positive aerobic bacteria, as well as *Streptococcus pneumoniae*, within a 4 to 10-hour timeframe. Some laboratories still utilize the older, less automated Vitek1 system, which is more limited with a 45-well card and does not include testing for *Streptococcus pneumoniae* (Jorgensen & Ferraro, 2009).

## 3.6 Bacterial Genomic DNA extraction

Genomic DNA from *Klebsiella pneumoniae* was extracted using the CTAB method. To start, centrifugation of 1 ml of an overnight bacterial culture was carried out for 5minutes at 13,000 rpm. The resulting pellet at the bottom of the tube was resuspended in 567 µl of TE buffer with gentle vortexing. Next, step was the addition of 30 µl of 10% SDS and 3 µl of proteinase K, and the resulting mixture was incubated at 37°C for 1 hour. Following incubation, addition of 100 µl of 5M NaCl and 80 µl of CTAB/NaCl solution (0.7M NaCl, 10% CTAB) was performed, and incubation of the mixture at 65°C was done for 10 minutes. After this, to the tube an equal volume of chloroform alcohol (24:1) was added and was uniform mixing was done. The mixture was then subjected to centrifugation for 5 minutes at 13,000 rpm. The aqueous layer i.e. upper layer was carefully without mixing two different layer was transferred to a new sterile tube. Addition of 600 µl of isopropanol was done inorder to precipitate the DNA. The tube was mixed until DNA strands were visible. This solution was centrifuged again at 13,000 rpm for 5 minutes. Finally, the supernatant i.e. the liquid portion was thrown, and the DNA pellet present at the bottom of the tube was the washed using 1 ml of 70% ethanol. This was followed by another round of centrifugation at 13,000 rpm for 5 minutes in oder to wash DNA pellet. After removing supernatant, the pellet was left to air dry to remove any ethanol if left after discarding the supernatant. Finally, after complete drying of the DNA pellet, it was resuspended in TE buffer (50 µl) and was stored at 4°C.

## 3.7 Amplification of 16srRNA gene of *Klebsiella pneumoniae*

The 16S rRNA gene amplification process was conducted to molecularly confirm the bacterial sample. We utilized pre-optimized PCR conditions and reaction volumes for this amplification step.

**Table 5: Forward and reverse primer for 16SrRNA Sequencing**

| Primer name | Sequence |
|---|---|
| 27F | 5'-AGAGTTTGATYMTGGCTCAG-3' |
| 515R | 5'-TTACCGCGGCKGCTGGCAC-3' |

**Table 6: Thermocycling condition for PCR amplification**

| SN | Steps | Temperatue | Time | Cycle |
|---|---|---|---|---|
| 1 | Initial Denaturation | 94 | 3min | 1 |
| 2 | Denaturation | 94 | 30sec | 28 |
| | Annealing | 60 | 40sec | |
| | Extention | 72 | 1min | |
| 3 | Final Extention | 72 | 7min | 1 |
| 4 | Final Hold | 4 | ∞ | |

The PCR reaction mixture was prepared in a PCR tube, which was then subjected to PCR amplification. After amplification process, the amplified product obtained was analyzed with the help of agarose gel electrophoresis (1%) in Trsi Acetate EDTA buffer. For DNA staining, Ethidium bromide (EtBr) was used. The band of DNA on the gel was seen with the help of UV transilluminator, and image of DNA band was captured using the Geldoc system. Thus prepared PCR product was sent to the Center for Molecular Dynamics Nepal (CMDN) for 16S rRNA sequencing. Various bioinformatics tools were employed to assess the sequence obtained from the lab. Chromas was utilized for chromatogram file analysis, and a direct BLAST search was conducted to determine the level of sequence similarity between our sequences and sequence found in the nucleotide database.

## 3.8 Molecular amplification of resistance gene (blaNDM)

PCR of blaNDM gene was done in following PCR program (Table 8) using following set of primer (Table 7).

**Table 7: Primer used to amplify blaNDM gene.**

| | | |
|---|---|---|
| **Forward primer sequence** | **AATGCTGAATAAAAGGAAAACT** | **(Tm = 47.6ºC)** |
| **Reverse Primer sequence** | **GGCAGATTGGGGGTGA** | **(Tm = 51.8ºC)** |
| **PCR product length** | **869 bp** | |

**Table 8: PCR condition of amplification of blaNDM gene**

| S.N. | Steps | Temperature(˚C) | Time |
|---|---|---|---|
| **1.** | Enzyme Activation | 95 | 2minutes |
| **2.** | Initial Denaturation | 95 | 30seconds |
| **3.** | Annealing | 56.7 decrease 0.5 per cycle | 30seconds |
| **4.** | Extension | 72 | 90seconds |
| **5.** | Step | (2-4) 14 cycle | |
| **6.** | Denaturation | 95 | 30seconds |
| **7.** | Extension | 72 | 90seconds |
| **8.** | Steps | (6-8) 19 cycle | |
| **9.** | Final Extension | 72 | 5 minutes |
| **10.** | Final Hold | 4 | ∞ |

The PCR reaction mixture was prepared in a PCR tube and subsequently PCR amplification was carried out. Afterward, 5µl of the amplified product was analysed via 1% agarose gel electrophoresis. The DNA band on the gel was visualized using a UV transilluminator, and its image was captured utilizing the Geldoc system.

## 3.9 Sewage sample collection and processing

Sewage sample was collected from Bagmati River near Balkhu, as it is one of the most polluted rivers from Kathmandu valley. The collection of sample was done from stagnant water rather than running water. Sample was collected in 50ml falcon and transported

to Center Department of Biotechnology where further processing was done. Firstly, the collected sewage sample was subjected to centrifugation at 4000rpm for exactly 30 minutes to eliminate larger and unwanted cellular particle along with other contaminant present. Then, after centrifugation upper portion i.e. supernatant was collected in new sterile tube and then filtration was done through a syringe filter of size 0.22 µm (PES Filter Media, Whatman™). The resulting filtrate, collected in a sterile Falcon tube, was used as the phage source for further experiments.

**Figure 4: Water sample collection site, Balkhu, Kathmandu**

## 3.10 Bacteriophage Isolation

Isolation of bacteriophage was conducted using protocol described by Adams (1951). The protocol uses double layer agar assay (DLAA) where two layers of agar is used battom layer of hard agar (1.5%) and upper layer is soft agar(0.5%). This method is commonly used to isolate bacteriophages from various sources such as water samples, sewage, dairy waste, and soils. In the DLAA, a base layer of hard agar (1.5% tryptic soy agar) is used, while a top layer consists of tryptic soy broth (TSB) with 0.5% agar. To begin, 1 ml of the processed filtrate was placed in a sterile Falcon tube. After this, 100 µl of log-phage culture of bacteria was added to the falcon tube and it was left undisturbed for 5 minutes to allow for phage attachment. After the attachment period, soft agar(3ml) which is TSB containing 0.5% agar having temperature of about 50°C was poured to the tube. Then gentle mixing was performed and whole contain was poured gently over the TSA plate containing hard agar layer. The plate was then gently mixed by making eight on the flat surface. The plates were then allowed to dry completely. Overnight incubation of plates is done at 37˚C. Next to overnight incubation, the presence of any plaques was seen. Observations included the clarity or turbidity of the lysis, the number of plaques, and the shape and size of the plaques.

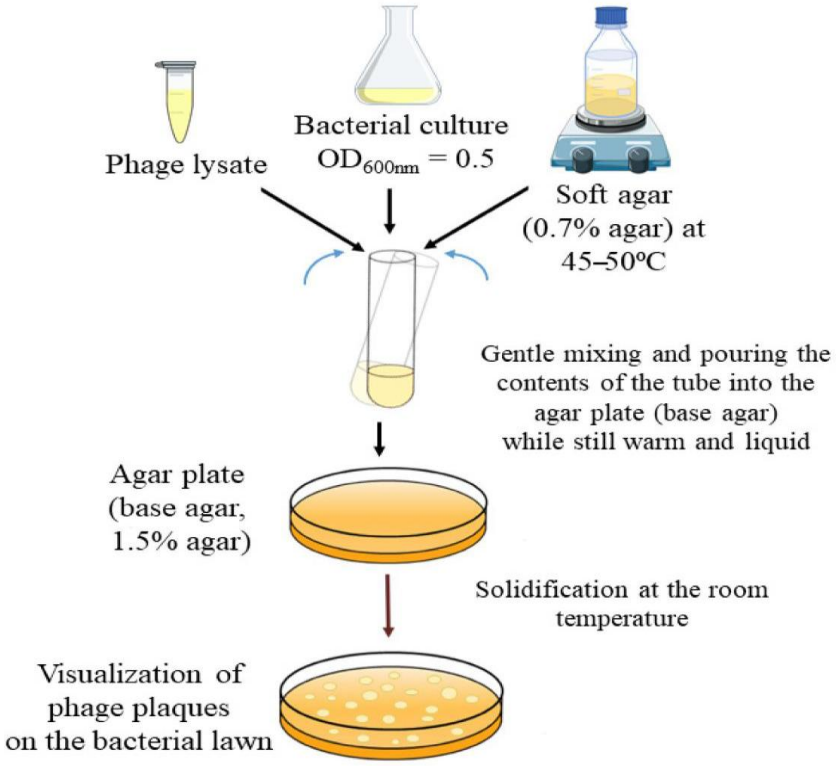



**Figure 5: DLAA technique for phage isolation and culture (Stachurska et al., 2021)**

## 3.11 Clonal Purification of bacteriophage

The plaques that are initially seen on the Petri dish may be of different phages. For the characterization and further processing the phage need to be in pure form. Continuous streaking method was used to obtain the pure form of phage from the heterogeneous populations of phages. First of all, a single isolated plaque was picked using a sterile inoculating loop and then continuously streaked onto a TSA (1.5%) plate. The host bacterial culture in active log phase (100 µl) was mixed with 3ml of semisolid TSB (0.5%) and poured in the streaked Petri dish from the end point and the plate was allowed to hardened. Overnight incubation of plates at 37˚C was carried out. Observation for the clear lysis and plaque formation was done after overnight incubation. Three subsequent round of streaking were performed for purification.

## 3.12 Preparation of phage Stock

The phage stocks were prepared by amplifying the phages on Petri dish. A single plaque was streaked on eight TSA plate by using continuous streaking technique. After that 100µl of bacterial culture (host bacteria in log phase) was mixed with 3ml of TSB and poured onto the streaked plates. After solidification of agar, overnight incubation was done at 37˚C. Following overnight incubation, 4 ml of SM buffer (Sodium Magnesium buffer was added to each plate. The SM buffer aids in absorbing and detaching the

phage particles from the agar. The Petri dishes were then agitated in shaker for 2hours at 120 rpm. After this, the upper layer was scrapped and collected in sterile falcon tube. The mixture collected in the falcon tube was subjected to vortexing for about 10minutes. After vortexing, the tube was centrifuged at 4000rpm for thirty minutes. The filtration of resulting supernatant was was done using a syringe filter having size of 0.22 µm and was gathered in a sterile Falcon tube. The filtrate was then stored at 4°C.

## 3.13 Determination of Phage titer

The phage titer assay is used to determine the amount of bacteriophage particles present in stock solution of bacteriophage. For the titer assay, the phage stock was serially diluted up to 10^15 dilution. For this, 900 µl of SM buffer were distributed into Eppendorf tubes labeled from 10^-1 to 10^-15. Then 100µl of phage from the stock was transferred to eppendorf tube labeled as 10^-1 and mixed properly. Subsequently serial dilution was performed up to 10^-15 dilution changing the tips in each dilution. Now, for spot assay, the grid was drawn with the help of scale at the bottom of agar plate and labeled with the designations for phage dilutions from 10^-1 to 10^-15 and negative control (NC). Initially, lawn of bacterial culture was created. This was done by mixing 3ml of semisolid TSB and active log phase host bacteria and then overlaying the content over TSA plate. The plates were left undisturbed for allowing the solidification of agar. Next, 5 µl of each respective phage dilution was aseptically transferred onto designated grids on the TSA plate. The droplets were allowed to fully absorb into the agar, which was followed overnight incubation at 37˚C. A clear zones indicating bacteriophage activity were observed post-dilution.

## 3.14 Determination of phage stock concentration

After spot assay, highest titer which showed the clear lysis was selected and DLAA was performed for those dilutions. For DLAA, 1ml of serially diluted phage and 100µl of host bacteria in active log phase was mixed and allowed to stand for attachment for at least 5minutes. Then, 3 ml of soft agar was added on top of the TSA plate and overnight incubation at 37˚Cwas done. Following overnight incubation, plate with countable plaques number was selected and the same dilution was taken for the determination of titer.

The total number of plaques forming units per milliliter i.e. pfu/ml of phage solution was calculated by the help of formula mentioned below:

$$\text{pfu/ml} = \frac{\text{Number of phages}}{\text{volume of phages} \times \text{dilution}}$$

Where, pfu/ml=plaque forming unit per milliliter of sample

## 3.15 Host Range Analysis

The host range of isolated bacteriophage was determined by spot assay. Both interspecies and intra species host range was determined. For this, lawn cultures of different bacteria were prepared. This was done by mixing log phase bacteria (100µl) and soft agar (0.5%, 3ml) and pouring onto the 1.5% TSA plate. After solidification 5µl of phage stock was added to the lawn and 24 hours incubation was done at 37˚C. Following that, careful examination for potential bacterial culture lysis was carried out.

For intra specific host range five available strain of *Klebsiella pneumoniae (Klebsiella8, Klebsiella9, Klebsiella12, Klebsiella13, Klebsiella14*) were used which were collected from Shukraraj Tropical and Infectious Disease Hospital (STIDH). Similarly, different strain of the bacteria like *Pseudomonas, Acinetobacter, Staphylococcus* and *Escherichia* species were used to determine interspecies host range.

## 3.16 Protein profiling by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Analysis of bacteriophage protein using SDS-PAGE was done by direct heating method. Initially, 15 µl of phage solution was combined with 20 µl of loading dye (62.5Mm Tris HCL, 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromophenol blue). The mixture prepared was subsequently heated for 10 minutes at 95°C. The prepared sample was then set for gel electrophoresis.

For SDS-PAGE, two gels were prepared, one was resolving gel (12%) and other was stacking gel (5%). The gel was cast in a glass plate cascade and carefully placed into the electrophoresis tank. Electrophoresis was conducted using 1X Tris-glycine running buffer (39 mM Tris, 48 mM glycine, and 0.1% SDS). Subsequently, 10 µl of the prepared sample and 5 µl of protein marker were loaded into the well, and the gel was electrophoresed at 120 volts for 2 hours. Then after electrophoresis process, the gel is taken out from the tank and stained using a staining dye, Coomassie brilliant blue and left in the shaking incubator overnight for the proper observation of the band. After complete staining procedure, the gel was then placed in the destaining solution (75% Glacial acetic acid, 5% methanol and double distilled water) in order to eliminate any excess stain. The gel was then examined to detect various protein bands of phage. The Genei protein molecular weight marker- Broad range (50 lanes, ranging from 3.5 KDa to 205 KDa) was utilized as a reference standard.

## 3.17 Physiochemical characterization of bacteriophage

To consider phage as antibacterial agent it must be stable at different temperature and pH .Thus, phage needs to be exposed to various physiochemical conditions. So, the pH and thermal stability was tested in laboratory.

## 3.18 Thermal stability

For determining the thermal stability of phage, 1ml of phage stock (10^-8 dilution, pfu/ml) was aliquoted in six eppendorf tube labeled 10, 20, 30, 40, 50, 60minutes each. The tubes were then incubated at 37˚C. After 10minutes, tube labeled as 10 minutes was withdrawn and DLAA was performed immediately. Same procedure was applied for other tubes as the time labeled. Overnight incubation was performed. Plaques seen after incubation was counted and expressed as pfu/ml (plaque-forming units per milliliter).

The described procedure was repeated for all the temperature for studying the effect of different temperature on bacteriophage. Subsequently, a graph correlating pfu/ml with various incubation times was plotted.

## 3.19 Stability of bacteriophage against different pH

Bacteriophage was exposed to various pH ranges in order to determine its survivability at different pH. Using 1M HCl and 1M NaOH, pH of LB was adjusted so as to prepare the pH gradient. Then, 300µl of bacteriophage stock having 10^8 dilution and 700µl of pH adjusted media were mixed together maintaining the final volume 1ml. Incubation at room temperature for 1hour was performed. Then, 100µl of active log phase host bacteria and pH treated phage solution were mixed. Immediately, after mixing DLAA was performed. The plates were then subjected to overnight incubation. Following this, number of plaques were counted. Subsequently, a graph correlating pfu/ml with various pH values was then constructed.

## 3.20 One step growth curve analysis and burst size determination

First, 1000 µl of high titer phage (10^-8 dilution) was taken in each tube. Adding of 100 µl of log phase host bacterial culture was done next. Both were mixed properly. All tubes were incubated at 37°C. The tube labeled "5min" was removed after 5 minutes and centrifugation was carried out for five minutes at 12000rpm. The supernatant which contain unabsorbed phage was discarded. The phage which are absorbed on bacterial host are found in pellet at the bottom of the tube. This was resuspended in SM buffer(100µl). DLAA was performed after resuspention. Above process was repeated to all the tubes at their respected time. Following overnight incubation pfu/ml was calculated and then graph was constructed between pfu/ml and incubation time.

