PHYSIOCHEMICAL AND GENOMIC CHARACTERIZATION OF BACTERIOPHAGE AGAINST COLISTIN RESISTANT *Acinetobacter baumannii* TO ASSESS ITS POSSIBLITY FOR PHAGE THERAPY

M.Sc. Thesis

(2024)

Submitted to:

CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
Kirtipur, Kathmandu, Nepal

For the partial fulfillment of Masters of Science in Biotechnology

Submitted by:

Shobha Amagain
Exam Roll No: BT 615/075
TU Registration No: 5-2-0282-0174-2013

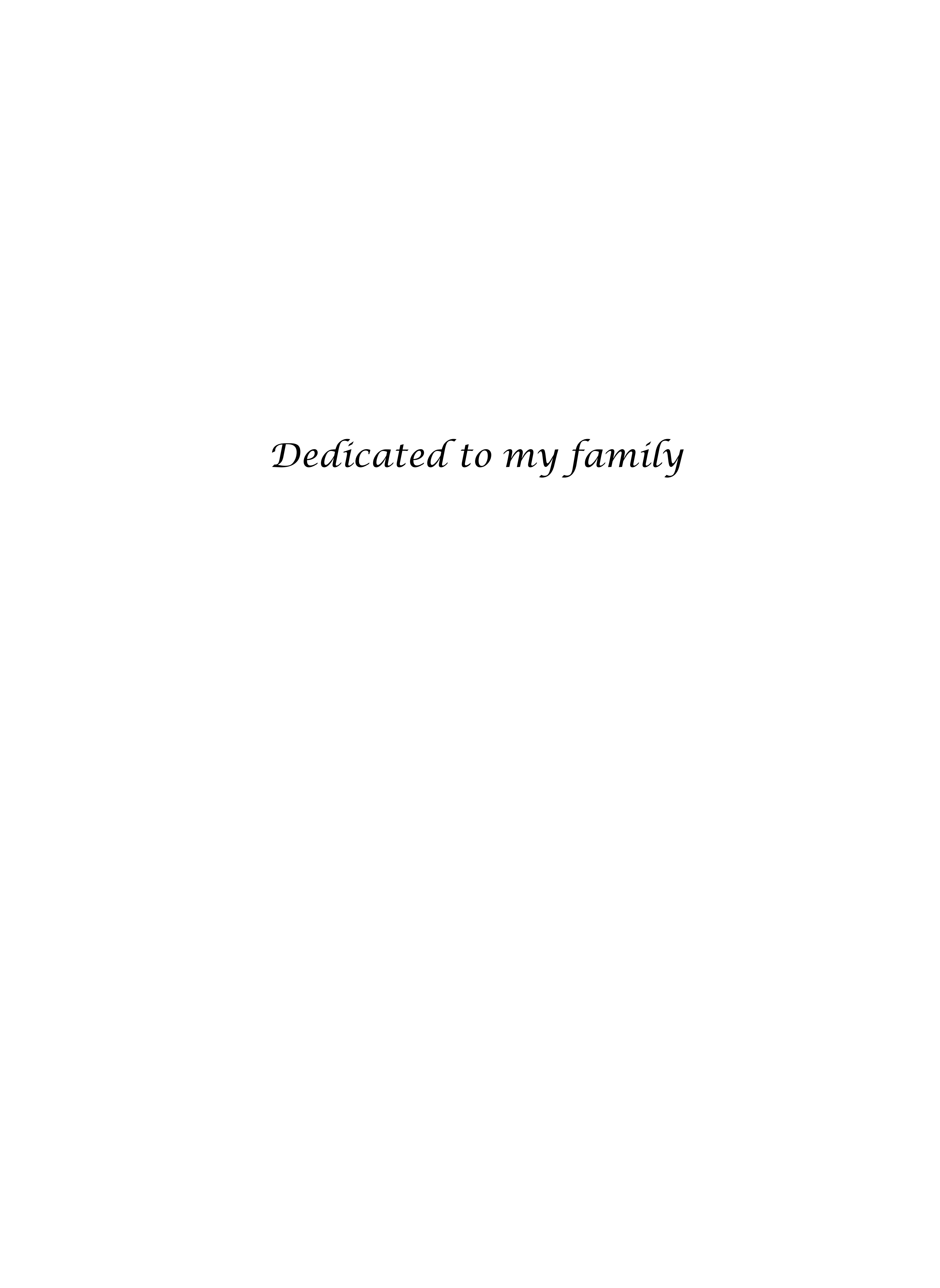
Supervisors:

Asst Prof. Pragati Pradhan
Prof. Rajani Malla

Tribhuvan University
Central Department of Biotechnology
Kirtipur, Kathmandu, Nepal

Co-Supervisors:

Mr. Rajindra Napit
Mr. Dipendra Mandal



Dedicated to my family

ACKNOWLEDGEMENT

At first, I am deeply grateful to my supervisors **Asst. Prof. Pragati Pradhan and Prof. Dr. Rajani Malla**, for guiding me throughout my dissertation with continuous encouragement. This thesis would not have been completed without their invaluable effort and suggestion.

I am thankful to Mr. **Rajindra Napit** and Mr. **Roshan Nepal** for their support and advice throughout this period. Their vision towards any research work was the continuous source of inspiration for me.

I am also grateful to the Head of the Department **Prof. Dr. Krishna Das Manandhar**, Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal for approving my research project.

I would like to recognize all faculty members. I am grateful to Mr. **Dipendra Mandal** and Mr. **Gorakh Giri** along with all teaching and non-teaching staffs of Central Department of Biotechnology.

I am thankful to all members of CDBT who helped me either through their sound suggestion or by granting access to lab equipment for completing many of my research experiments. I extend my heartfelt gratitude to Ms. **Roji Raut** for her invaluable support.

I extend my warmest thanks to my co-researchers Ms. **Puja Dahal** and Ms. **Pragya Sapkota** for their endless support throughout my dissertation.

I am thankful to my friends of 10th Batch, CDBT, along with my all seniors and juniors.

Finally, I am heartily thankful to **my parents**, **my whole family** and **my friends**, whose love and support helped me get through all of the toughest moments to perform better.

……………………………

SHOBHA AMAGAIN

ACRONYMS

µg:	microgram
µl:	microliter
AST:	Antibiotic Sensitivity Test
AMR:	Antimicrobial Resistance
BLAST:	Basic Local Alignment Search Tool
CDC:	Centers for Disease Control and Prevention
CFU:	Colony Forming Unit
DLAA:	Double Layer Agar Assay
dsDNA:	double stranded Deoxyribonucleic Acid
FDA:	Food and Drug Administration
gDNA:	genomic Deoxyribonucleic Acid
ICTV:	International Committee on Taxonomy of Viruses
MDR:	Multidrug Resistance
MHA:	Muller Hilton Agar
MOI:	Multiplicity of Infection
NGS:	Next Generation Sequencing
NCBI:	National Center for Biotechnology Information
NPHL:	National Public Health Laboratory
ORF:	Open Reading Frame
Pfu:	Plaque forming unit
PHASTEST:	Phage Search Tool Enhanced Release
SM buffer:	Sodium Magnesium buffer
WGS:	Whole Genome Sequencing
ZOI:	Zone of Inhibition

TABLE OF CONTENTS

ACKNOWLEDGEMENT **i**

ACRONYMS **ii**

TABLE OF CONTENTS **iii**

LIST OF FIGURES **vii**

LIST OF TABLES **ix**

ABSTRACT **x**

CHAPTER 1: INTRODUCTION **1**

1.1 Background 1

1.1.1 Bacteriophage 2

1.1.2 Bacteriophage History 4

1.1.3 Bacteriophage Lifecycle 5

1.1.4 Bacteriophage classification 7

1.1.5 Phage Therapy 7

1.1.6 *Acinetobacter baumannii* 8

1.1.7 Virulence of Acinetobacter baumannii 9

1.1.8 Mechanism of resistance in *Acinetobacter baumannii* 10

1.1.9 Resistance to polymyxins B and E (colistin) 10

1.2 Rationale of the study 11

1.3 Objectives 12

1.3.1 General Objective 12

1.3.2 Specific Objectives 12

1.4 Research Hypothesis 12

1.4.1 Null Hypothesis (Ho) 12

1.4.2 Alternative Hypothesis (H1) 12

CHAPTER 2: LITERATURE REVIEW **13**

2.1 Antimicrobial Resistance 13

2.2 *Acinetobacter baumannii* and Its MDR Property 13

2.3 Bacteriophages and Discovery of Bacteriophages 14

2.4 Interaction between phage and bacteria 15

2.5 Phage Therapy and Its Advantages 16

2.6 Resurgence of Phage Therapy 16

2.7 Advantages of Phage Therapy Over Antibiotics 18

2.8 Current Studies and Recent Advancements in Phage Research 19

2.9 Phage-Encoded Enzymes for the Treatment of *A. baumannii* 21

2.9.1 Endolysins 21

2.9.2 Depolymerases 21

2.10 Phage Enumeration Method 21

2.11 Molecular Detection of Bacteria 22

2.12 Whole genome sequencing 22

2.13 MLST (Multilocus Sequence Typing) 23

CHAPTER 3: MATERIALS AND METHODS **24**

3.1 Laboratory Setting 24

3.2 Collection of Bacterial Strain and Preservation 24

3.3 Identification of bacteria by Gram Staining and Biochemical Tests 24

3.4 Antibiotic Susceptibility Testing (AST) 25

3.5 Bacterial Genomic DNA Extraction 26

3.6 Molecular Detection of Bacteria by VITEK 26

3.7 Genomic Confirmation of Bacteria by MLST 27

3.8 Sewage Sample Collection and Processing 27

3.9 Clonal Purification of Phage (Phage Streak Protocol) 27

3.10 Preparation of Phage Lysate 28

3.11 Phage Titer Assay: Spot Assay and DLLA Method 29

3.12 Titration of Phage 29

3.13 Host Range Analysis: Spot Assay 30

3.14 Characterization of Phage 30

3.15 Effect of temperature on phage viability 31

3.16 Effect of pH on Phage Viability 31

3.17 Growth Curve 31

3.18 Phage DNA Extraction 32

3.19 Isolation, Qualitative and Quantitative Analysis of gDNA 33

3.19.1 Library preparation of phage 34

3.20 Whole Genome Sequencing 34

CHAPTER 4: RESULTS AND DISCUSSION 35

4.1 Identification of Host Bacteria 35

4.2 Gram Staining 35

4.3 Biochemical Test of Host Bacteria 36

4.3.1 Catalase test 36

4.3.2 Oxidase test 37

4.3.3 IMViC test 37

4.3.4 Growth at 44°C 39

4.4 Antibiotic Sensitivity Test (AST) 39

4.4.1 Antibiogram Assay 39

4.4.2 Confirmation of Antibiotic susceptibility using Vitek Compact System 2 41

4.5 Genomic DNA Extraction 41

4.6 Quantification and purity check of isolated Bacterial DNA 42

4.7 Molecular Identification of Host 42

4.8 Whole Genome Sequencing of Host ACB-1 43

4.9 BLAST Result 43

4.10 Genome analysis of ACB-1 by PROKSEE 44

4.11 MLST profile of *Acinetobacter baumannii* (ACB-1) 44

4.12 PHAGE ISOLATION, SCREENING AND MANIPULATIONS 45

4.12.1 Sample Collection and Isolation of Bacteriophage by DLAA Method 45

4.12.2 Clonal Purification of Bacteriophage 47

4.12.3 Determination of Phage Titer/ Spot assay 48

4.13 Physiochemical characterizations of phage 49

4.13.1 Multiple Host range analysis of Phage 49

4.14 Stability of phage at different pH range 50

4.15 Stability of the phage at different temperature 51

4.16 One step growth curve of phage 52

4.17 Whole Genome Sequencing 54

4.17.1 Isolation, Qualitative and Quantitative Analysis of gDNA: 54

CHAPTER 5: SUMMARY 61

CHAPTER 6: CONCLUSION 63

CHAPTER 7: RECOMMENDATION 64

REFERENCES 65

APPENDIX 71

LIST OF FIGURES

Figure 1.1 Diagrammatic Representation of Bacteriophage 2

Figure 1.2 Life Cycle of Bacteriophages 6

Figure 2.1 Phage therapy reports and phage studies by year listed 17

Table 3.1 Antibiotic Susceptibility Test; Antibiotics and Concentration of Discs 25

Figure 4.1 ACB-1 on NA 35

Figure 4.2 Gram Staining of ACB-1 36

Figure 4.3 Catalase Test with Positive Result for ACB-1 37

Figure 4.4 Oxidase test with negative result for ACB-1 37

Figure 4.5 IMVIC Test of ACB-1 38

Figure 4.6 Growth of ACB-1 at 44°C 39

Figure 4.7 Antibiogram Assay of ACB-1 41

Figure 4.8 Gel Electrophoresis of Extracted DNA viewed under UV light 41

Figure 4.9 Molecular Identification of Host by Vitek Method 42

Figure 4.10 FASTA file analyzed using the BLAST Program 43

Figure 4.11Circular diagram of Host Bacteria genome using PROKSEE 44

Figure 4.12 MLST profile of ACB-1 44

Figure 4.13 Sample Collection Site (Bishnumati River, Gongabu) 46

Figure 4.14 Purification of Phage 47

Figure 4.15 Determination of Phage Titre of ACB-1 48

Figure 4.16 Host Range Analysis 50

Figure 4.17 Stability of the ACB-1 phage at different temperatures 52

Figure 4.18 One-step Growth Curve of Phage 54

Figure 4.19 Electrophoresis of phage DNA 55

Figure 4.20 PHASTEST generated circular Genome Map 56

Figure 4.21 PHASTEST generated Linear Genome Map 57

Figure 4.22 PHASTEST generated circular Genome Map 58

Figure 4.23 PHASTEST generated Linear Genome Map 58

Figure 4.24 PHASTEST generated circular Genome Map 59

Figure 4.25PHASTEST generated Linear Genome Map 59

LIST OF TABLES

Table 3.1 Antibiotic Susceptibility Test; Antibiotics and Concentration of Discs 25

Table 4.1 Biochemical Tests of ACB-1 36

Table 4.2 Result of IMVIC Test of ACB-1 38

Table 4.3 Antibiotic Susceptibility Pattern of ACB-1 bacteria 40

Table 4.4 Concentration Determination of Extracted DNA 42

Table 4.5 Phage and their site of isolation 45

Table 4.6 Plaque morphology of Phage ACB-1 47

Table 4.7 Phage Titre of ACB-1 48

Table 4.8 Multiple host range of ACB-1 Phage 49

Table 4.9 Concentration Determination of Extracted DNA of ACB-1 phage 55

Table 4.10 PHASTEST generated circular Genome summary of ACB-1 phage 1 56

Table 4.11 PHASTEST generated Genome summary of ACB-1 phage 2 57

Table 4.12 PHASTEST generated genome summary of ACB-1 Phage 3 59

Graph 4.1 pH stability of Phage ACB-1 50

Graph 4.2 Thermal stability of Phage ACB-1 51

Graph 4.3 One Step Growth Curve of ACB-1 Phage 53

ABSTRACT

Introduction: Antimicrobial drug resistance in pathogenic bacteria has become a global concern, with *Acinetobacter baumannii* posing a significant threat in hospital-acquired infections. Designated as a Priority-1 critical pathogen by the WHO since 2017, there's a pressing need to develop new antimicrobial agents against it. Both Europe and America have been exploring bacteriophages as potential remedies for antibiotic-resistant infections in recent years. In the current scenario, antibiotic resistance has become prevalent across various antibiotics, including the last resort options like carbapenems and colistin. As a result, utilizing phages in therapeutic applications to combat antimicrobial resistance emerges as a potential solution to the increasing global threat posed by multidrug-resistant bacteria. Of significant concern is the prevalence of multidrug-resistant *Acinetobacter baumannii*, a pathogen commonly associated with hospital-acquired infections. This study aims to isolate and comprehensively characterize a phage, both in terms of its physical and genetic properties, with the objective of harnessing its potential for phage therapy against colistin resistant *Acinetobacter baumannii.*

Methodology: A single strain of multidrug-resistant *Acinetobacter baumannii* was obtained from the hospital and identified it using MLST (Multilocus Sequence Typing) profiling. Colistin resistance was identified using the Vitek Compact System 2 analyzer. Lytic phage was isolated from sewage samples by using double-layer agar assay method against the collected host strain. The phage was purified through successive sub-culturing of single plaques and standard spot assays. The potent phage was morphologically and physiochemically characterized, including assessment of its life cycle, biological features, host range, and sensitivity to temperature and pH. Further characterization included determining the phage's latent period and burst size through 'One Step Growth Curve' experiments. The MiSeq Illumina platform was employed for genome sequencing, facilitating a thorough examination for the existence of toxin genes or virulence factors. Advanced bioinformatics tools like PHASTEST and Proksee were utilized for detailed genomic analysis.

Results: The bacterial strain was confirmed through genomic analysis to be *Acinetobacter baumannii* type 2 using MLST profiling. The isolated host, ACB-1, exhibited resistance to more than three antibiotics, indicating multidrug resistance. Notably, resistance to colistin, a last-resort antibiotic, was also observed emphasizing the urgent need for alternative treatment options. A single bacteriophage targeting *Acinetobacter baumannii* was isolated from various sewage samples. Physiochemical characterization of phage ACB-1 revealed a burst size of 32 virions per bacterium. Furthermore, the phage showed stability at temperatures of up to 60°C and remained viable after exposure to 70°C for 10 minutes. Moreover, it exhibited a limited host range. In an attempt to sequence the entire genome

of the phage DNA, difficulty was encountered due to the inadequate number of reads gathered for a comprehensive analysis of the bacteriophage. Consequently, the analysis was directed towards the integrated phage within the bacterial genome, ultimately resulting in the identification of three integrated phages within the bacterial genome.

Of the three phages studied, one was classified as a lysogenic phage. Its genome encodes 64 intact proteins, with a calculated GC content of 40.60%. Moreover, the circular genome map of the prophage generated by PHASTEST measures a length of 49,208 base pairs. The other two phages are likely to be lytic phages, meaning they infect bacteria and cause them to lyse without integrating their genetic material into the host's genomes. From the PHASTEST annotation, the region length of the second infecting phage genome was determined to be 35.5kb, encoding 47 intact proteins. The GC content of the genome was calculated to be 40.60%. Similarly, the genome of the third phage was found to be 23.3 kilobases in length, coding for 27 intact proteins. Its GC content was calculated to be 43.20%.

Conclusion: Bacteriophage ACB-1 exhibited a limited host range and prominent stability across a broad spectrum of temperature and pH conditions. Furthermore, its lytic activity was observed on plates. Despite the lack of genomic analysis for ACB-1 phage due to insufficient reads, the examination of *Acinetobacter baumanii*'s genome revealed the presence of lytic phages within the bacterial genome, which suggested a potential impact on phage therapy. However, additional research and studies are necessary to continue exploring the potential of phage therapy with the isolated phage.

Keyword: Bacteriophage, Phage Therapy, Colistin resistant, Whole genome sequencing

CHAPTER 1: INTRODUCTION

1.1 Background

Antibiotic resistance occurs when a microorganism can thrive or survive even in the presence of an antibiotic concentration that would typically inhibit or kill organisms of the same species. This resistance can arise through two main mechanisms: intrinsic resistance, where microorganisms naturally possess traits that make them resistant to certain antibiotics, and acquired resistance, where microorganisms develop resistance after exposure to the antibiotic. Acquired resistance can occur through mutation, where changes in the microorganism's genetic material led to resistance, or through the direct transfer of genes encoding resistance mechanisms from other bacteria or microorganisms. These mechanisms allow microorganisms to adapt and survive in the presence of antibiotics, posing a significant challenge to effective treatment of bacterial infections and highlighting the need for careful antibiotic use to mitigate the development and spread of resistance (Sabtu et al., 2015).

Antimicrobial resistance (AMR) stands as a formidable threat to humanity, with projections indicating dire consequences if action is not taken. A study has predicted that by 2050, approximately 10 million people could die annually to untreatable bacterial infections if viable alternatives and novel antibiotics are not developed and made accessible. This grim outlook emphasizes the urgency of addressing AMR through comprehensive strategies that involve sensible antibiotic use, enhanced infection prevention measures, and the prioritization of research and development efforts to discover new antimicrobial agents (Williams, 2016).

The intensifying threat of antibiotic resistance is placing considerable strain on the healthcare system, prompting researchers to explore novel or modified antibiotics as potential solutions. Yet, the quest for new antibiotics is a daunting attempt that often spans several years, and even upon discovery, the possibility of bacteria evolving resistance to these newly developed treatments emerges large. Bacterial mechanisms exhibit remarkable agility, allowing them to develop resistance to antibiotics in relatively short periods. This inherent adaptability underlines the ongoing challenge in combating antibiotic resistance and emphasizes the need for sustained research efforts and innovative approaches to stay ahead of evolving bacterial threats (Loganathan et al., 2021).

The term commonly employed by the Centers for Disease Control and Prevention (CDC) to denote a concerning microbial isolate is "multidrug resistance," indicating resistance to at least one antibiotic within three or more distinct drug classes. This phenomenon arises through various mechanisms, notably the aggregation of resistance genes on plasmids or

transposons denoted as resistance (R) elements. These genetic elements harbor genes that confer resistance to specific antimicrobial agents, thereby facilitating the development of multidrug resistance. Additionally, multidrug resistance can arise through the operation of multidrug efflux pumps, which expel multiple types of drugs from bacterial cells. These efflux pumps serve as formidable defense mechanisms for bacteria, enabling them to evade the action of diverse antimicrobial agents and contribute to the widespread dissemination of multidrug-resistant strains (CDC, 2019).

Consequently, there has been a growing recognition within the global scientific community of the necessity to explore new therapeutic avenues that extend beyond conventional antibiotic treatments to address the escalating challenge of antibiotic resistance. Among these alternatives, phage therapy has emerged as a promising option. Phages, or Bacteriophages, are viruses that naturally target and infect bacteria, ultimately leading to their destruction. Despite being one of the oldest known therapeutic approaches, the use of phages in Western medicine diminished following the beginning of antibiotics in the 1940s. Antibiotics quickly became the basis of antibacterial therapy due to their efficacy and versatility. However, the persistent pressure exerted by antibiotics has fueled the development of antibiotic-resistant bacteria, steering in the post-antibiotic era in the late 2000s. As rates of treatment failure and deaths due to antibiotic-resistant infections continue to rise, there has been a renewed interest in phage therapy. The ability of bacteriophages to selectively target and eliminate specific bacterial strains, without disrupting the body's beneficial microbiota, makes them an attractive alternative for combating multidrug-resistant infections. Consequently, there is a resurgence of research and clinical interest in harnessing the therapeutic potential of bacteriophages to confront the growing threat posed by antibiotic resistance (Abedon et al., 2011).

1.1.1 Bacteriophage

Figure 1.1 Diagrammatic Representation of Bacteriophage
(Source: https://www.freepik.com)

Bacteriophages are viruses that penetrate bacterial cells. The term "bacteriophage" originates from the combination of "bacteria" and the Greek word "phagein," which means "to devour." Bacteriophages were the initial type of viruses to be identified. FW Twort and Félix d'Herelle both made independent discoveries of bacteriophages in 1915 and 1917, respectively, leading to its alternate names such as the Twort-d'Herelle phenomenon or Bacteriophage phenomenon (Singh et al., 2024). Bacteriophages are obligate intracellular parasites that replicate within bacteria by utilizing the host biosynthetic machinery. The shortened term "phage" is commonly used to refer to them. Despite being considerably smaller than the bacteria they infect; bacteriophages are widely recognized as the most abundant and diverse forms of life in the biosphere. Phages are omnipresent and can be discovered in all environments inhabited by bacterial hosts, such as soil or animal intestines. Sea water is recognized as one of the richest natural sources for phages and other viruses (Trempy & Trun, 2003)

Phages can have either DNA or RNA as their nucleic acid, but not both. Their genetic material often includes uncommon or modified bases, which protect the phage nucleic acid from enzymes that degrade the host's genetic material during infection. Simple phages may have as few as 3-5 genes, while complex ones can possess over 100 genes. Some phages are known to utilize single-stranded DNA as their genetic material. (Rao, 2006). Bacteriophages exhibit a wide array of sizes and shapes.

Size - T4 is one of the largest phages, with dimensions around 200 nm in length and 80-100 nm in width. Other phages vary in size, with most falling between 24-200 nm in length.

Head or Capsid - Every phage possesses a head structure, which can vary widely in size and shape. Some have an icosahedral shape with 20 sides, while others are filamentous in structure. The head or capsid is constructed from numerous copies of one or more proteins. Enclosed within the head is the nucleic acid, serving as a protective enclosure for it.

Tail - Several phages, though not all, have tails connected to their heads. Usually, the tail forms a hollow tube through which the phage's genetic material travels during infection. Tail sizes can vary, and certain phages lack a tail structure altogether. In more complex phages such as T4, the tail is encased in a contractile sheath that contracts during infection of bacteria. At the end of the tail, these advanced phages like T4 have a base plate with one or more tail fibers attached to it. These components play a role in binding the phage to the bacterial cell. However, in phages that lack base plates and tail fibers, other structures enable the phage particle to attach to the bacterium (Trempy & Trun, 2003).

1.1.2 Bacteriophage History

Microbiologists frequently noted the spontaneous breakdown of bacterial cultures in the 19th century, often attributing it to autolysis, a process that can occur independently of viral involvement. During that period, the literature on autolysis was extensive; however, none of the authors proposed the presence of infectious agents affecting bacteria (Letarov, 2020).

In 1896, the English chemist Ernest Hankin published two articles in the Annales de l'Institut Pasteur detailing microorganisms found in rivers in India. The second article, titled "The bactericidal action of waters of Jumna and Ganges on the cholera microbe," has been widely recognized by several authors as an early description of this phenomenon of phages (Abedon et al., 2011). He was serving as the Bacteriologist and Chemical Engineer to the Government of the United Provinces and of the Central Provinces of India when he showed that the waters of the Indian rivers Ganga and Yamuna contained a biological component capable of destroying cultures of cholera-causing bacteria. This substance had the ability to permeate millipore filters, which are typically effective at retaining larger microorganisms like bacteria (Wittebole et. al, 2014).

