PHYSIOCHEMICAL AND GENOMIC CHARACTERIZATION OF BACTERIOPHAGE AGAINST URINARY TRACT INFECTING *Pseudomonas aeruginosa* TO ASSESS ITS POSSIBLITY FOR PHAGE THERAPY

M.Sc. Thesis

2023

Submitted to:

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

For the partial fulfillment of Masters of Science in Biotechnology

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Recommendation

This is to certify that the research work entitled "PHYSIOCHEMICAL AND GENOMIC CHARACTERIZATION OF BACTERIOPHAGE AGAINST UTI INFECTING *Pseudomonas aeruginosa* TO ASSESS ITS POSSIBLITY FOR PHAGE THERAPY" was successfully carried out by Ms. Pragya Sapkota under our supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the Course code BT621. The result presented here is her original findings. We hereby, recommend this thesis for final evaluation.

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ACKNOWLEDGEMENT

I would like to express my deepest vow and gratitude towards my supervisor prof. Mrs. Rajani Malla for her continuous supervisions and support for the completion of the entire work with immense love and care as well as the necessary arrangement of the equipment and materials for the entire work. I am also indebted to my supervisor Asst Prof Mrs. Pragati Pradhan for her continuous guidance supervision and motivation towards the entire work. Despite my own effort, it would not have been possible without their constant motivation love and support.

I would also like to acknowledge the Head of Department prof. Dr. Krishna Das Manandhar for his encouragement and necessary efforts for providing comfortable space and environment to complete Thesis in the department. I am also extremely Thankful to Dipendra Mandal sir and Elisha Regmi mam for providing necessary support, motivation and dedication towards us for the successful completion of our work.

I'd also want to thank the entire department Professors for their demanding instructions which consistently inspired me to put my knowledge and abilities into Practice in order to produce my finest work. My deepest gratitude to the laboratory and the office workers for all of their assistance with the arrangement of necessary equipment, tools and chemicals required for the work as well as for their cooperation. I would also like to express my deepest gratitude towards my seniors, Juniors and to my friends who directly or indirectly helped me to complete this work. I would like to appreciate my friends Suruchi Karna, Sudip Timalsina, Puja Dahal, Shobha Amagain and Suzeeta Maharjan for their encouragement and support throughout this research work.

I would also want to acknowledge my family members for their endless love, support and sacrifices and also to those who have helped me directly and indirectly. Thanks to my husband Mr. Kapil Khanal and brother Pratik sapkota for the necessary assistance and unfailing support. Lastly, thanks to all the well-wishers who directly or indirectly helped me for the completion of the journey.

GLOSSARY ACRONYMS

AST : Antibiotic susceptibility tests

BMD : Broth Microdilution

CDC : Centre for Disease Control

CDS : Coding Sequence

CLSI : Clinical laboratory standard institute

CTAB : Cetyltrimethylammonium bromide

DD : Disk Diffusion

DLAA : Double Layer Agar Assay

DTP_s : Distal Tail Proteins

DNA : Deoxyribonucleic acid

EOP : Efficiency of Plating

EPS : Exopolysaccharides

ESBL : Extended Spectrum Beta lactamase

FAD : Flavin adenine dinucleotide

FMN : Flavin Mononucleotide

ICTV : International Committee on Taxonomy of Viruses

LPS : Lipopolysaccharides

OD : Optical Density

ORF : Open reading Frame

MALDI- TOF : Matrix Associated Laser Desorption Ionization – Time of Flight assay

MBL : Metallo- beta Lactamase

MHA : Muller Hinton agar

MRVP	:	Methyl red and Voges Proskauer test
NA	:	Nutrient agar
NGS	:	Next Generation Sequencing
PBS	:	Phosphate buffered saline
PFU	:	Plaque forming unit
PHASTER	:	Phage enhanced search tool release
RAST	:	Rapid Annotation using Subsystem Technology
RNA	:	Ribonucleic acid
SDS- PAGE	:	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SIM	:	Sulphur Indole Motility
SM	:	Sodium Magnesium buffer
STIDH	:	Shukraraj Tropical and Infectious Disease Hospital
TAE	:	Tris Acetate EDTA
TSIA	:	Triple Sugar Iron agar
WHO	:	World Health Organization
TSA	:	Tryptic Soya Agar
LB broth	:	Luria Bertani broth
UTI	:	Urinary Tract Infections
UV	:	Ultra-Violet
ZOI	:	Zone Of Inhibition

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ABSTRACT

Introduction: Emerging antibiotic resistance against widespread Uropathogen has grown to be a significant therapeutic concern for UTI in recent years particularly in low- and middle-income nations by the reckless uses of antibiotics. In present context, resistance is seen in almost all antibiotics even in the last line Carbapenems and colistin. Thus, using phages in therapeutics to fight against antimicrobial resistance can be a solution to the global threat caused by these resistant bacteria. This study aims to isolate and characterize the phage both physiochemically and genetically to combat this global issue.

Methodology: Host bacterial species was identified by biochemical test and 16SrRNA sequencing. Antibiotic susceptibility test was performed by disc diffusion method and confirmed by Vitek Compact System 2 analyzer. Bacteriophage isolation was done by Double Layer Agar Assay (DLAA). pH and temperature stability of phage was analyzed. Latent period, burst size and host range was determined. Whole genomic sequencing of phage was done. Bioinformatic analysis to assess for any kind of toxin gene or the virulence gene of the bacterial origin using different tools such as Ugene, RAST, PHASTER and Proksee.

Results: Lytic bacteriophage against *Pseudomonas aeruginosa* was isolated. The phage was maximally stable at temperature 37˚c and pH 7. The latent period was 30 min and burst size was 96 virions per bacterium. Isolated phage 6661 showed intraspecific host range with *Pseudomonas* (P1) whereas no any activities with other *Pseudomonas*. Protein profiling of the phage through SDS- PAGE shows the four distinct band of protein in the gel. The size of DNA was found to be greater than 10kb. Phage length and GC content was determined by whole genome sequencing which was 43212 kb and 53.79% respectively. No any toxin or virulence gene was determined on bioinformatic analysis. The isolated phage significantly reduces the biofilm by 50.73%.

Conclusion: *Pseudomonas* phage 6661 showed good stability to various physiochemical factors and on genomic characterization, presence of endolysin absence of toxin or virulence gene and integrase enzyme favors the therapeutic potential of the virus.

Keywords: Antibiotic resistance, Bacteriophage, Burst size, Colistin, Phage Therapy

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Golden age of antibiotics" started with the discovery of antibiotic Penicillin by Sir Alexander Fleming. Antibiotics were believed to be at the peak from 1950's to 1970's. (Aminov, 2010) Despite the fact that the discovery of antibiotics revolutionized the treatment of prevailing infectious diseases (Diarrhoea, Pneumoniae cholera, Typhoid fever) at that era, improper use of antibiotics resulted in the development of multidrug resistance in variety of bacteria. Multi- drug resistant (MDR) in bacteria is the phenomenon of acquiring resistant to at least one agent in three or more antimicrobial categories (Magiorakos *et al.,* 2012). The golden age of antibiotics come to an end, as did the enormous concerns posed by the antibiotic resistant organisms. Antimicrobial resistance is a serious issue in health care affecting mortality, service quality and financial harm. Nowadays, in this present era, various pattern of antimicrobial resistance is being identified in the healthcare associated infections. One of them is MDR. The next is extensively drug resistant organism (XDR) and Pan drug resistant (PDR) bacteria. XDR is defined as resistance to at least one agent in all antimicrobial categories except two or fewer similarly, PDR is defined as resistant to all commercially approved and available antimicrobials (Magiorakos *et al.,* 2012). Many pervasive sociocultural and financial practice have made the resistance management extremely difficult. Several potential avenues for the transmission of the resistant bacteria and their resistance genes in soil and surrounding systems and ecosystems have been identified, including hospital effluent, agricultural waste and waste water treatment facilities (Irfan *et al.,* 2022). Since, the antimicrobial resistance is rising at high level, nowadays even the simpler infection is becoming harder to treat with the available antibiotics.

Nowadays, several therapeutic techniques are being tested in order to address antibiotic resistance crisis. These tactics can be divided into three categories i) Naturally occurring strategies ii) synthetically created strategies and iii) biotechnology-based strategies. (Ghosh *et al.,* 2019). However, no strategies are unacceptable unless it is safe, effective and affordable. In this context, using naturally occurring bacteriophages as bacteriophage therapy to remove certain bacterial pathogens can be an exciting strategy. Bacteriophage

therapy is one of the alternative treatments that has undergone the most investigation and has the longest history. Over a century of research into Phage in medicine has led to the development of Phage as a rejuvenated therapy. The use of Phage in therapeutics is the subject 0f numerous ongoing studies, raising the possibility that they could be the powerful weapon in the fight against antimicrobial resistance. More understanding of phages and their mode of bacterial infection before they can be exploited as a treatment (Allen *et al.,* 2017).

1.2 Antibiotic resistance a global phenomenon

Globally, the spread of multidrug -resistant bacterial strain has posed a serious risk to the public's health when it comes to the regular management of infectious diseases. Despite the worldwide upsurge in such resistant germs, the development of new antibiotics has slowed down over the past few decades. Therefore, it is imperative to determine a promising alternative to treat infectious diseases for the prevention of the spread of the antibiotic resistance among the organisms (Bhetwal *et al.,* 2017).

According to the CDC estimates, each year in the United States, over two million individuals get sick with antibiotic resistant illness, which cause at least 23000 fatalities. The antibiotic era has come to an end due to the bacterial development of resistance to multiple antibiotics as a result of antibiotics overuse and misuse. In the post antibiotic era that we are currently living in simple diseases or small wound might become lethal (WHO, 2014). According to the recent ant studies, there are more than 2.8 million antibiotic resistant infections in the US each year, and as a result around 35,000 people die each year (Centers for Disease Control,2019). According to Cassini et al. 2019, 33,000 individuals in Europe, each year pass away from diseases that are resistant to antibiotics. The world Health organization predicts that drug resistant disease could result in the death of around 10 million people by the year 2050. To prevent such a dire situation, the search and development of novel antibacterial agents is urgently needed and bacteriophage may play a significant role in resolving this world-wide dilemma (Pires *et al.,* 2020). The current use of common antibiotics such as β- lactams, aminoglycosides, chloramphenicol and Tetracycline is seriously jeopardized by the existence of the antibiotic resistance gene that code for bacterial resistance to these drugs. These genes

also appear to be prevalent in the environment. In particular the hard- to -treat nosocomial infections brought on by the ESKAPE pathogens (*Enterococcus* faecium*, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa and Enterobacter spp*) pose a risk due to the fact that many antibiotics that are losing their effectiveness against common antibiotics (Lin *et al.,* 2017).

A return to "the pre- antibiotic era" is increasingly being warned about, and regulatory agencies like the Centre for Disease Control (CDC) and WHO have declared antibiotic resistance threat to the world health (Lin *et al.,* 2017). Due to the prevalence of Multidrug resistant illnesses and the prevalence of resistance to nearly every known antibiotic, the CDC has dubbed the current period the "post antibiotic age" (Schooley *et al.,* 2017**).**

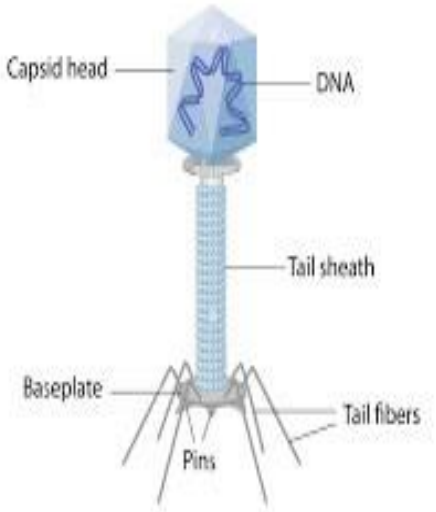


Figure 1: Structure of bacteriophage

1.3 Bacteriophage

Bacteriophage are the type of virus that only targets and replicates within a bacterial cell**.** They are structurally simple and the most divergent living creatures assisting the management of the bacterial populations in the natural ecosystems all around the world. Additionally, it was claimed that once every 24 hours, they are in charge of eliminating 20 to 40 percent of the bacteria on marine surfaces (Soressa Bakala & Motuma, 2022). They are approximately 50 times smaller than the bacteria (Ly-Chatain, 2014)**.** They can attack the target bacteria without harming the commensal flora and can be spontaneously removed once the bacteria have been eradicated. because of this specificity they are also coined as intelligent antimicrobials". Bacteriophage are regarded as incomplete organism because of the absence of the cell structure and the enzyme system required for the food uptake, protein synthesis and the formation of the new particles. Hence, also known as bacterial parasites. Twort in 1915 discovered bacteriophages as unidentified molecules that inhibit bacterial growth, but later in 1917 D'Herelle isolated and characterized phages and he was regarded the first person to develop phage treatment (Wernicki *et al.,* 2017).

Several significant genetic traits in phage show their lengthy evolutionary history. Phages have a very diversified genome, to start 2000 genome that have been sequenced provide an example of this. Phage infecting a single strain of Mycobacterium smegmatis, containing around 30 distinct genomic types with little gene similarity between them and significant heterogeneity within each type. The average phage gene size is only around two third of the bacterial gene because phage genomes are frequently densely packed with overlapping protein- coding and occasionally RNA- encoding genes and ad are packed with comparatively small gene of unknown function. Although several of the unknown function gene may affect the generation of the phage particles and the phage Host dynamics, they are typically not necessary for the lytic growth. Finally, phage genomes are largely mosaic and contain only one gene found in diverse genomic setting in phages that are unrelated to one another. This has probably come out as a result of sequence independent recombination event over a lengthy evolutionary period of time (Strathdee *et al.,* 2023).

1.4 History of Bacteriophages

William Twort first identified bacteriophages in 1915, and Felix D 'Herelle determined they had the capacity to eradicate bacteria in 1917. After a pre antibiotic era, they were essentially recognized as important therapeutic agent in the west mostly because of the ease in administration of the antibiotics (Clokie *et al.,* 2011). In 1923, certain parts of the Soviet Union and Eastern Europe had difficulty in access to antibiotics during World War II, which led to the development of phage therapy. Phage therapy was frequently recommended under the Soviet Union and is still practiced today in Russia and other Eastern European nations, particularly at the Eliava institute which was founded by d' Herelle in Tbilisi Georgia (Silva *et al.,* 2021).

A program to treat phage patients with suppurative infections was created in 1952 at the Hirszfed Institute. By the 1980s, the growing concern of antibiotic resistance had started to alter western perceptions about phage therapy. The English scientific literature began to publish controlled animal research in the 1980s. Herbert Williams smiths, a British veterinarian and Experimental scientist at the institute in Animal Disease Research in Houghton, Cambridgeshire was one of the first to study phage therapy in the west. (Smith,

Huggins 1982, 1983, 1987; Datta) In the west Some Western European nations have started approving therapeutic use in recent years. Paul Turner and colleague have revealed in 2016 that they have isolated a phage that could help multidrug resistant *Pseudomonas aeruginosa* regain its antibiotic sensitivity. A patient with a persistent aortic graft infection who had not responded to repeated surgical procedures and rigorous antibiotic Therapy was later treated with this phage (Silva *et al.,* 2021).

The development of phage-based biotechnologies for human health, animal health or the bio- control in the agri-food industry occurred in parallel with the widespread rejection by a portion of infectious diseases community in the late 1990s and early 2000s. Indeed, some projects, have been successful in the later sector. The Canadian Environmental protection agency EPA and FDA have each approved two anti- Listeria Phage mixture since 2007. Later they were given permission by the European food safety agency EFSA. Phages are also employed in conventional and organic farming to defend Tomato and Pepper crops against the diseases *Xanthomonas pestris* and *Pseudomonas Syringae* **(**Brives & Pourraz, 2020).

1.5 Structural organization of Bacteriophage

Phages are the ubiquitous creatures, extremely diverse in size, morphology and genomic organization. All however, include a nucleic acid genome wrapped in a shell of phage encoded capsid proteins that mediate the passage of genetic materials into the following host cell (E. White & V. Orlova, 2020)**.** Numerous phage type now visualized through electron microscopy, some of which even appear to have like “heads”, “legs” and “tails”. Despite of their appearance, phages are non -motile and rely on Brownian motion to reach their targets. (Kasman LM, Porter LD. Bacteriophages [Updated 2022, sep26]**.** Viruses with tail are the most common as well as studied phage which is further classified into viruses with short tail (podoviridae), long non- contractile tails (siphoviridae), and contractile tails (myoviridae) (Aksyuk & Rossmann, 2011).

Bacteriophages are distinguished by high sensitivity to bacteria at infection. Numerous morphologies of phage can be identified including filamentous phage, phages with lipid containing envelope and phages with lipid in the particle shell. They have a single or double stranded genome that can be made of either DNA or RNA and contain information

about protein that make up the particles, extra protein that flip cellular molecular mechanism in favor of viruses, and therefore information on the self- assembly process. The genome which is housed inside the capsid can be either single or multipartite (E. White & V. Orlova, 2020).

The size of the viral genome corelates strongly with the size of its capsid, which in turn shows the complexity and organization of the virus. Similar organization can be found in several eukaryotic and bacterial viral capsids. As predicted by Crick and Watson (CRICK & WATSON, 1956) and later confirmed by Caspar (CASPAR, 1956), multiple copies of viral capsid proteins are put together into icosahedral cells. An icosahedral particle can be created with a minimum of the 60 copies of capsid proteins, in which all of the subunits have same identical environments. On the pentameric vertex of the capsid lies a portal protein, also known as connector. The portal or the connector as the name imply, is necessary for the DNA entry and release as well as for the attachment of the neck protein to the head. Additionally, by copolymerizing the scaffolding and capsid proteins, the phage head assembly is likely to be started from portal vertex (Aksyuk & Rossmann, 2011).

Depending on how viral DNA was copied, DNA packaging can start at any time. DNA concatemer or Head to tail multimers produced by large number of phages, are utilized by lambda, T4 and P22 as packaging substrate. In such case the ATPase breaks the concatemer and package the genomes length. As a result, the packaging ATPase is referred to as terminase. To distinguish it from small terminase, which does not cleave DNA, this enzyme is sometimes known as large terminase. The terminase complex is replaced by neck proteins once the head has been packaged with DNA (Black, 1989). The proteins along with the connector create the “gatekeeper complex”, which prevents early DNA leakage from the head and may even start DNA exit upon attachment to the host bacteria (Aksyuk & Rossmann, 2011). Many phages add small or pilot proteins into the head as they assemble the head. These proteins are not necessary for the virus, hence are present in the low copy number, however these are important for viral infectivity **(**Aksyuk & Rossmann, 2011).

The distinctive organelle known as ‘’tail” distinguishes all tailed bacterial virus and is responsible for releasing DNA from the head and contain cell recognition proteins. Once the head is assembled, there is different tail assembly branch in the assembly route of

siphoviridae and myoviridae phages, which enable the preformed tail to bind to the head via the neck proteins (Aksyuk & Rossmann, 2011)**.** Distal tail proteins (DTPS), which are present in the majority of siphoviridae phages, create an oligomeric ring that is joined to the last ring of the tail tube. Typically, the DTP act as the mechanism to identify and link to the receptor binding proteins; occasionally tail fibers present in T4, T5 and other phage facilitate this contact (E. White & V. Orlova, 2020). Following that, the preassembled fibers are attached in the case of T4. Following the head to tail interactions, gp wac bind to the neck area to create whiskers. The pre-assembled long tail fibers must be attached and this is made possible with the help of whiskers. This places the proximal part near the baseplate attachment site and align the fibers along the length of the tail. Furthermore, the attachment of the fibers via the whiskers prevents any fiber from adhering towards the free tails. If the PH and ionic strength are unfavorable for phage growth, the fibers are known to remain in the retracted state, inhibiting infection (Yap & Rossmann, 2014).

1.6 Research Hypothesis

This study aims to assess the activity of bacteriophage against *Pseudomonas spp.*

Null Hypothesis (Ho): Lytic bacteriophage does not show activity against *Pseudomonas spp.*

Alternative Hypothesis (H1): Lytic bacteriophage show activity against *Pseudomonas spp.*

1.7 Objectives

1.7.1. General objective

Physiochemical and genomic characterization of potential lytic phage isolated from the waste water sample against multidrug resistant *Pseudomonas aeruginosa.*

1.7.2. Specific objective

i. To confirm host bacteria by biochemical test and 16S rRNA sequencing.
ii. To confirm multidrug resistant in host bacteria by antibiotic susceptibility test.
iii. To isolate and purify lytic bacteriophage against multidrug resistant bacteria from the waste water sample.

iv. To determine the interspecific and intraspecific host range analysis of the isolated phages using different types of multidrug resistant bacteria.
v. To determine the stability of the phage against PH and temperature and growth curve analysis of phage.
vi. To investigate the protein profile of the isolated phage using SDS PAGE.
vii. To determine the interspecific and intraspecific host range analysis of the isolated phages using different types of multidrug resistant bacteria.
viii. To identify phage morphologically using Transmission electron microscope.
ix. To analyze the whole genome sequence of phage using Miseq Illumina platform.

1.8 Rationale of the Study

Among various alternatives, bacteriophage have growing frequency of multidrug resistance in pathogenic microorganism against existing antibiotics has pose the worldwide health service a global challenge. Bacteria are changing at faster rate resulting in an outbreak of superbugs. WHO in 2017 has also listed *Pseudomonas aeruginosa* as critical priority pathogen for the research and new drug development antibiotic resistant *Pseudomonas aeruginosa* are mostly associated with the hospital acquired infections such as burn wound infection, skin infection, urinary tract infections and also the patient with cystic fibrosis. Resistant genes even to last resort of antibiotics, carbapenems and colistin has also disseminated globally. Thus, it is of utmost importance to search for the possible alternatives to fight against this antimicrobial resistance crisis.

Thus, Bacteriophage therapy has proven to be more efficient, specific and perhaps the less expensive technique than others. Phage therapy which employs lytic bacteriophage has long history of its usage and has become one of the most important therapeutic options in this antimicrobial resistance era. With the knowledge and advancement in technology such as whole genome sequencing and electron microscopy, previous limitations from the past can be readdressed and its diversity can be easily investigated. Besides that, this technology has also enabled us to study and check for harmful and toxic gene in the genome prior to application.

This study was aimed to investigate and evaluate the possible applicability of lytic bacteriophage against multidrug resistant *Pseudomonas aeruginosa* both physiochemically and at level which will form the basis for evaluating phage for potential therapeutic application.

CHAPTER 2: LITERATURE REVIEW

2.1 Multidrug resistant in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, a leading nosocomial pathogen is responsible for majority of the hospital acquired infections particularly in ill or immunocompromised patient. Because of the limited antimicrobial susceptibility and the frequent evolution of antibiotic resistance during therapy, the infections caused by the organism are often severe and life threatening. In 2017, world Health organization (WHO) identified carbapenem resistant *Pseudomonas aeruginosa* as most critical pathogen and listed it as most prioritized pathogen for new research and development (WHO, 2017). Drug resistant in the organism mainly increased due to both patients to patient transmission of resistant organism and the de novo generation of resistance in particular organism following antimicrobial exposure. According to various preliminary studies, Multidrug resistant was characterized as the inability to be treated by at least three antibiotics from different classes, primarily aminoglycosides, antipseudomonal penicillin, cephalosporin, carbapenems and fluroquinolones.

Multidrug resistant in *Pseudomonas aeruginosa* may develop as a result of the accumulating resistant brought on by repeated exposure to different antibiotics and the cross resistant between different drugs (Aloush *et al.,* 2006). Besides, several processes including multidrug efflux system, enzyme synthesis, loss of the outer membrane protein porin, and target mutation may contribute to multidrug resistant phenotype in *Pseudomonas aeruginosa* (Hirsch & Tam, 2010).

The high level of intrinsic resistance in Pseudomonas aeruginosa is due to reduced outer membrane permeability (about 12-100-fold lower than in *E. coli*), the existence of antibiotic efflux system and the development of the endogenous inactivating enzymes. Horizontal gene transfer (acquisition of the aminoglycosides modifying enzyme and lactamases enzyme) or mutational event that result in the overexpression of efflux pump or lactamase that decreased expression or the modification of the target sites and porins causes acquired resistance mechanisms. External stimuli (antibiotic exposure) activate

adaptive resistance mechanisms, which then becomes inactive when the external stimulus is removed (Kunz Coyne *et al.,* 2022).

Because of the temporary changes in gene expression in response to an environment stimulation, adaptive resistance also improves *Pseudomonas aeruginosa* ability to the toleration of the antibiotic attack. The best characterized adaptive resistance mechanism in Pseudomonas aeruginosa are biofilm formation and the development of the persister cells which result in the prolonged infection and a poor prognosis in the patients especially with Cystic fibrosis (Kunz Coyne *et al.,* 2022).

2.2 Metallobeta lactamase producing bacteria

In the treatment of severe infections brought on by Gram Negative bacteria (GNB), Carbapenems have been employed as last option drug. The development of carbapenems' in particular Metallo – β-Lactamases (MBL) has put the clinical use of Carbapenems in danger. Penicillin, Cephalosporins and Carbapenems, among many other β- Lactam drugs and can all be hydrolyzed by MBL. Many chelators, such as ethylene-diamine tetra acetic acid (EDTA) and thiol- based substances block MBLs. MBL- encoding genes have been discovered in Gram negative bacteria including *Pseudomonas species, Acinetobacter species* and members of the *Enterobacteriaceae* family, all over the world. MBLs can rapidly spread via the plasmids and are the root of outbreaks and nosocomial infections. Patients who are admitted to intensive care units with many comorbidities are most commonly affected by such infections. Therefore, early diagnosis and identification of MBL producing organisms are essential for the treatment- adequate prevention of nosocomial infections (Panchal *et al.,* 2017).

Bacterial resistance to beta lactam antibiotics typically comes through alteration of the target Penicillin binding proteins, decreased intracellular concentration as a result of reduced permeability or efflux or inactivation of antibiotic from the β-lactamases, which is the most frequent mechanism. Some pathogens have innate resistance to β- lactams. Because of this intrinsic resistance, entire species of bacterial pathogens have evolved as a result of repeated exposure to antibiotics. Furthermore, the introduction of novel treatment has occasionally had the unintended effect of changing the etiology of some prevalent hospital infections to species that are inherently more resistant pathogen than

they replaced. In many intensive care units, for instance (ICU) Carbapenemase producing *Acinetobacter baumanii* and *Stenotrophomonas maltophilia* have become widespread as a result oof the increased use of carbapenems especially among the people with mechanical ventilation.

IMP, VIM, SPM, GIM and SIM are five different forms of MBLs whose prevalence is rising quickly. IMP and VIM are the two that predominate among these. Due to the global rise in prevalence and variety of MBLs, early detection is beneficial for reaping the benefits of stringent infection control procedures and alternative antimicrobial treatments (Deshmukh *et al.,* 2011).

Polymerase chain reaction (PCR) based MBL gene detection typically yields accurate and satisfactory result, but is only marginally useful for regular diagnostic microbiology laboratories due to its high cost. Therefore, a quick and low cost-testing technique is required to identify MBL Producers (Panchal *et al.,* 2017)**.** The Imipenem (IMP)-EDTA combination disc test is one of the straightforward and less expensive techniques available for assessing MBL production. For the identification of metallo-beta-lactamase in MBL producing *Pseudomonas* species and *Acinetobacter* species, Yong et al. found that the Imipenem (IMP) 10g and EDTA (750g) Combination disc test has 95.7% sensitivity and 91% specificity. (Deshmukh *et al.,* 2011)

2.3 Urinary tract infection and *Pseudomonas aeruginosa*

One of the most frequent bacterial illness affecting people of all ages is urinary tract infection. This bacterial disease results about more than 8 million people visiting doctors, 1.5 million emergency room visits and about 300,000 hospital admissions each year alone in the United state annually. UTIs are the most prevalent urological condition in the United state and the second most common infection of any organ system, with a total annual cost of more than 3.5 billion. More than half of all women have at least one UTI episode in their lifetime, with antibiotic therapy being the most common treatment. Furthermore, roughly 20–40% of women who have already had an initial UTI will develop a return of infection within 3–4 months (Zalewska-Piątek & Piątek, 2020). One of the most frequent factors that predisposes the host to the severe urinary tract infection is urinary tract catheterization. Catheter implantation may cause mucosal layer injury by disrupting the

natural barrier and allowing bacterial colonization. *Escherichia coli*, *proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae and Streptococcus faecalis* are the most common pathogen inflicted with catheter associated UTIs. However, the third most frequent infection linked to UTIs caused by catheter in hospital is *Pseudomonas aeruginosa* (Mittal *et al.,* 2009).