## 3.21 Phage DNA extraction

Extraction of bacteriophage DNA was done using the kit available at the laboratory. The procedure was followed according to the manual provided with the kit. The kit used was Norgen Biotek Corp Phage DNA Isolation kit. Foremost 15ml of sterile falcon tube was taken, to which 1ml of bacteriophage lysate was added along with 500µl of lysis buffer. Inorder to mix them properly, the tube was vortexed vigorously for about 10 seconds. Then incubation at 65˚C for 15minutes was carried out. Water bath was maintained at 65˚C to perform incubation. In between the incubation period the tube was mixed by up and down motion for 2 to 3 times. After incubation, next step was addition of 320µl of isopropanol. The tube was vortexed once again to mix the content properly. The collection tube provided with the kit was set with the spin column which was also found in the kit. Then, 650µl of the lysate prepared earlier was loaded onto the column and centrifugation was carried out for one minutes at 8000rpm. Then, the flowthrough which was collected in the collection tube was discarded and the both spin column and the spin column was reassembled. Same procedure was repeated till the time all the lysate on the tube were being passed through the column. Next step was washing step. Firstly, the column was washed with the help of wash solution A. Wash solution A (400µl) was added to the spin column. Centrifugation was then carried out for 1minute at 8000rpm. Washing process was repeated two more times. Afterward, the spin column was centrifuged at 8000rpm for 2 minutes to dry it. Finally, after complete washing the collection tube was discarded. The column was then fit with the elution tube which was also found in the kit used. Next step was the addition of Elution buffer B inorder to elute bacteriophage DNA into the elution tube. For this 75µl of elution buffer B was added to the spin column. Centrifugation was carried out next at 8000rpm for 1minutes. After the centrifugation, bacteriophage DNA was collected in the elution tube. For proper storing, extracted bacteriophage DNA was kept at -20˚C.

## 3.22 Electrophoresis of phage DNA

To conduct electrophoresis of phage DNA, firstly agarose gel(1%) was prepared. Inorder to prepare 1% gel, 0.5 grams of agarose powder was mixed properly with 50ml of 1X TAE buffer solution. So as to allow proper mixing of the powder, the mixture was boiled properly. Then the mixture was allowed to cool to get it to about 50˚C and the ethidium bromide was added and thoroughly mixed. After proper mixing, the prepared gel was slowly poured into a casting tray. This was allowed to solidify without any disturbance. Next, the bacteriophage DNA (5µl) which was to be visualized is mixed with loading dye (1µl). The mixture was mixed properly and was then loaded onto the well. A Fermentas O'GeneRuler 1KB DNA ladder was also run with the bacteriophage DNA. After this, gel electrophoresis was carried out for about one hour at 70 volts. A UV transilluminator was used to visualize bacteriophage DNA bands.

## 3.23 Whole genome sequencing of Bacteriophage

Due to the unavailability of sequencer in the laboratory, the bacteriophage DNA was sent to Center for Molecular Diagnosis Nepal(CMDN) which is located in Thapathali, Kathmandu inorder to perform the sequencing process.

Before sequencing, preparation of library is necessary. The entire DNA was fragmented into smaller pieces, after which adapters were attached. Attachment was done at both side of fragmented DNA. Next step was indexing. This was conducted to distinguish between different samples. Subsequently, using the Illumina platform, the generated DNA fragments were sequenced.

The fragmented DNA after the preparation of library, for sequencing was loaded onto NGS MiSeq Illumina Platform.

The fragmented DNA was immobilized on a flow cell, where polymerase chain reaction of segments of DNA occurs via bridge amplification. Following the amplification, there was generation of clusters of DNA.

The process of sequencing on the Illumina platform utilizes reversible dye terminator chemistry, where single bases are sequentially added. Each base is fluorescently labeled, emitting a signal upon addition that is recorded by the computer to determine the added base. After each addition cycle, bases which were excess were washed away, deblocking step was done after this, and there is repeatation of this cycle till the whole fragments of DNA are sequenced. Initially stored as BCL files, the sequencing data is then converted to FastQ files using computer software. FastQ files are crucial during downstream analysis. This is crucial but different errors like that of base calling errors, low-quality reads, contamination of adaptor, and errors in sequencing may occur. To mitigate these issues, a quality control step is performed. FastQC is employed to assess and correct read quality, while FastP filters the reads. FastP, noted for its speed due to being written in C++, efficiently filters reads compared to other tools. Once filtering is complete, filtered short reads are obtained. These short reads are subsequently assembled into contigs using a de novo genome assembler such as SPAdes. Contigs represent DNA segments with overlapping ends, enabling reconstruction of the original sequence.

From the assembled contigs, viral sequences can be detected using tools such as Virfinder, which employs machine learning based on k-mer frequency rather than relying on homology-based searches or gene finding methods. Once viral sequences are identified, their genes can be annotated using tools like Prokka, designed for prokaryotic annotation. Prokka identifies protein coding regions and annotates their products by searching sequentially in user-provided annotated proteins, UniProt bacterial proteins, RefSeq finished bacterial genomes, and the Hidden Markov Model Database. Proteins

not found in these databases are labeled as 'Hypothetical protein'. Prokka generates output files in formats such as genebank (gbk), Fasta (fna), and text (txt). The output files from Prokka (genebank and Fasta) are further analyzed using tools like UGENE, Phaster, and Prokka itself to extract information from the viral genome. These tools aid in exploring the structure, function, and characteristics of viral genetic material during phage genome analysis.

## 3.24 Biofilm production

The biofilm production was done using the protocol which was described by Christensen et al. 1995. The protocol developed by them is now considered as gold standard method (O'Toole, 2011). The method was employed to check the biofilm production with slight modification. The bacteria were firstly inoculated in Luria Bertani broth and incubation was done for 24hours at temperature 37˚C. Then, dilution was done using fresh Luria bertani media in the ratio 1:100 to meet the optical density 0.5. Now 200 µl of diluted culture was added to sterile 96 well polystyrene tissue culture plates. Sterile Luria bertani broth was used as negative control.Incubation was then done for 24 hours at temperature 37˚C. After the completion of incubation, the content from each well was discarded by gentle tapping of the plates. After this, washing step was performed by using phosphate buffer saline (pH 7.2) atleast for four times. This helps in removal of free floating bacteria. Next step is fixation step, where the biofilm that are formed on the wall of plates are fixed using 2% sodium acetate and then the biofilm was stained with the help of crystal violet(0.1%). After the staining step, the plate was washed by the helpof deionized water and then it was allowed to air dry. After complete drying, 95% ethanol was poured in each well. ELISA reader was then used to measure the optical density of the biofilm that were attached to the wall. The OD was measured at 620nm. This experiment was conducted in triplicate. This is required in order to maintain consistency and also to make sure the experiment is reliable. The optical density cut off (ODc) was determined using the formula given below.

Optical density cut off value= an average optical density of negative control + 3× standard deviation of negative control

Being based with the optical density cutoff value (ODc), the classification of bioflim forming bacteria was done. They were classified as strong, moderate and weak biofilm producer.

A bacteria was considered as strong biofilm producer if , $OD > 4ODc$

Similarly they was categorized as moderate biofilm producer if, $2ODc < OD \leq 4ODc$

And are considered weak biofilm producer if, $ODc < OD \leq 2ODc$

## 3.25 Disruptions of biofilm by the use of bacteriophage

Biofilm disruption was carried out following the procedure outlined by Forti et al. (2018). Initially, inoculation of bacteria was done in luria bertani broth which was followed by the overnight incubation at 37˚C.Then dilution of bacterial culturewas performed in fresh LB media at a ratio of 1:100 to achieve an optical density of 0.5. Then, 200µl of the bacterial culture was added to sterile 96-well polystyrene tissue culture plates. Overnight incubation was done at temperature 37˚C to ensure the formation of biofilm. Following the incubation process, the liquid portion was discared and washing was performed with 200µl of LB broth two times. Following the washes, 200µl of phage lysate was added to each well and incubation of 4 hours was done. Next step was washing step. This was done after empting the wells and with help of PBS. Washing step was performed twice. All the bacteria that were adhered to the well were fixed by adding 200 µl of sodium acetate and incubating at 60°C for 30 minutes. The contents were then discarded, and staining was performed with the help of crystal violet. For this 200µl of 0.1% of crystal violet was used. The staining was done for 10 minutes. The stained in excess was removed and the plate was rinsed using deionized water. The plates were then allowed to air dry. After proper drying of each well, 95% of ethanol was added. Finally, at 620nm using ELISA reader optical density was measured.LB broth was used as a negative control in this experiment.

## 3.26 Synergistic effect of bacteriophage and antibiotics in biofilm reduction

**A) Minimum Inhibitory Concentration determination of bacteria**:

**i) Culture Preparation:**

A bacterial culture of *Klebsiella pneumoniae* was grown overnight. The turbidity of the culture was adjusted to match that of a 0.5 McFarland standard solution. One milliliter of this adjusted culture was used for determining the Minimum Inhibitory Concentration (MIC).

**ii) Preparation of antibiotic solutions and its ranges**:

First, a stock solution of the antibiotic was made by solubilizing 0.2 grams of the antibiotic to 20 ml of distilled water, resulting in a concentration of 10,000 mg/L. Next step is the preparation of stock of 1000mg/l which was by mixing 1ml of stock solution of 10,000 mg/l with 9ml of distilled water. Further 100mg/l of stock was prepared. For this dilution of 100µl of the 10,000 mg/ml stock solution was done with 9.9ml of distilled water. Proper sterility was maintained to avoid any contamination.

After preparing the required stocks, antibiotic solutions were prepared with concentrations of 0, 1, 2, 4, 8, 16, 32, 64, 128, and 256 mg/L.

**iii) Incubation:**

Next, step is incubation step. For this, 1ml of all the antibiotics prepared were taken in separate tubes with proper labeling. Then, 1ml of overnight bacterial culture of *Klebsiella pneumoniae* which were already prepared were added to each tubes. The the tubes were subjected to overnight incubation. Any presence or absence of visible growth in each tube was observed.

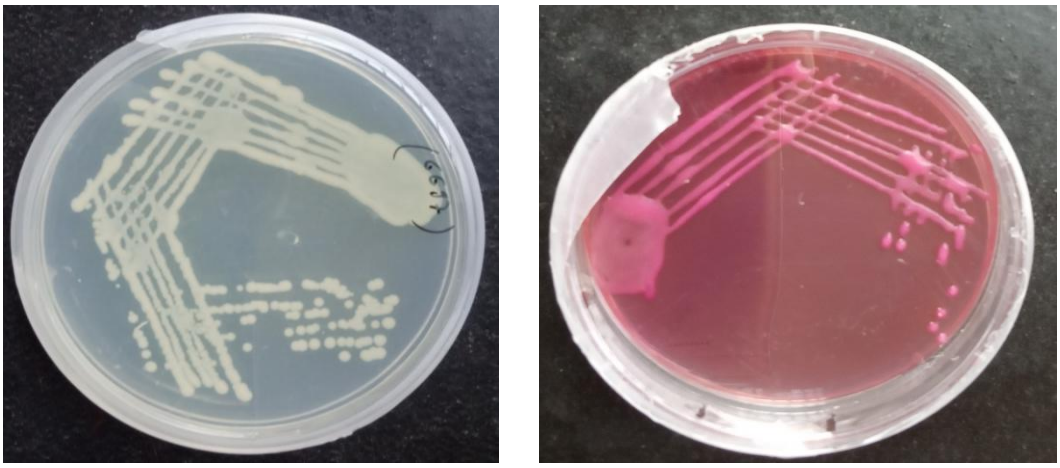
**B) A synergistic effect of bacteriophage and antibiotics in biofilm reduction:**

First step is the preparation of bacterial culture. For this, the bacteria i.e. *Klebsiella pneumoniae* was cultured overnight using LB broth. Subsequently, the dilution of the culture was carried out to achieve an optical density (OD) of 1. Then, 200 µl of the bacterial culture was inoculated into microtiter plates. Incubation of plates were done overnight for allowing the biofilm formation. After this, excess LB broth was discarded; each well using fresh LB broth was properly washed twice. Subsequently, bacteriophage lysate and the antibiotic solution matching the minimum inhibitory concentration were mixed in equal volume. Next, 200 µl of the prepared mixture of bacteriophage and antibiotic was added to each well. Then incubation of four hour was performed. For further step, PBs was used to wash the well. Washing was done for two times. This washing step helps in removal of any unattached bacteria. The bacteria attached to the plate wells were fixed with sodium acetate at 60°C for 30 minutes. After discarding the solution, staining was done using crystal violet. Staining was done for at least 10 minutes. Deionized water was used to remove any excess stain. Following this, 95% ethanol was added to each well to extract the stained cells, and the optical density (OD) using ELISA reader was measured. Luria bertani was used as negative control.

# Chapter 4 RESULTS AND DISCUSSION

## 4.1 Identification of host bacteria

The bacterial sample when cultured on Nutrient agar produced large, mucoid, and white colour colonies. On MacConkey agar, produced large, mucoid and pink colored colonies. These colony characteristics closely resembled those of *Klebsiella pneumoniae*.



(A) (B)

**Figure 6: Bacterial streaking on Nutrient agar(A) and MacConkey agar(B)**

A single isolated colony from the nutrient agar plates was selected, and a Gram staining was performed. On gram staining the bacteria was found to be gram-negative rod-shaped bacterium.

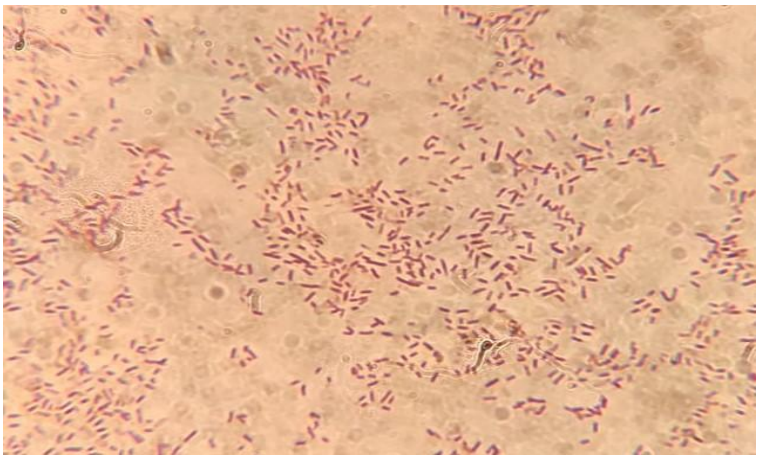


**Figure 7: Gram staining of bacteria**

## 4.2 Biochemical Test of Host bacteria

To confirm the identity of the bacterium, further biochemical tests were performed.

**Table 9: Biochemical Test for confirmation of bacteria**

| S.N. | Test Performed | Result Obtained | Inference |
|---|---|---|---|
| **1.** | Indole Test | Negative | *Klebsiella pneumoniae* |
| **2.** | Methyl Red Test | Negative | |
| **3.** | Voges Proskauer Test | Positive | |
| **4.** | Citrate Utilization Test | Positive | |
| **5.** | Triple Sugar Iron Agar Test | Red/Red, Gas-positive,H2S-negative | |
| **6.** | Oxidative Fermentative | Fermentative | |
| **7.** | Urease | Positive | |

The result showed Indole negative, MR negative, VP positive, Citrate positive, TSIA red/red, Urease positive and fermentative. Thus, confirming the identification of the bacteria *Klebsiella pneumoniae*.

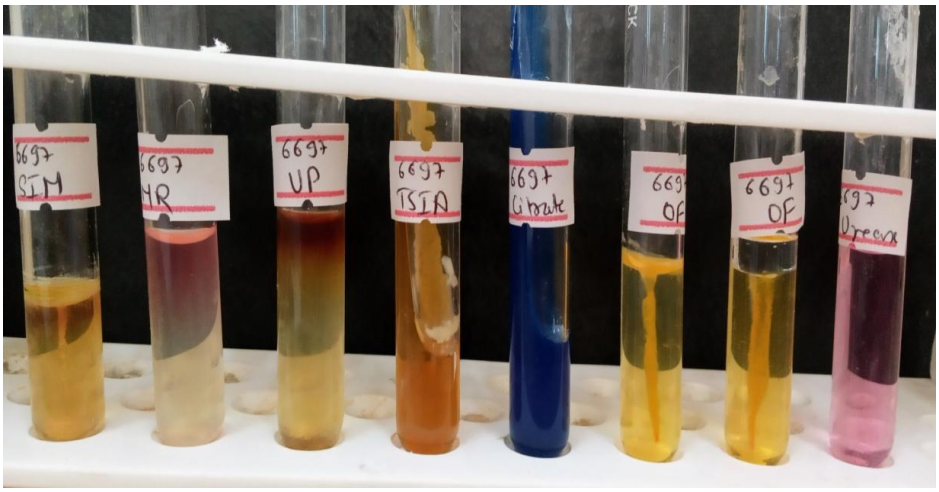



**Figure 8: Biochemical test of Klebsiella pneumoniae**

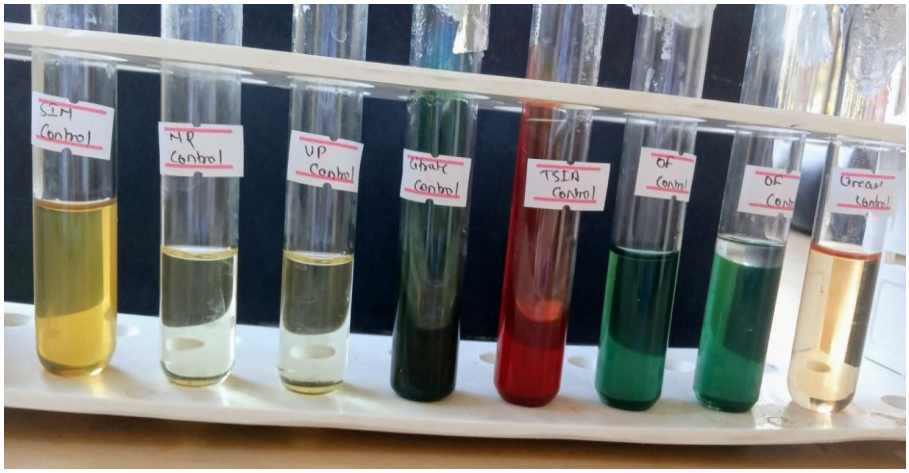



**Figure 9: Control for Biochemical test**

For the additional confirmation, a molecular test was conducted.