After their initial prominence before the discovery of antibiotics, bacteriophages were largely neglected as important therapeutic agents in Western countries due to the convenience of antibiotics. However, research and the use of bacteriophages persisted in countries like Georgia (part of the former USSR), where they were regularly isolated and used to treat various diseases. As a result, bacteriophage research shifted to focus on several model phages, mostly infecting *E. coli*. These studies formed the basis of modern molecular biology, revealing crucial principles like the genetic basis of inheritance and the triplet nature of the genetic code (Clokie et al., 2011).

In 1915, Frederick Twort, a British microbiologist, noticed during his research on vaccinia virus growth on cell-free agar media that certain "pure" bacterial cultures seemed to be associated with a transparent material capable of passing through filters. This material could completely disintegrate bacteria within a culture into granules. Twort referred to this material as a "filterable agent" and demonstrated its ability to infect fresh cultures of micrococci. Independently, Félix d'Herelle made a similar discovery in 1917 while investigating patients with bacillary dysentery. He isolated a "microbe" from recovering patients' stools that could cause lysis of *Shiga bacilli*. This microbe was later identified as an obligate bacteriophage. While Twort's research in this area was limited due to lack of funding and other obligations, d'Herelle pioneered the clinical use of bacteriophages and conducted numerous trials worldwide. The exact nature of bacteriophages remained uncertain until the advent of the electron microscope, which allowed researchers like Helmut Ruska to visualize and characterize phage particles. Subsequent studies confirmed

the viral nature of bacteriophages and their interactions with bacterial hosts (Wittebole et al., 2014).

For many years, only a handful of phages were deeply investigated. However, the renewed interest in phage biology is driven by a greater understanding of their abundance in environments dominated by bacteria. This awareness has been facilitated by advanced microscopy techniques and molecular studies (Clokie et al., 2011). Phage therapy regained attention in the 1980s and saw human experimentation starting in the 2000s. Interest in phage biology resurged in the early 21st century, as evidenced by the Phage Summit in 2004. Today, research on bacteriophages spans numerous linguistic and geographic domains, with thousands of publications contributing to a comprehensive understanding of these microbes (Wittebole et al., 2014).

1.1.3 Bacteriophage Lifecycle

The initial phase of the infection process involves the attachment of the phage to the bacterial cell. This attachment is facilitated by tail fibers or similar structures found on phages lacking tail fibers. Phages bind to specific receptors located on the outer surface of the bacterial cell, including proteins, lipopolysaccharides (LPS), pili, and lipoproteins. This attachment process is reversible initially. However, irreversible binding of the phage to the bacterium is facilitated by one or more components of the base plate. After the irreversible binding of the phage to the bacterium, the sheath contracts (if the phage has one), and the hollow tail fiber penetrates the bacterial envelope. Certain phages carry enzymes that can break down different components of the bacterial envelope. The genetic material of the phage exits the head, travels through the hollow tail, and enters the bacterial cell. The rest of the phage remains outside the bacterium, resembling a "ghost." Interestingly, even bacteria that aren't naturally susceptible to infection can be artificially infected by injecting phage DNA, a process called transfection. Phages can be either lysogenic (temperate) or lytic (virulent), depending on their life cycle,. Lytic phages cause cell death in the host cells they infect, while temperate phages establish a long-lasting infection within the cell without causing immediate death. In the lytic cycle, subsequent steps involve the synthesis of phage components, assembly, maturation, and ultimately the release of new phages (Rao, 2006).

In the lytic cycle, upon attaching to the host surface, the phage injects its DNA into the host bacterium. This triggers a shift in the host bacterium's protein machinery, leading to the replication of phage DNA and the production of 50-200 new infectious phages. Consequently, the host weakens and eventually ruptures, releasing the new phage particles. The lytic life cycle is vital for biogeochemical cycling as it releases organic molecules into nature through the degradation of bacterial cells.

During the lysogenic phase of the phage life cycle, phage DNA becomes integrated into the host genome, potentially persisting as a plasmid. The resulting new phages carry a combination of phage DNA and host genome (Naureen et al., 2020). This integration allows for the continuous replication of viral genetic material without causing immediate harm to the infected host. Consequently, the phenotype of the infected bacteria may change, potentially affecting its pathogenicity (Singh et al., 2024). Phage genes may revert to the lytic cycle under certain conditions, leading to the release of fully assembled phages.

Additionally, there are two less common but significant phage life cycles known as pseudo-lysogenic and carrier state. Phage nucleic acid neither forms a stable long-term connection nor induces a lytic response in the pseudo-lysogenic cycle. Instead, it remains inside the host cell in an inactive form, allowing phages to avoid starvation and removal when nutrients are limited and bacterial hosts are scarce.

In the chronic carrier state of the phage life cycle, the phage establishes a persistent infection within the bacterial host, continuously releasing progeny either from the cell or through asymmetric transmission to daughter cells. (Naureen et al., 2020)

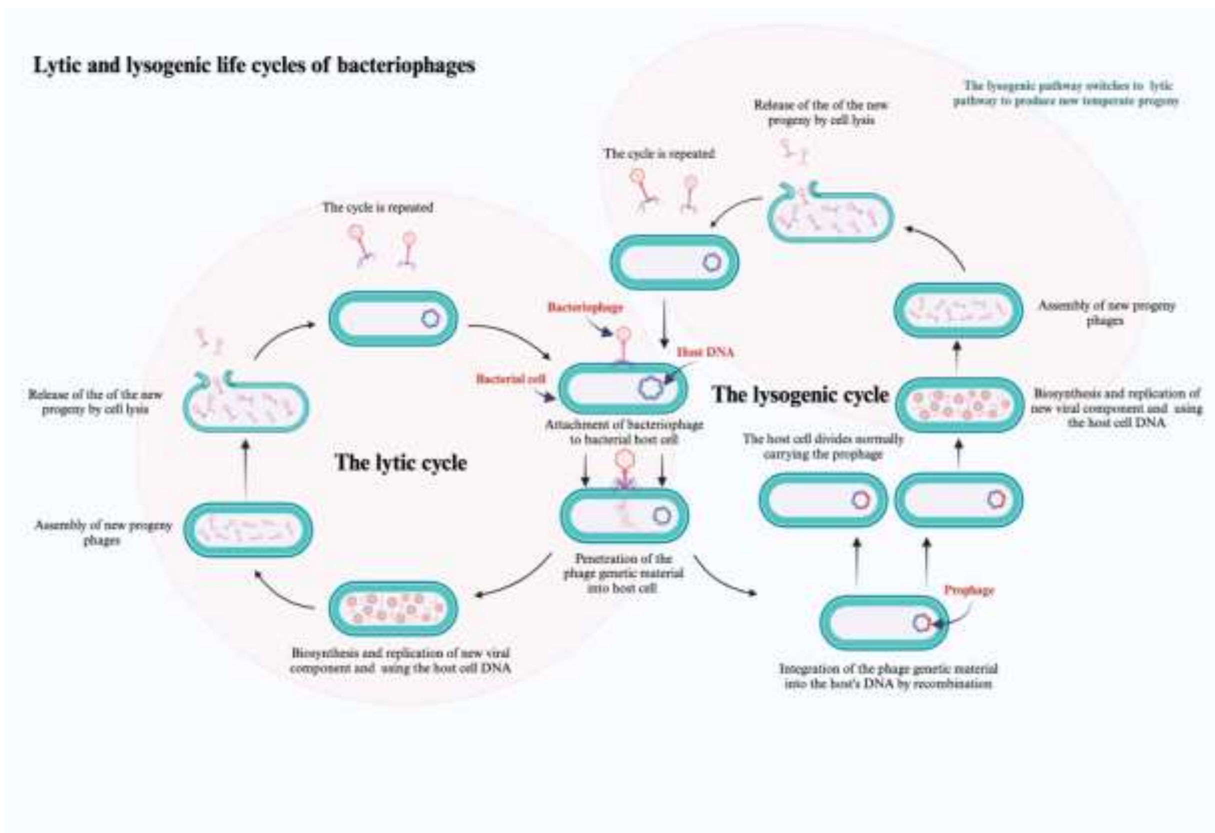


Figure 1.2 Life Cycle of Bacteriophages (Source: BioRender.com)

1.1.4 Bacteriophage classification

The International Committee on Taxonomy of Viruses (ICTV) and the Bacterial and Archaeal Subcommittee (BAVS) within the ICTV oversee the taxonomic classification and naming of viruses, including phages. This classification system considers various properties of phages, such as the molecular composition of the virus genome (single-stranded or double-stranded DNA or RNA), the structure of the virus capsid, host range, pathogenicity, and sequence similarity. The ICTV has established a valuable taxonomy framework widely accepted in the scientific community. To assist with the complex task of phage taxonomy, Adriaenssens and Brister have published comprehensive guidelines (Chibani et al., 2019).

However, with the advent of Next Generation Sequencing (NGsS) technologies, there is an abundance of genomic and metagenomic sequence data available, including complete or fragmented genomes of previously unknown phages. Unfortunately, systematic classification of these genomes into the ICTV scheme is challenging due to the lack of corresponding biological and experimental data. Therefore, taxonomic characterization based on phage genome sequence information has become essential (Chibani et al., 2019).

1.1.5 Phage Therapy

The ineffectiveness of antibiotics and chemotherapy in eradicating bacteria has compelled scientists to seek alternative biological methods. Among these, the utilization of lytic bacteriophages against pathogenic bacteria has emerged as a promising approach. Felix d'Hérelle, credited as the discoverer of bacteriophages, initially proposed the concept of phage therapy at the onset of the 20th century (Alexander Sulakvelidze, 2005). Phage therapy, therefore harnesses the inherent predator-prey dynamic between phages and their target bacteria. It utilizes a refined cocktail of phages, administered directly to patients, where only lytic phages undergo exponential replication within bacteria following immediate infection. This characteristic is crucial for therapy, as it mitigates the risk of transduction potential (Raza et al., 2021).

Phage therapy emerges as a promising alternative to address the shortcomings of antibiotics and their diminishing efficacy. Extensive research on phage therapy against a range of bacterial diseases indicates that phages offer a robust defense against resilient, antibiotic-resistant bacterial strains. Their therapeutic potential lies in their ability to replicate within the target host, exerting bactericidal effects. This distinct pharmacokinetic property of self-replication sets phages apart from conventional antibiotics and chemical drugs, rendering them preferable. Moreover, many phages have been isolated to specifically target these strains with extended spectra and have been patented. (Qadir et al., 2018).

In the beginning, trials of phage therapy didn't always work well enough to replace antibiotics. Despite this, some US pharmaceutical companies still made commercial phage products in the 1930s. Later, the Soviet Union developed phage therapy for treating diseases such as diarrhea and wound infections(Sulakvelidze et al., 2001).

Phage therapy harnesses the lytic capabilities of bacteriophages for treating bacterial infections. Since their discovery, bacteriophages have been utilized in infection treatment. Initially, their efficacy as a therapeutic agent was tested in animals like pigs, cows, and chickens. Once promising results were observed in animal studies, scientists began using bacteriophages to treat humans, leading to the origin of the term "phage therapy"(Summers, 2012).

Phage therapy as a therapeutic intervention began in 1919, shortly after its discovery, for treating dysentery, and continued until the 1940s, addressing various infections over that period. Despite the many benefits of phages compared to antibiotics, industrial production of phages as a commercial product has largely ceased in most Western European countries. However, due to the increasing antibiotic resistance and the ineffectiveness of antibiotics against bacterial biofilms, there is renewed international interest in using phages as powerful antibacterial agents. Numerous studies published globally indicate that bacteriophages could be applied in phage therapy for humans, animals, and the food industry. (Azizian et al. 2013).

1.1.6 *Acinetobacter baumannii*

TAXONOMY AND CLASSIFICATION:

KINGDOM : Bacteria

PHYLUM : Proteobacteria

CLASS : Gammaproteobacteria

ORDER : Pseudomonadales

FAMILY : Moraxellaceae

GENUS : *Acinetobacter*

SPECIES : *baumannii*

(Whiteway et al., 2022)

Acinetobacter baumannii is a gram-negative bacillus, strictly aerobic, non-motile and pleomorphic in nature. The Dutch microbiologist Beijerinck achieved the first isolation of the organism in 1911 from soil. It often acts as an opportunistic pathogen, posing a significant threat to immunocompromised individuals, especially those with prolonged hospital stays exceeding 90 days. In recent years, *Acinetobacter baumannii* has garnered

attention as a "red alert" human pathogen due to its widespread antibiotic resistance spectrum, raising concerns within the medical community (Howard et al. 2012). Skin and soft tissue infections, secondary meningitis, urinary tract infections, wound infections, ventilator-associated pneumonia, and bloodstream infections are most commonly associated with *Acinetobacter baumannii* (Antunes et al., 2014). *Acinetobacter baumannii* stands out for its distinct characteristics in terms of pathogenesis, transmissibility, and mechanisms of resistance. This microorganism possesses an exceptional capacity to develop resistance to commonly employed hospital antibiotics, further compounded by its robust survivability and rapid dissemination within healthcare settings. *Acinetobacter baumannii*'s adeptness at acquiring resistance traits, combined with its ability to thrive in the hospital environment, underlines its formidable nature as a microbial threat. These attributes highlight the urgent need for enhanced surveillance and infection control measures to mitigate the impact of *Acinetobacter baumannii* infections and prevent their further spread within healthcare facilities (Itani et al., 2023). *Acinetobacter baumannii*, originally regarded as a low-level pathogen, has undergone a significant transformation in its clinical significance, emerging as a prominent culprit in both hospital and community-acquired infections (Asif et al., 2018).

Acknowledging the pressing demand for novel treatments, the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have identified *Acinetobacter baumannii* as a Priority-1 critical focus for research (Itani et al., 2023).

The *Acinetobacter* genus comprises diverse group of organisms, encompassing 17 named species and an additional 15 species lacking valid names. Among these, *Acinetobacter baumannii*, along with closely related species 3 and 13TU, are considered clinically significant (Dijkshoorn et al., 2007).

1.1.7 Virulence of Acinetobacter baumannii

The pathogenicity of *Acinetobacter baumannii* is facilitated by a combination of virulence factors that collectively aid to its capacity to cause infections. These factors include the formation of biofilms, supported by the production of biofilm-associated protein (BAP), which enhances adherence to both abiotic and biotic surfaces, aiding in colonization of host tissues and medical devices. Additionally, *Acinetobacter baumannii* relies on efficient metallic homeostatic systems, particularly iron uptake and zinc acquisition, for colonization in various tissue environments. The K1 capsular polysaccharide enables the bacterium to evade phagocytosis by macrophages, promoting survival and proliferation in human body fluids. Several proteins, such as Omp38, phospholipases C, RecA protein and phospholipases D, contribute to pathogenesis by inducing host cell apoptosis, enhancing survival under stress conditions, and facilitating invasion of epithelial cells. Notably, the outer membrane protein 'A' (OmpA) targets bacterial mitochondria, further promoting

epithelial cell apoptosis. Lipopolysaccharides (LPS) in the bacterial cell envelope can lead to septic shock upon bloodstream entry. Furthermore, *Acinetobacter baumannii* produces verotoxins (vtx-1 and vtx-2), that inhibit protein synthesis by targeting the cell ribosome machinery, thereby enhancing virulence. Understanding the multifaceted nature of these virulence factors is essential for devising effective strategies to combat *Acinetobacter baumannii* infections (Gautam et al., 2021).

1.1.8 Mechanism of resistance in *Acinetobacter baumannii*

Acinetobacter baumannii employs genetic plasticity as a key mechanism for generating resistance, facilitated by its ability to undergo rapid genetic mutation and recombination. Additionally, *Acinetobacter baumannii* can form biofilms, extending its survival on medical devices like endotracheal tubes and catheters. This combination of genetic adaptability and biofilm formation enhances 's resilience and poses challenges for infection control in healthcare settings (Reina et al. 2022).

Acinetobacter utilizes enzyme-mediated degradation (beta-lactamases), genetic modifications (mutations, gene acquisition or loss, and gene expression regulation), and efflux pumps as strategies to evade antibiotic destruction. Beta-lactamases break down antibiotics, genetic changes alter resistance profiles, and efflux pumps expel antibiotics from the bacterial cell. These mechanisms collectively confer resistance, complicating treatment and contributing to multidrug-resistant *Acinetobacter* infections (Asif et al., 2018).

1.1.9 Resistance to polymyxins B and E (colistin)

Acinetobacter baumannii's resistance to polymyxins B and E (colistin) stems from its unique external membrane composition, which regulates the influx of essential nutrients and the elimination of harmful substances. Lipopolysaccharides (LPS) on the membrane's surface feature lipid A, a hydrophobic, negatively charged component that interacts with the cations of polymyxins. This interaction disrupts the membrane, allowing polymyxins to enter the periplasmic space and increase membrane permeability. Although the precise mechanism remains unclear, the hydrophobic portion of polymyxins likely induces membrane damage akin to a detergent-like effect. Mechanisms of resistance include genetic mutations altering lipid A, mutations in genes crucial for lipid A biosynthesis leading to lipid A deficiency, defects in membrane permeability and osmotic resistance, inadequate cofactors for LPS formation essential for polymyxin sensitivity, and efflux pumps expelling the antibiotics. These multifaceted resistance mechanisms contribute to elevated minimum inhibitory concentrations (MIC) of polymyxins, posing challenges for effective treatment against *A. baumannii* infections (Reina et al., 2022).

1.2 Rationale of the study

The intensifying prevalence of antimicrobial drug resistance among pathogenic bacteria has emerged as a critical global challenge. Multidrug-resistant superbugs represent a leading cause of mortality in contemporary society. Among them, *Acinetobacter baumannii* stands out as a significant pathogen responsible for hospital-acquired infections. Recognized as a Priority-1 critical pathogen for research and development of new drugs by the World Health Organization in 2017, combatting this resilient superbug is imperative. To curtail the spread of antimicrobial drug resistance among pathogens and effectively eradicate this formidable threat, there is an urgent need for swift, cost-effective, and reliable alternatives.

Phages have a rich history of combatting pathogens and saving lives ever since their discovery. Initially, their use as antimicrobials was restricted due to limited knowledge and inadequate tools for studying them. However, with the advancements in scientific technology and intensive research in phage therapy, many uncertainties have been addressed. As a result, phages now present enormous potential in the fight against antimicrobial resistance.

In Nepal, the alarming rise in antibiotic resistance has emerged as a significant public health issue. Unfortunately, there has been a lack of productive efforts towards finding alternatives to antibiotics, with very few studies published on Bacteriophages. This study seeks to investigate, identify, and characterize lytic bacteriophages to assess their potential as antimicrobial agents against *Acinetobacter baumannii.*

1.3 Objectives

1.3.1 General Objective

Physiochemical and genomic characterization of potential lytic phage isolated from the sewage sample against colistin resistant *Acinetobacter baumannii.*

1.3.2 Specific Objectives

- Molecular identification of colistin resistant strain of the *Acinetobacter baumanii* by VITEK
- Isolation, screening and purification of bacteriophage against colistin resistant *Acinetobacter baumannii* from different sewage samples of Kathmandu
- Determination of phage-host range
- Physio-chemical and molecular characterization of the phage
- Analysis of the whole genome sequence of phage using Miseq Illumina platform

1.4 Research Hypothesis

This study aims to assess the activity of bacteriophage against colistin resistant *Acinetobacter baumannii*.

1.4.1 Null Hypothesis (Ho)

The lytic bacteriophage displays no significant activity against strains of *Acinetobacter baumannii.*

1.4.2 Alternative Hypothesis (H1)

The lytic bacteriophage displays significant activity against strains of *Acinetobacter baumannii.*

CHAPTER 2: LITERATURE REVIEW

2.1 Antimicrobial Resistance

Antimicrobial agents, which encompass antibiotics, antifungals, antivirals and antiparasitics, play a critical role in preventing and managing infections in humans, animals, and plants. However, the emergence of Antimicrobial Resistance (AMR) occurs when bacteria, viruses, fungi, and parasites evolve to resist these treatments. This adaptation diminishes the effectiveness of antibiotics and other antimicrobial drugs, complicating the treatment of infections and sometimes rendering them untreatable. AMR represents a significant global health challenge, contributing to the spread of infections, severe disease outcomes, and increased mortality rates (Facts, 2015).

The increase in antimicrobial resistance (AMR) is driven by multiple factors, including the inappropriate use of antibiotics in sectors such as food production, animal agriculture, and healthcare. Moreover, there is a shrinking pipeline of new antibiotics, as pharmaceutical companies are less engaged in their discovery and development. Certain pathogens naturally resist antibiotics, complicating treatment with existing medications. Without substantial changes in current approaches, forecasts indicate that by 2050, AMR could cause at least 10 million deaths annually and result in economic costs exceeding one trillion dollars per year, largely due to reduced productivity (Nimer et al., 2016).

Common bacterial infections such as urinary tract infections, sepsis, and sexually transmitted infections are increasingly becoming resistant to antibiotics worldwide, posing significant challenges for treatment. For instance, antibiotics like ciprofloxacin, commonly prescribed for urinary tract infections, are facing resistance rates ranging from 8.4% to 92.9% in *Escherichia coli* and 4.1% to 79.4% in *Klebsiella pneumoniae*. *Klebsiella pneumoniae*, a bacterium found in the intestines, is now resistant globally to last-resort treatments like carbapenem antibiotics, leading to severe infections. Additionally, resistance to fluoroquinolone antibiotics in *E. coli*, often used for urinary tract infections, is widespread across many countries. Methicillin-resistant Staphylococcus aureus (MRSA) infections are particularly concerning, as patients are 64% more likely to die compared to those with infections susceptible to drugs. Furthermore, global monitoring of antibiotic resistance in pathogens such as E. coli and MRSA is crucial to address the growing threat of antimicrobial resistance (Facts, 2015).

2.2 *Acinetobacter baumannii* and Its MDR Property

Acinetobacter baumannii is an opportunistic bacterial pathogen often associated with infections acquired in hospital settings. There has been a recent increase in its occurrence, especially among combat troops returning from conflict areas, and a significant rise in

strains that are resistant to multiple drugs (MDR), highlighting its growing significance as an emerging opportunistic pathogen (Howard et al., 2012). *Acinetobacter* exhibits high resistance to treatment, making it challenging to initiate effective empirical therapy in clinical practice. Infections caused by *Acinetobacter baumannii* can affect multiple anatomical regions with varying degrees of severity, impacting patient outcomes significantly.

Once viewed as a low-virulence opportunistic pathogen, *Acinetobacter baumannii* has evolved due to its robustness, facilitating cross-transmission, and its acquired resistance to numerous antimicrobial agents. These attributes give *Acinetobacter baumannii* a distinct advantage over many other hospital-acquired bacteria. Infections caused by this bacterium, particularly those involving resistant strains, are linked to increased morbidity and mortality rates, prolonged hospitalizations, and higher healthcare expenses compared to infections caused by susceptible strains (Abbo & Carmeli, 2007). *Acinetobacter baumannii* is frequently present as a colonizer on human skin and in the respiratory tract, posing a risk for severe infections including septicemia, endocarditis, pneumonia, meningitis, and wound infections (Alrahmany et al., 2021).

Acinetobacter baumannii exhibits a wide range of antibiotic resistance mechanisms, such as the production of carbapenem-hydrolyzing beta-lactamases (carbapenemases) that often lead to carbapenem resistance. Resistance can also result from the increased activity of efflux pumps that expel antibiotics and changes in outer membrane porins that block antibiotic entry. Apart from its antibiotic resistance, *Acinetobacter baumannii* possesses several potential virulence factors, including systems that acquire iron through siderophores and the ability to form biofilms. These factors have the potential to impact clinical outcomes, complicating the management of infections caused by this bacterium (Lemos et al., 2014).

Resistance to colistin, an antibiotic considered a last resort, first appeared with colistin-resistant *Acinetobacter* identified in the Czech Republic in 1999. Since then, its prevalence has been steadily reported worldwide. In the USA, resistance levels range from 2.1% to 7.1%, while in Europe, reports indicate resistance rates between 7% and 11%. India shows the highest resistance at 53%, followed by Iran (48%), Spain (40.7%), and Korea (30%) (Asif et al., 2018).

2.3 Bacteriophages and Discovery of Bacteriophages

Bacteriophages are bacterial viruses discovered over a century ago that possess the ability to infect and destroy bacterial cells. Similar to most viruses, infections start when virions attach to specific cell-surface receptors, leading to intracellular reproduction (Chan et al., 2013). For bacteriophages to be considered effective therapeutic agents, they must exhibit low immunogenicity, a strictly lytic lifestyle, lack of toxins or antibiotic resistance genes, a

broad host range targeting multiple isolates of the pathogen, and the capability to limit the emergence of phage-resistant mutants (Yang et al., 2020). In 1896, Ernest Hanbury Hankin reported the presence of an antibacterial agent against cholera-causing bacteria in two Indian rivers, the Ganga and Yamuna (Wittebole et al.,2013). He proposed that an unidentified substance, which could pass through fine porcelain filters and was sensitive to heat, was responsible for this phenomenon and helped limit the spread of cholera epidemics (Sulakvelidze et al., 2001). Ernest Hankin observed the presence of particles capable of lysing cholera-causing bacteria in the waters of the Ganges and Yamuna rivers, but he did not isolate or formally discover bacteriophages. He suggested the existence of these particles. The official discovery of bacteriophages was credited to Félix d'Hérelle, a French-Canadian microbiologist at the Pasteur Institute in Paris in 1910. D'Hérelle identified them while studying fecal filtrates from soldiers infected with Shigella. He observed zones of bacterial death on culture plates and proposed these agents as ultraviruses. Frederick Twort, a British bacteriologist, also independently observed similar bacterial lysing agents in 1917 while studying cultures of micrococci, but he did not identify them as viruses. Eventually, the discoveries of both Twort and d'Hérelle were widely accepted by the scientific community, leading to the term "Twort d'Hérelle phenomenon," later recognized as the bacteriophage phenomenon.