Pseudomonas aeruginosa multifactorial virulence factors has been attributed to exoenzymes or secretory virulence factors like protease, elastase, phospholipases, pyocyanin, exotoxin A, exotoxin S, hemolysins (rhamnolipids) and siderophores in addition to cell associated factors like alginate, Lipopolysaccharides, flagellum, pilus and non-pilus adhesins (Matheson *et al.,* 2006)**.** In contrast to isolate from other infections such as burn wound infection, skin wound infections and acute pneumonia, Woods et al's research found out that urinary tract infection strains produce more elastase and protease (Woods *et al.,* 1986). However, Hamood et al, performed quantitative analysis for elastase, phospholipase C, toxin A and exoenzyme S by the strain of *Pseudomonas aeruginosa* in the various site of infections. Hamoods *et. al* also observed that during urinary tract infection *Pseudomonas aeruginosa* strain produces significantly higher level of exoenzyme S than other enzymes during infection (Hamood *et al.,* 1996). The association between the generation of virulence factor and the site of infection was studied by Ciragil and Soyletir. These researchers isolated *P. aeruginosa* strain from the lungs, urine and blood of cystic fibrosis patient and non -cystic fibrosis patients. It was shown that when compared to other isolates, urinary isolates produced least quantity of alginate and most amount of alkaline protease. Cystic fibrosis patients had noticeably lower amount of alkaline protease than other isolates while the elastase levels were not significantly different between the *Pseudomonas aeruginosa* species (Ciragil *et. al.,* 2004).

Development of the virulence factor was examined in the patient with urinary tract infection caused by different strains of *Pseudomonas aeruginosa* by Visca *et. al* and it was discovered that *Pseudomonas aeruginosa* uropathogenic strain produced at least one form of siderophores namely pyochelin or Pyoverdine however, not all *Pseudomonas aeruginosa* produced both siderophores (Visca *et al.,* 1992).

2.4 Life cycle of viruses

Bacteriophages are very host specific and typically infect only one bacterial species or even a particular strain within a species. A bacteriophage can pursue either lytic or lysogenic replicative cycle once it becomes attached to the susceptible host. Lytic cycle of bacteriophage starts with the attachment of the phage to the receptive host bacterium, insertion of the genome into the cytoplasm of the host cell and further utilization of the host ribosomes to synthesize its proteins. The existence of the particular receptors that the phage can attach on to the surface of the bacterial cell is necessary for the attachment phase to occur. Phage use random diffusion method to get attached to the host bacterium (Soressa Bakala & Motuma, 2022). Resources from the host cells are then quickly transferred into the viral genome and capsid protein which assemble together to form numerous copies of the original phage. The phage then gets lysed actively or passively once the host cell decomposes, releasing the new bacteriophage to infect another host cell. Phage implicates its lytic enzyme (endolysin or lysin) to degrade the bacterial peptidoglycan layer resulting in the lysis of the host cell.

During the lysogenic replication cycle, the phage gets attached to the receptive host bacterium, inserts its genome into the cytoplasm of the host cell. Instead, the phage genome is either preserved as an episomal element or incorporated into the chromosome of the bacterial cell, where it is duplicated and transmitted into the offspring bacterial cells without killing them. Thus, integrated phage genome is known as prophage and the bacteria harboring those prophages are termed as lysogens. Lysogenic mechanism of the bacteriophages results in the vertical transfer of virus genetic material to the bacterias' daughter cells with the ultimate expression of viral genes and proteins (Soressa Bakala & Motuma, 2022). Prophage encoded genes becomes accessible to the host when they are incorporated into the bacterial genome. One bacterial genome can contain up to 18 prophages as seen in the food pathogen Escherichia coli 0157: H7 sakai, and prophages encoded genes can make up as much as 20% of bacterial chromosomal content. Prophages gene which can encodes for virulence factors, metabolic genes and antibiotic resistance genes such as lactamases can be advantageous to the bacterial host (Lin *et al.,* 2017). Prophages have the ability to switch back to the lytic cycle and kill their host, most frequently in response to shifting environmental factors. (Kasman LM, Porter LD.

Bacteriophages [Updated 2022, sep26]. The life cycle of the temperate phage also referred to as non-virulent phage is lysogenic and does not include the destruction of bacterial cell (Alsubhi, 2021).

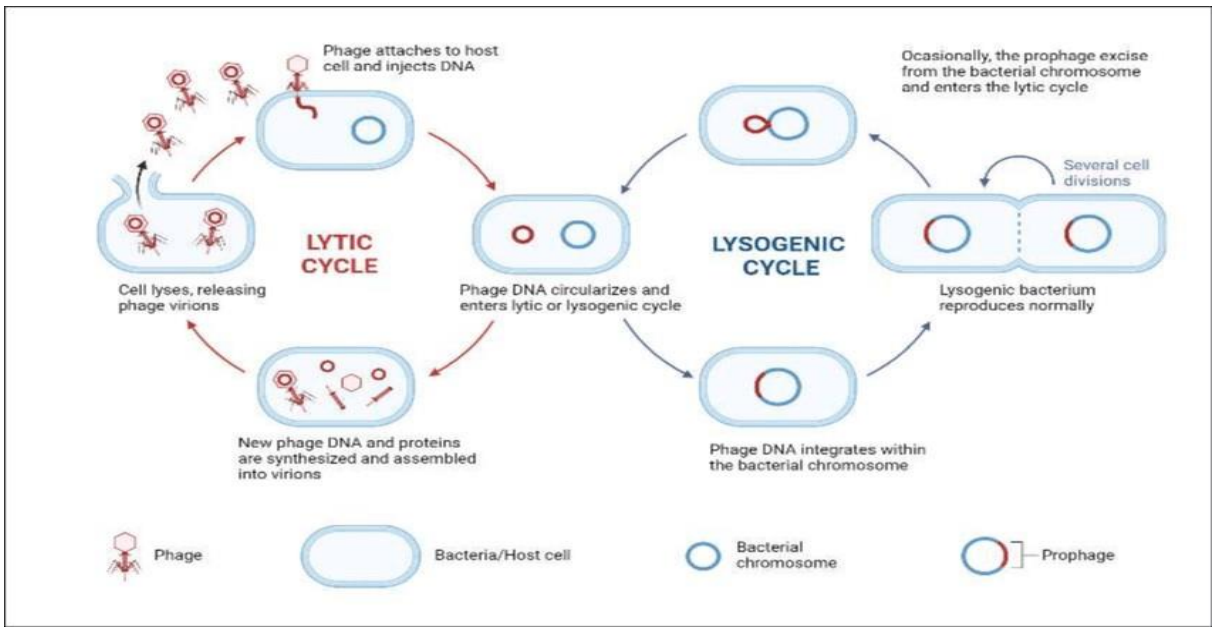


Figure 2: Lytic cycle and Lysogenic cycle of bacteriophage (Alsubhi, 2021)

2.5 Classification of Bacteriophage

Bacteriophage classification was first proposed by Ernst Ruska by classifying the viruses into three morphological types. Later, phages are classified as suborder virale by Holmes in 1948 based on the host range which was never widely accepted. In 1962, Lwoff, Horne and Tournier later proposed that viruses could be classified by their nucleic acid type (DNA or RNA), capsid shape, presence or absence of an envelope and the number of capsomeres. In 1966, International committee on taxonomy of viruses (ICTV) was founded and proposed that viruses should be classified based on their properties of the virion and nucleic acid content rather than host range and pathogenicity. At present phage classification is based on the scheme proposed by Bradley in 1967 exemplified by phages T4, λ, T7, ϕX174, MS2, and fd. Phage classification is open ended process because of the daily discovery of phage and ICTV Is far behind its schedule (Ackermann, 2009).

An organized approach for classifying viruses has been developed by the International Committee on Taxonomy of Viruses (https://talk.ictvonline.org/taxonomy/)(E. White & V. Orlova, 2020). Phages include viruses with double-stranded DNA (dsDNA; the vast

majority), single-stranded DNA (ssDNA), single-stranded RNA (ssRNA), and double-stranded RNA (dsRNA; very rare). Most virions (96%) are tailed; other types (herein called CFP) are “cubic,” filamentous, or pleomorphic (∼200 representatives, less than 4%). Tailed phages come under the three families (Myoviridae, Siphoviridae and Podoviridae). Polymorphic viruses fall under the family of (Microviridae, corticoviridae, Tectiviridae, SHI*, STIV*, Leviviridae and Cystoviridae). Filamentous phage falls under the family of (Inoviridae, Lipothrixviridae and Rudiviridae). Pleomorphic viruses fall under the family of (Plasmaviridae, fuselloviridae, Guttaviridae, Ampullaviridae and Bicaudaviridae (Ackermann, 2009). Tailed phages on the order Caudovirales (Myoviridae, Siphoviridae and Podoviridae) are the most studied group of phages. Myovridae family have a complex contractile tail; Siphoviridae family have a long non- contractile tail; and Podoviridae family have a short non-contractile tail. Mostly the tailed phages are encapsidated by double stranded genome. The genome is encapsulated inside the head shielded by the icosahedral capsid and a tail designed for delivering DNA inside the bacterial host**.** Tailed phages are also the most diverse phage in terms of size, structure DNA content, genome, protein structure antigenicity and biological characteristics.

Cubic phages or the phages from family microviridae are small non-enveloped viruses whose DNA are almost similar to the filamentous phage. The phage replicates by rolling circle mechanism. Corticoviridae virions have circular DNA with multilayered lipid containing capsid. Tectiviridae member consist of outer proteinic shell and inner lipoprotein vesicle and are capable of forming “pseudo-tail” of about nearly 60 nm in length during the infection process. SH1 phage have the same structure as Tectiviridae and contain lipid. STIV viruses have the structural similarity with Tectiviridae and is characterized by the apical protrusions. Leviviridae are the SSRNA virus with the characteristic’s resemblances to the polio viruses. Cystoviridae are the enveloped viruses with the segmented genome and character of infecting only the *Pseudomonas syringae* strain (Ackermann, 2009).

Filamentous phage or the Inoviridae family replicates by roiling circle mechanism and generate double stranded DNA intermediate. Lipothrixviridae virions are characterized by their structure of long rods with lipoprotein envelope whereas Rudiviridae virions are known for their straight rod like appearance without envelope and resemble the Tobacco

Mosaic virus. Pleomorphic phages have only one member and found in mycoplasma only. Plasmaviridae member excrete by budding and they possess envelope and nucleoprotein granule (without capsid). Fuselloviridae virion are spindle shaped without capsid. Salterpro virus constitute a floating genus with no assigned family. Guttaviridae Virions are droplet-shaped and have a unique beehive-like structure with a “beard” of fibers. Ampullaviridae family are characterized with their bottle shaped mantle, cone shaped inner body and a helical nucleocapsid. Bicaudaviridae are the largest of all the archaeal viruses found in hot springs (Ackermann, 2009).

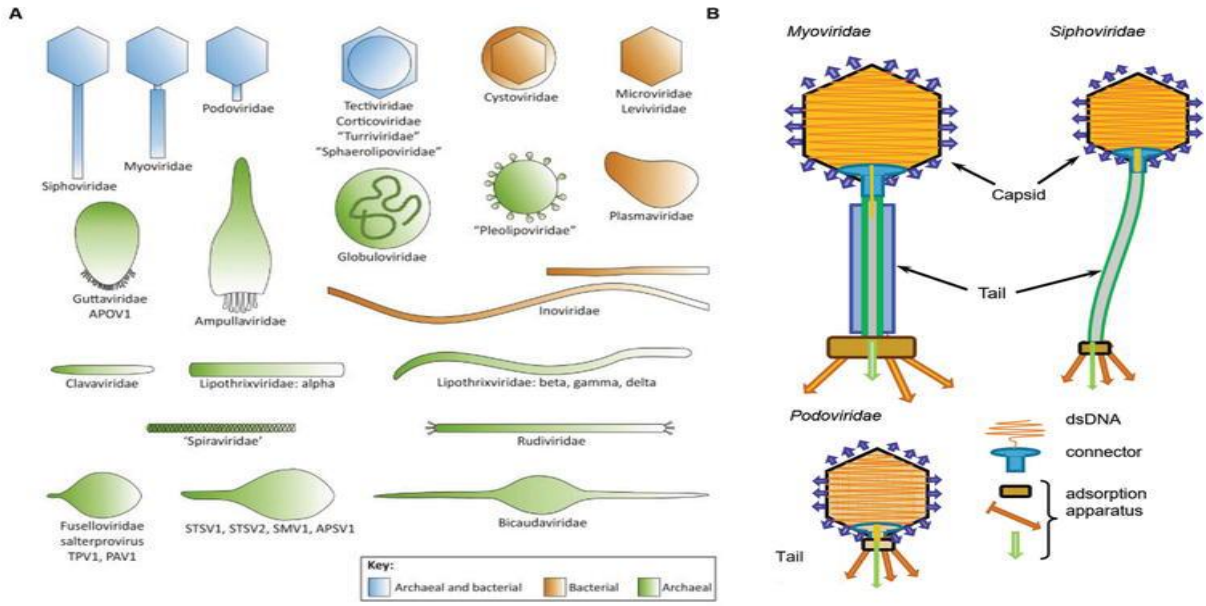


Figure 3: Classification of viruses (E. White & V. Orlova, 2020)

2.6 Biofilm in Pseudomonas aeruginosa

Biofilm is an architecture composed primarily of autogenic extracellular polymeric substances that act as scaffold to encase bacteria on surfaces, protect them from environmental stresses, obstructs phagocytosis, and thus confers the ability to colonize and persists for long period of time. It is one of the approaches taken by the bacteria during unfavorable living conditions such as temperature fluctuation, oxygen gradient, PH changes and nutrient unavailability. statistics shows that bacterial biofilm formation may be responsible for up to 80% of recurrent chronic and microbiological illness in humans (Liu *et al.,* 2022). Biofilm comprises of self- secreted matrix including water (97%), proteins (2%), DNA (1%), polysaccharides (1-2%) and RNA (1%). The three exopolysaccharides Psl

and Pel are in charge of creating the biofilm's matrix essential elements. They perform a variety of biological tasks, notably when it comes to the human immune system and protecting the bacterial cells from drugs. When compared to the identical strain cultivated in planktonic culture, bacteria that develop in biofilms frequently show a wide range of phenotypic variations. These variations include altered motility, occasionally higher level of extracellular polysaccharide synthesis and elevated antibiotic resistance (Tuon *et al.,* 2022). In comparison to their planktonic counterparts, bacteria within a biofilm are 1000 times more resistant to antibiotic treatments and can evade host immune response (Thi *et al.,* 2020)**.**This results from altered metabolic activity and protein synthesis, as well as failure of antibiotics to penetrate the complex polysaccharide matrix (Tuon *et al.,* 2022).

The complex process of bacterial biofilm formation can be broken down into five key stages. There are four phases of bacterial attachment (i) reversible attachment phase, where bacteria attach to surface aided by intermolecular force and hydrophobicity without specific purpose; (ii)irreversible attachment phase, where bacterial adhesins like fimbriae and lipopolysaccharides (LPS) are used to interact between bacterial cell and surfaces; (iii) production of the extracellular polymeric substances (EPS) by the resident bacterial cells; and (iv) biofilm maturation phase, during which bacterial cells produce and release signaling molecules to detect one another, promoting the development of microcolonies and biofilms; and (v) the dispersal/ detachment phase, during which the bacterial cells leave biofilm and resume an independent planktonic lifestyle.(Muhammad *et al.,* 2020).

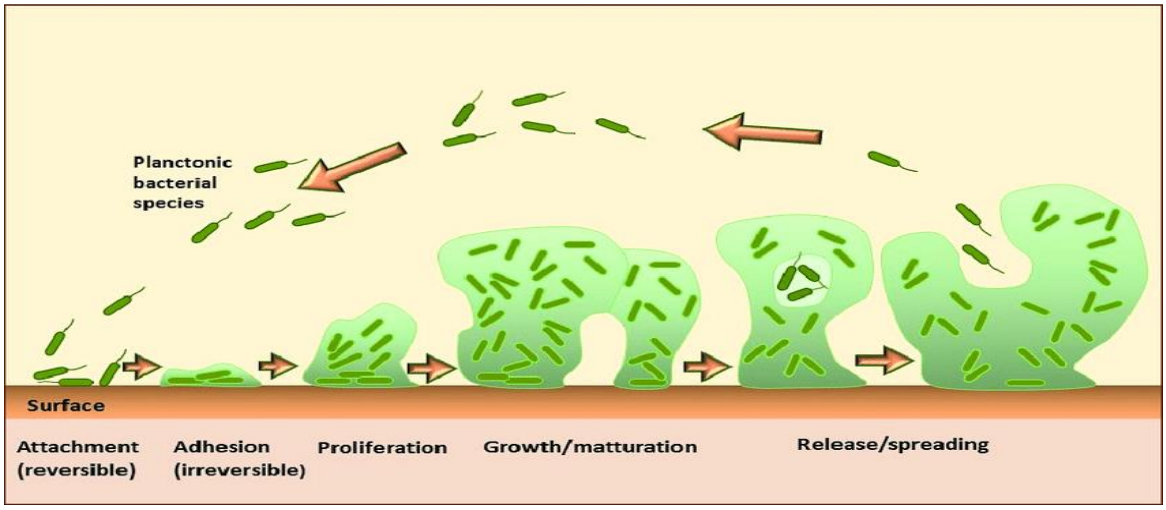


Figure 4: Process of biofilm formation (Rukavina & Vanić, 2016)

Pseudomonas aeruginosa is widely known for producing biofilm, making it an excellent model to study biofilm formation. In particular, the ability to build biofilm gives the bacteria a significant advantage when trying to infect susceptible hosts with illness like urinary tract infections, ventilator associated pneumonia, cystic fibrosis in lungs etc (Tuon *et al.,* 2022). Pseudomonas *aeruginosa* needs a strong biofilm environment to compete, survive and rule in the polymicrobial environment of lung cystic fibrosis. Pseudomonas aeruginosa can also successfully colonize the surfaces of medical devices such as urinary catheters, implants and contact lens and also the food related machinery such as mixing tanks, vat and tubing. For the development of efficient method to manage, prevent and most importantly eradicate the biofilm associated infections, a deeper understanding of the composition and structure of the biofilm as well as the molecular mechanism underlying the antimicrobial tolerance of the bacteria growing within the biofilm is essential (Thi *et al.,* 2020).

2.7 Bacteriophage to treat biofilms

Phage are the most ubiquitous organism in the nature commonly found in the places teeming with bacterial communities such as Wastewater, dirt and the guts of animals. Being, the natural predators of the bacteria, they can act on target bacterial cells and destroy biofilms through variety of mechanisms. The ability of the phage to encode range of enzymes including depolymerase and lysins, to breach the host bacteria's defensive barrier during infections is one of the most important mechanisms. (Liu *et al.,* 2022) For instance, Pires *et al.,* have identified that there are 160 putative depolymerases in 143 phages, which can be divided into two main classes: hydrolases, including sialidase, levosidase, xylosidase, glucanase, rhamnosidase as well as peptidase; and lyases, including hyaluronidase, alginate lyase as well as pectin/pectin lyase (Pires *et al.,* 2016)**.** These depolymerases are typically present in the form of free enzymes or tail spikes protein of phages, and they have the ability to recognize bind and specifically digest the exopolysaccharides (EPS) of the host bacterial cells in order to disrupt the biofilm structure and facilitate access to the deep cells within the inner biofilm layers. Lysins, also known as endolysins, are highly evolved peptidoglycan produced towards the end of lytic cycle of phage infection. They kill host cells by causing cell lysis and death by cleaving

peptidoglycan in the bacterial cell wall and releasing mature phage progenies from host cell (Liu *et al.,* 2022).

2.8 Phage Therapy

Phage treatment is an established method of treating bacterial illness that makes use of bacterial viruses. Typically, phage therapy uses naturally occurring phages to infect and kill bacteria at the infection site. Although phage therapies have been largely abandoned in western Nations, interest in them as alternatives to conventional antibiotic treatments has recently surged due to the development of drug -resistant to multi-drug resistant bacterial diseases (Durr & Leipzig, 2023). In addition, to the long running phage therapy programs in the republic of Georgia and Poland, established programs are now available in the United States, Belgium, France and Sweden. In order to use therapeutic uses, cooperative endeavors in Europe and Australia were effective in standardizing phage Therapy regimens. Recent news from UK also stated that compassionate use phage Therapy will there be taken into the account by National Health Services (Strathdee *et al.,* 2023).

Revitalization of the phage therapy in the recent years is due to the phage desirable properties over antibiotics some of which are mentioned below.

- Phage act as the effective bactericidal agent because bacteria that are once infected by obligately lytic phage cannot regain their vitality.
- Phages during the bacterial killing process are only able to multiply when suitable hosts are present and they themselves choose the phage dose depending on the relative bacterial densities.
- Phages are inherently nontoxic as they consist mostly nucleic acid and proteins however can interact with immune response resulting in harmful immune response which can be avoided with formulation of highly purified phage preparation in phage therapy.
- Phage only have a negligible effect on health protecting regular flora bacteria because of their host specificity, which can range from ability to infect only few bacterial species, more incredibly, the capability to infect more than one very closely related bacterial genus.

- The number of bacteria with which specialized phage resistance mechanism can occur is constrained by the comparatively limited host range exhibited by most phage.
- Specific antibiotic resistance mechanism does not interfere with phage resistance mechanism because the methods by which phage infect and kill is different from those of antibiotics. Hence, phage can be easily employed to treat the infections that are caused by multidrug resistant bacteria.
- It is simple to find phages against many harmful bacteria, frequently from sewage and other waste items that have high bacterial, phages that exhibit little or no toxicity and identified against majority of the targeted bacteria in contrast to antibiotics which can be harmful.
- Phage can be formulated in varieties of ways, just like antibiotics, including by mixing with specific antibiotics. In addition to being appropriate for the majority of administration routes, they are also adaptable in terms of application form including liquids, impregnated solids creams etc. additionally different phages are combined to form as “Cocktails” to enhance their quality which often leads to a broader spectrum of antibacterial activity overall. (Loc-Carrillo & Abedon, 2011)

An adequately defined phage is one of the most crucial factors influencing the success of phage Therapy. The absence of integrase, toxin related genes ad virulence gene in phage make it prime option for safe Phage therapy (Ong *et al.,* 2020). It is important to investigate the phage genetic makeup to determine whether any gene responsible for lysogeny (integrase and transposons), virulence, toxicity and antibiotic resistance are present. Large burst size, high adsorption rate, enhanced thermal stability, lytic cycle has made phage an attractive candidate for therapeutic application. (Piracha *et al.,* 2014) A phage used in phage therapy is usually selected on the basis of given characteristics including the ability to completely remove the target bacterium from a culture, host range, an additional phage virulence indicator transduction potential, toxic gene screening and obligatory lytic growth or absence of lysogenic capacity. (Hyman, 2019).

2.9 Current Studies and recent advancements in phage research

Currently, antibiotic resistance is a significant public health issue worldwide and if antibacterial tactics are not created right once, the consequences could be disastrous for global health (Pires *et al.,* 2020). A variety of bacterial illnesses, including *Staphylococcal* infections, dysentery and cholera were all experimentally treated with phage throughout the 1920s with different degree of effectiveness. To be used in these early applications, phage had to be separated from their environmental origins, grown on bacterial hosts and purified using available technologies. McCallin *et al*. stated that in order to establish phage therapy as a workable substitute for antibiotics clear efficacy information from randomized control clinical studies is required. He also highlighted the importance of "compassionate use of phage therapy "which refers to the use of experimental phage outside of clinical trials when all other approved therapeutic option has been explored for a patient (McCallin *et al.,* 2019).

Weber Dabrowska *et al.,* make use of the cocktail therapy through the combined (oral and topical) use of phage to treat the skin infections in burn patients caused by pathogenic bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and proteus. Bacteria were not found in the topical skin and 85% of these individuals made a full recovery. The clinical symptoms dramatically decreased even though germs were still typically found in the remaining individuals (Weber Dabrowska *et al.,* 2001).

New approaches in phage therapy typically includes bioengineered phages and purified phage lytic proteins to the repertoire of the potential phage treatments. New possibilities for the phage therapy have been opened up by the gene editing tool CRISPR/cas. Using a bioengineered phage to deliver a CRISPR/cas programmed to break antibiotic resistance genes and antibiotics resistance plasmids is pone examples of this. Hospital surfaces may be treated with these phages to lessen the occurrence and spread of antibiotic resistance genes. Though it is still in its infancy, the science of bioengineered phage will definitely produce many priceless inventions like this one (Lin *et al.,* 2017). Tom-Patterson, who was infected in Egypt by a toti- resistant strain of *Acinetobacter baumanii*, was brought back to the United States in a critical condition ana treated at university of California San Diego

with phages that cured him has become the subject of the many articles and the bestselling book in the United States (MacDonald, 2020).

Nowadays, combination therapy of the bacteriophage and antibiotics therapy are also coming into practice due to the better curative efficacy. Comeau et al. first coined the term bacteriophage synergy in 1958 to describe the sub -lethal concentration of antibiotics that aid in lytic bacteriophage replication and considerably enhance their antibacterial action, Additionally, there may be significant order impact when antibiotics and bacteriophage are used together, bacteriophage treatment carried out prior to the medication treatment results in maximum bacterial death (Ling *et al.,* 2022).

Recently, a number of encouraging clinical trials and case reports of tailored phage therapy have been released. In order to evaluate the effectiveness of a combination of anti- *Pseudomonas aeruginosa* phages for treating burn wound infections a randomized, multicenter, open label phase I/II clinical trial was conducted in Europe in 2015. Patient did benefit clinically from this treatment regimen even though natural lytic *anti Pseudomonas aeruginosa* bacteriophage reduced bacterial burden in burn wounds more slowly than usual therapy (Jault *et al.,* 2019).

2.10 Choosing highly efficient phages for phage Therapy

Not all phages can be the good therapeutic agent. For utilizing them as a promising alternative agent, we should consider the four different categories such as phage selection, phage host -range limitations, uniqueness of phage as pharmaceuticals and the unfamiliarity with phages (Loc-Carrillo & Abedon, 2011).

Good therapeutic phage should have high capacity for locating and killing the bacteria whereas the limited capacity to alter the environment in which they are administered. For application, the phage should be obligately lytic and stable at usual condition of storage and temperature, it should be subjected to sequencing for assessing the safety and the efficacy phage and also the presence of the undesirable gene such as toxin carrying gene. A phage that is obligately lytic means the phage is incapable of demonstrating the lysogenic cycle and is only released after the lysis of the cells. The use of temperate phages as treatment is difficult since they encode bacterial virulence factors such as toxins, and

also display the superinfection immunity which also renders the phage sensitive bacteria as phage insensitive (Loc-Carrillo & Abedon, 2011).

Phage characterization aims to remove those phages that exhibit low killing potential against target bacteria in addition to exclusion of temperate or the toxin carrying phages. Such low virulence can be the result of the inadequate adsorption abilities, a limited capacity to circumvent bacterial defenses or poor replication capabilities. Phages that have poor pharmacokinetics, poor adsorption, poor distribution and poor in-situ survival are less appropriate for the therapeutic uses. Additionally, the phages should have little capacity for the transduction the movement of the bacterial genes between the bacteria. Phage characterization aims to remove those phages that exhibit low killing potential against target bacteria in addition to exclusion of temperate or the toxin carrying phage (Loc-Carrillo & Abedon, 2011).

Moreover, adsorption kinetics, latent period and burst size are the few characteristics of lytic phage replication cycle that may be preferred for optimizing therapeutic phage killing. A powerfully adsorbing phages effectively optimizes fast and irreversible adsorption to sensitive cells to one or more binding sites on the cell surfaces. To obtain more round of infections per unit of time, latent period – the time between the phage adsorption and release of new cells by lysis should be decreased. Large burst size of phages is also advantageous because they produced up to millions of newer phage particles increasing the possibility that fewer phages will be able to dominate the target bacterial populations. Finally, phage stability is essential for the long-term preservation of phages and may improve virus longevity when given to the patient **(**Oromí-Bosch *et al.,* 2023).