## 4.3 Mass spectrometry analysis for the bacterial conformation

For bacterial confirmation, bacterial sample "6697" was sent to the National Public Health Laboratory. The bacteria were identified as *Klebsiella pneumoniae*, and the confirmation method utilized Biomerieux Vitek-MS. The results are included in the appendices section (1) of this thesis.

## 4.4 Antibiotic Susceptibility Test (AST) / Antibiogram Assay

The sample bacteria(*Klebsiella pneumoniae*, 6697) was found to be resistant to more than 3 classes of antibiotics thus the bacteria was inferred as multidrug resistant. The antibiotics used were of different generation and various classes such as quinolones, aminoglycosids and beta lactams. The bacteria were also resistant to carbapenem class of antibiotics that is Imipenem and meropenem as a result it was concluded as carbapenem resistance.

(A) (B) (C)

**Figure 10: Antibiotic susceptibilty test by Kirby Bauer disc-diffusion method. A,B,C showing AST of *Klebsiella pneumoniae*(6697). The clear zones indicate zone of lysis of bacteria.**

**Table 10: Antibiotic Susceptibility pattern of *Klebsiella pneumoniae***

| SN | Antibiotics Used | Concn of discs | Reference Zone | | | Zone of inhibition (mm) | Result |
|---|---|---|---|---|---|---|---|
| | | | S | I | R | | |
| **1** | Ciprofloxacin | 5 mcg | ≥25 | 19-24 | ≤18 | 0mm | Resistant |
| **2** | Gentamicin | 10 mcg | ≥15 | 13-14 | ≤12 | 0mm | Resistant |
| **3** | Cotrimoxazole | 14 mcg | ≥16 | 11-15 | ≤10 | 0mm | Resistant |
| **4** | Ceftazidime | 14 mcg | ≥18 | 15-17 | ≤14 | 0mm | Resistant |
| **5** | Amoxicillin | 10 mcg | ≥18 | 14-17 | ≤13 | 0mm | Resistant |
| **6** | Meropenem | 10 mcg | ≥19 | 16-18 | ≤15 | 0mm | Resistant |
| **7** | Tigecycline | 15 mcg | ≥19 | 15-18 | ≤14 | 13mm | Resistant |
| **8** | Doxycycline | 30 mcg | ≥14 | 11-13 | ≤10 | 14mm | Sensitive |
| **9** | Cefoperazone sulbactam | 75/30 mcg | ≥21 | 16-20 | ≤15 | 0mm | Resistant |
| **10** | Imipenem | 10 mcg | ≥19 | 16-18 | ≤15 | 0mm | Resistant |
| **11** | Cefepime | 30 mcg | ≥25 | 19-24 | ≤18 | 0mm | Resistant |
| **12** | Nalidixic acid | 10 mcg | ≥19 | 14-18 | ≤13 | 0mm | Resistant |
| **13** | Piperacillin tazobactam | 100/10 mcg | ≥21 | 15-20 | ≤14 | 0mm | Resistant |
| **14** | Nitrofurantoin | 300mcg | ≥17 | 15-16 | ≤14 | 13mm | Resistant |

## 4.5 Confirmation of Antibiotic susceptibility using Vitek Compact System 2

Antibiotic susceptibility test was further confirmed by Vitek Compact System 2 analyzers. For this bacterial sample was send to Siddhi Poly Path Lab. The result was similar to the test we performed in the laboratory. Additionally it was found sensitive to Fosfomycin, amikacin and colistin. Full table is included in the appendix portion.(2)

## 4.6 Detection of Extended Spectrum beta lactamase (ESBL) and Metallobeta lactamase (MBL) production

### 4.6.1 Extented spectrum beta-lactamase (ESBL)


**Figure 11: ESBL detection of *Klebsiella pneumoniae***

The test yielded a positive result if the area of inhibition surrounding either ceftazidime or ceftazidime clavulanic acid exceeded 5mm. Since no inhibition zones were detected around the sample bacteria, the bacteria were classified as ESBL negative.

### 4.6.2 Metallobeta lactamase detection test (MBL)

The difference in zone of diameter between imipenem and imipenem EDTA is greater than or equal to 7 mm was considered as positive result for metallobeta lactamase (MBL) production. Since, the difference was greater than 7mm, the bacetrial isolate was confrimed as MBL positive.


**Figure 12: MBL detection of *Klebsiella pneumoniae***

## 4.7 Molecular amplification of carbapenem resistance gene (blaNDM)

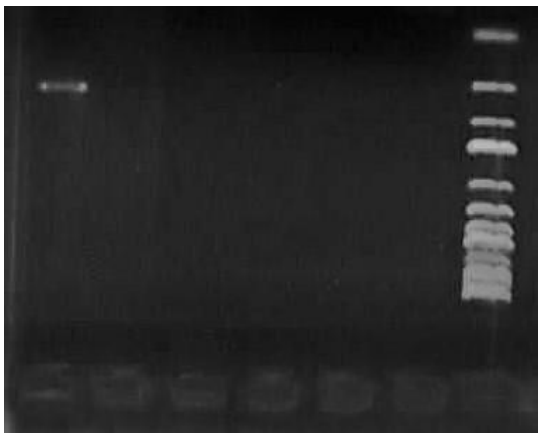


Figure 13:Molecular amplification of blaNDM gene

Carbapenem resistant gene blaNDM was found to be positive in *Klebsiella pneumoniae.*

## 4.8 Genomic DNA Extraction of *Klebsiella pneumoniae*

The DNA extraction from Klebsiella pneumoniae was conducted using the CTAB method. Subsequently, gel electrophoresis was performed on the extracted DNA, and the isolated DNA band was visualized under a UV transilluminator.

Figure 14: DNA band of *Klebsiella pneumoniae*

## 4.9 Molecular identification of the host bacterial strain using 16srRNA gene

16srRNA gene amplification was conducted for the isolated host bacterial species. After sequencing, the gene sequence was obtained in a chromatogram file accessible via the Chromas program. Subsequently, the chromatogram file was searched using the BLAST program (nucleotide BLAST) in the NCBI database to find sequences with similarity to our obtained sequence.The Maximum match was seen with partial sequence of *Klebsiella pneumoniae* subsp. pneumoniae strain SU101 which is 99.57%.

| Description | Scientific Name | Common Name | Taxid | Max Score | Total Score | Query Cover | E value | Per. Ident | Acc. Len | Accession |
|---|---|---|---|---|---|---|---|---|---|---|
| Klebsiella pneumoniae subsp. pneumoniae strain SU101 16S ribosomal RNA gene, partial seque... | Klebsiell... | NA | 72407 | 839 | 839 | 37% | 0.0 | 99.57% | 1102 | MT640265.1 |
| Klebsiella pneumoniae strain 7604 16S ribosomal RNA gene, partial sequence | Klebsiell... | NA | 573 | 839 | 839 | 37% | 0.0 | 99.57% | 1413 | MT516157.1 |
| Klebsiella pneumoniae strain 1k2 16S ribosomal RNA gene, partial sequence | Klebsiell... | NA | 573 | 839 | 839 | 37% | 0.0 | 99.57% | 885 | MT476960.1 |
| Klebsiella pneumoniae strain CEMTC_3425 16S ribosomal RNA gene, partial sequence | Klebsiell... | NA | 573 | 839 | 839 | 37% | 0.0 | 99.57% | 706 | MT439082.1 |
| Klebsiella pneumoniae strain CEMTC_2634 16S ribosomal RNA gene, partial sequence | Klebsiell... | NA | 573 | 839 | 839 | 37% | 0.0 | 99.57% | 703 | MT439072.1 |
| Klebsiella pneumoniae strain B17KP0020 chromosome, complete genome | Klebsiell... | NA | 573 | 839 | 6577 | 37% | 0.0 | 99.57% | 5308267 | CP052503.1 |
| Klebsiella pneumoniae strain B16KP0198 chromosome, complete genome | Klebsiell... | NA | 573 | 839 | 6682 | 37% | 0.0 | 99.57% | 5309291 | CP052520.1 |
| Klebsiella pneumoniae strain B16KP0183 chromosome, complete genome | Klebsiell... | NA | 573 | 839 | 6682 | 37% | 0.0 | 99.57% | 5307940 | CP052522.1 |
| Klebsiella pneumoniae strain E16KP0287 chromosome, complete genome | Klebsiell... | NA | 573 | 839 | 6610 | 37% | 0.0 | 99.57% | 5278060 | CP052265.1 |
| Klebsiella pneumoniae strain E16KP0235 chromosome, complete genome | Klebsiell... | NA | 573 | 839 | 6643 | 37% | 0.0 | 99.57% | 5184326 | CP052278.1 |
| Klebsiella variicola strain k8 16S ribosomal RNA gene, partial sequence | Klebsiell... | NA | 244366 | 839 | 839 | 37% | 0.0 | 99.57% | 1009 | MT355809.1 |
| Klebsiella pneumoniae strain SRG8 16S ribosomal RNA gene, partial sequence | Klebsiell... | NA | 573 | 839 | 839 | 37% | 0.0 | 99.57% | 1443 | MK743987.1 |
| Klebsiella pneumoniae strain KPBIQ19 16S ribosomal RNA gene, partial sequence | Klebsiell... | NA | 573 | 839 | 839 | 37% | 0.0 | 99.57% | 1402 | MT102627.1 |
| Klebsiella pneumoniae strain C2414 chromosome, complete genome | Klebsiell... | NA | 573 | 839 | 6677 | 37% | 0.0 | 99.57% | 5588118 | CP039819.1 |
| Klebsiella quasivariicola strain 18 16S ribosomal RNA gene, partial sequence | Klebsiell... | NA | 2026240 | 839 | 839 | 37% | 0.0 | 99.57% | 1025 | MN733164.1 |

**Figure 15: BLAST result for 16S rRNA sequencing**

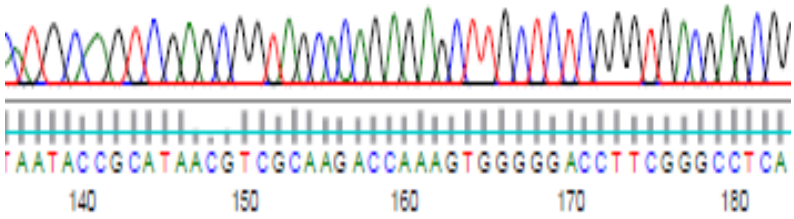



**Figure 16: Chromatogram of 16SrRNA sequence of *Klebsiella pneumoniae*. Different peaks with different colors shows different nucleotide sequences (ATCG)**

## 4.10 Bacteriophage isolation, manipulation and processing

A total of three different types of bacteriophage were isolated against two strains of UTI causing *Klebsiella pneumoniae* and one strain of *E.col* respectively. Although the water sample was collected from different places in Kathmandu valley, bacteriophage was mostly obtained from Balkhu River. This could be because the water from Balkhu river was more polluted with hospital wastes than other rivers. The initial plate consisted of a mixed population of phages displaying diverse variations in plaques morphology and lysis characteristics. For strain 6697, which was used for further characterization, only 3 plaques were seen.

**Table 11: Initial Screening of bacteriophage**

| Source of sample | Bacterial host | Name of bacteriophage | Initial plaque number | Plaque opacity | Morphology of plaque |
|---|---|---|---|---|---|
| **Balkhu River** | *Klebsiella pneumoniae* (6697) | 6697 P1 | 3 | Clear | Large Bull's eyes |
| **Balkhu River** | *Klebsiella pneumoniae* (5615) | 5615P2 | 72 | Clear | Large Bull's eyes |
| **Balkhu River** | *E.coli* (2778) | 2778P3 | 28 | Clear | Large Bull's eyes |

The plaques formed were of different morphology. Mostly bull's eyes plaques were obtained. The different size and morphology of plaques indicates presence of different types of phages in water sample. Various factors including adsorption rate, lysis time, virion morphology also affects plaque size. A high adsorption rate leads to larger plaque size. A greater and smaller lysis time results in smaller plaque size. Smaller lysis times are correlated with small burst size, which means fewer phage particles available for infecting nearby cells, as a result small plaques are formed. Additionally, larger virion size leads to longer diffusion times and smaller plaque size (Gallet et al., 2011).

Similary, the plaque formed were round, clear with distinct peripheral halo as seen in figure below. These plaque halos which diffuses around the lysis area indicates the existence of depolymerase enzyme which is capable of degrading capsular exopolysaccharides and biofilm produced by *Klebsiella pneumoniae*. Earlier studies illustrated that certain *Klebsiella pneumoniae* produced depolymerase enzyme at the time of phage proliferation. These enzymes where then subsequently released from the infected bacteria that target capsular polysaccharide (CPS) of other bacteria (Adams & Park, 1956).


(A) (B) (C)

**Figure 17: Initial plaques of bacteriophage obtained in DLAA plate. A. Mixed plaques obtained against host 6697 in the initial screening plate. B. Bull's eye plaques observed against host sample 5615 in initial plate. C. Bull's eye plaques observed against host s**

The phages were isolated in high titer without any enrichment protocol indicating that the river of Kathmandu are highly polluted with hospital wastes as the bacterial sample we used were clinical sample. Another major concern is the presence of multidrug resistant bacteria in the river flowing around Kathmandu valley which may easily be transmitted to people who comes in contact with the river.

## 4.11 Purification and amplification of isolated phages

The initial plate of bacteriophage may contain mixed type of bacteriophage, as we cannot differentiate phage types with our naked eyes. However, to perform physiochemical characterization and whole genome sequencing, we require a single, pure strain of bacteriophage.

The bacteriophage was purified using the continuous streaking technique. A single bull's-eyes plaque with clear lytic property was selected for purification. Following the phage streaking process, the formation of uniformed sized plaques was observed as seen in figure below. The plaques were abundant in the starting line but their frequency gradually decreased towards the end. Progeny plaque derived from the single mother plaque was used to prepare phage stock. This stock was then stored at 4˚C and used for further research.

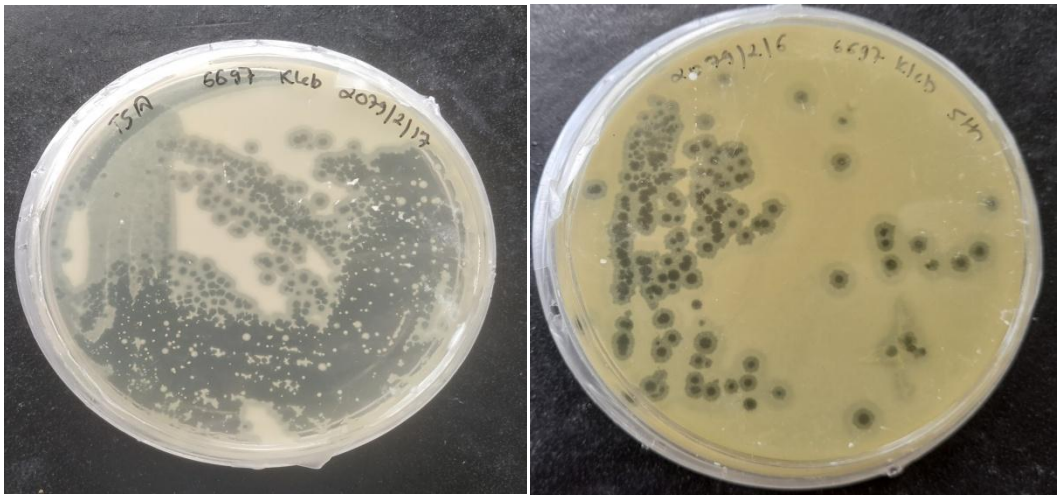


**Figure 18: Purification of phage by continuous streaking method. A and B showed the isolated plaques obtained after streaking the single plaques on TSA plate.**

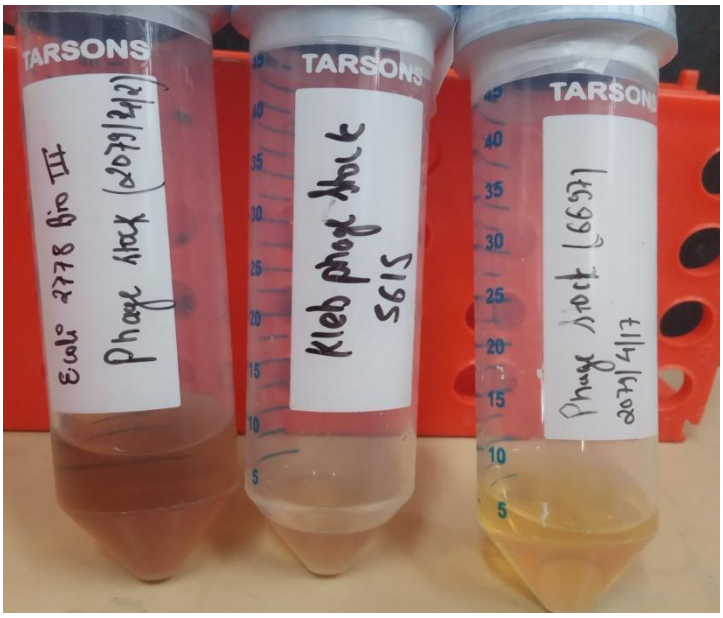



**Figure 19: Stock of phage in SM buffer**

The preparation of pure phage lysate is crucial for the characterization of phages. Various techniques can be employed to purify bacteriophage, some of which are clarification with organic solvents, polyethylene glycol (PEG) precipitation, ultracentrifugation, and chromatography. These are also techniques which are widely used (Bonilla et al., 2016). There is a risk of phage inactivation with various techniques, so we used filtration technique to reduce any harm in preparation of bacteriophage lysates. The process of purification is a crucial step as it is required for the elimination of any contaminant that might be originating from the bacterial host which includes flagella, proteins, proteins, lipopolysaccharides, etc. For lipid-enveloped phages sensitive

to chloroform, filtration through 0.22 µm pore filters is a suitable method (Jooczyk-Matysiak et al., 2019).