2.4 Interaction between phage and bacteria

Phages, or bacteriophages, are omnipresent in nature and are most commonly found in environments where bacteria are prevalent. They are especially abundant in oceans, soil, wastewater treatment facilities, hot springs, and the gastrointestinal tracts of animals. (Womack & Colwell, 2000; Prigent et al., 2005; Srinivasiah et al., 2008). Phages are categorized based on their size, structural composition, genome organization, and the specific host they infect (Ackermann, 2009). The interaction between a host and a phage particle begins when the phage identifies specific receptors on the bacterial cell wall. The tail proteins of the phage recognize the receptor proteins on the bacteria and inject their own DNA into the host cytoplasm, initiating either a lytic or lysogenic lifecycle. Phages exhibit two distinct life cycles. In the lytic lifecycle, the phage injects its DNA into the host cell and multiplies by hijacking the host's replication machinery. After replication, the phage lyses the bacterial cell, releasing progeny virus particles into the environment. In contrast, during the lysogenic lifecycle, the phage DNA integrates into the bacterial genome, replicates along with the bacterial DNA, and is passed on to the progeny of the host cells. (Parmar et al., 2017).

2.5 Phage Therapy and Its Advantages

Bacteriophages, also known as phages, are viruses that target and destroy bacteria through lysis. Phage therapy, which utilizes phages to treat bacterial infections, was pioneered by Félix d'Herelle around 1920, predating the practical use of penicillin, the first antibiotic, by about two decades. When initially discovered, phage therapy showed promise as a potential treatment for bacterial infections (Liu, 2014). Research has demonstrated several advantages of phage therapy over traditional chemical antibiotics. According to Abedon & Abedon (2010), a single dose of phage therapy can effectively treat bacterial infections, achieving significant bacterial clearance. Unlike many chemical antibiotics, phage therapy minimally disrupts the resident gut flora due to its specificity towards bacterial hosts. This specificity also reduces the likelihood of developing resistance by limiting the range of bacteria susceptible to phage-resistance mechanisms (Hyman & Abedon, 2010).

From an economic standpoint, producing phages is relatively inexpensive compared to chemical antibiotics, making therapeutic phage products more accessible to the general population than some costly antibiotics available today (Skurnik et al., 2007). Furthermore, enhancing the host-range of phages can improve the efficacy of phage therapy, as success largely depends on the phage's ability to attach to bacterial surface receptors. This range can be narrow, targeting only specific strains within a species, or broad, encompassing multiple bacterial genera (Hyman & Abedon, 2010).

2.6 Resurgence of Phage Therapy

In the past five years, a resurgence of interest in phage therapy has been seen, driven by the escalating issue of antimicrobial resistance (AMR) and the declining supply of new antibiotics. This renewed focus has been bolstered by numerous notable cases where phage therapy has effectively treated severe, multidrug-resistant bacterial infections. Advances in high-throughput sequencing, genetic engineering, metagenomics, and synthetic biology have overcome previous limitations that hindered the field's progress.

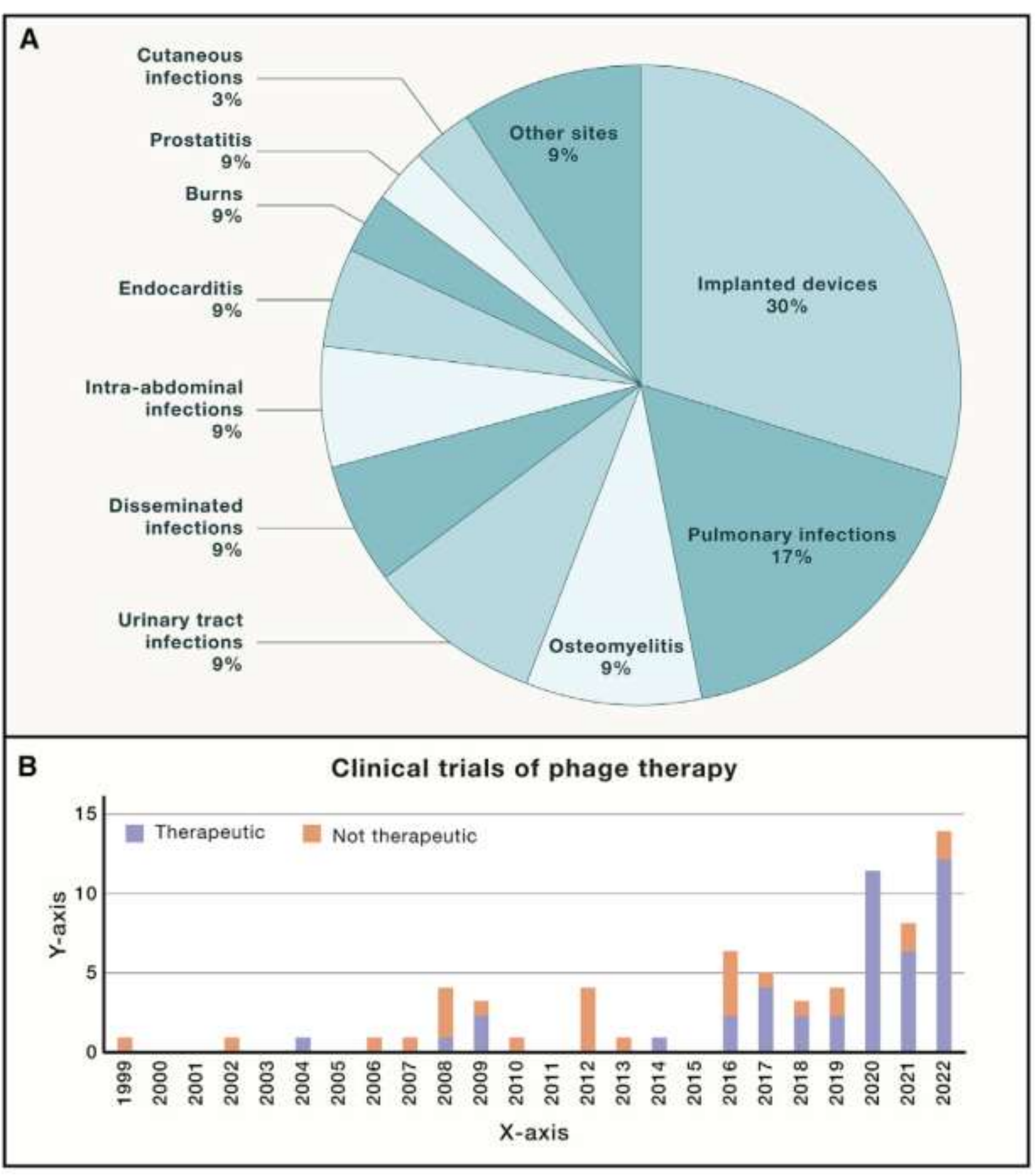


Figure 2.1 A. Case studies on phage therapy from 2000 onwards; B. Clinical trials of phage therapy documented on ClinicalTrials.gov since 1999 (Sthrathdee et. al 2022)

Until recently, the targeted modification of phage genomes has faced challenges due to the lack of widely applicable techniques suitable for both virulent and temperate phages. However, a novel in vivo technology has surfaced, originally designed to introduce genetic modifications into bacterial genomes, known as recombineering. This method has been adapted for effective manipulation of both temperate and lytic phages, termed bacteriophage recombineering of electroporated DNA (BRED). Initially developed by Marinelli et al. for Mycobacterium phages, BRED can be tailored for use with other phages. The technique involves boosting the frequency of homologous recombination between

phage DNA and the targeted DNA substrate by inducing bacterial overexpression of plasmid-encoded recombination genes (Nobrega et al. 2015).

Over the past decade or so, advancements in technology have made it possible to prepare therapeutic phages that meet near-Good Clinical Practice (GCP) standards. As a result, phage therapy is now more commonly administered intravenously. A recent extensive review of phage administration to humans and animals found that it is generally well tolerated, regardless of the route of administration.

Non-parenteral routes of delivery, such as oral or topical, allow for the use of phages that may not have undergone as rigorous preparation. However, delivering phages efficiently to infection sites through these routes can be challenging due to various factors.

Using naturally occurring phages for therapeutic purposes can be hindered by several factors. Firstly, the only phages available with the desired ability to target specific bacteria may be temperate. Secondly, phages that infect the target bacteria may not efficiently kill them. Lastly, some phages may carry genes that could potentially be harmful. To overcome these challenges, it may be necessary to engineer phages with improved therapeutic properties, safety profiles, and broader host ranges. There are two main strategies for phage engineering: modifying existing phage genomes to change their properties or designing phages from scratch using synthetic genomics based on known phage biology principles. While phage synthetic genomics is still in its early stages, it offers great potential as it is not limited by the constraints of naturally occurring phages.

Recent studies have highlighted the use of RNA-targeting CRISPR-Cas13a for engineering phage genomes. By combining this approach with homologous recombination, researchers have been able to modify a wide range of phages. These modifications include introducing single codon deletions and adding fluorescent tags to large jumbo phages with genome sizes ranging from 200 to 500 kbp. In the future, a combination of CRISPR-based tools such as programmable base editors, transposases/recombinases, nucleases, and prime editors may enable the creation of precise, genome-scale changes in various phages. (Strathdee et. Al 2022)

2.7 Advantages of Phage Therapy Over Antibiotics

Phage therapy offers several advantages over traditional antibiotics. Paul Gulig from the University of Florida, Gainesville, FL, USA, explains that yeast infections and diarrhea are common side effects of antibacterial therapy because beneficial bacteria in the genital tract and intestines are also killed, disrupting the ecological balance and allowing other pathogens to thrive and cause disease. In contrast, bacteriophages target specific bacterial strains, sparing patients from the side effects associated with destroying natural flora (Pirisi, A. 2000).

Another benefit of phage therapy is auto-dosing. Phages have the ability to increase in number specifically where their bacterial hosts are located during the bacterial-killing process. However, this process has some limitations, such as dependence on relatively high bacterial densities (Loc-Carrillo, C. 2011). While bacterial resistance remains a concern, phages possess a unique advantage: they can mutate in step with evolving bacteria. Additionally, if bacteria develop resistance to one phage, there is a natural abundance of phage species that could potentially attack even newly resistant strains (Pirisi, A. 2000).

In the era after antibiotics, personalized phage therapy emerges as a promising alternative for treating human infections, tailored to each patient's specific needs. This approach could potentially bridge the gap between antibiotic failures and the need for effective clinical treatments. Additionally, with the rise of zoonotic pathogens in the food chain, the use of phages to combat these pathogens is becoming increasingly justified (Zalewska-Pią̨tek, 2023).

Beyond treating bacterial infections, phage therapy shows promise in addressing chronic diseases where bacteria play a role in pathogenesis. For instance, the microbiome gut-liver axis is implicated in inflammatory responses linked to conditions like alcoholic and non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), and irritable bowel syndrome (IBS). While the exact causal pathways are still being studied, preclinical research is encouraging, and clinical trials are planned to investigate whether phage therapy can selectively target bacteria such as *Enterococcus faecalis, Klebsiella pneumoniae*, and invasive *E. coli* associated with Crohn's disease (Strathdee et al., 2022).

2.8 Current Studies and Recent Advancements in Phage Research

Recent advancements in phage research have propelled this field into the spotlight of microbiology and biotechnology. Phages, or bacteriophages, are viruses that infect and replicate within bacteria, making them a promising avenue for combating bacterial infections, particularly those resistant to antibiotics. Let's delve into the current studies and breakthroughs in this dynamic area of research.

Phage Therapy: This therapy involves the therapeutic use of phages to treat bacterial infections. Recent studies have exhibited the efficacy of phage therapy in treating antibiotic-resistant bacterial infections, including those caused by pathogens like Staphylococcus aureus, Pseudomonas aeruginosa, and Escherichia coli. Clinical trials are underway to further explore the safety and effectiveness of phage therapy in humans (Schooley et al., 2017)

Phage Engineering: Researchers are actively engaged in engineering phages to enhance their therapeutic potential. This includes modifying phage genomes to improve host

range, increase specificity, and evade bacterial resistance mechanisms. Advances in synthetic biology and genetic engineering have facilitated the design of custom phages tailored to target specific bacterial strains (Lu and Koeris, 2011).

Phage-Driven Evolution: Phages play a significant role in driving bacterial evolution through processes such as horizontal gene transfers and selection pressure. Understanding the coevolutionary dynamics between phages and bacteria is crucial for developing effective phage-based therapies and predicting the emergence of bacterial resistance (Kroskella and Brockhurst, 2014).

Phage-Derived Enzymes: Phages encode various enzymes that can degrade bacterial cell walls, disrupt biofilms, and modulate bacterial metabolism. These phage-derived enzymes, such as endolysins and depolymerases, have therapeutic potential as antimicrobial agents for treating infections or as biocontrol agents in agricultural and food industries (Bramkamp and Baarle, 2009).

Phage Display Technology: Phage display is a powerful technique for screening peptide or protein libraries displayed on the surface of phage particles. Recent advancements in phage display technology have enabled the discovery of novel peptides and antibodies for applications in drug development, diagnostics, and targeted therapy (Dolgin, 2019).

Phage Ecology and Diversity: The study of phage ecology and diversity has revealed the immense genetic and structural diversity of phages in diverse environments, including oceans, soil, and the human microbiome. Metagenomic approaches have provided insights into the ecological roles of phages and their potential as sources of novel antimicrobial agents (Breitbart, M. et al., 2002).

Phage-Based Biocontrol: Phages offer a promising alternative to chemical pesticides for controlling bacterial pathogens in agriculture. Research is focused on harnessing phages as biocontrol agents to combat plant diseases, reduce antibiotic use in livestock farming, and mitigate foodborne pathogens in the food industry (Buttimer C. et al., 2017).

A recent study has shown the effectiveness of phage therapy in treating secondary *Acinetobacter baumannii* infections in COVID-19 patients (Wu et al., 2021). The finding highlights the potential of phage therapy as a treatment option for secondary bacterial infections in individuals with COVID-19 (Khan, A. 2022).

In conclusion, recent advancements in phage research have expanded our understanding of phage biology, evolution, and applications in medicine, biotechnology, and agriculture. Phages hold immense potential as versatile tools for combating bacterial infections, addressing antibiotic resistance, and promoting sustainable practices in various fields. Continued research and innovation in phage biology and biotechnology are poised to revolutionize healthcare, agriculture, and environmental science in the coming years.

2.9 Phage-Encoded Enzymes for the Treatment of *A. baumannii*

2.9.1 Endolysins

The phage-produced hydrolases, endolysins, play a crucial role in the replication cycle of bacteriophages by lysing bacterial cell walls, facilitating the release of progeny phages. These enzymes exhibit high specificity towards bacterial species or subspecies, making them advantageous over traditional broad-spectrum antibiotics. This specificity minimizes interactions with surrounding microbial cells, reducing the risk of disrupting beneficial microbiota. Furthermore, endolysins have shown effectiveness against biofilms and mucosal surfaces, areas where traditional antibiotics often struggle. Their ability to target specific pathogens reduces the development of resistance, and they can be used in combination with other antibacterial agents for synergistic effects. Overall, endolysins offer a promising alternative for combating bacterial infections with greater precision and fewer adverse effects on the microbiome (Zhang, 2022).

2.9.2 Depolymerases

The formation of biofilm involves bacterial cells enclosed by extracellular polymers (EPSs), that can hinder phage penetration and increase bacterial resistance to antimicrobial agents. *Acinetobacter baumannii*'s EPSs contribute to its resistance and can lead to persistent, challenging-to-treat infections. Depolymerases, phage-derived enzymes, play a crucial role in phage infection by breaking down extracellular bacterial proteins. These enzymes can be part of the phage virion or secreted in a soluble form during bacterial cell lysis. Depolymerases' ability to precisely target and degrade EPSs and related biofilm components makes them a promising tool for controlling pathogens (Zhang, Y. 2022).

2.10 Phage Enumeration Method

Phage enumeration is essential for various applications in industries, medicine, and other fields. The double-layer agar (DLA) plaque assay is a classical method used for isolating, detecting, and enumerating phage particles in a solution. This method, along with spot tests, direct plating plaque assays, and small drop plaque assays, is crucial for phage enumeration and isolation of phage-resistant mutants.

While the DLA method is widely used for both large and small plaque-forming phages, it can be challenging to precisely enumerate small plaque-forming phages because of the large area of analysis and also poor visibility, that can lead to errors in phage enumeration. Earlier studies reveal that visualizing plaques formed by small plaque-forming phages was a significant challenge (Chhibber, S. 2018).

2.11 Molecular Detection of Bacteria

The VITEK II compact system is an automated method used for the identification and classification of microorganisms such as bacteria and fungi (Kareem et al., 2022). It utilizes growth-based technology for microbial analysis. This system supports colorimetric reagent cards, which undergo automatic incubation and interpretation for microbial identification. It includes features such as compliance with 21 CFR Part 11 for electronic records and signatures, as well as a specialized colorimetric reagent card (BCL) for identifying spore-forming Gram-positive bacilli like Bacillus and related genera. Similarly, other colorimetric reagent cards (GN, GP, YST) are applicable across all system formats, serving both industrial and clinical laboratory needs (Pincus, 2010).

2.12 Whole genome sequencing

The advancement of DNA sequencing has brought about a significant transformation in the field of biological sciences, making it simpler to uncover the functions of genes. The primary DNA sequencing method adopted widely was Sanger sequencing, which emerged in the 1980s and initially involved manual procedures (Sanger, Nicklen, & Coulson, 1977). In the 1990s, advances in DNA sequencing techniques led to automated sequencing, enabling the sequencing of entire genome(Heather & Chain, 2016)

Whole genome sequencing (WGS) is a lab method that figures out all the DNA sequences in an organism's genome simultaneously. It includes finding out the order of bases in the organism's complete set of DNA. This process relies on automatic DNA sequencing methods and computer tools to manage and organize the large amount of genetic data (Yin, Kwoh, & Zheng, 2018).

Whole genome shotgun sequencing was already being utilized by 1979 for small genomes, typically ranging from 4000 to 7000 base pairs in size (Staden, 1979). *Haemophilus influenzae* genome was sequenced for the first time in 1995.

The sequencing of the complete genome of the bacteriophage øX174 in 1977 marked a significant milestone in scientific progress. This breakthrough revolutionized our understanding of genetic information. By decoding the polynucleotide sequence of genes within the genetic material, scientists gained the ability to predict the proteins synthesized by these genes. This predictive capability is invaluable for taxonomically classifying viruses into orders, families, subfamilies, genera, and species. Furthermore, since genetic material varies widely among bacteriophages, this achievement opened up avenues for understanding the diversity and evolution of viruses at a molecular level. Thus, the sequencing of the øX174 genome not only provided fundamental insights into the biology of viruses but also laid the groundwork for further advancements in molecular biology and virology.

2.13 MLST (Multilocus Sequence Typing)

Multilocus Sequence Typing (MLST) analyzes fragments from seven essential genes to identify microorganisms at the strain level. These genes are vital for cell function. By examining sequences of about 450-500 base pairs from these genes, MLST reveals diverse allelic profiles, indicating differences between strains. Each sequence observed at each gene locus is given a unique allele number, collectively forming an allelic profile for strain characterization. Like 16S rDNA sequencing, online data sharing supports MLST development. Central databases allow users to submit strain information and compare sequence profiles for identification. MLST is increasingly valuable for research, outbreak tracking, environmental monitoring, and confirming strains in manufacturing processes (Mitchell, 2018).

CHAPTER 3: MATERIALS AND METHODS

3.1 Laboratory Setting

All the works required for the research were conducted in Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

The duration of research was 12 months, from March, 2021 to February, 2022.

Experimental Bacteria: *Acinetobacter baumannii*

3.2 Collection of Bacterial Strain and Preservation

The multidrug-resistant bacterial strain was collected from the Sukraraj Tropical and Infectious Disease Hospital, Teku. Bacterial samples were streaked onto sterile Nutrient agar plates. These plates were incubated for 24 hours at 37°C for bacterial growth. Bacterial samples were freshly sub-cultured, before each experiment. The cultures used for experiments were taken from 3-4 hours cultures in the active-log phase. This ensures that the bacteria are actively growing and in a suitable phase for experimentation.

Glycerol stocks were prepared for long-term preservation of bacterial strains. A single colony of the desired bacterial strain was grown overnight in Luria Bertani (LB) broth at 37°C. The next day, a pellet of bacterial cells was collected from the overnight culture. To prepare the glycerol stock, 700µl of autoclaved 50% glycerol and 300µl of autoclaved LB broth were added to the bacterial pellet. This mixture was then kept at 37°C overnight. The following day, the mixture was stored at 4°C temporarily and transferred to -80°C for long-term storing. This methodology ensures that the bacterial strains are maintained in a viable state for future experiments and research. The use of glycerol stocks and appropriate storage conditions (such as -80°C) helps preserve the genetic and phenotypic characteristics of the bacterial strains over extended periods.

3.3 Identification of bacteria by Gram Staining and Biochemical Tests

Acinetobacter baumannii (ACB-1) isolated from Sukraraj Tropical and Infectious Disease Hospital, Teku was sub cultured in Nutrient Agar (NA) followed by a series of biochemical tests to characterize its properties. These tests included catalase, oxidase, citrate utilization, SIM (Sulfur Indole Motility) test, and growth observation at 44°C. For each test, specific media were prepared in test tubes, and the bacteria was inoculated into these media. Then, the inoculated tubes were incubated overnight at 37°C to allow for bacterial growth and metabolic activity. During the tests, observations were made for various indicators of bacterial behavior. The catalase test involved observing the formation of

bubbles, indicating the presence of the catalase enzyme. The oxidase test involved detecting a color change in response to the presence of cytochrome c oxidase. In the citrate utilization test, a color change was observed as an indicator of citrate utilization by the bacterium. For the SIM test, observations were made for hydrogen sulfide (H_2S) production, indicated by blackening of the medium, as well as for motility, observed by growth spreading away from the point of inoculation. Finally, the growth of the bacterium at 44°C was assessed by observing colony development on petri plates incubated at this temperature. These tests collectively provide valuable information about the biochemical and physiological characteristics of *Acinetobacter baumannii*, aiding in its identification and further understanding of its pathogenicity.

3.4 Antibiotic Susceptibility Testing (AST)

A single colony of the multidrug-resistant bacteria was picked and grown overnight. The overnight culture was diluted to match the turbidity of 0.5 McFarland standards. This ensures a standardized bacterial inoculum for testing. A sterile cotton swab was used to spread the diluted bacterial culture evenly across the surface of agar plates. The plates were allowed to air dry for a few minutes. Antibiotic discs, representing different classes of antibiotics such as beta-lactams, carbapenems, aminoglycosides, fluoroquinolones, etc., were placed on the surface of the agar plates, maintaining proper spacing between each disc to prevent interference. These plates were incubated for 24 hours at 37°C for bacterial growth and antibiotic diffusion. After incubation, clear zones around the antibiotic discs, indicating inhibition of bacterial growth, were observed. These zones were measured using a scale. Larger zones indicate higher susceptibility to the antibiotic, while smaller or absent zones indicate resistance. The diameter of the zone of inhibition was compared to interpretive criteria provided by the CLSI (Clinical and Laboratory Standard Institute) for each antibiotic. Based on these criteria, results were classified as sensitive (S), intermediate (I), or resistant (R).

This method allows for the determination of the susceptibility profile of the multidrug-resistant bacterial strains to various antibiotics, helping guide appropriate antibiotic therapy and further research into antimicrobial resistance mechanisms.

Table 3.1 Antibiotic Susceptibility Test; Antibiotics and Concentration of Discs

SN	Antibiotics used	Concentration of discs (mcg)
1	Ciprofloxacin	5
2	Gentamicin	10
3	Cotrimoxazole	14
4	Amikacin	30
5	Ceftazidime	14
6	Meropenem	10

7	Imipenem	10
8	PolymyxinB	300
9	Nalidixic acid	10
10	Cefepime	30
11	Nitrofurantoin	300
12	Cefoperazone Sulbactam	75/30

3.5 Bacterial Genomic DNA Extraction

The genomic DNA extraction from a bacterial strain, specifically *Acinetobacter baumanni* was conducted using the CTAB method. Initially, 1 ml of an overnight bacterial culture was transferred into an Eppendorf tube and then centrifuged for 5 minutes at 13,000 rpm to collect the cells. The supernatant was discarded, and the pellet containing the cells was then resuspended in 567µl of TE buffer through gentle pipetting or using vortex.

Subsequently, a mixture comprising 30µl of 10% SDS and 3µl of proteinase K was added to the resuspended cells and, were incubated at 37℃ for approximately 1 hour. Following this incubation, 100µl of 5M NaCl and 80µl of CTAB/NaCl solution (containing 0.7m NaCl and 10% CTAB) were added and thoroughly mixed. The resulting solution was then incubated at 65℃ for 10 minutes. After incubation, an equal volume of Chloroform:Isoamyl alcohol (24:1) was added and mixed well, and centrifugation was done at 13,000 rpm for 5 minutes. The upper aqueous layer containing DNA was carefully transferred to a new Eppendorf tube, and 600µl of isopropanol was added to precipitate the DNA. Following another centrifugation at 13,000 rpm for 5 minutes, the isopropanol was removed, and the DNA pellet was washed with 1ml of 70% ethanol, followed by another round of centrifugation. Ethanol was then discarded, and the DNA pellet was allowed to air dry.

Once dried, the DNA was resuspended in 50µl of TE buffer and stored at 4℃. Subsequently, the extracted DNA underwent analysis using a Nanodrop spectrophotometer to determine its concentration and ensure purity.