2.11 Challenges of phage therapy

There are various challenges for the successful implication of phage therapy in this current era. some of which are mentioned below.

- **Quality and safety requirements:** One of the major challenges for the successful implication of phage therapy is its safety and quality control. The safety of phage preparation is crucial to the effectiveness of phage therapy which present issues in manufacturing and formulation. Phage would need to be generated in vast

quantities using Good Manufacturing practices (GMP) that should be certified by the regulatory bodies for the use in a wide range of applications (Pires *et al.,* 2020). However, according to Mutti and Corsini (2019), no precise rules have yet been developed specifically for the phage production (Mutti & Corsini, 2019). To address the problem, phage researchers have established some safety and quality standards for long lasting phage therapy products. avoiding phages that code for virulence, lysogeny, virulence factors or antibiotic is one of the pre-requisites. However, this may restrict the utility of phage therapy in some fastidious bacteria such as *Clostridium difficile,* for which no longer strictly virulent phages have been discovered. Endotoxin and other contaminants in phage preparations are ought to be prevented or kept to a minimum. To remove these harmful components, a number of purifications techniques have been created and optimized (Pires *et al.,* 2020).

- **Stability of the phage preparations:** A potential phage candidate for the therapy should have a good shelf life, which means it should be stored in a formulation that ensures activity without a noticeable drop in phage titer during processing and long - term storage such as its decrement might compromise the treatment success (Malik et al. 2017). The emergence of spontaneous mutations in phage stocks that have been kept for a long time have accumulated during phage manufacture, and manufacturing which might reduce viral fitness is another problem with phage stability. Although challenging, it would be useful to foresee phage evolution throughout the production in order to set up a manufacturing method that would reduce the rate of phage genome mutation (Garca et al. 2019).

- **Fast screening method for phages:** Large phage collection must be frequently screened so as to identify a phage that targets a specific strain because of the great selectivity of the phage activity. Double layer agar is the most widely used technique in order to identify phage activity against a strain. The DLA approach is not practical in therapeutic situation when prompt diagnosis is essential because findings may take 48 hrs. to manifest, depending on the growth rate of the specific strain to the target. To find the phage that can effectively infect the target

strain(s), it is preferable to use high throughput and screening techniques (Pires *et al.,* 2020).

Among few of the numerous techniques for the phage detection or the direct or indirect measurement, Real-time PCR is one of the quick and sensitive techniques for the identification of the infection by the detection of the rising phage concentrations. However, when testing vast and (rapidly developing phages collection against a target strain, qPCR require a set of primers and set of optimal conditions for (nearly) every phage which is neither high throughput nor practical (Martín *et al.,* 2008).

According to Pires et.al (2020), flow cytometry has also been utilized to identify phage infection by identifying cells with low-density cell walls. The approach is low- throughput and probably not applicable to all bacterial species and/or phages but it does enable quick and early detection of phage infection (Pires *et al.,* 2020).

By monitoring the release of the enzyme from the bacterial cells as a result of the phage induced cell-lysis, certain additional studies have indirectly discovered phage multiplication, adenylate kinase, adenosine 5' triphosphate and β- galactosidase are the examples of the intracellular enzyme that have been used to quantify the level of E. coli phage infection. When a certain substrate is broken down, an enzyme release is indicated by the production of a color or bioluminescence signal. These essays are extremely sensitive; even when starting with a little amount of phage, they can produce a detectable signal in about three hours. Though they might theoretically operate with any phage and are compatible with high throughput, these techniques need to be tailored (enzyme/ substrates) chosen for each species of bacteria (Guzmán Luna *et al.,* 2009).

- **Evolution of Bacterial resistance to phages:** Regarding phage therapy, one of the main concerns is the potential for the bacteriophage insensitive mutants (BIMs) to arise which could negatively impact therapy's efficacy. Phage resistant's mutants are common and nearly inevitable, according to the research conducted in recent years on the subject of bacterial resistant to phages. The following are some of the resistance mechanisms that bacteria uses to counter attack phage evasion: i) preventing phage adsorption by losing or changing bacterial receptors; ii) preventing phage DNA entry by superinfection exclusions systems, iii)

degrading phage DNA by CRISPR-CAS systems, restriction- modification systems and other related systems iv) using abortive infection systems that prevent phage replication, translation or transcription or iv) anti-phage system based on cyclic oligonucleotides (Pires *et al.,* 2020).

2.12 Application of the bacteriophage other than phage therapy

Despite the Western world's mistrust of the uses of phages for human medicine, bacteriophages are now being considered for alternative biocontrol methods.

- **Bacteriophage for the waste water treatment plant:** Bacteriophage have been considered as a reliable candidate for waste water treatment plant and signs of bacterial contamination. The use of bacteriophages as Pathogen tracers to enhance and monitor disinfection techniques is justified by the specificity of phages, which is why they are utilized as indicators. Instead, the direct application of bacteriophages for the decontamination has been suggested for the removal of the filamentous bacteria in Active sludge Process (ASP) system (Universal aerobic treatment widely used to remove the amount of organic matter by using microorganisms such as *Aeromonas spp*, *Pseudomonas spp* and *campylobacter spp*, as well as for the control of foam. However, a thorough knowledge of microbial population is necessary for the treatment of biocontrol plant. It is crucial to choose and use particular phages that can target undesirable pathogen of this.

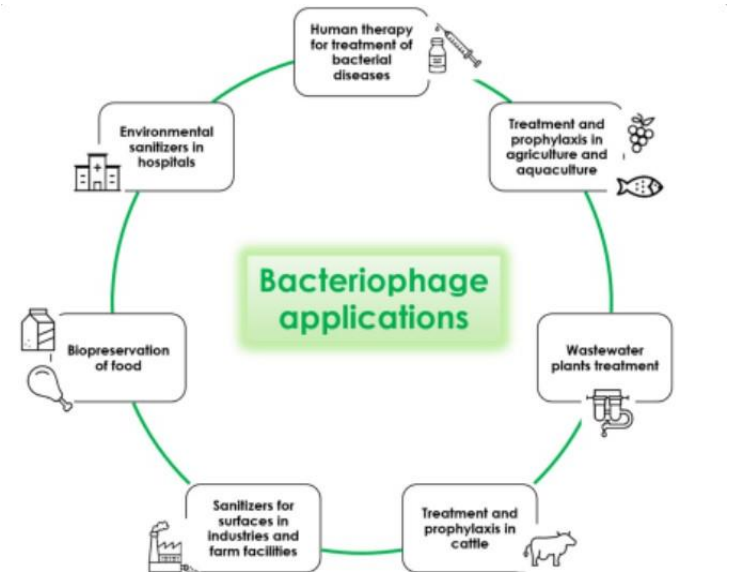


Figure 5: Application of bacteriophages (D'Accolti et al., 2021)

Due to the scale and the complexity of phage systems, a significant number of phages are required for the treatment to be effective analyzing polyvalent phages with broad host range could have unfavorable effects also by attacking beneficial bacteria (D'Accolti *et al.,* 2021).

- **Environmental sanitizers in Hospital:** Based on the potential of phages to decontaminate in various contexts, their application in sanitizers has also been speculated. One of the most common and significant complications for hospitalized patients in all healthcare facilities around the world is so called health care associated infections, which are primarily transmitted by persistent bacterial contamination of surfaces. On the basis of numerous studies, some of the pathogens associated with healthcare associated infections are *E.coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Surface contamination is difficult to remove because of the frequent recontamination brought on by the presence of colonized or infected patients. Additionally, chemicals have drawbacks such as temporary effects and potential to cause resistance to both chemical and antibiotics. The spread of pathogenic frequently drug-resistance bacteria is facilitated by the indiscriminate killing of microorganisms by disinfectants, which also target the potentially helpful bacteria that are typically sentinel on surfaces (D'Accolti *et al.,* 2021).
- **Bacteriophage application in aquaculture and agriculture:** In agriculture, plant infections that lower production and product quality can result in significant economic losses. Phages can be effective in controlling those pathogens. Recent years have seen the publication of a numerous studies demonstrating the utility of phages in agricultural setting with a particular emphasis on the ability of phage to manage bacterial plant diseases. Based on this a phage product (Agri phage created by company Omnilytics, Inc, Sandy, Utah) containing particular bacteriophages against *Xanthomonas campestris Pv. Vesicotaria* and *Pseudomonas syringae* bacteria responsible for bacterial spot and speck in peppers, has been readily available since 2005. Additionally, since 2019, a new product (Agriphage fire by Omnilytics Inc, Sandy Utah and Certis, USA, Columbia USA) has been certified for use against Fire blight on apple and pears (caused by

Erwinia amylovara). These products were demonstrated to greatly boost yields when compared to the common copper compounds used commercially and to prevent bacterial spot effectively (D'Accolti *et al.,* 2021).

Phage research has also been done on reducing infectious diseases in aquaculture and diseases linked to significant economic losses in addition to agriculture. The primary bacterial pathogen of the cultivated fish and shellfish are actually several species of the bacteria from the families *Lactococcus, Pseudomonas, Aeromonas* and *Vibrio*. These bacteria can cause sickness when consumed by human in the form of contaminated foods. The impact of phages on fish bacterial pathogens and their capacity to reduce bacterial infections in aquaculture especially when supported by MDR organisms have been the subject of several invitro and invivo investigations (D'Accolti *et al.,* 2021).

2.13 Next Generation Sequencing

Next generation sequencing, deep sequencing and massively parallel computing are all the related phrase that are used collectively for DNA sequencing technology that are used to transformed genomic research (Behjati & Tarpey, 2013). These DNA sequencing technologies have been around since the early 1970s, but at first their cost, complexity and need for the radioactive and the toxic reagents restricted their applications to the research settings. The main sequencing technology between the 1975 and 2005 was the Sanger sequencing method (also known as the first-generation technology) and is regarded as the gold standard for DNA sequencing. Sanger sequencing generates DNA segments that are somewhat lengthy (500-1000) bp and are of the excellent quality (Besser *et al.,* 2018).

The "next generation sequencing" revolution was launched in 2005 with the development of pyrosequencing technology by 454 life sciences without the requirement for cloning. This high throughput approach enabled the synthesis and detection of thousands to millions of a short sequencing read in a single machine run. Since then, a wide variety of additional NGS method have appeared, producing short (50-400) bp and long reads (1-100kb). This short-read technology that are frequently in use are termed as second-generation sequencing. In second generation sequencing also called next generation sequencing, each genome is sequenced numerous times in small, arbitrary sections to

obtain very massive databases, resulting in billions of nucleotide sequences throughout each run. The workflows use similar steps even though platforms have different biochemistry and arrays Which includes (i) DNA extraction (ii) library preparation includes shearing of DNA either mechanically or enzymatically, adding adaptors/barcode indexes, and amplification (iii) template preparation, done either by bridge amplification or emulsion PCR and iv) automated sequencing (Besser *et al.,* 2018).

According to the Wadapurkar & Vyas, 2018, currently different NGS platforms are running such as Roche 454 and Ion Torrent, Life technologies SOLID and Illumina HiSeq/MiSeq. Illumina HiSeq/MiSeq is one of the most affordable NGS technology developed till now (Wadapurkar & Vyas, 2018). With the development of these next generation technologies, the cost of the sequencing has drastically decreased. Over the preceding seventeen years, the cost of a DNA Sequence per mega base fell from approximately $10,000 to less than $0.1. Illumina's platforms are widely used among all the platforms in the market (Liu *et al.,* 2019).

Illumina technology, originally created by Solexa and Lynx therapeutics is by far the most significant player in the sequencing market. Illumina uses "sequencing by synthesis" technology. Based on a process called bridge – amplification, illumine sequencing uses DNA molecules roughly (500bp) as substrates for the repeated amplification synthesis reactions on a solid support (glass slides) that contain oligonucleotide sequences complementary to a ligated adapter. When the DNA is subjected to numerous rounds of amplification, the oligonucleotide in a slide is spaced in such a way that it forms clonal clusters with roughly 1000 copies of each oligonucleotide fragment. Millions of parallel clusters reactions can take place on a single glass slide. Exclusively modified nucleotides with fluorescent labels that corresponds to each of the four bases are introduced into the synthesis reaction and then detected. The nucleotides also serve as synthesis terminators for each reaction which are unblocked following the detection of subsequent round of synthesis, for at least 300 iterations the responses are repeated. Contrary to camera-based imaging, the use of fluorescence detection accelerates detection by direct imaging (Slatko *et al.,* 2018). The use of Illumina spans genomics, transcriptomics and epigenomics. The company provides a variety of adaptable equipment (MiniSeq, MiSeq,

NextSeq, HiSeq and HiSeqX) to fulfill various needs. With greater than 99% accuracy, the read length spans from 150 to 300bp (Del Vecchio *et al.,* 2017).

2.14 Bacteriophage world and Next generation sequencing

Phage genome have played a significant role throughout the development of sequencing technology. When the bacteriophage MS2's (3569 nucleotide) RNA genome was sequenced in 1976, it marked the beginning of the genomics. First whole genome of PhiX174 that infected *E. coli* was discovered by F. Sanger and colleagues in 1977 using their sequencing method. In the early 2000s, hardware platform for next generation sequencing were developed as a result of the continued development and automation of the sequencing technologies. Even now, PhiX174 is one of the most frequently used positive control for sequencing including NGS(Babkin & Tikunova, 2023). So, it can be said that the emergence of next generation technologies has created the exciting opportunities for whole genome sequencing of numerous organisms (Babkin & Tikunova, 2023).

The isolation, complete genome characterization and optimization of bacteriophages that can infect a wide range of hosts are some of the characteristics that have recently been examined in the modern phage research. It is preferable to complete the entire genome sequence for the phage application. To better understand phage-encoded proteins and biomolecules, particularly phage lytic enzymes (holin, lysin) that are involved in lysis and death of the bacterial cells, a new and reliable way is to sequence the entire genome sequence of a bacteriophage. With a genome size of 143,625 (bp) and endolysin activity against stationary phage cells as well as biofilm cell reduction, Melo et al. isolated and described LM12, a broad host range bacteriophage against *Staphylococcus aureus* **(**Melo *et al.,* 2018).

For regulatory approval of any phage or phage product application in the health care or food +industries, whole genome sequencing is now required. Even though, phage genomes are order of magnitudes smaller; phage whole genome sequencing has not been able to keep up the trend with the sequencing of the bacteria and other organism. The number of the published entire genome is only slowly rising. There are currently 600

Caudovirales genomes in the NCBI genome database, along with others unidentified phage genome (Klumpp *et al.,* 2012).

Phage genome sequencing is difficult due to various factors, including the difficulty of acquiring pure phage genomic material, biases in PCR amplification and complexity of the genetic materials, which includes repetition and methylated bases that are challenging to sequence and assemble.

2.15 Genome annotation

Through the use of Next Generation Sequencing (NGS), a massive amount of DNA sequence data from a wide variety of lifeforms has been produced in an incredibly short length of time. To learn more about the organism itself and evolution in general, information that is contained in each sequence must be extracted. Annotation is a technique for locating data that has been encoded in the several unique sequence patterns of four nucleotides (Ejigu & Jung, 2020). The detection of similarity between newly identified genes and proteins and previously annotated sequence is the basic for genome annotation. In general, genes predicted in newly sequenced genomes or metagenomes are translated and compared to reference database to detect homologues. A sufficiently long open reading Frame (ORF) is one of the most typical indicators of gene's presence especially in organisms where splicing is rare. It is common practice to record ORFs that are only as long as the shortest usual proteins (50-100 amino acids) in order to avoid missing possible relevant genes. It is commonly recognized that only a very small fraction of these is truly likely to be a protein coding region (FICKETT, 1995).

Proteins are encoded using Open Reading Frames (ORFs) which have start and stop codons. Most ORFs are rather short. It is well established that the distribution of total ORF length and GC content of the genome correlate, with stop codons typically being AT rich. Overlapping gene configuration in viruses have long been known. When two genes are integrated into one another or share a DNA locus, there are significant overlaps. Multiple overlapping genes that are incorporated in bacteriophages have been discovered. According to the alternative frame in which they appear, we were able to show that shorter than normal ORFs can also be meaningful in contrast to the typical approaches,

which only considers the largest ORF as the candidate for the protein coding genes (Mir *et al.,* 2012).

Despite the exponential increase of sequence databases and the continuous addition of annotation data to reference databases, large percentage of coding sequences in well studied sequences remains functionally unannotated. When predicted protein sequences cannot be functionally annotated, they are commonly labelled as “hypothetical proteins” (Lobb *et al.,* 2020). Unknown functional roles are present in considerable proportion of newly discovered genes, particularly when they are specific to particular lineage or Organism. Currently, categorized as “hypothetical”, these genes may support vital cell functions and one day may could be the focus of medical, diagnostic or pharmacogenomic research. A critical issue for the scientific community is connecting these newly discovered genes with a biological function that can be confirmed through experimental screens. In the absence of sequence or structural homology to known genes, must rely on cutting - edge biotechnological methods such as DNA- chips and protein-protein interaction as well as computational tools to assign potential role to these genes. As a result of advances in genomic sequencing, a staggering number of novel genes have been found, yet their biological functions remain unknown (Karaoz and colleagues (2004)).

The term complement refers to a group of genes that work together to produce effect that are distinct from one another. Two or more complements can work together to provide a single separate function. There are examples of separable functional domain in proteins connected to intragenic complementation. It was further implied that connection between polypeptide chains exist in proteins oligomeric complexes. Example have demonstrated that how a gene mutation might prevent another gene from functioning properly. This suggest that the outcome of two or more interacting gene is engaged in same function (Hays *et al.,* 1989).

CHAPTER- 3: METHODOLOGY

This study was carried out in Central Department of Biotechnology (CDBT), Institute of Science and Technology, Nepal.

3.1 Bacterial strain collection and preservation

Urinary bacterial samples used in this study were collected from Shukraraj Tropical and Infectious Disease Hospital and the samples were then stored at 4℃ in the bacteriophage laboratory at Central Department of Biotechnology. Out of these collected sample *Pseudomonas aeruginosa* 6661 was processed for further detailed characterization.

Table 1: List of collected bacterial samples and their codes

SN	Bacterial strains	Bacterial code
1	*Pseudomonas aeruginosa*	6661
2	*Pseudomonas aeruginosa*	209205
3	*Pseudomonas aeruginosa*	P3

3.1.1 Preparation of media and subculture of bacteria for Identification and Preservation

Nutrient agar (NA) as a general-purpose media was selected for the further sub culture of collected bacterial samples. Single isolated colonies were obtained by streaking method. The isolated colonies thus obtained was then further used for the preparation of glycerol stock and further identification of the bacteria.

3.1.2 Preparation of Glycerol stock

For long term preservation of the collected bacterial sample, Glycerol stock of the sample was prepared. Hence for this preparation a pure colony was cultured in LB broth and incubated at 37℃ for 24 hrs. One milliliter of overnight culture was taken in cryovial and centrifuged at 12000 rpm for 5 min. The supernatant was discarded and the pellet was

resuspended with 300µl of fresh LB broth. Then 700µl of 50% autoclaved glycerol was added and incubated at 37℃ for 3 hrs. After incubation the prepared tubes were then finally transferred to -80℃ for long term storage of the bacterial samples**.**

3.2 Identification of bacterial isolates

For the identification of bacterial sample, bacteria were cultured in the Nutrient Agar (NA), and cetrimide agar (selective medium for the *Pseudomonas isolates*) and morphological analysis was carried out. Gram staining and biochemical test was performed in order to identify the host bacterial strain. Catalase and oxidase test were also performed so as to confirm the bacteria.

Biochemical tests are based on the ability of microorganism to metabolize certain biomolecules resulting in the formation of useful product for themselves. SIM (Sulphur Indole Motility), MRVP (Methyl Red and Voges Proskauer), Citrate, TSIA (Triple sugar Iron Agar), OF (Oxidative Fermentative), and Urease test medium were done for the biochemical characterization of the bacteria. To carry out the test, respective test medium was prepared in the test tube and the bacteria were cultured in these medium. After overnight incubation of cultured bacteria at 37℃, the test tubes were observed for color change (MRVP, Citrate, TSIA, OF, Urease). SIM medium was observed for motility as well as H2S production.

3.2.1 Matrix Assisted Laser Desorption Ionization – Time of flight (MALDI-TOF) Analysis Assay

Bacterial identification in clinical laboratory in today 21st century is not only limited to phenotypic traditional phenotypic identification (morphological characteristics, staining and biochemical tests. Development of automated microbiology techniques (VITEK®, bioMe´rieux; BD Phoenix™ Automated Microbiology System; MicroScan®) has revolutionized the field of diagnostic microbiology. These automated systems combine the capabilities of mass spectroscopy and bioinformatics analysis to yield novel methodology for both bacterial and fungal identification. (Patel, 2013). MALDI-TOF mass spectroscopy for the bacterial identification starts with the isolation of single pure colony of the bacteria in the culture plate. Thus, isolated colony is then picked to a spot on

MALDI- TOF mass spectrometry plate containing a defined number of spot area. A thin layer of matrix (e.g.: 1-2µl of α- cyano-4hydroxy-cinnamic acid diluted in 50% acetonitrile solution and 2.5% trifluoracetic acid) is placed over organism on the plate, and the plate is dried usually for 5 min or less. On different spots, identification, calibration standard(s), and reagent contamination control are evaluated. The MALDI-TOF mass spectrometer's chamber is then subsequently filled with the plate. The procedure is automated with the mass spectrum of the test isolate created and evaluated against a database of reference spectra. Software evaluates the test isolates spectrum similarity to the known spectra kept in the system library, producing a ranking (for example percentage or score), list of potential organisms and a degree of identification assurance. The software performs this operation in a manner similar to doing a google search, thus the technologist is not necessary to undertake a formal mass spectrum interpretation. It is possible to test one or several isolates at once. The turnaround time for analysis is three minute per isolation. Comparing this technology to more conventional biochemical based automated instruments it is more "green" technology because of the requirement of less disposables and reagents and the plates are also reusable (Patel, 2013).

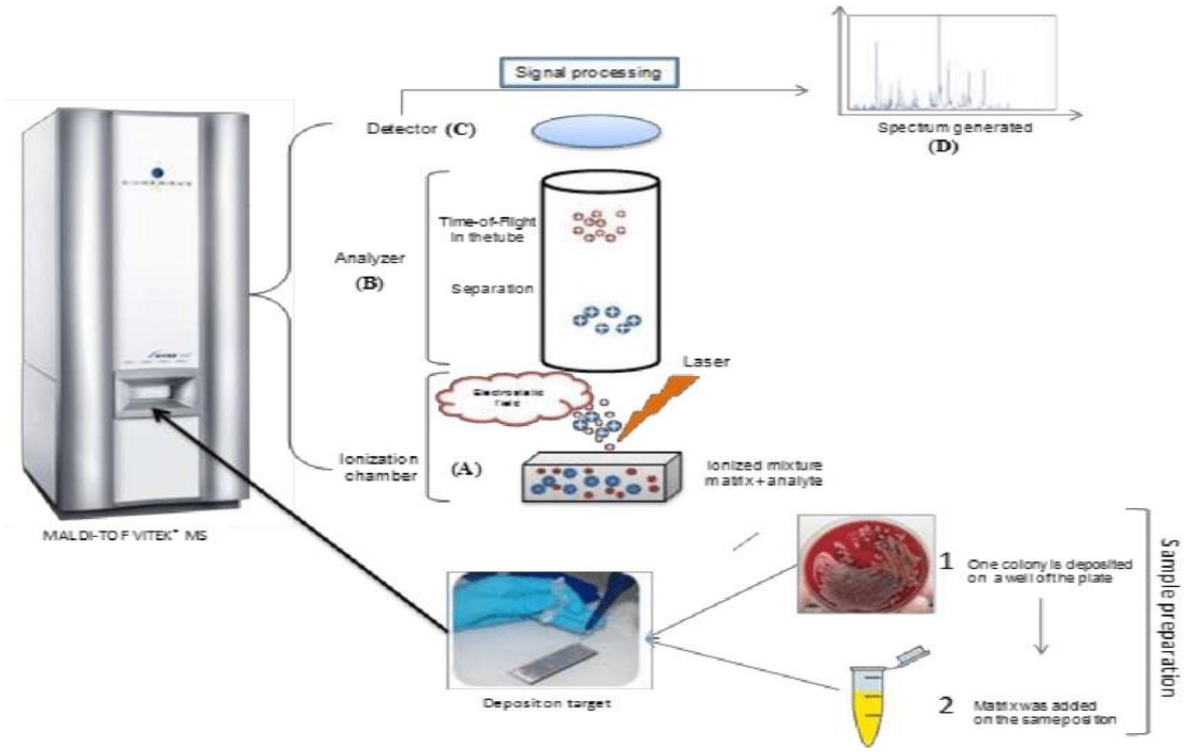


Figure 6: MALDI TOF mass spectrometry analysis for the identification of bacteria (Lo *et al.,* 2017)

3.3 Antibiotic Susceptibility Test (AST)

Antibiotic susceptibility pattern of the bacteria *(P. aeruginosa)* was analyzed using Kirby Bauer disc diffusion technique. All antibiotics used in this study were further classified as Sensitive, Resistant and Intermediate according to the Clinical and Laboratory Standard Institute (CLSI) guideline (2020).

Lawn culture of Log phase bacteria was prepared on Muller Hinton Agar (MHA) using sterile cotton swab and left for air drying. After air drying, antibiotic discs were placed on the lawn culture and incubated at 37℃ for 24 hrs. After incubation, zone of inhibition against confluent lawn of bacterial growth was observed and measured using calibrated ruler.

Table 2: Antibiotics and their code used for AST

SN	Antibiotics used	Code	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	Polymyxin B	PB	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	Amikacin	AK	30 mcg
15	Cefixime	CFM	5 mcg
16	Piperacillin	PIP	100/10 mcg

3.3.1 For resistance detection (ESBL AND MBL)

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

Table 4: Metallobeta lactamase detection

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

3.3.2 Antibiotic susceptibility test using vitek compact system 2

Most clinical microbiology laboratory in the United States employ commercially available automated methods for bacterial identification and susceptibility testing. They are frequently favored method over the more labor- intensive clinical and laboratory standard institute reference method of Broth Microdilution (BMD) and disk diffusion (DD) due to their simplicity of use and affordability (Bobenchik *et al.,* 2014).

The Vitek2 compact system (bioMierieux) is a highly automated system that makes use of very small plastic reagent cards (about the size of a credit card) which are 64 well formatted and contain microliter quantities of test media and antibiotics. Throughout a brief incubation time, the vitek 2 continuously monitors the bacterial growth using turbidimetry. 30-240 tests may be run simultaneously on the instrument depending on the configuration. The susceptibility card enables testing of typical, rapidly growing gram positive and gram-negative aerobic bacteria as well as *Streptococcus pneumoniae* during a period of 4 to 10 hours. Some laboratories continue to employ an older, less automated vitek 1 system. The system is more constrained with a 45- well card and excludes *Streptococcus pneumoniae* **(Jorgensen & Ferraro, 2009).**

3.4 Genomic DNA extraction of *Pseudomonas aeruginosa*

Genomic DNA extraction of bacterial strain (*Pseudomonas aeruginosa*) was carried out by using CTAB method. In this method, 1 ml of overnight bacterial culture was taken in Eppendorf tube. The culture was then centrifuged at 13000 rpm for 5 minutes. The cells were then harvested by discarding the supernatant. To the remaining pelleted cells 567µl of TE buffer was added and mixed gently by repeated pipetting or vertexing for resuspension. Then 30µl of 10% SDS and 3µl of proteinase K was added to the resuspended cells, mixed gently and incubated at 37℃ for about 1 hour. After incubation, 100µl of 5M NaCl followed by 80µl of CTAB/ NaCl (0.7m NaCl, 10% CTAB) was added and well mixed. Thus, prepared solution was incubated at 65℃ for 10 minutes. After incubation, equal volume of Chloroform: Isoamyl alcohol (24:1) was added to the solution, well mixed and then centrifuged at 13000 rpm for 5 min. After centrifugation, upper aqueous layer was transferred to the new Eppendorf tube and 600µl of isopropanol was added and mixed gently till the DNA was precipitated and centrifuged at 13000 rpm for 5 minutes. The isopropanol was removed after centrifugation and 1ml of 70% ethanol was added so as to wash DNA and centrifuged for 5 minutes. Ethanol was discarded after the centrifugation process and it was left for air drying. After the drying process, DNA was then resuspended in 50 µl of TE buffer and stored at 4℃. Thus, extracted DNA was then subjected to Nanodrop for determining the concentration of DNA in the sample for purity check.