## 4.12 Spot assay of phage and titer determination.

Phage activity was observed up to $10^{-8}$dilution while performing spot assay. Countable number plaques were seen in dilution $10^{-8}$and$10^{-9}$. Dilution $10^{-8}$ was selected as only 3 plaques were observed in case of dilution$10^{-9}$. The spot assay is essential to determine up to which dilution phage could lyses the host.

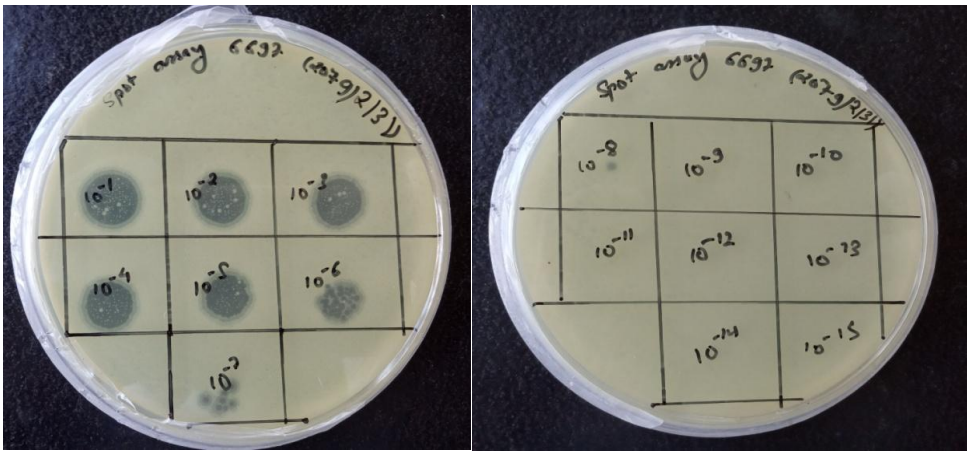


**Figure 20: Spot Assay of phage**

**Figure 21: DLAA plates showed countable plaques in different dilution A: Spot assay of Phage $10^{-7}$ dilution, Fig B: spot assay of phage 10-15 dilution**

Phage titer was calculated using formula below and was found to be 29× $10^{8}$pfu/ml.

$$\text{Pfu/ml} = \frac{\text{Number of plaques formed}}{\text{Dilution} \times \text{volume of sample}}$$

$$= \frac{29}{10^{-8} \times 1} = 29 \times 10^{8} \text{pfu/ml}$$ where pfu/ml=plaques forming unit per milliliter

A phage titer is the process of determining the concentration of bacteriophage through dilution and plating with susceptible cells. Determination of phage titer is important as it helps to assess the viable phage particle in phage stock. High phage titer is required for

process like whole genome sequencing and transmission electron microscopy. It is important to select suitable dosage of phage

Determining the phage titer in a sample is a critical step in phage research, particularly in phage therapy for bacterial infections, where selecting an appropriate dosage is crucial. A commonly used method for quantifying phage concentration is the plating assay, specifically the plaque assay.

## 4.13 Multiple host range analysis

There was no any lysis seen against any inter or intraspecific host used during host range analysis of phage. Altogether five intraspecific host were used. While 11 interspecific host including *Pseudomonas*, *E.coli*, *Enterobacter*, *Acinetobacter* were used. All samples used were UTI samples brought from Sukraraj Tropical and Infectious Disease Hospital (STIDH)

**Table 12: Host range analysis by Bacteriophage**

| Bacteria | Phage 6697 | Phage 6697 and Phage 5615 |
|---|---|---|
| *Klebsiella8* | No | No |
| *Klebsiella9* | No | No |
| *Klebsiella12* | No | No |
| *Klebsiella13* | No | No |
| *Klebsiella14* | No | No |
| *Pseudomonas 1* | No | No |
| *Pseudomonas 2* | No | No |
| *Pseudomonas-sho* | No | No |
| *209205 (Pseudomonas)* | No | No |
| *Entero3* | No | No |
| *E. coli 5* | No | No |
| *E. coli 10* | No | No |
| *Acinetobacter4* | No | No |
| *Acinetobacter (Acb1)* | No | No |
| *Pseudomonas 3* | No | No |
| *Pseudomonas 4* | No | No |

## 4.14 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) for protein profiling

Protein profiling of the bacteriophage was carried out using SDS-PAGE. Three distinct band of protein were observed.

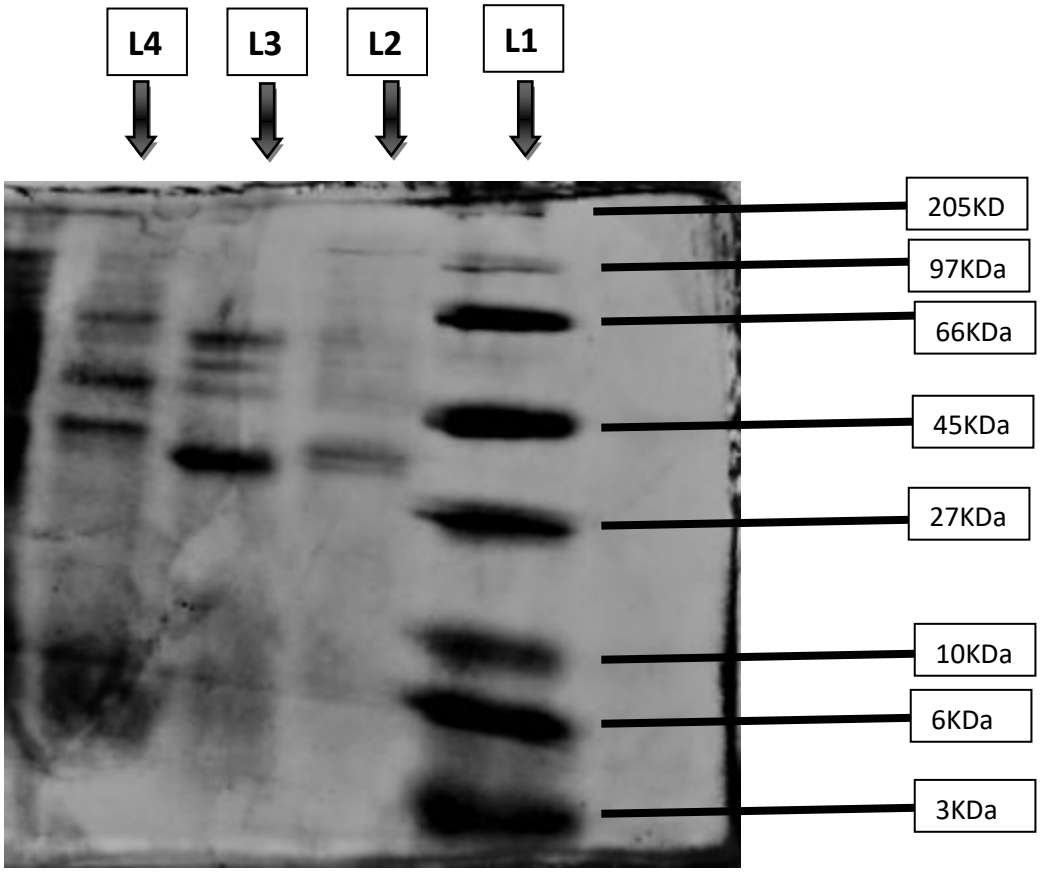



**Figure 22: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis(L1: marker, L2: Phage TU_ Pse _1B, L3: Pseudomonas phage 6661 L4: Klebsiella phage 6697)**

The observed size of the protein was found to be 40KDa, 55KDa and 66KDa respectively. The larger protein size may be the tail fiber protein, while the smaller proteins are likely capsid proteins and internal virion proteins.

## 4.15 Phage stability at different temperature

The thermal stability experiment was conducted in order to assess the heat resistance of the bacteriophage. The result showed that the isolated bacteriophage was not stable above 80˚C as the plaques were not seen after 10 minutes of incubation at 80˚C. Plaques were seen up to 30 minutes after incubation at 70˚C whereas survivability at 60˚C was seen even at 60minutes though the titer was seen decreasing as the time increased. Maximum luti activity was seen at 37˚C.

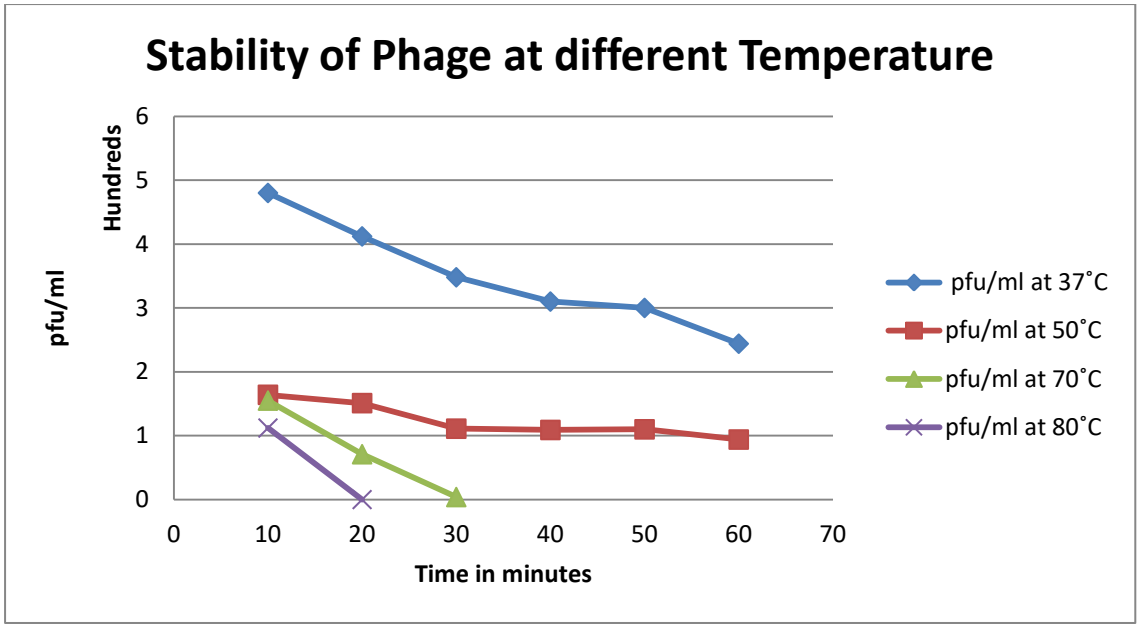



**Figure 23: Stability of phage at different temperature for different time interval**

The ability of phages to maintain their stability when exposed to varying temperatures serves as a crucial model for understanding their adaptability to new environments. Temperature plays a fundamental role in influencing key aspects of the phage life cycle, such as during attachment process, penetration of host, multiplication within the host. At a temperature that is lower than optical temperature, less amount of phage genetic material enters bacterial host resulting in fewer number of phages participating in the multiplication phase. While higher temperature extends the duration of latent phase (Jończyk et al., 2011). Studying the effects of temperatures on phages gives insights into how they behave and survive. This helps in understating how phages interact with various hosts and environment.

## 4.16 Phage stability at different pH

Phage stability is strongly influenced by its exposure to either alkaline or acidic environment. The pH stability test showed that isolated phage is stable from pH 4 to 12. However, it exhibits the highest stability between pH 5 to 10 after 1hour of incubation. At pH seven, the phage showed most lytic activity with the plaque count of 119. Below pH 4, the phage loses its lytic activity as no plaques were observed. Based on this observation, it can be inferred that the phage displays stability across a broad pH range.

**Table 13: pfu/ml of bacteriophage at different pH**

| pH | pfu/ml |
|---|---|
| 2 | 0 |
| 3 | 0 |
| 4 | 26 |
| 5 | 53 |
| 6 | 74 |
| **7** | **119** |
| 8 | 65 |
| 9 | 64 |
| 10 | 46 |
| 11 | 30 |
| 12 | 25 |

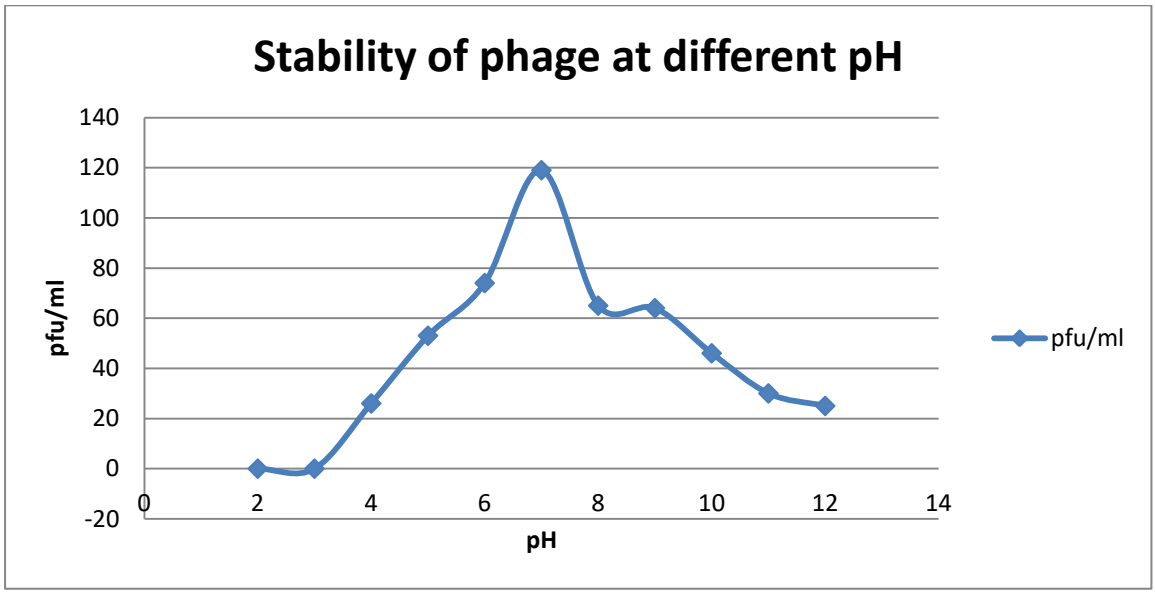



**Figure 24: Stability of phage at different pH**

Phage viability and survivability are highly influenced by factors like acidity and alkalinity. These factors lead to alternation in phages like in nucleic acids and also the structural components. Notably, low pH levels have been observed to significantly decrease the phage titer and proliferation. The instability of phages under low pH conditions hinders its effectiveness in phage therapy, acidic pH can cause irreparable damage. The low pH

promotes phage aggregation due to the protonation effect, which negatively impacts the phage activity (Ly-Chatain, M. H. 2014).

## 4.17 One step growth curve analysis and burst size determination

The various aspects of bacteriophage growth cycle, including a latent period, an eclipse period, a rise period and also the burst size was identified from this study. Our study showed that 119 virions per bacterium was the brust size of the bacteriophage and 20 minute was the latent period for our isolated bacteriophage.

**Table 14: pfu/ml produced by phage at different time interval**

| Time | Pfu/ml |
|---|---|
| 5 | 8 |
| 10 | 12 |
| 20 | 31 |
| 30 | 150 |
| 40 | 170 |
| 50 | 184 |

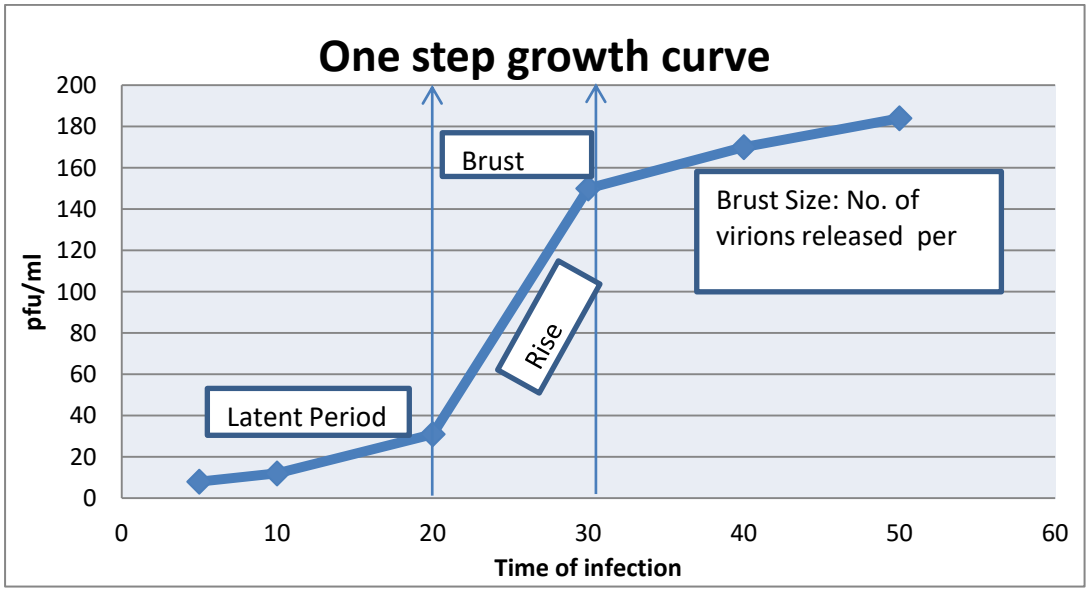



**Figure 25: One step growth curve of phage**

Various factors affect the brust size of bacteriophages. In the study conducted by Choi et al. in 2010, it was found that smaller cells generally have smaller burst sizes compared to larger cells exhibit higher burst size (Choi et al., 2010). Brust size also depends on

nutritional status of bacterium and also the phage coded functions such as polymerase and regulatory proteins (Gadagkar & Gopinathan, 1980). In their study Gadagkar and Gopinathan found that brust size also depends on multiplicity of infection. The finding showed that the burst size was inversely correlated with MOI (Gadagkar & Gopinathan, 1980).