3.6 Molecular Detection of Bacteria by VITEK

The host bacterium was sent to Siddhi Poly Path Lab for molecular verification, employing Vitek Compact System 2 analyzer. The Vitek2 compact system by bioMérieux is a highly automated platform utilizing small plastic reagent cards,which is approximately the size of a credit card, containing 64 wells with microliter quantities of test media and antibiotics. Through a brief incubation period, the Vitek2 continuously monitors bacterial growth using turbidimetry. Depending on the configuration, the instrument can run 30 to 240 tests simultaneously. The susceptibility card allows testing of common, rapidly growing

gram-positive and gram-negative aerobic bacteria, as well as Streptococcus pneumoniae, within 4 to 10 hours (Jorgensen & Ferraro, 2009).

3.7 Genomic Confirmation of Bacteria by MLST

Using Multilocus Sequence Typing (MLST) for genomic confirmation of bacteria involves analyzing multiple genetic regions to identify and classify bacterial strains. This method provides detailed information about the genetic makeup of bacteria, aiding in understanding their diversity, evolution, and transmission patterns. Whole genome sequencing of bacteria was performed in CMDN (Center for Molecular Dynamics Nepal) and accordingly MLST data were analysed for the genomic confirmation of the host bacteria.

3.8 Sewage Sample Collection and Processing

Sewage samples were gathered from various sites along rivers within the Kathmandu and Lalitpur Valley. Specifically, the most heavily polluted site was targeted to maximize the likelihood of capturing phage particles. The collected sewage was carefully transferred into a 50 ml Falcon tube. The Falcon tube containing the sewage sample was promptly transported to the Central Department of Biotechnology for further processing and analysis. Upon arrival at the laboratory, the sewage sample was centrifuged at 4,100 rpm for a duration of 30 minutes. This step aimed to separate the suspended solids and debris from the liquid phase, facilitating the concentration of phage particles. Following centrifugation, the supernatant from the centrifuged sewage sample was filtered through a 0.22µm syringe filter. These filters, typically made of polyether sulfone (PES) material, helped to further remove unwanted materials and particles from the water samples. This filtration step effectively removed any remaining particulate matter, bacterial cells, and larger debris, yielding a clarified filtrate. The filtrate, now devoid of larger contaminants, was collected in a sterile Falcon tube and promptly stored at 4°C. This refrigeration ensured the preservation of the phage particles present in the sample for subsequent analysis and experimentation. The phage sample labeled as AcB1 was likely prepared for sequencing.

3.9 Clonal Purification of Phage (Phage Streak Protocol)

A clonal purification technique known as the plaque-streak assay was employed to obtain a pure culture of phage. This method, similar to streaking for single bacterial colonies, helped isolate individual phage strains from a sample containing potentially heterogeneous populations. The procedure unfolded as follows:

The sample was plated onto a Petri dish containing 1.5% TSA (tryptic soy agar) to allow for the formation of plaques. Plaques represent areas of bacterial lysis caused by individual

phage particles. Using a sterile loop, a single isolated plaque was touched and streaked onto fresh TSA. This step aimed to separate and purify individual phage strains. The host bacterial culture was mixed with semisolid agar and poured over the streaked Petri dish, covering the entire surface. The dish was then incubated inverted overnight at 37°C. After incubation, 2 ml of SM buffer (the buffer used for phage storage and maintenance) was poured onto the plates containing plaques. The Petri dishes were agitated in a shaker for 3 hours at 120 rpm to elute the phage particles from the agar. Following agitation, the SM buffer along with the top agar was transferred to a Falcon tube and vortexed for approximately 15 minutes to ensure thorough mixing. The solution was then subjected to centrifugation at 4,100 rpm for 20 minutes to pellet any bacterial debris and agar particles. The supernatant, containing the phage particles, was carefully filtered through a 0.22µm syringe filter to remove any remaining debris and bacterial cells.

The filtrate, now containing purified phage particles, was collected in a sterile Falcon tube and stored at 4°C for further analysis. By employing this plaque-streak assay, individual phage strains were isolated and purified, allowing for subsequent morphological, physical, and molecular characterization of the phage population.

3.10 Preparation of Phage Lysate

Phage amplification was conducted on TSA plates by streaking single plaque onto several plates, followed by the preparation of phage stocks through the extraction of phage solution from these streaked plates. After streaking isolated plaques onto multiple plates and overnight incubation, plaques were observed along the streaking lines. The upper layer of top agar containing plaque remnants was collected by scraping with sterilized pipette tips into Falcon tubes, to which SM buffer was added in a ratio of 2ml per plate. Following vigorous mixing via vortex, centrifugation was carried out for 20 minutes at 4100rpm, and the resulting supernatant was collected. Syringe filtration was then performed to eliminate any bacterial debris, yielding a pure phage stock. Phage titer was determined through serial dilution of the phage solution and the DLAA method, typically resulting in the collection of 50ml phage solutions with an average concentration ranging from 10^{10} to 10^{12} pfu/ml. Alternatively, in some cases, stocks were prepared using DLAA plates, where selected plaques were carefully excised using a pipette tip and immediately dissolved in 2mL of SM buffer. Subsequently, the tube underwent a minimum of 2 hours of shaking with chloroform (at a concentration of 50µl/ml) for extraction. Following centrifugation at 4000 rpm, the supernatant was filtered through a 0.22 syringe filter to obtain a purified phage stock.

3.11 Phage Titer Assay: Spot Assay and DLLA Method

The phage titre assay, utilizing both the spot assay and the Double Layer Lysate Assay (DLLA) method, serves as a fundamental technique for determining the concentration of phage particles within a solution and facilitating successive rounds of purification for individual phages. Initially, only one completely isolated plaque per plate was selected for further analysis, ensuring the purity of subsequent studies. The process commenced with the serial dilution of the filtrate upto 10^{10}, employing sterile tips for each dilution step to prevent contamination. For the spot assay, a grid was drawn onto the bottom of an agar plate, delineating designations for phage dilutions ranging from 10^1 to 10^{10}, while a negative control comprised solely of SM buffer was included. Subsequently, a bacterial lawn was prepared by mixing 3 ml of semisolid medium with 100 µl of active log-phase host bacteria, ensuring uniform spread across the labeled plate. Following this, the plate was allowed to dry completely. In the next step, 5 µl of all phage dilutions were aseptically transferred onto the respective blocks on the grid. These droplets were allowed to permeate the agar until no liquid remained on the surface. The plates were then inverted and placed into a 37ºC incubator for a duration of 24 hours. Post-incubation, plaques or clear zones were observed, serving as indicators of phage activity.

This assay procedure provides crucial insights into the concentration and activity of phage particles within a given solution, thereby aiding the purification and subsequent study of individual phages.

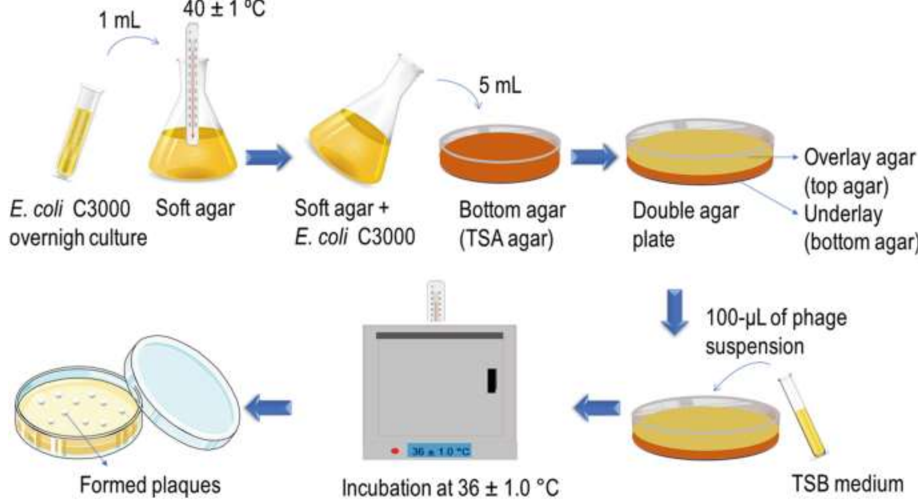


Figure 3.1 Phage Titre Assay: DLLA Method

3.12 Titration of Phage

Following the spot assay, the phage sample exhibiting the highest titre, characterized by clear lysis, was chosen for further analysis via the Double Layer Lysate Assay (DLLA), using the same bacteria as the initial host. In the DLLA procedure, 1 ml of serially diluted phage

sample was mixed with 100 µl of host bacteria and allowed to attach for 5 minutes. Subsequently, DLLA was conducted, involving a series of dilutions and plating. After an overnight incubation period, the plates were examined for plaque formation. A plate containing a plaque that could be individually distinguished was selected for the determination of phage titre. The number of plaques present on this plate was then counted.

To calculate the titre of the stock solution, the following formula was employed:

$$\text{Titre (PFU/ml)} = \frac{\text{Number of Plaques (PFU)}}{(\text{Dilution} \times \text{volume of phage added to plate in ml})}$$

This formula utilizes the counted plaque number to determine the titre of the stock solution, accounting for the dilution factor and the volume of phage added to the plate. By using this method, the concentration of plaque-forming units (PFU) per milliliter of the original phage sample can be accurately determined, providing valuable insights into the activity and potency of the phage preparation.

3.13 Host Range Analysis: Spot Assay

Spot Assay was used to determine host range of purified phage stock. For this a lawn culture of hosts bacteria were prepared by mixing 100µl of log phage bacterial culture with 3ml of soft agar and the mixture was then poured into 1.5% TSA plates and, allowed to set. After the solidification of agar, 5 µl of phage stock of dilution 10^{-8} was poured over the lawn culture and the plates were incubated inverted overnight.

3.14 Characterization of Phage

Testing the pH and thermal stability of phages is indeed crucial when considering them as potential antibacterial agents. The stability of phages under varying physiological conditions can affect their efficacy in targeting bacterial pathogens.

To test the pH stability of phages, a range of pH conditions should be prepared using buffers, and the phages should be incubated at these different pH levels for a specified period. The ability of the phages to maintain their infectivity or viability under different pH conditions can then be assessed through plaque assays or other infectivity assays.

Similarly, for thermal stability testing, phages can be subjected to different temperatures for varying durations, and their infectivity can be measured over time. This can provide insights into the temperature range within which the phages remain active and effective.

By conducting these stability tests, researchers can determine the robustness of the phages under conditions they might encounter in different environments, such as within

the human body or in various industrial or agricultural settings. This information is vital for the development and application of phages as antibacterial agents (Jin, Li et al. 2012).

3.15 Effect of temperature on phage viability

The thermal stability of the phage was assessed by aliquoting 1 ml of a phage stock (at a dilution of 10^{-8}, x PFU/ml) into Eppendorf tubes and subjecting them to varying temperatures ranging from 25°C to 90°C for different time intervals (10, 20, 30, 40, 50, and 60 minutes). Following each incubation period, a double-layer agar (DLLA) assay was performed to quantify the viable phage particles, allowing for the determination of plaque-forming units (PFU/ml). Subsequently, the average PFU/ml values were calculated for each temperature and time combination. Plotting the data revealed trends in thermal stability, with the graph illustrating the relationship between time and PFU/ml at each temperature. The analysis of the graph provided insights into the phage's ability to withstand thermal stress, highlighting potential temperature ranges where the phage maintains its activity. These findings are crucial for understanding the phage's suitability as an antibacterial agent, particularly in environments with fluctuating temperatures, and can inform further research and development in phage therapy.

3.16 Effect of pH on Phage Viability

A series of different experiments were conducted to assess the survival rate of bacteriophage particles in an LB medium whose pH was manipulated to cover a range from 1 to 14. This manipulation involved the addition of 1M HCl and 0.5M NaOH to adjust the pH levels accordingly. Following the adjustment, 100 microliters of a bacteriophage suspension at a 10^{-8} stock dilution, equivalent to x pfu/ml, were introduced into 1.5ml of the pH-adjusted medium. The mixture was then subjected to incubation at 37°C for a duration of 1 hour. Following incubation, the surviving bacteriophage particles were promptly enumerated using the DLAA method. The results were tabulated and used to construct a graph illustrating the relationship between plaque-forming units (pfu) and time. This experiment provides valuable insights into the stability and survivability of bacteriophages under varying pH conditions, contributing to our understanding of their behavior in different environmental settings.

3.17 Growth Curve

One step growth curve analysis of the bacteriophage was carried out, aiming to determine two key parameters: burst size and latent period. Bacterial growth typically involves five sequential stages in its life cycle, and each cycle typically spans an hour for completion. To analyze the phage's growth curve, the protocol developed by Adams and Wassermann in 1956 with few modifications was applied. This adaptation allowed for a detailed

examination of how the virus proliferates within bacterial hosts (Adams & Wassermann, 1956). The procedure began with the preparation of seven sterile Eppendorf tubes, each labeled with a different time interval ranging from 5 minutes to 60 minutes. Next, 1000µl of high titre phage stock (10^{-8} pfu/ml) was added to each tube, followed by the addition of 100µl of log-phase host Acinetobacter culture (with an optical density of 0.4). The tubes were then placed in a 37℃ incubator for incubation.

After 5 minutes of incubation, the tube labeled as "5 min" was removed from the incubator and centrifuged at 12000 rpm for 5 minutes to separate the phage-infected bacterial cells. The supernatant was discarded to remove unabsorbed phage particles, and the pellet containing the infected cells was resuspended in 100µl of SM buffer. This suspension was then mixed with 3ml of soft agar and overlaid onto a TSA plate using the double-layer agar assay (DLAA) technique. The same process was repeated for the remaining six tubes according to their respective incubation times. Once all plates were prepared, they were allowed to solidify before being incubated at 37℃ for 24 hours. After the incubation period, plaques (clear zones indicating bacterial lysis due to phage infection) were counted on each plate. The number of plaques counted was used to determine the phage concentration, expressed in pfu/ml (plaque-forming units per milliliter). This method allowed for the assessment of phage infectivity and replication dynamics over different time intervals (Adams & Wassermann, 1956). From the plotted graph of pfu/ml against the time of incubation, the latent period of the virus was identified as the duration between the initial adsorption of the phage and the first noticeable increase in plaque count. Additionally, the burst size of the virus was calculated by dividing the average number of plaque-forming units (pfu) per infected cell during the post-rise phase of the growth curve by the average number of pfu per infected cell during the pre-rise phase of the growth curve. This allowed for a comprehensive understanding of the virus's replication dynamics and its behavior during different stages of infection (Ellis & Delbrück, 1939).

3.18 Phage DNA Extraction

Phage DNA extraction was carried out using the Norgen Biotek Corp Phage DNA isolation kit. Initially, 1 ml of phage lysate was transferred into a 15 ml tube, followed by the addition of 500 microliters of lysis buffer B. The mixture underwent vigorous vortexing for 10 seconds before being incubated for 15 minutes at 65°C. Throughout the incubation period, the lysate was intermittently mixed by inverting the tube 2-3 times. Subsequently, 320 microliters isopropanol were added and vortexed briefly. A spin column was assembled onto a collection tube provided in the kit, and 650 microliters of the lysate were applied to the column. Centrifugation at 8000 RPM for 1 minute facilitated the separation of the DNA, with the flow-through being discarded. This process was repeated until the entire

lysate had passed through the column. Following this, 400 microliters of wash solution A were added to the column, which was centrifuged again for 1 minute at 8000 RPM. The wash step was repeated twice more to ensure purity. The column was then spun for 2 minutes at 14000 RPM to dry it thoroughly, and the collection tube was discarded. Then, the column was placed into an elution tube which was provided in the kit, and 75 microliters of elution buffer B were added. Centrifugation at 8000 RPM for 1 minute eased the collection of phage DNA in the elution tube. Finally, the DNA sample was stored at -20°C for subsequent analysis and experimentation. This method provides an efficient means of extracting phage DNA for further molecular studies.

3.19 Isolation, Qualitative and Quantitative Analysis of gDNA

Genomic DNA was done from the phage sample using the Norgen Phage DNA Isolation Kit (Cat. 46800, 46850). To assess the quality of the genomic DNA (gDNA) sample obtained, 5 microliters of the isolated DNA were loaded onto a 1% agarose gel. The gel electrophoresis was conducted at 90V for 60 minutes to separate the DNA fragments and visualize the presence of an intact band, indicative of high-quality genomic DNA. Additionally, to further evaluate the purity of the DNA sample, one microliter was loaded onto a Nanodrop 8000 spectrophotometer to determine the A260/280 ratio. This ratio provides insight into the level of contamination by proteins or other organic compounds, with a ratio close to 1.8 indicating pure DNA. By employing these methods, the integrity and purity of the genomic DNA extracted from the phage sample were assessed, ensuring its suitability for downstream molecular biology applications such as sequencing or PCR analysis.

A 1% agarose gel was prepared, by mixing 0.75 grams of agarose in 50 ml of 1X TAE buffer, which was heated until the agarose has completely dissolved. Subsequently, 0.25 microliters of ethidium bromide (EtBr) were added to the solution to aid in DNA visualization. The molten agarose gel was carefully poured into a casting tray and allowed to solidify. Following gel preparation, 5 microliters of phage DNA, along with 1 microliter of loading dye, were mixed and carefully loaded into a well on the gel. Additionally, a DNA ladder, specifically Fermentas O' Gene Rule 1 KB DNA ladder, was run alongside the sample to serve as a size reference. Electrophoresis was then conducted at 70 volts for one hour to separate the DNA fragments based on size. Upon completion of electrophoresis, the gel was examined under a UV illuminator to visualize the DNA bands, allowing for analysis of the phage DNA sample and comparison with the DNA ladder to determine fragment sizes. This method serves as a fundamental technique in molecular biology for analyzing DNA samples based on their size and migration pattern within the gel matrix.

3.19.1 Library preparation of phage

The genomic DNA of the Phage was purified using AMPure beads (Beckman Coulter, United States) in a 1:1 ratio, followed by quantification using Qubit (Thermo Fisher Scientific, United States). The sample was then normalized to a concentration of 0.2 ng/µL. Next, the normalized sample underwent tagging and indexing using the Nextera Indexing Kit (Illumina, United States). After indexing, the library was purified using AMPure beads at 0.8X concentration and quantified using the HS kit (Thermo Fisher Scientific, United States). The library's quality was assessed using a bioanalyzer and subsequently normalized to a final concentration of 4nM. To enhance diversity, 5% of a 4 nM PhiX spike-in was added to the normalized library. Following this, the library was denatured using NaOH. The denatured library was then diluted to 20 pM and subsequently to 10 pM using hybridization buffer. Finally, the resulting 10 pM library was loaded onto the Illumina MiSeq platform using the MiSeq Reagent Kit V3 600-cycles (Illumina, United States).

3.20 Whole Genome Sequencing

After confirming the presence of phage DNA through gel electrophoresis, the entire genome sequencing of the phage ACB-1 DNA samples was sent to the Center for Molecular Diagnosis (CMDN) located in Thapathali, Kathmandu for sequencing analysis. Before sequencing could commence, the DNA underwent library preparation, which involved fragmenting the DNA into smaller pieces and attaching adaptors to both ends. Indexing was then performed to distinguish between different samples. The prepared DNA samples were subsequently processed on the Illumina platform for sequencing.

During sequencing on the NGS MiSeq Illumina platform, DNA fragments were loaded onto a flow cell where bridge PCR amplifies the DNA segments, generating clusters of DNA. Sequencing was carried out using Illumina's Sequencing by Synthesis method, where each base was added individually, and fluorescently labeled bases emit signals upon addition. These signals were recorded by the computer. After each cycle, excess bases were washed away, and the process continued until the entire DNA fragment was sequenced. The sequencing signals were stored in BCL files, which were then converted to FastaQ files for downstream analysis. After identifying viral sequences, annotation of viral genes was carried out using tools like PHASTER and Proksee to derive information from the viral genome.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Identification of Host Bacteria

Acinetobacter baumannii, ACB-1 (named from the original isolate), was revived in Nutrient Agar. It formed small, smooth, and round cream-colored colonies in Nutrient agar plates.

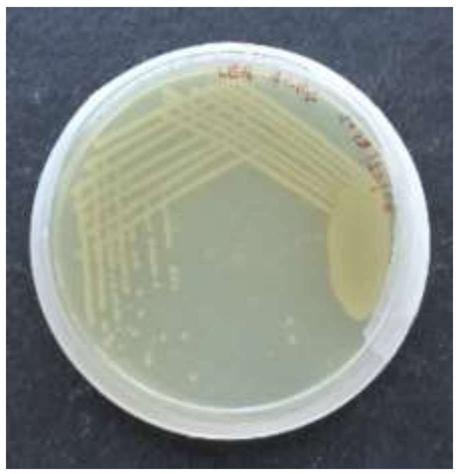


Figure 4.1 ACB-1 on NA

4.2 Gram Staining

After selecting a single isolated colony from a nutrient agar plate, Gram staining was conducted, revealing that the bacterium was gram-negative and exhibited a coccobacilli morphology. Gram staining serves as a fundamental technique in microbiology for distinguishing between different types of bacteria based on their cell wall composition. Gram-negative bacteria typically possess a thinner layer of peptidoglycan in their cell walls compared to gram-positive bacteria, along with an outer membrane containing lipopolysaccharides. Gram-negative rod becomes spherical at the stationary phase of growth.

The cellular structures crucial for maintaining cell shape in bacteria include key components of the bacterial cytoskeleton, notably FtsZ, MreB, and penicillin-binding proteins. Among these, MreB plays a significant role in the shift from rod-like to spherical cell shapes, though it may not always be present in this transition phase (Howard et al. 2012).

ACB-1 as coccobacilli bacteria was characterized by their shape, appearing as short, oval, or round rods, often resembling cocci. This classification provided important preliminary information about the bacterium, laying the groundwork for further identification and characterization through additional tests, such as biochemical assays or molecular

techniques, to determine its specific genus or species and understand its biological characteristics.

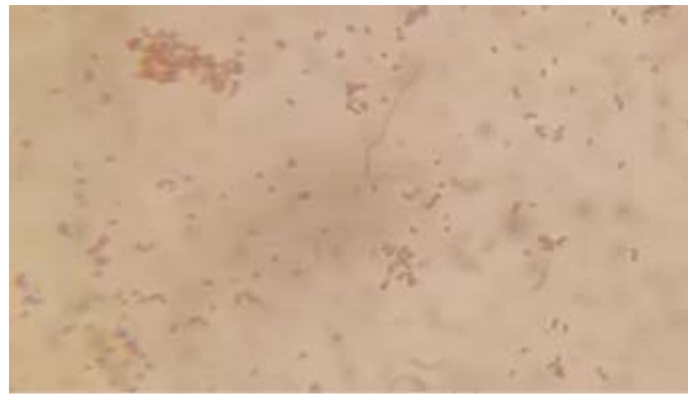


Figure 4.2 Gram Staining of ACB-1

4.3 Biochemical Test of Host Bacteria

After gram-staining, biochemical tests of ACB-1 were performed. Some key biochemical tests that were performed for the identification of ACB-1 included catalase, oxidase, motility and citrate test. It was further tested for growth at 44°C predicting the bacteria to be *Acinetobacter baumannii*. It is the only bacterium in the genus that can grow at 44°C (Asif et al., 2018).

Table 4.1 Biochemical Tests of ACB-1

Tests	Result	Inference
Catalase	Positive	The detected bacteria were indicative of *Acinetobacter baumannii*
Oxidase	Negative	
MR	Negative	
VP	Negative	
Motility	Negative	
Citrate	Positive (Blue)	
Growth at 44°C	Positive	

4.3.1 Catalase test

Catalase test is used to identify organisms which produce the catalase enzyme. The production of oxygen gas represented by resulting bubbles clearly indicated a catalase positive result by ACB-1. The catalase enzyme detoxifies hydrogen peroxide by breaking it

into water and oxygen gas. Thus, the enzyme helps organisms to neutralize harmful forms of oxygen that are generated during metabolism.

Figure 4.3 Catalase Test with Positive Result for ACB-1

4.3.2 Oxidase test

The oxidase test is a common biochemical method used in microbiology to determine whether a bacterial strain possesses cytochrome c oxidase activity. This enzymatic activity is vital in indicating the organism's capability to perform aerobic respiration. For example, bacteria are recognized as oxidase-negative, meaning it doesn't contain the cytochrome c oxidase enzyme.

Figure 4.4 Oxidase test with negative result for ACB-1

As a result, when ACB-1 underwent the oxidase test, it showed a negative outcome. This was indicated by no color alteration or the emergence of a pink to purple shade within a set timeframe after applying the oxidase reagent. This method helped in correctly categorizing ACB-1 strains, facilitating their identification within a varied microbial environment.

4.3.3 IMViC test

The four IMViC series tests ACB-1 was subjected to were Indole production, Methyl Red test, Voges-Proskauer test, and Citrate utilization test.

Table 4.2 Result of IMVIC Test of ACB-1

S.No.	Test	Result
1.	Indole Production	Negative
2.	Methyl Red Test	Negative
3.	Voges-Proskauer Test	Negative
4.	Citrate Utilization Test	Negative

ACB-1 depicted a negative indole test result. Indole tests are commonly used to differentiate bacterial species based on their ability to produce indole, a metabolic byproduct of the breakdown of the amino acid tryptophan. *Acinetobacter baumannii* is generally known to be indole-negative, meaning it does not produce indole.

ACB-1 yielded a negative result in the methyl red test, indicating it did not produce significant amounts of stable acidic end products from glucose fermentation. The methyl red test is a biochemical test which is used to determine the ability of an organism to perform mixed acid fermentation of glucose, producing stable acidic yielded end products such as lactic acid, acetic acid, and formic acid.

ACB-1 showed a negative result in the VP test, which indicated no production of significant amounts of acetoin. The Voges-Proskauer (VP) test is a biochemical test used to detect the presence of acetoin, a metabolic product produced by some bacteria during glucose fermentation.

ACB-1 was negative for citrate utilization, meaning it could not use citrate as a carbon source. The citrate utilization test was done to determine whether an organism can utilize citrate as its sole carbon source for growth.