3.5 Amplification of 16srRNA gene of *Pseudomonas aeruginosa* (6661)

16srRNA gene simplification was carried out for the molecular confirmation of the samples to determine whether they are bacteria or not. Pre-optimized PCR conditions and reaction volume were employed for this amplification.

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	Temperature	Time	Cycle
1	Step 1: Initial Denaturation	94°C	3 min	1
2	Step 2: Denaturation	94°C	30 sec	
	Annealing	60°C	40 sec	28
	Extension	72°C	1 min	
3	Step 3: Final extension	72°C	7 min	1
4	Step 4: Final hold	4°C	∞	

PCR reaction mixture was prepared in PCR tube and was subjected to PCR amplification. 5µl of the amplified product was analyzed by 1 % agarose gel electrophoresis on Tris Acetate EDTA buffer containing 0.5µg/ml Ethidium bromide as a DNA staining solution. DNA band on a gel was viewed on a UV transilluminator and the image was captured with the help of Geldoc system. Thus, prepared PCR product was then sent to Center for Molecular Dynamics Nepal (CMDN) for the 16S rRNA sequencing. Several bioinformatics tools were used to evaluate the sequence that was obtained from the lab. Chromas was used to analyze the chromatogram file, and a direct BLAST search was conducted to determine the degree of sequence similarity between our sequences and the sequences in the nucleotide database.

3.5 Water Sample Collection for Bacteriophage Isolation

Waste water samples were collected from Balkhu river among different river of Kathmandu valley. The sample was collected from stagnant rather than free flowing running water. Water samples were collected in 50ml sterile Falcon tubes and transported to the laboratory of Central Department of Biotechnology (CDBT), TU. The sample was then centrifuged at 4000rpm for 30 min in order to remove unwanted waste particles or cell debris. After that the supernatant was transferred to another falcon tubes without disturbing the cell debris. The supernatant was then subjected to filtration with the aid of 0.20 µm syringe filter (PES Filter Media, Whatman ™) and the filtrate (free of unwanted contamination and cell debris) was then collected in a sterile falcon tube. Thus, prepared sample was ready to use as a phage source in our further experiment.

Figure 7: Water sample collection site, Balkhu, Kathmandu

3.5.1 Bacteriophage Isolation

Double Layer Agar Assay (DLAA) was used as the standard method for the isolation of bacteriophages (Kropinski *et al.,* 2009). The double agar overlay plaque assay is also known as the "soft agar overlay," "double agar layer" or "double layer" method of plaque assay. In this assay, a hard agar plate of Tryptic Soya Agar (TSA) (1.5%) was prepared. The hard agar supports the growth of host bacterium and form the base layer of the plate. Also, soft TSA agar (0.7%) was used which was generally poured on the top of hard agar. To the 1 ml of processed/filtered water sample ,100µl of log phase host bacterium was added and left for attachment in sterile falcon tube. Then after few minutes 3ml of soft molten agar was added to the tube. The mixture was then poured into the already prepared Tryptone Soya plates (1.5%), swirled gently and left to solidify. After solidification of the media, the plates were then incubated at 37°Cfor 24 hrs. After the incubation procedure the plates were observed for the formation of the plaques. Plaques were further characterized on the basis of shape, size and turbidity. Plaque showing clear lysis was further used for processing (Kropinski *et al.,* 2009).

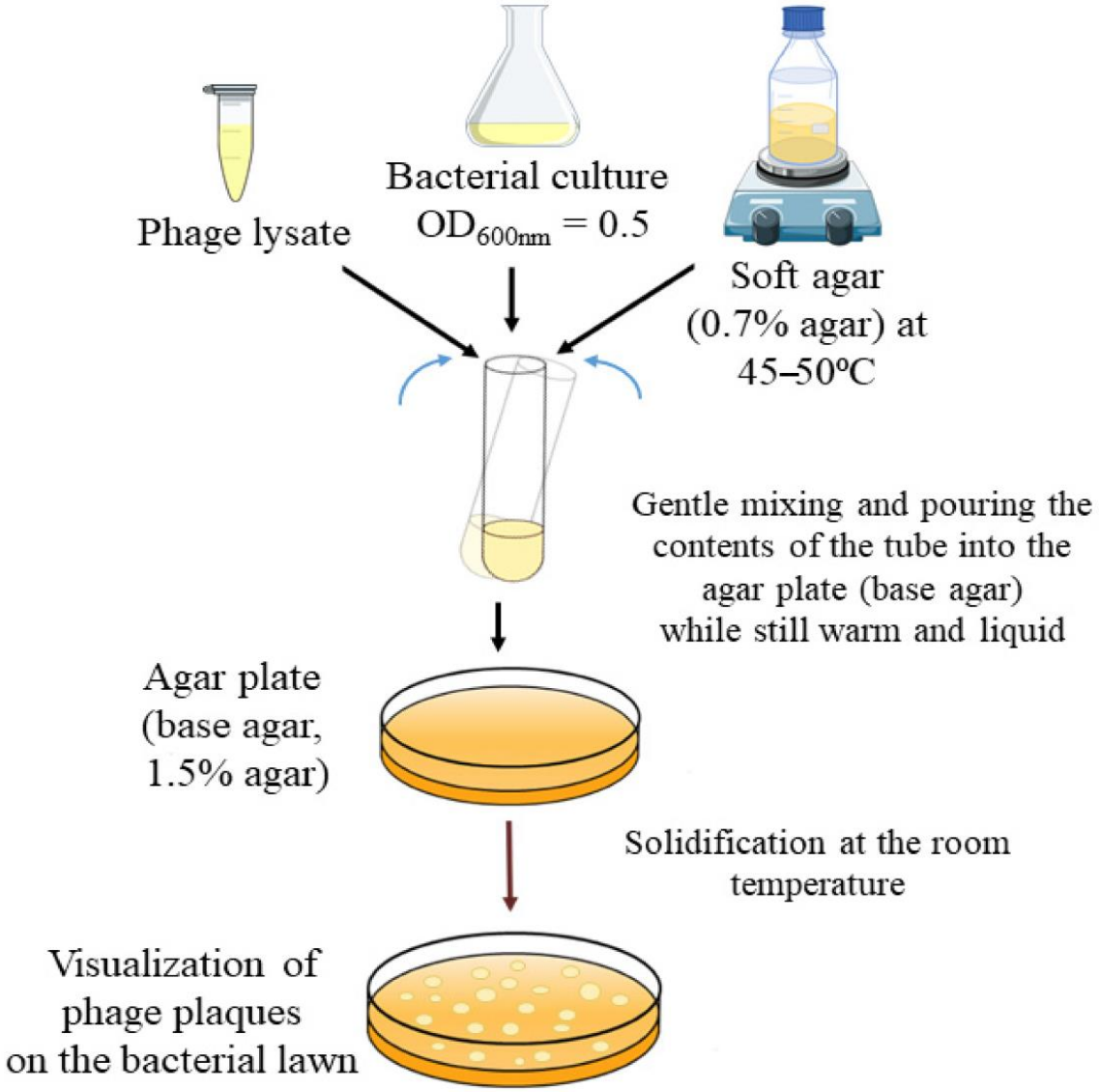


Figure 8: DLAA technique for phage isolation and culture (Stachurska et al., 2021)

3.5.2 Bacteriophage Purification

Bacteriophage purification was done by continuous streaking method. Plaque obtained from the isolated plates may be of different types. In order to isolate a pure phage, single isolated plaque showing clear lysis was touched in the middle portion and further sub cultured in another TSA plate in a similar manner to bacterial streaking protocol. Firstly, a primary inoculum is made with the loop touching the middle portion of the plaque. The primary inoculum site and the ending (diluted) portion of the streaking zone was marked. Then, 100µl of the log phage host bacteria was mixed with 3ml of soft agar (0.7% TSA) and poured through the diluted portion of the streaking zone. The mixture was then allowed

to spread across the plate from the diluted portion to the concentrated area of the plate uniformly which was then left to solidify. After solidification, the plate was then incubated at 37 ℃ for 24 hrs. After overnight incubation, clear plaques on the streaking line were observed. Three rounds of continuous streaking were carried out so as to obtain the pure phage showing single plaque morphology.

3.5.3 Bacteriophage stock/lysate Preparation

Bacteriophage lysate solution was prepared by amplifying phage in several plates. Amplification of phage was done through streaking of the single type of plaque in multiple plates. Altogether phage was streaked in five different TSA plates in a similar manner as done in the bacteriophage purification protocol. After incubation and the visualization of the plaques, then about 5 ml of Sodium Magnesium buffer (SM) was poured on all of the streaked plates containing plaques. SM buffer aids in the adsorption and detachment of phage particles from the media. Subsequently, the plates were then rotated in shaker at about 100 rpm for about one hour. In the succeeding step, soft agar in the plates containing plaques were scraped out and SM buffer of the plates were then poured on the sterile falcon tubes and centrifuged at 4000 rpm for about 30 minutes. Centrifugation steps helps to settle down all the debris present in the solution. The supernatant after centrifugation steps was filtered with the aid of the syringe filter having pore size of 0.22 µm. This eliminates all of the bacterial debris from the solution. Thus, prepared phage lysate solution was ready to use for the characterization. For future use the lysate solution was stored at around 4℃. For more stock preparation all of the above steps were repeated**.**

3.5.4 Phage Titer determination assay: Spot Assay

Phage titer of the stock solution was determined through a process called spot assay. Spot assay forms the basis for determining the concentration of phage particles in a pure phage stock solution. For spot assay, Bacterial lawn culture was prepared in TSA plate by mixing 100µl of log phase bacteria and 3ml of TSA (soft agar) media. Phage stock solution was then diluted from 10^{-1} to 10^{-15}. For the dilution 100µl of phage stock solution was mixed with 900µl of SM buffer taken in a 1.5ml Eppendorf tube. Then 100µl of diluent was withdrawn from the first tube and mixed with the 900µl SM buffer of the second tube.

The process was repeated until the dilution reach 10^{-15} with the changing of pipette tips for every single dilution. Grids were drawn on the outward side of the plate marking the space for each dilution. Then around 4.5µl of the respective prepared dilution was transferred in the plate containing marked grid for each dilution. The droplet of respective diluent was then allowed to soak into the agar and the plate was incubated at 37℃ for 24 hrs. After 24hrs, clear spot of the plaques was observed.

For determining the concentration of the phage stock solution, the last three dilution which showed clear lysis on the spot assay were used. Double layer agar assay was done for those three-dilution taken from the spot assay. In this procedure, 1ml of serially diluted phage solution was mixed with 100µl of log phase host bacteria and let for attachment for about 5min. To the mixture 3ml of soft agar was added and overlaid on the TSA plate. The plates were then incubated at 37℃ for 24 hrs. After overnight incubation, the plates were then observed for the countable number of single type of plaques for the phage titer determination. The plaque number on each plate were counted and plaque forming unit per milliliter of the sample was calculated by using the following formulae. This gives the phage stock concentration.

$$\text{PFU/ml} = \frac{\text{number of plaques observed}}{\text{Volume of sample} \times \text{dilution of phage}}$$

3.6 Characterization of phages

The isolated bacteriophage which shows consistent bacterial lysis was then subjected to further physiochemical characterization. The characterization of bacteriophage was done by adsorption assay, growth curve analysis, assessment of stability against temperature and pH. Protein profiling of the isolated phage by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE).

3.6.1 Host Range Analysis

Host range of a bacteriophage is defined as the ability of the virus to lyse particular bacterial genera, species and strains and is considered one of the distinguishing biological characteristics of a particular bacterial virus. (Kutter, 2009) Inter and intra specific host range analysis was done by spot assay along with DLAA method. For intra specific host range five available strain of *Pseudomonas aeruginosa* which were collected from

Shukraraj Tropical and Infectious Disease Hospital (STIDH) were used. For spot assay all the collected *Pseudomonas* strains were grown aseptically to the active log phase. 100µl of bacterial culture was then mixed with 3ml of soft (TSA) agar and poured on properly labelled Tryptic Soya Agar plate. Petri plates were then swirled gently so as to uniformly distribute the agar all over the plates and left for few minutes to solidify. Isolated *Pseudomonas* phage and one cocktail of *Pseudomonas* which was previously isolated in the department was used to analyze the host range. SM buffer was used as a negative control. For this separate grid were marked on the bottom side of the plate. To the solidified plate about 4.5 µl of the high titer phage stock was correspondingly placed on the marked area and left for about few minutes for adsorption. The plates were incubated for 24 hrs. at 37℃. After overnight incubation, plates were then checked for the presence of clear spot of lysis. Double layer agar assay was done for those host strain which shows intra host range during the spot assay and the number of plaques were counted after DLAA to calculate the Efficiency of Plating. (EOP).

For interspecies host range analysis, different strain of the bacteria *Klebsiella, Acinetobacter, Staphylococcus* and *Escherichia* species were collected and revived. Intraspecies host range analysis was also carried out by using the spot assay following the similar protocol as explained above in the intra specific host range analysis. Isolated phage and one cocktail of Pseudomonas were tested. Then, Double layer agar assay for phage showing interspecies Host range analysis so as to assess the Efficiency of Plating.

3.6.2 One Step Growth Curve analysis of Phage

One step growth curve analysis of phage was carried out for determining the burst size and latent period of the virus. Bacteria follows 5 steps for the completion of its life cycle. Bacteria normally takes one hour for the completion of its life cycle. The protocol of Adams and Wassermann, 1956 was employed with some modification so as to analyze the growth curve of the virus (Adams & Wassermann, 1956).

For the procedure, seven different sterile Eppendorf tubes were taken and labelled as 5min, 10min, 20min, 30min, 40min, 50min and 60min respectively. To the labelled tube 1000µl of high titer phage stock 10^9 pfu/ml was transferred on each tube and 100µl of log phase host *Pseudomonas* (OD0.4) was mixed on each seven different tube and incubated

in 37℃ incubator. After 5min incubation, the tube in which 5 min was labelled was taken out from the incubator and centrifuged at 12000 rpm for 5 min. Supernatant was discarded so as to remove the unabsorbed phage particle and pellet was resuspended with 100µl of SM buffer. Then 3ml of soft agar was mixed to the dissolving solution and overlaid on the TSA plate following the DLAA technique. The same workflow was repeated for other 6 different tubes in their respective time and the plates were left to solidify. After solidification the plates were incubated at 37℃ for 24 hrs. and the plaques were counted and expressed in pfu/ml (Adams & Wassermann, 1956).

Graph was plotted with pfu/ml against time of incubation. From graph, the latent period of the virus was determined as the time period between the adsorption of phage and the initial rise in plaque number. Also, the burst size of the virus was determined by dividing the average pfu/ infected cell in the post- rise period of the growth curve by the average of pfu/ infected cell in the pre- rise period of the growth curve (Ellis & Delbrück, 1939).

3.6.3 Stability of phage against pH

Bacteriophage stability test against pH was done by adjusting the pH of fresh 10 ml Luria Bertani (LB) broth in the range of pH (2-12). Desired acidic pH of the LB broth was maintained by adding 1M HCL dropwise whereas the desired basic pH was adjusted with the aid of 1M NaOH. All of the pH adjusted broth were then autoclaved. Adjacently on the other side 10^{-9} phage dilution was prepared. Then ,700µl of different pH adjusted media was aliquoted in respective well labelled Eppendorf tube. To those respective tube, 300µl of phage suspension were then added and mixed properly. The mixed solution is then incubated for 1 hour at room temperature. After incubation 100µl of log phage host bacteria was mixed with all of the pH treated phages and DLAA was done for the tubes of respective pH. The plates were then incubated at 37℃ for 24hrs. After incubation no of plaques were counted and phages were expressed as pfu/ml and plotted against the pH values.

3.6.4 Stability of phage against temperature

Bacteriophage stability test against temperature was done by exposing the phage against different temperature and time. Firstly, for the assessment of stability of phage against the temperature, phage stock was diluted to different dilution in SM buffer and 10-9

phage diluted solution was taken at which countable plaques of phage were found. 1ml of 10^{-9} diluted phage solution were kept in different sterile Eppendorf tubes and were exposed to temperature of 37℃, 40℃,50℃,60℃ and70℃ for different time 10min, 20min, 30min,40min, 50min and 60min. temperature exposure to phage was maintained in dry heat equipment such as incubator, heating mantle and hot air oven. After incubation in different time and temperature the tubes were withdrawn from the temperature at their respective time. Heat treated phage solution were then mixed with 100µl of log phage host *Pseudomonas* and DLAA assay was performed for the all of the time at all of the temperatures. The plates were then incubated at 37℃ for 24 hrs. After overnight incubation the no plaques were counted and expressed as pfu/ml. Then graph was plotted Pfu/ml against temperature values.

3.6.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis of protein

Phage sample for SDS – PAGE analysis was prepared by heating method. 10µl of phage solution was mixed with 15µl of the protein 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 was heated at 95℃ for10 min. Thus, prepared sample was then ready for gel run.

For SDS- page, two gel were prepared, resolving gel (12%) containing acrylamide bisacrylamide in the ratio (29:1) and other 5% stacking gel containing acrylamide bisacrylamide (29:1). The gel was prepared in glass plate cascade and properly fitted with the electrophoresis tank. 1X Tris-glycine (running buffer) (39mM Tris, 48Mm glycine and 0.1% SDS) was then added to the tank. Then 10µl prepared sample and 5µl protein marker were loaded on the well and the gel was run at 120 volts for 2 hours. After completion of the electrophoresis, the gel is removed from the tank and stained with 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) to remove the excess stain. The gel was then visualized for the presence of different phage protein band and compared against the Genii protein molecular weight marker-Broad range (50 lanes, 3.5KDa-205KDa) so as to determine the size of the protein separated.

3.7 Biofilm Production

The quantitative test described by Christensen's et al. is considered as the gold standard method for the determination of biofilm production (O'Toole, 2011). Bacteria *Pseudomonas aeruginosa* was inoculated in LB broth of about 5ml overnight at about 37℃. The bacteria were then diluted (1:100) with fresh medium so as the bacteria reaches the optical density of about 0.6. Then 200µl of fresh diluted culture was then added to the 96 well polystyrene plates (Nunclon™ Delta Surface) in triplicate manner. Also, 200µl of fresh LB medium was used as control and 200µl of other biofilm positive species. (*Klebsiella pneumoniae)* was used as positive control. The microtiter plate containing the bacterial cultures was then incubated overnight at 37℃. After incubation content of each well were removed by gentle tapping. The wells were then washed with 200µl of 1X phosphate buffered saline (PBS) four times for the removal of the free-floating bacteria. Adherent biofilm culture was then fixed with 200µl of 2% Sodium Acetate and left for about 30 min at 60℃. The adherent biofilm cells were then stained with 200µl of 0.1% crystal violet solution and left for about 10 min for staining. Excess stain was then washed with deionized water. After washing, the plate was air dried completely and 95% ethanol was added to all the well containing biofilm of sample bacteria, Negative control (LB medium), and positive control (*Klebsiella pneumoniae*). Then optical density of stained adherent biofilm was taken at wavelength of 620nm using Elisa plate reader. The experiment was performed in triplicate manner and optical density cut off value was determined. On the basis of those cutoff value bacteria was then classified into moderate, strong and weak biofilm producers.

ODc = Average OD of negative control + 3 X SD of Negative control

OD > 4ODc = Strong Biofilm Producer

2 ODc < OD ≤ 4 ODc =Moderate Biofilm Producer

ODc < OD ≤ 2 ODc = Weak Biofilm Producer

3.8 Biofilm disruption

Disruption of the biofilm was carried out using the method described by Forti et al. 2018. Bacteria were grown overnight at about 37℃in Luria – Bertani broth. The bacterial broth

was then diluted with fresh culture medium (LB) broth to obtain the optical density of 1. 200 µl of the diluted broth was then inoculated into polystyrene plate in triplicate manner. The plate was then incubated at 37℃ for 24 hrs.' for the biofilm formation. After incubation, broth was removed from the well and the well were washed with 200µl LB broth for two times. Then 200µl of phage lysate was added. It was then incubated for 4 hours at 37℃. After incubation, contents of each well were removed by gentle tapping. The wells were then washed with 200µl of 1x phosphate buffered saline (PBS) for two times. Bacteria adhering to the wall of polystyrene plates were then fixed with 2%sodium acetate for 30 minutes at 60℃. The content of each well was then discarded and adhered cells in the well were stained with 200µl of 0.1% crystal violet solution for 10 minutes. Excess of the stain was removed by washing with deionized water. Plates were kept for drying. After drying 95% ethanol was added to each well and optical density of bacteria was measured using Elisa plate reader. LB was used as negative control (Forti *et al.,* 2018).

3.9 Bacteriophage DNA Extraction

Norgen Biotek Corp Phage DNA isolation kit was used to isolate the phage DNA. Firstly ,1 ml of phage lysate was added to the clean and sterile Eppendorf tube. To the tube, 500µl of lysis buffer B was added and vortexed vigorously for about 10 seconds. The resulting solution was then incubated at 65℃ for 15 minutes. During incubation, the lysate solution was thoroughly mixed by the inversion of the tube 2-3 times. Then, 320µl of isopropanol was added and again vortexed briefly. Following that step, a spin column was assembled to one of the collection tubes provided in the kit and 650µl of the above lysate was applied to a column. It was then centrifuged at 8000 RPM for 1 minute. The flow through was then discarded. The column and the collection tube were then reassembled. The above process (from spin column assembly) was repeated till the entire lysate passed through the column. 400 microliter of wash solution A was then added to the column and centrifuged at 8000 RPM for 1 minute. The flow through was discarded and the spin column was reassembled with its collection tube. The column was then again washed for a second and third time by wash solution A by above mentioned procedure. After that step the column was spin for 2 minutes for drying at 14000 RPM. The collection tube was then discarded. Again, the column was placed into an elution tube provided with the kit and 75µl of elution buffer B was added in the column. it was then centrifuged at 8000 RPM for 1

minute. Finally, the phage DNA was collected in elution tube. The DNA sample thus collected was then stored at -20^{o}C.

3.10 Electrophoresis of phage DNA

For the electrophoresis of phage DNA, 1 percent of agarose gel was prepared. 0.5 gram of agarose powder was mixed with 50 ml of 1X TAE buffer. The mixture was then heated till boiling so as to completely dissolve the agarose powder. Then, 0.2 microliter of EtBr was added to the solution and mixed thoroughly. The gel was then poured into the casting tray and was allowed to solidify. 5 microliter of phage DNA was mixed with 1 microliter of loading dye and resulting mixture was loaded into the well. DNA ladder (Fermentas O' Gene Rule 1 KB) was also run along with the sample. Gel electrophoresis was performed at 70 volts for about one hour. After completion of electrophoresis, the gel was observed under UV transilluminator for visualization of the DNA band.

3.11 Transmission electron microscopy of phage

Pseudomonas phage (6661) which was characterized physiochemically was selected for Transmission electron microscopy (TEM). Phage titer of appropriate concentration that is (10^8-10^9) was prepared and transported to All India Institute of medical science Hospital-New Delhi (AIIMS), India in a cold chain. Thus, prepared phage sample were fixed with fixatives (2.5% glutaraldehyde and 2% Paraformaldehyde) prepared in 0.7M Sodium phosphate buffer (pH 7.2). An equal volume of fixative and Phage lysate were added, stirred and allowed to sit overnight for fixation. The fixed phages were centrifuged at high rate of speed (35,000 g) for three hours on the next day. The phage pellet was placed on a distinct 300 mesh carbon- coated copper grid, which was then flooded with 2% (w/v) Uranyl acetate (pH 4.5). The excess stain was removed with blotting paper after two minutes. After drying, the copper grid was investigated using a Transmission electron microscope. The micrograph thus obtained allowed us to observe the morphology of Phage.

3.12 Whole Genome sequencing of phage DNA

Whole genome sequencing of the phage DNA was performed following the confirmation of phage DNA by gel electrophoresis**.** phage DNA was delivered to the Center for Molecular Diagnosis (CMDN), Thapathali, Kathmandu for sequencing.

Prior to the sequencing procedure, Library preparation must be completed. The process involves breaking down of DNA into smaller fragments. Adaptors were then added to the both ends of the DNA fragments. Indexing was performed so as to identify the samples. Thus, prepared DNA sample was then processed to illumine platform for sequencing.

Following library preparation, DNA fragments are loaded onto the NGS MiSeq Illumina platform for sequencing. A flow cell holds the DNA fragments together. Amplification of DNA segments using bridge PCR takes place inside the flow cell. Following PCR, a cluster of DNAS will be generated. After this sequencing of DNA was performed. Illumina performs sequencing by Synthesis. It is based on the chemistry of the reversible dye terminators. Each base is added separately one at a time. The bases are fluorescently labelled which after addition excited to release signal. The computer will record the released signal after the addition of the particular base. This step is followed by the washing of the extra bases and the deblocking step. Then the same cycle continues until the whole DNA fragment is sequenced. BCL files are used to store the sequencing signal. These BCL files are converted using computer software to FastaQ files, the standard format needed for downstream analysis. Also, these FastaQ files contains error including low quality reads, base calling error, adaptor contamination and sequencing errors. Prior to analysis, these mistakes must be fixed which is called quality control. FastQC evaluates and corrects the quality of reads whereas the FastaP performs the filtering of reads. FastP is written in C $^{++}$ and comparatively faster than other tools. After filtering, filtered short reads will be obtained. These short reads are then assembled into contigs by the de novo genome assembler (SPAdes). Contigs are the DNA segments with the overlapping ends. Now we can recognize the viral sequences from these contigs. This can be accomplished by using Virfinder tool. Virfinder is based on the machine learning method and employs k-mer frequency to detect the viral sequences. It is independent of the homology-based searches and gene finding.

Now, with the use of technologies like Prokka, we can annotate the viral genes. Prokka refers to the prokaryotic annotation. Prokka annotate bacterial and viral genes by identifying the first protein coding region and then its product which is normally done by comparing with a database normally in a hierarchical manner. Proteins are first searched in the user- provided set of annotated proteins, then in the bacterial proteins found in the

Uniprot, and finally in all of the finalized bacterial proteins in Hidden Markov Model Database, followed by genome (RefSeq) for a specific genome. If the protein cannot be found, then it is termed as “Hypothetical Proteins”. Ten files are produced and placed in the output directory. As a result, files are created with ten distinct suffixes including gbk, fna, txt etc. Prokka is dependent on third- party tools like Prodigal. These output files (Genebank and Fasta) are then further analyzed by other additional tool to derive information from viral genome. The phage analysis tools employed were UGENE, PHASTER, RAST and Proksee.

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Identification of the Host bacteria

The bacterial sample collected was firstly replicated on Nutrient agar and Cetrimide agar. Nutrient agar is the general-purpose medium whereas Cetrimide agar is the selective and differential medium for *Pseudomonas* species. This medium contains cetrimide (Cetyltrimethylammonium bromide), a quaternary ammonium-based surfactant, a selective agent against other alternative microbial species because of its detergent properties. Cetrimide also increases the production of Pseudomonas pigments such as pyocyanin and pyoverdine, which have a characteristic blue-green and yellow-green color. Pseudomonas aeruginosa produces a number of water-soluble irons chelating agents, including yellow-green or yellow-brown fluorescent pyoverdine. When pyoverdine combines with water-soluble blue pyocyanin, the characteristic bright green color of Pseudomonas aeruginosa is created. Growth on the cetrimide agar confirms the bacteria to be *Pseudomonas* spp.