## 4.18 Phage DNA extraction

After gel electrophoresis, a distinct band of bacteriophage DNA was observed when viewed under UV transilluminator. A DNA ladder Fermentas o Gene Rule 1KB was also run alongside bacteriophage DNA. Based on band observed, it was found that the size of the DNA was greater than 10kb.

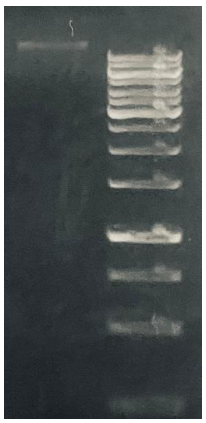


Figure 26: Electrophoresis of bacteriophage DNA showing distinct bands

## 4.19 Whole genome sequencing of phage DNA

The results from whole genome sequencing of the bacteriophage genome(DNA) were gathered in two file formats i.e. FASTA and GenBank formats. The sequence was interpreted using various tools, including UGENE and PHASTEST.

### 4.19.1 Genomic analysis by using UGENE

**General Statistics**

Table 15: General Information of bacteriophage genome

| | |
|---|---|
| **Length** | **45,288nt(45kb)** |
| **GC content** | 53.97% |
| **Molecular weight** | 27985314.46 |
| **Extinction coefficient** | 720395602l/(mol*cm) |
| **Ug/OD260** | 38.85 |
| **Melting temperature** | 86.99°C |

After the complete genome sequencing it was revealed that the phage genome consists of 45,288 basepair (45kb) having GC content of 53.92%. The bacteriophage genome comprises of 54 predicted ORF (open reading frames) whereas there was no any tRNA gene observed.

The DNA has a melting temperature of 86.99 °C. The molecular weight of the double-stranded DNA is 27,985,314.46 Da. The extinction coefficient (molar attenuation coefficient) of the DNA is 720,395,602liter/mole/cm. This coefficient indicates how strongly the light is being absorbed by DNA at a specific wavelength and considered as an intrinsic characteristics of the DNA molecule (Banihashemian et al., 2013).

**Table 16: Character occurrence and their percentage**

| Nucleotides | Number | Percentage |
|---|---|---|
| **Adenine** | 11047 | 24.4% |
| **Guanine** | 13196 | 29.1% |
| **Cytosine** | 11175 | 24.7% |
| **Thymine** | 9775 | 21.6% |

Total number of adenine Nucleotide in the genome of phage was 11047 with percentage count 24.4%. Guanine accounts for the highest number of nucleotide which was 13196 with percentage 29.1%. Total number of cytosine nucleotide in the phage genome was 11516 which was 24.7% of the total nucleotide in the phage genome. Thymine accounts for 21.6% of the total nucleotide percentage which is lowest among all.

**Circular map of phage genome using UGENE showing annotated proteins**

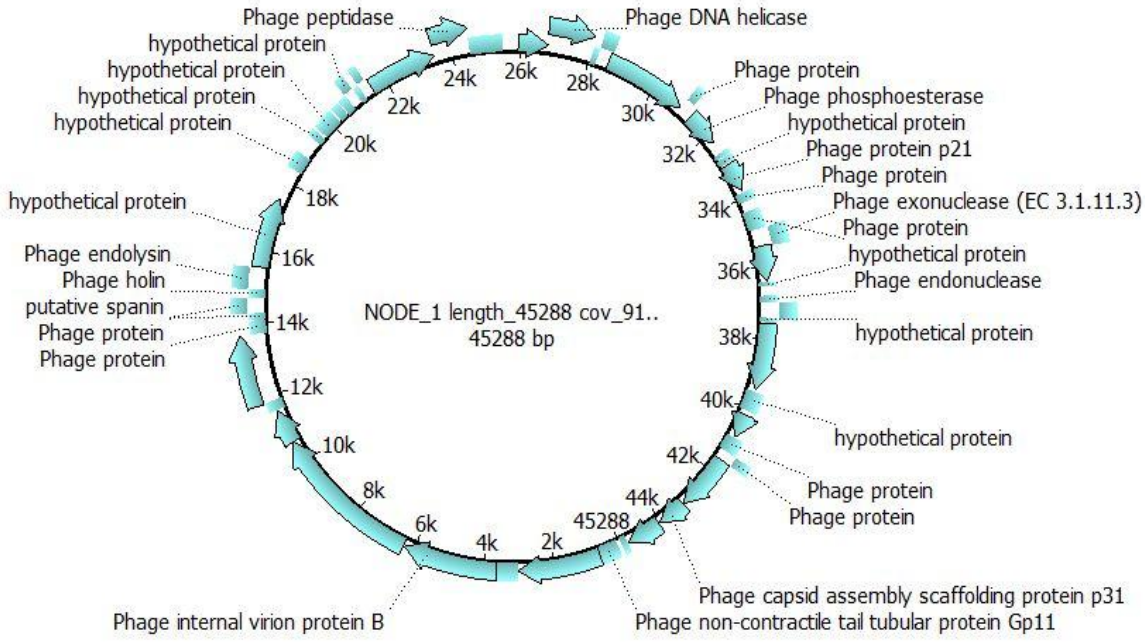



**Figure 27: Circular form of whole genome generated from UGENE software**

UGENE software was used to generate circular form of phage. This circular form of DNA showed different protein present in phage genome along with their name and respective position. Some important protein like Phage endolysin, phage holin and phage spanin can also be seen in the figure above.

### 4.19.2 Complements gene

The gene bank format of whole genome includes detailed information about 54 CDS (Coding sequence) found in the phage. Among the 54 CDS documented, 14 were named as hypothetical as their functions were unknown. Hypothetical proteins are those which are predicted to be expressed in an organism with no evidence of their existence. Notably, the phage genome did not contain any toxic or integrase genes. After bioinformatics analysis, some were found to be structural protein coding genes. Some gene like Phage terminase large subunit, phage DNA Polymerase, DNA primase, DNA helicase were associated with DNA replication, recombination, repair and packaging. Some gene like dehydrogenase, Deoxycytidylate deaminase were found to be associated with transcription, translation and nucleotide metabolism. Endolysin and holin, two important phage protein which are essential for breaking down their host cells were also present. Endolysins facilitate the breakdown of bacterial peptidoglycan post-phage replication, and helps in the release of new phage particles (Lu et al., 2020).

### 4.19.3 Genome analysis through PHASTEST

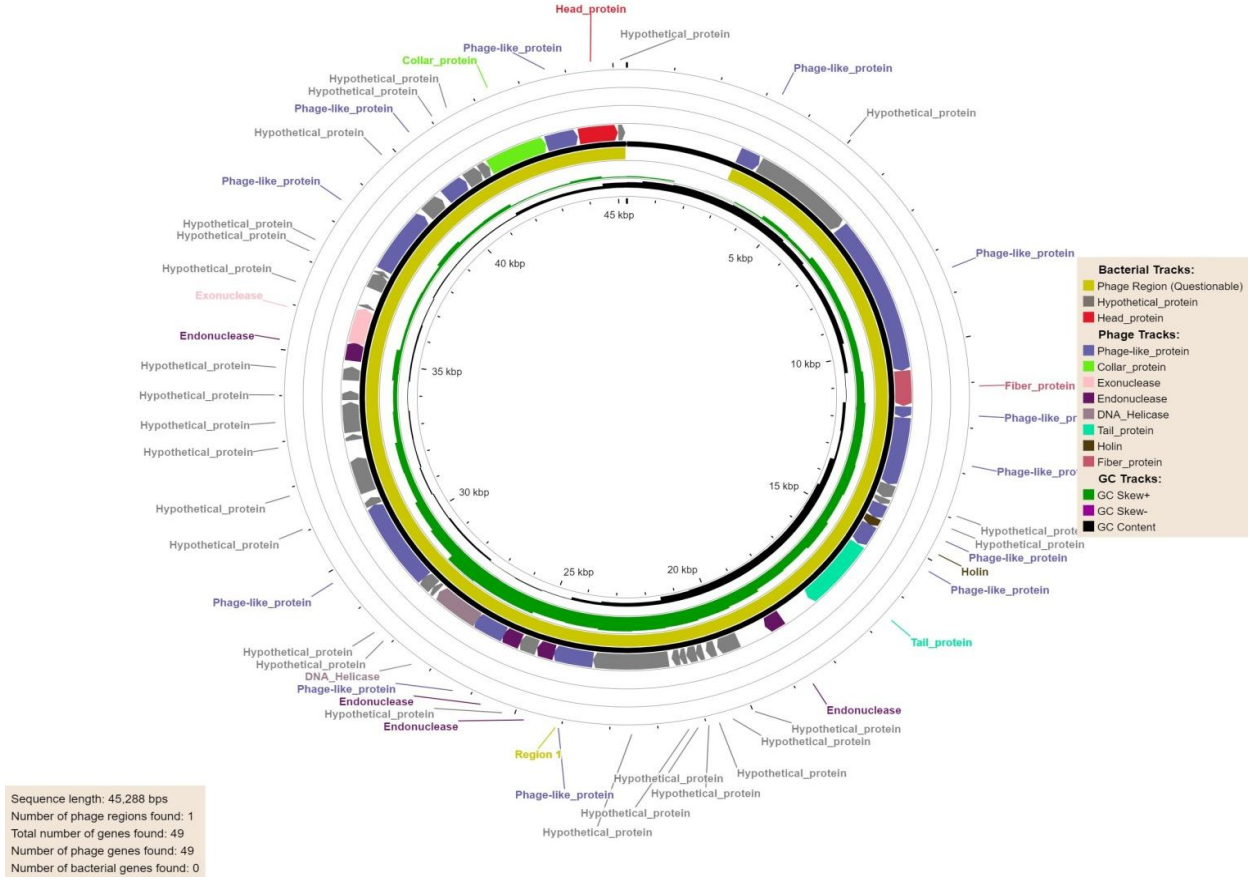



Figure 28: Circular view of genome map using PHASTEST

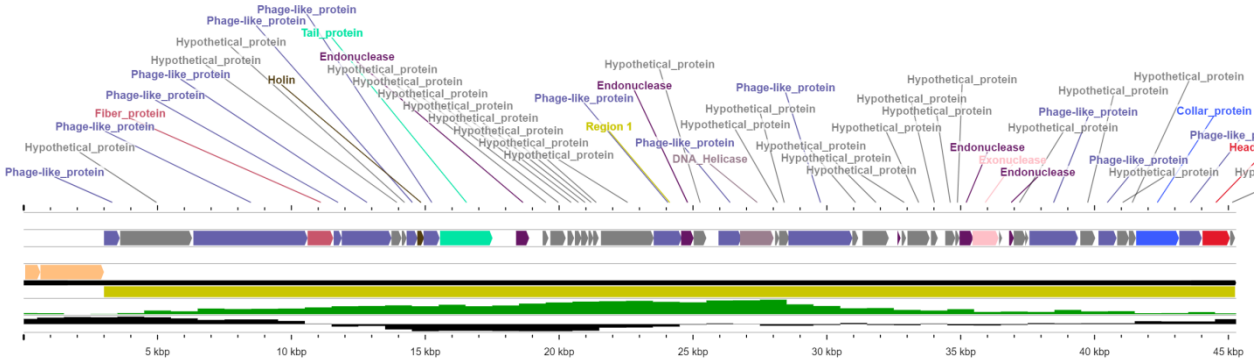



Figure 29: Linear form of phage genome generated using PHASTEST

PHASTEST( PHAge Search Tool with Enhanced Sequence Translation), is an online platform for has been designed for quickly detecting, annotating, and presenting prophage sequences discovered in bacterial genomes and plasmids. Furthermore, PHASTEST enables comprehensive annotation and interactive display of different gene

categories, such as protein-coding regions, tRNA sequences, and rRNA sequences, found within these genomes. Circular aswell as linear genome map of phage 6697 was generated with genome size of about 45288 bp. GC content in the genome was found to be 53.81%. Total number of phage gene found was 49. There was no any bacterial gene found in our phage genome. Our phage showed highest similarity with the PHAGE_Klebsi_vB_KpnP_KpV48_NC_047783(16).CDS position along with their blast hit result is being included in the appendix portion. (010)

### 4.19.4 Genome Annotation

Nucleotide blast was performed for the obtained fasta sequence against NCBI nucleotide database using BLAST. Figure below show the blast hit result of the phage. It can be seen that our phage have maximum genome similarity with Klebsiella phage myPSH1235 and Bacteriophage sp.

| Description | Scientific Name | Max Score | Total Score | Query Cover | E value | Per. Ident | Acc. Len | Accession |
|---|---|---|---|---|---|---|---|---|
| Klebsiella phage myPSH1235, complete genome | Klebsiella phage myPSH1235 | 20943 | 49959 | 79% | 0.0 | 92.15% | 45135 | NC_047944.1 |
| Bacteriophage sp. isolate 1078_68233, complete genome | Bacteriophage sp. | 12421 | 47573 | 81% | 0.0 | 86.59% | 45208 | OP073663.1 |
| TPA: Caudoviricetes sp. isolate ctbqY9, partial genome | Caudoviricetes sp. | 10179 | 45842 | 76% | 0.0 | 92.87% | 43643 | BK038280.1 |
| Klebsiella pneumoniae phage strain 5899STDY8049207 genome assembly, chromosom... | Klebsiella pneumoniae phage | 10050 | 47503 | 78% | 0.0 | 92.59% | 44964 | OU509534.1 |
| Klebsiella phage SKP1, complete genome | Klebsiella phage SKP1 | 10008 | 47969 | 78% | 0.0 | 92.69% | 44006 | OP114731.1 |
| Klebsiella phage BUCT86, complete genome | Klebsiella phage BUCT86 | 9913 | 47510 | 77% | 0.0 | 92.45% | 44542 | OL474125.1 |
| Klebsiella phage VLCpiA1m, complete genome | Klebsiella phage VLCpiA1m | 9823 | 46667 | 78% | 0.0 | 92.22% | 43756 | ON602727.1 |
| Klebsiella phage VLCpiA1k, complete genome | Klebsiella phage VLCpiA1k | 9806 | 46463 | 74% | 0.0 | 91.96% | 43278 | ON602739.1 |
| TPA: Caudoviricetes sp. isolate ctC9y1, partial genome | Caudoviricetes sp. | 9801 | 43149 | 72% | 0.0 | 91.91% | 43511 | BK046074.1 |
| Klebsiella phage CX1, complete genome | Klebsiella phage CX1 | 9780 | 48400 | 78% | 0.0 | 92.10% | 45013 | MT090077.2 |
| Klebsiella phage mtp6 genome assembly, chromosome: 1 | Klebsiella phage mtp6 | 9780 | 46608 | 76% | 0.0 | 92.05% | 43492 | OX335428.1 |
| Klebsiella phage cp48 genome assembly, chromosome: 1 | Klebsiella phage cp48 | 9780 | 46608 | 76% | 0.0 | 92.05% | 43569 | OX335379.1 |
| Klebsiella phage NTUH-K2044-K1-1 DNA, complete genome | Klebsiella phage NTUH-K2044-K1-1 | 9755 | 47618 | 79% | 0.0 | 91.99% | 43871 | NC_025418.1 |
| Klebsiella phage VLCpiA1b, complete genome | Klebsiella phage VLCpiA1b | 9729 | 33842 | 68% | 0.0 | 88.92% | 44598 | ON602752.1 |
| Klesiella phage xx20, complete genome | Klebsiella phage xx20 | 9708 | 48075 | 79% | 0.0 | 91.88% | 44287 | OQ871562.1 |
| Klebsiella phage pKP-M212-2.1, complete genome | Klebsiella phage pKP-M212-2.1 | 9653 | 45125 | 76% | 0.0 | 91.74% | 42673 | OQ734493.1 |
| Klebsiella phage SRD2021, complete genome | Klebsiella phage SRD2021 | 9601 | 48076 | 78% | 0.0 | 91.61% | 45212 | MZ208805.1 |

**Figure 30: BLAST result of the FASTA sequence of phage**

## 4.20 Multiple Sequnece alignment and phylogenetic tree construction

NCBI's BLAST search engine was used inorder to search other similar bacteiophage whole genome which showed similarity with isolated bactriophage. Search using BLAST gave a list of *Klebsiella pneumoniae* bacteriophage which possesses similar identity with isolated bacteriophage. From this only top ten phages showing highest percentage similarity was selected. Highest percent identity (92.15%) was seen against *Klebsiella* phage myPSH1235.For performing multiple sequence alignment, the top ten bacteriophage was selected and their fasta sequence were then downloaded . Then, the

multiple sequence alignment was done before the construction of phylogenetic tree. Using the above downloaded 10 FASTA format of the phages, phylogentic tree was constructed with the help of UGENE platform. The phylogenetic relationship between our phage genome and other 10 phage genome is depicted in the picture below (Figure 31).