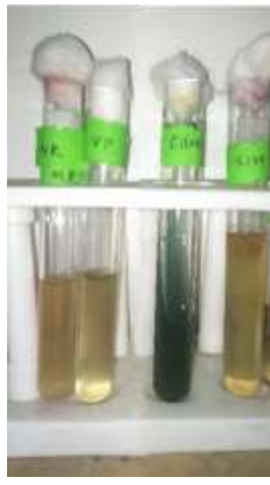


Figure 4.5 IMVIC Test of ACB-1

4.3.4 Growth at 44^{0}C

ACB-1 exhibited very little growth at 44°C. This result led us to the preliminary confirmation of bacteria to be *Acinetobacter baumannii*. *Acinetobacter baumannii* typically does not grow well at elevated temperatures like 44°C. This bacterium is considered mesophilic, meaning it thrives at moderate temperatures typically found in the environment or the human body, such as around 35°C - 37°C. At temperatures significantly higher than its optimum range, *Acinetobacter baumannii* may experience reduced growth rates or even fail to grow altogether.

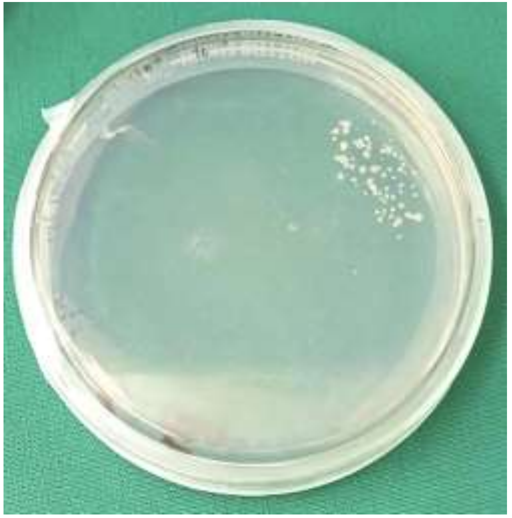


Figure 4.6 Growth of ACB-1 at 44°C

Several studies have examined the survival of *Acinetobacter baumannii*, primarily focusing on clinical isolates. As per a study, clinical strains of *A. baumannii* can grow within a temperature range of 25 to 45°C, with optimal growth occurring at 37°C (Antunes et al. 2011).

The above result was comparable to the studies made by the team of Shristi Raut and researchers, in which *Acinetobacter baumannii* was confirmed by using different phenotypic tests including: Gram-negative coccobacilli, oxidase negative, catalase positive, non-fermentative (oxidative), non-fastidious, gas negative, growth at 44°C, H_2S negative, urease negative, methyl red positive, indole negative, Voges-Proskauer negative and citrate positive (Antunes et al. 2011).

4.4 Antibiotic Sensitivity Test (AST)

4.4.1 Antibiogram Assay

From the antibiotic susceptibility test (AST) by using Kirby Bauer Disk diffusion method the bacteria ACB-1 showed resistance to more than 3 antibiotics, and thus were found to be multidrug resistant. The bacteria displayed Multidrug Resistance (MDR) when they were

exposed to a range of antibiotics spanning different generations and classes, including quinolones, aminoglycosides, and β-lactams.

Table 4.3 Antibiotic Susceptibility Pattern of ACB-1 bacteria

S.N.	Bacterial sample	Antibiotics	Conc. Mcg	Zone of resistivity (mm Standard	Resistant	Sensitive
1.	*A. baumannii*	Ciprofloxacin	5	≤18	Resistant	
		Gentamicin	10	≤12	Resistant	
		Cotrimoxazole	14	≤10	Resistant	
		Amikacin	30	≤14	Resistant	
		Ceftazidime	14	≤14	Resistant	
		Meropenem	10	≤15	Resistant	
		Imipenem	10	≤15	Resistant	
		Amoxicillin	10	≤13	Resistant	
		Nalidixic acid	10	≤13	Resistant	
		Cefepime	30	≤18	Resistant	
		Nitrofurantoin	300	≤14	Resistant	
		Cefoperazone Sublactam	75/30	≤15	Resistant	

The antibiotics used were of a different generation and various classes and the host bacterium showed resistance to all the tested antibiotics.

The study conducted in Saudi Arabia suggests that *Acinetobacter baumannii* is becoming more resistant to multiple classes of antibiotics. A retrospective study at Riyadh Military Hospital, between 2005 and 2010, found an increase in the number of *A. baumannii* strains isolated from blood cultures that were resistant to several antibiotics (Aldali, 2023).

The overuse of broad-spectrum antibiotics, including carbapenem, has been identified as one of the primary factors contributing to the worldwide increase in multidrug-resistant (MDR) *A. baumannii.* In Nepal, the easy availability of antimicrobials over the counter (OTC) poses a significant challenge and further exacerbates the development of antimicrobial resistance (Karna & Khanal, 2020). According to a study conducted in Iran, the prevalence of multidrug-resistant *A. baumannii* increased from 50% to 94% between 2001 and 2011 (Moradi, Hashemi, & Bahador, 2015). Several studies have demonstrated a 57–77% cure rate of colistin in severely ill patients infected with *Acinetobacter* species (Maragakis & Perl, 2008).

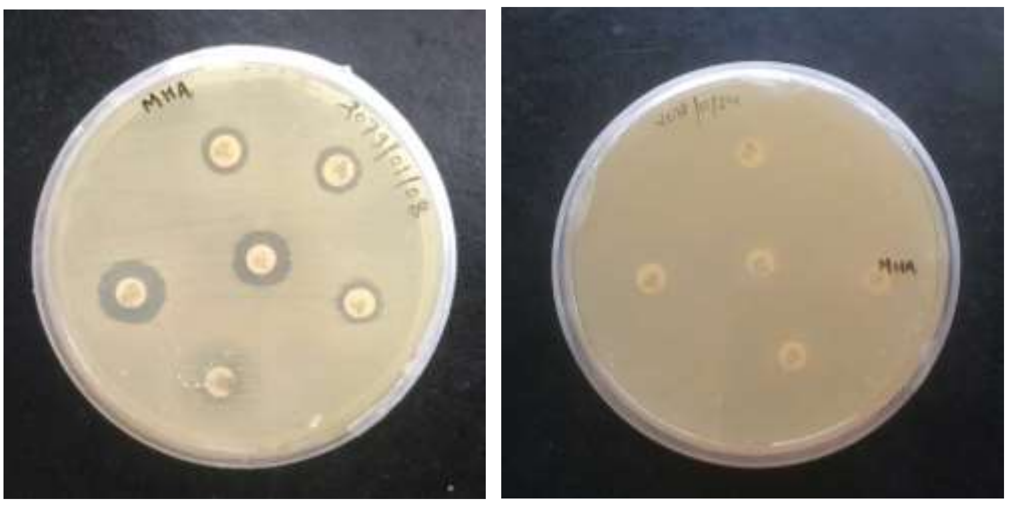


Figure 4.7 Antibiogram Assay of ACB-1

4.4.2 Confirmation of Antibiotic susceptibility using Vitek Compact System 2

The confirmation of antibiotic susceptibility testing was conducted using VITEK Compact System-2 analyzers, yielding results consistent with our laboratory tests. Furthermore, the assessment revealed resistance to other antibiotics and also to Colistin, a last-resort antibiotic. Colistin resistance presents a serious concern since it's one of the limited options available for tackling multidrug-resistant bacteria. When bacteria develop resistance to antibiotics such as Colistin, it significantly hampers our ability to manage infections effectively. This emphasizes the vital need for cautious antibiotic usage and the pressing exploration of new antibiotics or alternative treatments to address the challenge of antibiotic resistance.

4.5 Genomic DNA Extraction

Genomic DNA of ACB-1 was extracted by CTAB method. The purified phage was used to infect the host bacterium ACB-1, and then the bacterial genome was extracted. The extracted DNA was run through gel electrophoresis and viewed under UV illuminator, to observe distinct band of DNA.**Figure 4.8** Gel Electrophoresis of Extracted DNA viewed under UV light

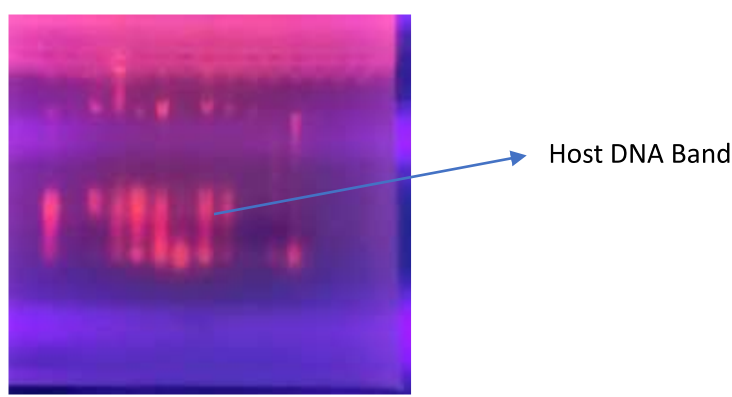


Figure 4.9 Gel Electrophoresis of Extracted DNA viewed under UV light

4.6 Quantification and purity check of isolated Bacterial DNA

Table 4.4 Concentration Determination of Extracted DNA

Sample ID	Concentration (ng/µl)	A260/280
ACB-1	343.7	1.88

4.7 Molecular Identification of Host

The Vitek Compact System 2 was employed for the molecular detection of bacteria. The VITEK 2 automated compact system is a fully automated platform designed for high-throughput bacterial identification and antimicrobial susceptibility testing (AST). It achieves bacterial identification through biochemical analysis using colorimetry. This technique enables efficient processing of large numbers of samples, making it valuable for laboratory workflows. It detected bacteria to be *Acinetobacter baumannii.*

SIDDHI POLY PATH LAB

NABL ISO 15189:2012 Accredited

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

DR. ISWAR LAL SHRESTHA
CONSULTANT HAEMATOLOGIST/PATHOLOGIST

DR. BISHWO SHRESTHA
CONSULTANT PATHOLOGIST

Name	Mr. RAMESHWOR DAS	Lab No.	107935997
Age/Gender	48 Yrs Male	Registered date	08/01/2023 18:34:39
Phone	9841408810	Collected Date	08/01/2023 18:38:18
Address	JANAKPUR	Reported date	11/01/2023 15:31:06
Referred by			

Urine Culture & Sensitivity Test

Specimen .... Urine

Organism Isolated .... > 10^5 CFU/ml of Acinetobacter baumannii complex

Identification Information	Analysis Time:	9.95 hours	Status: Final
Selected Organism	88% Probability Bionumber:	Acinetobacter baumannii complex 0041002303500202	
ID Analysis Messages			

Susceptibility Information	Analysis Time: 15.57 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Piperacillin/Tazobactam	>= 128	R	Ciprofloxacin	1	S
Ceftazidime	>= 64	R	Levofloxacin	1	S
Cefepime	>= 64	R	Tigecycline	4	I
Imipenem	8	R	Colistin	4	R
Meropenem	8	R	Trimethoprim/Sulfamethoxazole	160	R
Gentamicin	>= 16	R			

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

Culture Identification and Susceptibility testing done on Fully Automated Vitek 2 compact system, bioMerieux, France

Interpretation of S I R:

S - Sensitive

I- Intermediate

R- Resistant

Note:Please correlate clinically.

(Dr. Bishwo Shrestha, MD)

Mr. Ajay Kumar Chaurasiya
M.Sc.CM, Clinical Microbiologist
NHPC : 122 Med. Microbiology

(Dr. I. L. Shrestha, MD)

Page No: 1 of 2

In pursuit of scientific excellence since 1981 ...

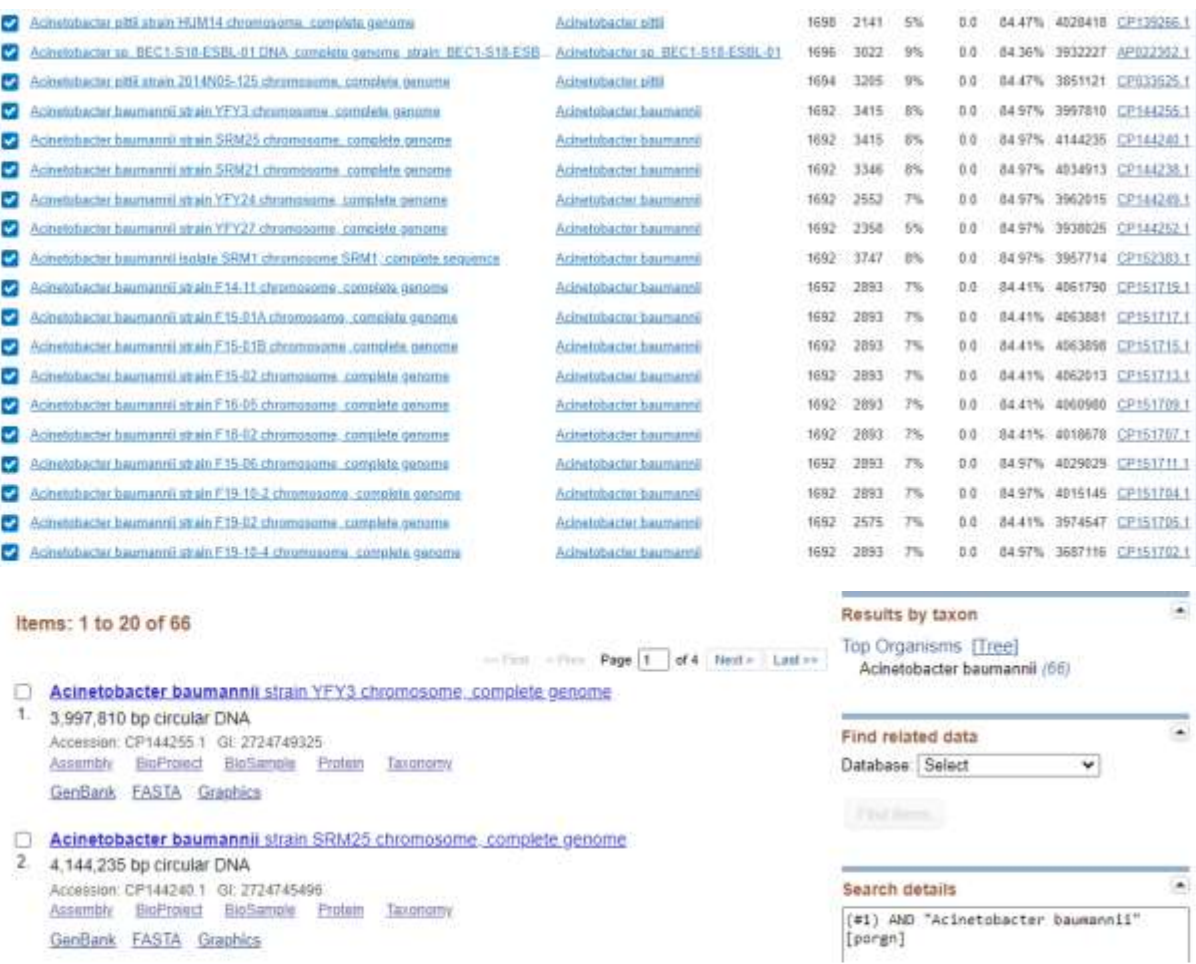
Figure 4.10 Molecular Identification of Host by Vitek Method

4.8 Whole Genome Sequencing of Host ACB-1

Whole-genome sequencing (WGS) of the host bacterium ACB-1 was conducted to determine the complete DNA sequence of this bacterium's genome. WGS provided detailed information about the genetic makeup of the bacterium.

4.9 BLAST Result

The sequences of the genes were examined using the Chromas program, with the results stored in a fasta file. This file was then analyzed using the BLAST program to compare it with sequences in the NCBI database. The closest match was found with the sequence of *Acinetobacter baumannii.* Among 100 similar organisms, 66 of the matches were identified as *Acinetobacter baumannii.*



Acinetobacter pittii strain HUM14 chromosome, complete genome	Acinetobacter pittii	1698	2141	5%	0.0	84.47%	4028418	CP139266.1
Acinetobacter sp. BEC1-S18-ESBL-01 DNA, complete genome, strain: BEC1-S18-ESB...	Acinetobacter sp. BEC1-S18-ESBL-01	1696	3022	9%	0.0	84.36%	3932227	AP022362.1
Acinetobacter pittii strain 2014N05-125 chromosome, complete genome	Acinetobacter pittii	1694	3205	9%	0.0	84.47%	3851121	CP033525.1
Acinetobacter baumannii strain YFY3 chromosome, complete genome	Acinetobacter baumannii	1692	3415	8%	0.0	84.97%	3997810	CP144255.1
Acinetobacter baumannii strain SRM25 chromosome, complete genome	Acinetobacter baumannii	1692	3415	8%	0.0	84.97%	4144235	CP144240.1
Acinetobacter baumannii strain SRM21 chromosome, complete genome	Acinetobacter baumannii	1692	3346	8%	0.0	84.97%	4034913	CP144238.1
Acinetobacter baumannii strain YFY24 chromosome, complete genome	Acinetobacter baumannii	1692	2552	7%	0.0	84.97%	3962015	CP144249.1
Acinetobacter baumannii strain YFY27 chromosome, complete genome	Acinetobacter baumannii	1692	2358	5%	0.0	84.97%	3938025	CP144252.1
Acinetobacter baumannii isolate SRM1 chromosome SRM1, complete sequence	Acinetobacter baumannii	1692	3747	8%	0.0	84.97%	3957714	CP152383.1
Acinetobacter baumannii strain F14-11 chromosome, complete genome	Acinetobacter baumannii	1692	2893	7%	0.0	84.41%	4061790	CP151719.1
Acinetobacter baumannii strain F15-01A chromosome, complete genome	Acinetobacter baumannii	1692	2893	7%	0.0	84.41%	4063881	CP151717.1
Acinetobacter baumannii strain F15-01B chromosome, complete genome	Acinetobacter baumannii	1692	2893	7%	0.0	84.41%	4063898	CP151715.1
Acinetobacter baumannii strain F15-02 chromosome, complete genome	Acinetobacter baumannii	1692	2893	7%	0.0	84.41%	4062013	CP151713.1
Acinetobacter baumannii strain F16-05 chromosome, complete genome	Acinetobacter baumannii	1692	2893	7%	0.0	84.41%	4060980	CP151709.1
Acinetobacter baumannii strain F18-02 chromosome, complete genome	Acinetobacter baumannii	1692	2893	7%	0.0	84.41%	4018678	CP151707.1
Acinetobacter baumannii strain F15-06 chromosome, complete genome	Acinetobacter baumannii	1692	2893	7%	0.0	84.97%	4029029	CP151711.1
Acinetobacter baumannii strain F19-10-2 chromosome, complete genome	Acinetobacter baumannii	1692	2893	7%	0.0	84.97%	4015145	CP151704.1
Acinetobacter baumannii strain F19-02 chromosome, complete genome	Acinetobacter baumannii	1692	2575	7%	0.0	84.41%	3974547	CP151705.1
Acinetobacter baumannii strain F19-10-4 chromosome, complete genome	Acinetobacter baumannii	1692	2893	7%	0.0	84.97%	3687116	CP151702.1

Figure 4.11 FASTA file analyzed using the BLAST Program

4.10 Genome analysis of ACB-1 by PROKSEE

PROKSEE (https://proksee.ca/) is a popular tool in bioinformatics used for annotating bacterial and archaeal genomes. It predicts genes and other genomic features and provides a standard output format for downstream analysis. In the circular diagram we could visualize the rec genes. In Acinetobacter baumannii, the recB and recC genes are part of the bacterial DNA repair system. These genes encode proteins involved in the homologous recombination pathway, which is crucial for repairing DNA damage, such as double-strand breaks (Peng V et al., 2012). The UGENE software revealed that the complete genome sequence of ACB-1 DNA spans 3,319,573 base pairs.

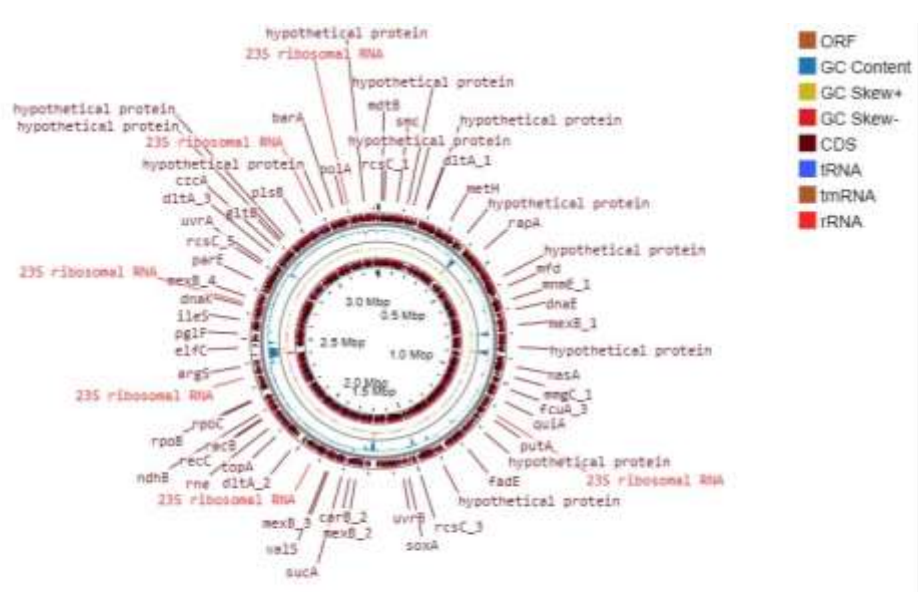


Figure 4.12 Circular diagram of Host Bacteria genome using PROKSEE

4.11 MLST profile of *Acinetobacter baumannii* (ACB-1)

Based on the Multi-Locus Sequence Typing (MLST) analysis, the bacterium was identified as *Acinetobacter baumannii*. MLST is a technique used to characterize and classify bacteria based on the sequences of several housekeeping genes. By comparing these sequences to a database of known sequences, researchers can determine the species or strain of the bacterium. In this case, the MLST profile matched known profiles for *Acinetobacter baumannii*, confirming its identity.

bactACB1_cns.fasta abaumannii_2 - Pas_cpn60(~405) Pas_fusA(~204) Pas_gltA(~328) Pas_pyrG(100) Pas_recA(399) Pas_rplB(112) Pas_rpoB(187)

Figure 4.13 MLST profile of ACB-1

4.12 PHAGE ISOLATION, SCREENING AND MANIPULATIONS

4.12.1 Sample Collection and Isolation of Bacteriophage by DLAA Method

Sample Collection

Sewage sample was collected from the most polluted section of the Bishnumati River at Gongabu. The sample was collected in a 50 ml Falcon tube and then transported to the laboratory for further analysis.

Isolation of Bacteriophage by DLAA Method

Using MDR ACB-1 as the host strain, a previously unidentified phage was isolated from sewage water and designated it as *Acinetobacter* phage i.e. ACB-1 phage.

A single bacteriophage was isolated against the strain of *Acinetobacter baumannii* collected from hospital. Although sewage/ water samples were collected from various places of Kathmandu and Lalitpur, we were able to isolate phages from the water sample of Bishnumati river, Gongabu. This might be because of the polluted water in the respective river compared to others.

Table 4.5 Phage and their site of isolation

S.N.	Name of sample collection site	Host 1
1	Bagmati (Balkhu)	-
2	Bagmati (Teku)	-
3	Bishnumati (Gongabu)	+
4	Karmanasa (Hattiban)	-
5	Nakkhu Khola (Bhaisepati)	-

Single type of plaque was isolated from Bishnumati river flowing through Gongobu. The plaque morphology observed were small pin-headed clear plaque. A single clear plaque was selected for further processing. There were different sized but similar morphological plaques which indicated the presence similar phages in the water sample. The size of a plaque correlates with how effectively the phage attaches to host cells, the duration of its latent period, and the number of progeny phages released during the burst.

Variability in plaque sizes can occur when phages infect cells at different stages of bacterial growth; those that attach early tend to create larger plaques than those attaching later. Additionally, the physical size of the phage itself influences plaque size: smaller phages diffuse more readily through semi-solid agar, creating larger zones of lysis, whereas larger phages struggle to diffuse and therefore form smaller plaques. A phage that replicates

slowly, producing fewer progeny, typically results in smaller plaques compared to fast-replicating phages. A clear plaque indicates that the host cell is fully susceptible to the phage.

Likewise, reduced lytic efficiency may result from aging of the bacterial lawn, leading to larger microcolonies within the lawn, or from phenomena such as lysis inhibition observed in certain phages like the T-even phages. The halo zone surrounding a plaque indicates that the phage has released enzymes, like depolymerases, that break down the bacterial host's capsule.

Phages were isolated following double agar assay. It helped to form greater uniformity of plaque and form greater size plaque due to high rate of phage diffusion in soft agar. During phage extraction/storage SM (sodium chloride and magnesium sulphate buffer was used. The gelatin present in SM buffer helped to stabilize the phage particle while storage. Chloroform maintained the sterility of phage stock by obstructing Bacterial growth not causing any harm to phage. The visualization of visible plaques denoted the positive result for phage isolation. Plaques are clear zones which are formed in a lawn of cells because of lysis by phage. The phages were isolated in high titre without enrichment which indicated that the rivers of Kathmandu valley are heavily polluted with hospital waste because *Acinetobacter baumannii* is a pathogen normally causing tissue infection. Another major concern is that there is the presence of multidrug resistant pathogens in river flowing around Kathmandu valley which may result in MDR pathogen infection in people.

Figure 4.14 Sample Collection Site (Bishnumati River, Gongabu)

4.12.2 Clonal Purification of Bacteriophage

The initial isolation plate may contain a mixture of different phage strains, making it necessary to purify a single strain for further study. This purification process involved streaking individual plaques (clear zones where bacterial cells have been lysed by phages) onto fresh plates multiple times until a pure culture of a single phage strain was obtained. Once a single plaque was obtained, it represented a population of phages derived from a single phage particle. From this plaque, progeny plaques (plaques resulting from the infection of bacterial cells by phages from the original plaque) were selected for further propagation. The progeny plaques selected from the single mother plaque were used to prepare a phage stock. This involved transferring the phages from the progeny plaques into a liquid medium, such as a buffer or bacterial culture, to create a concentrated stock of the purified phage. The progeny plaques selected from the single mother plaque are used to prepare a phage stock.