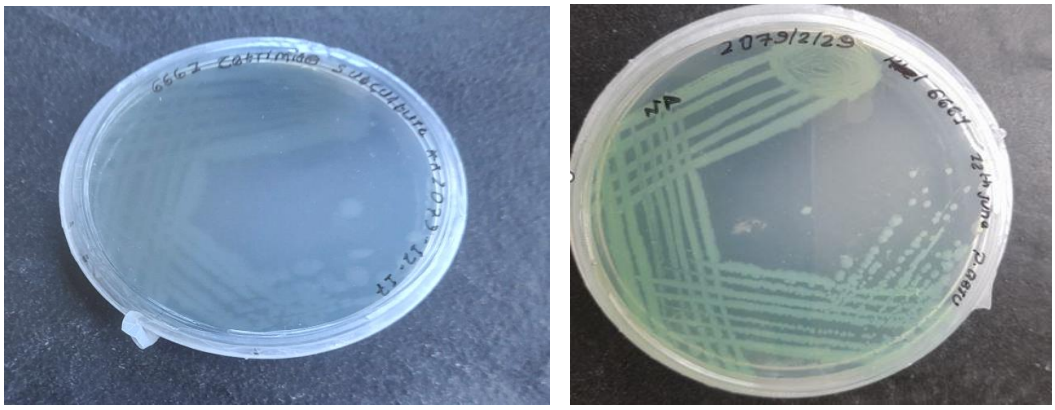


Figure 9: *Pseudomonas aeruginosa* on Cetrimide and Nutrient agar

On Cetrimide agar *Pseudomonas aeruginosa* produced medium sized colonies with irregular growth due to the swarming nature of the bacterium. The pigment production is enhanced and diffused in the medium imparting characteristic Greenish blue coloration to the medium. In Nutrient agar, medium colonies are irregular with distinct musty or earthy smell in the medium.

Single isolated colony from the nutrient agar plates was taken and gram staining was performed. On gram staining the bacteria was found to be gram-negative rod-shaped bacterium.

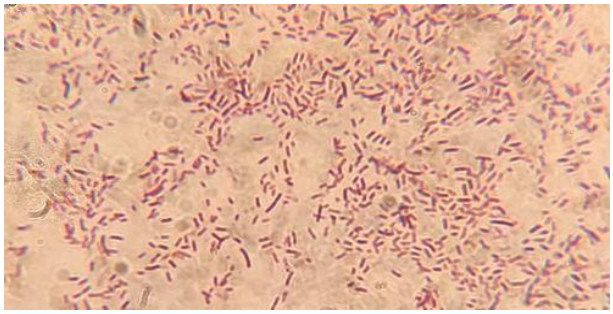


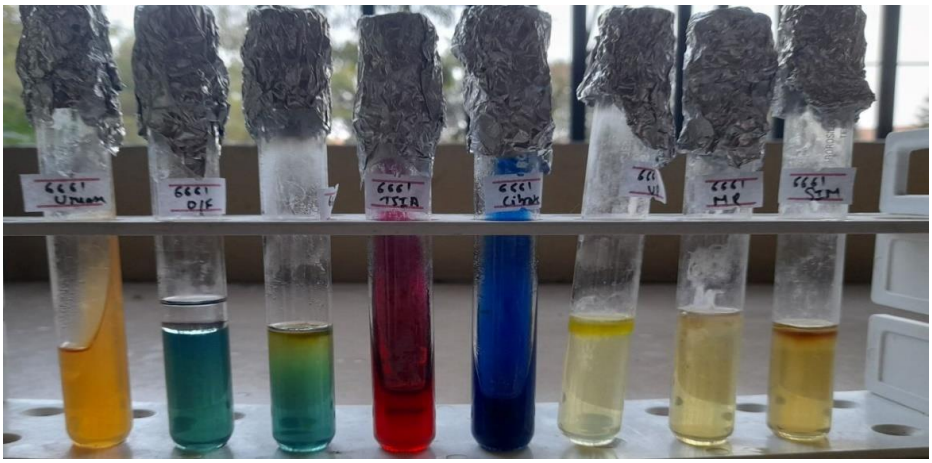
Figure 10: Gram staining of bacteria

4.1.1 Identification of Bacteria by biochemical test

The biochemical test of *Pseudomonas aeruginosa* are as follows:

Table 7: Biochemical test of *Pseudomonas aeruginosa*

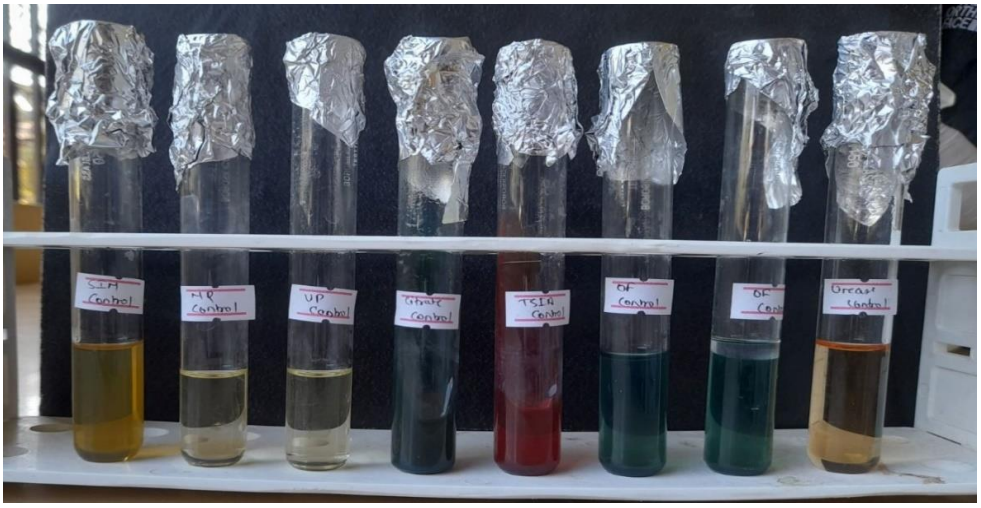
SN	Test performed	Results	Inference
1	Sulphur Indole Motility test	Motile, Indole – ve, sulphur –ve	Bacteria inferred as *Pseudomonas aeruginosa*
2	Methyl red test	Negative	
3	Voges Proskauer test	Negative	
4	Citrate utilization test	Blue	
5	Triple sugar iron agar test	Red/Red	
6	Urease test	Negative	
7	Oxidative/Fermentative test	Oxidative	



Tube 1: urease test ,Tube 2 and 3: oxidative fermentative test, Tube 4: TSIA test

Tube 5: Citrate utilization test, Tube 6 and 7: MR and VP test, Tube 8: Indole test

Figure 11: Biochemical test for *Pseudomonas aeruginosa*



Tube 1: Indole (control) Tube 2 and 3: MR and VP test Tube 4: Citrate test Tube 5:TSIA Test Tube 6 and 7: Oxidative Fermentative test Tube 8: Urease test

Figure 12: Biochemical test (control)

The Sample bacterium was found to be both catalase and oxidase positive. Oxidase positive organism delineates the property of the organism containing Cytochrome oxidase to oxidize the artificial electron donor provided and the color of the oxidase discs turned out dark purple. Catalase positive organism infers that the organism is able to produce enzyme catalase which detoxifies hydrogen peroxide by breaking down into water and oxygen gas. The clear bubble in the catalase positive result indicates the production of oxygen gas.

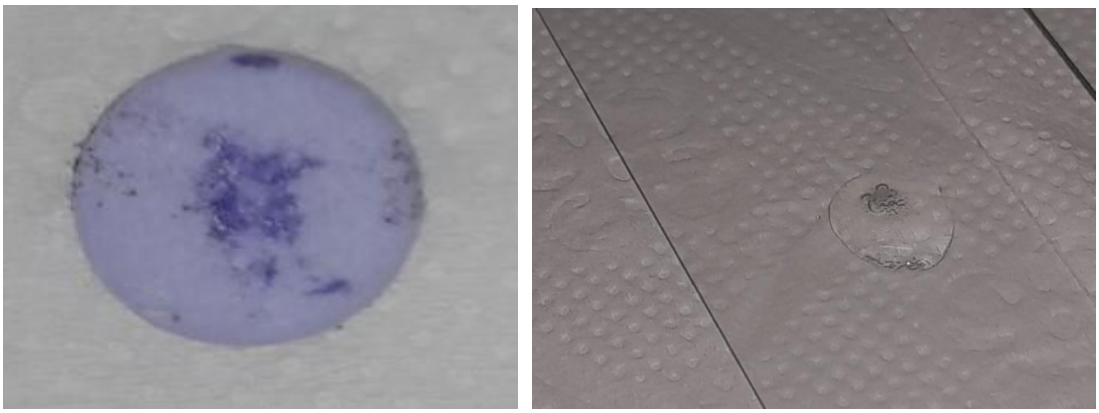


Figure 13: (A) Oxidase test, Fig B) Catalase test

4.1.2 Mass spectrometry analysis for the confirmation of bacteria

For bacterial confirmation, Bacterial sample (6661) was sent to National Public health Laboratory. The bacteria were confirmed as *Pseudomonas aeruginosa* and the method of the confirmation was based on Biomerieux Vitek -MS based on Matrix assisted Laser Desorption Ionization Time of Flight Spectrometry. The result was included in the appendices portion of this thesis.

4.2 Antibiotic Susceptibility Tests

The collected bacterial sample was subjected to all the available antibiotics in the laboratory. Metallo beta lactamase and Extended spectrum beta lactamase resistance was assessed. The sample bacteria (*Pseudomonas aeruginosa 6661*) was found to be resistant to more than 3 classes of antibiotics thus the bacteria can be inferred as Multidrug resistant. The bacteria was also resistant to carbapenem class of antibiotics that is Imipenem and meropenem which confers carbapenemase resistant. The clear zone around the antibiotic discs indicates the zone of inhibition. The diameter of the zone of inhibition (ZOI) was measured and expressed in millimeter (mm). The measured value was compared with guideline provided by Clinical Laboratory Standard Institute and interpreted as Resistant, Sensitive and intermediate.

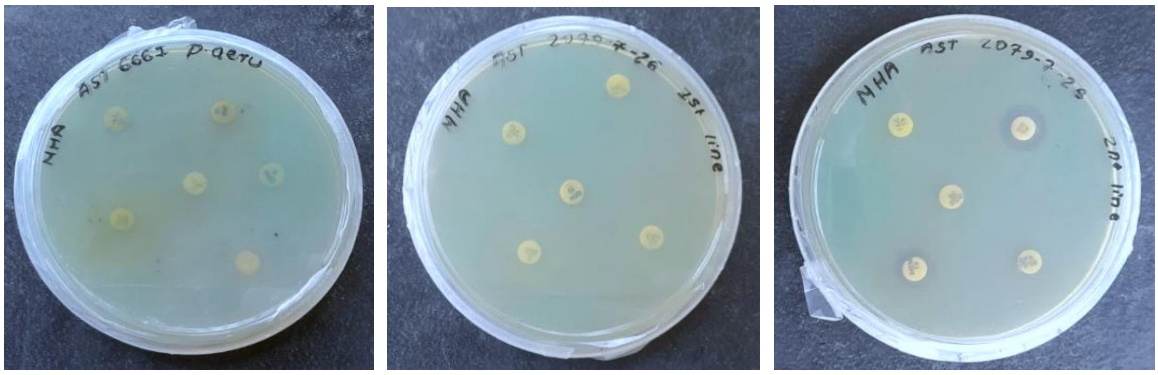


Figure 14: Antibiotic susceptibility test of *Pseudomonas aeruginosa*

Table 7: Antibiotic susceptibility pattern of host bacterial species

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	Amikacin	30 mcg	≥17	15-16	≤14	0mm	Resistant
5	Ceftazidime	14 mcg	≥18	15-17	≤14	0mm	Resistant
6	Amoxicillin	10 mcg	≥18	14-17	≤13	0mm	Resistant
7	Meropenem	10 mcg	≥19	16-18	≤15	10mm	Resistant
8	Tigecycline	15 mcg	≥19	15-18	≤14	0mm	Resistant
9	Doxycycline	30 mcg	≥14	11-13	≤10	0mm	Resistant
10	Cefoperazone sulbactam	75/30 mcg	≥21	16-20	≤15	0mm	Resistant
11	Imipenem	10 mcg	≥19	16-18	≤15	0mm	Resistant
12	Cefepime	30 mcg	≥25	19-24	≤18	0mm	Resistant
13	Nalidixic acid	10 mcg	≥19	14-18	≤13	0mm	Resistant
14	Piperacillin tazobactam	100/10 mcg	≥21	15-20	≤14	0mm	Resistant
15	Nitrofurantoin	300mcg	≥17	15-16	≤14	0mm	Resistant
16	PolymyxinB	300mcg		11-17		12mm	Intermediate

4.2.1 Confirmation of Antibiotic susceptibility using Vitek Compact System 2

Antibiotic susceptibility test was further confirmed by vitek compact system 2 analyzers. The result was similar to the test we performed in the laboratory. Additionally, Colistin resistance was determined where our bacteria seem to be susceptible only to Colistin, the last resort of antibiotics.

4.2.2 Extended spectrum beta lactamase detection test (ESBL)

For the initial detection of extended spectrum beta lactamase detection, both the ceftazidime and ceftazidime clavulanic acid was used in combination. The test was considered positive when the zone of inhibition either around ceftazidime or ceftazidime clavulanic acid was greater than 5mm. In case of the sample bacteria, no zone of inhibition was observed, so the bacteria can be considered ESBL negative.

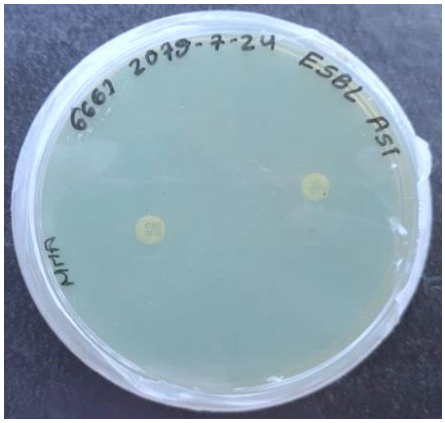


Figure 15: ESBL detection of *Pseudomonas aeruginosa*

4.2.3 Metallobeta lactamase detection test (MBL)

Combined disc test was performed for the initial Metallobeta lactamase detection. In this test imipenem and imipenem EDTA was used in combination. The zone size of both the antibiotic was measured. In our study, the zone size around the imipenem disc was 10 mm and the zone size around the imipenem EDTA disc was 22mm. The zone diameter difference in our case is 12mm. A zone diameter difference between Imipenem and Imipenem EDTA disc greater than or equal to 7 mm was interpreted as positive result for MBL production.

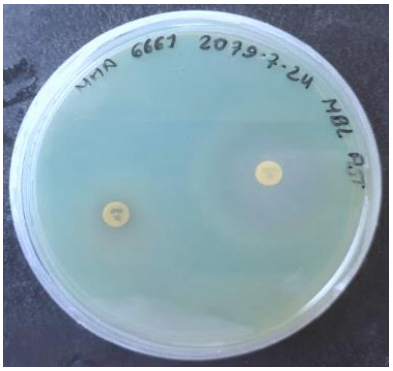


Figure 16: Metallobeta lactamase of *Pseudomonas aeruginosa*

4.3 Genomic DNA Extraction of *Pseudomonas aeruginosa*

DNA extraction of *Pseudomonas aeruginosa* was carried out by CTAB method. Gel electrophoresis of extracted DNA was performed and the isolated DNA band was visualized under UV transilluminator**.**

Figure 17: Genomic DNA extraction of *Pseudomonas aeruginosa* (6661)

4.3.1Quantification and purity check of isolated Bacterial DNA

Table 8: concentration determination of extracted DNA

Sample ID	**Concentration (ng/µl)**	**$A_{260/230}$**	**$A_{260/280}$**
6661 (*P. aeruginosa*)	288.4	2.28	1.98

4.4 Molecular identification of the host bacterial strain using 16SrRNA gene

16SrRNA gene amplification was conducted for the isolated host bacterial species that is *Pseudomonas aeruginosa* (6661). There are two primary subunits of bacterial ribosomal RNA: the large subunit, 50S and the small subunit,30S. 16SrRNA is small subunit component particularly useful for the identification of the bacteria as well as for the reconstruction of the bacterial evolutionary relationships. In our study, the bacteria was amplified by 16SrRNA universal primer and was sent to Center for Molecular Dynamics Nepal (CMDN) for sequencing.

After Sequencing the genes sequence was found in chromatogram file that was accessible via the Chromas program. After that, the chromatogram file was searched using BLAST

program (nucleotide BLAST) to see whether the sequence in the NCBI database were similar. The Maximum match was seen with partial sequence of *Pseudomonas aeruginosa* NBRC 12689 species that is 99.80 %.

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download | Select columns | Show 100

select all *100 sequences selected* — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudomonas aeruginosa strain NBRC 12689 16S ribosomal RNA, partial sequence	Pseudomonas aeruginosa	898	898	38%	0.0	99.80%	1461	NR_113599.1
Pseudomonas aeruginosa strain ATCC 10145 16S ribosomal RNA, partial sequence	Pseudomonas aeruginosa	898	898	38%	0.0	99.80%	1489	NR_114471.1
Pseudomonas aeruginosa strain DSM 50071 16S ribosomal RNA, partial sequence	Pseudomonas aeruginosa	898	898	38%	0.0	99.80%	1527	NR_117678.1
Pseudomonas aeruginosa strain DSM 50071 16S ribosomal RNA, complete sequence	Pseudomonas aeruginosa	837	837	37%	0.0	98.33%	1537	NR_026078.1
Pseudomonas citronellolis strain ATCC 13674 16S ribosomal RNA, partial sequence	Pseudomonas citronellolis	826	826	38%	0.0	97.14%	1499	NR_112069.1
Pseudomonas panipatensis strain Esp-1 16S ribosomal RNA, partial sequence	Pseudomonas panipatensis	826	826	38%	0.0	97.15%	1419	NR_044209.1
Pseudomonas delhiensis strain RLD-1 16S ribosomal RNA, partial sequence	Pseudomonas delhiensis	822	822	38%	0.0	96.95%	1505	NR_043731.1
Pseudomonas citronellolis strain NBRC 103043 16S ribosomal RNA, partial sequence	Pseudomonas citronellolis	817	817	38%	0.0	96.53%	1462	NR_114194.1
Pseudomonas otitidis strain MCC10330 16S ribosomal RNA, partial sequence	Pseudomonas otitidis	815	815	38%	0.0	96.73%	1531	NR_043289.1
Pseudomonas citronellolis strain DSM 50332 16S ribosomal RNA, partial sequence	Pseudomonas citronellolis	815	815	38%	0.0	96.73%	1510	NR_026533.1
Pseudomonas guariconensis strain PCAVU11 16S ribosomal RNA, partial sequence	Pseudomonas guariconensis	813	813	38%	0.0	96.55%	1524	NR_135703.1

Figure 18: BLAST result for 16S rRNA sequencing

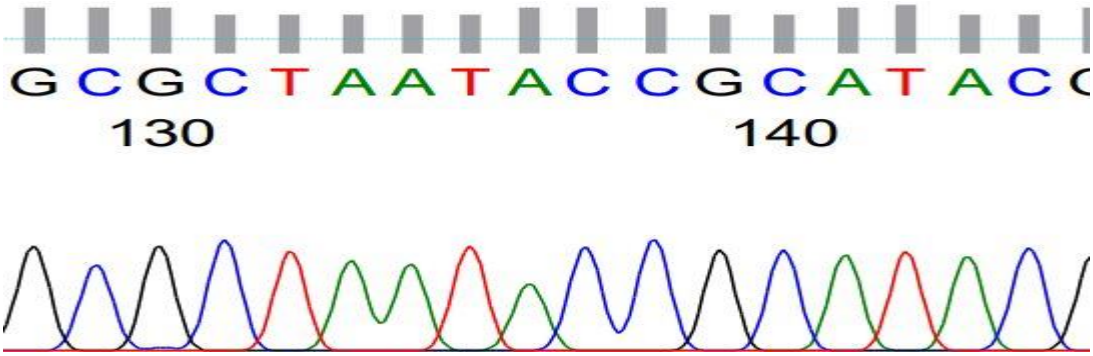


Figure 19: chromatogram of 16SrRNA sequence of *Pseudomonas* (6661). Different peak with different color shows different nucleotide sequences (ATCG)

4.5 Bacteriophage isolation and purification

4.5.1 Bacteriophage Isolation

Bacteriophage against the identified bacterial sample was isolated from the waste water sample collected from the Balkhu river by Double layer agar technique (DLAA). Single plaque of phage was isolated at the beginning which was further multiplied. Thus, isolated phage was then subjected to initial screening for its morphology, opacity before further characterization.

Table 9: Isolated phage and its characteristics

Sample source	Host Bacteria	Name of Phage	Initial no of plaques	Plaque Opacity	Plaque Morphology
Balkhu river	*Pseudomonas Aeruginosa*	6661	1	Clear	Small, Pin head

4.5.2 Bacteriophage purification

Bacteriophage purification was done by continuous three rounds of streaking of single plaque. Bacteriophage containing clear lytic plaque morphology was selected for purification. Phage streaking results in the formation of plaque of uniform size as the single phage was replicated to multiple numbers through streaking process. High number of plaques were formed in the starting line than the ending line.

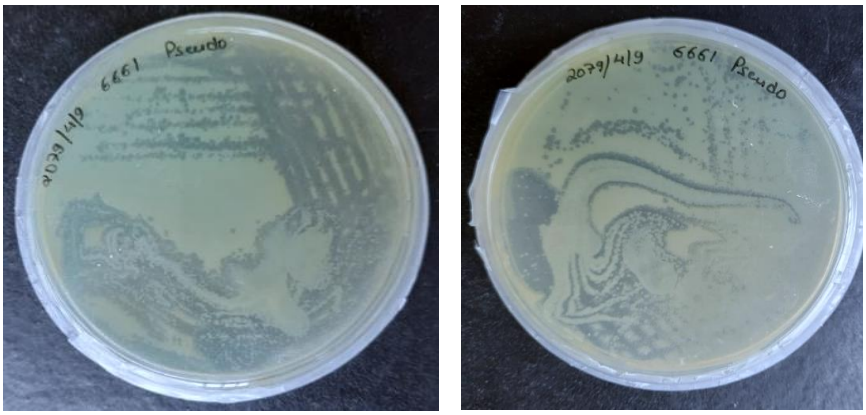


Figure 20: Purification of bacteriophage by streaking method

Figure 21: Phage stock in SM buffer

4.6 Spot assay of phage for titer determination

Spot assay along with Double Layer Agar Assay (DLAA) was implicated for the tittering of phage or phage titer determination. Both of the assay is regarded as reference technique and they both work on the premise of subjecting the overnight development of the lawn of bacteria on an agar matrix to the known quantity of phage (Daubie *et al.,* 2022). In our assay, clear large spot was observed up to 10^{-7} dilution and small pin point lysis zone was observed in 10^{-8} and 10^{-9} dilution. So as to determine the titer of the phage we take the three most diluted phage up to where lysis was observed (10^{-7},$10^{-8}$and 10^{-9} dilution) and it was again plated using DLAA method. On plating, countable no of plaques was observed up to 10^{-9} dilution. 10^{-9} dilution was taken for further procedures. The concentration of phage lysate solution was then determined by using the following formula.

$$\text{PFU/ml} = \frac{\text{No.of plaques}}{\text{Volume of phage} \times \text{Dilution of phage}}$$

$$= \frac{56}{1\text{ml} \times 10 - 9}$$

$$= 5.6 \times 10^{10} \text{ PFU/ml}$$

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

The spot assay was used to determine the bactericidal activity of the phage isolated and is expressed as PFU/ml. Applying phage at high titer fail to discriminate between the ability of the phage to multiply within or merely kill the test strain since phage infection and lysis, abortive infection all generate the similar result. The singly diluted lower phage concentration is more efficient than high phage titer because the level of productive phage infection is required to produce a death signal. Plaque formation through Efficiency of plating (EOP)is the better indicator of productive phage infection, since it is the multiple rounds of infection, lysis, and release of progeny. Efficiency of plating (EOP)counts the number of plaques formed by a phage on a test strain relative to its titer observed on its original host. **(Xie *et al.,* 2018)**

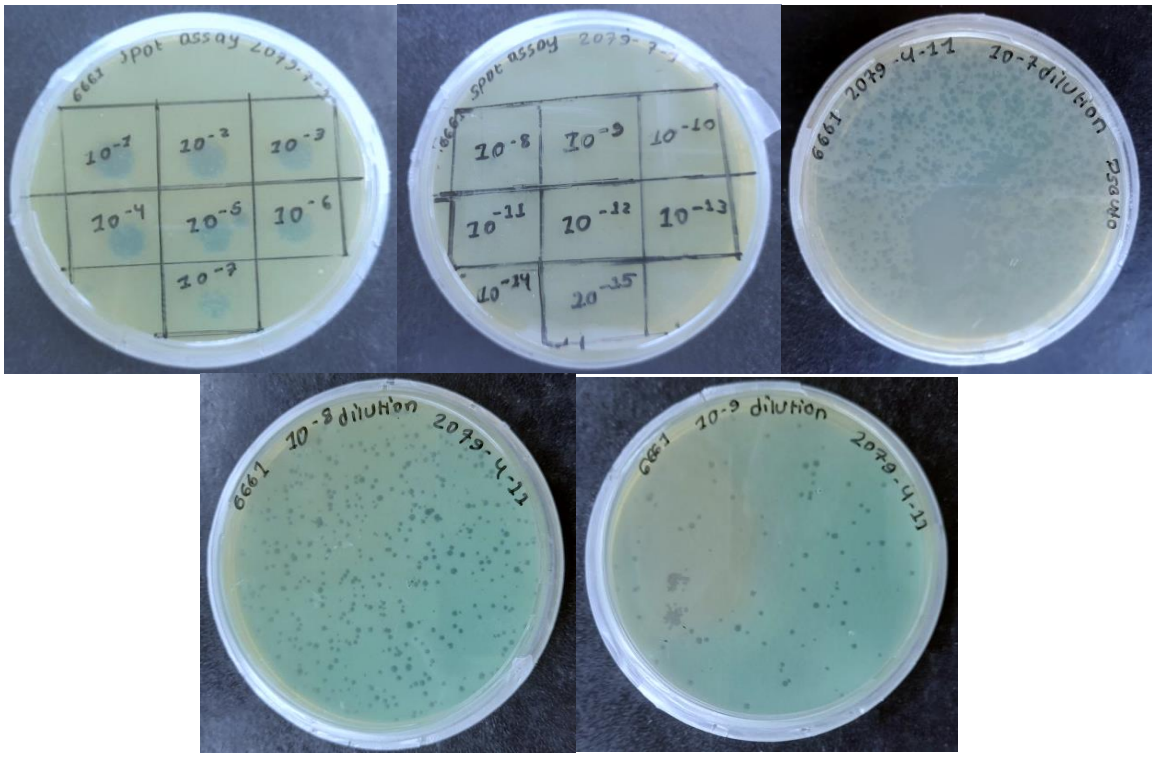


Figure 22- A: Spot assay of Phage 10-7 dilution, Fig B: spot assay of phage 10-15 dilution, Fig C, D, E: Plaque in 10-7, 10-8 ,10-9 dilution

4.7 Physiochemical characterization of phage

4.7.1 Multiple Host range analysis of Phage

"Host range" describe the limitation of bacteriophage strain to the specific strain of the host bacteria. Different bacteria can be infected by certain phages, while some phages are restricted to a single species. Due to the host phage binding specificity (RBPs) discovered by Hyman and Abedon (2010), it is believed that practically all bacteriophage may infect a limited range of bacterial strains. (Hyman & Abedon, 2010). Some phage has the wide host range, which is defined as the phages capacity to infect a variety of bacterial species and strains of identical species (Ross et al. 2016). For instance, coliphage like the Mu phages can infect two host group because they have two sets of tail fibers according to the recent study by Hyman (2019) (Hyman, 2019).

Lysis was seen against *Pseudomonas*1 in host range analysis of phage. Phage 6661 showed lysis activity against this *Pseudomonas* species showing intraspecific host range while there was no lysis activity against other *Pseudomonas* species. Yehl et al., 2019 stated that the reason for this might be due to the recognition of the receptor by the phage as the common receptor might be present between the various strain of same bacterial species

(Yehl et al., 2019). Also, Lenski & Levin, 1985 also adds the interplay of evolutionary dynamics between bacteria and phage significantly influence the host range as they both constantly try to evolve according to their adaptive circumstances (Lenski & Levin, 1985).

Lysis was not observed for other bacterial genus. Phage 6661 did not show interspecific Host range.