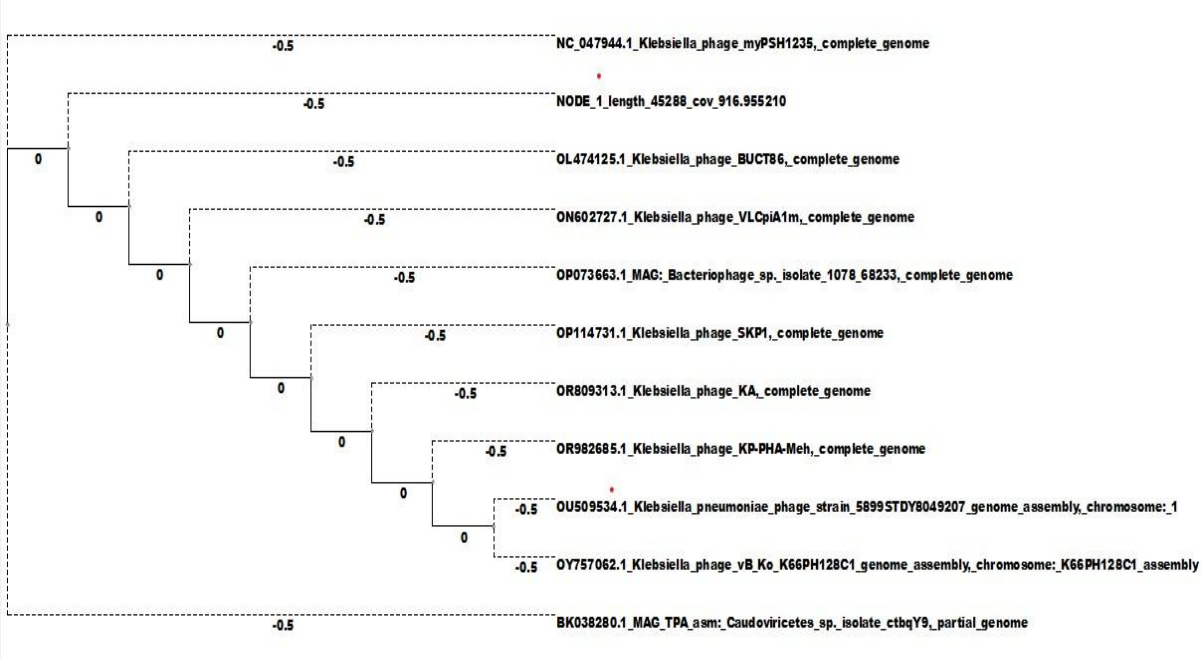



**Figure 31: Phylogenetic analysis of *Klebsiella* phage**

Isolated phage genome showed closest phylogenetic relation with Klebsiella_phage-myPSH1235 as seen in above figure. Further, we also evaluated the phylogenetic relationship between the different phage using two major protein Phage terminase large subunit Gp19 and Phage non-contractile tail tubular protein Gp11. Tree elucidating the evolutionary history of the phage is shown in the picture below (Figure 32).

Phage terminase large subunit Gp19

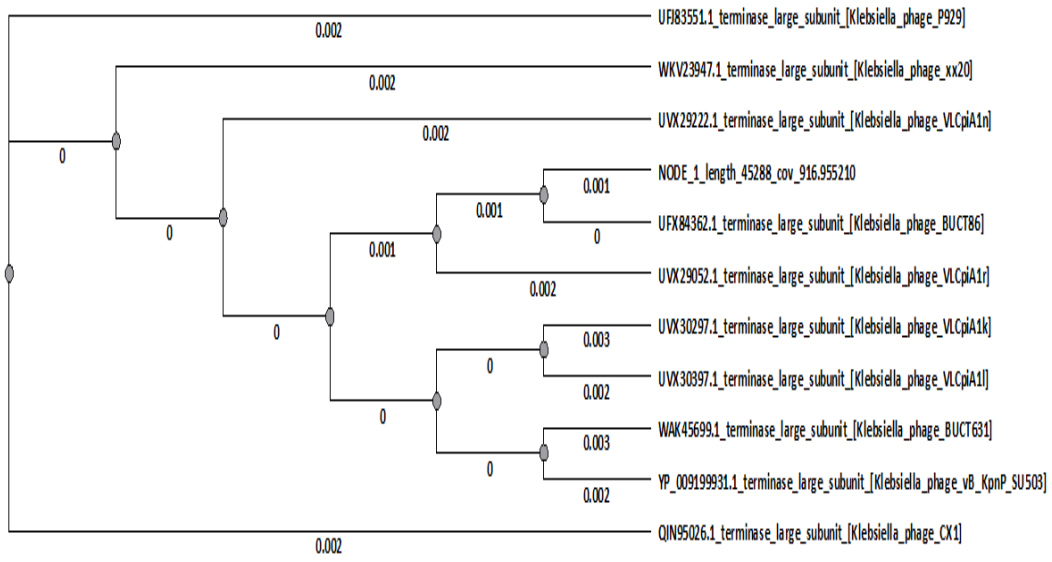



**Figure 32: Phylogenetic analysis of Phage terminase large subunit Gp19**

Phage non-contractile tail tubular protein Gp11

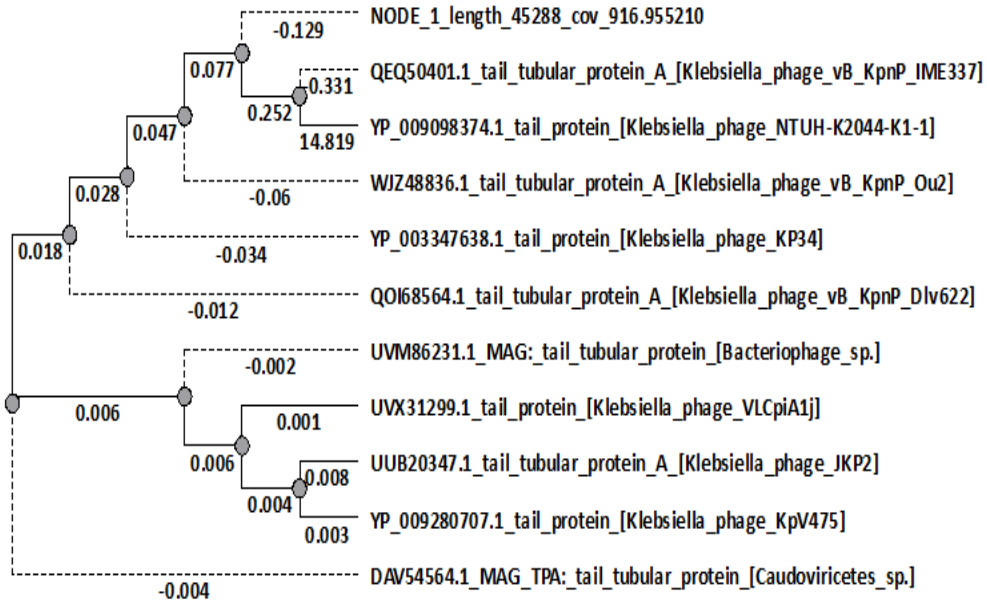



**Figure 33: Phylogenetic analysis of Phage non-contractile tail tubular protein Gp11**

Phylogenetic trees of terminase large subunit showed that phage protein showed homology with other *Klebsiella* phages Klebsiella_phage_BUCT86 while tail tubular protein showed homology with Klebsiella_phage_v8_KpnP_IME337 and Klebsiella_phage_NTUH-K2044-K1-1.

## 4.21 Formation of Biofilm

For determining the biofilm formation process developed by Stepanovic et al, was followed with slight modification. The ability of bacteria *Klebseilla pneumoniae* to form biofilm was analyzed using 96 well polystyrene tissue culture microtiter plates. Inorder to determine this, firstly ODc i.e. optical density cut off value should be calculated.

ODc was calculated using formula provided :

ODc = An average OD of negative control (NC) + 3× Standard deviation of NC

An average OD of Negative Control (NC) = 0.1498

A standard deviation of negative control =0.031125

ODc= 0.1498+3×0.03112

=0.24317

2ODc=0.4863

4ODc=0.9727

Optical density of Klebsiella (6697)= 2.21825

The OD of 6697 was found to be 2.2185 which were greater than the ODc. Since, the OD of bacteria was found to be more than 4ODc, the bacteria was interpreted as strong biofilm producer. Thus, the biofilm disruption experiment was carried out using this bacterium.

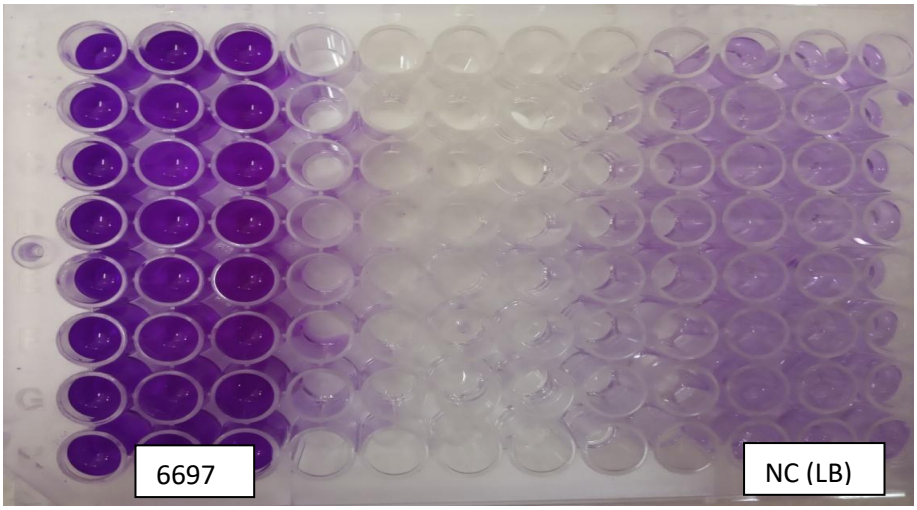



**Figure 34: Microtiter plate showing the assessment of biofilm formation by *Klebsiella pneumoniae***

## 4.22 Biofilm disruption

Biofilm disruption interpretation was carried out in accordance with the methodology proposed by Forti et al. For biofilm disruption, firstly bacteria (*Klebsiella pneumoniae*)

inorder to develop biofilm , was grown in 96 well microtiter plate overnight. Then the plate was exposed to bacteriophage and the optical density was recorded using ELISA reader. For control, bacteria without phage treatment was used.

Average OD of phage untreated bacteria= 2.0447

Average OD of phage treated bacteria= 1.1575

Therefore, Percentage reduction of biofilm = 43.39%

Hence isolated phage was able to reduce the biofilm by 43.39% .Though not completely phage was able to reduce the bacterial biofilm significantly and to the same extent.

The biofilm producing bacteria have high pathogenicity due to the presence of extra virulence factor of biofilm-forming extracellular polymeric substance (EPS) compared to non biofilm producer. Also, bacterial biofilm presents a significant challenge to the healthcare system due to their involvement in various human diseases and their heightened resistance to antimicrobial agents (Zhao et al., 2023). So, disrupting the biofilm while simultaneously eliminating the pathogen becomes crucial for effective therapy. In this study, phage showed promising result in disruption of bacterial biofilm.

## 4.23 Biofilm scanning electron microscopy

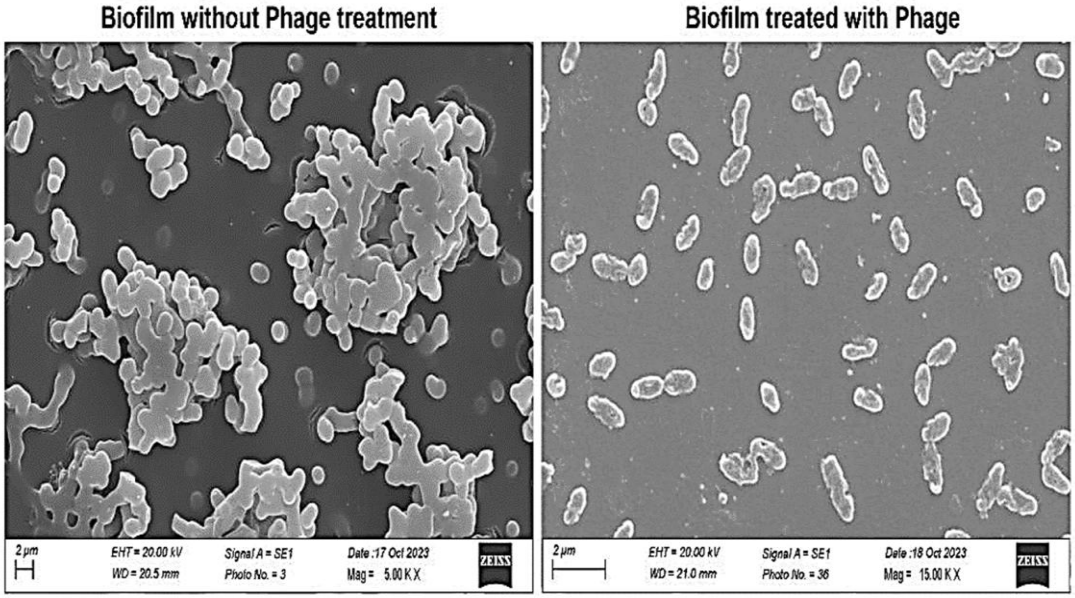



Figure 35: Biofilm observed under SEM at AIIMS, India

Scanning electron microscopy was conducted to closely examine *K. pneumoniae* biofilms, their physical characteristics and the impact of phage on these biofilms. Figure above presents representative scanning electron micrographs of biofilms. Clear distinctions between phage treated bioflim and phage untreated bioflim can be observed. There is significant reduction in bioflim when phage treatment is applied.

## 4.24 Pre treatment with bacteriophage

Pretreatment with bacteriophage before the formation of biofilm showed promising results. The average OD of the positive control was found to be 1.625. Whereas the OD of phage treated well was found to be 0.433. Average percentage reduction in bacterial load after 24hours was 75.75%.

## 4.25 Synergistic effect of antibiotic and phage in biofilm reduction

Two antibiotics Meropenem and Ceftriaxone were used for observing the synergistic effect between phage and antibiotics. The average OD of the well when only phage was used was found to 1.444. Similarly, the OD of phage and Meropenem treated well was found to be 1.081. For the well contaning the phage and Ceftriaxone, the average OD was 0.992. A significant reduction in OD can be seen in the well with the phage and antibiotics than in the well with only bacteriophage i.e., by 0.363 (25.138%) incase of phage and Meropenem combination and by 0.448(31.30%) incase of phage and Ceftriaxone combination. There was further reduction of the biofilm by 25.138% and 31.30% with the combination of phage and antibiotic.

The average optical density (OD) of the positive control was 2.084, while 0.347 was the the optical density of the negative control (LB).

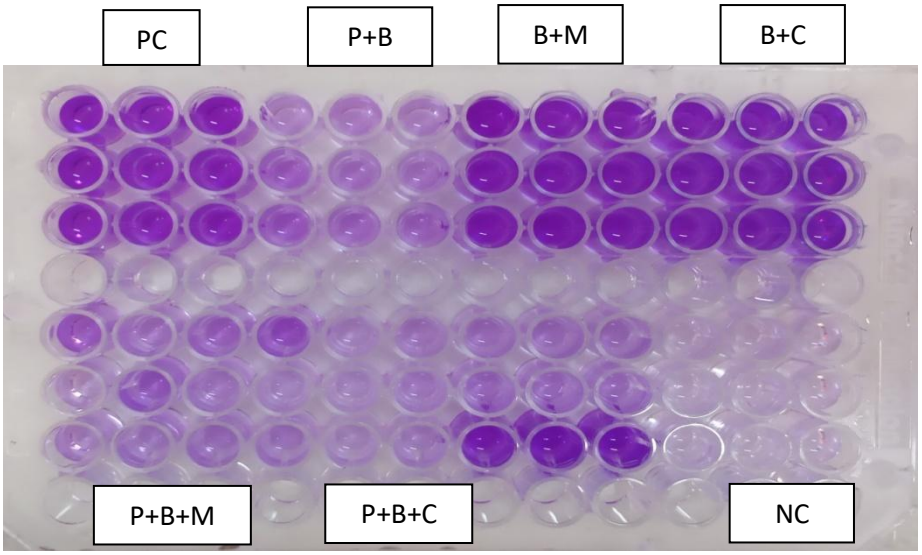



**Figure 36: Microtiter well showing positive contol (PC), phage treated well (P+B), bacteria and antibiotic treated well(B+M AND B+C), bacteria plus phage and antibiotic treated well(P+B+MAND P+B+C) and negative control (NC)**

Bacteriophage plays a crucial role in disrupting biofilms, which often hinder the effectiveness of antibiotic treatment. When combined with antibiotic, its effectiveness has been found to be more enhanced in eliminating bacteria within biofilm. Also, it was

found that many bacterium embedded within the biofilm could be removed using phage with antibiotic together instead of using them individually. Additionally, it has been observed that certain antibiotics exhibit improved performance at lower doses when administered in conjunction with phages, as highlighted by Chaudhry et al., 2017. The research conducted by Yilmaz et al., 2013, showed that using phage Sb-1 alone reduced bacteria in *S. aureus* biofilms but didn't fully eliminate them. Combining phage Sb-1 with rifampicin/daptomycin eradicated the biofilm by breaking down its structure. Staggered treatment with phage Sb-1 and antibiotics was also seen effective against biofilms (Yilmaz et al., 2013).