Figure 4.15 Purification of Phage

Pin- headed plaque formation, characterized by a clear inner zone surrounded by a turbid outer zone, can arise due to various factors affecting the efficiency of phage-induced bacterial lysis. This phenomenon may occur more prominently during the late phase of plaque development, suggesting changes in phage-host interactions over time. Factors such as the aging of the bacterial lawn and associated increases in colony size can impact the distribution and efficiency of bacterial lysis by phages. Additionally, certain phages, like T7 phages, may exhibit lysis inhibition, further influencing plaque morphology. The presence of different phage types within a sample can also contribute to the formation of plaques with varying degrees of transparency and sizes, reflecting the diverse interactions between phages and their bacterial hosts.

Table 4.6 Plaque morphology of Phage ACB-1

S.N.	Name of phage	Plaque morphology	Lysis
1.	ACB-1 Phage	Pin head	Clear

4.12.3 Determination of Phage Titer/ Spot assay

Countable plaques were observed in dilution 10^{-10} in the DLAA plates. Phage titer was calculated using formula given below and found to be 1.1×10^{11} pfu/ml.

$$\text{Pfu/ml} = \frac{\text{Number of plaques observed}}{Volume\text{ of phage sample} \times \text{Dilution}}$$

$= 96 / (1\text{ml} \times 10^{-8})$

$=. \; 9.6\times10^{9}$

Where, Pfu/ml = Plaque forming unit per milliliter of the sample

Table 4.7 Phage Titre of ACB-1

S.N.	Name of the phage	Dilution	Pfu/ml = $\frac{\text{No of plaques observed}}{\text{Dilution} \times \text{Volume of sample}}$
1.	ACB-1 phage	10^{-8}	9.6×10^{9}

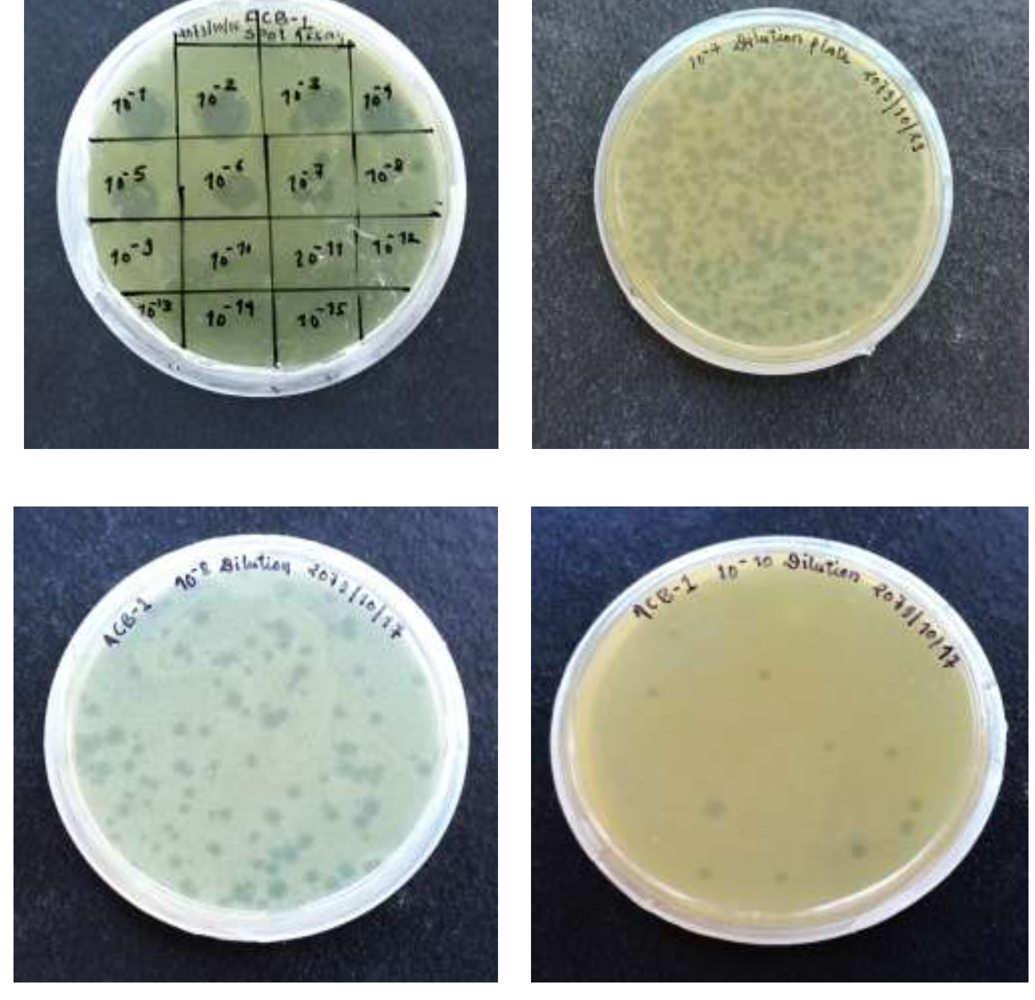


Figure 4.16 Determination of Phage Titre of ACB-1

4.13 Physiochemical characterizations of phage

4.13.1 Multiple Host range analysis of Phage

The phages were tested against several other gram-negative microorganisms, including carbapenem and colistin-resistant *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *E. coli*, and *Salmonella* spp. Based on the spot test, *Acinetobacter* phages did not show any inter host range property results as depicted in table below.

Table 4.8 Multiple host range of ACB-1 Phage

S.N.	Bacteria	Phage	Lysis
1.	Pseudomonas 6661	ACB-1	Negative
2.	Klebsiella 6667		Negative
3.	E. coli 5		Negative
4.	E. coli 10		Negative
5.	Acinetobacter (ACB)		Negative
6.	Pseudomonas 3		Negative
7.	Pseudomonas 4		Negative

The ability of phages to infect and destroy their bacterial hosts determines their host range. Molecular studies often emphasize the attachment phase in broad-host range phages. However, after attaching to the host, phages must successfully replicate, which is influenced by their genetic and regulatory mechanisms. Furthermore, the modulation of host range can also be heavily influenced by various proteins, including host receptor binding proteins (RBPs), and their adaptations.

Several studies have demonstrated the potential to broaden the host range of phages by genetically modifying tail fiber proteins, resulting in improved therapeutic efficacy against clinical multi-drug resistant (MDR) bacterial infections. Spontaneous mutations in tail tubular structures may be common in Acinetobacter phages, enabling the expansion of phage host ranges and enhancing their ability to overcome resistance during treatments (He et al., 2024).

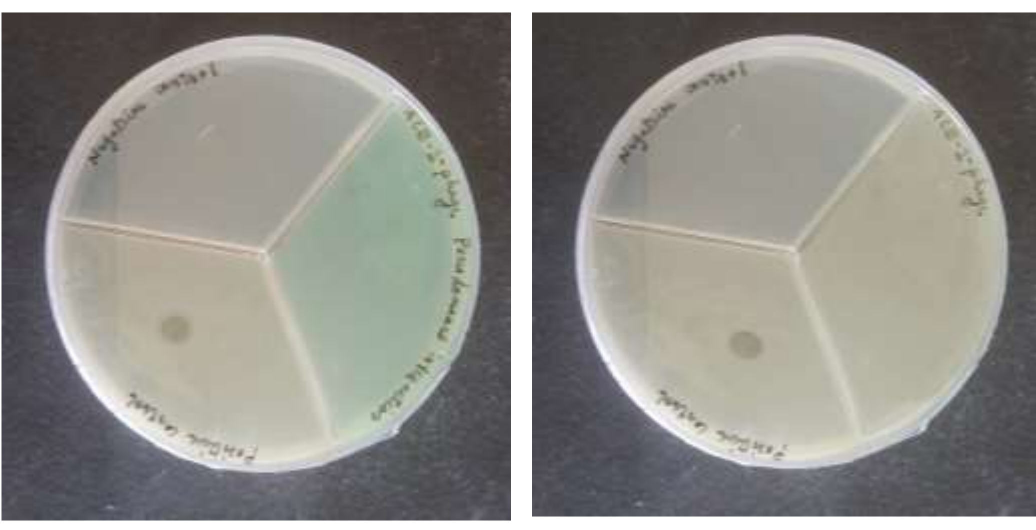
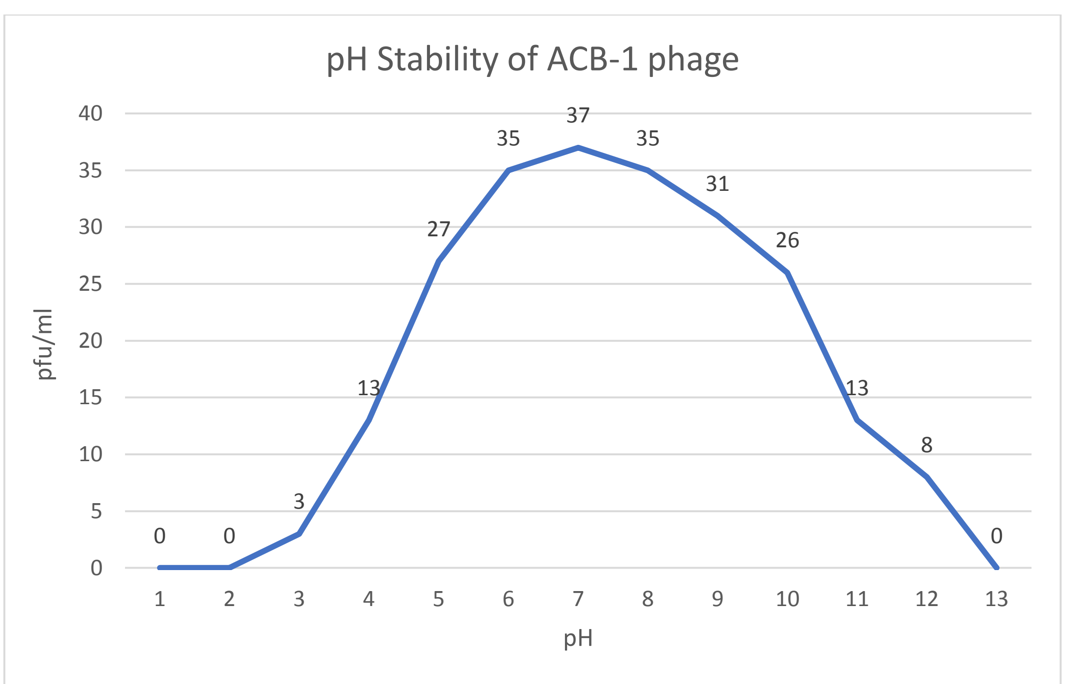


Figure 4.17 Host Range Analysis

4.14 Stability of phage at different pH range

A pH stability test was conducted to determine the optimal pH of ACB-1 Phage at various pH levels for 1 hour at room temperature. The phage titre decreased at lower pH levels and became inactive at pH 1 and 2. However, the phage titers then increased significantly at higher pH levels, demonstrating effective lysis up to pH 12.



Graph 4.1 pH stability of Phage ACB-1

As shown in graph, phage ACB-1 showed its lytic activity even at pH 12 where it was inactivated at pH 1 and 2 and its optimal pH was in range of 4 to 10.

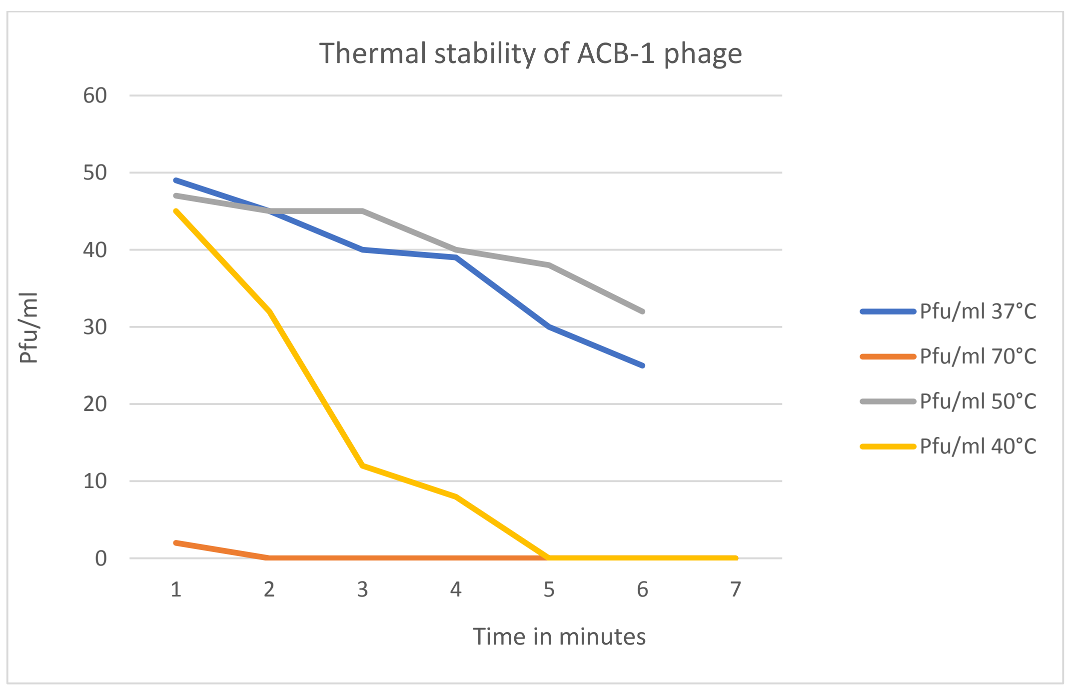
Many phages are relatively stable at neutral pH. They can maintain their infectivity and structural integrity under these conditions. Some phages are stable under acidic

conditions, while others may experience reduced stability or loss of infectivity. Acidic pH conditions can denature phage proteins and affect their ability to infect bacterial hosts. Phage stability can vary at basic pH levels. Some phages may remain stable, while others may lose infectivity or undergo structural changes. Basic pH conditions can also affect phage proteins and viral particles, potentially leading to loss of function. Extreme pH conditions (very low or very high pH) can often lead to the denaturation and inactivation of phages. However, some extremophile phages have evolved mechanisms to survive and remain stable in extreme pH conditions (Dekic, S. et al., 2018).

Obeidat et al. (2014) in his studies observed the survival of clinical *A. baumannii* isolates for up to 23 days in distilled, tap, and saline water environments, at temperatures ranging from 18 to 24°C and pH levels between 4.5 and 8.

4.15 Stability of the phage at different temperature

Thermal stability tests were conducted to assess the heat resistance of phage ACB-1. The phage's ability to withstand different temperatures over varying incubation periods was evaluated. It was observed that phages remained stable even when exposed to temperatures as high as 70°C for up to 10 minutes. The result showed that viability of phage diminishes as the temperature increases and as the temperature increases plaque morphology also changed to small size.



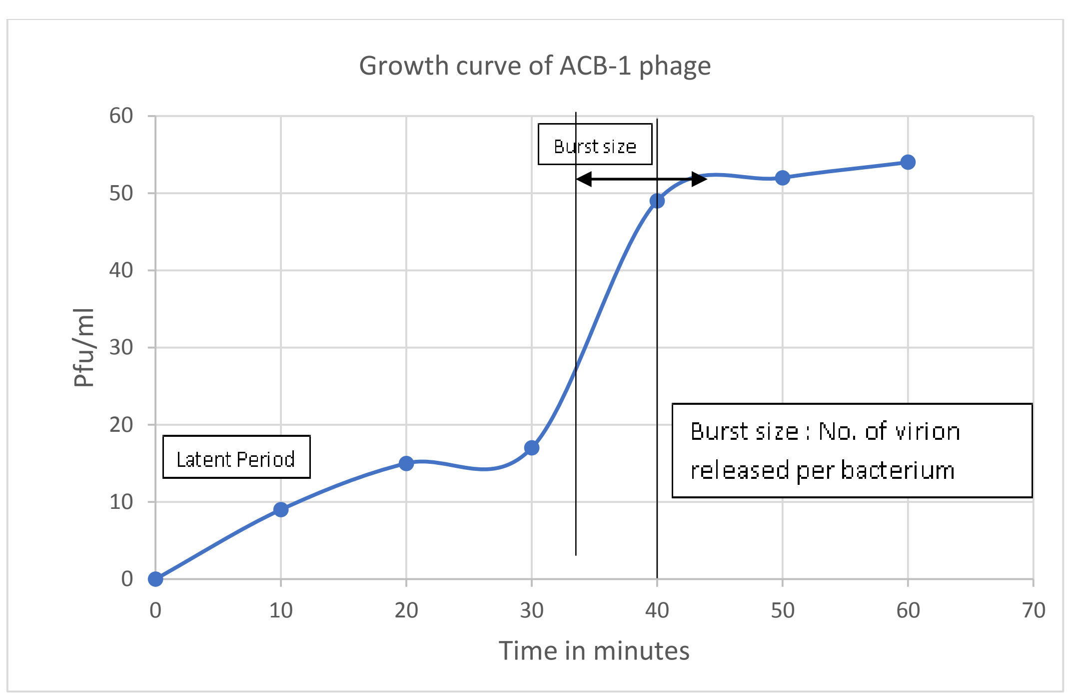
Graph 4.2 Thermal stability of Phage ACB-1

Figure 4.18 Stability of the ACB-1 phage at different temperatures

Bacteriophages are viruses that target and infect bacteria, and their stability can vary depending on the specific type. Generally, bacteriophages are stable within a certain temperature range, typically around the normal human body temperature of 37°C (98.6°F), which is often the optimum temperature for their stability and activity. Most bacteriophages can be stored in a refrigerator (2-8°C) for several months, and lyophilization (freeze-drying) is a common method for long-term storage. While some phages are stable at higher or lower temperatures, they may be sensitive to freeze-thaw cycles. For instance, phages found in extreme environments, such as hot springs, may be stable at high temperatures. However, some phages may be inactivated at temperatures exceeding 60°C. Apart from temperature, pH is another critical factor affecting phage stability, with most phages stable in a pH range of 6 to 8. The structure of the phage, particularly its capsid, is essential for stability, as some phages have a more robust capsid, allowing them to withstand harsher conditions. While the stability of phages in environmental conditions such as salinity and exposure to UV light may vary, it's essential to consult specific literature or data provided for a particular phage to determine its stability in various conditions (Harada et al., 2020; Moelling et al., 2018; Schooley et al., 2017).

4.16 One step growth curve of phage

A one-step growth curve of the phage was conducted to ascertain both the latent period and burst size of the phage. This enabled the determination of how the phage number varies throughout one replicative cycle.



Graph 4.3 One Step Growth Curve of ACB-1 Phage

After the initial steady period, an increase in phage titer was seen after 30 minutes. Hence, the latent period for ACB-1 phage was identified as 30 minutes. Each phage has a characteristic burst size, and different phages require varying amounts of time to complete one growth cycle. We determine successful reproduction of a phage when we can detect plaques or circular areas with minimal or no bacterial growth on an agar plate, which are covered with a thin layer of bacteria.

The burst size for the phage ACB-1 was computed to be 32 phage particles per infected cell. The latent period was determined by the time between absorption of phage and the initial rise in plaque number. Burst size was determined by dividing the average number of plaque-forming units (pfu) per infected cell during the post-rise phase of the growth curve by the average number during the pre-rise phase.

The infection dynamics of phage ACB-1 were characterized using a one-step growth curve, revealing a brief latent period. By the 40-minute mark, the phage had reached a plateau stage, achieving a burst size of 32 pfu per cell. A smaller burst size may be due to factors such as limited cellular resources necessary for viral replication, competition among viruses within the same cell, or mechanisms that restrict viral production to prevent early cell lysis, ensuring the survival of the viral population. These results indicate that phage ACB-1 has a low replication capacity and lytic activity (Ackermann, 2003).

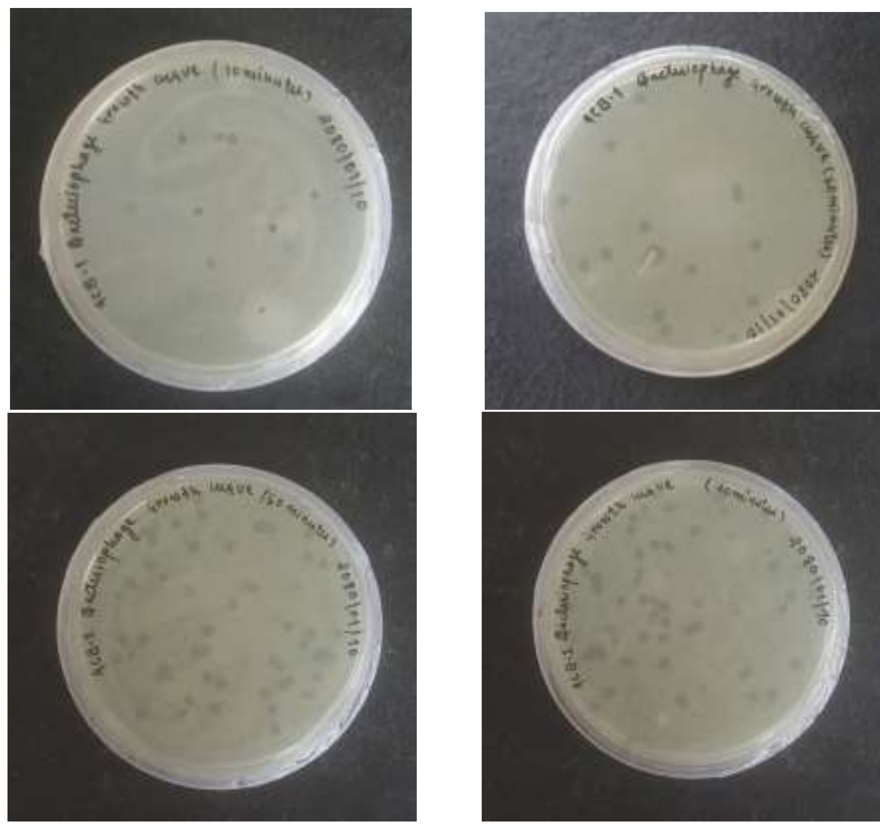


Figure 4.19 One-step Growth Curve of Phage

4.17 Whole Genome Sequencing

4.17.1 Isolation, Qualitative and Quantitative Analysis of gDNA:

A. Gel electrophoresis of phage DNA

A fundamental requirement for all DNA sequencing technologies is the need for high-quality nucleic acid preparations, devoid of contaminating RNA, proteins, or solvents. Genomic DNA was extracted by breaking the phage capsid with heat and proteinase K using the Norgen Phage DNA Isolation kit, and subsequently purified. The DNA of Phage ACB-1 displayed A280/260 values of x and formed a faint band on agarose gel electrophoresis. Following gel electrophoresis, the genomic DNA of the phage was found to be y base pairs when compared with the ladder.

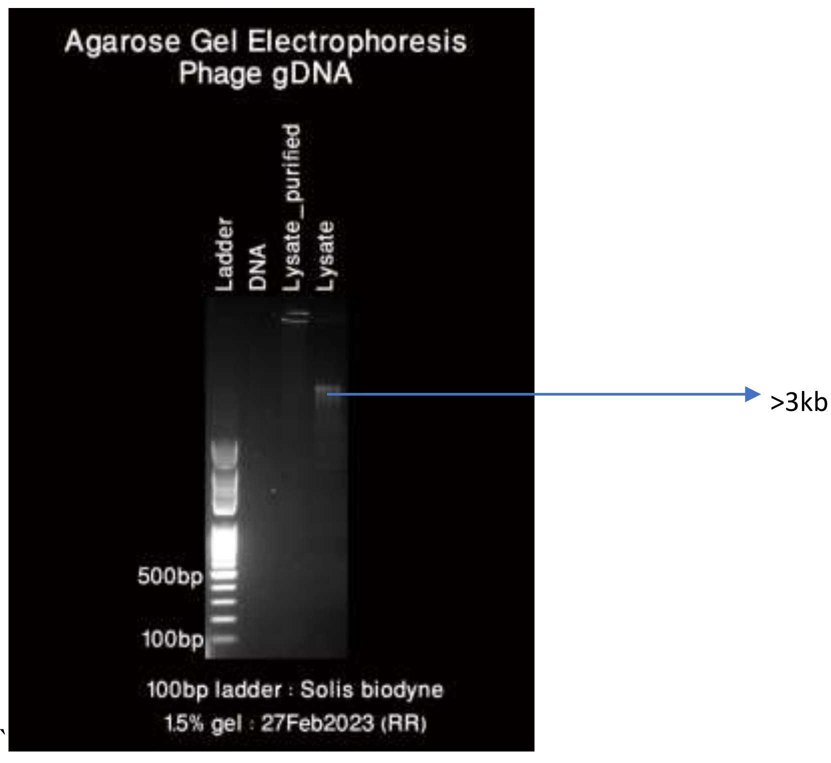


Figure 4.20 Electrophoresis of phage DNA

In this study, gel electrophoresis was employed as an initial step to assess the quality of Phage DNA prior to whole genome sequencing. However, the DNA extracted initially exhibited a notably low quantification, prompting further investigation. Subsequent attempts utilizing direct cell lysis resulted in an excessive smear pattern and fragmented DNA, indicative of impurities. To address this issue, the lysate underwent purification using 0.5X AMPure beads, a critical refinement step that ultimately yielded a significant improvement. Through this purification process, we achieved a substantial increase in DNA concentration, particularly in high molecular weight fragments, thus enhancing the quality and suitability of the DNA for subsequent sequencing analyses.