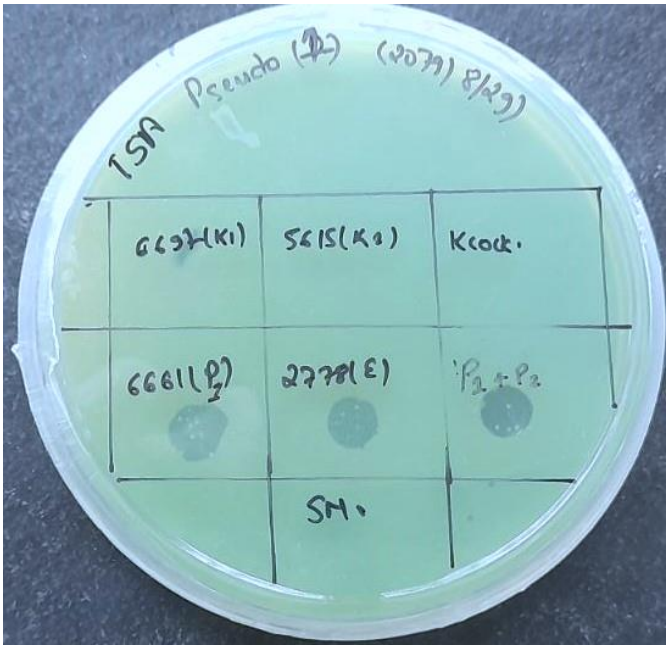


Figure 23: *Pseudomonas aeruginosa* Showing intraspecific host range

4.7.2 Stability of phage at different PH range

pH stability of phage 6661 under severe condition was assessed by exposing the phage to different pH level, acidic to basic pH (PH 2-12). The PFU/ml of the phage was counted to identify the pH range up to which phage can survive.

Table 10: Host range analysis by phage 6661

Bacteria	Phage 6661	Phage 6661and PhageTU- Pse-1B Cocktail
Pseudomonas 1	Yes	Yes
Pseudomonas 2	No	No
Pseudomonas-sho	No	No
209205 (Pseudomonas)	No	No
Klebsiella8	No	No
Klebsiella9	No	No
Entero3	No	No
Klebsiella12	No	No
Klebsiella13	No	No
Klebsiella14	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

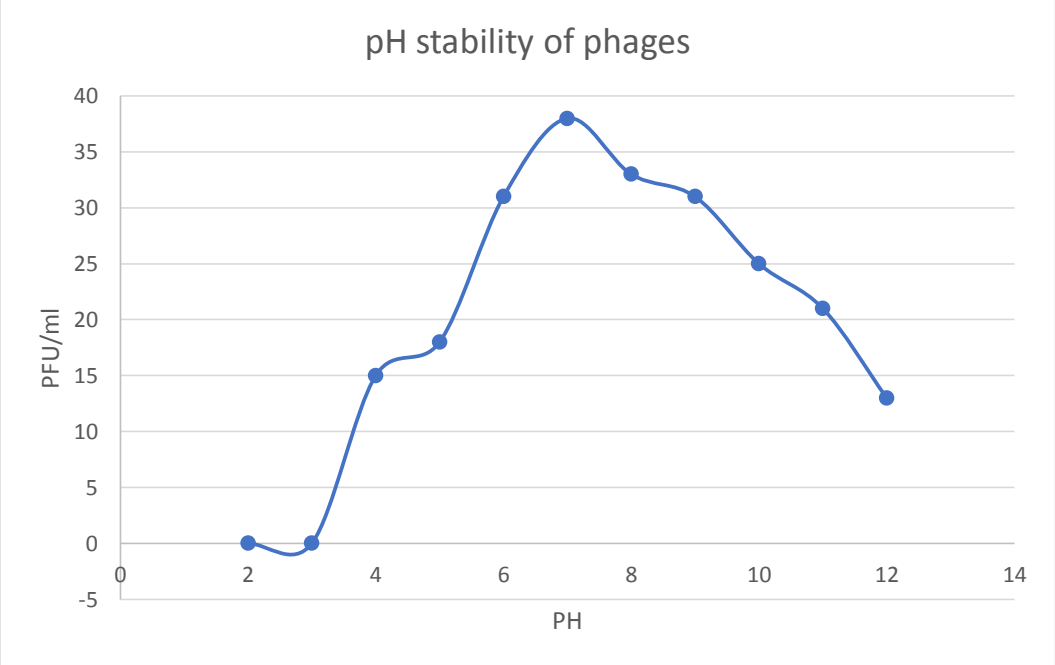


Figure 24: pH stability of phage 6661

No plaques were observed at extremely acidic pH that is pH 2 and 3. However, *Pseudomonas* phage 6661 was seem to be stable at pH 4 to 12. The maximum stability of the *Pseudomonas* phage 6661 can be observed at pH 7. From pH 8, the stability of the isolated phage was then decreased up to pH 12. Although, the stability was decreased but it was able to survive even at the extremely basic pH. Inactivation of the Phage at pH 2 and 3 may be due to denaturation of the protein in extreme acidic condition. Also, aggregation process can cause the phages to decrease in acidic conditions (Majdani & Ghahfarokhi, 2022). Santos et al. isolated two phages against *Pseudomonas aeruginosa* P2S2 and *Pseudomonas aeruginosa* P5U5 which exhibit significant stability within the pH range 4 to 11 (Santos *et al.,* 2011).

4.6.3 Stability of the phage at different temperature

Phage 6661 was subjected to various temperature for varying lengths of time in order to assess its thermal stability or temperature stability. The capacity of phage to survive at various temperatures was determined by plotting the plaque forming unit per milliliter (PFU/ml) vs time.

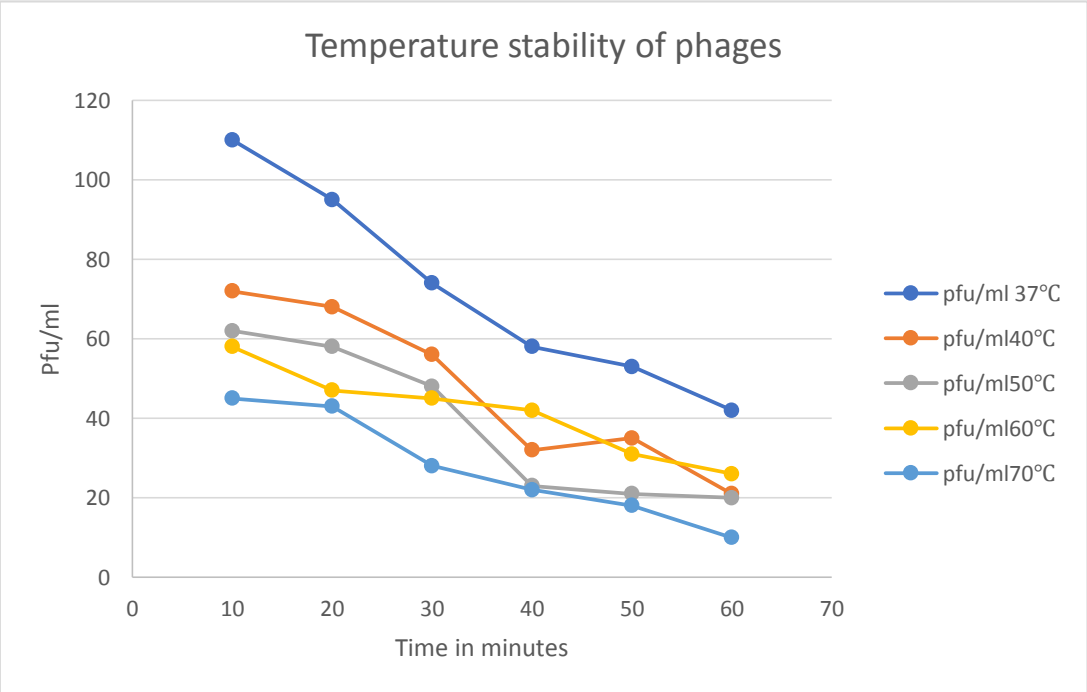


Figure 25: stability of phage at different temperature for different time interval

Phage was found to be stable even at 70℃ up to 60 minutes. But as shown in the graph, the phage titer decreased with the increment in temperature and vice versa. So, it can also be said that the infectivity of the phage decreases with increase in temperature.

4.7.3. One step growth curve of phage

One step growth curve of the phage was performed so as to determine the latent period and the burst size of the phage. The variation of the phage number in one replicative cycle was determined.

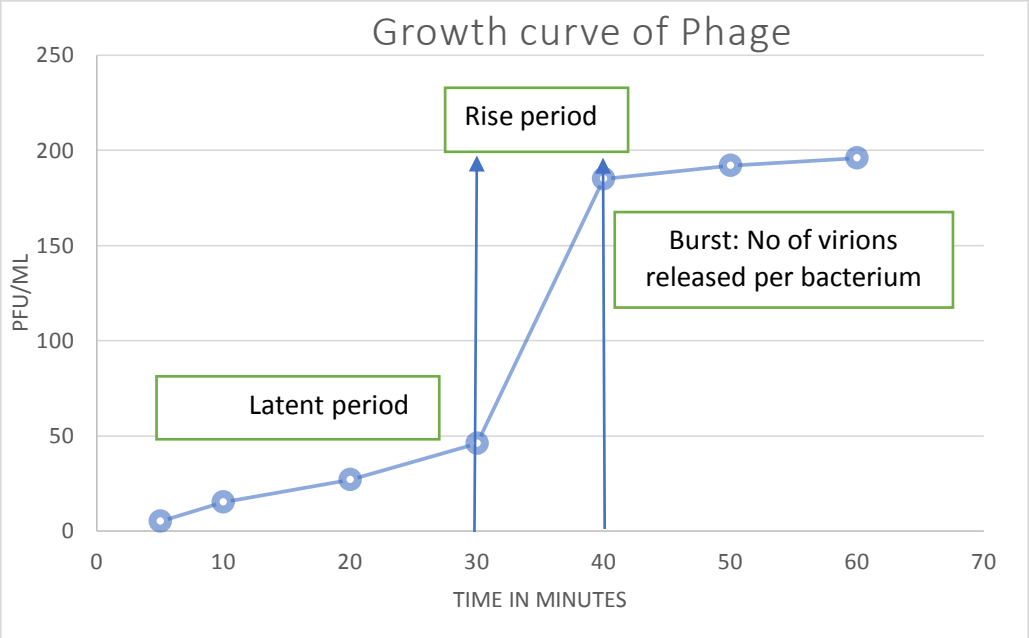


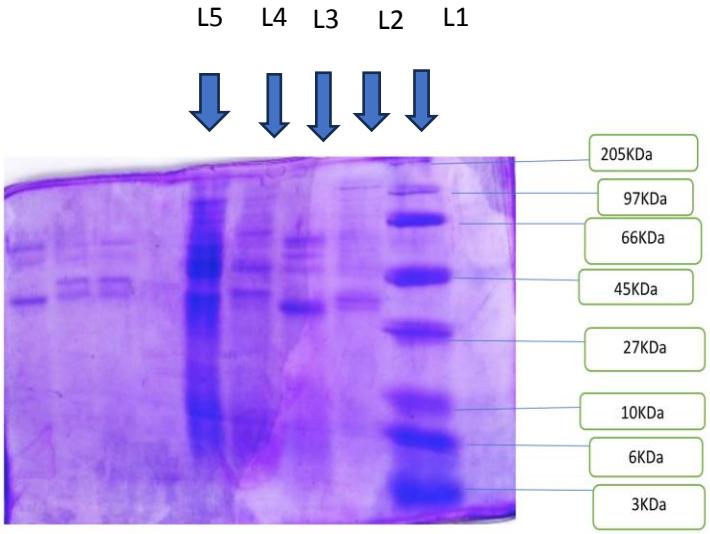
Figure 26*:* One Step growth curve of Phages

The spike in phage titer was observed after initial 30 min. Thus, the latent period for the viruses was found to be 30 min. The burst duration was discovered to be 10 min and the burst size was calculated to be 96 virus particles per infected cells. Hyman and Abedon (2009) determined the latent period and burst size of the viruses. The time between the phage adsorption and initial rise in plaques was used to determine the latent period. The burst size was calculated by dividing the average of pfu per infected cells in the post rise period by the average of pfu per infected cells in the pre rise period. Eclipse phase refers to the stage of the bacteriophage growth curve when it begins to attach to the surface of the host cell and mature using the components produced by host cell's biosynthesis. Depending on the bacteria, it's unique phage, phage host interaction and the external environment normally it took 5 to 10 minutes. During this stage, plaque development may

or may not occur. No plaques formed in our investigation up to 5 minutes and formed in 10 minutes. Differences in the medium, environmental conditions such as PH and temperature, and host cell can also affect the latent period and burst size of phages (Majdani & Ghahfarokhi, 2022).

4.7.4 Sodium Dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE)

By using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page), the protein profile of the bacteriophage was examined. Phage 6661 showed four distinct band of proteins.



L1: marker, *L2: Phage TU_ Pse_1B, L3: Pseudomonas phage 6661*

L4: Klebsiella *phage 6697*

Figure 27: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The observed size of the one protein was found to be 30 KDa whereas the size of other proteins bands were found to be of the size 50Kda, 55KDa and 60KDa respectively. Faint band in SDS could be the result of the mistake made during the SDS PAGE experiment such as excessive heating, incorrect mixing of the dye and breakdown of the protein from the excessive heat. Some other factors that result in unclear band might be due to the improper concentration of the protein in the sample, large sample volumes, inappropriate gel concentration, usage of the old gel, high voltage and running time and insufficient resolvation of the proteins.

4.8 Transmission electron microscopy of *Pseudomonas* phage (6661)

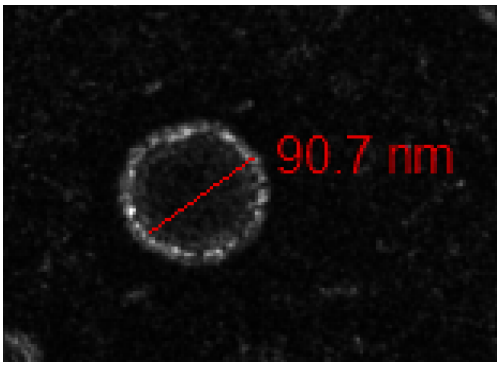


Figure 28: Transmission electron microscopy of a *Pseudomonas* phage

The transmission electron micrograph of *Pseudomonas* phage 6661 was visualized in a Image J viewer software. From the above micrograph result, we can predict that the capsid diameter of the phage was 90.7 nm. Since, from the whole genome sequencing data on protein BLAST of isolated phage it was confirmed that the phage isolated was tailed phage with the presence of phage long tail fiber proximal subunit, Phage tail tape measure and phage tail fibers, the isolated phage belongs to the order Caudovirales. However, In the figure since the phage tail was not seen we could not determine the family of the virus. The absence of tail in the micrograph of transmission electron microscopy might be due to the positive staining with Uranyl acetate which is undesirable byproduct of the negative staining and the capsid of this type of stained phage are always reduced and the capsomere and edge are always missing (Ackermann, 2013). Another reason for the tail breakage or tail loss of the viruses might be due to the complex steps of sample preparation and improper purification step.

In the other study conducted by Williamson *et. al*, 2011 he stated that Myovirus phages are more prone to tail breakage than Podovirus phage also tail morphology (contractile vs flexible and non-contractile tail) determine the loss or the breakage of the tail leading to the false interpretation in classification of the bacteriophage (Williamson *et al.,* 2011).

4.6.6 Electrophoresis of Phage DNA

Genomic DNA extraction of phage showed a clear band under 0.8% electrophoresis. Following the gel electrophoresis, a distinct band of phage DNA was seen, the phage DNA was run along with a Fermenta o Gene Rule 1 kb DNA Ladder. The size of the phage DNA was larger than 10kb.

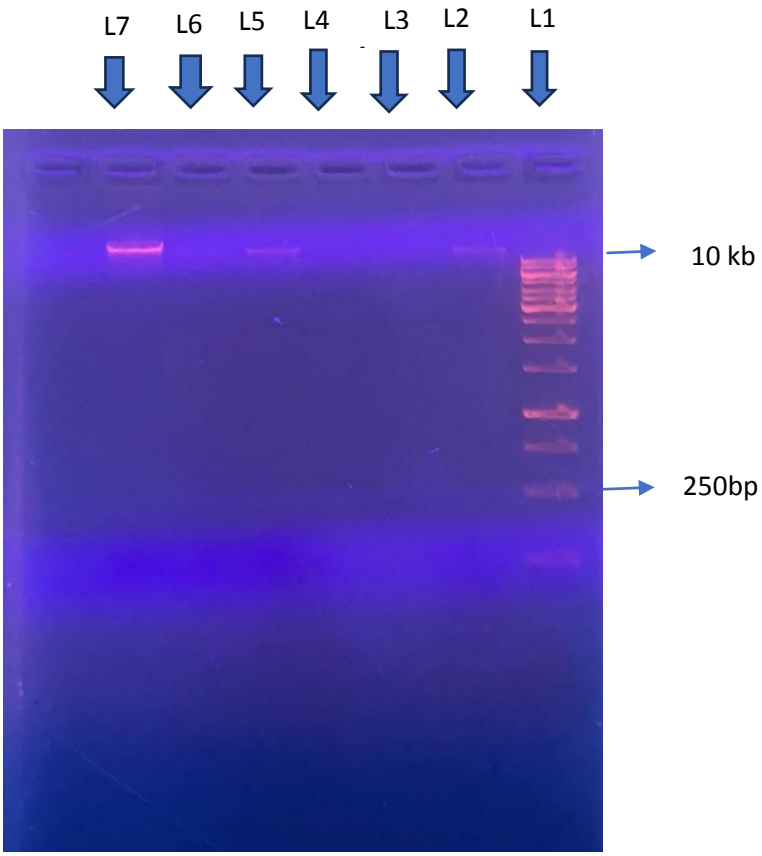


Figure 29: Electrophoresis of phage DNA

4.8 Whole genome sequencing of phage DNA

4.8.1 Genome analysis by ugene

Unipro Ugene is a bioinformatics tool kit that provides visualization modules for biological objects such as annotated genome sequences, Next Generation Sequencing (NGS) assembly data, multiple sequence alignments, phylogenetic trees and 3D structures.

Table 11: General statistics obtained from phage whole genome sequencing

Length	43,113nt
GC content	53.79%
Melting temperature	86.94°C
Molecular weight	26641230.50 Da
Extinction coefficient	679340757 l/ (mol * cm)
µg/OD_{260}	39.22

ORF	233
No of restriction sites	92
Restriction enzymes	EcoRI (31 sites), HindIII (60 sites), DraI (1 site)

The length of the whole genome sequence of *Pseudomonas* phage DNA was found out to be 43,113 nucleotides (43 kb) long. The GC content of bacteriophage DNA was 53.79 %. Bohlin *et al*., 2017 stated that higher GC content in phage genome is related to the stability of the genome. Higher GC content means higher the bond between the base pairs resulting in the structural stability in the phage genome. (Bohlin *et al.,* 2017) The melting temperature of the DNA was 86.94 °C. Molecular weight of the double stranded DNA was 26641230.50Da. Extinction coefficient (molar attenuation coefficient or molar absorption coefficient) of the DNA was 6793407571Liter/mole^{1-}cm^{1-}. The molar attenuation coefficient or molar absorption coefficient is a measure of how strongly a chemical species absorbs light at a given wavelength and it is an intrinsic property of the species (Banihashemian *et al.,*2013). The µg/OD260 for the DNA was 39.22. It means the concentration of DNA was 39.22 µg per ml. Upon visualization of the genome 233 ORF region was predicted. Total number of restriction sites predicted was 92. Three types of restriction sites was identified in our phage genome which are EcoRI, HindIII, DraI.

Circular map of phage genome using Ugene showing annotated proteins

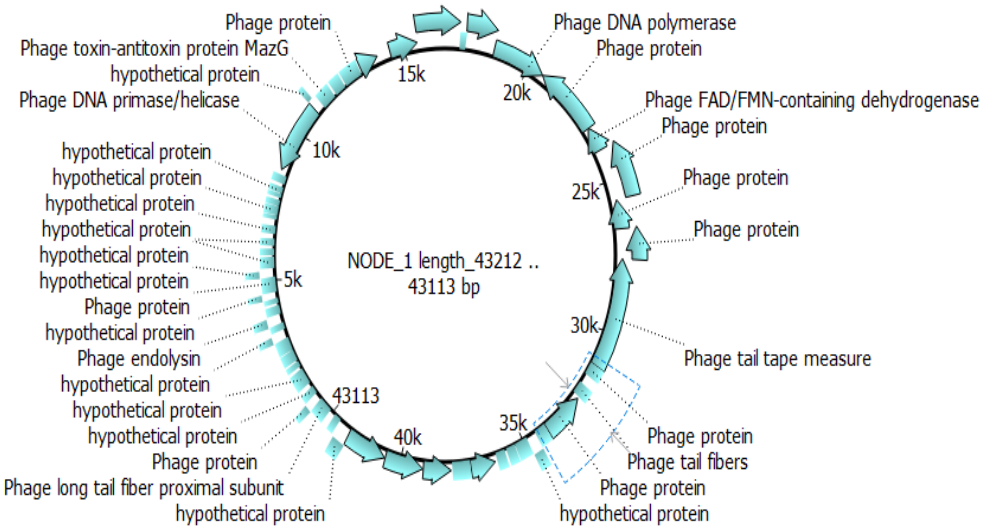


Figure 30: Circular map of Phage genome using Ugene Software

Circular map of phage genome was generated using Ugene software. The circular representation of the phage genome shows the name of different proteins present in the phage genome. Also, this software was useful for deriving the location of the particular types of the protein present in the phage genome. For example, the major lysis protein of phage, Phage endolysin was present between 5000 and 43000 base pairs. One bacterial protein, phage toxin- antitoxin protein MazG was identified in our phage genome which might have either been obtained from prophage element or may be through the horizontal gene transfer.

Table 12: Character Occurrence of Nucleotide and their percentage

Nucleotides	**Number**	**Percentage**
Adenine	9597	22.3%
Guanine	11516	26.7%
Cytosine	11673	27.1%
Thymine	10327	24.0%

Total number of adenine Nucleotide in the genome of phage was 9597. Its percentage was22.3%. Cytosine accounts for the highest percentage of nucleotide in the phage genome which was 27.1% and the number was 11673. Total number of Guanine nucleotide in the phage genome was 11516 which was 26.7% of the total nucleotide in the phage genome. Thymine accounts for 24% of the total nucleotide percentage and the number was 10327.

Table 13: Different amino acids and their total number in the Genome

Amino acids	**Number**	**Amino acids**	**Number**
Arginine (R)	11873	Asparagine (N)	3613
Alanine (A)	8390	Isoleucine (I)	3184
Serine (S)	6858	Glutamine (Q)	2943
Leucine (L)	6691	Glutamic acid (E)	2631
Glycine (G)	5088	Cysteine (C)	2556
Proline (P)	5086	Aspartic acid (D)	2157

Threonine (T)	4923	Tyrosine (Y)	1904
Valine (V)	4923	Histidine (H)	1213
Phenylalanine (F)	4346	Tryptophan (W)	937
Lysine (K)	3989	Methionine (M)	780

The amino acid with the highest occurrence was Arginine followed by Alanine and Serine whereas Methionine was the amino acid with lowest occurrence.

In the whole genome sequence of phage, 233 open reading frames were predicted. Different open reading frame code for the different gene. Among all ORF, 18 ORF code for hypothetical proteins,14 resemble phage protein and other code for functional gene products. On bioinformatic analysis, some are structural protein coding genes. Phage minor capsid protein, phage possible peptidoglycan binding protein, phage 62kDa structural protein, phage tail tape measure, phage tail fibers and phage long tail fiber proximal subunit are some of the structural protein presents in our phage. Some gene are associated with DNA replication, recombination, repair and packaging. Phage terminase large subunit, phage DNA Polymerase, DNA primase, DNA helicase is some of the protein related to above function. some gene are associated with Transcription, translation and nucleotide metabolism. Phage FAD/FMN containing dehydrogenase, Deoxycytidylate deaminase are some of the proteins associated with the above function. The gene associated with lysis module of the phage is phage endolysin. Besides one horizontally transferred region phage toxin antitoxin protein MazG is also present in our phage.

MazG protein has been proposed to increase phage survival by maintaining the host viability during phage propagation and it is expected to be a regulator of nutritional stress and programmed cell death. By lowering the cellular level of Guanosine Penta phosphate and Guanosine tetraphosphate also called alarmones (p)ppGpp, the Escherichia coli MazG can obstruct the MazEF toxin antitoxin system operation. (Gross *et al.,* 2005**)** A recent investigation however revealed that Cyanophage MazG has substantial hydrolytic activity towards dGTP and dCTP but no binding or hydrolysis activity against alarmone (p)ppGpp. Alarmones are the key regulators of the bacterial growth, stress adaptation, antibiotic tolerance and pathogenicity. This finding raised the possibility that MazG is involved in

hydrolyzing the rich G+C host genomes for phage replication. (Rihtman *et al.,* 2019) Investigations were needed to determine whether the MazG protein encoded by *Pseudomonas* phage 6661 play similar function to phage replication (Qin *et al.,* 2022).

4.7.4 Genome analysis through PHASTER

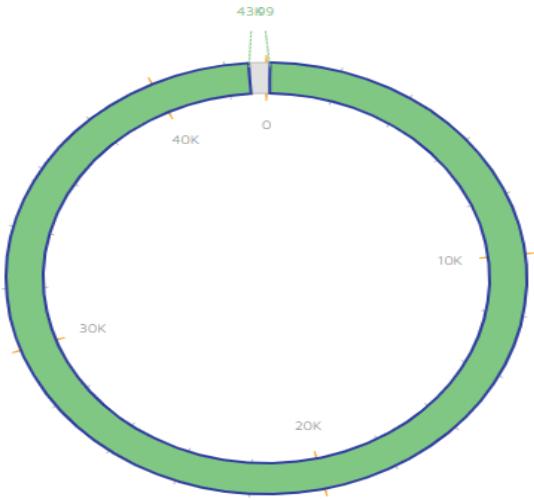


Figure 17: Complete genome of phage 6661

PHASTER (Phage Search Tool Enhanced Released) is an upgraded form of web server used for Phage genome annotation and analysis. PHASTER helps to generate circular genome of phage and provides information about the length of genome, GC content, completeness of genome, number of CDS region and start and end point of the prophage genome on the host genome. Circular genome of phage 6661 was generated with the genome size of about 43199 bp. GC content in the genome was found to be 53.77%. Total number of predicted proteins were 58. One intact prophage genome was identified on the host genome with the start site ranging from 99 to the end point of 42641. Our phage shows highest similarity with the PHAGE_Pseudo_vB_Pae_Kakheti25_NC_017864(52).

Region	Region Length	Completeness	Score	# Total Proteins	Region Position	Most Common Phage	GC %	Details
1	42.5Kb	intact	150	58	99-42641	PHAGE_Pseudo_vB_Pae_Kakheti25_NC_017864(52)	53.77%	Show

Intact (score > 90)
Questionable (score 70-90)
Incomplete (score < 70)

Region: The number assigned to the region.
Region Length: The length of the sequence of that region (in bp).
Completeness: A prediction of whether the region contains a intact or incomplete prophage based on the above criteria.
Score: The score of the region based on the above criteria.
 # Total Proteins: The number of ORFs present in the region.
Region Position: The start and end positions of the region on the bacterial chromosome.
Most Common Phage: The phage(s) with the highest number of proteins most similar to those in the region.
GC %: The percentage of GC nucleotides of the region.

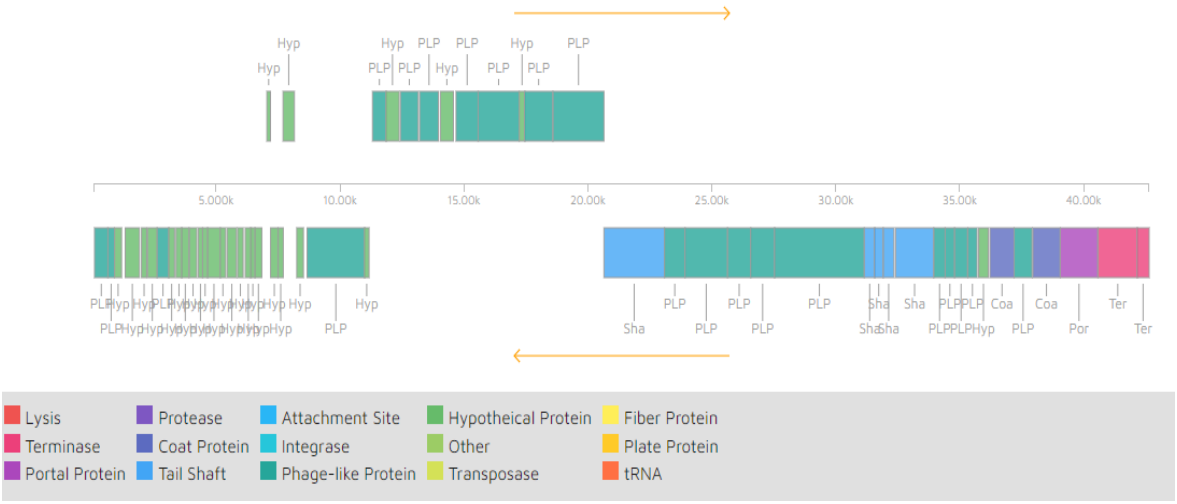


Figure 31: Different protein coding gene map of phage showing all of the proteins. Different color represents different protein present in phage such as integrase, terminase, lysis, coat protein, transposase etc.