Scientists have suggested that combining antibiotics with phages could be more effective than using either alone. This joint approach may offer benefits like improved bacterial suppression, better penetration into biofilms, and potentially lower chances of bacteria developing resistance to phages or antibiotics (Li et al., 2021). Combining phages and antibiotics aims to combat biofilms because bacteria can develop resistance to phages as well. The ongoing evolutionary competition between bacteria and phages results in the emergence of phage-resistant bacteria (Hasan & Ahn, 2022).

# Chapter 5 SUMMARY

Multidrug resistance bacterial sample was collected from Shukraraj Tropical and Infectious Disease Hospital. Identification of the bacterial culture was then done through biochemical test. The bacteria were further confirmed through Matrix Assisted Laser Desorption Ionization assay (MALDI-TOF). The bacteria were identified as *Klebsiella pneumoniae.* For determining the antibiotic resistant pattern of the host bacterium antibiotic susceptibility test was carried out. Thus, isolated *Klebsiella pneumoniae* was resistant to all sorts of antibiotics including Carbapenems class. Further confirmation of the Antibiotic Susceptibility test was done using Vitek Compact system 2. Metallo-beta-lactamase detection in bacteria was performed through disk diffusion test with the use of Imipenem and Imipenem- EDTA disc. The bacterium was found to be metallo-beta-lactamase positive.

Lytic bacteriophage against *Klebsiella pneumoniae* was isolated and further characterized. The ability of the bacteriophage to survive against various pH and temperature was also found. Phage was found to survive at wide range of pH that is from pH 4 to 11. Maximum stability was seen at pH7. Additionally, the isolated phage showed highest stability at 37°C. However the stability was seen decreasing with the increase in temperature. So this phage showed good thermal and pH stability. The latent period and burst size of the bacteriophage was found to be 20 minutes and 119 virion per bacterium respectively after one step growth curve analysis. Protein profiling of the bacteriophage revealed three distinct bands of protein of the size approximately equal to 40KDa, 55KDa and 66KDa respectively. Isolated phage did not show any intraspecific host aswell as interspecific host. The phage DNA was extracted with the help of a kit, and the length of the bacteriophage DNA was found to be greater than 10 kilobases (kb) after performing gel electrophoresis.

Genome analysis of phage revealed that the phage genome consists of 45,288 basepair (45kb) with the GC content of 53.92%. The total genes number was 54. Similarly, presences of many hypothetical proteins with unknown functions were also found in bacteriophage genome. PHASTEST analysis revealed that the phage genome lacks any tRNA, integrase gene and any other bacterial genome. On phylogenetic analysis our phage genome showed closest relationship with Klebsiella_phage-myPSH1235.The *Klebsiella pneumoniae* was found to be strong biofilm producer. Our phage was able to reduce the biofilm by 43.39%. The synergistic effect of phage and antibiotics was also analyzed. When phage plus antibiotic were used in combination, there was further reduction of the biofilm by 25.138% and 31.30%. Pretreatment of phage was found to be more effective.

# Chapter 6 CONCLUSION

1) A lytic bacteriophage against MDR *Klebsiella pneumoniae* was isolated from the polluted river of Kathmandu valley.
2) Isolated phage shoewed maximum stability at 37℃. However, the infectivity of the virus was seen decreasing with increase in temperature. The isolated phage also showed maximum stability at pH 7 and was able to survive at wide pH range i.e. 4-10.
3) The isolated phage was also found to be effective in the disruption of bacterial biofilm. Significant reduction of biofilm was seen after the application of bacteriophage. The combination of antibiotic and phage further improved the biofilm reduction. The pretreatment with the phage was seen more effective.
4) On whole genome analysis, it was confirmed that the isolated bacteriophage lacked any toxic or virulence in the genome. Presence of endolysin and absence of integrase enzyme also favors the therapeutic potential of the virus.
5) Based on this result, we can conclude that phages have raised the possibility of utilizing these phages in phage therapy as well as for biocontrol of pathogenic bacteria and clearance of biofilm.

# Chapter 7 RECOMMENDATIONS

In this study, the focus was on determining the morphological, physiochemical, and, to some extent, molecular characteristics of the phage. Additionally, the biofilm-forming capability of the bacteria and the biofilm-disrupting ability of the phage, along with the synergistic effect of the phage and antibiotic, were evaluated. However, certain limitations exist in this study, leading to the following recommendations.

1) Extensive analysis of the genome and submission of the entire genome to GenBank have not been completed yet. If the study could be conducted in collaboration with a bioinformatics expert, it would lead to better findings and conclusions.

2) Likewise, the endolysin gene, a critical enzyme in the host lysis mechanism, can be cloned, and an in-depth study can be carried out.

3) If the study of the synergistic effect could be conducted in an animal model, it would yield reliable and meaningful data to support the assertions made in our study.

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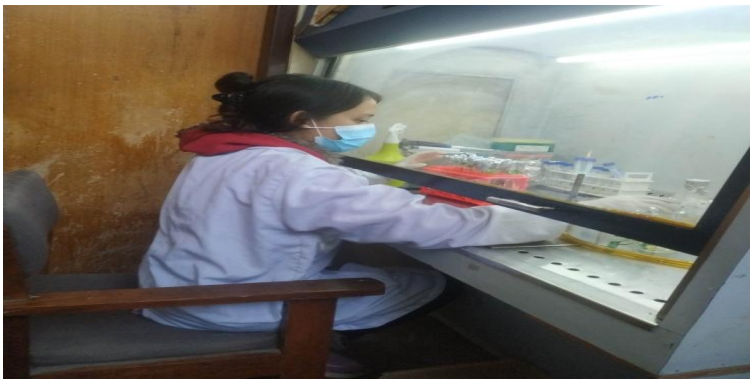
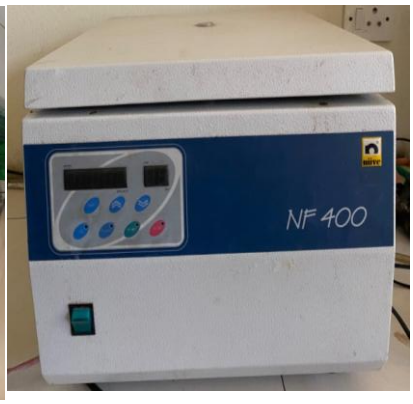
# APPENDIX

Figure: Performing SDS PAGE



Phage group (2075)




Different instrument used during thesis work

Preparation of reagents

SM buffer

| | |
|---|---|
| 1. Water | 800ml |
| 2. Sodium Chloride | 5.8 gram |
| 3. Magnesium Sulphate | 2 gram |
| 4. 1 Molar Tris Cl (pH 7.5) | 50 ml |
| 5. 2% gelatin | 5ml |

Final volume was made 1000ml.

Tris base composition 1 molar

| | |
|---|---|
| 1) Tris base | 12.11 gram |
| 2) Water | 80 ml |
| 3) HCL | 7ml |

Final volume was maintained 100ml

Composition for phosphate buffer saline

| Salt | Concentrations (g/L) |
|---|---|
| 1. Nacl | 8 |
| 2. Kcl | 0.2 |
| 3. Disodium Hydrogen Phosphate | 1.42 |
| 4. Potassium dihydrogem phosphate | 0.24 |

The final volume was maintained to 1000ml.

Tris Acetate EDTA buffer

| | |
|---|---|
| 1) Tris base | 242gm |
| 2) Acetic acid | 57.1ml |
| 3) 500 mM EDTA | 18.612 gram |

For the preparation of 50X solution of TAE buffer 242 gram of Tris base was dissolved in water. To this solution, glacial acetic acid and 500mM EDTA was added at the quantity mentioned above in the table, maintaining one liter as final volume. Thus, prepared stock solution is diluted to make 1X working solution in the ratio49:1.

**Preparation of SDS reagent**

30 % Acrylamide solutions

| Constituents | Weight in gram |
|---|---|
| 1. Acrylamide | 29 gram |
| 2. Bis acrylamide | 1 gram |
| 3. Distilled water | 100ml |

Upper Tris buffer (pH 6.8)

| | |
|---|---|
| 1) Tris base | 3.03 gram |
| 2) Distilled water | 50 ml |

Lower Tris buffer(pH 8.8)

| | |
|---|---|
| 1. Tris base | 18.17gm |
| 2. Distilled water | 100 |

Comassie - Brilliant Blue (500 ml)

| | |
|---|---|
| 1) Comassie Brilliant Blue | 500mg |
| 2) Glacial acetic acid | 25ml |
| 3) Methanol | 250ml |
| 4) Distilled water | 225ml |

Loading dye

| | |
|---|---|
| 1) Upper tris | 1.25ml |
| 2) 10% SDS | 3.0ml |
| 3) Glycerol | 4.75ml |

| | |
|---|---|
| 4) Beta Mercaptoethanol | 0.5 ml |
| 5) 0.1% Bromophenol | 0.5 ml |

Destaining solution (500ml)

| | |
|---|---|
| 1. 7.5% Glacial acetic acid | 37.5 ml |
| 2. Methanol 5% | 25 ml |
| 3. Distilled water | 437.5 ml |

A running buffer (1000 ml) having pH 8.4

| | |
|---|---|
| 1) 39mM Tris | 4.72 |
| 2) 48mM glycine | 3.603 |
| 3) 0.1% SDS | 0.37g |

5% Resolving gel (5 ml)

| | |
|---|---|
| 1. Water | 3.4ml |
| 2. 30% acrylamide | 0.83ml |
| 3. 1M Tris HCL having pH 6.8 | 0.63ml |
| 4. 10% SDS | 0.05ml |
| 5. 10% (NH4)2S2O8 10% | 0.05ml |
| 6. TEMED | 0.005ml |

12% Resolving gel (10ml)

| | |
|---|---|
| 1) Water | 3.3 ml |
| 2) 30% acrylamide | 4 ml |
| 3) 1 M Tris HCL PH (6.8) | 2.5 ml |
| 4) 10% SDS | 0.1ml |

| | |
|---|---|
| 5) 10% (NH4)2s2O8 | 01ml |
| 6) TEMED | 0.004ml |

TE buffer

| | |
|---|---|
| 1) Tris base 1M pH(10-11) | 1ml |
| 2) EDTA(0.5ml) | 0.2ml |

1. Confirmation of bacteria through Mass Spectroscopy analysis (4.3)

Government of Nepal
Ministry of Health and Population
Department of Health Services
**National Public Health Laboratory**
Teku, Kathmandu
ISO 15189:2012 Accredited Laboratory

Tel : 01-5352421
Fax : 4252375
E-mail: nphl@nphl.gov.np
ISO Certificate No: MLS-004

Date: 2079/08/29

**Confirmation of Bacterial Identification**

Here are the reports of bacterial isolates sent for confirmatory identification to National Public Health Laboratory from Sukraraj Tropical and Infectious Diseases Hospital (STIDH), Teku, Kathmandu, Nepal.

| Isolate No. | Isolate Id | Specimen | Isolation site | Presumptive Identification | Confirmatory Identification |
|---|---|---|---|---|---|
| 1 | 6661 | Urine | STIDH | Gram Negative Bacilli | *Pseudomonas aeruginosa* |
| 2 | 6697 | Urine | STIDH | Gram Negative Bacilli | *Klebsiella pneumoniae* |
| 3 | 6656 | Urine | STIDH | Gram Negative Bacilli | *Pseudomonas aeruginosa* |

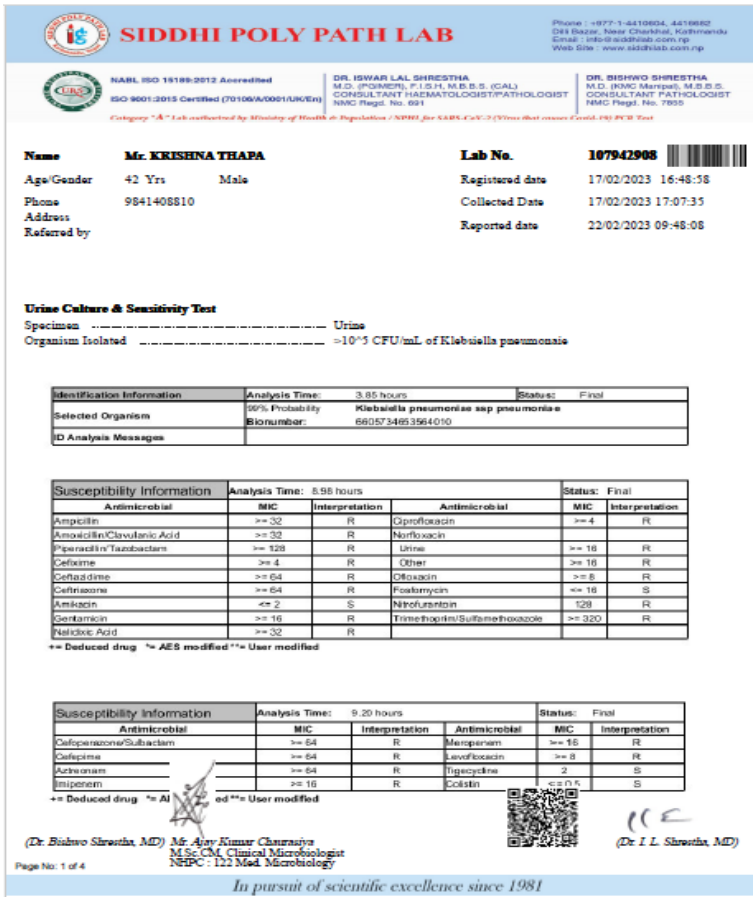
**Method of Confirmatory Identification:** BioMerieux VITEK-MS based on Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS).

Santosh Kumar Yadav
(Microbiologist)

*Not under the scope of accreditation.

Report : www.nphl.gov.np

2. Antibiotic susceptibility test using Compact Vitek system 2(4.5)



**SIDDHI POLY PATH LAB**

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NABL ISO 15189:2012 Accredited
ISO 9001:2015 Certified (70106/A/0001/UK/En)

DR. ISWAR LAL SHRESTHA
M.D. (PGIMER), F.I.S.H, M.B.B.S. (CAL)
CONSULTANT HAEMATOLOGIST/PATHOLOGIST
NMC Regd. No. 691

DR. BISHWO SHRESTHA
M.D. (KMC Manipal), M.B.B.S.
CONSULTANT PATHOLOGIST
NMC Regd. No. 7855

Category "A" Lab authorized by Ministry of Health & Population / NPHL for SARS-CoV-2 (Virus that causes Covid-19) PCR Test

**Name** Mr. KRISHNA THAPA
Age/Gender 42 Yrs Male
Phone 9841408810
Address
Referred by

**Lab No.** 107942908
Registered date 17/02/2023 16:48:58
Collected Date 17/02/2023 17:07:35
Reported date 22/02/2023 09:48:08

**Urine Culture & Sensitivity Test**

Specimen ..... Urine
Organism Isolated ..... >10^5 CFU/mL of Klebsiella pneumoniae

| Identification Information | Analysis Time: 3.85 hours | Status: Final |
|---|---|---|
| Selected Organism | 99% Probability | Klebsiella pneumoniae ssp pneumoniae |
| | Bionumber: | 6605734853564010 |
| ID Analysis Messages | | |

| Susceptibility Information | Analysis Time: 8.98 hours | | | Status: Final | |
|---|---|---|---|---|---|
| Antimicrobial | MIC | Interpretation | Antimicrobial | MIC | Interpretation |
| Ampicillin | >= 32 | R | Oprofloxacin | >= 4 | R |
| Amoxicillin/Clavulanic Acid | >= 32 | R | Norfloxacin | | |
| Piperacillin/Tazobactam | >= 128 | R | Urine | >= 16 | R |
| Cefixime | >= 4 | R | Other | >= 16 | R |
| Ceftazidime | >= 64 | R | Ofloxacin | >= 8 | R |
| Ceftriaxone | >= 64 | R | Fosfomycin | <= 16 | S |
| Amikacin | <= 2 | S | Nitrofurantoin | 128 | R |
| Gentamicin | >= 16 | R | Trimethoprim/Sulfamethoxazole | >= 320 | R |
| Nalidixic Acid | >= 32 | R | | | |

+= Deduced drug *= AES modified **= User modified

| Susceptibility Information | Analysis Time: 9.20 hours | | | Status: Final | |
|---|---|---|---|---|---|
| Antimicrobial | MIC | Interpretation | Antimicrobial | MIC | Interpretation |
| Cefoperazone/Sulbactam | >= 64 | R | Meropenem | >= 16 | R |
| Cefepime | >= 64 | R | Levofloxacin | >= 8 | R |
| Aztreonam | >= 64 | R | Tigecycline | 2 | S |
| Imipenem | >= 16 | R | Colistin | <= 0.5 | S |

+= Deduced drug *= Al ed **= User modified

(Dr. Bishwo Shrestha, MD) Mr. Ajay Kumar Chaurasiya
M.Sc.CM, Clinical Microbiologist
NHPC : 122 Med. Microbiology

(Dr. I. L. Shrestha, MD)