B. Quantitative analysis of gDNA

Table 4.9 Concentration Determination of Extracted DNA of ACB-1 phage

Sample ID	Concentration (ng/µl)	A260/280	A260/230
ACB-1 Phage	952.1	1.55	0.44

C. Genome analysis of Phage: PHASTEST annotation

PHASTEST, which stands for PHAge Search Tool with Enhanced Sequence Translation, represents the next evolution in the lineage of PHAST servers dedicated to prophage analysis. It is an online tool built to facilitate the speedy detection, annotation, and visualization of prophage sequences present in bacterial genomes and plasmids in either genebank or fasta format. It also enables quick annotation and interactive visualization of

all genes, such as protein-coding regions and tRNA/tmRNA/rRNA sequences, found in bacterial genomes. PHASTEST offers quicker and more precise prophage annotations compared to previous versions. It also enhances whole genome annotations and improves genome visualization, making it a potent tool for comprehensive genome annotation. Additionally, PHASTEST introduces a modern and responsive interface, enabling users to generate, edit, annotate, and interactively visualize high-quality genome maps (Wishart et al., 2023).

Table 4.10 PHASTEST generated circular Genome summary of ACB-1 phage 1

Region length	48Kb
Completeness	Intact
Score	150
Total proteins	64
Most common Phage	PHAGE_Acinet_YMC11/11/R3177_NC_041866(24)
GC%	40.60%

Region	Region Length	Completeness	Score	# Total Proteins	Region Position	Most Common Phage	GC %
1	48Kb	intact	150	61	560-48634	PHAGE_Acinet_YMC11/11/R3177_NC_041866(25)	40.60%

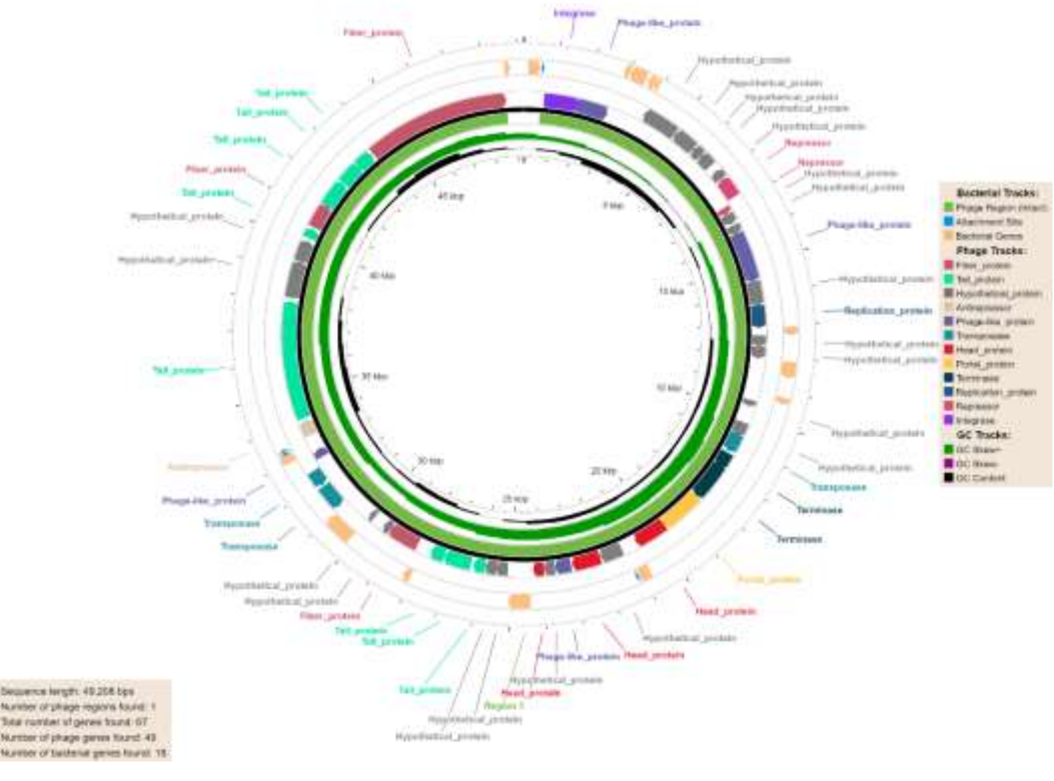


Figure 4.21 PHASTEST generated circular Genome Map

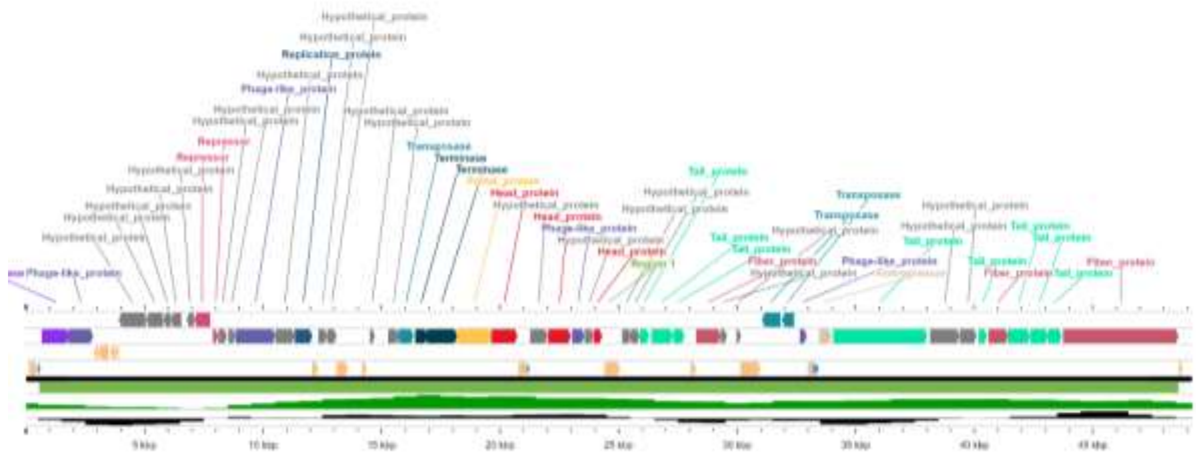


Figure 4.22 PHASTEST generated Linear Genome Map

Phage Genes

Bacterial Genes

Bacterial, total 2 CDS

#	CDS Position	BLAST Hit	E-Value
1	131..490	tmRNA Resume consensus sequence (at 89): tatagtcgcaaacgacga	0.0
2	48626..48805	PP_00062; hypothetical protein	0.0

Table 4.11 PHASTEST generated Genome summary of ACB-1 phage 2

Region length	35.4Kb
Completeness	Intact
Score	150
Total proteins	47
Most common Phage	PHAGE_Haemop_SuMu_NC_019455(12)
GC%	40.60%

Region	Region Length	Completeness	Score	# Total Proteins	Region Position	Most Common Phage	GC %
1	35.4Kb	intact	150	47	290-35780	PHAGE_Haemop_SuMu_NC_019455(13)	40.84%

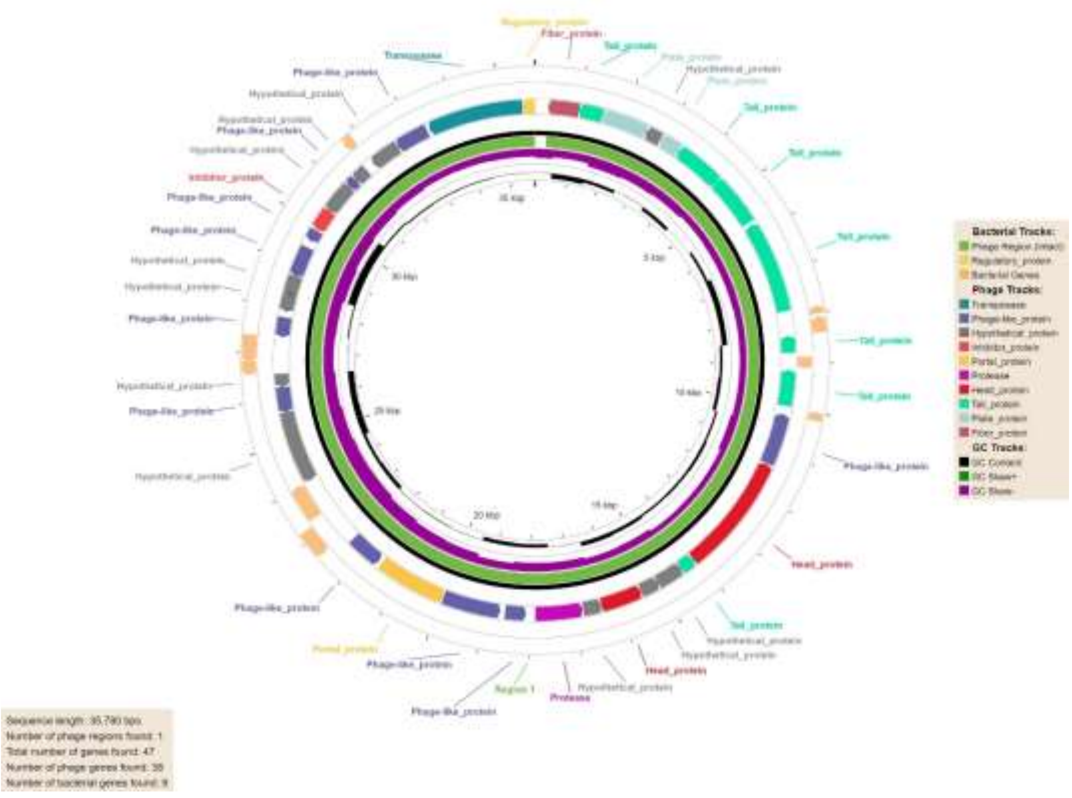


Figure 4.23 PHASTEST generated circular Genome Map

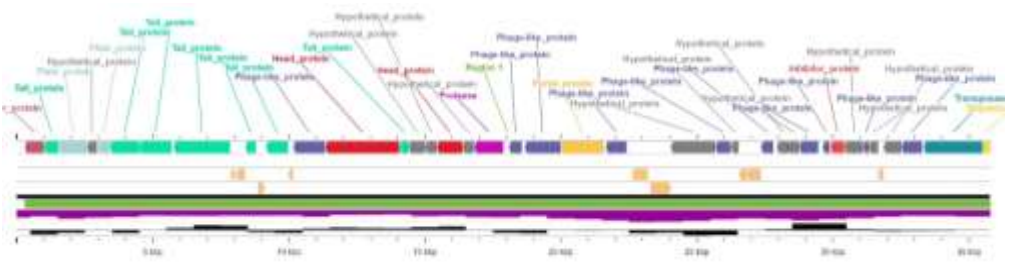


Figure 4.24 PHASTEST generated Linear Genome Map

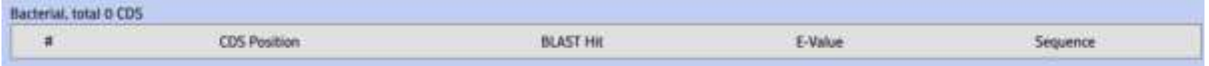
Phage genes

Fiber protein | Tail protein | Plate protein | Hypothetical protein | Phage-like protein
Head protein | Protease | Portal protein | Inhibitor protein | Transposase
Regulatory protein

intact
Region 1, total 38 CDS

#	CDS Position	BLAST Hit	E-Value
1	250..997	PP_00001 putative tail fiber protein phage PHAGE_Entero_phiP27_NC_003356	1.96e-13
2	994..1533	PP_00002 tail protein phage PHAGE_Shigel_SfIV_NC_022749	2.04e-05
3	1530..2579	PP_00003 baseplate J protein phage PHAGE_Mannhe_vB_MhM_3927AP2_NC_028766	8.89e-25
4	2581..2943	PP_00004 hypothetical protein phage PHAGE_Mannhe_vB_MhM_3927AP2_NC_028766	2.82e-11
5	2952..3425	PP_00005 baseplate assembly protein phage PHAGE_Mannhe_vB_MhM_3927AP2_NC_028766	8.15e-19
6	3425..4510	PP_00006 tail protein P phage PHAGE_Mannhe_vB_MhM_3927AP2_NC_028766	2.0e-32
7	4500..5687	PP_00007 tail DNA circulation protein phage PHAGE_Salmon_ST64B_NC_004313	3.82e-17
8	5792..7840	PP_00008 bacteriophage tail length determination protein phage PHAGE_Haemop_SuMu_NC_019455	9.96e-52

Bacterial genes



Bacterial, total 0 CDS

#	CDS Position	BLAST Hit	E-Value	Sequence

Table 4.12 PHASTEST generated genome summary of ACB-1 Phage 3

Region length	23.3Kb
Completeness	Intact
Score	100
Total proteins	27
Most common Phage	PHAGE_Psychr_pOW20A_ NC_020841(6)
GC%	43.20%

Region	Region Length	Completeness	Score	# Total Proteins	Region Position	Most Common Phage	GC %
1	23.3Kb	intact	100	27	1429-24798	PHAGE_Psychr_pOW20_A_NC_020841(6)	43.20%

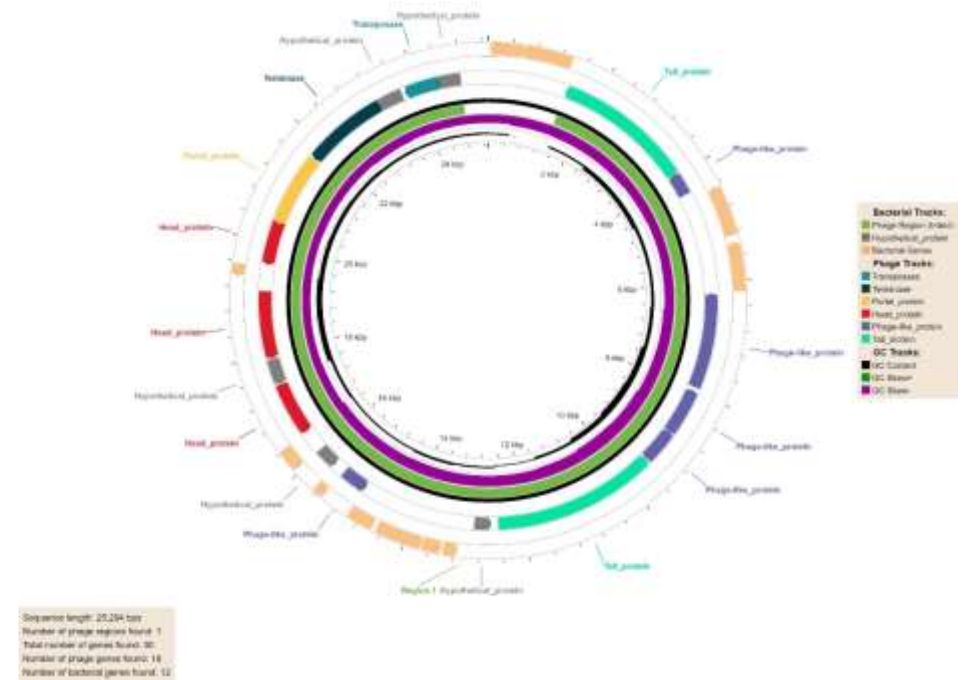


Figure 4.25 PHASTEST generated circular Genome Map

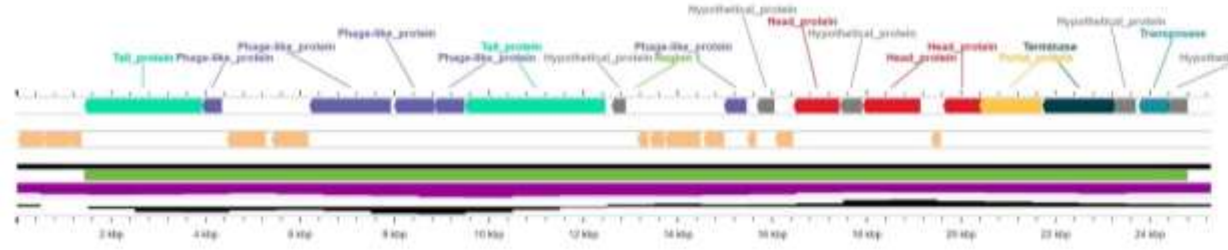
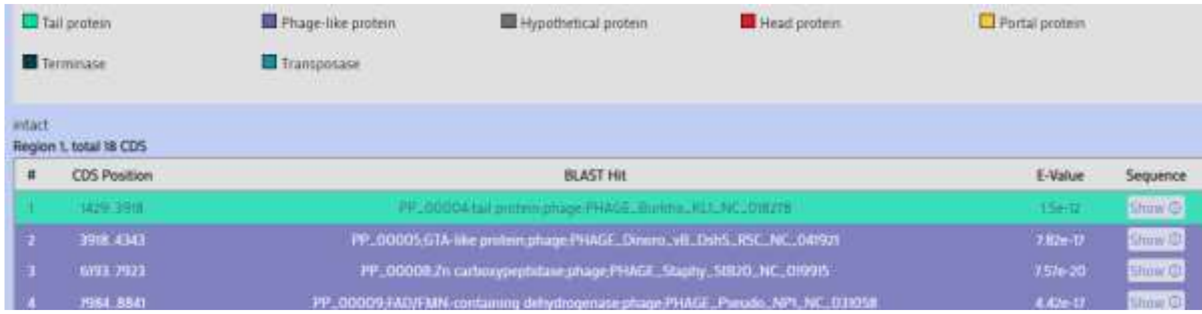


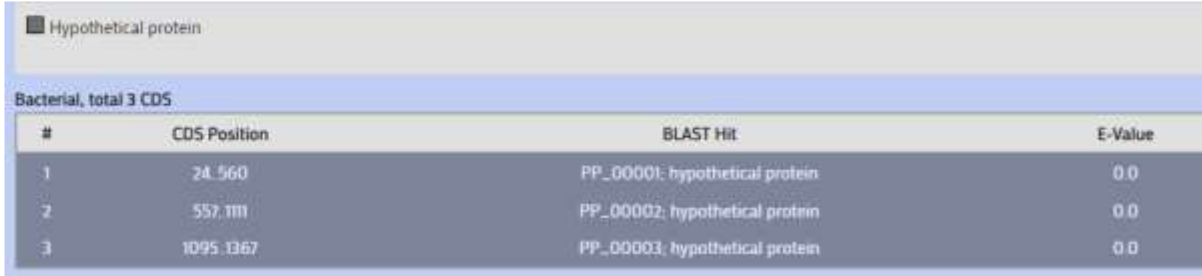
Figure 4.26 PHASTEST generated Linear Genome Map

Phage proteins



#	CDS Position	BLAST Hit	E-Value	Sequence
1	1429..3918	PP_00004;tail protein;phage;PHAGE_Burkho_KL1_NC_018278	1.5e-12	Show
2	3918..4343	PP_00005;GTA-like protein;phage;PHAGE_Dinoro_vB_DshS_R5C_NC_041921	7.82e-17	Show
3	6193..7923	PP_00008;Zn carboxypeptidase;phage;PHAGE_Staphy_SfB20_NC_019915	7.57e-20	Show
4	7984..8841	PP_00009;FAD/FMN-containing dehydrogenase;phage;PHAGE_Pseudo_NP1_NC_031058	4.42e-17	Show

Bacterial genes



#	CDS Position	BLAST Hit	E-Value
1	24..560	PP_00001; hypothetical protein	0.0
2	557..1111	PP_00002; hypothetical protein	0.0
3	1095..1367	PP_00003; hypothetical protein	0.0

Of the three phages studied, one was classified as a lysogenic phage. Its genome was found to encode 64 intact proteins, with a calculated GC content of 40.60%. Moreover, a circular genome map generated by PHASTEST revealed a length of 49,208 base pairs. The other two phages are likely to be lytic phages, meaning they are believed to be capable of infecting bacteria and causing them to lyse without integrating their genetic material into the host's genomes. From the PHASTEST annotation, the region length of the second infecting phage genome was determined to be 35.5 kb, encoding 47 intact proteins. The GC content of the genome was found to be 40.60%. Similarly, the genome of the third phage was found to be 23.3 kilobases in length, coding for 27 intact proteins. Its GC content was calculated to be 43.20%.

CHAPTER 5: SUMMARY

With the rising threat of antibiotic resistance, scientists are actively seeking effective alternatives to address this pressing issue. Bacteriophages are increasingly recognized as powerful solutions to combat the antibiotic crisis. Colistin-resistant *Acinetobacter baumannii* is listed as the top critical pathogen prioritized by the WHO, responsible for severe hospital-acquired infections. It is the most critical among multidrug-resistant bacteria, which presents a significant threat in healthcare settings such as hospitals and nursing homes. It particularly endangers patients reliant on medical devices like ventilators and blood catheters.

Due to the significant health threats posed by *Acinetobacter baumannii*, our research has concentrated on isolating and characterizing phages specifically designed to combat this highly resistant strain. This thesis presents the discoveries regarding the biological characteristics of these phages, including plaque morphology, virion structure, and their ability to lyse *Acinetobacter baumannii* strains. Additionally, genetic studies have been conducted, establishing a foundation for phage research with potential therapeutic applications.

Only one lytic phage was isolated, capable of infecting and lysing colistin-resistant bacteria originating from human clinical isolates. Therefore, the null hypothesis is rejected as that the lytic bacteriophage shows no activity against strains of *Acinetobacter baumannii*.

The bacterial strain was identified as *Acinetobacter baumannii* type 2 through molecular analysis using MLST profiling. The isolated host, ACB-1, showed resistance to more than three antibiotics, indicating multidrug resistance. Notably, it also exhibited resistance to Colistin, an antibiotic of last resort, underscoring the urgent need for alternative treatments. A bacteriophage, ACB-1, targeting *Acinetobacter baumannii* was isolated from various sewage samples. Physiochemical characterization of phage ACB-1 revealed a burst size of 32 virions per bacterium. Additionally, the phage demonstrated stability at temperatures of up to 60 degrees Celsius and across a wide pH range of 4 to 10, with an optimal pH of 7. Furthermore, it displayed a limited host range. Efforts to sequence the entire genome of a bacteriophage were hindered by insufficient reads, prompting a shift towards analyzing integrated phages within the bacterial genome. Eventually, three integrated phages were identified.

Among them, one was identified as a lysogenic phage, hosting a genome encoding 64 intact proteins with a calculated GC content of 40.60%. A circular genome map generated by PHASTEST revealed a length of 49,208 base pairs. The other two phages were classified as lytic, indicating their ability to infect bacteria and induce lysis without integrating into the host genome. The second phage's genome, according to PHASTEST annotation,

spanned 35.5kb and encoded 47 intact proteins, with a GC content matching the first phage at 40.60%. Similarly, the third phage's genome measured 23.3 kilobases in length, encoding 27 intact proteins, and boasting a slightly higher GC content of 43.20%. Bacteriophage ACB-1 had a limited ability to infect different hosts but showed impressive resilience to fluctuations in temperature and pH levels. Its ability to cause bacterial lysis was evident on agar plates. Although the genetic analysis of ACB-1 was not possible due to limited data, the investigation of the *Acinetobacter baumannii* genome revealed the existence of a lytic phage integrated within it, hinting at its potential relevance in phage therapy.

CHAPTER 6: CONCLUSION

- Multidrug-resistant (MDR) *Acinetobacter baumannii* was isolated from the hospital sample, demonstrating resistance to Colistin, the last-resort antibiotic.
- A lytic bacteriophage, named ACB-1, was isolated, demonstrating its ability to lyse multidrug-resistant *Acinetobacter baumannii*. This phage produced distinctive pinhead colonies on Tryptic Soy Iron agar.
- Phage ACB-1 displayed no host range, meaning it couldn't infect either closely related or different bacterial strains of *Acinetobacter baumannii*.
- The phage demonstrated its highest stability at 37℃. However, its ability to infect decreased as the temperature rose. Despite this, the phage could survive even at 70^{0}C although only for a short duration of 10 minutes.
- The isolated phage showed its highest activity at pH 7, with its optimal pH ranging from 4 to 10. Remarkably, phage ACB-1 displayed its lytic activity even at pH 12.
- The burst size of the virus was determined to be 32 virions per infected cell. This means that, on average, each infected cell releases 32 new virus particles during the lytic cycle.
- PHASTEST genomic analysis indicated a phage genome region length of approximately 48kb, 35.5 kb, and 23.3 kb for phage 1, 2, and 3 respectively.
- Within these regions, 64, 47, and 27 intact proteins were encoded for phage 1, 2, and 3 respectively.
- The GC content of the genomes was calculated to be 40.60%, 40.60%, and 43.20% for phage 1, 2, and 3 respectively.
- This study on colistin-resistant *Acinetobacter baumannii* could potentially be the first of its kind in Nepal. It aims to enhance our understanding of phage characterization, propagation, and adsorption, offering valuable insights into phage genomic analysis as well.
- Bacteriophage ACB-1 showed limited host range but strong stability across various temperature and pH conditions, with observed lytic activity on plates. Despite insufficient data for genomic analysis, examination of *Acinetobacter baumannii*'s genome indicated the presence of a lytic phage within, hinting at potential implications for phage therapy.

CHAPTER 7: RECOMMENDATION

This study primarily focused on examining the morphological, physiochemical, and to some extent molecular attributes of the phage. However, the study uncovered some limitations, which led to the following suggestions for future research.

1. The study should expand its scope beyond one bacterium and include multiple MDR bacterial hosts.
2. It is recommended to conduct extensive genome analysis and submit the whole genome to GenBank. Collaborating with a bioinformatics expert can enhance the findings and conclusions of the study.
3. Investigating the cloning of endolysin genes and modifying phage endolysin-based proteins for phage therapy represents an important area of research within the field of phage studies.
4. Utilizing animal models in the study would furnish reliable and valuable data to support the assertions posited. Initiate well-designed clinical trials to evaluate the efficacy and safety of phage therapy for treating bacterial infections in humans.

REFERENCES

Abbo, A., & Carmeli, Y. (2007). *Impact of multi-drug-resistant Acinetobacter baumannii on clinical outcomes*. (December 2004), 793–800. https://doi.org/10.1007/s10096-007-0371-8

Abedon, S. T., Thomas-abedon, C., Thomas, A., & Mazure, H. (2011). *Is or is not Hankin , 1896 , a phage reference ? 1*(3), 174–178.