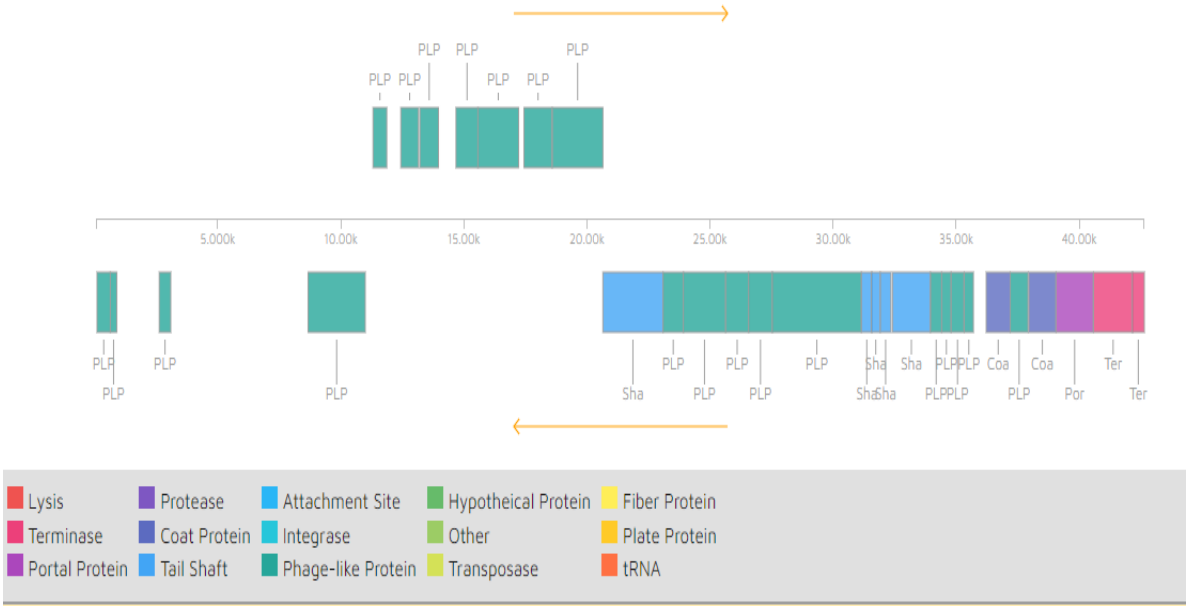


Figure 32: Protein coding gene map of phage showing only the annotated protein excluding the hypothetical protein of unknown function.

The upper arrow represents forward direction of ORF whereas lower arrow represents the backward direction/strands of ORF. The region position value predicts the location of prophage genome on host genome. PHASTER annotation also reveals the different protein coding region of phage such as lysis protein, terminase, coat protein, tail shaft protein, portal protein etc. various hypothetical protein was also observed in our phage. Hypothetical proteins are the proteins with unknown function. The presence of the gene

coding for the tail shaft confirms our phage to be tailed phage. Integrase coding gene and tRNA was absent in our phage.

4.8.5 RAST genome annotations

RAST (Rapid Annotation using Subsystem Technology) is a fully-automated service for annotating complete or nearly complete bacterial and archaeal genomes. It can be accessed via the link **https://rast.nmpdr.org/**.It provides high quality genome annotations for these genomes across the whole phylogenetic tree. The service normally makes the annotated genome available within 12-24 hours of submission. From the RAST genome annotation, the length of the phage genome was found to be 43,113 base pairs nucleotides. The GC content of the phage genome was found to be 53.8%. L50 Value and the number of contigs (with PEG) were 1. Only two subsystem category was found in the genome. Total number of coding sequence in the genome was 60. No RNAs were present in the phage genome. Typical features that can be found in the phage genome are protein coding genes, noncoding RNA gene, Transposable elements, insertion elements, direct and indirect repeats, origin of replication, attachment and integration sites. sites. RAST annotated the genes and gives information about Gene identification, tRNA identification, rRNA identification, functional annotation and subsystem assignment. The absence of virulence, disease and defense gene in phage is one of the important characters to be considered so as to implicate the isolated phage in phage therapy. Transposable elements are also absent in the phage genome which is also one of the desired properties of phage to be used in the phage therapy. Subsystems-based technologies were developed in the SEED with the view that the interpretation of one genome can be made more efficient and consistent if hundreds of genomes are simultaneously annotated in one subsystem at a time".

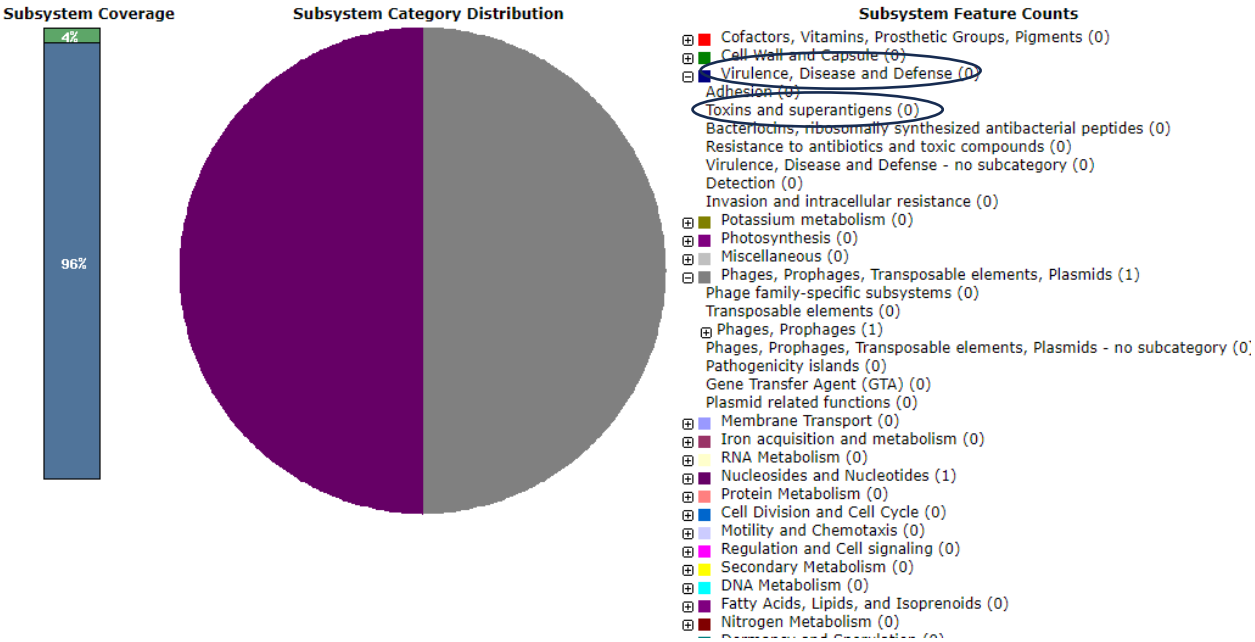


Figure 33: Subsystem category

Only two subsystems are present in the phage genome which falls in the category of phages, prophage, transposable elements and plasmid and other the nucleosides and nucleotides.

Category	Subcategory	Subsystem	Role	Features
all	all			
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage lysis modules	Phage endolysin	fig|6666666.979204.peg.10
Nucleosides and Nucleotides	Detoxification	Nucleoside triphosphate pyrophosphohydrolase MazG	MazG-related protein	fig|6666666.979204.peg.29

4.8.6 Construction of circular map of phage genome using Proksee

Proksee (https://proksee.ca/) is an online tool for Genome assembly, annotation and visualization. Proksee generates the circular map of genome showing all the annotated proteins of phage and also enables the user to visualize the GC content of the phage. The replication, recombination and repair sites of phage DNA was visualized using this tool. The stability, transfer and defense elements of phage was also visualized using this tool. one such type of site was identified in our phage genome. One horizontally transferred gene region was visualized in our phage which should be further assessed for its impact on the phage stability, efficacy and its impact on host killing mechanism by our phage. (Grant et al., 2023)

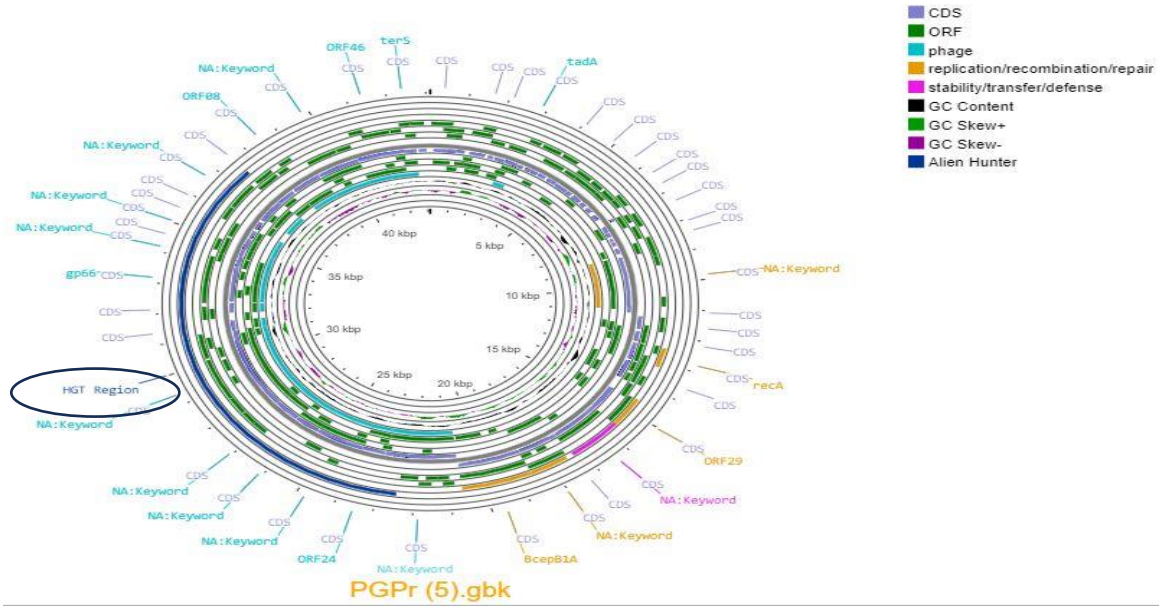


Figure 34: Genome analysis by Proksee

4.8.9 tRNA pred

For the prediction of Transfer RNA in our genome we use an online tool tRNAscan-SE. This web server(http://trna.ucsc.edu/tRNAscan-SE/) is easy to use means to identify tRNA gene in one or more query sequences. Furthermore, it allows user for the easy navigation of the result details and knowledge without requiring the familiarity with UNIX'S based command line or installation on one's own computer. Though we have already confirmed the absence of tRNA from the PHASTER annotation, we also visualize tRNA from this software. (Chan & Lowe, 2019). The result, thus obtained was similar to our previous findings that is the absence of tRNA in our phage genome.

4.9.10 Multiple sequence alignment and phylogenetic tree construction

Phylogenetic tree construction is one of the most widely used method to depict the evolutionary history of the phage. In order to find the phage genomes with similarity to our own, NCBI's BLAST search engine was used. Blast generated the list of pseudomonas phage that are similar to our phage genome. For multiple sequence alignment, the top ten phage genome with highest percentage similarity were chosen. These 10 phage genomes were then downloaded in the FASTA format. Prior to the construction of the Phylogenetic tree, multiple sequence alignment of our phage genome was performed with the above downloaded phage genome. After multiple sequence alignment,

phylogenetic tree was constructed using the above downloadable 10 FASTA format of the phages using UGENE platform. The phylogenetic relationship between our phage genome and other 10 phage genome is depicted in the picture below.

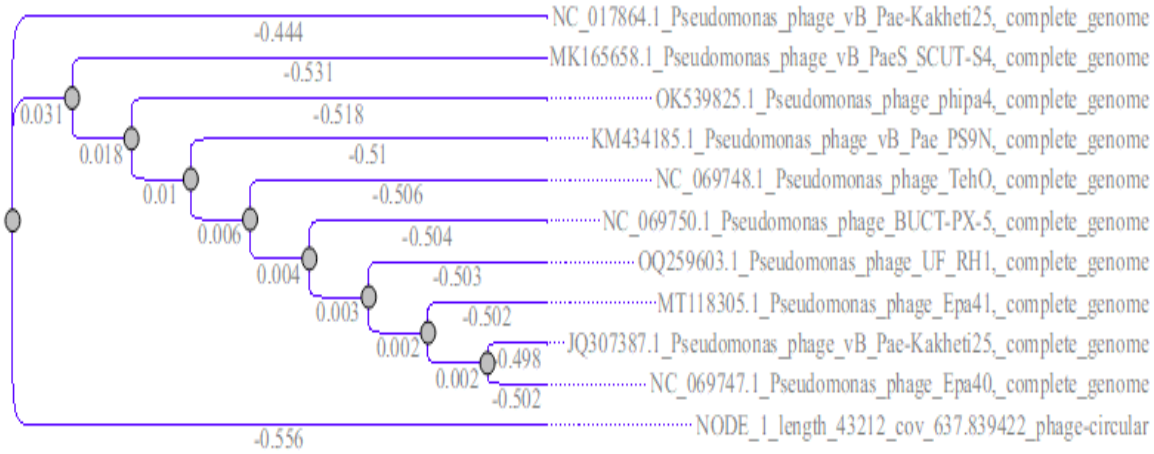


Figure 35: Phylogenetic analysis of *Pseudomonas* phage 6661

Given isolated phage showed the closest relationship with complete genome of *Pseudomonas phage Kakheti25* as it is connected with our phage through the single node. The number of nodes separating the two taxa represent the relatedness between the two taxa on phylogenetic analysis. (e.g. when there are three nodes separating two taxa, their relationship is more distant than when there are just two nodes (Dees *et al.,* 2014). Since our phage genome is not the complete genome so, we also evaluate the phylogenetic relationship between the different phage using two major protein one is major tail Protein and the other is capsid protein. Tree elucidating the evolutionary history of the phage is shown in the picture below.

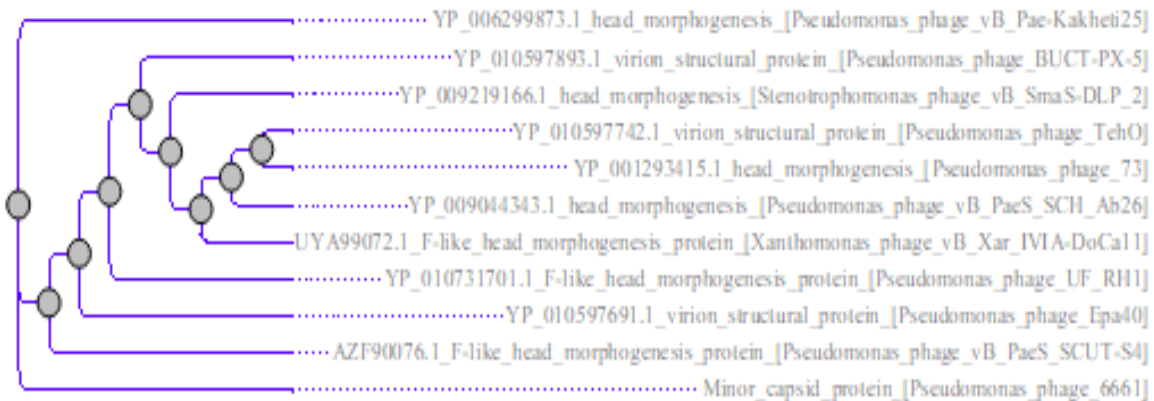


Figure 36: Phylogenetic analysis of Capsid proteins

4.10 Biofilm formation

Biofilm forming capability was assessed for the isolated phage 6661 and *Pseudomonas aeruginosa* (P1). Experiment was performed in a triplicate fashion in 96 well microtiter plate. With the aid of ELISA reader, OD was measured at 620 nm after the bacteria had been injected in the well for overnight. Only the culture media was employed as a negative control for the experiment. Stepanovic et al. protocols were employed for the assessment of the biofilm formation.

The data thus generated from the ELISA reader was interpreted on the basis of the following parameters.

OD>4 ODc = Strong Biofilm Producer

2ODc< OD≤ 4ODc = Moderate Biofilm producer

ODc< OD ≤ 2ODc = Weak Biofilm producer

OD ≤ ODc Non biofilm producer

To assess the biofilm forming ability, optical density cut off value (ODc) should be determined which is calculated using the following formulae.

ODc = Average OD of negative control + 3 × SD of negative control

Here, Average OD of negative control = 0.134

Also, Standard deviation of negative control =0.1214

ODc = 0.134 + 3 ×0.1214

=0.4982

2ODc= 0.9964

4ODc=1.9928

Avg OD of positive control bacteria OD=1.0436

Pseudomonas aeruginosa (P1) had an OD of 1.0436 which is greater than twice the optical density cutoff value but less than four times of (ODc). So, the *Pseudomonas aeruginosa* (p1) is a moderate biofilm producer whereas the isolated and characterized bacteria had

an OD of 0.134 which is less than ODc. Therefore, our isolated bacteria (Pseudomonas aeruginosa 6661) is weak biofilm producer. Since, on the host range analysis our isolated phage had shown the lytic activity against *Pseudomonas aeruginosa* (P1) so we selected the above bacteria for our biofilm disruption experiment.

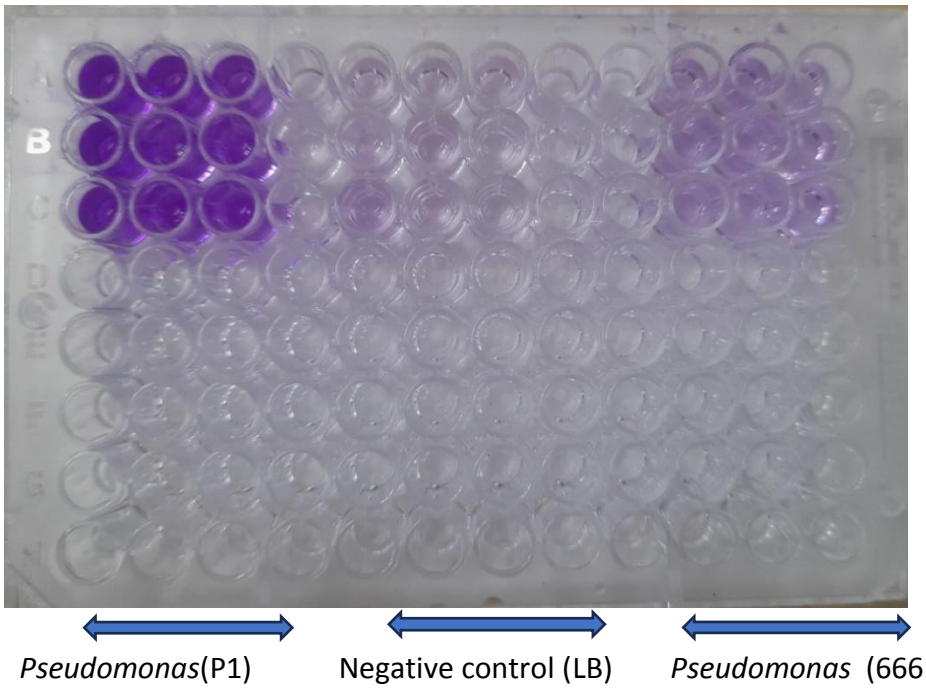


Figure 37: Microtiter plate showing the assessment of biofilm formation by *Pseudomonas* (P1) and *Pseudomonas* (6661)

4.11 Biofilm disruption

Biofilm disruption was interpreted according to protocol developed by Forti et al. To disrupt the biofilm of *Pseudomonas aeruginosa* (P1) we use the phage of *Pseudomonas aeruginosa* (6661) and analyzed the result. Firstly, the *Pseudomonas aeruginosa* (p1) was grown overnight in a microtiter plate and was hence treated with phage. Phage untreated bacterial well was used as control. Hence, the OD was measured at 620 nm in ELISA plate reader.

Average OD of phage untreated bacteria = 1.043

Average OD of phage treated bacteria = 0.517

Therefore, Percentage reduction of biofilm of P1 by Phage 6661 = 50.43%

Hence, the isolated phage was unable to completely eradicate the bacterial population however, it was able to reduce biofilm to some extent.

In this study, phage 6661 show promising result for the disruption of the biofilm formed by the Pseudomonas aeruginosa (P1) strain. Since, phages are highly diverse and they have adapted various mechanism to interact with various bacterial strains. Some of the properties of phages that aids in the biofilm disruption are as follows. These include biofilm matrix degradation by depolymerase enzyme, targeting specific surface structures, bacterial lysis mechanism and diversity of phage bacterial interaction. This property might have allowed for the targeting of the specific *Pseudomonas* strain responsible for the biofilm formation (Visnapuu *et al*., 2022). However, if we can understand the exact mechanism beside the interaction between the phage and bacteria, we can implicate the effective phage-based intervention in medical setting as well as other various areas to get rid of the biofilm forming Pathogenic bacteria (Chegini *et al*., 2020).

CHAPTER 5: SUMMARY

Multidrug resistance *Pseudomonas aeruginosa* was collected from Shukraraj Tropical and Infectious Disease Hospital which was further isolated in Central department of biotechnology Laboratory in different General-Purpose media. Identification of the culture bacteria was then done through biochemical test. The bacteria were further confirmed through Matrix Assisted Laser Desorption Ionization assay (MALDI-TOF) and 16S rRNA sequencing. On BLAST analysis of the Fasta sequence of phage, phage showed 98.22% similarity to *Pseudomonas* phage. Antibiotic susceptibility test was performed so as to determine the antibiotic resistant pattern of the bacterium. Thus, isolated *Pseudomonas aeruginosa* (6661) was resistant to all sorts of antibiotics including Carbapenems class. On further confirmation of the Antibiotic Susceptibility test using Vitek Compact system 2, the bacteria was found to be susceptible to only the last resort of the antibiotics that is Colistin. 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 *Pseudomonas aeruginosa* (6661) was isolated and further characterized. Phage 6661 was further purified and stock was prepared so as to preserve phage for future analysis. According to Ackermann et al. (2004), tailed phage was the most stable phage and demonstrate the longest survivorship: some of them continued to be viable even after 10-12 years at 4℃. pH and Temperature stability of the phage was determined. Phage was found to be stable at wide range of pH that is from pH 4 to 11 and maximally stable at pH of 7. Additionally, the isolated phage was found to be maximally stable at 37°c however the stability was in decreasing pattern with the increase in temperature. This was considered as one of the most significant parameters for the phage to be used as therapeutics. On one step growth curve analysis, burst size of the virus was found to be 96 viral particles per infected cell and the latent period of the virus was 30 min. Protein profiling of the bacteriophage revealed four distinct bands of protein of the size approximately equal to 30KDa, 45KDa, 50KDa and 55KDa respectively. Phage 6661 did not show intraspecific host range but showed intraspecific host range with another *Pseudomonas* strain (P1). Morphology of the phage was determined through Transmission electron microscopy and the phage was found to be of order Caudovirales.

Phage DNA was extracted and Agarose gel electrophoresis was performed so as to determine the size of phage genome. The size of phage genome was found to be greater than 10 kb.

Genome analysis of phage showed that the length of whole genome sequence of phage was found to be 43,113 nucleotide long with 53.79 % of the GC content. PHASTER analysis revealed that the phage genome lacks tRNA, integrase gene and any other Virulence gene in the genome whereas depicts the presence of the various functional gene such as lysis protein, tail shaft protein, portal protein and the capsid protein in the genome. Lack of integrase gene in the genome showed that the lytic phages are unlikely to integrate the phage genome for the transition into the Prophage state. Presence of the tail shaft protein in the genome suggest that the isolated phage is tailed phage. RAST genome annotation illustrate the absence of the toxic and the virulence gene in the genome. Different replication, recombination, repair protein is illustrated on Proksee analysis. Furthermore, various stability and defense protein are visualized along with one horizontally gene transfer region which should be further assessed for the Phage efficacy. On phylogenetic analysis our phage genome showed closest relationship with *Pseudomonas* phage Kakheti 25.

On Biofilm assessment of the phage, it was observed that Phage 6661 was unable to form biofilm however, it showed the lytic activity on the one *Pseudomonas* strain(P1) reducing the biofilm of the bacteria by 50.73 %.

CHAPTER 6: CONCLUSION

- Pathogenic MDR *Pseudomonas aeruginosa* was isolated from the hospital sample and was identified as Carbapenem resistant bacteria. Thus, isolated bacteria is only susceptible to last resort of the antibiotics that is Colistin.
- Lytic bacteriophage 6661 against MDR *Pseudomonas aeruginosa* was isolated possessing lytic property showing the pin head colonies on Tryptic soya Iron agar.
- Phage 6661 did not show interspecific host range but showed susceptibility to one *Pseudomonas* strain on host range analysis.
- The phage showed maximum stability at 37℃. However, the infectivity of the virus decreases with increase in temperature. The isolated phage thus seems to form plaques from pH range 4 to 11 and maximum stability to was shown at pH 7.
- Burst size of the virus was found to be 96 virions per infected cell and the isolated phage was found to be of order Caudovirales.
- The isolated *Pseudomonas aeruginosa* (6661) was non- biofilm producer however, it showed the activity against another *Pseudomonas* strain (P1) (Moderate biofilm producer and reduced it by 50.73%.
- On whole genome analysis, it was confirmed that the isolated virus lacks any toxic or virulence in the genome. Presence of endolysin and absence of integrase enzyme also favors the therapeutic potential of the virus.

CHAPTER 7: LIMITATIONS AND RECOMMENDATIONS

7.1 Limitations of the study

1. Out of three isolated *Pseudomonas aeruginosa*, only one strain was extensively studied due to constraint of time and resources.
2. Whole genome sequence analysis is time demanding and expertise requiring task but due to the limitations of the both, we cannot fully annotate our genome through bioinformatic analysis.
3. MazG protein was present in our phage genome which generally used to be present in the bacterial genome. Cloning and removal of that protein would have help us to know the utility or effect of that protein in our phage.

7.2 Future Recommendations

In this study only morphological, physiochemical and to some extent molecular characteristics of the phage were determined. Biofilm forming capability of the bacteria and biofilm disrupting ability of the phage was also assessed still there are some limitations of this study for which following recommendations are suggested.

1. Study should not be limited to one bacterium; it should be conducted in more than one host MDR bacteria.
2. Extensive genome analysis and whole genome submission to GenBank is yet to be done. Better findings and conclusions will have come from a study if it has been conducted in collaboration with a bioinformatics expert.
3. Since endolysin is a crucial enzyme involved in the host lysis mechanism. Lysin therapy might be improved much like phage- therapy, if in-depth-study on endolysin gene cloning could be conducted.
4. If animal model study could be conducted, it would produce trustworthy and significant data to the assertion that we had made through our study.