Page No: 1 of 4

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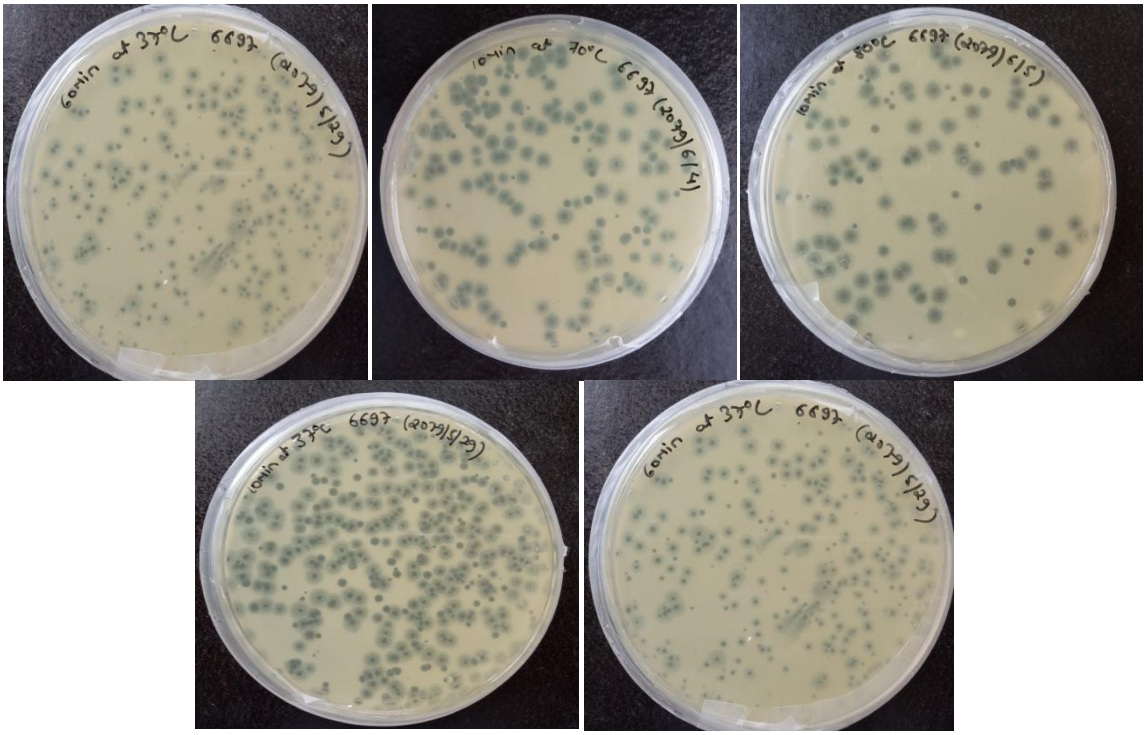
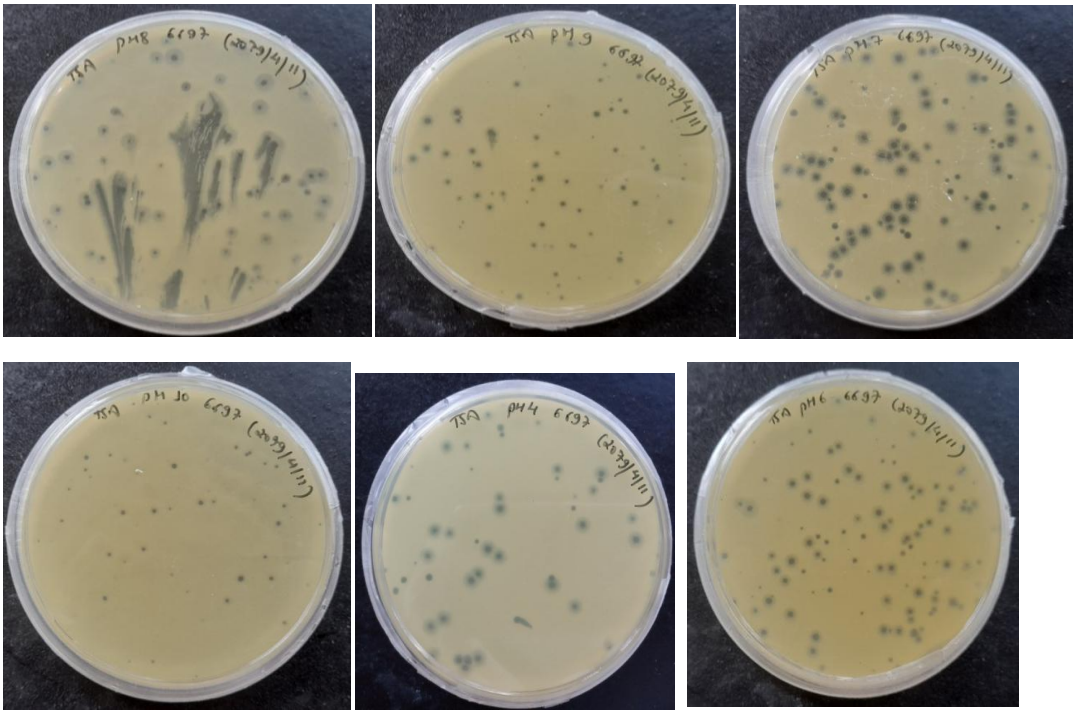


Bacteriophage at different temperature



Bacteriophage stability at different pH

CDS position along with their blast hit result (4.19.3)

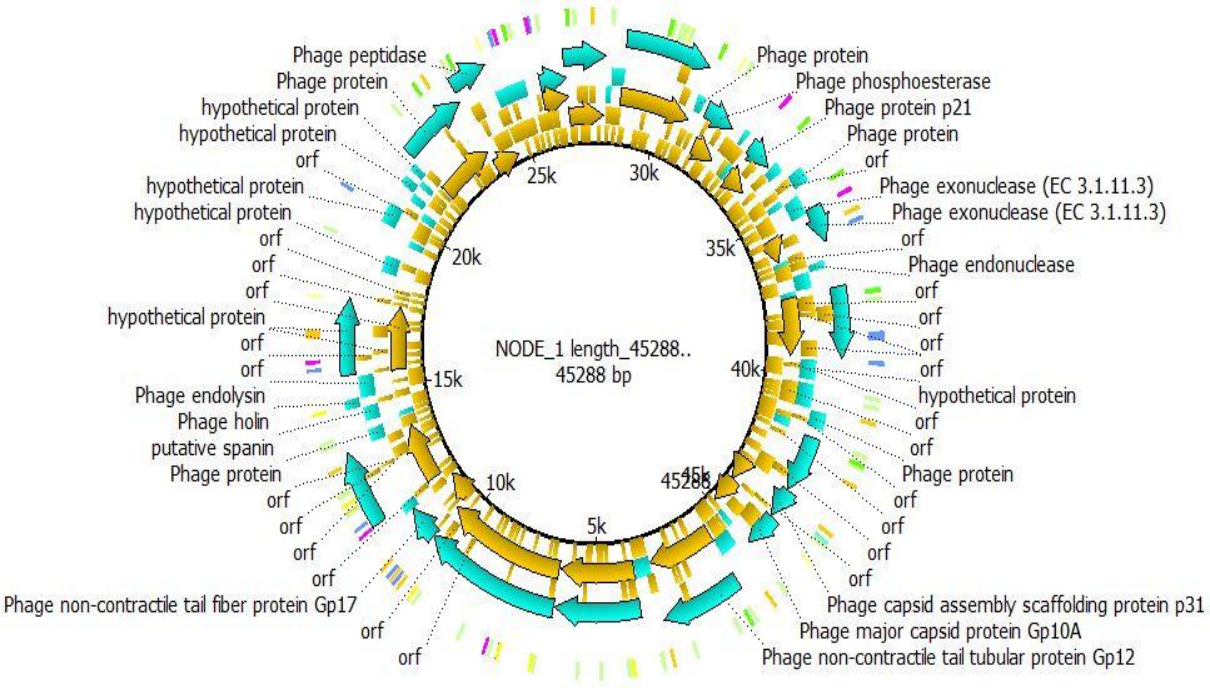
Phage-like protein | Hypothetical protein | Fiber protein | Holin | Tail protein
Endonuclease | DNA Helicase | Exonuclease | Collar protein | Head protein

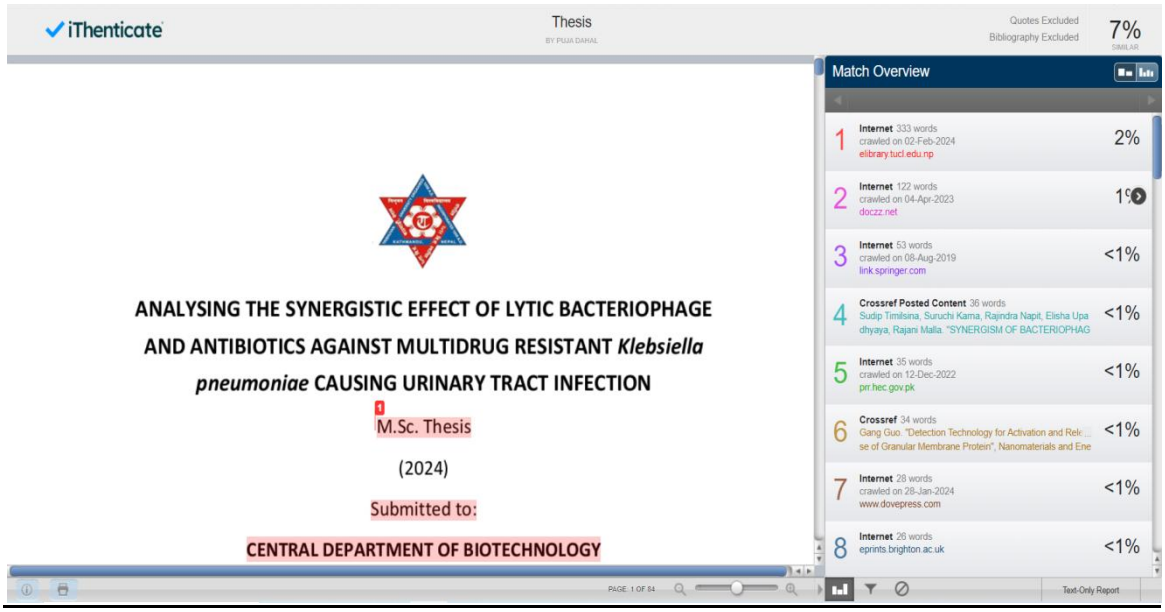
questionable
**Region 1, total 49 CDS**

| # | CDS Position | BLAST Hit | E-Value |
|---|---|---|---|
| 1 | 2999..3586 | PP_00003;internal virion protein;phage;-;PHAGE_Klebsi_AltoGao_NC_047849 | 4.52e-139 |
| 2 | 3603..6287 | PP_00004;hypothetical protein;phage;-;PHAGE_Klebsi_NTUH_K2044_K1_1_NC_025418 | 0.0 |
| 3 | 6338..10603 | PP_00005;putative internal core protein;phage;-;PHAGE_Klebsi_myPSH1235_NC_047944 | 0.0 |
| 4 | 10605..11561 | PP_00006;tail fiber protein;phage;-;PHAGE_Klebsi_AltoGao_NC_047849 | 0.0 |
| 5 | 11578..11880 | PP_00007;putative DNA maturase A;phage;-;PHAGE_Klebsi_vB_KpnP_KpV74_NC_047811 | 1.47e-64 |
| 6 | 11880..13736 | PP_00008;putative DNA maturase B;phage;-;PHAGE_Klebsi_vB_KpnP_SU503_NC_028816 | 0.0 |
| 7 | 13736..14110 | PP_00009;hypothetical protein;phage;-;PHAGE_Klebsi_myPSH1235_NC_047944 | 2.04e-83 |
| 8 | 14122..14304 | PP_00010;hypothetical protein;phage;-;PHAGE_Klebsi_KPV811_NC_047787 | 2.35e-31 |
| 9 | 14304..14708 | PP_00011;putative spanin;phage;-;PHAGE_Klebsi_phiKpS2_NC_047857 | 6.95e-89 |
| 10 | 14701..14952 | PP_00012;putative holin;phage;-;PHAGE_Klebsi_vB_KpnP_SU503_NC_028816 | 7.38e-54 |
| 11 | 14936..15535 | PP_00013;putative endolysin;phage;-;PHAGE_Klebsi_vB_KpnP_SU552A_NC_028870 | 2.32e-137 |
| 12 | 15553..17511 | PP_00014;tail spike protein;phage;-;PHAGE_Klebsi_May_NC_047900 | 1.29e-39 |
| 13 | 18391..18879 | PP_00015;putative HNH endonuclease;phage;-;PHAGE_Dickey_BF25/12_NC_047759 | 8.3e-19 |
| 14 | 19672..20247 | PP_00016;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_SU552A_NC_028870 | 7.21e-136 |
| 15 | 20313..20558 | PP_00017;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_SU503_NC_028816 | 2.29e-25 |
| 16 | 20636..20830 | PP_00018;hypothetical protein;phage;-;PHAGE_Shigel_SFN6B_NC_047929 | 1.15e-14 |
| 17 | 20823..21086 | PP_00019;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_KpV41_NC_028670 | 2.04e-56 |
| 18 | 21095..21274 | PP_00020;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_SU503_NC_028816 | 4.61e-34 |
| 19 | 21271..21477 | PP_00021;hypothetical protein;phage;-;PHAGE_Klebsi_myPSH1235_NC_047944 | 4.75e-42 |
| 20 | 21562..23532 | PP_00022;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_KpV41_NC_028670 | 0.0 |
| 21 | 23532..24575 | PP_00023;putative peptidase;phage;-;PHAGE_Klebsi_KP34_NC_013649 | 0.0 |
| 22 | 24556..25023 | PP_00024;HNH endonuclease;phage;-;PHAGE_Acinet_vB_AbaP_B1_NC_042003 | 7.31e-32 |
| 23 | 25026..25499 | PP_00025;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_SU552A_NC_028870 | 1.68e-78 |
| 24 | 25486..25989 | PP_00026;putative HNH endonuclease;phage;-;PHAGE_Dickey_BF25/12_NC_047759 | 2.03e-76 |
| 25 | 25953..26804 | PP_00027;putative DNA primase;phage;-;PHAGE_Klebsi_vB_KpnP_KpV48_NC_047783 | 0.0 |
| 26 | 26758..28014 | PP_00028;putative DNA helicase;phage;-;PHAGE_Klebsi_vB_KpnP_KpV71_NC_031246 | 0.0 |
| 27 | 28066..28221 | PP_00029;hypothetical protein;phage;-;PHAGE_Klebsi_myPSH1235_NC_047944 | 8.53e-29 |
| 28 | 28218..28589 | PP_00030;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_SU503_NC_028816 | 4.02e-72 |
| 29 | 28564..30954 | PP_00031;putative DNA polymerase;phage;-;PHAGE_Klebsi_vB_KpnP_SU552A_NC_028870 | 0.0 |
| 30 | 30951..31172 | PP_00032;hypothetical protein;phage;-;PHAGE_Klebsi_KP_Rio/2015_NC_047779 | 6.14e-43 |
| 31 | 31334..32314 | PP_00033;hypothetical protein;phage;-;PHAGE_Shigel_SFN6B_NC_047929 | 0.0 |
| 32 | 32796..32960 | PP_00034;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_KpV475_NC_031025 | 1.42e-24 |
| 33 | 33012..33836 | PP_00035;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_KpV475_NC_031025 | 0.0 |
| 34 | 33889..34143 | PP_00036;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_SU552A_NC_028870 | 2.13e-51 |
| 35 | 34422..34796 | PP_00037;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_KpV41_NC_028670 | 2.31e-68 |
| 36 | 34957..35463 | PP_00038;putative HNH endonuclease;phage;-;PHAGE_Entero_phiKDA1_NC_027980 | 2.82e-41 |
| 37 | 35438..36412 | PP_00039;putative 5'-3' exonuclease;phage;-;PHAGE_Klebsi_KP34_NC_013649 | 0.0 |
| 38 | 36436..36546 | PP_00040;hypothetical protein;phage;-;PHAGE_Klebsi_KP34_NC_013649 | 2.69e-18 |
| 39 | 36982..37425 | PP_00041;hypothetical protein;phage;-;PHAGE_Klebsi_F19_NC_023567 | 6.37e-73 |
| 40 | 37412..37528 | PP_00042;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_KpV71_NC_031246 | 4.05e-20 |
| 41 | 37567..39381 | PP_00043;DNA-dependent RNA polymerase;phage;-;PHAGE_Klebsi_phiKpS2_NC_047857 | 0.0 |
| 42 | 39468..40025 | PP_00044;hypothetical protein;phage;-;PHAGE_Pectob_PPWS2_NC_048103 | 4.72e-43 |

| | | | |
|---|---|---|---|
| 43 | 40147..40821 | PP_00045;putative DNA-dependent RNA polymerase;phage;-;PHAGE_Klebsi_vB_KpnP_KpV48_NC_047783 | 1.32e-160 |
| 44 | 40845..41282 | PP_00046;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_KpV74_NC_047811 | 5.43e-103 |
| 45 | 41282..41545 | PP_00047;hypothetical protein;phage;-;PHAGE_Klebsi_phiKpS2_NC_047857 | 1.23e-52 |
| 46 | 41555..43150 | PP_00048;putative collar protein;phage;-;PHAGE_Klebsi_vB_KpnP_KpV74_NC_047811 | 0.0 |
| 47 | 43165..44007 | PP_00049;putative scaffolding protein;phage;-;PHAGE_Klebsi_vB_KpnP_KpV48_NC_047783 | 0.0 |
| 48 | 44033..45052 | PP_00050;putative capsid protein;phage;-;PHAGE_Klebsi_vB_KpnP_KpV48_NC_047783 | 0.0 |
| 49 | 45064..45246 | PP_00051;hypothetical protein;phage;-;PHAGE_Klebsi_vB_KpnP_KpV475_NC_031025 | 1.83e-31 |

ORF prediction from Ugene(4.19.1)



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