Ackermann, H. W. (2003). Bacteriophage observations and evolution. *Research in Microbiology*, *154*(4), 245–251. https://doi.org/10.1016/S0923-2508(03)00067-6

Aldali, J. A. (2023). *Acinetobacter baumannii* A multidrug-resistant pathogen, has emerged in Saudi Arabia. *Saudi Medical Journal*, *44*(8), 732–744. https://doi.org/10.15537/smj.2023.44.8.20230194

Alrahmany, D., Omar, A. F., Harb, G., El Nekidy, W. S., & Ghazi, I. M. (2021). *Acinetobacter baumannii i*nfections in hospitalized patients, treatment outcomes. *Antibiotics*, *10*(6), 1–12. https://doi.org/10.3390/antibiotics10060630

Antunes, L. C. S., Visca, P., & Towner, K. J. (2014). *Acinetobacter baumannii:* Evolution of a global pathogen. *Pathogens and Disease*, *71*(3), 292–301. https://doi.org/10.1111/2049-632X.12125

Asif, M., Alvi, I. A., & Ur Rehman, S. (2018). Insight into *Acinetobacter baumannii:* Pathogenesis, global resistance, mechanisms of resistance, treatment options, and alternative modalities. *Infection and Drug Resistance*, *11*, 1249–1260. https://doi.org/10.2147/IDR.S166750

Azizian, R., Dawood, S., Nasab, M., & Ahmadi, N. A. (2013). Bacteriophage as a Novel Antibacterial Agent in Industry and Medicine. *Journal of Paramedical Sciences*, *4*(4), 93–101.

Bramkamp, M., & van Baarle, S. (2009). Division site selection in rod-shaped bacteria. Current opinion in microbiology, 12(6), 683–688. https://doi.org/10.1016/j.mib.2009.10.002

Breitbart, M., Salamon, P., Andresen, B., Mahaffy, J. M., Segall, A. M., Mead, D., Azam, F., & Rohwer, F. (2002). Genomic analysis of uncultured marine viral communities. Proceedings of the National Academy of Sciences of the United States of America, 99(22), 14250–14255. https://doi.org/10.1073/pnas.202488399

Buttimer, C., McAuliffe, O., Ross, R. P., Hill, C., O'Mahony, J., & Coffey, A. (2017). Bacteriophages and Bacterial Plant Diseases. Frontiers in microbiology, 8, 34.

https://doi.org/10.3389/fmicb.2017.00034

CDC. (2016). *The Whole Genome Sequencing (WGS) Process*. 264789.

Chan, B. K., Abedon, S. T., & Loc-Carrillo, C. (2013). Phage cocktails and the future of phage therapy. *Future Microbiology*, *8*(6), 769–783. https://doi.org/10.2217/fmb.13.47

Chhibber, S., Kaur, J., & Kaur, S. (2018). Liposome Entrapment of Bacteriophages Improves Wound Healing in a Diabetic Mouse MRSA Infection. Frontiers in microbiology, 9, 561. https://doi.org/10.3389/fmicb.2018.00561

Chibani, C. M., Farr, A., Klama, S., Dietrich, S., & Liesegang, H. (2019). Classifying the unclassified: A phage classification method. *Viruses*, *11*(2). https://doi.org/10.3390/v11020195

Clokie, M. R. J., Millard, A. D., Letarov, A. V., & Heaphy, S. (2011). Phages in nature. *Bacteriophage*, *1*(1), 31–45. https://doi.org/10.4161/bact.1.1.14942

Dekic, S., Hrenovic, J., Ivankovic, T., & Van Wilpe, E. (2018). Survival of ESKAPE pathogen *Acinetobacter baumannii* in water of different temperatures and pH. Water science and technology, 78(6), 1370-1376.

Dijkshoorn, L., Nemec, A., & Seifert, H. (2007). An increasing threat in hospitals: Multidrug-resistant *Acinetobacter baumannii*. *Nature Reviews Microbiology*, *5*(12), 939–951. https://doi.org/10.1038/nrmicro1789

Dolgin E. The secret social lives of viruses. Nature. 2019;570:290–292. doi: 10.1038/D41586-019-01880-6.

Facts, K. E. Y. (2015). *WHO, "Antimicrobials: Handle with Care" in 2020.* 1–6.

Gautam, D., Dolma, K. G., Khandelwal, B., Mitsuwan, W., Mahboob, T., Pereira, M. D. L., … Nissapatorn, V. (n.d.). *SYSTEMATIC / NARRATIVE REVIEW ARTICLE Acinetobacter baumannii : An overview of emerging multidrug-resistant pathogen*. 357–371.

Harada L. K., Silva E. C., Campos W. F., Del Fiol F. S., Vila M., Dąbrowska K., et al. (2018). Biotechnological applications of bacteriophages: state of the art. Microbiol. Res. 212–213 38–58. 10.1016/j.micres.2018.04.007

He, P., Cao, F., Qu, Q., Geng, H., Yang, X., Xu, T., … Luan, G. (2024). Host range expansion of *Acinetobacter* phage vB_Ab4_Hep4 driven by a spontaneous tail tubular mutation. *Frontiers in Cellular and Infection Microbiology*, *14*(February), 1–12. https://doi.org/10.3389/fcimb.2024.1301089

Heather, J. M., & Chain, B. (2016). Genomics The sequence of sequencers : The history of sequencing DNA. *Genomics*, *107*(1), 1–8. https://doi.org/10.1016/j.ygeno.2015.11.003

Howard, A., O'Donoghue, M., Feeney, A., & Sleator, R. D. (2012). *Acinetobacter baumannii* An emerging opportunistic pathogen. *Virulence*, *3*(3), 5. https://doi.org/10.4161/viru.19700

Itani, R., Khojah, H. M. J., Karout, S., Rahme, D., Hammoud, L., Awad, R., … El-Lakany, A. (2023). *Acinetobacter baumannii*: assessing susceptibility patterns, management practices, and mortality predictors in a tertiary teaching hospital in Lebanon. *Antimicrobial Resistance and Infection Control*, *12*(1), 1–17. https://doi.org/10.1186/s13756-023-01343-8

Jin, J., Li, ZJ., Wang, SW. et al. Isolation and characterization of ZZ1, a novel lytic phage that infects *Acinetobacter baumannii* clinical isolates. BMC Microbiol 12, 156 (2012). https://doi.org/10.1186/1471-2180-12-156

Jorgensen, J. H., & Ferraro, M. J. (2009). Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 49(11), 1749–1755. https://doi.org/10.1086/647952

Kareem, A., Al-Sahlany, S. T. G., Verma, D. K., Thakur, M., Mohapatra, B., Singh, S., … Banwo, K. (2022). Trends, Analytical Approaches, and Applications of the VITEK System for Identification and Classification of Bacteria and Yeasts. *Quantitative Methods and Analytical Techniques in Food Microbiology*, (January), 255–272. https://doi.org/10.1201/9781003277453-15

Karna, S., & Khanal, R. (2020). *Trend and Characteristics of Acinetobacter baumannii Infections in Patients Attending Universal College of Medical Sciences , Bhairahawa , Western Nepal : A Longitudinal Study of 2018*. 1631–1641.

Khan, A., Rao, T. S., & Joshi, H. M. (2022). Phage therapy in the Covid-19 era: Advantages over antibiotics. Current research in microbial sciences, 3, 100115. https://doi.org/10.1016/j.crmicr.2022.100115

Koskella, B., & Brockhurst, M. A. (2014). Bacteria-phage coevolution as a driver of ecological and evolutionary processes in microbial communities. FEMS microbiology reviews, 38(5), 916–931. https://doi.org/10.1111/1574-6976.12072

Lemos, E. V., de la Hoz, F. P., Einarson, T. R., Mcghan, W. F., Quevedo, E., Castañeda, C., & Kawai, K. (2014). Carbapenem resistance and mortality in patients with *Acinetobacter baumannii* infection: Systematic review and meta-analysis. *Clinical Microbiology and Infection*, *20*(5),

416–423. https://doi.org/10.1111/1469-0691.12363

Letarov, A. V. (2020). History of Early Bacteriophage Research and Emergence of Key Concepts in Virology. *Biochemistry (Moscow)*, *85*(9), 1093–1112. https://doi.org/10.1134/S0006297920090096

Loganathan, A., Manohar, P., Eniyan, K., VinodKumar, C. S., Leptihn, S., & Nachimuthu, R. (2021). Phage therapy as a revolutionary medicine against Gram-positive bacterial infections. *Beni-Suef University Journal of Basic and Applied Sciences*, *10*(1). https://doi.org/10.1186/s43088-021-00141-8

Lu, T. K., & Koeris, M. S. (2011). The next generation of bacteriophage therapy. Current opinion in microbiology, 14(5), 524–531. https://doi.org/10.1016/j.mib.2011.07.028

Maragakis, L. L., & Perl, T. M. (2008). *Acinetobacter baumannii*: Epidemiology, antimicrobial resistance, and treatment options. *Clinical Infectious Diseases*, *46*(8), 1254–1263. https://doi.org/10.1086/529198

Mitchell, V. (2018). *STRAIN COMPARISON AND DIFFERENTIATION USING MULTILOCUS SEQUENCE TYPING (MLST)*. *44*(0).

Moradi, J., Hashemi, F. B., & Bahador, A. (2015). Antibiotic resistance of *Acinetobacter baumannii* in Iran: A systemic review of the published literature. *Osong Public Health and Research Perspectives*, *6*(2), 79–86. https://doi.org/10.1016/j.phrp.2014.12.006

Naureen, Z., Dautaj, A., Anpilogov, K., Camilleri, G., Dhuli, K., Tanzi, B., … Bertelli, M. (2020). Bacteriophages presence in nature and their role in the natural selection of bacterial populations. *Acta Biomedica*, *91*(7), 1–13. https://doi.org/10.23750/abm.v91i13-S.10819

Nimer, N. A., Al-Saa'da, R. J., & Abuelaish, O. (2016). Accuracy of the VITEK® 2 system for a rapid and direct identification and susceptibility testing of Gramnegative rods and Gram-positive cocci in blood samples. *Eastern Mediterranean Health Journal*, *22*(3), 193–200. https://doi.org/10.26719/2016.22.3.193

Parmar, K. M., Gaikwad, S. L., Dhakephalkar, P. K., Kothari, R., & Singh, R. P. (2017). Intriguing Interaction of Bacteriophage-Host Association: An Understanding in the Era of Omics. Frontiers in microbiology, 8, 559. https://doi.org/10.3389/fmicb.2017.00559

Pincus, D. H. (2010). Microbial identification using the bioMérieux VITEK® 2 system. *Encyclopedia of Rapid Microbiological Methods*, 1–32.

Pirisi A. (2000). Phage therapy--advantages over antibiotics?. Lancet (London, England),

356(9239), 1418. https://doi.org/10.1016/S0140-6736(05)74059-9

Qadir, M. I., Mobeen, T., & Masood, A. (2018). Phage therapy: Progress in pharmacokinetics. *Brazilian Journal of Pharmaceutical Sciences*, *54*(1), 1–9. https://doi.org/10.1590/s2175-97902018000117093

Rao, S. P. N. (2006). *Sridhar Rao, P.N, Bacteriophage (2006), Einsichtnahme 21.08.2016, http://www.microrao.com/micronotes/bacteriophage.pdf*. (1915).

Raza, Ali, jamil, M., Tahir Aleem, M., Aamir Aslam, M., Muhammad Ali, H., khan, S., … Khan, S. (2021). Bacteriophage Therapy: Recent Development and Applications. *Scholars Bulletin*, *7*(3), 27–37. https://doi.org/10.36348/sb.2021.v07i03.003

Reina, R., León-Moya, C., & Garnacho-Montero, J. (2022). Treatment of *Acinetobacter baumannii* severe infections. *Medicina Intensiva*, *46*(12), 700–710. https://doi.org/10.1016/j.medin.2022.08.003

Sabtu, N., Enoch, D. A., & Brown, N. M. (2015). Antibiotic resistance: What, why, where, when and how? *British Medical Bulletin*, *116*(1), 105–113. https://doi.org/10.1093/bmb/ldv041

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, *74*(12), 5463–5467. https://doi.org/10.1073/pnas.74.12.5463

Schooley, R. T., Biswas, B., Gill, J. J., Hernandez-Morales, A., Lancaster, J., Lessor, L., … Hamilton, T. (2017). Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection. *Antimicrobial Agents and Chemotherapy*, *61*(10). https://doi.org/10.1128/AAC.00954-17

Singh, S., Nath, G., & Maheshwari, A. (2024a). *Bacteriophages*. (January). https://doi.org/10.5005/jp-journals-11002-0078

Singh, S., Nath, G., & Maheshwari, A. (2024b). *Bacteriophages*. (October 2023).

Staden, R. (1979). *Nucleic Acids Research*. *6*(7), 2601–2610.

Sulakvelidze, A. (2005). Erratum: Phage therapy: An attractive option for dealing with antibiotic-resistant bacterial infections (Drug Discovery Today (June 15, 2005) 10: 12 (808)). *Drug Discovery Today*, *10*(13), 877. https://doi.org/10.1016/S1359-6446(05)03510-5

Sulakvelidze, A., Alavidze, Z., & Morris, J. (2001). Bacteriophage therapy. *Antimicrobial Agents and*

Chemotherapy, *45*(3), 649–659. https://doi.org/10.1128/AAC.45.3.649-659.2001

Summers, W. C. (2012). The strange history of phage therapy. *Bacteriophage*, *2*(2), 130–133. https://doi.org/10.4161/bact.20757

Trempy, J. E., & Trun, N. (2003). Chapter7 of phages. *Fundamental Bacterial Genetics*, 105–125. Retrieved from http://www.blackwellpublishing.com/trun/pdfs/chapter7.pdf

Whiteway, C., Breine, A., Philippe, C., & Van der Henst, C. (2022). *Acinetobacter baumannii. Trends in Microbiology*, *30*(2), 199–200. https://doi.org/10.1016/j.tim.2021.11.008

Williams, D. N. (2016). Antimicrobial resistance: Are we at the dawn of the post-antibiotic era? *Journal of the Royal College of Physicians of Edinburgh*, *46*(3), 150–156. https://doi.org/10.4997/JRCPE.2016.302

Wishart, D. S., Han, S., Saha, S., Oler, E., Peters, H., Grant, J. R., … Gautam, V. (2023). PHASTEST: Faster than PHASTER, better than PHAST. *Nucleic Acids Research*, *51*(W1), W443–W450. https://doi.org/10.1093/nar/gkad382

Wittebole, X., De Roock, S., & Opal, S. M. (2014). A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Virulence*, *5*(1), 226–235. https://doi.org/10.4161/viru.25991

Yin, R., Kwoh, C. K., & Zheng, J. (2018). Whole genome sequencing analysis. *Encyclopedia of Bioinformatics and Computational Biology: ABC of Bioinformatics*, *1–3*, 176–183. https://doi.org/10.1016/B978-0-12-809633-8.20095-2

Zalewska-Piątek B. (2023). Phage Therapy-Challenges, Opportunities and Future Prospects. Pharmaceuticals (Basel, Switzerland), 16(12), 1638. https://doi.org/10.3390/ph16121638

Zhang, Y., Lin, Y., Galgano, S., Houdijk, J., Xie, W., Jin, Y., Lin, J., Song, W., Fu, Y., Li, X., Chui, W., Kan, W., Jia, C., Hu, G., & Li, T. (2022). Recent Progress in Phage Therapy to Modulate Multidrug-Resistant *Acinetobacter baumannii*, including in Human and Poultry. Antibiotics (Basel, Switzerland), 11(10), 1406. https://doi.org/10.3390/antibiotics11101406

APPENDIX

Media compositions and Reagent preparations

Nutrient Broth/Agar (NB/NA) – HiMedia

Ingredients	Grams / Liter
Peptone	10.000
Beef extract	10.000
Sodium chloride	5.000

pH (after sterilization) 7.3±0.1

Sodium Magnesium Buffer (SM Buffer)

Ingredients	Concentration
Sodium chloride	100 mM
Magnesium sulfate (heptahydrate)	10 mM
Tris-HCl (pH 7.5)	50 mM
Gelatin	0.01 % (w/v)

Mueller Hinton Agar (MHA) – HiMedia

Ingredients	Grams / Liter
Meat, infusion solids from	300g
Casein acid hydrolysate	17.5
Starch	1.500
Agar	17.000

Final pH (at 25°C) 7.3±0.1

Tryptic Soy Broth (TSB)/Soybean-Casein Digest Medium – HiMedia

Ingredients	Grams / Liter
Pancreatic digest of casein	17.000
Papaic digest of soyabean meal	3.000
Sodium chloride	5.000
Dextrose	2.500
Dibasic potassium phosphate	2.500

Final pH (at 25°C) 7.3±0.2

Luria Bertani Broth-HiMedia

Ingredients	Gram/liter
Casein enzymic hydrolysate	10g
Yeast extract	5g
Sodium chloride	10g

Phosphate Buffered Saline (PBS)

NaCl - 8.0 g

KCl - 0.2 g

Na_2HPO_4 - 1.44 g

KH_2PO_4 - 0.24 g

Table: Thermal stability of ACB-1phage at different temperatures

S.N.	Time(minutes)	Pfu/ml 37°C	Pfu/ml 50°C	Pfu/ml 60°C	Pfu/ml 70°C
1.	10	49	47	45	2
2.	20	45	45	32	0
3.	30	40	45	12	0
4.	40	39	40	8	0
5.	50	30	38	0	0
6.	60	25	32	0	0

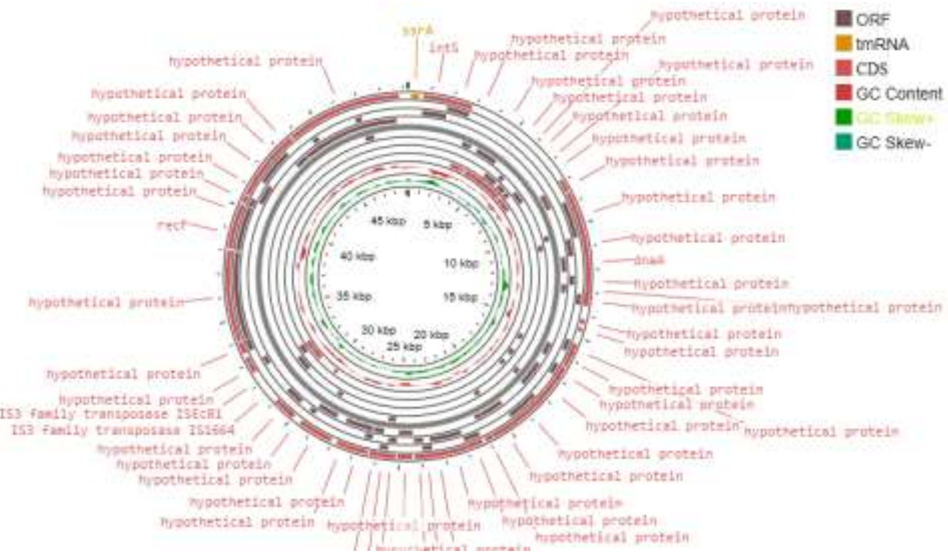
Table: One step Growth curve of phage ACB-1

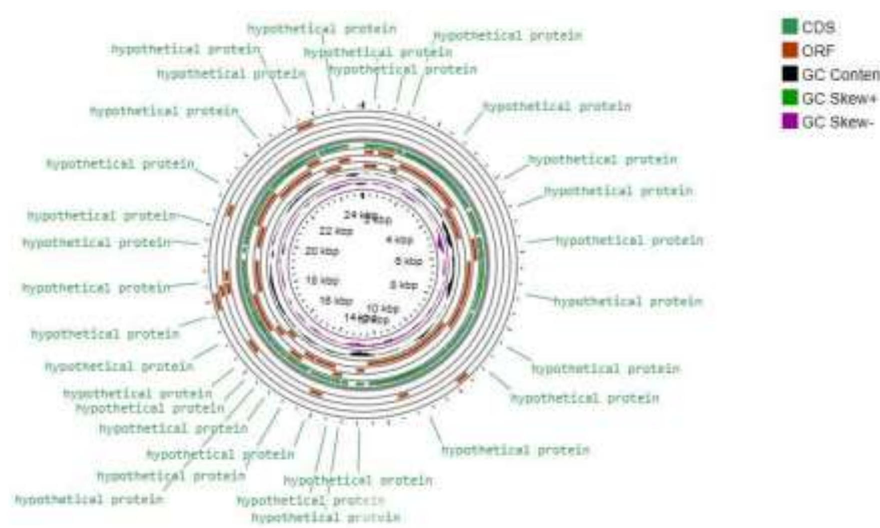
S.N.	Time (minutes)	Pfu/ml
1.	0	0
2.	10	9
3.	20	15
4.	30	17
5.	40	49
6.	50	52
7.	60	54

Table : pH stability of ACB-1 phage

S.N.	pH	Pfu/ml
1.	1	0
2.	2	0
3.	3	3
4.	4	13
5.	5	27
6.	6	35
7.	7	37

8.	8	35
9.	9	31
10.	10	26
11.	11	13
12.	12	8
13.	13	0





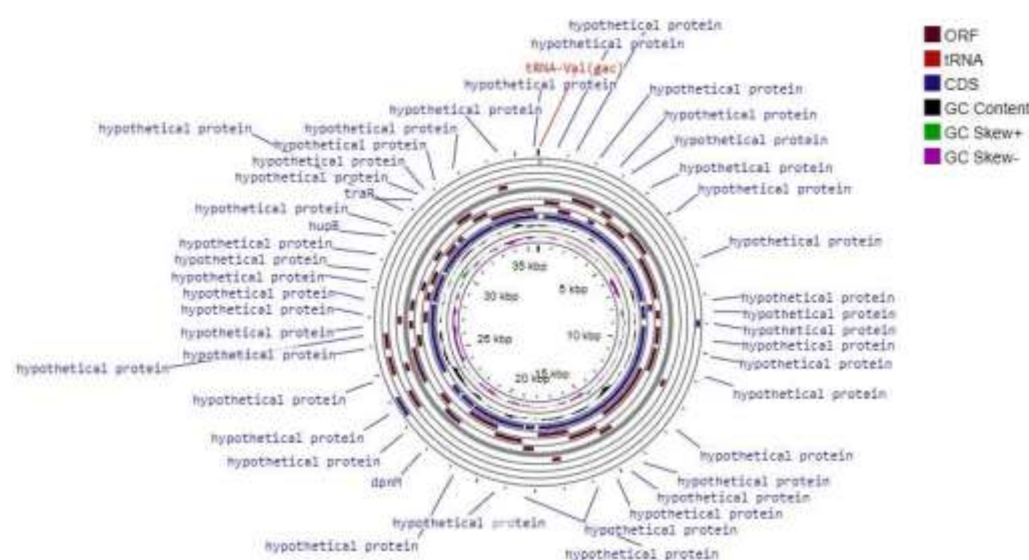


Figure: Proksee generated circular genome of phage ACB-1

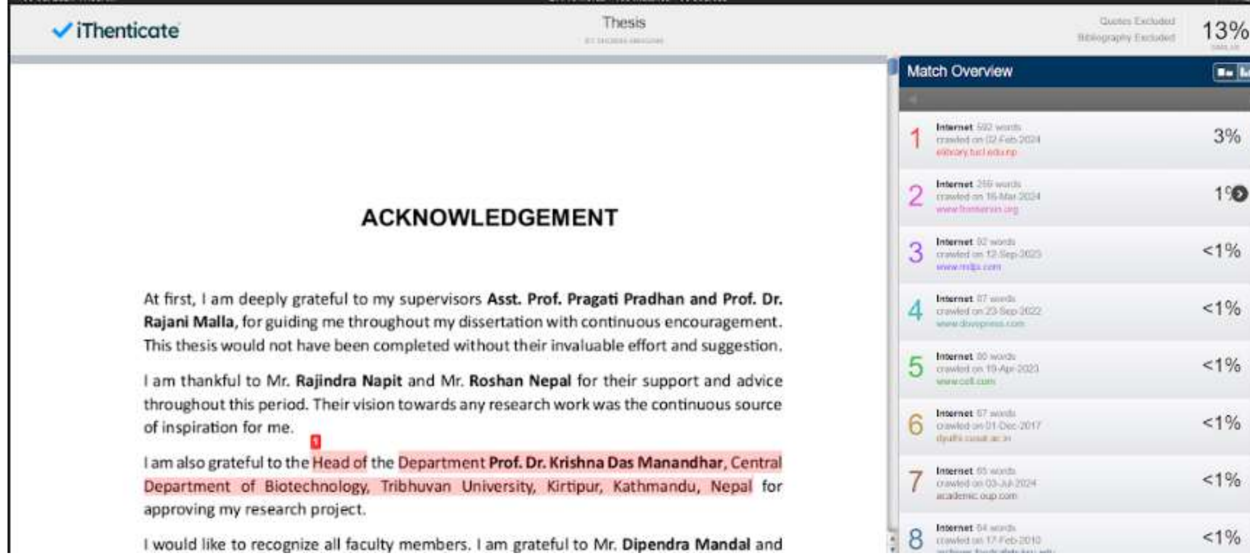
Figure: Circular Diagram generated by UGENE

Figure: Concentration of Phage ACB-1 DNA check on nanometer

Photograph: Sample Collection site

Photograph: Observing bacteria through microscope

Photograph: Team Phage



iThenticate

Document Viewer
Similarity Index
13%

Thesis

By: Shobha Amagain

As of: Jul 6, 2024 11:40:00 AM
21,716 words - 193 matches - 116 sources

sources:

295 words / 1% - from 02-Feb-2024 12:00AM
elibrary.tucl.edu.np
226 words / 1% - from 02-Feb-2024 12:00AM
elibrary.tucl.edu.np
54 words / < 1% match - from 02-Feb-2024 12:00AM
elibrary.tucl.edu.np
17 words / < 1% match - from 02-Feb-2024 12:00AM
elibrary.tucl.edu.np
98 words / < 1% match - from 16-Mar-2024 12:00AM
www.frontiersin.org
69 words / < 1% match - from 24-Oct-2023 12:00AM
www.frontiersin.org
60 words / < 1% match - from 17-May-2024 12:00AM
www.frontiersin.org