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PHOTOGRAPHS

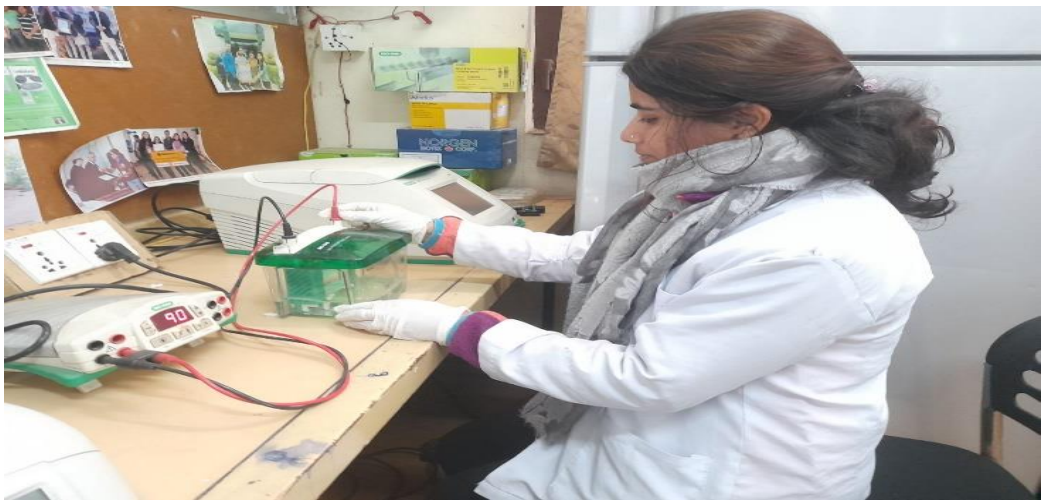


Figure: Performing SDS PAGE

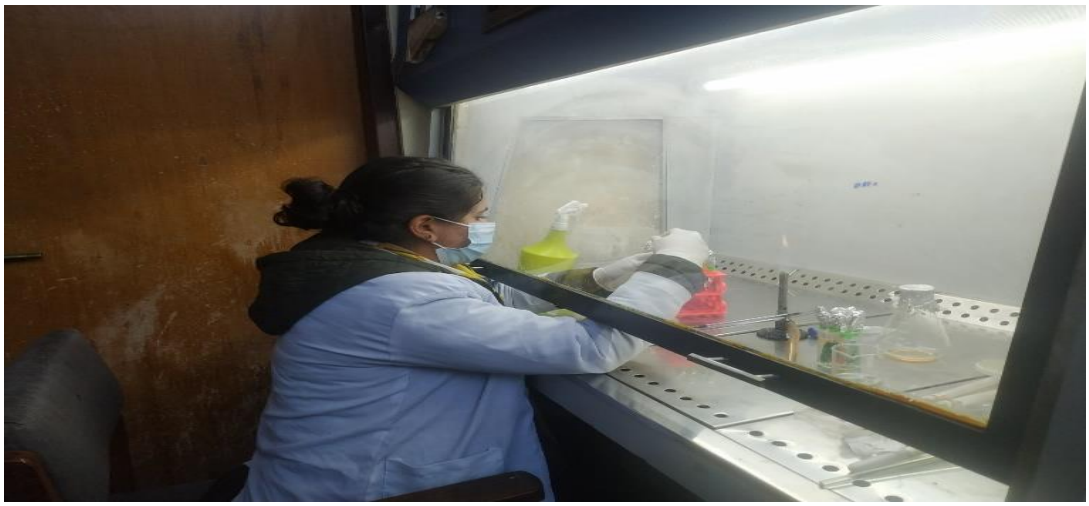
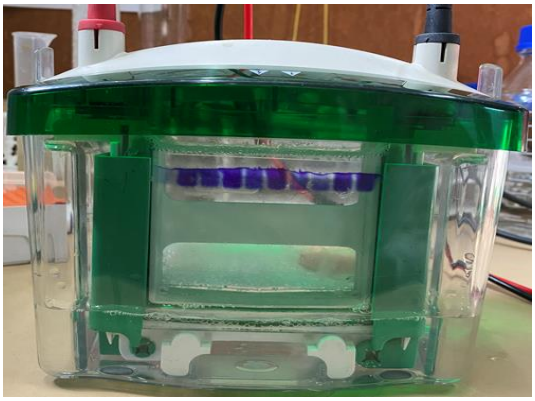


Figure: Performing biochemical test

Figure: Phage team (2075)

ELISA Plate Reader

Centrifuge



SDS electrophoretic tank

MIseq illumina sequencer

PCR

Preparation of Reagents

I) SM buffer

i)	Water	800ml
ii)	Sodium chloride	5.8 gram
iii)	Magnesium sulphate	2 gram
iv)	1 molar Tris cl PH (7.5)	50ml
v)	2% gelatin	5ml

Then the Final volume is made 1000 ml.

II) Phosphate buffered saline

Salt		Concentration (g/l)
i)	NaCl	8
ii)	Kcl	0.2
iii)	Disodium hydrogen phosphate	1.42
iv)	Potassium dihydrogen phosphate	0.24

All of the required components are dissolved in 800 ml of water and the final volume is maintained to 1 liter.

III) Molar Tris base HCL (for 100 ml)

i)	Tris base	12.11 gram
ii)	Water	80 ml
iii)	HCL	7ml

IV) Tris Acetate EDTA buffer

i)	Tris base	242 grams
ii)	Acetic acid	57.1 ml
iii)	EDTA	18.612 gram

50X solution of TAE buffer is prepared by dissolving 242 gram of Tris base in water. To this solution, 57.1 ml of glacial acetic acid and 100 ml of 500mM EDTA (PH 8.0) solution, maintaining the final volume to one liter. Thus, prepared stock solution is diluted in the ratio of 49:1 with water to make 1X working solution.

Preparation of SDS reagent

30 % Acrylamide solutions

Constituents		Weight in gram
i)	Acrylamide	29 gram
ii)	Bis acrylamide	1 gram
iii)	Distilled water	100ml

Upper Tris buffer (PH 6.8)

i)	Tris base	3.03 gram
ii)	Distilled water	50 ml

Lower Tris buffer (PH 8.8)

I)	Tris base	18.17 gram
II)	Distilled water	100

Comassie - Brilliant Blue (500 ml)

I)	Comassie Brilliant Blue	500mg
II)	Glacial acetic acid	25 ml
III)	Methanol	250 ml
IV)	Distilled water	225 ml

Loading dye

I)	Upper Tris (PH 6.8)	1.25 ml
II)	10% SDS	3.0 ml
III)	Glycerol	4.75ml
IV)	Beta Mercaptoethanol	0.5 ml
V)	0.1% Bromophenol blue	0.5 ml

Destaining solution (500ml)

I)	7.5% Glacial acetic acid	37.5 ml
II)	5% methanol	25 ml
III)	Distilled water	437.5 ml

Running Buffer (1000 ml) PH 8.4

39mM Tris	4.72
48mM glycine	3.603
0.1% SDS	0.37 g

5% Resolving gel (5 ml)

I)	Water	3.4 ml
II)	30% acrylamide	0.83 ml
III)	1 M Tris HCL PH (6.8)	0.63 ml
IV)	10% SDS	0.05 ml
V)	10% (NH4)2S2O8	0.05 ml
VI)	TEMED	0.005ml

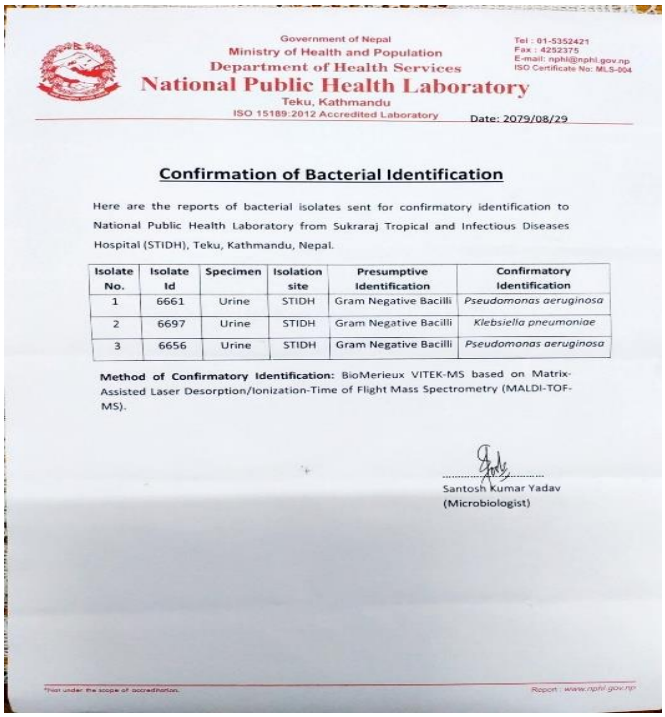
12% Resolving gel (10ml)

I)	Water	3.3 ml
II)	30% acrylamide	4 ml
III)	1 M Tris HCL PH (6.8)	2.5 ml
IV)	10% SDS	0.1ml
V)	10% (NH4)2S2O8	0.1 ml
VI)	TEMED	0.004 ml

TE Buffer

I)	Tris base (1M) PH 10-11	1 ml
II)	EDTA (0.5M)	0.2ml

Confirmation of bacteria through Mass Spectroscopy analysis



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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*

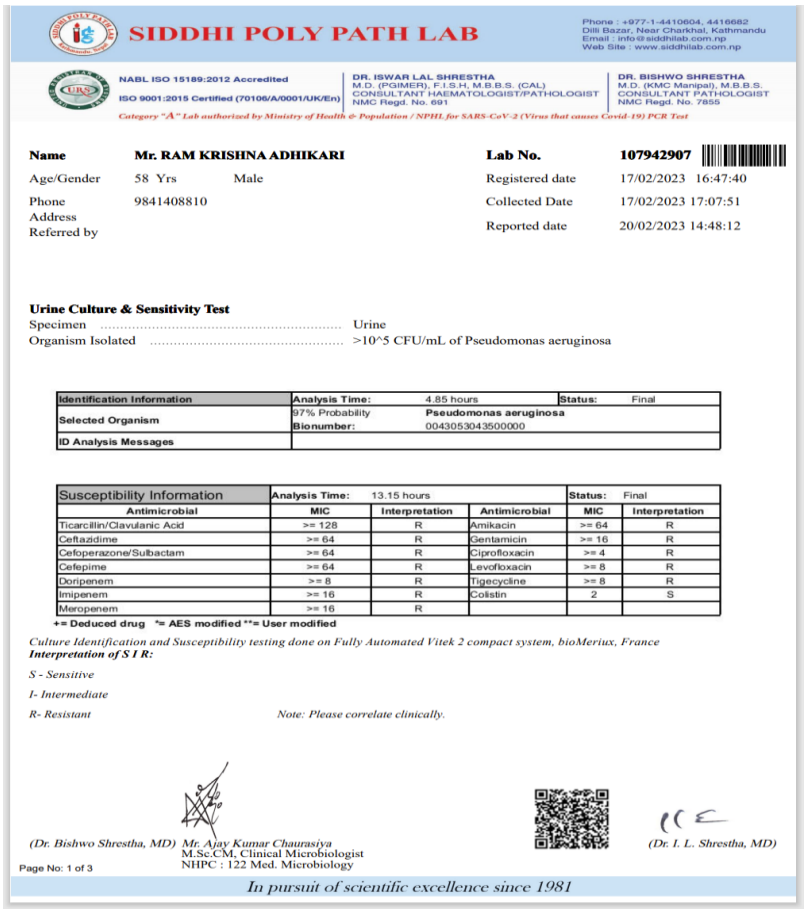
Method of Confirmatory Identification: BioMerieux VITEK-MS based on Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS).

Santosh Kumar Yadav
(Microbiologist)

*Test under the scope of accreditation.

Report : www.nphl.gov.np

Antibiotic susceptibility test using Compact Vitek system 2



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ISO 9001:2015 Certified (70106/A/0001/UK/En)

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

Name	**Mr. RAM KRISHNA ADHIKARI**	**Lab No.**	**107942907**
Age/Gender	58 Yrs Male	Registered date	17/02/2023 16:47:40
Phone	9841408810	Collected Date	17/02/2023 17:07:51
Address		Reported date	20/02/2023 14:48:12
Referred by			

Urine Culture & Sensitivity Test

Specimen ..... Urine
Organism Isolated ..... >10^5 CFU/mL of Pseudomonas aeruginosa

Identification Information	Analysis Time:	4.85 hours	Status:	Final
Selected Organism	97% Probability	**Pseudomonas aeruginosa**		
	Bionumber:	0043053043500000		
ID Analysis Messages				

Susceptibility Information	Analysis Time:	13.15 hours		Status:	Final
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Ticarcillin/Clavulanic Acid	>= 128	R	Amikacin	>= 64	R
Ceftazidime	>= 64	R	Gentamicin	>= 16	R
Cefoperazone/Sulbactam	>= 64	R	Ciprofloxacin	>= 4	R
Cefepime	>= 64	R	Levofloxacin	>= 8	R
Doripenem	>= 8	R	Tigecycline	>= 8	R
Imipenem	>= 16	R	Colistin	2	S
Meropenem	>= 16	R			

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

Culture Identification and Susceptibility testing done on Fully Automated Vitek 2 compact system, bioMeriux, France
Interpretation of S I R:

S - Sensitive
I- Intermediate
R- Resistant

Note: Please correlate clinically.

(Dr. Bishwo Shrestha, MD) *Mr. Ajay Kumar Chaurasiya*
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NHPC : 122 Med. Microbiology

(Dr. I. L. Shrestha, MD)

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Temperature stability curve

SN	Time	Temperature ℃				
		PFU/ml 37℃	PFU/ml 40℃	PFU/ml 50℃	PFU/ml 60℃	Pfu/ml 70℃
1	10	110	72	62	58	45
2	20	95	68	58	47	43
3	30	74	56	48	45	28
4	40	58	32	23	42	22
5	50	53	35	21	31	18
6	60	42	21	20	26	10

pH stability curve

SN	pH	PFU/ml
1	2	0
2	3	0
3	4	15
4	5	18
5	6	31
6	7	38
7	8	33
8	9	31
9	10	25

10	11	21
11	12	13

One Step growth curve analysis of phage

SN	**Time**	**PFU/ml**
1	5	5
2	10	15
3	20	27
4	30	46
5	40	185
6	50	192
7	60	196

Biofilm assessment of Phage

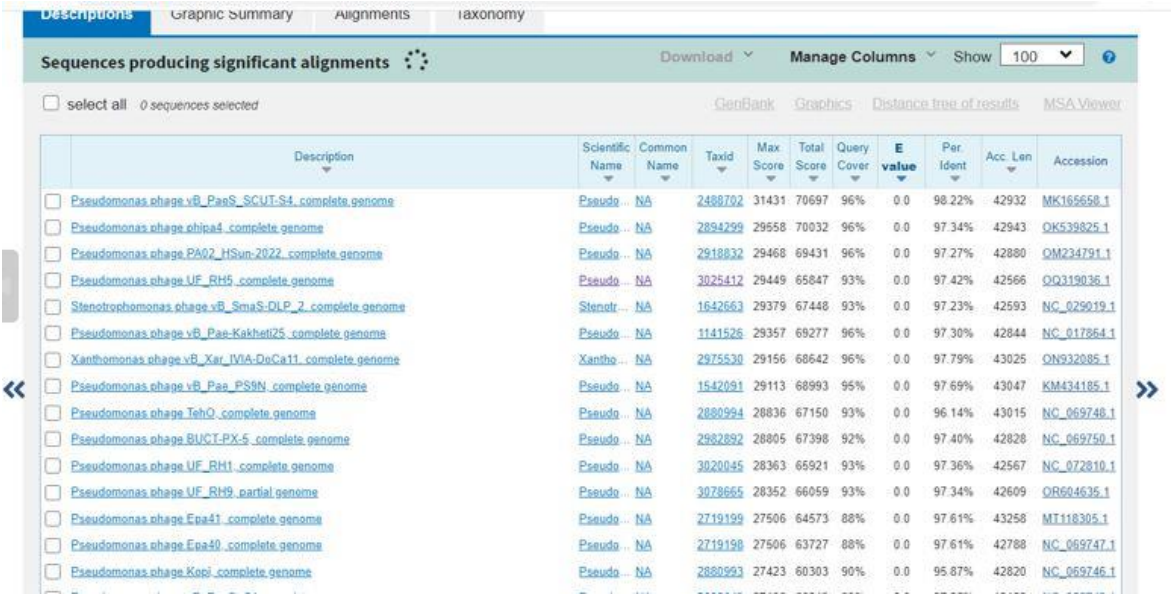
SN	OD (*Pseudomonas* 6661)			LB (Negative Control)			OD (*Pseudomonas* p1)		
1	1.415	0.987	0.808	0.122	0.136	0.202	0.182	0.202	0.137
2	1.506	0.884	0.709	0.147	0.133	0.078	0.233	0.277	0.201
3	1.257	0.943	0.884	0.191	0.115	0.085	0.221	0.221	0.236

Biofilm disruption by phage

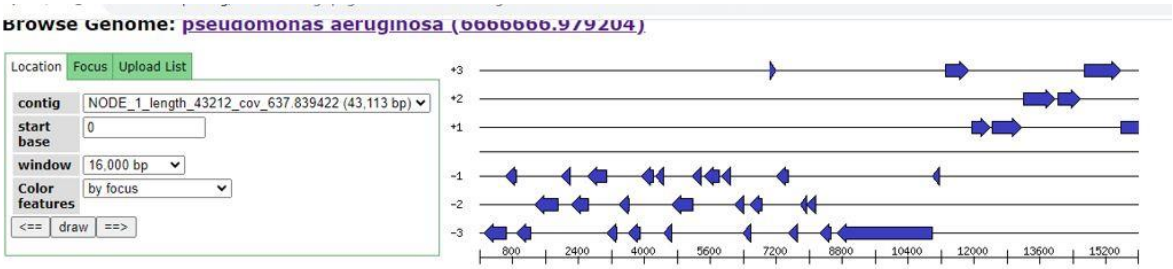
SN	**OD after Phage treatment Pseudomonas (P1)**		
1	0.625	0.458	0.427

2	0.415	0.549	0.534
3	0.671	0.503	0.471

BLAST result of the FASTA sequence of phage



RAST genome annotation of phage



Annotated components of phage

Feature ID	Type	Contig	Start	Stop	Length (bp)	Function	Subsystems	Region
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fig|6666666.979204.peg.2	CDS	NODE_1_length_43212_cov_637.839422	903	637	267	Phage protein	- none -	show
fig|6666666.979204.peg.3	CDS	NODE_1_length_43212_cov_637.839422	1232	909	324	hypothetical protein	- none -	show
fig|6666666.979204.peg.4	CDS	NODE_1_length_43212_cov_637.839422	1909	1337	573	Phage protein	- none -	show
fig|6666666.979204.peg.5	CDS	NODE_1_length_43212_cov_637.839422	2202	1993	210	Phage protein	- none -	show
fig|6666666.979204.peg.6	CDS	NODE_1_length_43212_cov_637.839422	2647	2237	411	Phage protein	- none -	show
fig|6666666.979204.peg.7	CDS	NODE_1_length_43212_cov_637.839422	3093	2626	468	dCMP deaminase (EC 3.5.4.12)	- none -	show
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fig|6666666.979204.peg.9	CDS	NODE_1_length_43212_cov_637.839422	3622	3389	234	Phage protein	- none -	show
fig|6666666.979204.peg.11	CDS	NODE_1_length_43212_cov_637.839422	4221	3922	300	Phage protein	- none -	show
fig|6666666.979204.peg.12	CDS	NODE_1_length_43212_cov_637.839422	4461	4282	180	hypothetical protein	- none -	show
fig|6666666.979204.peg.13	CDS	NODE_1_length_43212_cov_637.839422	4661	4458	204	hypothetical protein	- none -	show
fig|6666666.979204.peg.14	CDS	NODE_1_length_43212_cov_637.839422	5170	4661	510	Phage protein	- none -	show
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Feature ID	Type	Contig	Start	Stop	Length (bp)	Function	Subsystems	Region
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fig|6666666.979204.peg.19	CDS	NODE_1_length_43212_cov_637.839422	6575	6396	180	hypothetical protein	- none -	show
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fig|6666666.979204.peg.25	CDS	NODE_1_length_43212_cov_637.839422	8152	7946	207	hypothetical protein	- none -	show
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fig|6666666.979204.peg.32	CDS	NODE_1_length_43212_cov_637.839422	13214	13969	756	hypothetical protein	- none -	show

«first «prev displaying 31 - 45 of 58 next» last»

Feature ID	Type	Contig	Start	Stop	Length (bp)	Function	Subsystems	Region
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fig|6666666.979204.peg.35	CDS	NODE_1_length_43212_cov_637.839422	15571	17226	1656	ATP-dependent RNA helicase YejH	- none -	show
fig|6666666.979204.peg.36	CDS	NODE_1_length_43212_cov_637.839422	17231	17458	228	Phage protein	- none -	show
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fig|6666666.979204.peg.38	CDS	NODE_1_length_43212_cov_637.839422	18596	20647	2052	DNA polymerase B region	- none -	show
fig|6666666.979204.peg.39	CDS	NODE_1_length_43212_cov_637.839422	23089	20648	2442	Phage protein	- none -	show
fig|6666666.979204.peg.40	CDS	NODE_1_length_43212_cov_637.839422	23917	23093	825	Phage FAD/FMN-containing dehydrogenase	- none -	show
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fig|6666666.979204.peg.42	CDS	NODE_1_length_43212_cov_637.839422	26568	25636	933	Phage protein	- none -	show
fig|6666666.979204.peg.43	CDS	NODE_1_length_43212_cov_637.839422	27530	26568	963	Phage protein	- none -	show
fig|6666666.979204.peg.44	CDS	NODE_1_length_43212_cov_637.839422	31145	27531	3615	Phage tail length tape-measure protein	- none -	show
fig|6666666.979204.peg.45	CDS	NODE_1_length_43212_cov_637.839422	31585	31151	435	Phage protein	- none -	show
fig|6666666.979204.peg.46	CDS	NODE_1_length_43212_cov_637.839422	31910	31554	357	Phage protein	- none -	show
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Feature ID	Type	Contig	Start	Stop	Length (bp)	Function	Subsystems	Region
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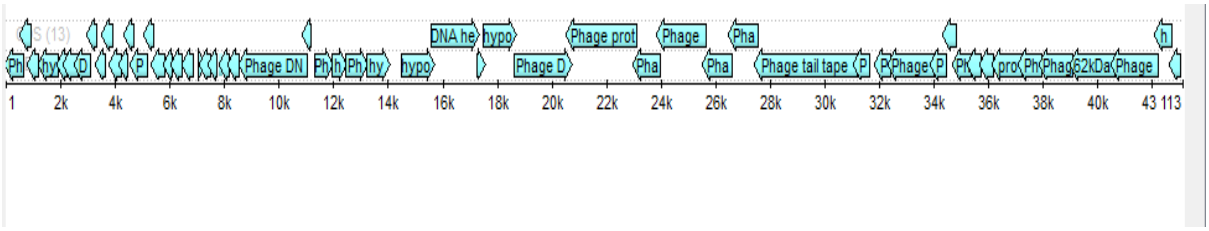
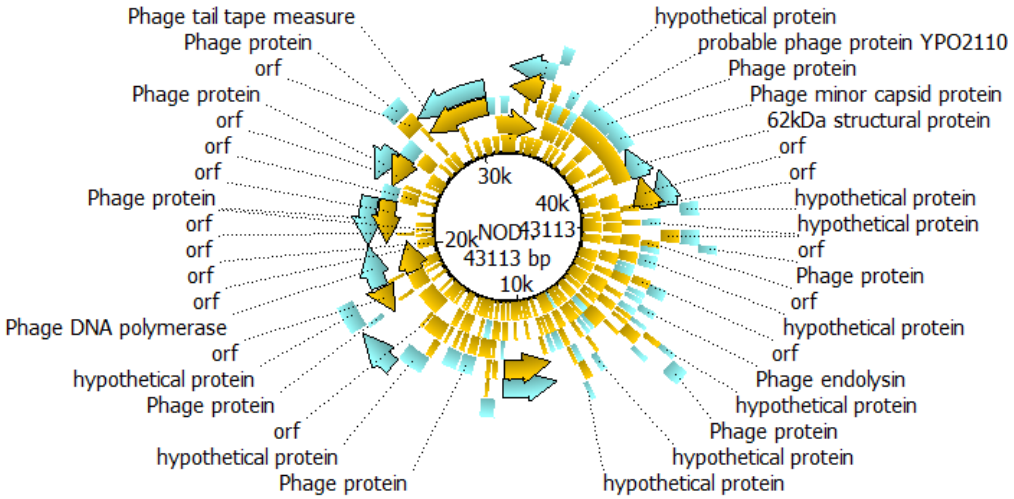
fig|6666666.979204.peg.34	CDS	NODE_1_length_43212_cov_637.839422	14682	15578	897	Phage protein	- none -	show
fig|6666666.979204.peg.35	CDS	NODE_1_length_43212_cov_637.839422	15571	17226	1656	ATP-dependent RNA helicase YejH	- none -	show
fig|6666666.979204.peg.36	CDS	NODE_1_length_43212_cov_637.839422	17231	17458	228	Phage protein	- none -	show
fig|6666666.979204.peg.37	CDS	NODE_1_length_43212_cov_637.839422	17442	18584	1143	Phage protein	- none -	show
fig|6666666.979204.peg.38	CDS	NODE_1_length_43212_cov_637.839422	18596	20647	2052	DNA polymerase B region	- none -	show
fig|6666666.979204.peg.39	CDS	NODE_1_length_43212_cov_637.839422	23089	20648	2442	Phage protein	- none -	show
fig|6666666.979204.peg.40	CDS	NODE_1_length_43212_cov_637.839422	23917	23093	825	Phage FAD/FMN-containing dehydrogenase	- none -	show
fig|6666666.979204.peg.41	CDS	NODE_1_length_43212_cov_637.839422	25632	23917	1716	Phage protein	- none -	show
fig|6666666.979204.peg.42	CDS	NODE_1_length_43212_cov_637.839422	26568	25636	933	Phage protein	- none -	show
fig|6666666.979204.peg.43	CDS	NODE_1_length_43212_cov_637.839422	27530	26568	963	Phage protein	- none -	show
fig|6666666.979204.peg.44	CDS	NODE_1_length_43212_cov_637.839422	31145	27531	3615	Phage tail length tape-measure protein	- none -	show
fig|6666666.979204.peg.45	CDS	NODE_1_length_43212_cov_637.839422	31585	31151	435	Phage protein	- none -	show
fig|6666666.979204.peg.46	CDS	NODE_1_length_43212_cov_637.839422	31910	31554	357	Phage protein	- none -	show
fig|6666666.979204.peg.47	CDS	NODE_1_length_43212_cov_637.839422	32347	31919	429	Phage tail fibers	- none -	show

Ugene genome annotation

Linear form of DNA obtained from Ugene

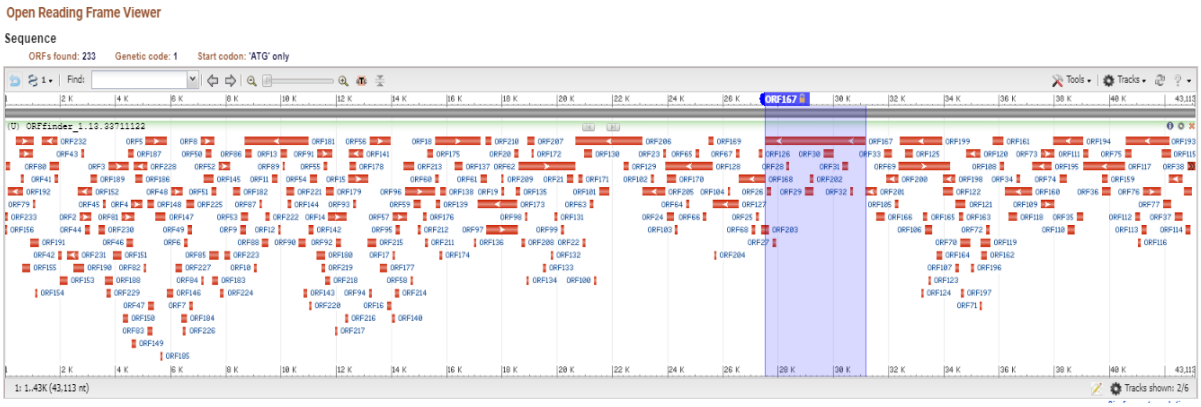


ORF prediction from Ugene



No of ORF predicted was found to be 197 taking minimum base length as 100.

Comparative analysis from ORF finder



No of ORF Predicted was found to be 233.

tRNA prediction from tRNAscan.se

Predicted tRNA genes	
tRNAs decoding Standard 20 AA	0
Selenocysteine tRNAs (TCA)	0
Possible suppressor tRNAs (CTA,TTA,TCA)	0
tRNAs with undetermined/unknown isotypes	0
tRNAs with mismatch isotypes	0
Predicted pseudogenes	0
Total tRNAs	0